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Biotechnology: Trends in Advancement of Life Science Research and Development in Nigeria

edited by

Oluyemi Akinloye, PhD

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SUMMARY

One challenge for those involved in the life science research, especially bio-physiological research is the emerging evolution of molecular technology. Advances in genetics and accumulated information in the genomics have made molecular technology and its application in life science the fastest growing tools in this millennium. Many Researchers in the developed countries are very much at home with this advancement and have taken advantage to develop their research interest and contribute more meaningful to their community. This technology is still new and just emerging in the developing countries. However, in recent years molecular technology tools is fast becoming popular in life science research in the developing world, especially in the field of agriculture, biology, biochemistry, medical science etc. Biotechnology tools are widely available and currently in use as research tools in most developed countries. The potentiality of its application in the developing countries, especially Africa has formed the motivation for seeking foreign fellowship on one hand and even returning home after the fellowship on the other hand by many young researchers. Organizing a scientific conference with focus or emphasis in this evolving area will give opportunity to researchers to take stock of how much has been acquired during the period of stay in the developed countries (especially Germany) and how much is applicable to research environment in the home country (Nigeria). Three days scientific conference was organized to achieve this goal. The scientific meeting was designed to stimulate collaboration and research interest in the emerging and revolutionary field of molecular biology among Humboldtians and other young researchers in Nigeria. The request from the participants and desire to preserve the high quality of papers and amazing scientific information presented during this conference motivated the publication of this book. The generosity of Humboldt Foundation to solely support both the conference and publication of this book, made possible the realization of our dream.

I also wish to deeply appreciate the support and partial review of the articles by Professor O. G Ademowo of the Institute of Advance Medical Research and Training, University of Ibadan and Dr. S. S. Taiwo of the Depatment of Medical Microbiology and Parasitology, Ladoke Akintola University of Technology, Ogbomoso, Nigeria.

Fuel Biotechnology: A Strategy for Sustainable Energy Production for Developing Nations

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ABSTRACT

The rapidly dwindling reserves of fossil fuels in recent years have stimulated a great interest in exploring alternative sources of renewable energy such as biogas. Biotechnology plays an important role in the production of biomethane from biological raw materials via anaerobic digestion technology. The development in biotechnology and engineering sciences in the last a couple of decades has provided many new possibilities to improve the performance of the technology .This study focused on the prospect of biomethane as a sustainable energy production for developing nations using Nigeria as a case study. An underground biodigester made of concrete was designed and constructed for a household in Ibadan .The digester was dug to a depth of 1.22m. The floor was made up of gravel, sand and cement. The digester was initially filled with 3300 litres of water. Cattle dung and water hyacinth were mixed with 1,100 litres of water to form slurry. A pH meter was used to measure the pH of the slurry. The slurry was used to charge the digester. The digester was subjected to anaerobic digestion for seven days after which the pressure rose indicating gas production in the digester. The biogas produced was stored in the digester. The gas was scrubbed to remove impurities, measured and monitored by using artisanal manometer. The digester produced biogas. The gas produced was flammable, odourless and smokeless. The gas generated was enough for domestic activities such as cooking a variety of staple foods by a household of six people. The household used an average of $0.30m^3$ of biogas per hour. The household preferred biomethane to other conventional ways of generating fuel since they could get the feedstock easily from a nearby farm thereby saving cost of buying fuel. Advancement in biotechnology is set to change our world. Biomethane technology using anaerobic digestion will play a vital role. The developing nations such as Nigeria which depends solely on fossil fuels needs a paradigm shift from conventional sources of generating fuel such as petroleum to unconventional ones that are renewable such as biomethane.

Keywords: Anaerobic digestion, Biomethane, Biotechnology, Cattle dung, Digester.

INTRODUCTION

There are to date more than 3.5 billion people, mostly in developing countries, who still rely on coal and biomass—such as wood, dung, and crop residues—as their main source of energy for both cooking and heating. This traditional sources of energy burned in simple stoves with no proper ventilation can be extremely polluting and cause serious environmental health problems (Smith, 2006). Women and children are at greater risk because of household responsibilities and increased exposure indoors (Dasgupta *et al.*, 2004).Furthermore, the rapidly dwindling reserves of fossil fuels in recent years have stimulated a great interest in exploring alternative sources of renewable energy. One of the best-established technologies available is Biogas Technology, where organic materials, e.g. animal manure, night soil, agricultural residues and industrial

effluents, are biologically fermented in the absence of oxygen to produce flammable gas consisting predominantly of methane and carbon dioxide. The use of cleaner fuels such as biogas or other modern biofuels can eliminate the current indoor and outdoor air pollution epidemic. Biogas technology not only provides pollution prevention, but also allows for sustainable energy, compost and nutrient recovery. Improving access to modern energy services – including electricity and modern cooking fuels and appliances – is essential if the world is to achieve the Millennium Development Goals (MDGs).The United Nations Millennium Project recommends an additional MDG "target to halve by 2015, the number of people without effective access to modern cooking fuels, and to make improved cooking stoves widely available".

Fuel biotechnology constituted production of energy-dense and convenient-to-use fuels like methane, ethanol, butanol, biodiesel and hydrogen from relatively diffuse and inconvenient-to-use source of energy-e.g. biomass and sunlight through the action of biological agents. Biologically produced fuels are called biofuels and often the names biomethane, bioethanol, biofuels, biodiesel and biohydrogen are used.

Technological progress - particularly in biotechnology - can and will help the energy needs of growing populations in the developing nations. Biotechnology is already helping to increase production of both food and biofuel. It is doing so by boosting agricultural yields and by increasing the efficiency of biofuel production from all feedstocks. Biotechnology also enables production of fuel from non-food feedstocks - switch grass, agricultural residues or trees - which are abundantly available in the developing world.

Biogas was aimed as a substitute for the non-renewable fossil fuels like petroleum, which are declining at an alarming rate. However, the ultimate source of energy for biomethane and fossil fuels is the sun via biomass. Biomass is the total cellular dry weight or organic material produced by an organism (usually from CO_2 and sunlight). In general, biofuels are aimed for use in transport as a substitute for the nonrenewable and rapidly declining fossil fuels derived from petroleum. Initially, biomass was the only source of energy available to and used by man but the development of fossil fuels (coal and oil) rapidly reduced the use of biomass as energy source, especially in the developed countries.

Biogas is a clean, abuandant and renewable energy source. They do not cause environmental pollution due to SO_2, CO_2, CH_4 etc. Low-cost substrates, often wastes including municipal waste can be converted to high-value product-the biomethane- together with the cleaning up of the environment. This is the mixture of gas produced by methanogenic bacteria while acting upon biodegradable materials in an anaerobic condition. Biomethane is mainly composed of 50 to 70 percent methane, 30 to 40 percent carbon dioxide (CO2) and low amount of other gases.

Biogas is about 20 percent lighter than air and has an ignition temperature in the range of 650° to 750° C. It is an odorless and colorless gas that burns with clear blue flame similar to that of LPG gas (Sathianathan, 1975). Its calorific value is 20 Mega Joules (MJ) per m^3 and burns with 60 percent efficiency in a conventional biogas stove.

Biogas technology, which converts biological waste into energy, is considered by many experts to be an excellent tool for improving life, livelihoods, and health in the developing world. Worldwide, about 16 million households use small-scale digesters, according to Renewables 2005: Global Status Report, a study by the World watch Institute. Biogas production by

anaerobic digestion is popular for treating biodegradable waste because valuable fuel can be produced while destroying disease-causing pathogens and reducing the volume of disposed waste products. The methane in biomethane combusts more cleanly than coal, and produces more energy with no emissions of carbon dioxide.

Biogas is a mixture of gases that is produced from biological waste material (termed biomass).Thus, through anaerobic bacterial decomposition, raw cow manure, human faeces, and water can be converted into a gas mixture primarily composed of methane gas that can subsequently be used as burning fuel. Biogas has a calorific value of 20 Mega Joules per cubic meter (FAO, 1997) and can be used for many domestic applications such as lighting, cooking, electricity generation, or fuel for modified internal combustion engines. It has been shown that one cubic meter of biometahne could cook 3 meals for a family of 5 or 6, generate 1.25 kilowatt hours of electricity, or power a one horse power internal combustion motor for 2 hours (ITDG, 2004[1]: 3). Biomethane can be produced by methanogenic bacteria in a digester. This physical structure, invented in the 1930s and developed in the 1950s and 1980s, is essentially an underground and airtight pit that can receive animal manure, human faeces, and water to produce biomethane and potent fertilizer. Once a suitable bacteria culture has been developed inside the Digester, biological waste is mixed with water in a 2:3 ratio and held at an optimal temperature of 30-40°C for approximately 50-60 days (FAO, 1997).

The recommended daily waste loading rate, for a conventional digester, should be approximately 6 kg of manure per cubic meter of biogas desired (FAO, 1997). It should be noted however, that given the long retention time of the manure (50-60 days), the underground chamber of the digester should have a total volume 50-60 times greater than the daily volume of manure added. In order to maximize the production of biogas, the input biological waste should have Carbon-Nitrogen (C/N) content ratios of 20 to 30 (FAO, 1997). Such C/N ratios can be obtained from cattle manure and other biological wastes. The gas output of the Digester varies depending on its size. However, it has been estimated that 50-70 percent of the raw materials fed in the Digester are eventually converted to biogas (Fry, 1973: 18). The remaining raw materials are converted into sludge (a liquid-solid mixture), which has been found to be a very potent fertilizer (Fry, 1973: 25).

The objective of this study is to produce biogas as a source of energy production for developing nations using a household in Nigeria as a case study.

METHODOLOGY

There are two main phases for the construction of the underground Chinese digester used for this research work; Phase I is basically the structural concrete work of the digester. This begins with the Planning/preparation stage to the final concrete plaster application to the inside of the digester /gas dome. The importance here is that all of the concrete work was carried out continuously, day to day to avoid any delays in the concrete work. Timing was taken into full consideration and the planning/preparation of the work to be done was fully understood so that nothing will interfere with gas production, durability. Each biogas unit meet specified technical requirements for it to be water and gas tight for the unit to function properly. After all of the main structural concrete work has been completed and the “curing” process of the concrete

properly stated, the Phase I construction is considered finished. The break between Phase I and Phase II is to allow for the proper curing of the concrete, a period of no less than 2-3 weeks.

The activities of Phase II are gas piping systems (from digester to the stove, manometer, gas meter, scrubbers) etc. and loading (testing for water and gas tightness).Furthermore, water and gas tightness was done before the actual loading of the digester

The Materials Used for the Construction

The different required materials were procured and all materials that were used were locally procured from material merchants in Ibadan, Nigeria. The Cow dung and its rumen were sourced from Bodija Market, Ibadan, Nigeria. However, the water hyacinth was sourced from University of Ibadan, Nigeria.The various materials used were shown in the table below:

Table 1.1: Materials for the digester

Bill of Engineering and Measurement

Quantity	**Materials**	**Unit Price**	**Amount**
25 Bags	Cement	1,700	42,500
2 Tipper	Sand	5,500	11,000
1 Tipper	Gravel	10,000	10,000
3(9 mm)	Iron rod	1,050	3,150
2	Quarter rod	900	1,800
6	1 kg silicate powder	1,500	9,000
1(13 ft)	PVC pipes	1,500	1,500
2	Labourers(block molding)	1,500	1,500
1	Carpenter(dome)	3,000	3,000
1	Iron bender(dome)	1,000	1,000
1	Materials (dome)	5,000	5,000
1	Materials for the inlet cover	4,000	4,000
1	Materials for the outlet cover	4,000	4,000
1	Wages for technician	80,000	80,000
1	Miscellaneous	20,000	20,000
		Total	**197,450**

Table 1.2: The Materials for the Piping System

Bill of Engineering and measurement

Qty	**Description of Materials**	**Unit Price**	**Amount**
10	½ inch elbow pipe	20	200
	½ inch T-pipe		
5	13 ft ½ inch pressure pipe	300	1,500
10	½ inch union	25	250
3	½ inch connector	20	60
4	Tap	200	800
2	Tongit Gum	400	800
1	Artisanal Manometer	2,000	2,000
1	Biogas Cooker	10,000	10,000
1	Scrubbers (Lime water, Finely divided iron, dessicator and activated carbon.	1,000	1,000
4	Scrubber's Containers	250	1,000
½Bag	Cement	1,000	1,000
		Total	**18,610**

The Sampling Site

The site was located at Alaka area, Orita Challenge, Ibadan.The site was considered suitable because the water table is not high and the area is not waterlogged. The town experiences two (2) seasons - rainy and dry. The raining season run from March through October, with temperatures ranging from 21°C to 34°C, rainfall from 8.4 cm to 8 cm and humidity ranging from 54 - 77%. The dry season is rather short extending from November through February. During this period, the prevailing temperature ranges from 20-35°C, rainfall from l cm to 4.6 cm and humidity from 43 -83%. The beginning of the dry season is characterized by cold humid mornings and warm afternoons, and is called "harmattan.

The Construction of the Digester

An underground biodigester made of concrete was designed and constructed following standard procedures for the household. The biodigester was dug to a depth of 1.22m. The floor was constructed with gravel, cement and sand. Poly-Vinyl-Chloride (PVC) pipes were used to connect the inlet and the outlet tanks to the digester. 3300 litres of water, 800kg of cattle and 30kg of water hyacinth were mixed together to form a slurry. A pH meter was used to measure the pH of the slurry. The slurry was used to charge the biodigester. The digester was subjected to anaerobic digestion for seven days after which the pressure rose indicating gas production in the biodigester. The biomethane produced was stored in the biodigester. The gas was monitored by with artisanal manometer.

RESULTS

The results of this research work could be divided into two phases which are; Phase I- the result of the designing and fabrication of the digester and Phase II- the result of biogas production.

Phase I: Designing and Fabrication of the digester

The Digester: The digester was inspected and no gas leakage was observed. The excavation work produced a cylindrical shaped underground digester. The flooring was saucer in shaped while the manhole cover has a dome shape. The sidewalls were made of blocks which help the digester to be water and air-tight.

i. **The Inlet Pipe:** The slurry (Cattle dung, and water hyacinth) flowed easily into the digester without any slurry leakage.

ii. **The Outlet Pipe:** The digestate flowed out through the outlet pipe without any leakage.

Phase II: Biogas Production

Gas Production: The digester produced 2.5m^3 of biogas per day. The gas produced was flammable, odourless and smokeless. The gas generated was enough for domestic activities such as cooking a variety of staple foods by a household of six people. The household used an average of 0.28m^3 of biogas per hour. The generated gas was found to be environmentally friendly as it produced a blue flame without any smoke. The household preferred biogas to other conventional ways of generating fuel since they could get the feedstock easily from a nearby farm thereby saving cost of buying fuel.

Gas Storage: The fixed dome beam top stored the gas produced and makes it convenient to store the gas since it was an inbuilt storage tank.

Gas meter: The gas meter measured the amount of biogas produced everyday. The digester produced 2.5m^3 of biogas daily.

Artisanal Manometer: The artisanal manometer checked pressure and monitored the gas produced. It indicated a low or negative value whenever there is little or no gas in the digester.

Gas Piping System: The pressure pipes experience no leakage as it passed through the digester to the kitchen where it was finally utilized.

Gas Scrubbers: The gas produced was scrubbed with lime water, finely divided iron, activated carbon and a dessicator which removed Co_2, H_2S NH_3 and water vapor respectively

Biogas Cooker: The biogas cooker produced a blue fame and was used for cooking a 3 square daily meal. The household prefers the biogas to the conventional ones.

Daily Gas Yield: The digester produced 2.5 m^3 of biogas per day. The gas was utilized by the household.

Daily Types of Food Cooked and Cooking Time: The household utilized 1.12m^3 of biogas everyday for four hours daily cooking from Monday to Friday while more biogas (1.68m^3) was used on weekends when six hours of cooking took place.

Fig. 1: The digging of the Digester

Fig. 2: The Construction of the digester walls and the fixing of the pipes.

Fig. 3: The underground bio-digester made of concrete with inlet and outlet tank.

Fig. 4: The slurry was poured into the bio-digester through the inlet tank.

Fig. 5: The Charging of the Digester

Fig.6: The Piping of the gas

Fig.7: The testing and production of bio-methane.

Fig. 8: The Use of the gas for Cooking

CONCLUSION

Advancement in biotechnology is set to change our world. Biogas technology using anaerobic digestion will play a vital role. The developing nations such as Nigeria which depends solely on fossil fuels needs a paradigm shift from conventional sources of generating fuel such as petroleum to unconventional ones that are renewable such as biogas.

Biogas is a well-established fuel for cooking and lighting in a number of countries. Biogas can provide a clean, easily controlled source of renewable energy from organic waste materials for a small labor input, replacing firewood or fossil fuels (which are becoming more expensive as supply falls behind demand). During the conversion process pathogen levels are reduced and plant nutrients made more readily available, so better crops can be grown while existing resources are conserved.

China and India which have about one-third of the world population have a well developed biogas programme. Nigeria which represents a significant population of the developing nations can provide a network of rural 'biogas service centres' to provide the infrastructure necessary to support dissemination, financing and maintenance. Other countries in Africa such as Egypt, South Africa, Ghana, Kenya e.t.c, could also develop active programmes as carbon emission levels are becoming of greater concern and as people realise the benefits of developing integrated energy supply options.

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Iso-electric focusing (IEF): a diagnostic tool for genetic purity evaluation and cultivar identification in seed analysis

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ABSTRACT

Till date variety verification and seed certification in Nigeria depends on field morphological inspection which is resource-intensive, time consuming and often inaccurate. Modern techniques for genetic purity analysis offer more precise information on genetic quality than field data. Molecular techniques involving amplification and electrophoresis of DNA provide precise data on genetic purity of seed lots when protocols are optimized for individual species, but the procedures are resource-intensive. A lesser cost intensive molecular diagnostic technique for seed genetic purity is using data of first dimension SDS-PAGE electrophoresis of seed storage proteins based on their charges. The procedure is termed Iso-Electric Focusing (IEF) and it is being promoted in international seed standards for evaluating genetic purity of seed varieties. In this paper, I present an overview on the applicability of IEF for evaluating genetic purity of commercial seed lots in Nigeria. The potential benefits of IEF analysis for upgrading seed quality standards and the Nigerian seed industry are highlighted in this paper. A proposal for capacity building for the application of IEF to seed analysis procedures in Nigeria as well as opportunities for enhancing IEF based research in Nigeria were discussed.

INTRODUCTION

Improved plant varieties and good quality seeds are essential for safe, reliable and efficient crop and food production. It had been estimated that improved crop varieties will provide up to 50% of the estimated crop productivity increases expected to meet food sufficiency targets in Africa (Pandey, 2008). In Nigeria, several breeding projects had led to the generation of improved crop varieties. For instance, breeder seeds of about 40 inbred lines of maize had been released by public agricultural research institutions to commercial seed companies for hybrid seed production in the past few decades (NACGRAB, 1997; Daniel and Adetumbi, 2004). Hybrid seed production seeks to explore hybrid vigour of F1 crops, it often involves crossing highly homogeneous inbred lines produced by several generation of selfing to produce heterogeneous hybrids as in maize or crossing the hybrid of a cytoplasmic male sterile line and a corresponding iso-nuclear maintainer line with a genetically diverse restorer line as in rice. However, recent reports indicate that production and performances of commercial hybrid seeds in Nigeria is decreasing, the most credible reason being hybrid breakdown (Olakojo, unpublished). Hybrid breakdown is a result of genetic contamination and low analytical purity of parental lines and the hybrids. Thus, assessment and maintenance of genetic purity of the parental lines and hybrids is crucial for the successful delivery of high quality hybrid seeds in Nigeria.

Presently, seed purity and variety identification in Nigeria is based on morphological evaluation of seeds and growing plants. The field inspection methods are rigorous, resource intensive, subject to bias and have very little precision. Molecular markers are potentially high precision assays of genetic purity of seed stocks (Cooke, 1988; 1989; Nandakumar, 2004). Applicable molecular testing methods are, electrophoresis of seed storage proteins by Isoelectric Focusing (IEF) and Sodium Dodecyl Sulphate (SDS) or acid-Polyacrylamide gel (Cooke, 1988), Polymerase chain reaction (PCR)-based techniques (Welsh and McClelland, 1990), Immunoassay e.g. ELISA (Skerritt et al, 1988) and herbicide bioassay. Compared to Immunoassay and PCR-based methods, electrophoresis by IEF offers the advantages of economy because it is less cost intensive to run and no sophisticated equipment are required, making it more affordable for laboratories with limited resources as in the case of Nigeria. IEF procedures are also known to be easy (clear, one-dimensional separation principle), efficient, fast and sensitive (Bio-analytix, 2003). IEF protocols are being developed in different parts of the world for cost effective assessment of variety purity for various crops, for example Nokolic *et al.* (2008) established IEF procedures and markers for sunflower seed storage proteins heliathinin. The objective of this paper is to examine the use of IEF for analyzing seed genetic purity in the Nigerian seed sector.

PRINCIPLES OF ISOELECTRIC FOCUSING

The successful exploitation of proteins for variety identification purposes is based on the fact that proteins are direct products of gene transcription and translation. Proteins are thus regarded as markers for the structural genes that encode them, thus methods for comparing protein composition provide a measure of genetic variation between individuals and populations. Thus proteins that exist in multiple forms *i.e.* polymorphic and are in abundance within seed samples can be used for variety discrimination. Potential candidates are seed storage protein like albumins, globulins, prolamins and glutelins.

The first dimension in a standard two-dimensional gel is the separation of proteins according to their isoelectric point thus IEF is a technique for separating different molecules by their electric charge differences. It is a type of zone electrophoresis, usually performed in a gel that takes advantage of the fact that a molecule's charge changes with the pH of its surroundings. A protein that is in a pH region below its isoelectric point (pI) will be positively charged and so will migrate towards the cathode. As it migrates, however, the charge will decrease until the protein reaches the pH region that corresponds to its pI. At this point it has no net charge and so migration ceases. As a result, the proteins become focused into sharp stationary bands with each protein positioned at a point in the pH gradient corresponding to its pI. The technique is capable of extremely high resolution with proteins differing by a single charge being fractionated into separate bands. Isoelectric focusing can resolve proteins that differ in pI value by as little as 0.01.

Isoelectric focusing takes place in a pH gradient and is limited to molecules which can be either positively or negatively charged (amphoteric molecules), like proteins, enzymes and peptides. Separation happens in a pH gradient which is formed by special amphoteric buffers (ampholytes) having high buffer capacities at their pI (isoelectric point). The pH gradient is produced by an electric field. Before an electric field is applied the gel has a uniform pH-value and almost all the carrier ampholytes are charged. When an electric field is applied, the negatively charged

ampholytes move towards the anode, the positively charged ones to the cathode and their velocity depend on the magnitude of their net charge. The carrier ampholytes align themselves in between the cathode and the anode according to their pI, and determine the pH of their environment. A stable gradually increasing pH gradient depending on the initial mixture of ampholytes is formed. Strips of filterpaper soaked in electrode solutions serve the purpose of stabilizing the gradient. An acid and a base are used as anolyte and catholyte respectively. When, for example, an acid carrier ampholyte reach the anode it acquires a positive charge from the medium and is attracted back towards the cathode.

Gels with large pores are usually used in this process to eliminate any "sieving" effects, or artifacts in the pI caused by differing migration rates for proteins of differing sizes. Gels are usually made of polyacrylamide or agarose. A number of amphoteric buffer solutions and pre-made gels are available covering broad and narrow pH ranges. High resolution is obtained when narrow pH-ranges are employed. In polyacrylamide gels pore size can be accurately controlled by the total acrylamide-concentration and degree of crosslinking (relationship between acrylamide and bis-acrylamide). When cross-linking is kept constant and total concentration increases pore size will decrease (and diffusion is reduced). Gel solution is made from appropriate amounts of acrylamide (~5%) ampholyte (~2%) double distilled water and riboflavin 5´P (for photopolymerization). Gels (approx 250x120x1mm) are mold between two glass-plates and polymerized overnight in UV-light (requires a pH in the solution >5-6).

Extraction of proteins and detection in IEF gel

A well-prepared protein sample should be free of salts, ionic detergents, non-protein macromolecules (nucleic acids, starch, lipids), particulate matter and prepared in a lyophilized or concentrated (> 1 mg mL^{-1}) state (Hurkman and Tanaka, 1986). Typically, if the sample has met these requirements it can be directly re-suspended in extraction media for IEF. For samples that do not meet the aforementioned criteria, further steps should be taken. Often, a single protein precipitation step (acetone, methanol or TCA) followed by thorough washing of the pellet (cold 80% acetone) is adequate for most samples, but for plant tissues like seeds rich in starch a phenol protein extraction step is required as reported in Hurkman and Tanaka (2004).

The most widely used protein stains are Coomassie Brilliant Blue R-250, Coomassie Brilliant Blue G-250 (colloidal Coomassie), silver staining and the fluorescent dye Sypro Ruby™. For highest sensitivity, silver and Ruby are preferred to the Coomassie dyes. But for reproducibility and quantitative comparisons, Coomassie and Ruby are superior to silver. It has been reported that the linear quantitative range for Ruby is at least three orders of magnitude (Berggren *et al.*, 2000). This is particularly important for quantitative comparisons as the dynamic range of most proteomes span multiple orders of magnitude. A recently developed alternative to Ruby is a related metal chelate fluorescent dye referred to as Ruthenium II (Rabilloud *et al.*, 2001). To minimize the accumulation of solvent waste, current staining methods recommend ultra-pure water for gel de-staining. For long-term storage, however, water must be replaced with 20% methanol to prevent microbial growth.

SEED TESTING APPLICATIONS

Hybrid purity testing

Hybrid varieties of crops offer improvement in plant vigour, yield, uniformity and disease tolerance over the open pollinated varieties. Since the production of F1 hybrids involves crossing highly inbred parent lines, it is most important to determine the success of the crosses made with them, hence the hybrid or genetic purity. Traditionally, this is done by growing out the seeds which is resource-intensive, time-consuming and liable to environmental influences. IEF and other electrophoresis procedures offer more precise and cost effective alternative for genetic purity analysis (Smith and Wych, 1986; McDonald, 1991). The application of electrophoresis for this purpose is based on the fact that protein or isozyme bands are inherited in a Mendelian fashion that is, when two inbred lines are crossed for F1 hybrid production the resultant progeny will express all of the protein bands from the parents. Thus impurities by self pollination of the female parent or pollination by an unintended male parent can be readily identified. An example for maize is shown in Figure 1, the first three lanes are protein banding patterns from seeds samples of the female parents, the three middle lanes are male parents and the last three lanes are the F1 hybrids. The banding patterns showed that the inbred lines are highly identical and thus genetically pure.

Distinctness testing, seed certification and variety registration

For most seed regulatory bodies in different regions of the world, approval for variety registration is based on three seed quality attributes: distinctness, uniformity and stability. Many countries have adopted the analysis of new varieties by electrophoresis procedures, many varieties have been granted distinctness on the basis of electrophoresis tests (Smith and Smith, 1992).

Seed testing and certification are legislative activities to enforce limits of hybrid purity and overall seed quality. It is not surprising therefore that IEF will play significant roles in seed certification procedures. Cooke (1988) demonstrated how IEF could be used for routine checking of identity of onion varieties as well as for the investigation of the origin of off-types. With the advent of increasing events of genetically modified (GM) crops, regulatory bodies of different countries need to adopt technologies that can effectively identify GM crops for effective control.

Quality control

Since modern industrial processing of agricultural raw materials are mechanized, high levels of uniformity is required of crop produce, hence uniformity of seed materials for crop production is an indispensable seed quality attribute (Nokolic *et al.* 2008). Thus precision in genetic purity of seed crops and the seed products for establishing commodity crops is an important quality control issue for growers and manufacturers. For example protein electrophoresis is now being routinely used in some parts of the world to check grain quality for processing specific brands of manufactured products. Besides uses in seed analysis, IEF analysis is applicable for quality control of other food products. For example, Schickle (2003) demonstrated the use of IEF to determine the conformity of cheeses from goat, ewe and cows to European reference standards as shown in figure 2.

CAPACITY BUILDING FOR IEF SEED ANALYSIS AT UNAAB

Capacity building in IEF for seed analysis will provide valuable services to the Nigerian seed sector. Specific areas of concentration are acquisition of facilities for IEF analysis, training for skill development, research for protocol development and networking.

At the University of Agriculture, Abeokuta, (UNAAB) Nigeria, capacity for molecular biology is increasingly building. The first phase of the central biotechnology building comprising of 5 laboratory spaces that can hold up to 40 students each was commissioned in early 2009. Infrastructural facilities like 50Kva power generator, sub-zero freezers, various thermo-cyclers (PCR machines), gel tanks, powers sources, water distillers, incubating chambers and centrifuges had been installed. This puts UNAAB at a vantage point as service provider for IEF analysis.

Besides the acquisition of equipment and facilities, UNAAB is involved in yearly summer courses in various aspects of biotechnology. This helps in capacity building in terms of human resources and skills acquisition. For IEF particularly, a PhD proposal is being developed to undertake a project with identification of Nigerian maize varieties so as to be able to develop reference protein markers for Nigerian maize seed industry.

Anticipated capacity building is in the aspect of enhancing research capabilities for IEF application to the numerous indigenous tropical African species, crops and commercial seed products. Till date very few of such species have protocols and reference markers for identifying varieties. Research potentials for IEF in Nigeria are therefore enormous.

The enormous research potentials for IEF cuts across different fields of biological applications, for example, IEF procedures can be used for several clinical applications like blood serum analysis. This undermines the need for extensive networking for IEF development in Nigeria. Networking can be in the area of service provision, comparative laboratory analysis of samples, research projects, co-supervision, staff training and exchanges.

CONCLUSION

In conclusion, this paper seeks to establish the potential role of IEF as a biochemical technique for easier, fast, efficient and sensitive evaluation of genetic purity of hybrid seeds. IEF should mitigate the problem of hybrid breakdown due to genetic impurities. Moreover, the use of modern techniques in plant breeding and the global availability of GM varieties together with the need for improved seed quality control will ensure that more advanced methods of seed purity assessment is made use of. No doubt capacity building in the IEF approach in Nigeria will enhance production of high quality hybrid seeds, and the ultimate benefit will be the promotion of seed standards to international quality and improvement of the Nigerian seed industry.

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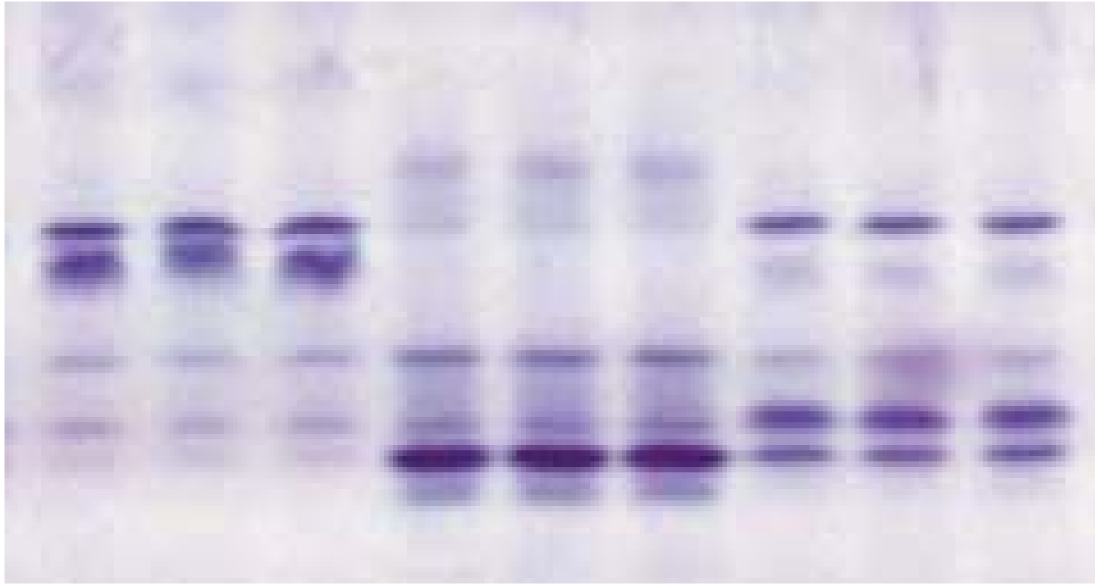

Fig. 1. IEF of maize seeds. From left to right side: female lines (first three lanes), male (second three lanes) and F1 generation seed (third three lanes).

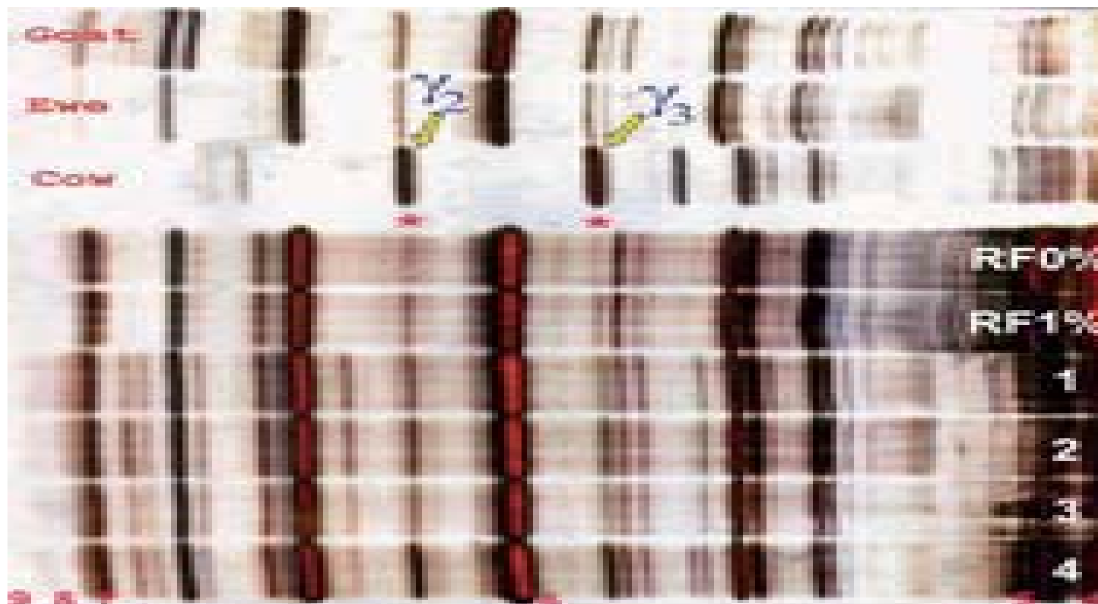

Fig. 2: Different ewes and goats milk cheeses (1,2,3,4) and European standard cheeses (RF 0%, RF 1%) separated by IEF and visualized by silver staining. Run on a ready-to-use gel (CleanGel Ultra, ETC GmbH) rehydrated in 3% high resolution ampholyte (5–7) and 7 M urea.

Detection of Low Genetic Variation in *Mansonia altissima* in Akure Forest Reserve, Nigeria

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ABSTRACT

Mansonia altissima (Sterculiaceae) is a timber tree species occurring naturally in lowland humid tropics of West Africa. A population genetic survey was conducted on the trees population in Akure Forest Reserve at Amplified Fragment Length Polymorphisms (AFLPs). With about 504 AFLP markers, the average Gene Diversity (H_e) of *M. altissima* in the Forest Reserve was 0.045 while Percentage Polymorphic Loci (PPL) and Band Richness (B_r) were 16.75 and 1.162 respectively. These results indicated that *M. altissima is* exceptionally low in genetic diversity within the study area and this suggests that genetic diversity of *M. altissima* is at risk in the forest reserve. This low level of genetic variation in *M. altissima* in this study area is in sharp contrast to results of AFLP analyses in other tropical trees, and since this is the first genetic study known to knowledge on *M. altissima*, the strikingly low genetic diversity prompted some logical questions which were addressed in this paper. These questions are; (1) Do laboratory errors prevent detection of genetic diversity? (2) Does the low genetic diversity linked with the mating system? (3) Is the genetic diversity is low as a consequence of its vegetative reproduction? (4) Is low genetic diversity is typical of *M. altissima*? Although further studies are needed to cover the entire range of the species, effective conservation and sustainable management programmes for the species in the forest reserve are urgent.

Keywords: AFLPs, *Mansonia altissima*, Genetic Diversity, Tropical rainforest.

INTRODUCTION

M. altissima (Sterculiaceae) occurs naturally in dry areas of lowland rainforests in West Africa, in Sierra Leone, Ivory Coast, Ghana, Benin, Nigeria and Cameroun (Keay, 1989). The tree can be up to 30 m in height and 80 cm in diameter at breast height (dbh). *M. altissima* is an hermaphrodite (Keay, 1989), and its winged and light seeds are easily dispersed by wind. Although there has been no documentation specifically on the species´ pollination biology, it is suspected to be pollinated by insects due to its fragrance flowers. Seeds of *M. altissima* are known to germinate in shade but their seedlings are light demanding (Veenendaal *et al.* 1996). The species has a high timber value because of its natural durability and good strength properties

of its wood. Currently, the remaining populations are affected by indiscriminate conversion of forests into agricultural lands and non-sustainable logging activities.

From literature search, there is no information on the level of genetic diversity in *M. altissima*. Successful strategies for the conservation of genetic diversity in species must include an understanding of the levels and distribution of genetic diversity (Hedrick, 2001; Zang *et al*. 2004). In the assessment of genetic variation both within and among plant populations, amplified fragment length polymorphism (AFLP) described originally by Vos *et al*. (1995) has proved to be reliable tool. The molecular marker technique can be used to analyze large numbers of loci distributed throughout the genome (Xue *et al*., 2005). The technique combines two strategies widely used for DNA fingerprinting: RFLP and the power of polymerase chain reaction. Due to the high multiplex ratio (number of polymorphic markers generated in a single PCR experiment), high reproducibility, and the small quantity and quality requirements for DNA (Jones *et al*., 1998; Pejic *et al*., 1998), AFLP has great potential to detect genetic variation in any part of the genome.

In this study, the goals are to test the utility of AFLP markers in assessing the level of genetic diversity in *M. altissima* and to estimate the level of genetic variation and genetic differences within and among populations of *M. altissima* throughout Akure Forest Reserve. This work will provide valuable information which can assist practical conservation initiative in tropical trees.

MATERIALS AND METHODS

Akure Forest Reserve

The study was carried out in Akure Forest Reserve located in Ondo State, South-western part of Nigeria. The Forest Reserve is a typical tropical moist rainforest. Adeduntan (2007) reported that the mean annual rainfall in the forest is about 4000 mm while the temperature ranges from about 20.6 ^{0}C to 33.5 ^{0}C annually. Akure Forest Reserve was selected for the study because its richness in Sterculiaecae (Akinnagbe, 2002)

Collection of Plant Materials

Leaf samples from 170 trees of *M. altissima* were collected from three natural populations and a plantation within Akure Forest Reserve, Nigeria. Information on the sampled plots is shown in Figure 1 while information on the geographic location, sample size, and density of sampled populations are summarised in Table 1.

Table 1: Geographic location and sample size of *M. altissima* populations

Population	Sample size	Latitude	Longitude	Altitude (m)	Plot size (tree/ha)	Density (trees/ha)
Primary Forest	44	7^0 12′12N	5^0 01′40E	282	2.8	18
Logged	43	7^0 16′27N	5^0 00′52E	296	5	12
Plantation	44	7^0 12′15N	5^0 01′40E	283	0.25	400
Isolated forest patch	39	7^0 13′47N	5^0 02′42E	281	5.5	6

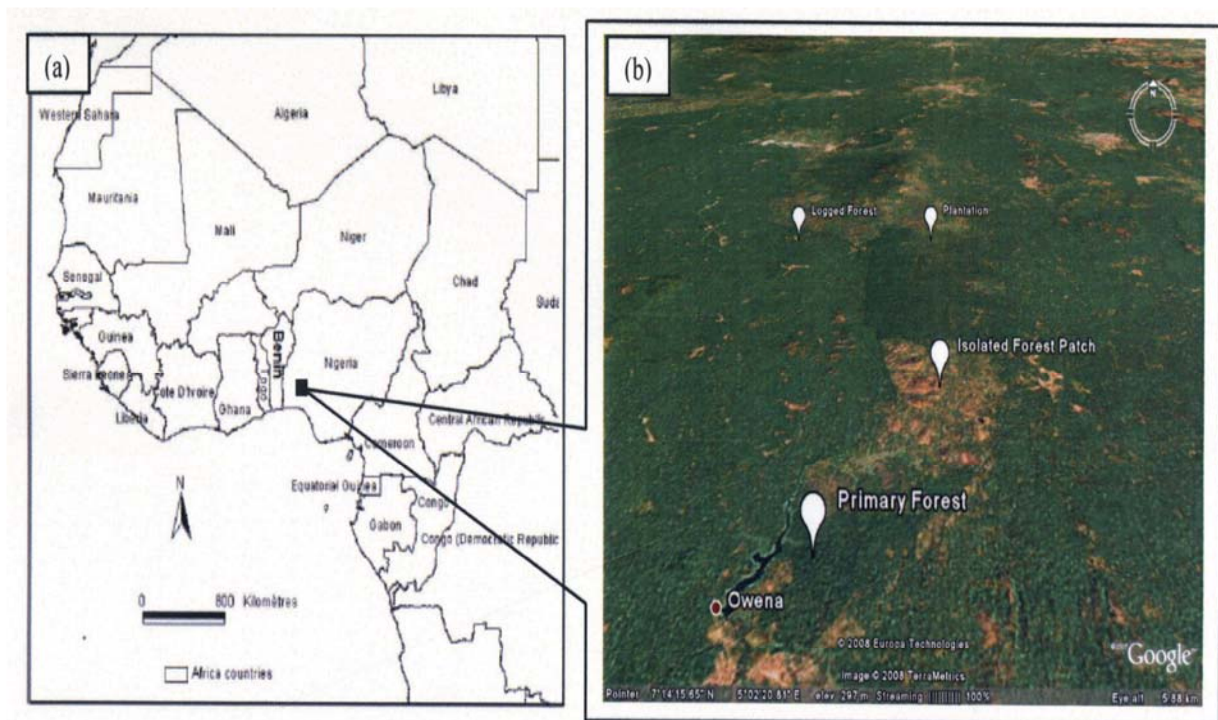

Fig 1: (a) Map of West Africa showing location of study area in Nigeria (b) Distribution of sampled plots in the study Area

DNA Isolation

In the isolating DNA from *M. altissima,* DNeasy® 96 Plant Kit protocol of QIAGEN (QIAGEN GmbH, Hilden, Germany) was followed. However, the DNeasy® 96 Plant Kit protocol was slightly modified as thus: (1.) Polyvinylpyrrolidone (PVP) was added to AP1 buffer to form a stock solution of 0.026 g/ml, (2.) a mixture of 100 ml AP1/PVP stock solution (for 2 x 96-plates) + 224 µl RNase and 224 µl DX was incubated at 65^0C in a water bath for 90 minutes. DNA quality and quantity was checked on a 0.8% agarose gel (at 100 V in Tris-acetate-ethylenediaminetetraacetic acid buffer) visualized by staining with ethidium bromide, and photographed in ultraviolet light. The extracted DNA was stored at −20°C.

AFLP Analyses

AFLP analyses were carried out according to the protocol of Vos *et al.* (1995). Total genomic DNA of each sample was digested simultaneously with the two restriction enzymes *Eco*RI and *Mse*I. Double-stranded *Eco*RI and *Mse*I adaptors were ligated to the ends of the restriction fragments to generate template DNA for polymerase chain reaction (PCR) amplification, which consists of two successive steps. In the pre-selective amplification, the restricted DNA fragments were amplified with the primer pair E01/M03 (each primer have one selective nucleotide).

The second step, which is the selective amplification, was performed with four primer pairs namely: E41/M63, E41/M64, E41/M72 and E35/M73 (each primer pair has three selective nucleotides). Nomenclature of the AFLP primers is according to the Keygene standard list of AFLP primers (http://wheat.pw.usda.gov/ggpages/keygeneAFLPs.html). For the purpose of visualisation during electrophoresis, primers E35 and E41 were labelled with the fluorescent dye 6-FAM. All PCR reactions were conducted in a Peltier Thermal Cycler (PTC-200 version 4.0, MJ Research). The amplified restriction products were resolved electrophoretically on an ABI Genetic Analyzer 3100 together with the internal size standard GeneScan 500 ROX (fluorescent dye ROX) from Applied Biosystems. AFLP fragments resolved by ABI Genetic Analyzer 3100 are shown in Fig 2.

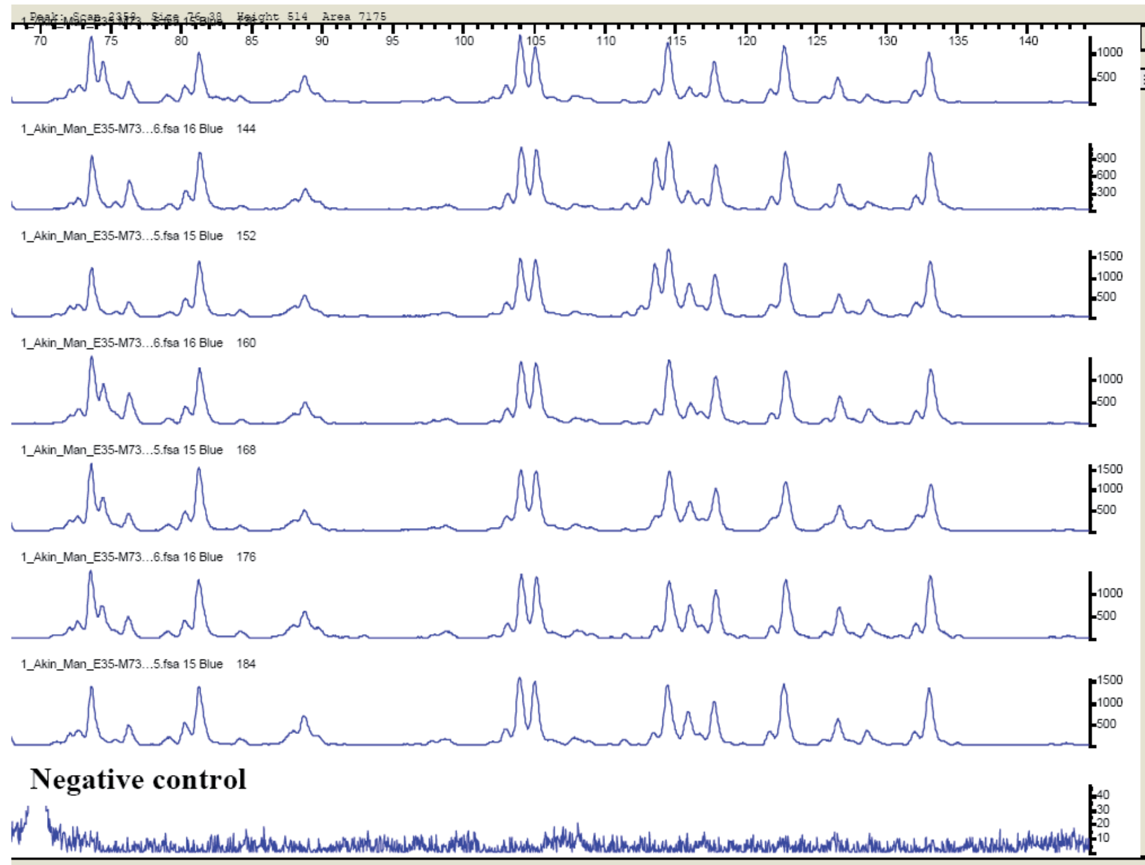

Fig 2: AFLP fragments of *M. altissima* as visualised by ABI Genetic Analyzer 3100

The size of the AFLP fragments was determined with the software packages GeneScan 3.7 and Genotyper 3.7 (Applied Biosystems). For a reproducibility test, the AFLP reactions from DNA restriction to selective PCR amplification were repeated for eight samples and two negative controls. Finally, a total of 504 fragments from the selective amplifications, with full reproducibility in the size range from 50 bp to 500 bp, were considered for further analyses.

Data Analyses

Each AFLP fragment was considered as a putative locus and assumed to be a dominant marker with two alleles. Fragments were manually scored for presence (1) or absence (0) in each sample. The binary data matrix generated from this scoring was then used for further analyses. Analyses of Molecular Variance (AMOVA) (Excoffier *et al.*, 1992) based on the pairwise squared Euclidean distances among molecular phenotypes was carried out using the program ARLEQUIN version. 3.01 (Excoffier *et al.,* 2006) to further partition the amount of genetic variation within and among populations. The significance levels of the AMOVA were evaluated using a permutation approach (1023 replications).

In addition, the software AFLP-SURV v.1.0 (Vekemans *et al.* 2002) was used to estimate allelic frequencies at AFLP loci applying the Bayesian approach with non-uniform prior distribution of allele frequencies (Zhivotovsky, 1999), which gives an unbiased estimate of allele frequencies from AFLP data (Zhivotovsky, 1999; Meudt and Clarke, 2007). These allelic frequencies were used as input for the computation of gene diversity (H_e) following the method described by Lynch and Milligan (1994), i.e. pruning of all loci with allele frequencies less than 0.05. The allelic frequencies obtained from AFLP-SURV v.1.0 were further used as input for the program AFLPDIV (Coart *et al.* 2005) to compute the percentage of polymorphic loci (PLP) and band richness (B_r) based on the rarefaction approach described by Petit *et al.* (1998) and Coart *et al.* (2005). In this approach, the rarefacted size is equalled to the lowest sample size in order to adjust for unequal sample sizes.

RESULTS

The AMOVA results presented in Table 2 indicate that the sampled populations are significantly differentiated from each other, but that about 96% of the total variation resided within the populations. The pairwise population F_{st} (Table 3) estimated as a follow-up to the AMOVA revealed that all the populations were significantly differentiated from each other with F_{st} ranging from 0.024 to 0.066. The lowest pairwise F_{st} values were recorded between Plantation and Logged Forest (0.024), and between Plantation and Isolated forest patch (0.025).

Table 2: Summary of Analysis of Molecular Variance (AMOVA) for AFLP phenotypes

Sources of variation	Df	SS	MS	Variance component	% Total	P value
Among Populations	3	23.880	7.96	0.111	3.9	< 0.001
Within Populations	185	504.247	2.726	2.726	96.1	< 0.001
Total	188	528.127				

Table 3:. Pairwise population differentiation (F_{st})

	Isolated Forest Patch	Logged Forest	Primary Forest	Plantation
Isolated Forest Patch	0.000	+	+	+
Logged Forest	0.028	0.000	+	+
Primary Forest	0.035	0.056	0.000	+
Plantation	0.025	0.024	0.066	0.000

Significance level: $p < 0.05$

Summary of the diversity estimates are presented in Table 4. The highest diversity estimates (PPL, B_r, H_j) were observed in the primary forest (H_e = 0.062; PPL = 21.00%; B_r = 1.204) while the lowest were observed in the isolated forest patch (H_e = 0.032; PPL = 09.00%; B_r = 1.089). PPL and H_e estimates in the isolated forest patch were almost twice as high as in the primary forest, logged forest and plantation.

Table 4: Genetic Diversity within populations of *M. altissima* in Akure forest reserve,

Population	Sample Size	PPL (%)	B_r	H_e
Primary Forest	44	21.00	1.204	0.062
Logged Forest	43	17.00	1.165	0.044
Plantation	44	20.00	1.190	0.042
Isolated Forest Patch	39	09.00	1.089	0.032
Mean		16.75	1.162	0.045

PPL = Percentage Polymorphic Loci; B_r = Band Richness; H_e = gene diversity; the program AFLP-SURV v1.0 was used to estimate H_e, while PPL and B_r were estimated using AFLPDIV

DISCUSSION

In this study, AFLP was used since sequence information on *M. altissima* was not available. The AFLP technique requires no sequence information prior to the generation of fingerprints (Cao *et. al,* 2006a), and can detect large numbers of polymorphic loci distributed throughout the genome (Powell *et al.* 1996). These attributes make AFLP technique useful in revealing genetic diversity within and among populations.

According to the analysis of molecular variance (AMOVA), the genetic diversity of *M. altissima* partitioned among populations was significant. In addition, most of the AFLP diversity (about 96%) resided within populations. This further affirms the fact that tropical trees tend to possess most of their genetic diversity within populations (Hamrick and Loveless 1989; Cao 2006). The pairwise differentiation among populations was low but significant, meaning that the populations used for the study were distinct populations. Such result is in accordance with the expectation of low diversity among populations in tropical trees (Loveless and Hamrick 1984).

The low level of genetic variation in *M. altissima* in this study is in sharp contrast to results of AFLP analysis in other tropical trees (see Table 5). Since this is the first genetic study known to knowledge on *M. altissima*, the strikingly low genetic diversity could prompt some logical questions like; (1) Do laboratory errors prevent detection of genetic diversity? (2) Does the low genetic diversity linked with the mating system? (3) Is the genetic diversity is low as a

consequence of its vegetative reproduction? (4) Is low genetic diversity is typical of *M. altissima*?

Do laboratory errors prevent detection of genetic diversity?

Anonymous markers like AFLP have been noted to be highly prone to contamination (Weising *et. al*, 2005) since they are universal markers. In this study, negative samples were used in order to ensure that the AFLP analysis is from contamination. It is expected that negative samples will produce no fragment, and in the case that negative fragment produces fragment pattern like other samples, it would mean that there is contamination. The negative sample in the study showed no fragment pattern (Figure 1), meaning that low genetic variation of *M. altissima* in this study is not as a result of laboratory contamination.

Does the low genetic diversity linked with the mating system?

Hamrick *et. al*, (1993) concluded in his study that woody species with outcrossing breeding systems are likely to have high genetic diversity. For *M. altissima*, it is still not clear whether the extremely low genetic diversity is closely linked to its mating system since there is presently no documented information on the nature of its mating system, although other tree species in the Sterculiaceae family like *Triplochiton Scleroxylon* (FRIN, 1977), *Theobroma cacao* (Cope, 1962; Sereno *et al.*2006), *Cola nitida* (Jacob 1973), *Sterculia chicha* (Taroda and Gibbs, 1982), and *Dombeya* spp. (Humeau *et. al.,* 1999) are all self-incompatible.

Is the genetic diversity is low as a consequence of its vegetative reproduction?

Contact of living branches or even whole stems of some trees with soil or mouldering logs leads to the growth of adventitious roots (Koop, 2004). This leads to clonal spreading of such trees. In the case of *M. altissima*, reproduction is only by seed (Adeyelu, pers. Com.) meaning that cloning in natural habitat is difficult. Therefore, the low diversity of *M. altissima* in this study cannot be associated with vegetative reproduction.

Is low genetic diversity is typical of M. altissima?

Similar to the result of this study, an extraordinarily low level of genetic diversity based on RAPD markers (Mosseler et al. 1992), and RAPD and ISSR markers (Zhang et al. 2005) have also been reported for *Pinus resinosa*, and *P. squamata*, respectively. For *P. resinosa*, historical genetic bottleneck during glacial episodes of the Holocene, fragmentation and selfing were given as reasons for the extremely low polymorphism in the species. In the case of *P. squamata*, it was proposed that strong bottlenecks experienced by the species during its long evolutionary history coupled with genetic drift and inbreeding in post-bottlenecked small populations, and human activities such as logging may have been the forces that contributed to its low genetic diversity.

In the case of *M. altissima*, it is difficult to assert that low genetic diversity is typical of *M. altissima* since the sampling did not cover the entire range of the species. However, results suggest that *M. altissima* might have a history of severe population bottlenecks in the study area. Levels of genetic diversities were higher in the primary forest when compared with other populations where there have been human impacts. In particular, the genetic diversity in the isolated forest patch was about half of the diversity in the primary forest (see Table 2). Further

investigations are still required to verify whether low genetic diversity is typical of the species or just limited to the study area.

CONCLUSION

This study has further affirmed the utility of AFLP in detecting the level of genetic variation within tree populations. In general, result of this study shows that genetic diversity of *M. altissima* in the study area is low. Although additional studies are needed to confirm whether the low level of genetic diversity is found across the natural range of the species or whether it is only limited to the study area, the low genetic diversity of *M. altissima* raises serious concern because the local department of forestry had already embarked on establishing *M. altissima* plantation using seeds collected from the forest reserve. Therefore, effective conservation and sustainable management programmes for the species are urgent. The scale of human impact in the study area could pose a serious threat to the future viability of the species if unchecked.

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Table 5: Genetic Diversity of some Tropical Tree Species based on AFLP Markers

Species	Sample Source	Ecozone	Pollinator	No. of primer pair	No. of Loci	PPL (%)	H_e	Sources
M. altissima	Akure FR, Nigeria	Afrotropic	Insect	4	504	16.75	0.045	Present Study
T. scleroxylon	Akure FR, Nigeria	Afrotropic	Insect	1	134	63.05	0.267	Akinnagbe (article in preparation)
Shorea leprosula	Indonesia	Indomalaya	Insect	1	56	53.32	0.161	Cao *et al.,* (2006b)
Shorea parvifolia	Indonesia	Indomalaya	Insect	1	56	51.79	0.138	Cao *et al.,* (2006b)
Araucaria. angustifolia	Brazil	Neotropical	Wind	1	166	77.80	0.270	Stefenon *et al.,* (2007)
Cordia africana	Ethiopia	Afrotropic	Insect	1	90	85.70	0.287	Derero (2007)
Hagenia abyssinica	Ethiopia	Afrotropic	Wind	1	109	92.70	0.200	Ayele (article in preparation)
Cedrela odorata	Peru	Neotropical	Insect	3	258	98.8	0.170	de la Torre *et al.*, (2008)

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Isolation and partial characterisation of root specific promoters for stacking multiple traits into cassava (*Manihot esculenta* CRANTZ)

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ABSTRACT

Cassava is capable of cultivation on impoverished soils with minimum inputs and its storage roots are a staple food for millions in Africa. However, these roots are low in bioavailable nutrients, low in protein content, contain cyanogenic glycosides, suffer from a very short post-harvest shelf-life and the plant is susceptible to viral and bacterial diseases prevalent in Africa. The demand for the improvement of cassava with respect to these traits comes from both farmers and national agricultural institutions. Genetic improvement of cassava cultivars by molecular biology techniques requires the availability of appropriate genes, a system to introduce these genes into cassava and the use of suitable gene promoters, the molecular switches that control the expression and tissue of gene activity in plants. This project involved the isolation of cassava gene promoters for targeted expression of genes in cassava. In silico analysis of the promoters sequences revealed putative cis-acting regulatory elements, including root-specific elements that may be required for its expression in vascular tissues. Research on the activities of these promoters is continuing with the development of plant expression cassettes for transformation into major African elite lines and farmers' preferred cassava cultivars to enable testing of tissue-specific expression patterns in planta. After testing, the promoters will be available for use by laboratories involved in the cassava improvement programme, including the Bio-Cassava Plus Community.

Key words: cassava, promoters, gene-walking, gene-expression

INTRODUCTION

The starchy storage roots of cassava provide the staple food for many millions of people in sub-Saharan Africa. In addition, they play a vital role as a famine reserve crop and are becoming increasingly important for processing into higher value products (Montagnac *et al.* 2009). Cassava is capable of cultivation on impoverished soils with minimum inputs and therefore an excellent staple food crop for small hold farmers (Cock 1985). However, it is far from being an ideal food and crop as its roots are low in bioavailable nutrients, low in protein content, contain cyanogenic glycosides, suffer from a very short postharvest shelf-life and the plant is susceptible to viral and bacterial diseases prevalent in Africa (Cock 1985, Puonti-Kaerlas 1998, Beeching *et al.* 1998, Restrepo *et al.* 2004, Verdier *et al.* 2004). The demand for the improvement of cassava with respect to these traits comes from both farmers and national agricultural institutions and is

articulated through international organisations such as the Cassava Biotechnology Network, IITA (International Institute of Tropical Agriculture) and CIAT (Centro Internacional de Agricultura Tropical), together with national institutes, such as KARI (Kenya Agriculture Research Institute), Kenya; NRCRI (National Root Crop Research Institute), Nigeria and NARO (National Agriculture Research Organisation), Uganda.

The ideal would be to identify and produce solutions to these various problems and to introduce several or most of these improved traits into the major African elite lines and farmers' preferred cultivars. One possible solution is the use of breeding to introduce these traits. However, this is largely not feasible due to the crop's clonal propagation, high heterozygosity and poor flowering ability (El-Sharkawy 2003). Genetic manipulation through molecular biology techniques is an alternative means to introduce these traits. This approach has been adopted in other crop plants with success and offers opportunities for African crops (Thomson 2008). The pyramiding of multiple traits into the major African elite lines and farmers of preferred cassava cultivars can be divided into two aspects: firstly, how these multiple traits can be readily introduced into a range of different cassava cultivars and, secondly, how to ensure that all the introduced transgenes express appropriately without interference. It is the second aspect that forms the problem tackled by this project. There is a critical need for root-specific promoters with a range of developmental and tissue specificities within the cassava storage root. Without access to such a tool-kit the transition from research to the release of cassava varieties improved for multiple traits will be delayed. It would be ideal to consider root-specific promoters from cassava, but, unfortunately, these are currently largely unknown and uncharacterised. The objective of this project was to isolate and characterise suitable gene promoters from cassava which could be used to target gene expression in this important food crop.

MATERIALS AND METHODS

Plant material and DNA isolation

Cassava plants (cultivar CM 2177-2) were grown in the tropical glasshouse at the University of Bath at 22-28° C, relative humidity of 40-80 % and a minimum light period of 12 hours per day under daylight, supplemented with 400 W Phillips highpressure sodium lights when necessary. High quality genomic DNA was extracted from young leaf samples of this cassava cultivar by the method of Dellaporta *et al.* (1983). Restriction enzyme digestion and adaptor ligation/ gene walker libraries construction Separate aliquots (100 ng) of cassava DNA were completely digested with four different restriction enzymes that leave blunt ends viz Dra I, Eco RV, Stu I and Pvu II in a total volume of 100 µl. Human genomic DNA aliquot was also digested with Pvu II to serve as positive control. Each batch of digested genomic DNA is purified and then ligated separately to the GenomeWalker^{TM} adaptor supplied with GenomeWalkerTM kit (Clontech Laboratories, Inc). The digestion and ligation of the DNA were carried out under the conditions specified by the kit manufacturer. The ligation mixtures were then diluted 10 folds. Each diluted adaptor ligated restriction enzyme digested genomic DNA constitutes a gene walker library.

Gene specific primers design and procurement

Sequences of the cDNA for the following cassava genes were mined from NCBI database: Late embryogenesis abundant protein gene, LEA (accession number DT 883604), Auxin repressed-like protein gene, ARP (accession number DB 924059) and Manihot esculenta allergenic-like protein gene, MEA (accession number EC 591287). Gene specific primers were designed from the cDNA sequences using the program PRIMERW. The primers were procured from Invitrogen, UK.

PCR based gene walking technique

Two set of PCR cycles, primary and secondary PCRs, were carried out in sequence. In the primary PCR, 1 μl of each of the libraries was amplified in a PTC 100 Programmable Thermal Controller (MJ Research Inc) using a set of gene specific primer (GSP1) and adaptor specific primer (AP1). The latter was supplied with the kit along with Advantage 2 polymerase enzyme. In the secondary or nested PCR, the product of the primary PCR (1 μl of 50 folds dilution) were used as templates using nested gene specific primer (GSP2) and adaptor specific primer (AP2).

Cloning procedures Amplified gene promoters were purified (Qiagen), ligated into pGEMR-T Easy vector (Promega) and used to transform competent *Escherichia coli* DH5α according to standard procedures (Sambrook *et al.* 1989).

DNA Sequencing and sequence analyses

PCR products or recombinant plasmid DNA molecules were sequenced on an ABI 337 automated dye primer sequencer using the GSP2, AP2 or universal primers for the cloning vector. Initial confirmation of sequence identity was by using BLASTN and TBLASTX searches against the GenBank non-redundant database using the default parameters (Altschul *et al.* 1997). Plant cis-acting regulatory elements (PLACE) database was used to determine plant cis-acting regulatory elements (Higo *et al.* 1999). Sequence data of DNA fragments were assembled with the program VECTOR NTI. Where necessary, internal primers were designed and used for further sequencing of a fragment.

RESULTS AND DISCUSSION

PCR amplified cassava promoters regions (DNA) Cassava gene promoters regions were amplified in the primary and secondary PCRs (as described in the materials and methods). Figure 1 represents amplification of ARP promoter region. The primary PCR produced many DNA bands which were reduced to few or even single band in the secondary PCR. In this way, gene specific amplifications were favoured. Amplified fragments in the size range (1.5- 2.0 kb) were sequenced. Sometimes, smaller fragments were also sequenced for comparison with the sequence from larger fragments in multiple sequence alignments. Figure 2 shows the ARP gene fragments sequence assembly.

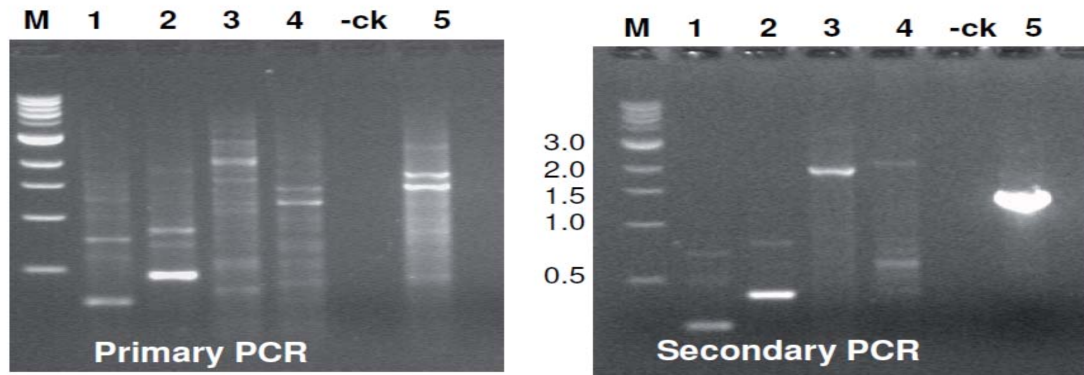

Figure 1: PCR amplification of cassava DNA (promoter regions) for Auxin repressed-like protein gene. **M** = 1 kb Marker, **1** = *Dra* I, **2** = *Eco* RV, **3** = *Stu* I, **4** = *Pvu* II and **5** = human/*Pvu* II libraries. While **–ck** = negative control with no DNA template

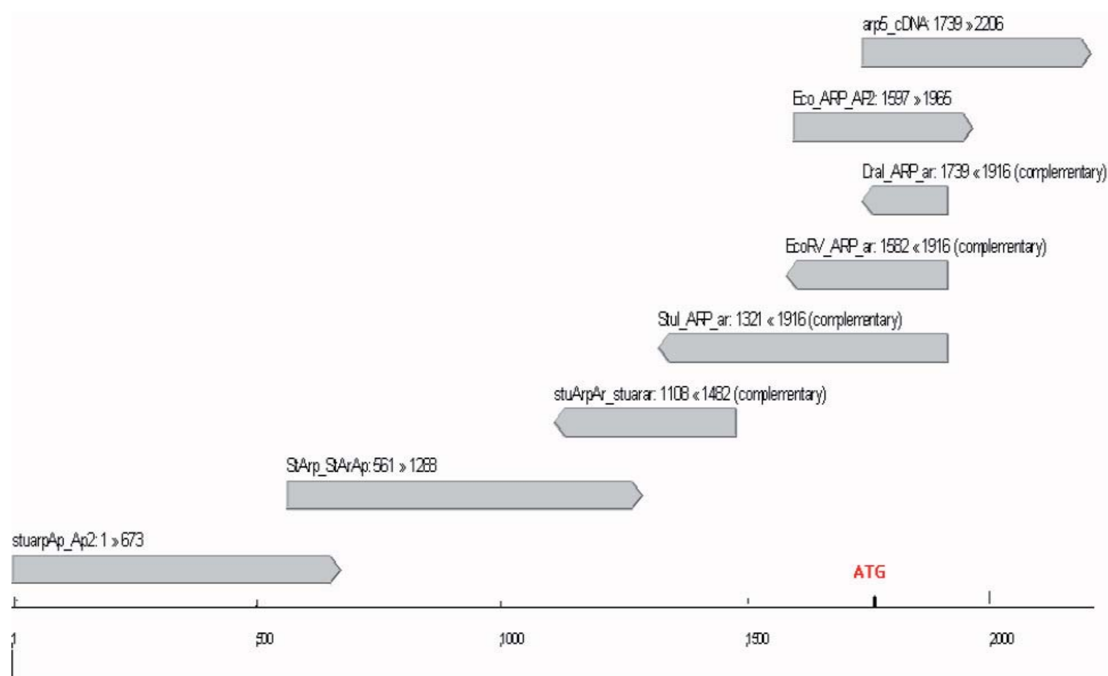

Figure 2: Scheme for the assembly of DNA sequence for Auxin repressed-like protein gene. Gene sequence fragments are represented by arrowed head bar showing the 5 →3 DNA fragment sequence direction. The name of a sequence fragment is a combination of the restriction enzyme for the gene walker library and the sequencing primer. Eco = *Eco* RV; Stu/St = *Stu* I. ATG is the initiation codon and ARP = Auxin repressed-like protein gene

Sequence analysis of the promoter region

The sequences of the amplified promoter regions were, in the first instance, screened for introns by comparison with the cDNA. Exon sequences are subjected to BLAST and TBLASTX searches to confirm identity with the original source gene cDNA and to identify amino acid sequence motifs. Figure 3 show the translation of sequences downstream of the initiation codon, ATG, and the promoter sequence of Auxin repressedlike protein gene (ARP). Amino acids sequence motifs typical of ARP are identified. Sequence analysis of the promoters showed that a TATA box (TATAA) is located 83 bp upstream the initiation codon ATG (Fig 3). De Souza *et al*. (2009) reported cassava *Mec1* promoter TATA box to be located 103 bp upstream from the initiator, ATG.

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AGGACACTTTCGGCGGCAGGTTCGGCGGCCGAAAGTCCCTCCAGAGCCGAAAGTCAGGC
AGGTTCGGCGGCACCTTCGGCGGCCGAAACTCCCAGACAGAGACGAAACTCATGCATGT
TCGGCGGCACCTTCGGCGGCCGAAACTGCCAGACAGAGACGAAAGTCTCCTTTCGGGGG
CAAGTTTCGGCAGCCGAAGGGCTGCCTCCCCAGCCATGTTCGGCGGCCGAAAGTTCCTT
CGGCTGCCGAACCTGGTTTCTGCCAAAGGGCAGAAACTTGGCTCCCAATGCACATTTCG
CCTCCAAACTTATCAAACATGCATCAAACCTATTCTACAACACACAAACGCAAGCATAC
ATGTTCCTAGGGGTCTCAAACCATCATAAACCCCATCTACAACACATCAAGCATCCACA
TTGTTCAAGAACACACATTATACCCATAAACACAACCATAACCTAAACATGCATTCTAA
CTCATAGATCTTGCATAAAACTTATTCAAAACATAAAACGAGCTTAAGATCGGCTCTTA
CCTCTTGAAGATCGAGAGAGACGACCCAAAAACTCGGAGTTGGGAGAGATTTGGTTCTT
GAACCTCCAAGCTCCAAAACTTTGCTCAAAAGCTTAAATCTTCAAAACCAAGTTAAAAC
AAGTGAAAATCTTGAAAGATTTAGAGGAAGAACATCAAAAATGGGTGAGGACGGCGGA
GAGCTCACCTGGGCCGAAAATGGGGAAAAGCTCGCCCGTTTTCGGCTAAGGGACCCTTT
TATAGTGGCTGGCCAGACCACGTTCGGGGGCCGAATGTGTCTCCGCATGCATGCCATGT
TCGGCGGCCGAACCTGGACTTCCCTCACTTATGCTTTCGGGGGCCTAAAGCACACCCGC
AACGCATGCATGTTCGGCGGCCGAACTTGAGGTTCGACGGCCGAACCTGAGTTTTCCTC
CAATGCTATTTTCATGCAAAAACTCATTTTCTTTCATGCTTAAAACATAAAACACATTA
AAATATTTCATAAAAACATGGTTTTACCCTACTAGAGACTTCCGACATCCGAGATTCCA
CCGGATGGTAGGAATTTCGATACCGGAGTCTAGCCGGGTATTACACTCATATAATAGAA
TTATCATCATTACACGAAATTTCTAGAAGAAAATATGCAATTCCAAAATCTTTACTGAA
ATTGAAAGAGTTTGCTGTCATTTTAGAGACCATGCATAACCCAATTAGTAGGAAGCTTT
TACGTTGCTCCCTTCGATTTTTTTTTTCTGCAGATATTTTCTGCAGCCAACTTACCAGC
CACTCAAACTAGGACTACCTAAGTAATATGTATTATTATGCTAAAATAACATCCTAGTA
CAAAATATTTCATATTTAAACATTGTTTAAAAATAAATATTTGCAATTCTTATCCTGTT
TTATAAGAAATGAAATATAAATTCTCATAGATATTGGATTTGCCACGTGGAACACCCAG
ACTGGCTATGGGGCAATAAATGAATCTAAGAACTTGGTGGTCCGAAAGGTGACGCGTAA
GATAGCCGACAAAAATCTTGAGCCTCCAATGAAATGAGAGTTCTAGATATCGAGAGATA
AGAAGCTTTGTGAAATCCTGGACGTGGGATTTTCAGCAAGATAAGAAAATCTACACTTA
ACTCATTATAAATGCCCACCAAATCCACTTCTCTAATTCACCACTTGAATCAAACAACA
TACAGAGGATTCAAACCCACACTCCTTTAAACA
1745 ATGgcagcctcaacaatggccctttcctccccttctcttgccggc
     M  A  A  S  T  M  A  L  S  S  P  S  L  A  G
1790 aaggcagtgaagctcacccccctctgcccctgagctcatgggcaat
     K  A  V  K  L  T  P  S  A  P  E  L  M  G  N
1835 ggccgtttttcaatgaggaaaactgccagcaaggctgtttcctct
     G  R  F  S  M  R  K  T  A  S  K  A  V  S  S
1880 ggaagcccatggtacggtccagaccgtgttaagtacttgggtcca
     G  S  P  W  Y  G  P  D  R  V  K  Y  L  G  P
1925 ttctctggtgagcccccatcctacttgactggcgaattccc 1965
     F  S  G  E  P  P  S  Y  L  T  G  E  F
```

Figure 3: Auxin repressed-like protein gene promoter region DNA sequence translation/analysis. Typical ARP protein amino acid sequence motifs are shown. The initiation codon (ATG) and TATAA sequence typical of eukaryotic promoter are shown in bold and underlined.

Promoter isolation and cloning

Primers were designed from the consensus sequence derived from the gene walking PCRs to amplify about 1.5 kb of the promoter sequence upstream the initiation codon, ATG (see Fig 3). Fig 4 shows the gel picture of amplified promoter for ARP. Restriction enzyme sites (*Bam* HI and *Nco* I) were incorporated into the primers to facilitate subsequent cloning in the plasmids.

The amplified promoters for LEA, MEA and ARP genes were then cloned in pGEM®-T Easy vector. Plasmids were isolated from bacterial culture and restriction enzymes digested to size the insert. Fig 5) and sequenced to confirm identity with the original sequence. The insert released from the restriction enzymes digestion of the pGEM-Teasy

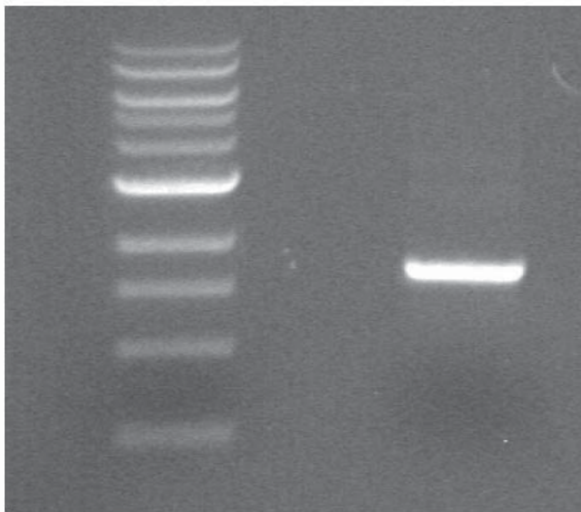

Figure 4: PCR amplification of cassava ARP promoter. The primers were designed to amplify just the promoter (ATG inclusive). The marker shown is NEB 1kb ladder (sizes details in Figure 1). About 1.5 kb size promoter was isolated.

The amplified promoters for LEA, MEA and ARP genes were then cloned in pGEM®-T Easy vector. Plasmids were isolated from bacterial culture and restriction enzymes digested to size the insert. Fig 5) and sequenced to confirm identity with the original sequence. The insert released from the restriction enzymes digestion of the pGEM-Teasy. Recombinant plasmids were cloned into pCAMBIA vector ready for transformation into cassava plants for tissue expression studies.

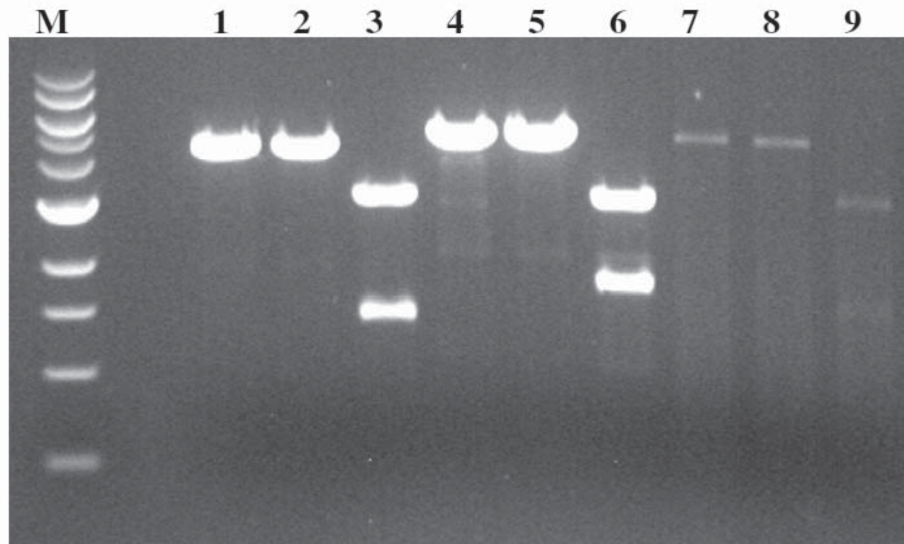

Figure 5: Restriction enzymes digestion of recombinant pGEM-Teasy plasmids with the cassava gene promoter inserts. **M** = 1kb ladder (sizes as shown in Figure 1); **1** = LEA/ *Pst* I, **2** = LEA/ *Nco* I, **3** = LEA/ *Pst* I & *Nco* I, **4** = MEA/*Bam* HI, **5** = MEA/ *Nco* I, **6** = MEA/ *Bam* HI & *Nco* I, **7** = ARP/*Bam* HI, **8** = ARP/ *Nco* I, **9** = ARP/*Bam* HI & *Nco* I

Analysis of the gene promoters' sequences with plant cis-acting regulatory elements (PLACE) identified important promoter motifs.

Using the program PLACE, many known regulatory elements were identified in the ARP promoter sequence such as light responsive elements, ABA-responsive element, stress responsive element, jasmonate- and elicitor-responsive elements, ACGT motif related to root expression and root specific elements among others (the motifs identified in ARP promoter are listed on Table I). Some of these regulatory elements have also been found in cassava *Mec1* promoter (de Souza *et al*. 2009) and *C54* promoter sequence (Zhang *et al*. 2003).

Table I: Putative *cis* regulatory motifs identified in cassava ARP promoter sequence by *in silico* analysis using plant cis-acting regulatory elements (PLACE).

Name	Function/Similar to	Sequence	Identity (%)
EIN3	Ethylene-insensitive 3 binding site	GGATTTGGTGGGCATTTATAATGAGTTA	76.9
GREGION	"G region" found in tobacco (N.t.) PRB	CAAAGCTTCTTATCTCTCGATATC	73.7
RSE	"RSE (root-specific element)" found in bean	GTTCCACGTGGCAAATCCAATATCTA	60.9
AGTA	"AGTA repeat" in pumpkin (C.s.) ascorbate oxidase	AAAAAATCGAAGGGAGCAACGTAAAAGC	69.2
SUREA	"SURE-a"; Sugar-responsive element	AAAACCAAGTTAAAACAAGTGAAAA	68.0
IDE2	IDE2 (iron-deficiency-responsive element 2	CTGAAATTGAAAGAGTTTGCTGTCATT	53.8
JERE	"JERE" (jasmonate- and elicitor-responsive element	TTCTTATCCTGTTTTATAAGAAAT	60.9
BOXC'PSAS1	Box C' in pea asparagine synthetase	TCCCAATGCACATTTCGC	70.6
IDE1	IDE1 (iron-deficiency-responsive element 1)	CGCAAGCATACATGTTCC	70.6
GLUTEBOX1	"Box 1" of rice (O.s.) glutelin Gt3 gen	TATCTTACGCGTCACCTTT	70.6

OCSENHAN	"OCS enhancer element" in octopine synthase	TAGGAAGCTTTTACGT	78.6
WAR	"WAR (wounding activating region)" in Brassica	TTTATGGGTATAATGTGTGT	55.0
OCSGMGH24	"OCS element" found in the soybean (G.m) GH2	AGGTTTGATGCATGTTTGATAAGT	55.0
PE1	PE1 Positively acting element	AGAATTGCAAATATTTATTTTT	68.4
REGION1	"region 1" ABRE-like sequence found in rice	CGGCGGCCGAACCTGG	68.8
AS1	"as-1 (activation sequence 1)" in CaMV 35S promoter	TGGTGGTCCGAAAGG-TGACGCGTAAGAT	60.9
GLUTEBP2	"Glutelin BP-2"; Binding with nuclear factor	TTGCTCAAAAGCT-TAAAT	77.8
BOX2	Box 2 of bean (P.v.) chs15 promoter; SBF-1	TTTTTATGAAATAT	76.9
LREBOXII	"BoxII"; Light responsive element (LRE)	TCCACGTGGC	100.0
BOXI	"Box I" found in the tobacco (N.t.) plastid	CATTCTAACTCATAGATCTTG	63.2
ABF	ABF (as-1-like box binding factor) binding site	AAATCTTTACTGAAATTGA	83.3
ALF2	"ALF-2 (as-1-like sequence binding factor 2	GGAGGCGAAATGTGCATT	61.1
LREBOX3	"LRE (light-responsive element) Box III	AAGATTTTCACTTGT	83.3
TEF1BOX	"tef1 box" found in the *Arabidopsis*	TATGAGTTAGAATGCATGTTTAGG	57.9
23BPZM	23 bp sequence found in the maize (Z.M)	AGCAAGATAAGAAAATCTACA	68.4
23BPUAS	"23 bp UAS (Upstream activating sequence)	TTTAGAGACCATGCATAACCCAA	55.0
AT1BOX	"AT-1 box (AT-rich element)" found in the promoter	AATATTTTAATG	83.3
SARE	Salicylic acid responsive element found in CaMV	CCCACGTCCAGGATTTCACAA	60.0
GLUTEBOX1	"Box 1" of rice (O.s.) glutelin Gt2 gene	CATGCATGAGTTTCGTCT	71.4
HSRE	"HSRE (HSR203 responsive element)" in tobacco	CAAAGTTTTGGA	83.3
TDBA12	TDBA12 binding site found in tobacco	TGACTTTCGGCT	90.0
ANAEROBIC CIS	20 bp anaerobic cis-regulatory sequence in maize	CGAAGGGAGCAACGTAAAAG	65.0

BOXICHS	"Box I consensus sequence in the promoters	ATGCATCAAACCTATT	76.9
COREOS	CORE (coordinate regulatory element for antioxidant)	AATATTTCATATTTAAACATT GTTTAAA	53.6
LEGUMINB OX	"legumin box" in legA 5' legumin gene	TCCCCAGCCATGTTCGGCGGC CGAAAGTT	60.7
DR5GMGH3	"DR5"; A highly active synthetic auxin responsive element	ACTTTCGTCTC	90.0
ABRE	ABA-responsive element	TCCACTTCTC	90.0
GAGA	"GAGA element" found in the promoter	AAGATCGAGAGAGACGAC	84.6
GAGA8	"GA octodinucleotide repeat"	AAGATCGAGAGAGACG	84.6
TCA1MOTI F	TCA-1 (tobacco nuclear protein 1) binding site	TCATTTTCTTT	90.0
TEFBOX	"tef-box" found in the Arabidopsis	GGGGGCCGAATGTGTCT	69.2
C1GMAUX2 8	"C1"; DNase I protected sequence	TAAAAACATGGTTTTA	68.8
SARE	Salicylic acid responsive element found in CaMV	TGGACGTGGGATTTTCAGCAA	52.6
WAR	"WAR (wounding activating region)" in Brassica	TCCAAACTTATCAAACATGC	83.3
JERE	"JERE" (jasmonate- and elicitor-responsive element	GAATTAGAGAAGTGGATTTG GTGG	47.6
ACGTROOT 1	"ACGT motif" related to root expression	TCCACGTGGC	90.0
SREN	Stress responsive element (SRE) in tobacco	TGGTAAGTTGGCT	69.2
TRANSTAR T	Plant consensus sequence for translation start	TAAACAATGTTT	83.3
ACGTROOT 1	"ACGT motif" related to root expression	GCCACGTGGA	100.0
ABREA	ABA-responsive element (ABRE A)	GCCACGTGGA	100.0
GLUTEBP2	"Glutelin BP-2"; Binding with nuclear factor	AGGCTCAAGATTTTTGTCG	66.7

The table shows the percentage identity of the cassava motifs/elements with others in the database.

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Plasma antioxidants and oxidative metabolic changes in pulmonary tuberculosis patients on chemotherapy with or without micronutrient supplementation

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ABSTRACT

Oxidative burst is the major cause of pathophysiologic changes in pulmonary tuberculosis (PTB). Significantly lower levels of antioxidants in PTB-patients have been associated with micronutrient deficiency. The present study determined the changes in levels of total antioxidant activity (TAA), total plasma peroxide (TPP), oxidative stress index (OSI), malondialdehyde (MDA), superoxide dismutase (SOD), reduced glutathione (GSH) and catalase (CAT) in PTB-patients on chemotherapy supplemented with (C+M) or without (C-M) micronutrient. Thirty-eight newly diagnosed PTB patients attending Chest Clinic, Oniyanrin, Ibadan, Nigeria were recruited for this study. Twenty of the PTB patients were treated with micronutrients and anti-tuberculosis drugs (C+M) while another eighteen PTB patients were treated with only anti-tuberculosis drugs (C-M) for a period of 4 weeks. The baseline values of each of these groups (C+M and C-M) served as control for the corresponding group. In C+M, values of MDA, TPP and OSI decreased significantly ($p < 0.05$) but the levels of TAA, SOD and CAT were significantly raised ($p<0.05$) at 2 week of treatment, while significantly increased level of GSH was observed at 4 week of treatment ($p=0.02$) when compared with the baseline values. In C-M, only MDA was significantly reduced ($p=0.02$) while significantly higher values of GSH and SOD were observed at 4 week ($p=0.00$ and $p=0.00$ respectively) of treatment when compared with the baseline values. Micronutrient supplementation enhanced increase in antioxidant activities and decrease in oxidative stress indices within 2 weeks of treatment in C+M. This is an indication that supplementation of anti-tuberculosis drugs with micronutrients should be considered in the management of PTB patients.

INTRODUCTION

During pulmonary inflammation, increased amounts of reactive oxygen species (ROS) and reactive nitrogen intermediated (RNI) are produced as a consequence of phagocyte respiratory burst (Kwiatkowska et al, 1999). Increased plasma levels of these free radicals cause an imbalance between the ROS / RNI and the antioxidant activities in favour of the former (Wiid et al, 2004). The effects of free radicals on protein molecules, such as hormones and enzymes are fragmentation, cross-linking, aggregation and denaturation. The free radical attack causes peroxidation of polyunsaturated fatty acids, oxidization of methionine, and deamination / nitration of the DNA (Ramanujam, 2004).

Vitamins A, C and E are scavengers of ROS, and prevent peroxidation of bio-membrane lipids. Vitamin E removes free radical intermediates to prevent generation of more free radicals. Antioxidant enzymes ultimately remove ROS from the system (Harma et al, 2003). The

synthesis and catalytic activities of major antioxidant enzymes are trace metal dependent (Bolukbas et al, 2005). Copper, zinc, manganese and iron are an integral part of superoxide dismutase (SOD). Iron is an integral part of catalase, while selenium (Se) is an integral part of Se-GPX (glutathione peroxidase). SOD catalyses the conversion of toxic superoxide anion to less toxic hydrogen peroxide which is there after converted to water and oxygen in two separate reaction catalyzed by GPX and catalase. Elevated values of ROS, MDA and OSI with reduced TAP have been established in PTB-patients by several workers (Kwiatkowska et al, 1999; Ramanujam, 2004). But none of these studies determined the post treatment levels of these parameters in PTB patients.

The present study was designed to bridge this gap by investigating the effect of micronutrients supplementation on the levels of antioxidants and markers of oxidative stress in Nigerian PTB patients on chemotherapy.

MATERIALS AND METHODS

Subjects

Thirty eight newly diagnosed PTB patients volunteered to participate in this study. All PTB patients were sputum smear, Acid Fast Bacilli (AFB) positive with radiological examination showing pulmonary inflammation. The PTB patients were negative to HIV-antibodies. Twenty of these PTB-patients were treated with micronutrient supplement and anti-TB chemotherapy (C+M) while eighteen PTB-patients were treated with only anti-TB drug (C-M). The multivitamin supplement contained vitamin A (1600 i.u), vitamin E (5mg) vitamin C (100mg), Vitamin D (100i.u), Fe (5mg), Zn (0.5mg), Mg (3mg), Se (70 mcg), calcium (75mg), phosphorus(58mg), molybdenum (0.1mg), cholin (3mg), biotin(3mcg), vitamin B1 (1.0mg) vitamin B2 (1.0mg) vitamin B6 (0.5mg) vitamin B12 (0.5mg) once per day for a period of four weeks.

Five (5) milliliters of blood was withdrawn from each patient into a lithium heparin bottle on three occasions; (1) the day of recruitment of PTB patient (baseline / pre-treatment evaluation), (2) after two weeks and (3) after four weeks of treatment (post treatment evaluations). 0.5 ml of the heparinized blood for erythrocyte SOD assay was washed in physiological saline three times, diluted up to 2.0ml with redistilled water and stored at -20^{0}C until analyzed. The remaining blood samples for CAT, GSH, TAA, TPP and MDA assay were separately centrifuged at each collection and the plasma stored at -20^{0}C until analyzed.

Methods

Estimation of TAA:

TAA was estimated with the method of Koracevic et al (2001). A standardized solution of Fe-EDTA complex reacts with hydrogen peroxide by a Fenton-type reaction, leading to the formation of hydroxyl radicals (OH). These reactive oxygen species degrade benzoate, resulting in the release of thiobarbituric acid reacting substance (TBARS). Antioxidants from the added sample of human fluid cause suppression of the production of TBARS. This reaction can be

measured spectrophotometrically and the inhibition of colour development defined as the antioxidant activity.

Estimation of S0D:

SOD estimation was carried out by using a colorimetric method as described by Suttle (1986) using a commercially prepared reagent manufacturer by Randox Laboratories Ltd, Antrim, UK. The principle was based on the fact that xantine and xantine oxidase generate superoxide radicals which react with 2 (4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrasodium chloride (INT) to form a red formazen dye. The superoxide dismutase activity is then measured by the degree of inhibition of this reaction. One unit of SOD is that which causes a 50% inhibition of the rate of reduction of INT under the condition of the assay.

Estimation of GSH:

The level of reduced glutathione (GSH) in the plasma was determined using the method of Beutler, *et al.*, (1963). The method is based on the development of a relatively stable light yellow colour when Ellman's reagent (5^1 5^1- dithiobis – (2-benzoic) acid) is added to a sulfhydryl compound. The chromophoric product resulting from the reaction of Ellman's reagent with the reduced glutathione (read colorimetrically at 412nm) is proportional to the concentration of GSH in the sample.

Estimation of CAT:

This method was according to Sinha (1972) which is based on the principle that catalase in the physiological sample is allowed to split H_2O_2 for different period of time. The reaction is stopped at a particular time by the addition of dichromate/acetic acid mixture to determine the remaining H_2O_2 by measuring spectrophotometrically (at wavelength between 570-610nm) the chromic acetate formed after heating the reacting mixture. The amount of H_2O_2 hydrolyzed was calculated for the CAT activity (Nwanjo, 2007).

Estimation of TPP:

Total plasma peroxide concentration was determined using FOX2 method (Miyazawa, 1989) with minor modification (Harma et al, 2003). FOX 2 test system is based on oxidation of ferrous ions to ferric ion plasma sample to produce a coloured ferric-xylenol orange complex which is measured spectrophotometrically at a wavelength of 560nm.

Oxidative stress index (OSI):

OSI, an indicator of the degree of oxidative stress, is the percent ratio of the total plasma peroxide to the total antioxidant activity (μMol/L) (Harma et al, 2003).

Estimation of MDA:

Level of lipid peroxidation was determined by measuring the formation of thiobarbituric acid reactive substances (TBARS) using the method of Varshney and Kale (58). The principle is based on the fact that malondiahydehyde (MDA) produced from the peroxidiation of membrane fatty acid reacts with the chromogenic reagent; 2-thiobarbituric acid (TBA) under acidic

conditions to yield a pink–coloured complex measured spectrophotometrically at 532 nm. 1, 1, 3, 3-tetramethoxylpropane was used as standard.

Statistical analysis

The quantitative data were presented in the form of mean and standard deviation. Student (t) test was used for comparison between groups. Significance was considered when p-values are less than 0.05.

RESULTS

The mean values of antioxidants and markers of oxidative stress in C+M patients are presented in Table 1. The plasma levels of TPP decreased significantly at 2 weeks (p=0.01) and 4 weeks (p=0.00) of treatment when compared with the baseline value. The levels of MDA at 2 weeks (p=0.04) and 4 weeks (p=0.00) of treatment decreased significantly when compared with the baseline value. The level of OSI at 2 weeks (p=0.02) and 4 weeks (p=0.00) of treatment decreased significantly when compared with baseline value. There were significant increases in the levels of TAA at 2 weeks (p=0.03) and 4 weeks (p=0.01) of treatment when compared with the baseline concentration. So also, the mean values of SOD increased significantly at 2 weeks (p=0.00) and 4 weeks (p=0.00) of treatment when compared with the baseline value. Mean values of CAT at 2 weeks (p=0.04) and 4 weeks (p=0.02) of treatment increased significantly when compared with the baseline concentration. There was no significant change in the value of GSH at 2 weeks (p=0.75) but the mean value increased significantly at 4 weeks (p=0.04) of treatment when compared with the baseline values.

In C-M patients (Table 2), there was no significant change in the values of TPP, OSI, CAT and TAA at 4 weeks (p>0.05) of treatment when compared with the baseline values. The mean value of MDA decreased significantly (p=0.04) while the mean values of SOD and GSH increased significantly (p=0.00) at 4 weeks of treatment when compared with the baseline values.

Table 1: Mean values of antioxidants and markers of oxidative stress in PTB patients on chemotherapy supplemented with micronutrients (C+M).

	Baseline	2 weeks	4 weeks	p^a	p^b
TPP (μmol H_2O_2 /L)	41.0±11.0	28.0±5.0	16.0±4.0	0.01*	0.00*
MDA (nMol / ml)	8.2±1.7	6.8±2.2	5.5±1.7	0.04*	0.00*
OSI (%)	2.7±0.9	1.9±0.6	0.9±0.8	0.02*	0.00*
SOD (u/ml)	127±30.3	165±24	199±18	0.00*	0.00*
GSH (ng/ml)	17.5±5.0	16.3±7.3	23.5±4.0	0.75	0.04*
CAT (μMol/L H_2O_2)	56.1±8.2	64.0±5.0	77.0±8.0	0.04*	0.02*
TAA (mMol/l)	1.6±0.4	1.9±0.8	2.2±0.7	0.03*	0.01*

P^a = Comparison between baseline values and values at 2 weeks post treatment.
P^b = Comparison between baseline values and values at 4 weeks post treatment.
* = significantly different from the baseline values

Table 2: Mean values of antioxidants and markers of oxidative stress in PTB patients on chemotherapy without micronutrient supplementation (C-M).

	Baseline	2 weeks	4 weeks	p^a	p^b
TPP (μmol H_2O_2 /L)	33.0±10.0	29.0±12	28.0±10.0	0.6	0.4
MDA (nMol / ml)	8.5±2.3	8.6±2.3	6.8±1.6	0.3	0.04*
OSI (%)	2.8±1.0	2.2±1.4	2.0±1.0	0.3	0.40
SOD (u/ml)	130±36	142±24	180±29	0.27	0.00*
GSH (ng/ml)	15.1±8.9	18.1±10.2	26.0±7.9	0.43	0.00*
CAT (μMol/L H_2O_2)	55.0±24.0	59.8±17.0	60.0±17.0	0.24	0.16
TAA (mMol/l)	1.6±0.6	1.6±0.4	1.5±0.6	0.73	0.62

P^a = Comparison between baseline values and values at 2 weeks post treatment.
P^b = Comparison between baseline values and values at 4 weeks post treatment.
* = significantly different from the baseline values.

DISCUSSION

Micronutrients deficiencies have been associated with antioxidants and immune dysfunctions in PTB patients (Bolukbas et al, 2005; El-Behairy et al 1997; Crowley et al, 2000). The synthesis and catalytic activities of major antioxidant enzymes as well as the bactericidal roles of polymorphonuclear cells depend on adequate micronutrient status (El-Behairy et al, 1997; Powell, 2000; Roughead et al, 1999). The role of micronutrients in the present study might have enhanced antioxidant enzymes activity for efficient neutralization of the toxic effects of free radicals in PTB patients. Manganese, copper and zinc are an integral part of superoxide dismutase (SOD) (Wiid 2004; El-Behairy et al 1997). Iron is an integral part of catalase, while selenium (Se) is an integral part of Se-GPX (glutathione peroxidase). SOD catalyses the conversion of toxic superoxide anion to less toxic hydrogen peroxide which is there after converted to water and oxygen in two separate reaction catalyzed by glutathione peroxidase and catalase. Catalase and glutathione peroxidase are major scavengers of H_2O_2, and this catalytic role makes them primary antioxidants (Ramanujam, 2004; Sies, 1993).

In C+M, the plasma levels of TPP, MDA and OSI declined significantly while the mean value of TAA increased significantly at 2 weeks of treatment. At 4 weeks of anti-TB chemotherapy in C-M patients, there were no significant changes in the levels of TPP, OSI, and TAA as opposed to when anti-TB chemotherapy was supplemented with micronutrient (C+M). The finding of insignificant change in the level of TPP in C-M is consistent with earlier report of Plit et al (1998) that TPP level is not affected by anti-TB chemotherapy. Vitamin A, C, E, and Cu, Zn, Mn, Fe, and Se supplements in C+M patients enhanced the synthesis of antioxidant enzymes and improved the antioxidant system of the PTB patients. The simultaneous increased rate of synthesis of antioxidant enzymes (SOD, CAT) and GSH in C+M therefore contributed to the lower levels of TPP, OSI and MDA with increased level of TAA observed in this study. This finding is in agreement with Weber et al (1997) that vitamin E supplementation enhanced decrease in the level of lipid peroxidation. Harma et al (2003) reported that high dosage of vitamin C was potentially efficacious for the lowering of physical stress. Luostarinen & Saldeen (1996) also demonstrated the reducing effect of dietary fish oil on the superoxide generation by human neutrophil, while Wild et al (2004) postulated that effective PTB control is subject to antioxidant status. The micronutrients included in the treatment of our PTB patients (C+M) could be responsible for the significant decline in TPP level either through direct scavenging of TPP by vitamins C and E or through the synthesis of micronutrients dependent antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase).

In conclusion, micronutrient supplementation enhanced the decrease in the mean levels of markers of oxidative stress (TPP, OSI and MDA) but enhanced the elevation of antioxidants (TAA, SOD, GSH and CAT) values after 2 weeks of treatment. Therefore, addition of micronutrients (synthetic antioxidants or naturally occurring ones such as fruits and vegetables) to anti-TB chemotherapy should be considered in the management of PTB patients.

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Adaptive responses of the small bowel to glutamine and honey following massive small bowel resection in local dogs

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ABSTRACT

The effects of honey, glutamine and honey/glutamine combination in the healing and adaptive process of the bowel following massive small bowel resection were studied in some Nigerian non-descript breeds of dogs. 24 dogs (3-4months old) of mixed sexes with mean body weight of 4.42±0.70 kg were studied. They were randomized into four treatment groups following 70% small bowel resection. Group A dogs were placed on glutamine treatment, Group B on oral glutamine/honey and group C on honey and group D normal saline (control). Their body weights were evaluated for 15 days and the pre- and post-post treatment gut biopsy samples were obtained and processed for morphometric evaluation. All groups exhibited signs of small bowel adaptation (Glutamine/honey>glutamine□ honey□ control) at the end of the experiment (4 weeks) with oral glutamine/honey combination showing the best overall effect based on intestinal mucosal growth and adaptation evidenced by increased in residual bowel Villi height (27.71μm), Villi weight (14.51μm), Crypt depth (11.25μm), and Villi density (3.40μm). Glutamine showed a better result with a significant increase in villi height (38.08μm), width (8.48μm) and crypt depth (40μm). Honey also showed a good effect with a significant increase in villi height (29.97μm), Crypt depth (12.91μm), Villi density (3.30μm) and a high but not significant Villi width (4.58μm). Our results showed that honey /glutamine combination had comparative therapeutic advantage over glutamine or honey and may be a preferred treatment for short bowel syndrome patients.

Key Words: Short bowel, adaptation, honey, Glutamine.

INTRODUCTION

A major physiologic derangement occurs in the small bowel secretory and absorptive functions when greater than 50% of its segments is resected in surgical management of gastrointestinal disease conditions such as small bowel neoplasia, mesenteric ischemia, radiation enteritis, inflammatory bowel disease, and mechanical obstruction -Intussusception, volvulus, torsion (Eyarefe *et al.*, 2001, Brown *et al.*, 2004, Eyarefe et al, 2008). This enteric derangement is characterized by rapid waves of smooth muscle contraction in the bowel, and propulsion of chyme (bowel hurry) which shortens the transit time and consequently affects absorption by limiting nutrient exposure to the brush-border hydrolytic enzymes as well as pancreatic and biliary secretions. These patho-physiologic events results in chronic maldigestion, malabsorption and diarrhea with consequent weight loss, dehydration, fatigue, lethargy, malaise, steatorrhea, anaemia, fluid and electrolyte imbalance, delayed wound healing, increased susceptibility to infection due to impaired immune function, gastric hypersecretion, and reduced pancreatic

enzyme secretion (Joy and Patterson, 1978, Fadrique *et al.*, 2001; Eyarefe *et a*l.., 2001, Utter and Duggan, 2003, Brown *et al.,* 2004, Eyarefe *et al.*, 2008).

Management of this clinical challenge (Short Bowel Syndrome) has been a major task compelling a multi-disciplinary therapeutic approach focused at promoting small bowel adaptation: which is a series of gradual gross and histological changes within the bowel wall and mucosal glandular epithelium to compensate for the shortened bowel length and to improve the functional integrity of the residual gut, thus preventing gastrointestinal insufficiency (Joy and Patterson, 1978 Tilson, 1980, Hays *et al.*., 1995, AGA, 2003; Brown et al,. 2004; Eyarefe *et al.*, 2008). Recently, we published our preliminary findings on the effects of honey and glutamine on small bowel adaptation following massive bowel resection in rabbits (Eyarefe *et al.*, 2008). In this paper we aim at reporting our observation that honey /glutamine combination therapy evoked a synergistic response on bowel wall and mucosa hyperplasia following 70% small bowel resection in some local dogs. Honey is a natural product that has gained popularity, in recent times, in therapeutic medicine. Its effects in the treatment of different types of wounds (Molan, 1999) and in management of upper gastrointestinal lesions have been documented (Haffejee and Moosa, 1985; Obi *et al.*, 1994, Swayeh and Ali, 1998,)**.** There is however, a dearth of data on the use of honey in promoting the gut adaptive process following massive small bowel resection. Moreover, the comparative advantage of honey/Glutamine over glutamine or honey in the gut adaptive process has not been reported in literature.

MATERIALS AND METHODS

Experimental Animals

Twenty four (24) non-descript local dogs, age 3-5 months, of either sexes with mean body weight of 4.42 ± 0.70 kg were studied. They were sourced from local communities in Ibadan, Nigeria, housed in the experimental animal unit, Department of Surgery and Reproduction, University of Ibadan, in individual cages that provided ample space for exercise and fed with balanced, compounded diet, and water ad libitum. The dogs were conditioned for three weeks and judged to be of good general health based on complete physical examinations before the commencement of the experiment.

Anaesthesia

Each animal was deprived of food 8 hours prior to surgery but had access to water ad-libitum. They were premedicated with 1ml injection of 3% Pentazocine (Fortwin, Ranbaxy pharmaceuticals Ltd, India) at the dose rate of 5mg/kg and 2% Xylazine (Kepro, Holland) at the dose rate of 0.5mg/kg. Surgical anaesthesia was achieved with 2.5% Thiopentone sodium at a dose of 10mg/kg through pre-placed scalp vein set via cephalic venupuncture.

Experimental design and Surgical Procedure

The ventral abdomen was aseptically prepared, and the intestine approached through a ventral midline abdominal incision. The intestinal loops were exteriorized over saline moistened laparotomy sponges. The Treitz ligament was located and the small bowel length was determined as earlier described (Eyarefe et al, 2001) from the Treitz ligament to the ileocolic junction.

Seventy percent (70%) of the small bowel length from 10cm distal to the Treitz ligament was resected. The residual bowel segment was apposed with Polyglactin 910 (Vicryl ® Ethicon, USA), in an end to end anastomosis as earlier described (Orsher et al, 1993). The laparotomy incision was closed using a standard surgical technique. A 3cm full thickness biopsy from sectioned segment was obtained and fixed in 10% formalin (Pre-treatment sample). Animals were placed in recovery cages, and maintained on intravenous dextrose solution until animals could drink and eat 24-48 hour post resection before being returned to the experimental animal cages. Following resection, the dogs were randomised into four treatment groups (A, B, C and D). Group A dogs (n=6) were placed on oral glutamine (33g/5kg per day), Group B dogs (n-6) on oral honey (10 ml) and glutamine (0.66g/ 5kg per day), Group C dogs (n-6) on oral honey (10ml), and Group D dogs water (control group). All groups were supplied with adequate food and water. 3cm full thickness post-treatment biopsy samples were obtained from two representatives of each group and fixed in 10% formalin following a 2^{nd} surgery at days 7' 14'and 28, and the residual small bowel segment evaluated for healing, and gross changes in length and size with Veneer caliper. The formalinized tissues were processed into histology slides for histomorphometric studies.

Body Weight evaluation

Animal body weights changes were evaluated at three days intervals for fifteen days to see the effects of the various treatments on body weight using a weighing scale.

Histomorphometric evaluation

A paraffin section of tissue samples was sliced at a thickness of 4µm and stained with haematoxylin and eosin. Measurements of mucosal villus height and crypt depth were taken using a micrometer rule, as described by Joaquim et al, (2005). For each parameter, at least 10 villi and crypts were evaluated and an average values calculated.

Statistical Analysis

All data are expressed as means ±SD. Differences among groups were evaluated by a one way analysis of variance (ANOVA) followed by a two-tailed Student's t-test. P-values less than 0.05 were considered to be statistically significant.

RESULTS

Effects of treatment on body weight

Animals in all the groups experience an initial fall in body weight from days 0-3 post surgery. Animal on Glutamine/honey combination, glutamine and honey had gradual increase in body weight from days 3-15 of weight evaluation. The control group, however, had a remarkable drop in body weight compared with other groups (Fig 1)

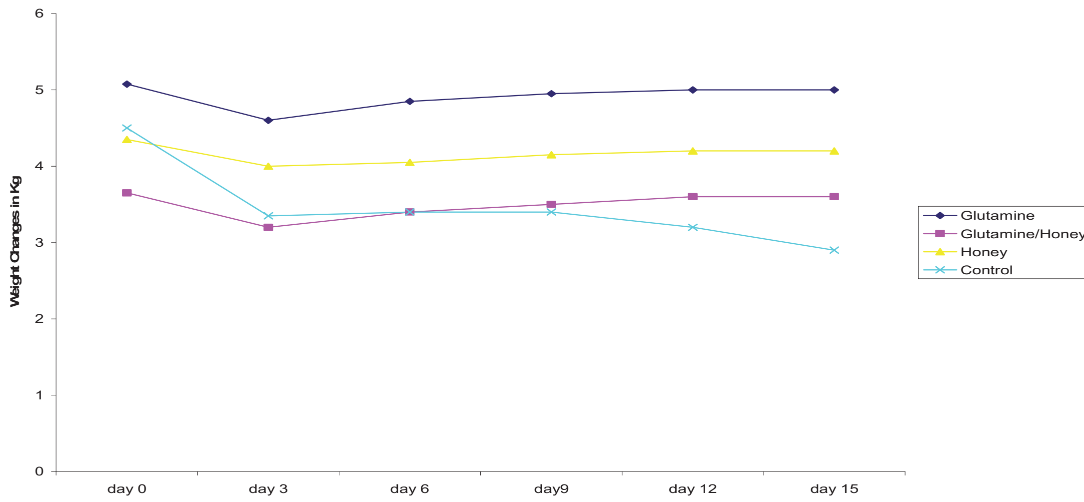
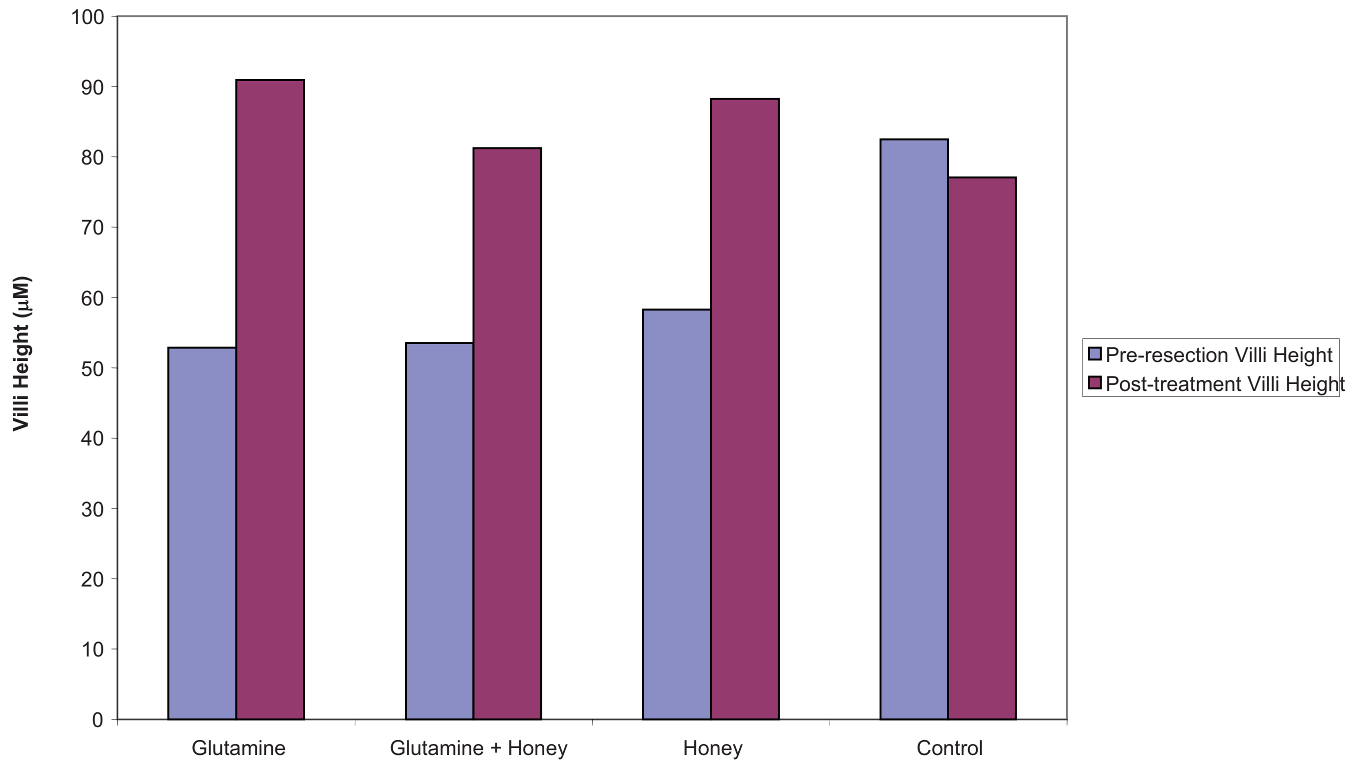


Fig 2



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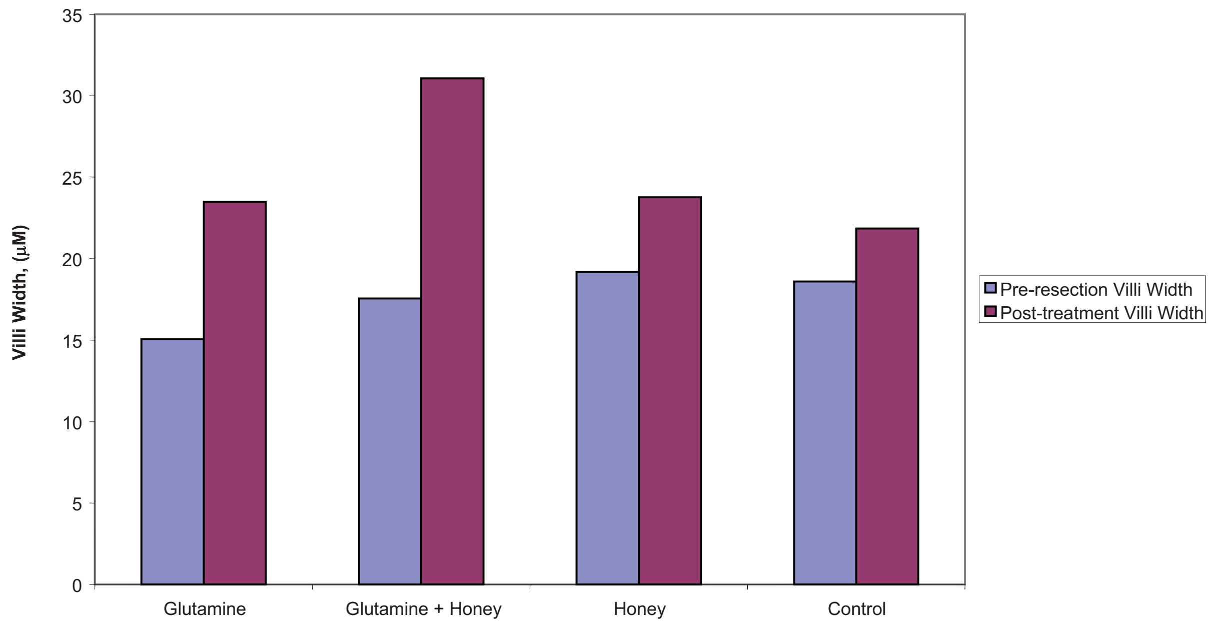

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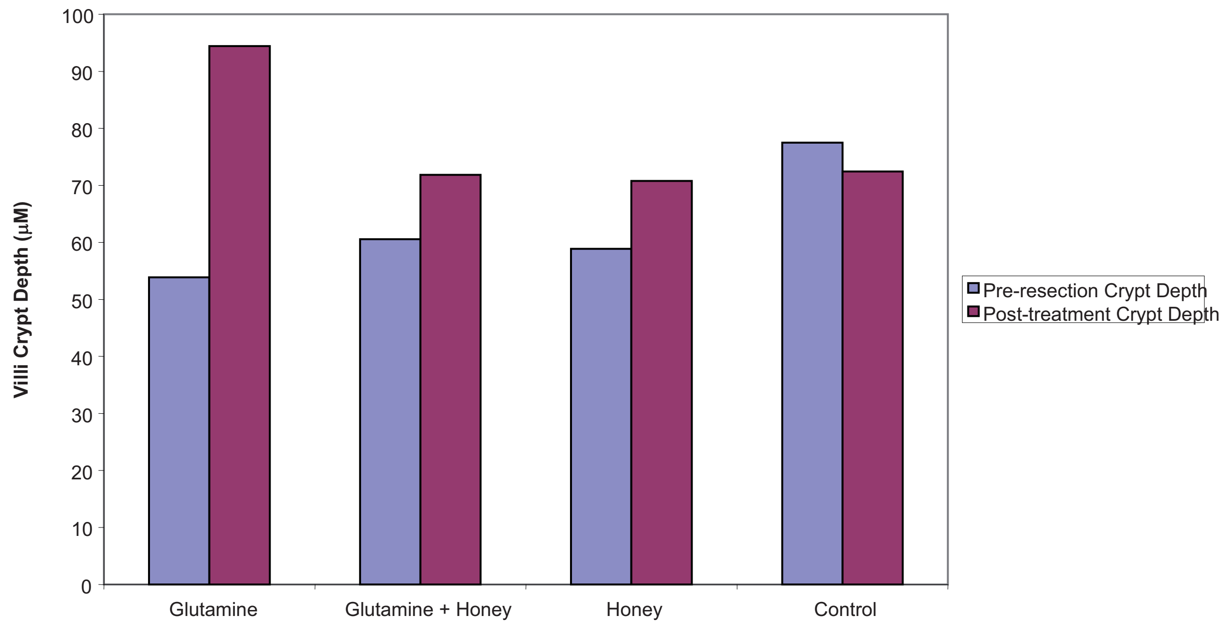

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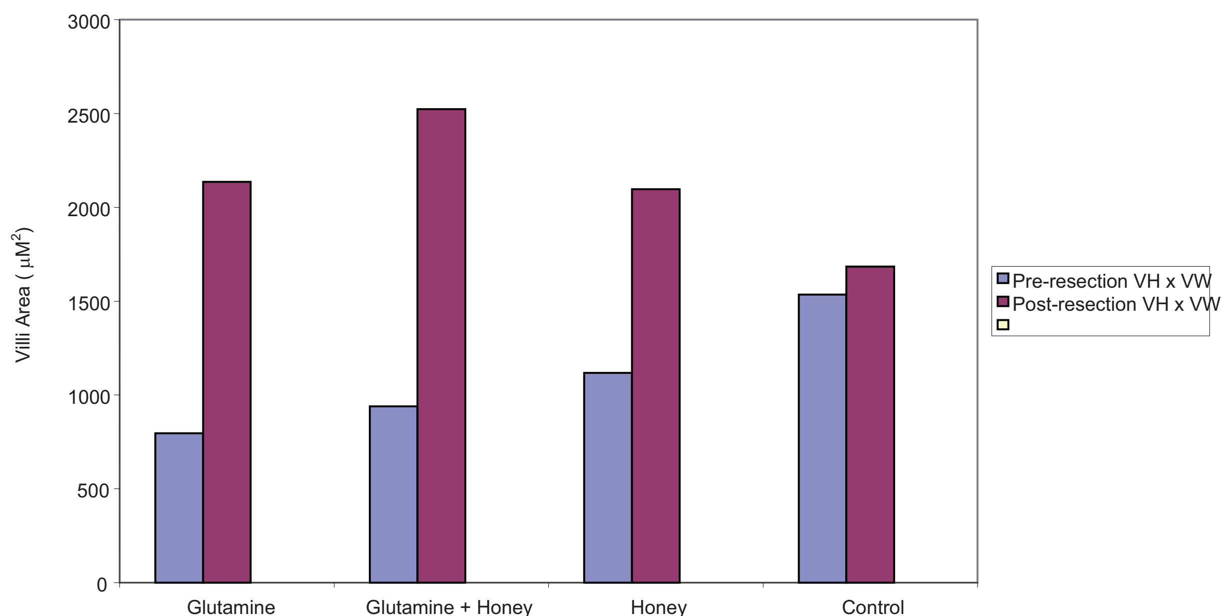

Effects of treatment on villi height

Animals in all the treatment groups experienced some increase in mucosal villi height. Those of glutamine, glutamine/honey and honey were significant at P<0.05 when pre-treatment and post treatment values were compared (fig2).

Effects of treatment on villi width

Animals in all the treatment groups experienced some increase in mucosal villi width. Those of, glutamine/honey, glutamine and honey were significant at P<0.05.(glutamine/honey> glutamine > honey) ((Fig 3).

Effects of treatment on cryptal depth

Animals in all the treatment groups experienced some increase in mucosal cryptal depth. That of glutamine was significant at P<0.05, while those of honey and glutamine /honey combination were remarkable but not significant at P< 0.05 (Fig 4).

Effects of treatment on villi density

A comparison of the villi densities the treatments show glutamine /honey combination expressing the best effects over glutamine, honey and control (Fig 5).

DISCUSSION

The result of this study shows that glutamine/honey, glutamine and honey have beneficial effects on intestinal adaptation process following massive resection. It also shows that glutamine/honey combination have a comparative therapeutic advantage over glutamine and honey. Eyarefe et al,. 2008 had earlier reported the effects of honey and glutamine on the gut adaptive process following 50 % small gut resection in rabbits. The novelty of this report is in the authentication of the initial finding, and the observed effects of the combination therapy which is the first to be reported in literature, showing the best trophic effects. The passing of diarrheic faeces among the groups between 1st to 3rd post operative days was consistent with findings in similar studies (Rombeau *et al.,* 1987, Eyarefe *et al.,* 2001, Eyarefe *et al.,* 2008). Resolution of the diarrhoea by the third post operative day was an indication that adaptive changes had began (AGA, 2003). Adaptive changes begin 12 -24 hours after massive intestinal resection and will continue for more than a year after resection, and with bowel compensation, diarrhoea and malabsorption are reduced (Rombeau et al, 1987, AGA, 2003). The initial weight loss observed in this study was consistent with those observed by other investigators Joaquim et al, (2005) in similar studies on massive bowel resection. It has been associated with surgical stress and tissue catabolism associated with wound healing (Eyarefe et al, 2001). It could also be attributed to the post resection diarrhea, and the reduced capability of the residual bowel to digest and absorb nutrients at the early post operative period (Lentze, 1989). The observed diarrhea subsided in all the groups from the third postoperative day. The correlated with the gradual increase in body weight gain observed in this study (fig 1). The increase in villi height, width, and cryptal depth in glutamine and honey treatments were observed in similar report using rabbits (Eyarefe *et al.*, 2008). Based on the results this study we therefore postulate that honey/glutamine combination have comparative therapeutic advantage over glutamine or honey and may be a preferred treatment for short bowel syndrome patients.

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Nutritional and Therapeutic Roles of Red Palm Oil in Human Health

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ABSTRACT

The link between dietary fats and cardiovascular disease has created a growing interest in dietary red palm oil research. Also, the link between nutrition and health, oxidative stress and the severity or progression of disease has stimulated further interest in considering the potential role of red palm oil (a natural antioxidant product) to improve oxidative status by reducing oxidative stress in patients with cardiovascular disease, cancer and other chronic diseases. In spite of its 50% saturated fatty acids, it has not been found to promote atherosclerosis and/ or arterial thrombosis. This is probably due to the ratio of its saturated fatty acid to unsaturated fatty acid content which is close to unity and its high concentration of antioxidants such as beta-carotene, tocotrienols, tocopherols and vitamin E. It has also been reported that the consumption of red palm oil reduces the levels of endogenous cholesterol and this seems to arise from the presence of the tocotrienols and the peculiar isomeric position of its fatty acids. The benefits of red palm oil to health include a reduction in the risk of developing arterial thrombosis and/ or atherosclerosis, inhibition of endogenous cholesterol biosynthesis, platelet aggregation, a reduction of oxidative stress and a reduction in blood pressure. It has also been shown that dietary red palm oil, taken at moderate levels by both experimental animals and humans, promotes the efficient utilization of nutrients, causes favourable body weight gains, activates the hepatic drug metabolizing enzymes, causes the haemoglobinization of red blood cells and improves the immune function in general. This paper provides comprehensive and current scientific information on the nutritional, physiological and biochemical roles of red palm oil in improving the wellbeing and quality of life of humans.

Key words: red palm oil, cardiovascular disease, antioxidants, chemo-preventive agent, natural supplement.

INTRODUCTION AND BACKGROUND

Red palm oil is an edible and natural oil produced from the fruit of the *Elaeis guineensis* tree, is known to have been enjoyed in the human diet for over 5000 years and is seen both as a nutritious food and as a valuable medicine (MacFarlane et al, 1984; Fife, 2007). It derives its colour from carotenes such as beta-carotene and lycopene, the same nutrients that give tomatoes and carrots as well as other fruits and vegetables their rich red and orange colours.

It is regarded as the second-most widely produced edible oil with over 28 million tons having been produced globally in 2004. It also serves as an important component of different soaps, washing powders and personal care products and has also been reported to be useful in treating wounds (United States Department of Agriculture, 2004).

For many years, the inhabitants of West African countries have recognized and used red palm oil, as a cooking oil. It has been reported that European merchants trading with West African countries sometimes purchased red palm oil for use in Europe. Available historical reports indicate that in the Asante Confederacy, state-owned slaves built large plantations of palm oil trees while in the Kingdom of Dahomey, King Ghezo passed a law in 1856 forbidding his citizens from cutting down palm oil trees. It was appreciated by the pharaohs of ancient Egypt as a sacred food. It is also known that red palm oil was regarded to be a highly valuable product by British traders for use as an industrial lubricant for their machines during Britain's industrial revolution and also in the manufacture of basic soap products. By 1870, palm oil constituted the primary export product of some West African countries such as Ghana and Nigeria. It is further believed to have originated from tropical Africa however it has now spread to most parts of the world. In many countries, it is found to be cheaper than other edible oils with the additional benefit of being it an important source of vitamin A in vitamin A deficient or marginally deficient vitamin A communities (Fife, 2007; United States Department of Agriculture, 2004).

The link between dietary fats and cardiovascular diseases has stimulated a growing interest in dietary red palm oil research. The link between nutrition and health in oxidative stress has created further interest in red palm oil and its potential ability to improve oxidative status by reducing oxidative stress in patients with cardiovascular disease, cancer and other chronic diseases. It is however important to note that too high intake of red palm oil could potentially induce toxicity to the liver and cause a loss of the cellular radial architecture and reduce the cell size which can be corroborated by increased alanine transaminase (ALT) and asparate transaminase (AST) activities. Interestingly, consumption of moderate amounts of red palm oil has been reported to reduce oxidative stress (Ebong et al, 1999; Edem, 2002; Farombi, 2003).

The aim of this review is to provide comprehensive and current scientific information on the nutritional, physiological and biochemical roles of red palm oil in improving the wellbeing and quality of life to humans.

NUTRITIONAL, PHYSIOLOGICAL AND THERAPEUTIC ROLES OF RED PALM OIL

Red palm oil is used as the basic source of dietary fat in various countries due to its nutritional and biochemical importance. In some countries, it is believed that red palm oil is an essential component in the diet of pregnant and nursing women for maintaining good health for both mother and child. Several studies have reported on the potential role of red palm oil in the treatment and prevention of malnutrition and vitamin deficiency diseases and some indicate that some governments of developing countries are currently incorporating red palm oil in the diets or snacks of children and pregnant women to eradicate deficiency diseases such as vitamin A deficiency which is found to be quite common in developing countries (Fife, 2007; Ebong et al, 1999; Edem, 2002; Farombi, 2003; Khosla, 2006). Red palm oil also supplies fatty acids which are essential for adequate growth and development and provides vitamins, antioxidants and other phytonutrients necessary to promote good health and quality of life (Edem, 2002; Farombi & Briton, 1999). Other reports indicate that red palm oil can adequately supply the fat and vitamin A precursors needed in the human diets. It is also regarded to be the richest dietary source of provitamin A carotenes, containing 15 times more provitamin A carotenes than carrots and 300

times more than tomatoes, thus making red palm oil a potential resource in the treatment of vitamin A deficiency. It is believed that one teaspoon of red palm oil per day could supply the daily recommended amount of vitamin A for children. Reports by Canfield et al (2001) and Balasundram et al (2005) show that the addition of red palm oil into the diet, can double or triple the amount of vitamin A in mother's milk.

Vitamin A deficiency is considered to be a public health problem in many developing countries (Oguntibeju et al, 2004). There is general consensus that food-based approaches are viable and sustainable options for addressing vitamin A deficiency in populations. One good example is the fortification of food, which if properly monitored, could make a significant contribution towards improving the vitamin A status of a population. It has also been shown that when incorporating red palm oil into different products, 15-20% of the recommended daily allowance (RDA) of beta-carotene per portion of product consumed, is provided (Benade, 2001).

Red palm oil is unique when compared with other dietary fats in that it contains the highest known concentrations of natural antioxidants, particularly provitamin A carotenes and vitamin E. It is known that both vitamins A and E contribute to the maintenance of good health and disease prevention and further that red palm oil vitamin E contains 70-80% tocotrienols (Ebong et al, 1999; Edem, 2002; van Stuijvenberg et al, 2001; Mclntyre et al, 2000). It is also known to contain more nutrients than any other dietary oil. In addition to beta-carotene, alpha-carotene and lycopene, it contains at least 20 other carotenes plus vitamin E, vitamin K, ubiquinone 10, squalene, phytosterols, flavonoids, phenolic acids, and glycolipids. The vitamin E, tocotrienols, carotenes and other antioxidants make red palm oil unique if not an essential source of antioxidant (Fife, 2007; Ebong et al, 1999; Kimmick et al, 1997; Sylvester & Shah, 2002; Narang, 2004).

Due to its nutrients and antioxidants, red palm oil is viewed as a natural dietary supplement. It is good for cooking and baking and consists of 50% saturated fatty acids, 40% monounsaturated fatty acids and 10% polyunsaturated fatty acids. The high saturated and monounsaturated fatty acid content makes red palm oil heat resistant and stable and the high saturated fat and antioxidant content make it resistant to oxidation and free-radical formation (Fife, 2007; Farombi, 2003; Khosla, 2006; Sylvester & Shah, 2002; Radhika et al, 2003; Khanna et al, 2005; Esterhuyse et al, 2006).

Studies have confirmed the nutritional value of red palm oil due to high monounsaturation at the 2-position of triacylglycerols, making it an even healthier oil than olive oil. It is also known that the contribution of dietary fats to blood lipid and cholesterol modulation is a consequence of the digestion, absorption and metabolism of the fats. Lipolytic hydrolysis of red palm oil glycerides containing mainly oleic acid at the 2-position and palmitic and stearic acids at the 1 and 3 positions, allows for the ready absorption of 2-monoacrylglycerols while the saturated fatty acids remain poorly absorbed. It has been observed that dietary red palm oil when employed in balanced diets, generally reduces blood cholesterol, low-density lipoprotein (LDL)-cholesterol and triglycerides while increasing the high-density lipoprotein (HDL) cholesterol. Improved lipoprotein(a) and apo-A1 levels have been observed in serum of persons who include red palm oil in their diet (Ong & Goh, 2002; Bayorh et al, 2005).

The effects of red palm oil on major plasma carotenoids, tocopherols, retinol and serum lipids were evaluated by Zhang et al (2003) in Chinese diets. In carrying out the study, the investigators

selected a red palm oil group (20 male subjects aged 18-32 years) and a soybean oil group composed of 22 male subjects of the same age group. The results showed that plasma alpha-carotene, beta-carotene and lycopene concentrations of the red palm oil (RPO) group who included the oil in their diet significantly increased over a 21 day period and at the end of the study (42 days), the alpha-tocopherol concentrations were found to be significantly increased. It was further observed that the serum concentrations of total cholesterol, triglyceride, high-density lipoprotein-cholesterol, apolipoprotein A1 and apolipoprotein B of all subjects showed no significant changes in the RPO group during the study. The results of this study suggest that RPO is a good source of carotenoids and vitamin E as used in the Chinese diet and also that it can significantly increase the plasma concentrations of alpha-carotene, beta-carotene, lycopene and alpha-tocopherol.

Canfield et al (2001) noted that despite vitamin supplementation programmes, vitamin A deficiency in children remains a public health concern in developing countries. A study conducted in the Honduras to investigate the effectiveness of short-term dietary supplementation of mothers with red palm oil as a strategy for improving their vitamin A status, reported that red palm oil supplementation in the maternal diet increased provitamin A carotenoids in breast milk and serum of both mother and infants.

Zangre et al (2002) carried out a study to demonstrate the feasibility and effectiveness of introducing RPO in non-consuming areas in Burkina Faso. RPO was obtained from women in the South-West region. It was then promoted and sold at project sites by community workers who have been trained in persuasive communication and social marketing techniques. Blood samples were later collected from the target population who bought and consumed the RPO. The analysis of serum and dietary data collected at baseline showed that about 65% of the target population had low serum retinol as dietary vitamin A. However, following consumption of RPO, it was reported that the percentage of mothers and children at risk of vitamin A deficiency had reduced significantly. The study thus indicated that the promotion of RPO was effective in improving vitamin A intake by the target population.

In a South African study, van Stuijvenberg et al (2001) determined the effect of a biscuit containing red palm oil as a source of beta-carotene on the vitamin A status of primary school children as compared to the effect of a biscuit containing beta-carotene from a synthetic source. The authors concluded that a biscuit with red palm oil as a source of beta-carotene is as effective as a biscuit with synthetic beta-carotene in improving the vitamin A status of primary school children. However, the additional qualities of red palm oil (no trans-fatty acids, rich source of antioxidants) make RPO an excellent alternative resource for addressing vitamin A deficiency and in our opinion, RPO can also be accepted to be an excellent resource in addressing vitamin E deficiency considering its high vitamin E content.

Sivan et al (2002) administered red palm oil (5 ml and 10 ml), groundnut oil fortified with 400 and 800 retinol palmitate and then groundnut oil (5 ml and 10 ml-no fortification with retinol palmitate) to six groups of preschool children in India (4 experimental and 2 control groups) in randomly assigned local porridge for a period of seven months. The study was designed to monitor the difference in the efficacy of the mode of supplementation and the optimum dose for improving vitamin A status. Results showed that the red palm oil groups had higher retinol and beta-carotene levels when compared to other groups (groundnut oil forfified with retinol

palmitate and groundnut oil without retinol palmitate) and observed that administration of 10 ml did not offer any substantial improvement over the 5 ml daily dose.

Other studies have shown that red palm oil supplementation protects against heart disease/reperfusion injury (Edem 2002; Esterhuyse et al, 2005; van Rooyen et al, 2008). To verify this, Esterhuyse et al (2005) fed rats with standard rat chow, plus cholesterol and/ or red palm oil supplementation for six weeks. The investigators determined functional recovery, myocardial phospholipid and cAMP/cGMP levels in isolated rat hearts subjected to 25 minutes of normothermic total global ischaemia. Their findings showed that dietary red palm oil in the presence of cholesterol improved aortic output recovery when compared to cholesterol only ($P<0.05$) and that improved functional recovery in hearts supplemented with red palm oil versus controls was preceded by elevation in the cGMP levels early in ischaemia. Based on these findings, they concluded that dietary red palm oil supplementation improves reperfusion aortic output through mechanisms that may include activation of the NO-cGMP and inhibition of the cAMP pathway.

In another animal study, Esterhuyse et al (2006) showed that dietary red palm oil may improve myocardial ischaemic tolerance by increasing bioavailability of nitric oxide (NO) and improving NO-CGMP signaling in the heart, suggesting a cardio-protective role of red palm oil to the ischaemic and reperfused heart. These findings call for further studies in both animal and human subjects to elucidate the mechanisms involved in the cardioprotective activity of red palm oil. Bester et al (2006) developed an oxidative risk-induced diet (ORD) rich in polyunsaturated fatty acids (PUFAs) and low in saturated fatty acids (SFAs) and a high saturated fat diet (HFD) rich in SFAs and low in PUFAs. The diets were either supplemented with red palm oil (experimental group) or not supplemented with red palm oil (control group) and the researchers investigated whether red palm oil could offer protection against oxidative stress, ischaemia and reperfusion injury. Their findings showed that red palm oil offered protection against reperfusion injury/oxidative stress in both ORD and in HFD fed rats as indicated by increased aortic output recovery and improved oxidative status following red palm oil supplementation for 14 weeks.

The signaling mechanisms responsible for the effects of red palm oil in the presence of cholesterol however remained to be elucidated. Therefore, Kruger et al (2007) examined the effects of red palm oil, with a high-cholesterol diet on mitogen-activated protein kinase (MAPK) phosphorylation and apoptosis. In this study, rats were fed control diets, which contained either 2% cholesterol or 25% cholesterol and 7 g red palm oil (CRPO) for 5 weeks. The hearts of the rats were excised and mounted on an isolated working heart perfusion apparatus. Cardiac function was measured after which hearts were freeze-clamped and used to assess MAPK phospohorylation and to evaluate apoptosis. It was observed that cholesterol supplementation caused a poor aortic output recovery when compared to the control group. However, in the presence of red palm oil, the percentage of aortic output increased significantly. It was further observed that the cholesterol group's poor aortic output was associated with a significant increase in p38-MAPK phosphorylation whereas the CRPO-supplemented group showed a significant reduction in p38-MAPK phosphorylation when compared to the cholesterol-supplemented group. The investigators also noted that the significant reduction in p38-MAPK was also associated with reduced apoptosis as indicated by significant reductions in caspase-3 and poly (ADP-ribose) polymerase cleavage. The study of Kruger et al (2007) provides a possible mechanism and alternative role for red palm oil as a non-pharmacological strategy to

protect the heart against ischaemia reperfusion-induced injury in the presence of cholesterol which is one of the risk factors associated with cardiovascular and ischaemic heart disease.

It has been shown that red palm oil supplementation improves reperfusion function however, the exact protective cellular mechanisms has not yet been established (Esterhuyse et al, 2006; Esterhuyse et al, 2005; Bester, 2006; Kruger et al, 2007). To determine a potential mechanism for cardiac functional improvement, Engelbrecht et al (2006) investigated the regulation of both mitogen-activated protein kinases (MAPK) and PKB/Akt in the presence and absence of dietary red palm oil supplementation in ischaemia/reperfusion-induced injury. The investigators used Wistar rats and fed them either a standard control diet or a control diet supplemented with red palm oil for 6 weeks. The investigators reported that dietary red palm oil supplementation caused differential phosphorylation of the MAPK and PKB/Akt during ischaemia/reperfusion injury. These changes in phosphorylation were associated with improved recovery and reduced cleavage of an apoptotic marker, suggesting that dietary red palm oil supplementation may in fact offer protection through the MAPK and PKB/Akt signaling pathways during ischaemia/reperfusion-induced injury.

Red palm oil contains the highest concentrations of natural tocotrienols which have been found to display potent anticancer activity at treatment doses and have little or no effect on normal cell growth or viability (Sylvester & Shah, 2002). Tocotrienols have been shown to induce apoptosis or programmed cell death in breast cancer. It is also known that morphological and biochemical characteristics of apoptosis, such as nuclear and cytoplasmic condensation and DNA fragmentation are mediated by the activation of cysteine proteases called caspases. Apoptosis is triggered by the activation of initiator caspases (caspase-8 or 9) that subsequently activate effector caspases (caspase-3, 6, and 7). A study conducted using a highly malignant mouse mammary epithelial cell line to determine if tocotrienol-induced programmed cell death is mediated through the caspase-8 or caspase-9 pathway, showed a significant increase in the activities of caspase-8 and-3 but not caspase-9. When tocotrienol, in combination with selected caspase-8 or caspase- inhibitors was administered to the same epithelial cell line, it was found that they completely blocked tocotrienol-induced apoptosis and activation of caspase-8 and caspase-3. These findings showed that tocotrienol-induced apoptosis in highly malignant mammary epithelial cells is mediated through caspase-8 activation and potentially provide important information required in understanding the health benefits of tocotrienol-rich red palm oil in preventing and or reducing the risk of breast cancer in women (Sylvester & Shah, 2002).

Previous studies have shown that tocotrienols and not tocopherols displayed potent anti-proliferative and apoptotic activity against breast cancer cells (Mclntyre et al, 2000; Mclntyre et al, 2002; Sylvester et al, 2001). These studies suggest that tocotrienols from red palm oil may have a significant value as therapeutic agents for breast cancer prevention and treatment.

Current available literature indicate that very little work has been done on the influence of red palm oil (RPO) which contain a higher concentration of carotenoids than refined, bleached and deodorized palm oil (RBO-PO). Wilson et al (2005) investigated the influence on hamster fed RPO or RBO-PO or RBO-PO plus a red palm oil extract on plasma cholesterol levels and aortic accumulation versus hamsters fed on coconut oil. Their findings showed that plasma total cholesterol and non-high-density lipoprotein-cholesterol (non-HDL-C) were significantly lower in the hamsters fed RPO compared to coconut oil-fed hamsters. Plasma HDL-cholesterol concentrations were higher by 14% and 31% respectively in hamsters fed the RBO-PO and RPO

compared to the coconut oil-fed hamsters. The coconut oil-fed hamsters also showed significantly higher plasma lipid hydroperoxide concentrations as compared to those fed RPO or RBO-PO and RPO-PO plus RPO. Also, the coconut oil-fed hamsters had significantly higher levels of aortic total, free and esterified cholesterol as compared to the hamsters fed the RPO. In conclusion, hamsters fed RPO, RBO-PO and RBO-PO plus RPO had lower total plasma cholesterol and non-HDL-cholesterol and higher HDL-cholesterol while accumulating less aortic cholesterol as compared to hamsters fed coconut oil. The total cholesterol content of the aorta of the hamsters fed coconut oil was significantly higher than that of the aorta of RPO-fed hamsters. The levels of free and esterified cholesterol were also higher in the coconut oil-fed hamsters. The ratio of free to esterified cholesterol was highest in aortas of hamsters fed RPO, a reflection of lowered ester deposition and thus, less severe atherosclerosis. This study clearly showed that RPO is less atherogenic than either RBO or reconstituted palm oil.

Red palm oil is often stigmatized as a hypercholesterolemic fat mainly because of its palmitic acid content. However, in the above study coconut oil was the most hypercholesterolemic while RPO was found to be less cholesterolemic than RBO-PO and RBO-PO plus PO (reconstituted RBO-PO). The result of the study is similar to that of Ng et al (1991) who showed that consumption of red palm oil after a 5 week intake decreased serum cholesterol concentrations in healthy volunteers. Previous work has shown that the presence of palmitic acid at the 2-position of a triglyceride molecule renders the triglyceride more cholesterolemic and atherogenic (Renaud et al, 1995; Kritchevsky et al, 1998; Kritchevsky et al, 2000). It should however be noted that although red palm oil contains 40% palmitic acid, only an estimated 3% of the palmitic acid is in the 2-position, thus making red palm oil less cholesterolemic. The study conducted by Sundram *et al.* also showed an increase in plasma HDL-cholesterol concentration in rats when fed RPO (Sundram et al, 1990).

Studies show that adding red palm oil to the diet can reduce plaque deposition in arteries and therefore, reverse the process of atherosclerosis. This has been demonstrated in both animal and human studies. In one such study, 50 atherosclerotic subjects were divided into two equal groups. At the beginning of the study, the degree of blockage of their carotid arteries were determined and found to range from 15 to 79 percent. Without any other changes to their diets or medications, half of the subjects began taking a daily red palm oil supplement. The other half received a placebo (control group). The degree of atherosclerosis was monitored using ultrasound scans over an 18 month period. In the group receiving red palm oil, atherosclerosis was found to have halted in 23 of the 25 participants. In seven of these participants, atherosclerosis was not only stopped but regressed. In comparison, none of the subjects in the control group showed any improvement and the condition in 10 of them in fact worsened (Tomeo et al, 1995). This and other studies as shown in this review clearly reveal that red palm oil is without doubt beneficial to human health.

A red palm oil diet has also been found to reduce platelet aggregation and decrease blood clotting (Khosla & Haves, 1996; Hornstra, 1988; Rand et al, 1988; Abevwarden et al, 1989), although there has been an isolated case report of enhanced platelet aggregability and thrombocytopenia which is thought to be associated with the red palm oil diet (Osim et al, 1992). However, in general most of the studies indicate a beneficial role of red palm oil in reducing the thrombotic tendency of platelets.

Red palm oil has been reported to maintain normal blood pressure. The high antioxidant content of the oil reduces free radicals and keeps inflammation under control. In one study, investigators induced inflammation in the arteries of test animals. Inflammation is known to cause swelling which narrows artery passageways, restricting blood flow to vital organs such as the heart. Half of the animals received red palm oil in their diet while the other half served as the control group. In the control group, artery passageways were severely constricted and 42% of the animals died. However, those that received the red palm oil showed far less inflammation and constriction resulting in a 100% survival rate (Fife, 2007). This study supports the hypotheses that red palm oil protects against heart disease. This has been confirmed in populations where red palm oil is regularly consumed. For instance, heart disease in Malaysia, Indonesia, Papua New Guinea and Nigeria (where red palm oil is a major if not the sole source of edible fat in the daily diet) is among the lowest in the world (Fife, 2007; Sron, 2005).

The powerful antioxidant properties of red palm oil have also been shown to be of benefit in the protection against neurological degeneration. Two of the most significant factors that affect brain function are oxidative stress and poor blood circulation (Rao & Agarwal, 2000). Oxidative stress generates free radicals that damage brain and nerve tissue and poor blood circulation which is known to affect the brain by restricting oxygen and glucose supplies which are vital for proper brain function. Some studies have found correlations between oxidative stress and reduced blood flow to the brain and senile dementia, Alzheimer's disease, Parkinson's disease, Huntington's disease and even schizophrenia. These conditions involve brain cell death. It is reported that red palm oil derived tocotrienols aids the brain by reducing oxidative stress and improving the blood flow. Glutamate-induced cell death in the presence of synthetic and dietary vitamin E has been reported and it is believed that synthetic and dietary vitamin E is probably not sufficiently potent to prevent glutamate-induced cell death. However, tocotrienols from red palm oil is believed to have the potential to reduce the destructive action of glutamate. Laboratory studies suggest that tocotrienol-treated neurons appear to maintain healthy growth and motility even in the presence of excess glutamate (Khanna, 2003).

Karaji-Bani et al (2006) reported that when supplementing rats' diet with red palm oil for 3 months a significant increase in HDL-cholesterol and significantly reduced triglyceride and LDl-cholesterol levels were observed. The authors also concluded that the use of red palm oil can be useful in preventing cardiovascular disease. In another study, investigators reported a 10% decrease in total cholesterol in 36 hypercholesterolemic subjects who received red palm oil capsules for 4 weeks. A follow-up study of 16 participants resulted in a 13% reduction in total cholesterol (Qureshi et al, 1995).

In another study, 31 participants were given a red palm oil supplement on a daily basis for 30 days. Participants were told to continue with their normal diets. Results showed that red palm oil supplementation reduced both total cholesterol and low density lipoprotein (LDL) levels in all the participants and the extent of reduction of total cholesterol ranged from 5 to 36% and the reduction of LDL ranged from 0.9 to 37%. Importantly, it was also shown that the red palm oil reduced the LDL to HDL-cholesterol ratio in 78% of the participants, demonstrating a highly significant and favorable response to red palm oil supplementation (Tan et al, 1991).

The vitamin E component of red palm oil is reported to provide a rich source of tocotrienols which have been shown to inhibit two human breast cancer cell lines *in vitro* (responsive MCF7 and unresponsive MDA-MB-231 cells). Nesaretnam et al (2000) reported that at low

concentrations, a tocotrienol-rich fraction of red palm oil and other individual fractions (alpha, gamma and delta) can also inhibit the growth of another responsive human breast cancer cell line (ZR-75-1).

According to a study by Yamanushi et al (2001), it was suggested that red palm oil carotene prevents lung tumorigenesis against active oxygen radicals by its protective effect on DNA. The authors also noted that apart from the chemopreventive effect, the growth of the tumour in a mouse model used in the study was inhibited by the administration of red palm oil carotene and the authors advocated for further studies in order to elucidate the mechanisms and potential effects of red palm oil carotenoids.

In another study Boateng et al (2006) compared the inhibitory effect of red palm oil (7% and 14% levels) and soybean oil (7% and 14%) on azoxymethane (AOM) induced aberrant crypt foci. Thirty-two male Fischer 344 rats were randomly assigned to four groups. Two groups received a control diet containing 7% and 14% soybean oil (SBO) respectively. Groups 3 and 4 received a treatment diet consisting of 7% and 14% RPO respectively. The rats received subcutaneous injections of AOM at 16 mg/kg body weight at 7 and 8 weeks of age. At 17 weeks of age, rats were sacrificed by CO asphyxiation. Numbers of ACF (mean+/- SE) in the proximal and distal colon were: 39+/-0.9, 53.8+/-2.8, 26.0+/-3.0, 27.5+/-1.5 and 118.2 +/-1.7, 125.6 +/-3.2, 41+/-7, 52.3+/-1.8 in rats fed 7% SBO, 14% SBO, 7%RPO and 14% RPO respectively. The results of this study showed that RPO intake reduced the incidence of AOM-induced aberrant foci and may therefore have a beneficial effect in reducing the incidence of colon cancer.

In another study, Marotta et al (2006) investigated the status of the reticulo-endothelial system as assessed by superoxide anion generation by blood monocytes, the erythrocyte oxidability and analyzed serum fatty acids and cytokines in 24 patients with chronic pancreatitis. A dietary questionnaire was used at entry and re-assessed at the end of the study using the model of a seven-day diet history. Patients were instructed not to consume any fish oil supplement and refrain from olive oil dietary consumption and then put on a 2-week wash out period from such use when present. Patients were then given a sample containing a specific highly-purified RPO at 40 ml daily for two weeks without a frying or heating process. The investigators reported that 22 patients fully complied with the supplementation protocol and that subjects reported good palatability of RPO and experienced no side effects. Body mass index (BMI) and waist-hip-ratio (WHR) as well as routine biochemical parameters remained stable throughout the study period being comparable to age-matched controls. Compared to baseline values, patients with chronic pancreatitis (CP) who were fed RPO, showed a significant improvement of the peak value of generated superoxide anions ($P<0.05$) and a reduced erythrocyte oxidability ($P<0.01$). RPO also significantly reduced alpha TNF and interleukin-6 ($P<0.05$). Taken as a whole, the results of the study suggest that a dietary enrichment with RPO is able to significantly improve the oxidative inflammatory profile in patients with chronic pancreatitis and also beneficially correct their fundamental deficiencies in essential fatty acids. Because of the multifactoriality of chronic pancreatitis and its subtle progressing clinical worsening condition, it would appear as if dietary intervention with red palm oil should be considered as an amenable integrative therapeutic tool.

CONCLUSION

As shown in this review, current research supports the hypothesis that red palm oil plays a beneficial role in improving the wellbeing and quality of life. As the second most used edible oil, it is rich in carotenoids, vitamin E, tocotrienols and other micronutrients. It has been shown to reduce the risk of atherosclerosis, can act as a therapeutic agent, enhances intestinal uptake of proteins, plays a role in the metabolism of sulphur amino acids and promotes reproductive capacity. It contains almost equal amounts of saturated and unsaturated fatty acids with negligible amounts of the hypercholesterolemic fatty acids, lauric and myristic acids while it is moderately rich in the hypocholesterolemic monounsaturated oleic acid and has an adequate amount of linoleic acid. It should be noted that the majority of the properties and effects linked to red palm oil are believed to be mediated via its main triglyceride and minor non-glyceride components but also by the peculiar stereochemical configuration of the fatty acid isomeric position. These unique properties make red palm oil a safe and edible oil which is rich in nutritional, physiological, therapeutic and biochemical benefits.

RECOMMENDATION

In view of this review, the following is recommended;

i. Moderate intake of red palm oil is recommended as excess may promote high cholesterol levels thereby increasing the risk of cardiovascular disease.

ii. Heating and frying is not encouraged as the practice of too high heating of red palm oil increases the possibility for deteriorative changes in the oil which include oxidation and the formation of toxic by-products. Heating and frying also affects the stability of red palm oil.

iii. Further research studies are needed in both areas of regular and iiegular consumption of red palm oil for comparative purposes.

iv. There is a need for future studies to examine the role of red palm oil in reducing oxidative stress and improving wellbeing in patients with HIV, TB and diabetes.

v. We recommend further studies on red palm oil should include a large sample size, longer duration and be randomized-controlled because of the limitations of observatory studies with a small sample size and short duration.

vi. There is a need to broaden the scope of research on the components of red palm oil.

vii. We recommend equivalent amounts of saturated fatty acids and polyunsaturated fatty acids in the diet with the remaining fatty acids being derived from monounsaturated fatty acids.

viii. Additional studies employing cell culture and rodent models are advocated to provide better understanding and greater insight into the potential therapeutic applications of red palm oil in humans.

ix. Further studies are needed to elucidate the mechanism of red palm oil carotenoids as a chemo-preventive agent.

x. The role of red palm oil tocotrienols as antioestrogen therapy needs to be investigated.

xi. Further investigations are necessary to elucidate the mechanisms involved in the cardio-protective role of red palm oil.

xii. More studies on the antioxidant activities (as a scavenger of free radicals, hydroxyl radicals and superoxides) of red palm oil is recommended.

xiii. More research studies are required to determine the exact receptors and ligands involved in mediating red palm oil tocotrienol-induced apoptosis.

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Multi Locus Sequence Typing (MLST): a useful tool for epidemiological mapping of MRSA and other antibiotic-resistant bacteria pathogens in Africa

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ABSTRACT

Molecular typing schemes based on a single gene locus sequencing approach are useful for bacteria that are clonal where genetic change occurs mainly by mutation with all parts of the genome evolving together, but most bacteria frequently undergo horizontal genetic exchange through recombination. Single locus sequencing scheme may mislead when studying evolutionary history of resistant clones. Multi-Locus Sequence Typing (MLST) was developed as a typing method by indexing the variation seen in the nucleotide sequences of multiple housekeeping genes located around the bacterial genome. It has been validated for *Neisseria meningitidis, Staphylococcus aureus,* Enterococcus, *Streptococcus pneumoniae* and a number of other bacteria species, as well as fungi. The scheme is highly discriminatory and characterizes bacterial isolates on the basis of the nucleotide sequence of ~450-bp internal fragments of 7 house-keeping genes and has ability to compare sequence data between laboratories via the internet. Every unique allele at each of the housekeeping loci is assigned an arbitrary number in the order of discovery, resulting in a MLST profile of seven numbers. Each unique profile, which is a combination of alleles, is then assigned a sequence type (ST) number and because there are many alleles at each of the 7 loci, isolates are highly unlikely to have identical sequence (allelic) profiles by chance. Isolates with the same ST are assigned as members of the same clone and STs sharing 100% genetic identity in at least 5 loci can be grouped into a clonal complex (CC). MLST has high resolution similar to Pulse Field Gel Electrophoresis (PFGE) but with easier interpretation and comparison. It also allows easy identification of genetic variations among isolates and is applicable where no isolate is available unlike PFGE. The application of MLST scheme, which is particularly ripe in Africa where evolutionary biologies of most bacteria are generally lacking, is discussed in this review.

Keywords: MLST, Evolutionary biology, Housekeeping genes, MRSA, Africa

INTRODUCTION

Epidemiological typing of bacteria is necessary to identify outbreaks and to study evolution and dissemination of resistant clones. Ideal typing method must have high discriminatory power, typeability, high reproducibility, applicable to all organisms of interest, easy to use, capable of generating data that can be easily transferred between laboratories, publish in research journal and the internet and should be cost effective (Struelens, 1996).

Typing methods could be based on phenotypic or genotypic characteristics. Methods based on phenotypic characteristics include biotyping, antibiogram, serotyping, protein electrophoresis including multi-locus enzyme electrophoresis (MLEE), phage and pyocin typing. Those based on

genotypic (DNA) characteristics may be non-polymerase chain reaction (non-PCR) method, PCR method and nucleotide sequencing method. Several of the non-PCR methods include; restriction fragment length polymorphism, restriction endonuclease analysis of plasmid or chromosomal DNA, ribotyping and pulse field gel electrophoresis (PFGE) of chromosomal DNA digest. There are several versions of the PCR based methods amongst which include arbitrary-primed PCR (AP-PCR), repetitive element PCR, amplified fragment length polymorphism (AFLP) and PCR-ribotyping. Nucleotide sequencing methods which are recently introduced could be based on sequence analysis of a single gene locus, multiple gene loci or a whole genome.

Molecular typing schemes based on a single gene locus sequencing approach are useful for bacteria that are clonal where genetic change occurs mainly by mutation and where all parts of the genome evolves together. But, most bacteria especially the rapidly dividing ones frequently undergo horizontal genetic exchange by recombination. In this case, single locus sequencing scheme may mislead when studying evolutionary history of resistant bacteria clones.

Multilocus sequence Typing

Multi-locus sequence typing was developed by Maiden *et al* (1998) as a typing method by indexing the variation seen in the nucleotide sequences of multiple housekeeping genes located around the bacterial genome. MLST has been validated for *Neisseria meningitidis* (Maiden *et al*, 1998), and for many other bacteria such as *Staphylococcus aureus, Enterococcus faecalis* and *Enterococcus faecium, Streptococcus pneumoniae* (Enright *et al,* 2000) and *Escherichia coli* (Lau *et al,* 2008). The list of bacteria for which MLST scheme has been validated is increasing. MLST is highly discriminatory and characterizes bacterial isolates on the basis of ~450-bp internal fragments of 7 house-keeping genes. It has ability to compare sequence data between laboratories via the internet.

MLST Procedural Steps

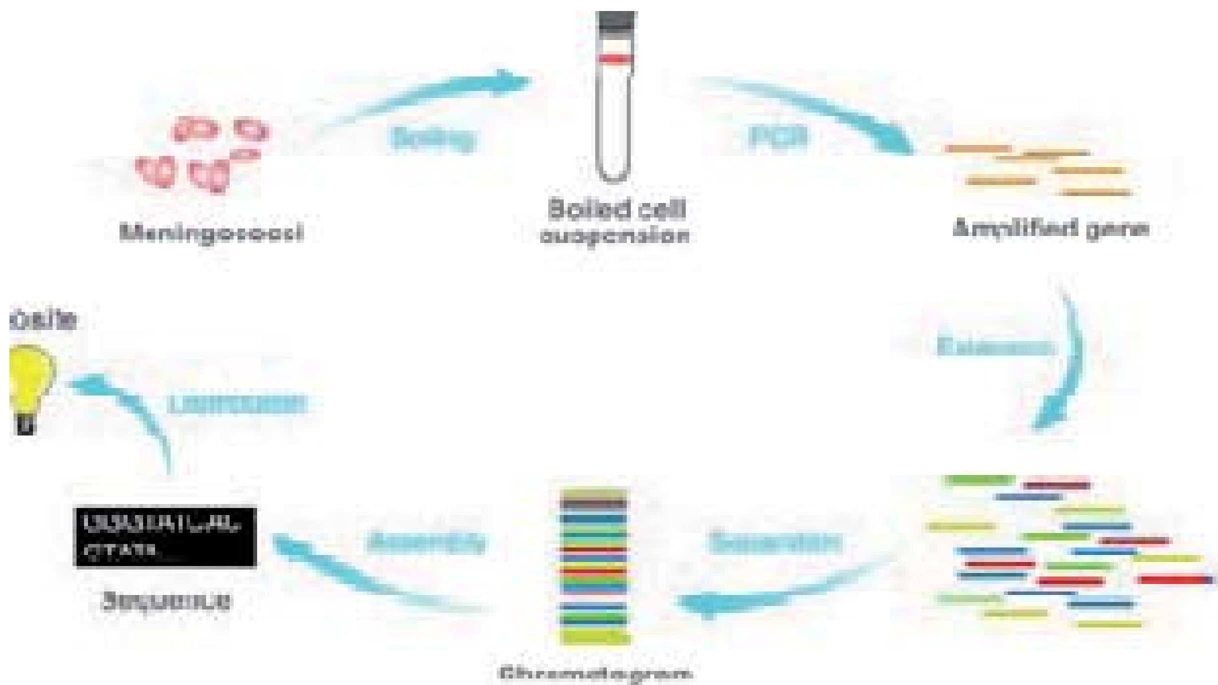
The MLST procedure is extremely simple and involves the following sequential steps (Maiden, 2006);

1. DNA Extraction. Different methods for rapid extraction of DNA are now available and an optimal extraction method is required.

2. PCR amplification of each housekeeping gene followed by purification and trimming of PCR amplicon using 2 set of primers designed for each gene

3. Cycle sequencing by dideoxy sequencing using the same primers used for initial PCR but with a mixture of deoxy nucleoside triphosphates (dNTPs) and a chain terminating dideoxy nucleoside triphosphates (ddNTPs). The ddNTPs are incorporated at different points resulting in different sized fragments. To enable the detection of the products the ddNTPs are labelled with 4 different fluorescent dyes.

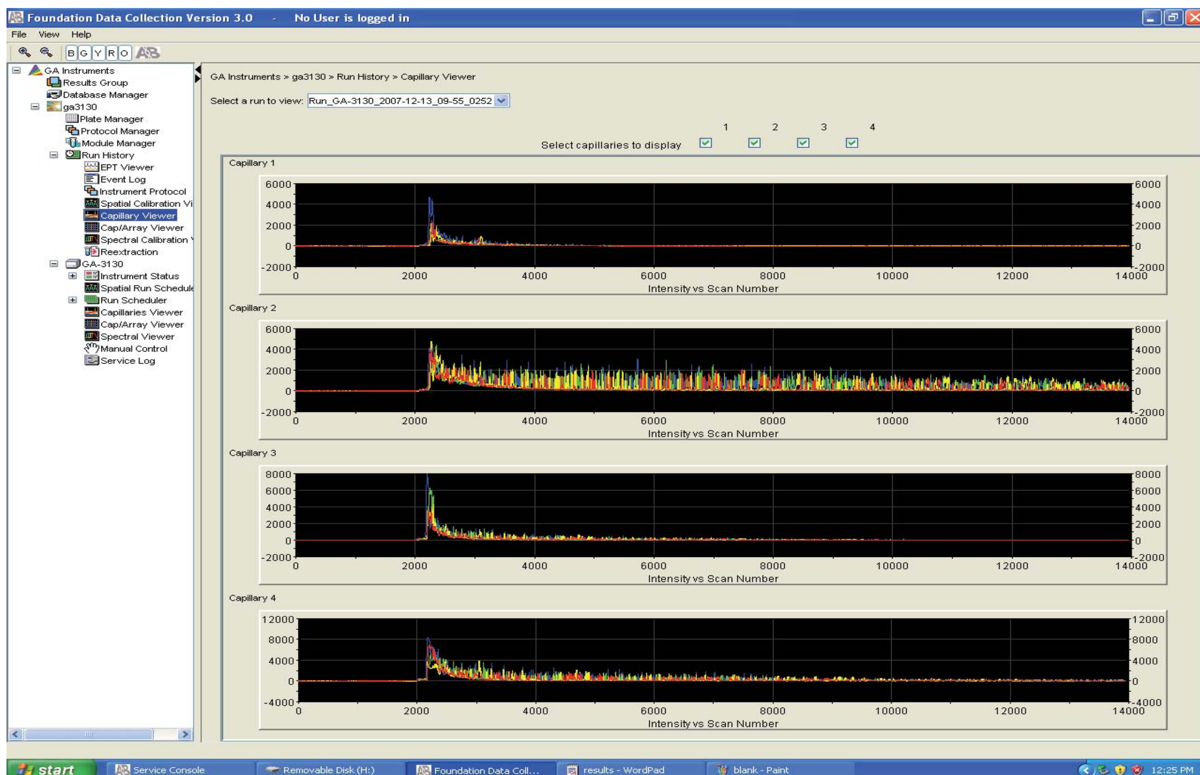
Fig 1: MLST procedural steps (Maiden, 2006)

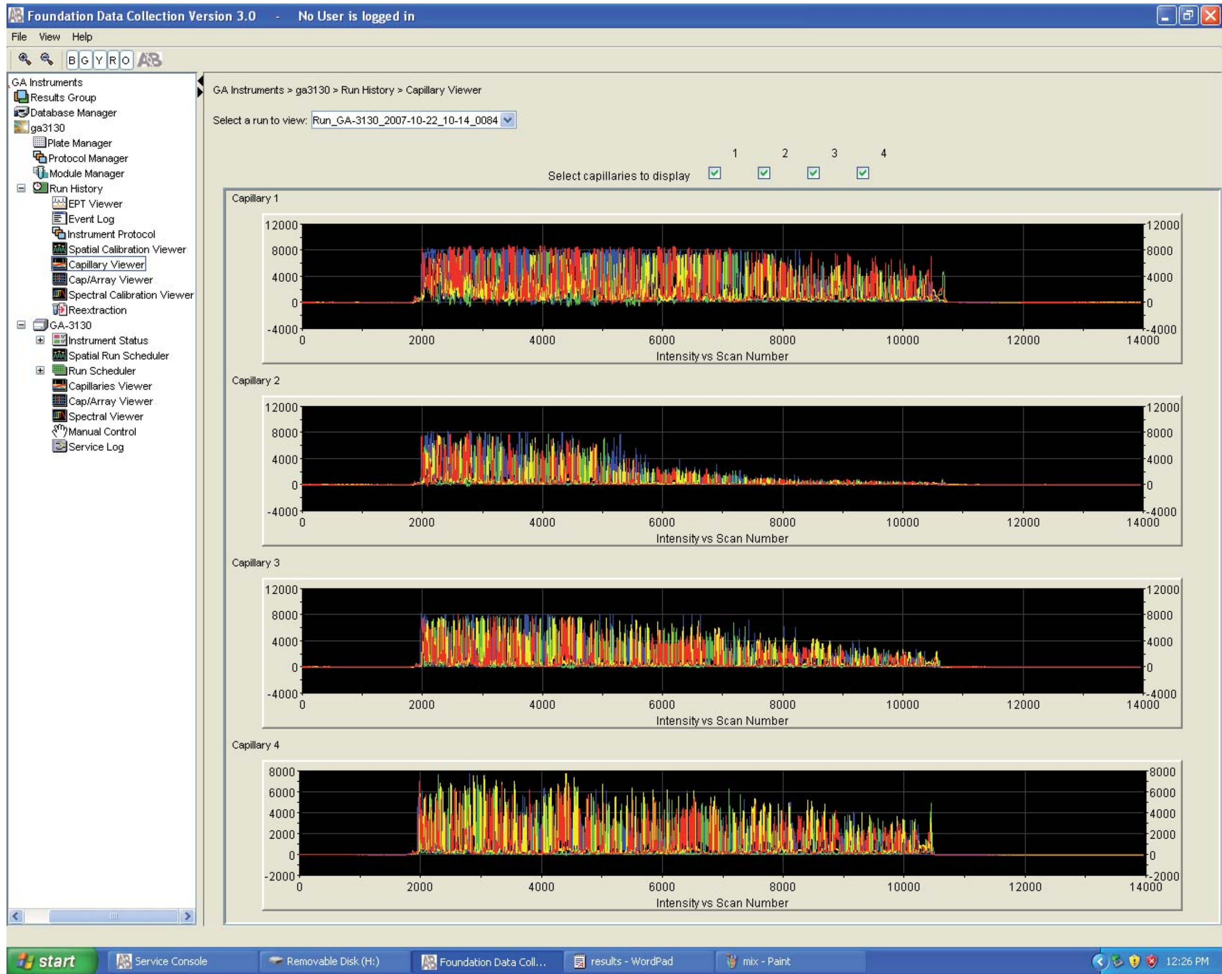


4. Precipitation, separation and detection of sequenced products are done using automated sequencer. Detection could be gel based (Applied Biosystem ABI 377), capillary detection now mostly in use (ABI 3730), progressive detection (Solexa/Illumina) and Next Gen parallel sequencing for large scale whole genome sequencing such as Roche 454 (www.454.com, Genome Sequencer FLX system), Illumina/Solexa (www.illumina.com, Genome Analyzer) and ABI (www.appliedbiosystem.com, SOLiD).

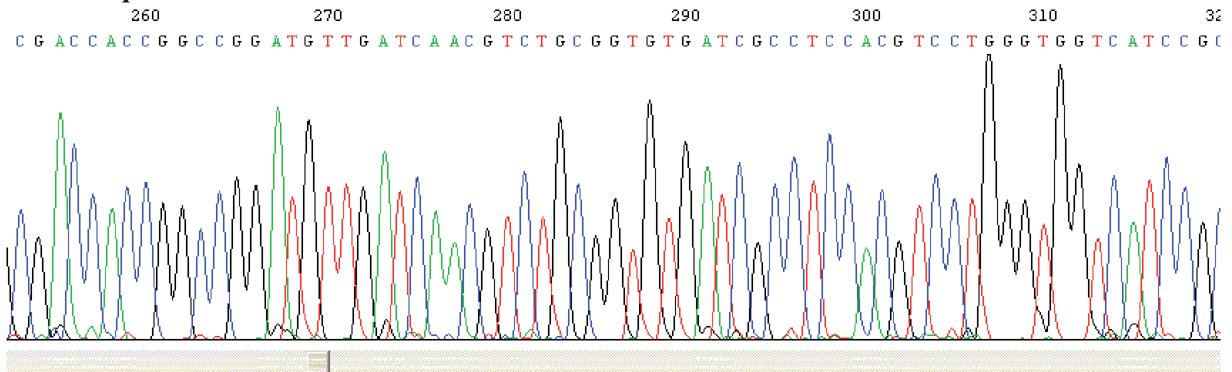
Fig2: ABI 3730 Sequencer

Fig 3: Chromatogram generated by the sequencer





Good sequence



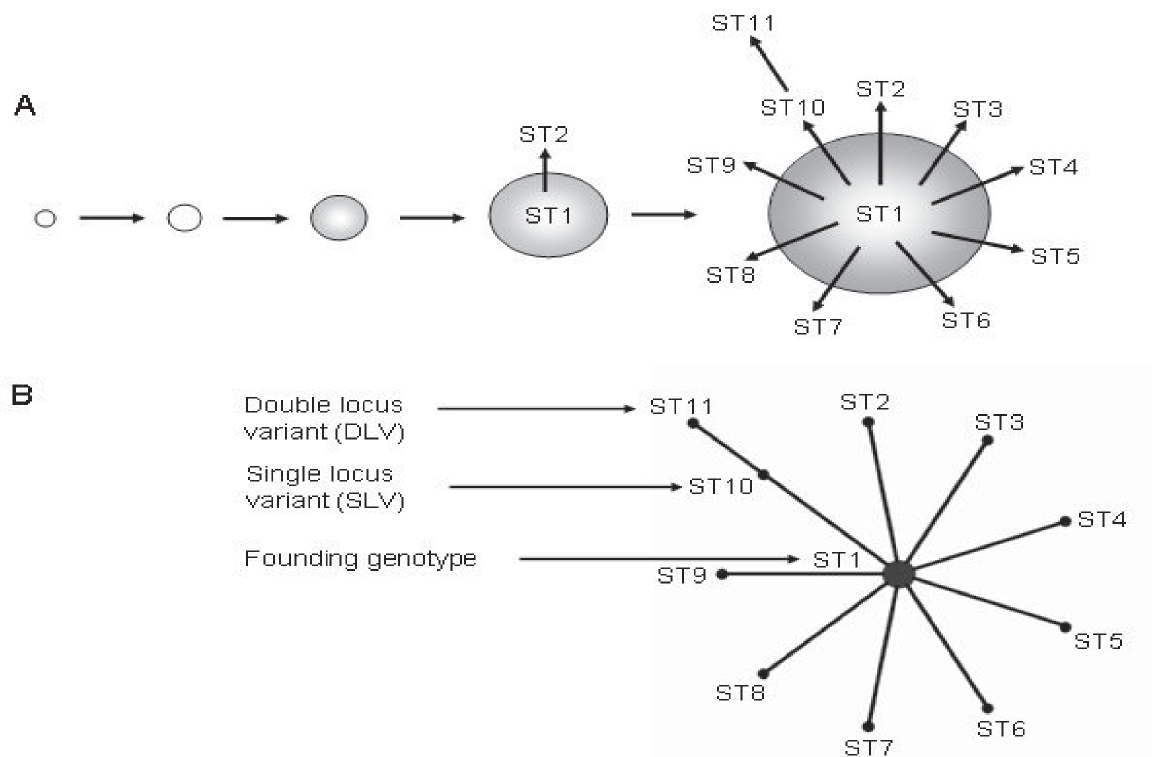
5. MLST sequence assembly, analysis and data base searches can be achieved using different software packages designed for genome analysis such as;

i. Staden (Pregap4, Gap4) package (http://staden.sourceforge.net/)
ii. BioNumerics (http://www.bioinformatics.org/sms/rev_comp.html)
iii. Finch TV (http://www.geospiza.com/finchtv.html)
iv. Bio-Linus 5.0 running on Ubuntu (http://nebc.nox.ac.uk)
v. Artemis and Artemis genome comparison tool (ACT) recently developed at the Pathogen Sequencing Unit (PSU) of Sanger Institute.

MLST data searches for different bacteria can be done on the public sites such as www.pubmlst.org, or on institution based sites such as http://www.mlst.net and http://web.mpiib-berlin.mpg.de

6. Interpretation of MLST scheme is done by assigning every unique allele at each of the housekeeping loci an arbitrary number in the order of discovery, resulting in a MLST profile of seven numbers. Each unique profile (combination of alleles) is assigned a sequence type (ST) number. As there are many alleles at each of the 7 loci, isolates are highly unlikely to have identical sequence (allelic) profiles by chance. Isolates with the same ST are assigned as members of the same clone. Relationship between closely related isolates of a bacterial species or population can be displayed using program such as BURST algorithm (http://eburst.mlst.net) for example STs sharing 100% genetic identity in $\geq 5/7$ loci can be grouped into a clonal complex (CC). The evolutionary descent from the founding genotype can be predicted and display as a radial diagram, centred on the predicted founding genotype (Fig 4)

Fig 4: e-BURST diagram from staphylococcus MLST website (www.mlst.net)



Why MLST in Nigeria and Africa?

Molecular epidemiological data on MRSA and most other bacteria in Nigeria and most parts of Africa and the evolutionary biologies of antibiotic resistant bacteria are generally lacking. Policy formulations on prevention and control of infectious diseases, especially bacteria, in Nigeria are lacking or when present are not based on sound molecular data. Nigeria is majorly excluded from global support for lack of data.

MLST has high resolution similar to PFGE in establishing relatedness of bacteria and detection of outbreaks but with easier interpretation and comparison (Feavers et al, 1999). The unambiguous nature of the DNA sequences which can be stored in internet based sites makes MLST portable and robust as a typing scheme. Identification of genetic variations among isolates is easy especially where recombination has occurred, a situation that makes PFGE band patterns very difficult to interpret.

MLST is more suitable for long term bacteria epidemiology and for monitoring evolution and spread of antibiotic-resistant clones. For example, the intercontinental dissemination of resistant clones of MRSA has been monitored using MLST. The International Union of Microbiological Societies subcommittee on *S. aureus* typing and the EU-funded Harmony project (Cookson et al, 2007) has adopted MLST/SCC*mec* typing for further characterization and assignment into clonal groups of representative isolates that have been identified by PFGE and/or Spa typing to be genetically related.

Unlike PFGE and most other typing schemes, MLST is applicable where no isolate is available. This is particularly important in Nigeria where bacteria from samples may be difficult to grow on cultures from previous unauthorized use of antibiotics which are often available over-the-counter to the populace. It is equally useful for fastidious bacteria such as *Neisseria meningitidis, Streptococcus pneumoniae, Haemophilus influenzae, Bordetella pertussis,* which are prevalent in Nigeria but are difficult to maintain in stored cultures because of fluctuating electricity. In this case, what is required is simply to boil the bacteria isolates in a tube to inactivate deoxyribonuclease (DNAse) while the DNA is preserved until use.

In most countries of Africa, the distributions of antibiotic resistant clones of most bacteria are unknown. At present, MLST appears to be the most appealing and on the long run the most economical to use in Africa.

What must we do in Africa?

There is need for collaborative works between different interest groups in different countries of Africa, organization of training and workshops on the use and application of MLST for different organisms, seek international support in term of grants, sponsorship and fellowship training, and networking with formation of African bodies for infectious diseases prevention/control, and Nigeria must be not be excluded.

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Molecular diversity among cassava bacterial blight caused by *Xanthomonas axonopodis pv manihotis* isolated from the Northern region of Nigeria

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ABSTRACT

Xanthomonas axonopodis pv manihotis (*Xam*) is the causal agent of cassava bacterial blight (CBB) in all cassava growing areas of the world. CBB disease is a major constraint to cassava cultivation, and losses can be extremely severe in regions where highly susceptible cultivars are grown. To build up a well-organized disease management approach, the genetic diversity of the pathogens population ought to be known. Information is scarce on the genetic diversity of *Xanthomonas axonopodis* pv *manihotis* population in Nigeria. We used AFLP (Amplified Fragment Length Polymorphism), a novel PCR-based technique, to characterize the *Xanthomonas axonopodis* pv *manihotis* isolates from the Middle belt and Northern States of Nigeria. Thirty-six strains *Xam* and 2 reference strains were tested with eight primers combination of AFLP. The 36 strains and two reference strains were analyzed with 8 primers of AFLP and the data generated were analyzed with NTSYS-PC version 2.0. Principal component analysis of the data was carried out using SAS version 8.2 to revealed a scatter-gram plot of the isolates. Numerical analysis differentiated the AFLP patterns into eight distinct clusters at 65 % similarity coefficient level. Cluster 1, 3, and 4 had the 73.7% of the total bacterial strains grouped together while the other clusters had two strains each shared the remaining 26.3 %. Cluster 3 had similar strains from different states together and had the 29% of the entire clusters while cluster 4 grouped strains from only two geographically related states together. The genetic analysis presented here contributes to understanding of the *Xam* population structure in the northern region of Nigeria and will help in developing resistant cassava cultivars for this agro-ecological zone.

Keywords: DNA, genetic diversity, dendrogram analysis, AFLP

INTRODUCTION

Bondar, (1912) was the first to report the presence of cassava bacterial blight in Brazil as a bacterial disease of cassava. It was thus believed that cassava bacterial blight (CBB) originated from Brazil and later spreads to other countries in South America, Africa and Asia (Lazano, 1973). The causal organism of cassava bacterial blight disease was first named *Bacillus manihotis* (Arthaud-Berthet) Starr (Lazano, 1973) then *Phytomonas manihotis* (Arthaud-Berthet and Bondar) viegas. The name was later changed to *Xanthomonas manihotis* (Arthaud-Berthet) Starr and one edition of Bergey's manual proved over a hundred different pathogens including *Xanthomonas cassavae* (yellow colony bacterium) and *Xanthomonas manihotis* as pathovars under *Xanthomonas campestris* (PNAS, 1978). Most recent nomenclature categorised *X. campestries pv manhotis* as a synonym of *X. axonopodis* pv *manihotis* (Vauterin et al, 1995).

Xanthomonas axonopodis pv manihotis (*Xam*) is the causal agent of cassava bacterial blight (CBB) in all cassava growing areas of the world. CBB disease is a major constraint to cassava cultivation, and losses can be extremely severe in regions where highly susceptible cultivars are grown. The disease has a wide spectrum of symptoms including angular leaf sport, blight, wilting, defoliation, vascular necrosis of the stem, stem lesions, production of exudates, tip die-back and plant death (Maraite, 1993; Verdier, 1988; Lazano, 1986). The combination of cassava bacterial blight symptoms mention above is unique among disease caused by plant pathogenic bacteria. Primary symptoms resulting from planting infected material are wilting of the young germinated sprouts; follow shortly by die back. Secondary symptoms are usually angular leaf sports followed by blight, defoliation, wilting of the immature shoot and finally die back. Initially, leaf spots are water-soaked, angular lesions clearly distinguishable on the abaxial surface of the leaves (Verdier et al, 1994). These sports become brown and are sometime surrounded by distinct yellow halos depending on susceptibility of the cultivars. The disease is a major biotic constraint for cassava production in all cassava-cultivating regions of the world (Lazano, 1986). Effective breeding for resistance to this disease requires information on the diversity and geographical distribution of the pathogen. In Africa, *Xam* populations were considered to be homogenous (Verdier et al, 1993), though recently, some genetic variation has been detected with RAPD markers (Assigbetsé et al, 1998; Ogunjobi et al, 2006). There is dearth of information on the genetic diversity of *Xanthomonas xonopodis* pv *manihotis* population in Nigeria. Genetic relationships among 36 isolates of *Xanthomonas axonopodis* pv. *manihotis* from the same geographic regions were determined by PCR method to ascertain the homogeneity of the Bacterial population within this region in Nigeria.

MATERIALS AND METHODS

All the *X. axonopodis* strains were obtained from the field collection in the survey of all agroecological zones of Nigeria (Ogunjobi et al 2001). The bacteria were isolated and identified using basic microbiological technique and biochemical test. The results were compared with Bergey's manual determinative bacteriology (Buchanam & Gibbons, 1974). The Isolates were inoculated on susceptible cassava cultivar to determined their ability to initiate infection with similar symptoms of CBB on cassava plant an indication that they are *Xanthomonas axonopodis* pv. *manihotis*

Bacterial Strains and DNA Isolation:

We used 36 field isolates collected in Western Nigeria in year 2000 and two reference strains from German Collection of Microorganisms and Cell Cultures (DSMZ). The bacteria were cultured on yeast extract dextrose peptone agar (YDPA) containing 5g yeast extract, 10g dextrose, 5g peptone and 15g agar per litre of distilled water (pH 7.2).

Extraction of genomic DNA of bacteria:

The cells of the bacteria were harvested from 1.5ml suspension of cell by low speed centrifugation at 1200xg for 2 minutes. The pellet was washed in 1ml of 5M NaCl and once in 1ml TE buffer (10mM Tris-HCl, 1mM EDTA (pH8)). Genomic DNA was extracted by the

method of Sambrook & Russell (2001). The DNA quality was checked on agarose and quantified with DNA fluorometer (model TD-700).

Amplified Fragment Length Polymorphism (AFLP) Restriction Endonuclease Digestion:

The Genomic DNA was quantified and diluted to 50ng/µl. AFLP marker was assayed as previously described by Restrepo et al (1999), with some modification. About 15µl of the diluted DNA was digested with EcoR1 and Mse1 restriction endonuclease simultaneously. EcoR1 has a 6-bp recognition site while Mse1 has a 4-bp recognition site. When used together, these enzymes generate small DNA fragments that will amplify well and are in the optimal size range (<1kb) for separation on denaturing polyacrylamide gels. The digested samples were then ligated at 20^{o}C for 2 hours to the respective adapters (Ogunjobi, 2005). Ten microlitre of the ligated mixture was added to 90µl TE buffer and mixed well. Five microlitre of the ligation reaction dilution was pre-amplified for 20cycles at 94^{o}C for 30sec; 56^{o}C for 60sec; 72^{o}C for 60sec and soaked temperature was 4^{o}C. In the first reaction, genomic DNA was pre-amplified with AFLP primers with no selective nucleotides (Primers E+0; M+0) [GIBCO BRL products]. The PCR products of the pre-amplification reaction were diluted in ratio 1:50 and 5µl of this was used as the template for the second amplification using two AFLP primers containing either zero, one or two selective nucleotides. This reaction mixture was introduced into PCR machine and run 23 cycles of 94^{o}C for 30sec; 56^{o}C for 30sec; 72^{o}C for 60sec. After the selective amplification the PCR products were mixed with equal volume of loading buffer (formamide dye; 98% formamide, 10mM EDTA, bromophenol blue, xylene cyanol). This was heated in the PCR machine to denatured DNA at 95^{o}C for 3min and immediately placed on ice to chill (Ogunjobi, 2005).

Six percent polyacrylamide gel (20: 1 acrylamide: bis; 7.5 M Urea; 0.5X TBE buffer) with 0.4 mm spacers and sharks-tooth combs was prepared on glass plate gel. The gel was pre-electrophoresed at constant power (Bio RAD, Model: 3000Xi computer controlled electrophoresis power supply) for about 20 - 30 minutes. About 5µl of each sample were loaded on the gel and electrophoresed at constant power until xylene cyanol (slower dye) is two-thirds down the length of the gel which takes about two hours. Thereafter the gel was stained in silver staining solution and de-stained in double distilled H_2O. The plates were thereafter dry at room temperature overnight. The gel was thereafter scored for polymorphism on fluorescence light pack and scanned for record purposes.

Data Analysis

AFLP gels were scored usually for the presence or absence. A band was considered polymorphic if it was absent in one or more of the isolates studied. The data generated was analyzed using Numerical Taxonomy and multivariate analysis system (NTSYS-PC 2.0 version) computer software. This involved clustering, using Unweighted Pair Group Method of Arithmetic Average (UPGMA) to obtain a phylogenetic tree or dendrogram. Principal component analysis of the data was carried out using SAS version 8.2 of 2004, which revealed the closeness and variations that existed among the bacterial population in a scatter-gram plot

RESULTS

The sampling location where the bacteria were isolated from diseased cassava leaves is shown in Fig 1. All the states in the middle belt and northern regions were represented in the survey. AFLP analysis with the combination of restriction endonucleases *EcoR1* and *Mse1* revealed a considerable degree of genomic heterogeneity among the 38 *Xanthomonas* strains tested. When the 141 polymorphic AFLP bands were used for the cluster analysis using the un-weighted pair group arithmetic means analysis (UPGMA) program of NTSYS-PC (version 2.0), a high level of polymorphism was shown in the pathogen population. The dendrogram in Fig. 2 shows the genotypic variability among the Xam strains analysed with AFLP.

Numerical analysis differentiated the AFLP patterns into eight distinct clusters, representing strains from different state grouped together in one cluster. The similarity patterns of different clusters showed less than 50 % in similarity coefficient. Cluster 3 is the largest containing 29 % of the entire bacterial strains from 5 different states within the northern region of Nigeria. Cluster 1 gather 9 bacterial strains representing 23.7% from 7 states within the study area. Cluster 4 grouped 8 bacterial strains from 4 states. The *Xanthomonas campestris* pv *malvacerum* used as reference organisms were also grouped in cluster 5 and were very closely related than the pathogen of cassava, having a similarity coefficient level of 96 % and were cluster separately. Clusters 2, 6, 7, and 8 had only two strains each from the same state or geographically closely related states. The principal component analysis revealed a different clustering from the phylogenetic tree. Five clusters were identified in the PCA analysis, grouping Adam 124B with the reference bacteria from Germany in cluster 1. Cluster 3 also had the largest number bacteria grouped together in this scatter plot. It is instructive to know that cluster 1 and 4 had three bacteria each similar to cluster 5 to 8 in the dendrogram which contain the same number of strains.

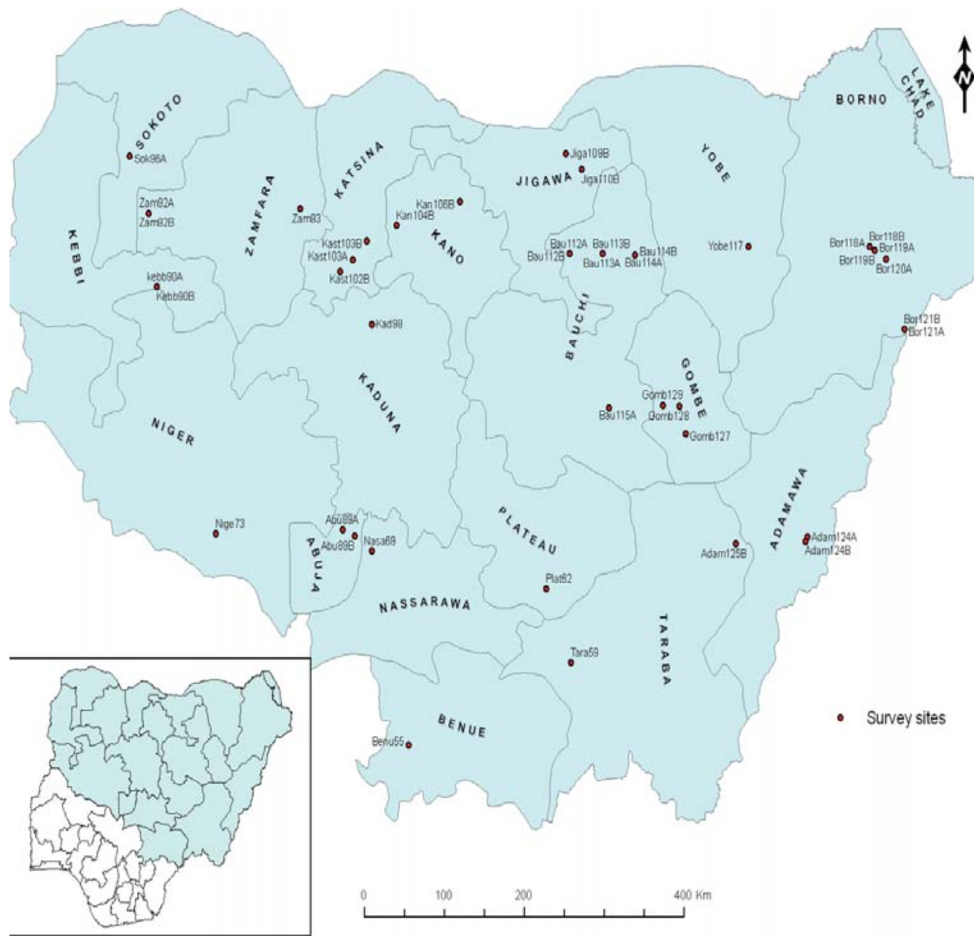

Fig 1: Nigerian Map showing the Northern region and location where samples were obtained for the study

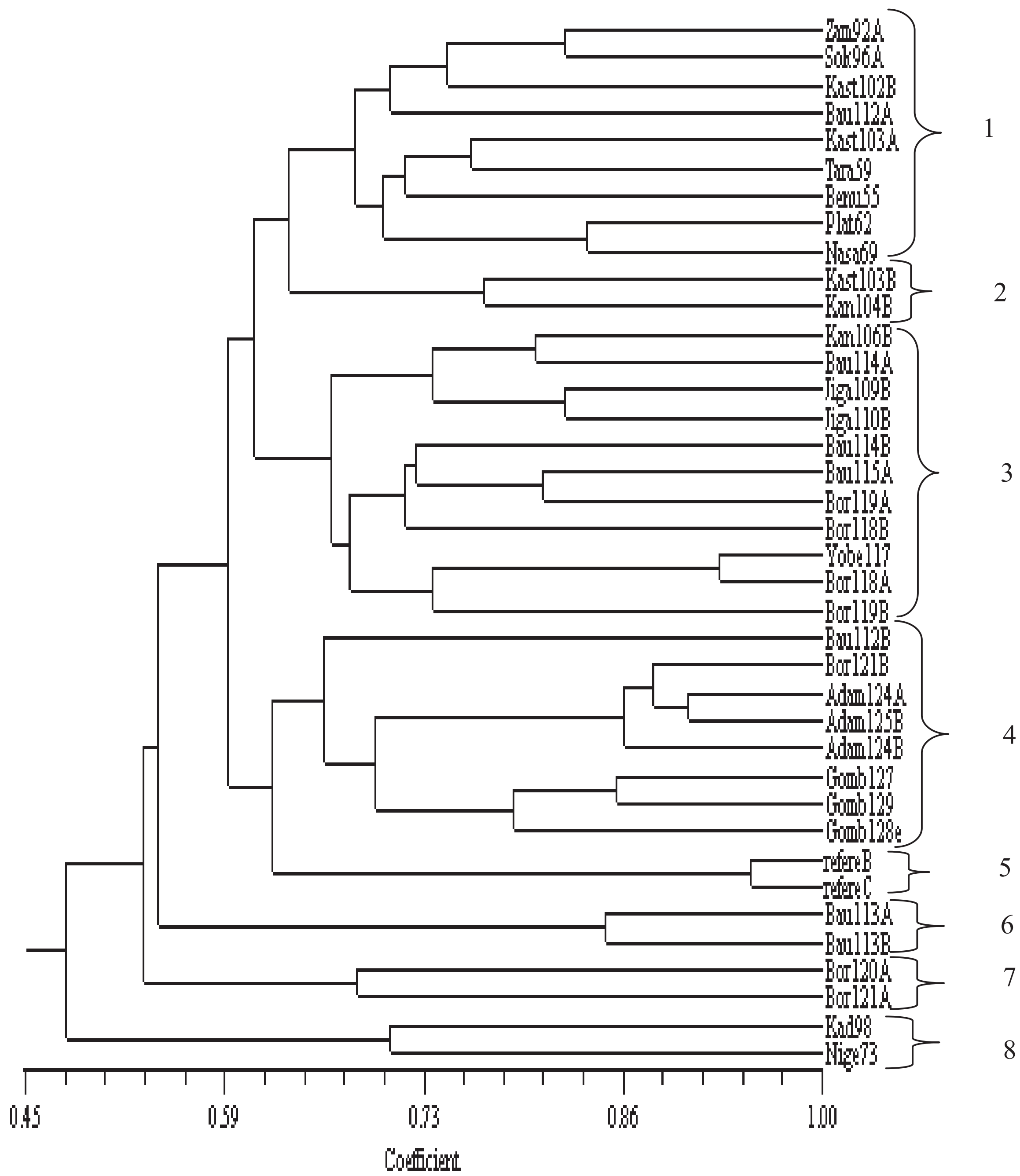

Fig 2: The dendrogram generated from the AFLP data analysis showing the relationship of the of the pathogen population from the Northern region of Nigeria

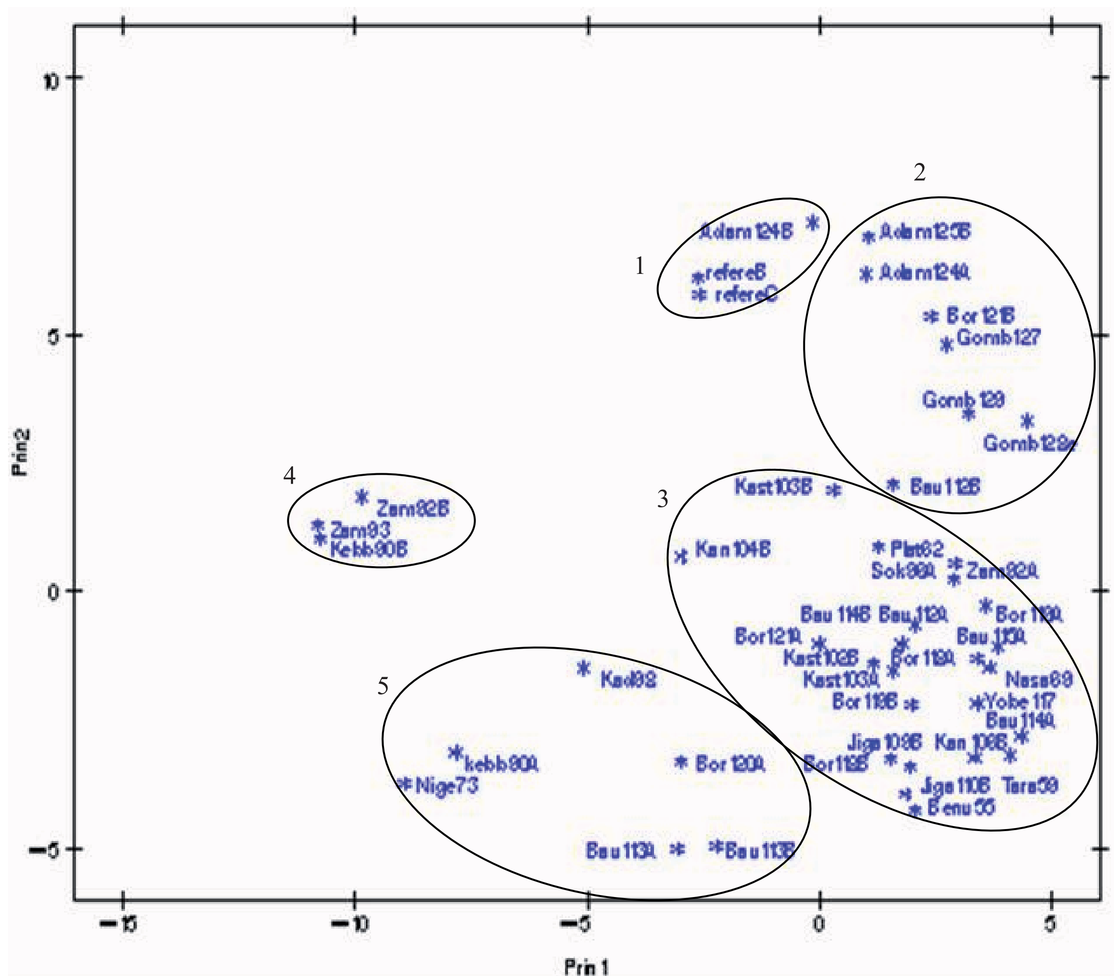

Fig 3: Principal component analysis of the Amplified Fragment Length Polymorphism banding patterns of *Xam* isolates showing the level of relatedness and diversity among the bacterial populations in Northern regions of Nigeria.

DISCUSSION

The genetic analysis presented here contributed to our effort in understand the genetic structure of the *Xam* population in Northern region of Nigeria and will help in selecting strains of the pathogen for screening cassava germplasm for resistance to the disease within the ecological area. Our study showed that the regional *X. axonopodis* pv. *manihotis* population had a high degree of genetic variability. The results of the AFLP analysis effectively and efficiently provided quantitative estimates of genetic similarities related to the distribution of variability among *Xanthomonas axonopodis* pathovars. A higher level of polymorphisms was shown in the pathogen population structure with this molecular technique. This is similar to the recent work

carried out on the same bacteria in other parts of Nigeria with different molecular maker (Ogunjobi et al, 2006; Ogunjobi et al, 2007).

The *Xam* strains were grouped into eight separate clusters in this study at a similarity coefficient level of 65 %. This signifies that the *Xam* populations in these ecological areas of Nigeria are not identical as it had reported earlier by some workers who reported the uniformity in the population of the bacteria in African and that heterogeneity was limited to Southern American countries where cassava was said to be indigenous (Ikotun, 1995; Persley, 1980; Verdier, 1988; Restrepo & Verdier, 1997). The PCA also grouped the bacteria into five clusters revealing that the bacteria genomic composition is not identical. AFLP technique proved to be an extremely useful and reliable method for detecting polymorphism in bacterial populations and its reproducibility has been reported to be very high (Vos et al, 1995).

A complex and informative fingerprint can become useful by making small changes in the primer sequence. Restrepo et al (1999) confirmed that a suitable choice of restriction enzymes and the number and base composition of selective bases determined the usefulness and applicability of AFLP fingerprints in diversity studies. AFLP genomic fingerprinting analysis has been shown to be an accurate approach for determining bacterial taxonomy (Restrepo et al, 1999; Ogunjobi, 2005) and the phylogenetic structure of bacteria, the discriminatory ability of the technique is confirmed in this study in which *Xam* that has been shown to be very difficult at biochemical and physiological methods (Ikotun, 1995; Ogunjobi, 2005) are discriminated by this technique.

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Nasal carriage of multi-drug resistant *Staphylococcus aureus* among HIV sero-positive patients

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ABSTRACT

Nasal carriage of *Staphylococcus aureus* appears to play an important role in the epidemiology and pathogenesis of infection in patients with human immunodeficiency viral (HIV) infection. *S. aureus* carriage is an important risk factor for invasive disease. The objectives of this study are to determine the nasal carriage rate of *S. aureus* and antibiotic susceptibility patterns among asymptomatic HIV patients in Mainland General Hospital out patient's clinic, Lagos Nigeria. Nasal swabs were collected from 187 HIV patients (36 male and 151 female between the ages of 18-60 years) after obtaining ethical approval and informed consent. *S. aureus* was isolated after culture in appropriate culture media and incubated at 37°C overnight. The resultant colonies were identified by Gram stain, catalase, coagulase and mannitol fermentation, and confirmed by PCR amplification of COAG 1&2 gene followed by agarose gel electrophoresis. Antibiotic susceptibility test was performed on pure cultures of isolated organism using disk diffusion method according to the National Committee for Clinical Laboratory Standards guidelines. Descriptive analysis was done and results analysed using SPSS version 14.0. A total of 65 (34.8%) samples yielded *S. aureus,* 12 (18.5%) from male and 53 (81.5%) from female ($P > 0.503$). Genotypic identification of COAG 1&2 gene shows two PCR products of 600 and 900 bp. The isolates were resistant to ampicillin (100%), penicillin (92.3%), cotrimoxazole (91.4%), tetracycline (76.3%), amoxycillin (73.5%), cloxacillin (73.3%), chloramphenicol (64.4%), gentamycin (50.6%), augmentin (49.2%), streptomycin (38.7%) and erythromycin (28.6%), but were susceptible to ceftazidine and ciprofloxacin (100%), vancomycin (96.7%) and methicillin (78.7%). The results shows that carriage rate of resistant *S. aureus* is common in HIV patients and cotrimoxazole currently used as prophylactic against opportunistic infections in HIV patients may be the cause of increase in cotrimoxazole resistance in this population. The findings suggest that ceftazidime and ciprofloxacin may be the best antibiotic of choice in cases of serious *S. aureus* infection in HIV patients.

INTRODUCTION

Staphylococci are considered as one of the most common cause of bacterial infections in patients with acquired immune deficiency syndrome (AIDS). HIV 1 and 2 are the causative agents of AIDS, but most of the morbidity and mortality in AIDS cases result from opportunistic infections. Identification of such pathogen is very important for clinicians and health planners to tackle the AIDS epidemic in a more effective manner (Singh, 2003).

Staphylococci are ubiquitous micro-organisms present in the respiratory tract and on the skin of high percentage of adults (Dale *et al.*, 1997). Carriage rates of *Staphylococcus aureus* have been demonstrated to be higher in patients infected with HIV-1 than in HIV negative (Weinke *et al.*,

1992). Nasal carriage of *S. aureus* has been linked to infection with this organism in the settings of haemodialysis, peritoneal dialysis, post operative surgical wound infections and nursing homes (Kluytmans *et al.,* 1996). HIV infection has been associated with an increased incidence of nasal colonization with *S. aureus* (Holbrook *et al.,* 1997) and it has also been observed that HIV/AIDS patients who have weakened immune system are at special risk of staphylococci infection (CDC, 2003). Nasal carriage in patients with AIDS and AIDS-related complex (ARC) increased the rates of *S. aureus* bacteremia, deep soft tissue infections as well as recurrent infections (Jacobson, 1988).

Nasal carriage plays a key role in the developments of *S. aureus* infections despite the use of cotrimoxazole prophylaxis. These populations of people still come down with one form of opportunistic infection or the other and determining the carriage rate in HIV patients and antibiotic susceptibility is important, so that high risk patients, who may benefits from preventive strategies can be identify and properly managed.

MATERIALS AND METHOD

Study design

A cross sectional study in which nasal swabs were collected aseptically from HIV patients attending the outpatient's clinic of Mainland General Hospital in Lagos, Nigeria after obtaining ethical approval and informed consent. One hundred and eighty seven HIV patients were recruited with 36 (19.3%) from male and 151 (80.7%) from female in the age range 18 - 60years.

Sample collection

The nasal swabs were collected from anterior nare using the modified method by Falanga et al (1985). This is done by removing the swabs from the plastic packaging, and the tip of the swab inserted into the vestibule (anterior nare) to the nasal mucosa of about 2cm from the nostril and this was gently rotated three times clockwise and three times anticlockwise to collect the secretions. The swabs returned into plastic transport container and immediately, 0.5ml of sterile saline is added to the sample.

Laboratory procedures

The samples were cultured on Chocolate agar, MacConkey agar and Mannitol salt agar and incubated at 37°C aerobically overnight. The resultant colonies were identified as *S. aureus* by Gram stain, catalase, coagulase and mannitol fermentation (Cowan and Steel, 1993).

Chromosomal DNA was extracted using the modification of a simple protocol described by Ravinder *et al.*, (2008). Cells were grown on Mueller-Hinton broth overnight and then harvested, washed twice in normal saline and centrifuged at 13,000 rpm at 10°C for 15 minutes. 200 µL of a solution containing 10 mM Tris-HCl (pH 8), 2.5 mg/ml of lysozyme and 2 µg/ml lysostaphin was added, and the mixture was mixed by vortexing and incubated at 37°C for 2 hours. After incubation, 400 µL of lysis buffer (50 mM Tris-HCL, 100 mM EDTA, 1% SDS, pH 8) and 1mg/ml of proteinase-K were added to the cell mixture. The samples were mixed by inverting tubes and incubated at 50°C for 1 hour in a water bath. Digestion with proteinase-K was

followed by the addition of 500 µL of a mixture of phenol: chloroform (1:1). This was mixed by vortexing and the debris was removed by centrifugation at 13,000rpm at 4°C for 15 minutes. The supernatant was transferred into a fresh sterile tube and extracted twice with chloroform: isoamyl alcohol (24:1) by centrifugation at 13,000rpm at 4°C for 15 minutes. The supernatant was again transferred into another tube and 50µl of 5M NaCl and 1ml of absolute ethanol were added. The solution was allowed to precipitate overnight on ice. This was followed by centrifugation at 13,000rpm for 15 minutes. The supernatant was discarded and the pellets were allowed to dry, and then dissolved in 50 µL of sterile distilled water, incubated at 45°C for the inactivation of DNAse and stored at -20°C until use. The purity and concentration of the extracted DNA was checked using a NANO drop spectrophotometer.

PCR amplification of CoA gene was done using the primers COAG1 (CGAGACCAAGATTCAACAAG) and COAG2 (AAAGAAAACCACTCACATCA) described by Goh et al (1992). The PCR was carried out in a 25µl reaction mixture containing 1X PCR buffer (Promega), 1.5Mm Mgcl2, 200µM of each dNTP, 50pMn of each primers and 1.25µl Taq DNA polymerase (Promega). Amplification was carried out in an eppendorf Master cycler gradient. The cycling parameter consist of an initial denaturation of 94ºC for 2 minutes, followed by 30 consecutive cycles of 94ºC for 30sec, 45ºC for 2 minutes and 72ºC for 4 minutes. This was followed by a final extension of 72ºC for 7 minutes. The PCR products were separated on a 2% agarose gel at 80V for I hour 30 minutes and viewed under the UV light after ethidium bromine staining.

Antibiotic susceptibility testing was performed on pure colonies of isolated *S. aureus* using the disk diffusion method (Bauer *et al.*, 1966) and diameter of zone of inhibition was compared with the NCCLS table with *S. aureus* ATCC 25923 used as control strain.

Data analysis

Descriptive analysis was done and results analysed with SPSS version 14.0.

RESULTS

A total of 65 (34.8%) samples yielded *S. aureus,* 12 (18.5%) from male and 53 (81.5%) from female (P> 0.503). Genotypic identification of COAG 1&2 gene shows 2 PCR products of 600 and 900 bp as represented in Figure 1.

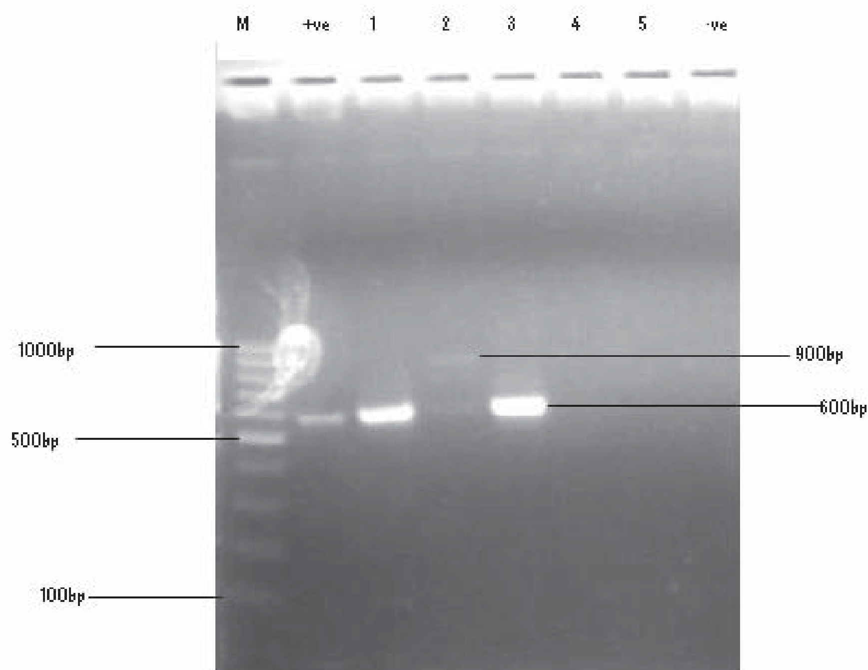

Figure 1: PCR identification of coA gene

The susceptibility results shows the isolates were resistant to ampicillin (100%), penicillin (92.3%), cotrimoxazole (91.4%), tetracycline (76.3%), amoxycillin (73.5%), cloxacillin (73.3%), chloramphenicol (64.4%), gentamycin (50.6%), augmentin (49.2%), streptomycin (38.7%) and erythromycin (28.6%). The isolates were susceptible to ceftazidime (100%), ciprofloxacin (100%), vancomycin (96.7%) and methicillin (78.7%) as represented in the Table below:

Table: Percentage susceptibility of *S. aureus* isolates

ANTIBIOTICS	% RESISTANT	% SENSITIVE
Ampicillin	100	0
Penicillin	92.3	7.7
Cotrimoxazole	91.4	8.6
Tetracycline	76.3	23.7
Amoxycillin	73.5	26.5
Cloxacillin	73.3	26.7
Chloramphenicol	64.4	35.6
Gentamycin	50.6	49.4
Augmentin	49.2	50.8
Streptomycin	38.7	61.3
Erythromycin	28.6	71.4
Ceftazidime	0	100
Ciprofloxacin	0	100
Vancomycin	3.3	96.7
Methicillin	21.3	78.7

DISCUSSION/CONCLUSION

In the present study, 34.8% of patients were nasal carriers of *S. aureus* which is a sensitive indicator of staphylococcal colonisation and is considered a source of subsequent infection. Our study shows that staphylococci infections are incriminated in people with HIV who are also more frequently prone to several other infections. Nasal carriage of *S. aureus* has been found to cause local and invasive infections such as bacteremia which may be followed by endocarditis or other responses (Kovac et al; 1997). *S. aureus* colonisation of the anterior nares is an important risk factor for infection in HIV patients which is the most common etiologic agent causing blood stream infection among this population (Tumbarello *et al.*, 2002; Nguyen et al, 1999).

Studies by other investigators have also demonstrated an increase in frequency of *S. aureus* nasal carriage among HIV infected patients (Weinke *et al.,* 1992). The result of this study shows cotrimoxazole has a resistant of up to 91.4% and that is the prophylactic agents used against opportunistic infection in HIV patients. This is contrary to the study in Israel conducted by Bishira et al, (2003) showing an increase in Cotrimoxazole susceptibility between 1988 and 1997. We can deduce from these present study and the Israel study that increase in cotrimoxazole resistance might be as a result of frequent usage of cotrimoxazole as prophylaxis in HIV patients. This might be the reason for this current high resistance. From our findings, ciprofloxacin has the high sensitivity. There is an urgent need to develop suitable new treatment, taking account of the emerging antibiotic resistance. In view of these findings, further study should be carried out to compare the results with HIV negative individual who are on cotrimoxazole and to study the polymorphism of gene and *in vitro* resistance of *S. aureus* in both population.

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Emergence of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) in Africa

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ABSTRACT

Knowledge of epidemiology of bacterial infections is very important for appropriate decision-making in the treatment of infections, such as septicaemia, wound and postsurgical infections. *Staphylococcus aureus* is an important pathogen in human infections and is implicated in a wide variety of infections. In Nigeria *S. aureus* constitutes significant epidemiologic and therapeutic problems. Ibadan is the capital of the Southwest province Oyo with a population density of 3.6 million and is the largest in geographical area. Over the last 20 years the incidence of both community-acquired (CA) and hospital-acquired (HA) *S. aureus* infections have increased, while antibiotic treatment is increasingly hampered by the spread of *S. aureus* strains, which are resistant to multiple antibiotics including methicillin. The African data on *S. aureus* are extremely limited, although methicillin-resistant *S. aureus* (MRSA) has disseminated in African countries as well. Between 1996 and 1997 the prevalence of MRSA was determined in eight African countries and was relatively high in Nigeria, Kenya, and Cameroon (21 to 30%) and below 10% in Tunisia and Algeria, although in Algeria this rate creased to 14%. The isolates were highly sensitive to ciprofloxacin, except in Kenya, Morocco, and Tunisia, where relative resistance to this drug has been described. Moreover, the results of four years' studies from a number of hospitals in Kenya have shown that 90% of patients admitted in burn units were colonized or infected with MRSA. The increasing prevalence of MRSA infections in non-hospitalized patients due to the emergence of unique community-associated *S. aureus* strains became also a Nigerian problem as it is global. Pheno-, genotypic and toxin gene analysis has not been done yet all in one for Nigerian *S. aureus* population. This study provides a comprehensive overview on the molecular epidemiology and genetic diversity of *S. aureus* at the largest university clinic in Ibadan**.** At the University College Hospital in Ibadan 346 non-duplicate *S. aureus* isolates were obtained during the 1-year-surveillance in 2007. All isolates underwent antibiotic susceptibility testing, toxin gene analysis, MLST (multilocus sequence typing), *agr* group and *spa* typing. And for MRSA SCC*mec* typing was performed. Among the isolates 20.23% were methicillin-resistant. 33 patients' isolates fulfilled the definition criteria for CA-MRSA according to the review of the medical charts. The majority of MRSA strains analyzed were isolated from surgical and paediatric patients. The commonest types of infection identified with HA-MRSA were surgical site infections whereas for CA-MRSA were conjunctivitis and otitis and accidental skin and subcutaneous tissue infection. The MSSA strains were heterogeneous by pheno- and genotypic analysis. The CA-MRSA strain ST88 was resistant to trimethoprim-sulfamethoxazole besides to penicillin and oxacillin. CA-MRSA infections are rapidly increasing among young patients with ophthalmic and otic infections. Urban regions of lower socioeconomic status and evidence of overcrowding appear to be at higher risk for the emergence of this clone. To the best of our knowledge, this is the first report of detection and genetic characterization of ST88 strain in Nigeria. Future prospective studies may further elucidate possible epidemiologic risk factors associated with acquiring CA-MRSA infection.

INTRODUCTION

Staphylococcus aureus is an important pathogen in human infections and is implicated in a wide variety of infections. Prior to introduction of penicillin in 1940, the mortality rate for invasive staphylococcal infection was as high as 90%. This improved remarkably with introduction of penicillin. *S. aureus* became resistant to penicillin very rapidly and this was first demonstrated in 1944. The resistance that was initially hospital based later spread into the community. Human beings are a natural reservoir of *S. aureus*, asymptomatic colonization being far common than clinical infection. Colonization of nasopharynx, perineum or skin occurs shortly after birth and may recur anytime thereafter. Carriage rates are higher in intravenous drug users, persons with insulin-dependant diabetes, patients with dermatological conditions, those with long term indwelling catheters and health care workers (Tenover and Gaynes 2000; Lowy, 2003; Holmes et al 2005).

In Nigeria *S. aureus* constitutes significant epidemiologic and therapeutic problems. Nigeria is the highest populated African country and Ibadan is the capital of the Southwest province Oyo with a population density of 3.6 million and is the largest in geographical area. Over the last 20 years the incidence of both community-acquired (CA) and hospital-acquired (HA) *S. aureus* infections have increased, while antibiotic treatment is increasingly hampered by the spread of *S. aureus* strains, which are resistant to multiple antibiotics including methicillin. Presently, patients having community-acquired MRSA show a distinct pattern in having little or no risk factors for acquisition of the organism. The community-acquired MRSA infections now serve as a source for nosocomial outbreaks. They are generally more susceptible to multiple antibiotics which are in contrast to the typical, multiple-drug resistant HA-MRSA isolates (Lowy, 2003; Witte *et al.*, 2007; Chambers and DeLeo, 2009).

The African data on *S. aureus*, particularly antibiotic susceptibilities, are extremely limited (Ako-Nai et al, 1991; Taiwo et al, 2005), although methicillin-resistant *S. aureus* (MRSA) has disseminated in African countries as well. Between 1996 and 1997 the prevalence of MRSA was determined in eight African countries and was relatively high in Nigeria, Kenya, and Cameroon (21 to 30%) and below 10% in Tunisia and Algeria, although in Algeria this rate creased to 14% (Kesah *et al.*, 2003; Ramdani-Bouguessa *et al.*, 2006). All MRSA isolates were sensitive to vancomycin. The isolates were also highly sensitive to ciprofloxacin, except in Kenya, Morocco, and Tunisia, where relative resistance to this drug has been described (Kesah *et al.*, 2003). Moreover, the results of four years' studies from a number of hospitals in Kenya have shown that 90% of patients admitted in burn units were colonized or infected with MRSA (Muthotho *et al.*, 1995). The increasing prevalence of MRSA infections in non-hospitalized patients due to the emergence of unique community-associated *S. aureus* strains became also a Nigerian problem as it is global. Due to the fact that the genetic analysis of indigenous *S. aureus* strains is limited in Nigeria we aimed to study the genetics, prevalence and dissemination of such strains in Ibadan, one of the biggest university hospitals in Nigeria (Okesola et al, 1999; Adesida *et al.*, 2006).

The objectives of this study were (1) to determine antibiotic susceptibility profiles, genotypes and toxin profiles of methicillin-susceptible *S. aureus* and MRSA from two hospitals in Ibadan, Nigeria; (2) to determine the prevalence; (3) to characterize the genetic determinants of CA-MRSA strains on hospital admission; and (4) to compare the prevalence of CA-MRSA in other African countries and with word-wide spread of CA-MRSA.

METHODOLOGY

Antimicrobial susceptibility testing

The identification and susceptibility testing (penicillin, oxacillin, trimethoprim/ sulfamethoxazole, tetracycline, erythromycin, clindamycin, ciprofloxacin, moxifloxacin, gentamicin, vancomycin, teicoplanin, linezolid, quinupristin/dalfopristin, fosfomycin, fusidic acid, nitrofurantoin, norfloxacin, levofloxacin, rifampicin, tobramycin) were performed by the automated VITEK 2® system (*bio*Mérieux, Marcy-l'Etoile, France). The results were interpreted in accordance to the current Clinical Laboratory Standards Institute (CLSI, 2005) guidelines: breakpoints for oxacillin susceptibility were used: MICs of 2 mg/l indicated susceptibility and MICs of 4 mg/l indicated resistance. Details have been previously described (Ghebremedhin *et al*., 2007).

DNA extraction

Chromosomal DNA was isolated from overnight cultures grown on blood agar at 37 °C. Genomic DNA was extracted by using the Qiagen® DNA extraction kit according to the manufacturers suggestions (Qiagen, Hilden, Germany) with the modification that 20µl of lysostaphin (Sigma; 1mg/ml) and 20µl lysozyme (Qiagen; 100mg/ml) were added at the cell lysis step. The concentration of the DNA was assessed by a spectrophotometer (Ghebremedhin *et al*., 2007).

agr group-specific multiplex PCR, PVL gene and toxin gene detection

Extracted genomic DNAwas used as a template to amplify specific *agr* alleles. For multiplex PCR one primer set was prepared to amplify the four specific *S. aureus agr* alleles using the primers as described by Lina *et al.* (2003). Details were given previously (Ghebremedhin *et al*., 2007). Genes for *sea-e*, *seg-h, tsst-1*, *eta*, *etb, hlgA, hlgCB*, *lukE-lukD, lukS-lukF-PV*, were detected by PCR as described previously (Ghebremedhin *et al*., 2007). Using PCR, we determined the presence of specific staphylococcal virulence genes and detected sequences specific for staphylococcal enterotoxin genes (***sea-e, seg, seh, sei, sej***), as well as the toxic-shock syndrome toxin gene (***tst***), *PVL genes* (***luk****S-PV–****luk****F-PV*), *LukE–lukD leukocidin genes* (***lukE–lukD***), and hemolysin genes (***hlg***). The positivity of the genes *pvl* and *tst* was confirmed by sequencing of their PCR products.

PCR for analysis of SCCmec type

SCC*mec* types were determined by use of a multiplex PCR strategy that generated a specific amplification pattern for each SCC*mec* structural type, according to the method described by Oliveira and de Lencastre. The analysis of the SCC*mec* type was performed according to previously described procedures (Oliveira and Lencastre, 2002).

spa gene typing

The polymorphic X region of the protein A gene (*spa*) was amplified from all *S. aureus* isolates as described previously by Harmsen *et al.* (2003). All sequencing reactions were carried out with an ABI Prism BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems,

Foster City, CA). The *spa* type was assigned by using Ridom StaphType software (version 1.4; Ridom GmbH, Würzburg, Germany).

Multilocus-Sequence-Typing (MLST)

MLST was performed according to previously published protocols (Enright *et al*., 2000). Briefly, standard DNA amplification and sequencing of the seven housekeeping genes (arcC**,** aroE**,** glpF**,** gmk**,** pta**,** tpi**,** yqiL**)** was performed on all *S. aureus* isolates. Nucleotide sequences were determined for both strands using published primers and compared to existing sequences in the MLST database (http://www.mlst.net) for assignment of allelic numbers. The isolates were assigned a sequence type (ST) number according to their allelic profiles. Clonal complexes were defined as isolates that were identical by 5 or more alleles. Phylogenetic relationships among the MSSA and MRSA were then assessed by cluster analysis, the unweighted-pair group method using average linkages and minimal spanning tree (MST) algorithm of the BioNumerics software applied to the MLST sequence data. The sequences for the variable sites from the seven gene fragments were concatenated into a single sequence.

RESULTS

SITUATION IN SOUTHWEST OF NIGERIA

Antibiotic resistance of S. aureus isolates

A total of 346 *S. aureus* isolates were investigated during the study period, out of which 70 were methicillin-resistant, giving an MRSA prevalence rate of 20.23% among the entire subject population. The age range for patients with HA-MRSA is 2-57 years whereas the age range in the CA-MRSA was 6-20 years. All studied *S. aureus* isolates were uniformly sensitive to vancomycin, teicoplanin, and fusidic acid. We found different resistance phenotypes (Ghebremedhin *et al*., 2009) using a panel of antibiotics (penicillin [PEN], oxacillin [OXA], trimethoprim/sulfamethoxazole [SXT], tetracyline [TET], erythromycin [ERY], clindamycin [CLI], gentamicin [GEN], vancomycin [VAN], teicoplanin [TEI], linezolid [LIN], fusidic acid [FUS], ciprofloxacin [CIP], rifampicin [RIF]). The overall susceptibility patterns of the CA-MRSA to the following antibiotics were as follows: 9.5% for SXT, 66.7% for TET, GEN, CIP and ERY, and 100% for FUS, LIN, RIF, TEI and VAN. The higher prevalence of resistance to trimethoprim-sulfamethoxazole in this environment could be due to widespread, indiscriminate use of these antibiotics.

MLST and relatedness of the clonal clusters

Among the 14 identified STs ST88 (*n*=32), ST241 (*n*=7) and ST250 (*n*=30) were representatives for MRSA strains. A minimum spanning tree was constructed by applying the software Bionumerics. Figure 1 depicts the clustering of the STs detected in Nigeria together with representatives of the international *S. aureus* clones which indicate the relatedness between MSSA in this study and MRSA pandemic clones (Fig. 1). The sequence types ST8-MSSA, ST250-MRSA/MSSA and ST241-MRSA strains were closely related and formed the clonal cluster CC8.

MSSA and MRSA strains and clinical significance

Among the MRSA strains ST88-MRSA-IV – as community-associated clone - was detected in outpatients from the University College Hospital (*n*=26) and Oluyoro Hospital (*n*=7) in Ibadan with respective diseases and no healthcare history in previous 1.5 years. These were detected within within 48 hours of admission to the hospital without permanent indwelling catheter and percutaneous medical device (e.g. tracheostomy tube and other catheter) within one year of MRSA detection. Additional criteria were no known positive culture for MRSA and no history of hospitalization, surgery, dialysis, or long-term care facility. The patients were presented predominantly conjunctivitis, cataract, otitis, pyomyositis and posttraumatic wound infections, whereas ST241-MRSA strains were common in hospitalized burn patients. ST250-MRSA and ST508-MSSA isolates were detected in diabetic patients and those with urinary tract infection (UTI). ST80-MSSA was mostly associated with cellulitis and postsurgical infections whereas ST30-MSSA isolates were obtained from different diagnosed patients with traumatic head injury, osteomyelitis, cataract, postsurgical wound infections and accidental skin infections. ST5-, ST7- and ST8-MSSA strains were isolated from patients with diabetic foot, UTI, gun-shot injury, multiple fractures, postsurgical wounds and polytraumatized patients.

Toxin gene detection and agr groups

None of the *S. aureus* isolates were positive for the toxin genes *sed*, *see*, and *seh*. Furthermore, none of the MRSA isolates were positive for *sea* gene whereas 32 out of 276 MSSA isolates were *sea* positive. 44 out 276 MSSA isolates (ST1, ST121, ST15, ST25, ST30, and ST5) were positive for *seb* and 6 MRSA isolates (ST250) were positive for *seb*. All the MSSA isolates which were typed as ST508 (*n*=9) and few others (ST5 (*n*=2), ST5 (*n*=2), ST7 (*n*=2)) were positive for *sec* whereas none of the MRSA isolates expressed this toxin gene. MSSA isolates from skin lesions or eye and wound infections were more likely to produce toxins (81% and 68% respectively) than nasal isolates (11%). Those belonging to *agr* III were preferentially positive for *luk-PV* expression, especially the CA-MRSA ST88 (*n*=33), besides the MSSA ST30 (*n*=30). PVL positive were also the MSSA isolates typed as ST1 (*n*=22 out of 22), ST121 (*n*=21 out of 38), and ST80 (*n*= 8 out of 8). Forty-two (52.85%) of the 70 MRSA isolates were hospital-acquired (HA). And the 33 patients' isolates (47.15%) were acquired in the community (CA) and these patients fulfilled the definition of having CA-MRSA according to the review of the medical charts. The majority of MRSA strains analyzed were isolated from surgical and paediatric patients. The commonest types of infection identified with MRSA isolates during the period of study were surgical site infections (>70%) whereas for CA-MRSA were conjunctivitis and otitis in 19 patients (57.6%) and accidental skin and subcutaneous tissue infection in 14 patients (42.4%). The expression of the *tst* gene was detected in 8 of the 70 MRSA isolates (five ST250, two ST88 and one ST8) and in 54 of the 276 MSSA isolates (all ST1 and ST508, four ST30 and eight ST5).

SCC*mec typing of MRSA isolates*

The sequence types MRSA-ST88 (*n*=33) and MRSA-ST241 (*n*=7) were grouped in SCC*mec* type IV whereas MRSA-ST250 (*n*=30) was typed as SCC*mec* type I attributing to HA-MRSA.

SITUATION IN NORTH-EASTERN OF NIGERIA

CA-MSSA in north-eastern Nigeria

A total of 96 *S. aureus* isolates obtained between January and December 2007 from clinical specimens in six tertiary-care hospitals located in north-eastern Nigeria were characterized (Okun *et al.*, 2009). All the isolates were susceptible to vancomycin, fusidic acid, and mupirocin, and 12 (12.5%) were resistant to methicillin. The genotypic analysis showed a low level of diversity among the 12 MRSA isolates, which belonged to a single cluster, while a high level of diversity among the MSSA isolates was observed. A total of 12 sequence types (STs) were observed among the 16 isolates analyzed by MLST. The MRSA cluster belonged to ST 241, while the predominant MSSA cluster was grouped into ST152. However, a PVL-positive CA-MRSA clone ST152 has been observed in the Balkans and Central Europe. The presence of PVL-positive MSSA isolates (ST152) in north-eastern Nigeria and Mali supports the hypothesis that the ST152-MRSA clone originated in Africa, migrated throughout central Europe, and acquired methicillin-resistance (Ruimy *et al.*, 2008). The above stated key-results underline and emphasize the needs of more comprehensive studies and surveillances.

SITUATION IN OTHER AFRICAN COUNTRIES

CA-MRSA in Algeria

The study of Ramdani-Bouguessa *et al.* (2006) indicates a very high prevalence of PVL-positive MRSA in Mustapha Pacha Hospital in Algiers. These strains were resistant to multiple antibiotics, including gentamicin and ofloxacin. The PVL genes were harbored by a major clone CA-MRSA ST80 accounting for 44 of the 45 PVL-positive isolates. This clone harbours *agr* type III and SCC*mec* type IV, and is resistant towards kanamycin (86%), fusidic acid (73%) and tetracycline (25%). Among the Algerian CA-MRSA isolates, a minor clone (ST37, *agr* type III, SCC*mec* IVa) was detected in two patients and was found to harbor the *tst* gene. Ramdani-Bouguessa *et al.* (2006) concluded that Poor infection control procedures in such an institution may have enabled community-acquired ST80 MRSA to supplant true hospital-acquired MRSA (ST5, ST239, ST241, and ST637).

CA-MRSA in Egypt

The prevalence of CA-MRSA expressing PVL in Egypt was 19.04% (Enany *et al.*, 2009). They belonged to different genetic clones with multi-locus sequence types (MLST) 30, 80, and the novel type 1010. ST80 strains showed unique antibiotic resistance profile that was distinguishable from the European ST80 clone: they had no resistance to tetracycline and fusidic acid. Two non-synonymous substitutions in the *lukS-PV* gene region and two haplotype variants among these isolates were detected.

CA-MRSA in Tunisia

Ben Nejma *et al.* (2008) characterized 64 Tunisian CA-MRSA by *agr* typing, PCR analysis for 20 virulence genes, SCC*mec* typing, PFGE, MLST, and *spa* typing. All isolates were positive for *luk-PV* and *etd*. They harbored SCCmec type IV and belonged to *agr* group III. The typing results revealed that all isolates had a *spa* type (t044) and a common sequence type (ST80). The isolates susceptible to the majority of antibiotics, but were resistant to kanamycin, erythromycin,

and tetracycline, and intermediate resistance to fusidic acid. The study by Ben Nejma *et al.* (2008) indicates that the Tunisian MRSA isolates were non-multiresistant and belonged to a single clonal type ST80.

DISCUSSION

Antimicrobial agents were recognized decades ago as "magic bullets" for killing microbes. This impression was thoroughly reinforced when penicillin and other antibiotics came into clinical use in the 1940s. However, shortly after the introduction of these magic bullets in clinical practice, it was discovered that the bacteria were capable of developing resistance to the antimicrobials. The full magnitude of the resistance problem was not appreciated during the first decades of chemotherapy. The bacteria became more resistant, new types of bacteria developed resistance, resistance genes spread among different bacteria, and resistant organisms spread to new geographical areas. Particularly serious resistance problems such as multidrug-resistant tuberculosis, methicillin-resistant *Staphylococcus aureus* and extended-spectrum beta-lactamase (ESBL) producing Gram-negative bacteria emerged and spread to most parts of the world. Inappropriate use of antibiotics, use of broad-spectrum antibiotics, insufficient hygiene, immunosuppression and prolonged hospitalization may promote antimicrobial resistance (Fischbach and Walsh, 2009).

The use of antimicrobials of poor quality may contribute to emerging resistance and is a huge problem in countries with poor regulatory capacities and resources. While antimicrobial resistance affects all countries, it has potential for doing more harm in developing countries since second-line antimicrobial drugs are often neither available nor affordable to those who need it. Diseases we have thought of as curable, such as pneumonia, bloodstream infections, typhoid fever and tuberculosis, may again become killers of people of all ages. If this scenario becomes real, developing countries may be where the harm will be felt first.

The knowledge of epidemiology of bacterial infections is very important for appropriate decision-making in the treatment of infections, such as septicaemia, wound and postsurgical infections. Iroha *et al.* (1998) investigated in Lagos conjunctivitis cases of neonates in a prospective study. The incidence of conjuctivitis in the newborn was 18 per 1000 live births. *S. aureus* (37.4%) was the most predominant aetiologic pathogens among the other bacteria (coagulase negative staphylococci (12.3%), *Klebsiella pneumoniae* (12.9%) and *Pseudomonas aeruginosa* (8.2%). The incidence of S. aureus-caused eye infection is consistent with our study, in particular in out-patients with CA-MRSA-positive conjunctivitis. This might be an indication that antibiotics are now more extensively used in the community besides hospital-settings, especially broad-spectrum antibiotics. *S. aureus* (61.2%) was the dominant cause of septicaemia and mortality in neonates according to Udo *et al.* (2008). Another study investigated the bacteriology of non-surgical wound infections in Ibadan. *S. aureus* (38%) was the predominant pathogen followed by the gram-negative bacteria (each 7-19%). High rates of antibiotic resistance were recorded among these isolates (Okesola and Kehinde, 2008). Thanni *et al*. (2003) determined the prevalence of bacterial pathogens in wounds from various units of a Nigerian tertiary hospital orthopedics and traumatology department. In this retrospective study from 1995 to 2001 *Pseudomonas aeroginosa* was the most common pathogen among the inpatients whereas

S. aureus was more common among outpatients. The rate of isolation of Gram-positive bacteria in general decreased while that of *S. aureus* in particular increased Thanni *et al*. (2003).

The MRSA prevalence in our study was moderate (20.23%) as compared to previous studies in South-western Nigeria, which ranged from 1.4% to 50% (Ako-Nai et al, 1991; Okesola et al, 1999; Kesah et al, 2003; Taiwo et al, 2005; Adesida et al, 2005). However, it should be considered that the detection of the *mecA* gene, which is the "gold standard" for determining methicillin resistance, was not investigated in some of these previous studies. A recent multicenter study in Southwestern Nigeria confirmed resistance to methicillin by the detection of the *mecA* gene by PCR and reported a lower prevalence rate of 1.4% (Adesida et al, 2005). Despite the low MRSA rate in our study, it is evident that multi-resistant MSSA occurred frequently in Southwestern Nigeria (Ghebremedhin *et al.*, 2009). However, the MRSA isolates were predominantly associated with infections (87%) as elsewhere observed (Taiwo *et al.*, 2005). Nevertheless, the prevalence of community associated MRSA (47%) was higher in our study as compared to that (29%) of Taiwo *et al.* (2005) The prevalence of MRSA among the *S. aureus* isolates at the University College Hospital, Ibadan, Nigeria in 1999 was generally set at 27% which was higher than 1972 value of 1%. Forty-one percent of the MRSA isolates were from inpatients while 59% were from outpatients. The high incidence of MRSA in outpatients was unusual at that time (Okesola *et al.*, 1999). A survey of MRSA at a teaching hospital in Ilorin, Nigeria suggested a similar MRSA prevalence rate at 34.7% (Taiwo et al, 2005). Many African countries have an extraordinary tradition of herbal or traditional medicine (Ogunshe *et al.*, 2006). Thus, the low cost and high acceptance and ease of access to such traditional 'therapies' make them the most common form of African alternative medicine. Therefore, the antibiotics in the community do not play the major role and hence the resistance problem is rather low for oxacillin or methicillin. In the hospital environment, the acquisition of the SCC*mec* (in its various forms) by multi-resistant MSSA could make infection control measures extremely difficult and could have serious consequences. The resistances to sulfonamides and tetracycline were remarkable in MRSA clones and sulfonamides were recommended and administered to treat MRSA infections in Nigeria (Taiwo et al, 2005). Hence, these antibacterial agents should not be considered anymore as first-line drugs for treatment of MRSA infections in Nigeria.

MLST of *S. aureus* strains in Nigeria indicated that certain major clones of MSSA are extremely successful in Nigeria (Adesida *et al.*, 2005). They include ST25, ST30, and ST120/121 which have been recognized as internationally well-disseminated clones (Enright *et al.*, 2002) along with the ST8 MSSA clone, which appeared to possess some epidemic potentials and had acquired the *mecA* gene (Adesida *et al.*, 2005). These STs were also detected in our study. MSSA isolates are often more genetically variable and have commonly been the subject of more general surveillance studies, but relatively few studies with molecular typing. Goering *et al.* (2008) reported ST121 as the most common PVL-positive MSSA clone (pulsed-field type USA1200), which was found primarily in South Africa and the Russian Federation. In our study ST121 clone was also prevalent and was positive for *luk-PV* gene. Another MSSA clone in our study belonging to the clonal complex CC1 was ST1 which was detected in predominantly PVL-negative isolates originating in India, South Africa, the United States, and Germany, with the difference that the Nigerian clone expressed *luk-PV* and *tst* genes. The MSSA clone ST5 (CC5) and ST30 (CC30) were identified in South Africa, the United States, and Germany as well according to Goering *et al.* (2008). In comparison to our study isolates, Adesida *et al.* (2006)

identified in their MSSA isolates from Lagos different lineages. Among their 17 MSSA isolates the *spa* typing revealed nine different types with predominance of *spa* t007 and t454. Considering the global and dynamic nature of MRSA in HA and CA infections, continued surveillance is important for clearer understanding of the epidemiology of these organisms.

To date, most SCC*mec* IV CA-MRSA isolates have had MLST allelic profiles that are not found in studies of HA-MRSA. However, CA-MRSA has the potential to move into the hospital setting and cause outbreaks. This study reiterates that the detection of SSC*mec* type IV CA-MRSA was associated with the PVL production. In contrast to the MRSA strains in Algeria (Ramdani-Bouguessa *et al.*, 2006) our MRSA isolates were not resistant to gentamicin (Ghebremedhin *et al.*, 2009). In the Algerian study the treatment options in the case of multiple-antibiotic-resistant MRSA strains included cotrimoxazole (SXT) for minor infections and glycopeptides for severe infections whereas SXT is not recommended for the Nigerian MRSA strains in Ibadan anymore as the resistance rate to SXT is approx. 53%, nearly all ST88-MRSA isolates and ST241-MRSA isolates, but none of the ST250-MRSA isolates were SXT resistant. Grim *et al.* (2005) stated that clonal outbreaks of MRSA resistant to SXT have been described; of these, the Brazilian clone has more often been resistant to SXT than the Iberian clone. Rates of SXT resistance were particularly high in institutions serving large numbers of patients infected by the human immunodeficiency virus, due to increased exposure for *Pneumocystis* prophylaxis.

Vandenesch *et al.* (2003) described continent-specific PVL-positive CA-MRSA clones - mainly on an *agr group III* background and characterized them by their sequence type (ST). The main European clone, ST80, was detected in France, Switzerland, the Netherlands, England, Belgium, and Germany, but also in northern Europe (e.g. Denmark), where MRSA strains are rare in hospitals. ST80-MRSA clone is usually resistant to tetracycline (mediated by the *tetK* gene), intermediate to fusidic acid (*far1* gene), and resistant to kanamycin (*aph3´-III* gene). We observed the prevalence of ST80-MSSA isolates in Nigeria which were tetracycline resistant as well, but susceptible towards fusidic acid. One of the most prevalent PVL-positive CA-MRSA clones in the United States (USA300) belongs to ST8; other US clones include USA400 (ST1), USA1000 (ST59), and USA1100 (ST30). ST30 is also a major clone in Asia and Oceania and is referred to as the South West Pacific MRSA clone. This sequence type was prevalent in Nigeria as a MSSA strain. In Singapore, as an international travel hub, several clones belonging to ST80, ST30, and ST59 have been reported. The prevalence of PVL-positive CA-MRSA varies considerably from continent to continent. In the United States, MRSA were isolated from 50% of patients with skin and soft-tissue infections seen in emergency departments of 11 cities (97% of isolates belonged to clone USA300). In Europe, the prevalence of CA-MRSA is lower, at ≈1–3% and in Africa this needs more investigational study.

In our study the MRSA strains were less “toxigenic” as compared to the MSSA strains (ST5, ST7 and ST30) which were more positive for PVL and *tst* (Ghebremedhin *et al.*, 2009). In contrast to our finding the MRSA isolates in a Japanese study evaluated that both MRSA and MSSA isolates carried a number of superantigenic toxin genes, but the MRSA isolates harboured more superantigenic toxin genes than the MSSA isolates (Hu *et al.*, 2008). Hu *et al*. (Hu *et al.*, 2008) compared the prevalence of superantigenic toxin genes in MRSA and MSSA and concluded that some of their MRSA isolates were *sec*, *seg* and *tst* positive. In our study none of the MRSA isolates shared these genes together, only one MSSA isolate typed ST508 was positive for *sec*, *seg* and *tst* together. This notable higher prevalence in Japanese MRSA isolates

indicated that possession of the *sec* and *tst* genes in particular appeared to be a habitual feature of MRSA (Hu *et al.*, 2008), unlike to the Nigerian MRSA (Ghebremedhin *et al.*, 2009).

This study provides a comprehensive overview on the molecular epidemiology and genetic diversity of *S. aureus* at the largest university clinic in Nigeria. First, it shows high prevalence of PVL-positive MSSA. Second, high heterogeneity of MSSA with broader resistance profiles was seen as compared to the MRSA strains (ST250 and ST88) which are homogenous. ST88 was resistant to trimethoprim-sulfamethoxazole besides to penicillin and oxacillin whereas ST250 as hospital MRSA was additionally resistant to tetracycline, ciprofloxacin and gentamicin (Ghebremedhin *et al.*, 2009). Trimethoprim-sulfamethoxazole and tetracycline are listed among antibacterial agents that have been rendered ineffective or for which there are serious concerns regarding bacterial resistance in Nigeria (Okeke, 2003). Therefore, the formulation and implementation of a national drug policy by governments are fundamental to ensure rational drug use. Control of community-acquired *S. aureus* will still remain a challenge for some regions in Nigeria, since the transmission is linked to migration and touristic reasons.

Two types of SCC*mec* were found with CC8 complex, the ST241-MRSA-IV strain and ST250-MRSA-I (Fig. 1). ST250-MSSA is most probably putative ancestor of ST250-MRSA by the insertion of the SCC*mec* I. ST250-MSSA itself might have derived from putative ST8-MSSA ancestor, common to all strains belonging to CC8 (Hu *et al.*, 2008). In this study we showed that in most of the clinical departments the less toxigenic MRSA strains circulate together with the more toxigenic MSSAs, e.g. ST30 and ST1 (Ghebremedhin *et al.*, 2009). This might be benefiting for the toxin gene transfer which could occur among the methicillin-susceptible and -resistant strains (Layer *et al.*, 2006).

In conclusion, the application of the different typing methods to our Nigerian strains provided important information on their clonal relationship and might also strengthen the representation of the population of *S. aureus* circulating in Nigeria. Moreover, this study summarizes comprehensive epidemiologic data and use of other genetic marker to investigate outbreak situations in such healthcare setting.

To the best of our knowledge, this is the first report of detection and genetic characterization of ST88 strain in Nigeria (Ghebremedhin *et al.*, 2009). ST88 was also isolated in Asia, Europe and South America (Aspiroz *et al.*, 2009) with the significant difference that the Nigerian clone is sensitive to tetracycline and fusidic acid, in contrast to the European CA-MRSA clones (Witte *et al.*, 2007). Therefore, epidemiological studies on the clonal relationship of MRSA strains in Nigeria with worldwide clones would be useful and important in understanding the global dissemination of such clones.

Limitations to our study include the limited number of patients. Thus, the prevalence of the MRSA in general and CA-MRSA might be underestimated. Future prospective studies may further elucidate possible epidemiologic risk factors associated with acquiring CA-MRSA infection.

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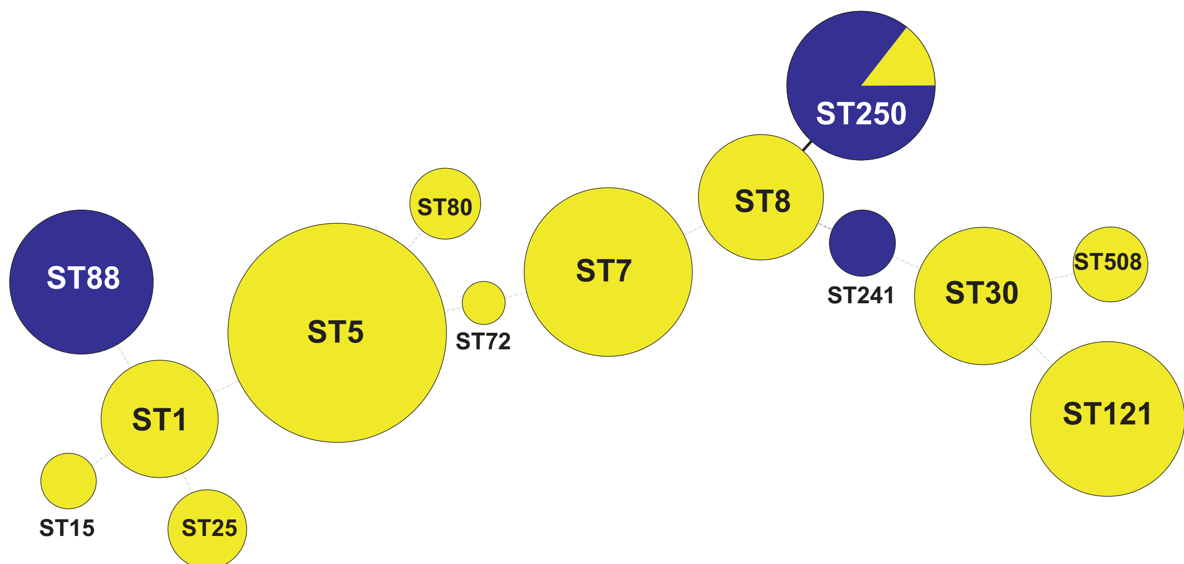
Fig. 1: The Minimum spanning tree based on MLST indicates the estimated relationships of the 14 STs, including representatives of the clonal complexes (CC1, CC5, CC7, CC8, CC30, and CC88). ST88, ST241, and ST250 represent MRSA strains (although some MSSA strains also belong to ST250). Each ST is represented by a circle whose size is proportional to the number of isolates belonging to the ST. Blue circles, MRSA; yellowish circles, MSSA. The relationships between the strains are indicated by the connections between the isolates and the lengths of the branches linking them.



Recovery of seeds from feces of cattle, sheep and goats consuming *Albizia saman* pod and silage effects on dry matter digestibility by West African dwarf rams

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ABSTRACT

Ruminants in the tropics are faced with challenges of scarce forage in the dry season and largely depend on browse plants for foliages, fruits, seeds and pods. *Albizia saman* pod is abundant in the production season, high in crude protein and relished by ruminants but the seeds are hard to digest. Against this background, two trials were conducted to determine the potential digestibility of the seeds of *Albizia saman* pods and processing method using ensiling to softening hardiness of the pods as feed for ruminants. Trial one assessed the number of seeds in 1 kg pod. A predetermined amount of pods were respectively fed once to cattle, sheep and goats in triplicates. Feces were collected twice daily for presence of the seeds. In trial two, 10, 20, 30 and 40% pods inclusion were ensiled for 42 d with Guinea grass as basal diet. The ensiled material was fed to ram in triplicates for determination of dry matter intake (DMI), organic matter intake (OMI), dry matter digestibility (DMD) and organic matter digestibility (OMD). About 1,000 seeds were contained in 1 kg pods of Albizia saman. The percentage of seeds hydrolyzed by cattle, goats and sheep were 49, 40.7 and 42.2 respectively. Seeds were recovered until 8th day and progressively decreased after 24 h of ingestion. Presence of undigested seeds was not noticed in the feces of rams fed ensiled *Albizia saman* pods. However, DMI and OMI were enhanced up to 30% pod inclusion after which it declined. DMD and OMD respectively ranged from 65.54 g in 40% to 70.16 g in 20% pods, 65.03 g in 40% to 69.40 g in 20% pods. Results indicated that seed of *Albizia saman* is hard to break by ruminants but when ensiled can easily be digested and utilized by sheep.

Keywords: *Albizia saman* pods, Seed recovery, Silage, digestibility, ruminants

INTRODUCTION

In livestock management, Albizia trees, also known as rain tree are occupying a progressive vital role in the tropics either as supplement or basal diets during the production season. In Nigeria, there are more than ten species of Albizia but most common amongst them are *Albizia saman*, *Albizia lebbeck* and *Albizia rhizonse*. Both the leaves and the seeds of these multipurpose trees are naturally endowed with high protein (Babayemi *et al*., 2004a). *Albizia saman* foliage is often rejected by ruminants possibly due to the presence of anti-nutrient factors but its pods (Figure 1) are relished and freely consumed during the production season (Otukoya and Babayemi, 2007). The tegmen of the pod is sugary and probably contains high carbohydrate and therefore, can serve as source of energy. High percentage of protein is inherent in the seed (Babayemi *et al.*, 2004b) but a high proportion of the seeds escape mastication, being hard and are passed out in the feces of cattle (Jolaosho *et al*., 2006) and that of goats (Otukoya, 2007) undigested. Knowledge of extent of breaking down and utilization of seeds from *Albizia saman* pod by goats, sheep and cattle under free choice intake is not known. In the present study, the first objective was to determine the extent of recovery of the seeds from sheep, goats and cattle feces and the

second was to determine the dry matter digestibility of ensiled *Albizia saman* pods with Guinea grass mixtures by WAD rams.

MATERIALS AND METHODS

Collection of Albizia saman pod

Fallen pods amounted to 1,000 kg were obtained between January and February, 2006 from more than forty trees around the University of Ibadan campus. Each day collection which lasted for two weeks was sun cured for an upward of two weeks on a raised platform. Relatively dried pods were kept in a plastic basket and stored in a ventilated room temperature.

Seed recovery trial

Determination of seed number of Albizia saman pod

One kilogram of the pod was weighed in triplicates such that pods were randomly picked from the sun-cured material. Each pod was split open by hand and the seeds were carefully removed. The number of seeds in each kg of pod was counted five times by different people and thereafter the weight was noted. Amount of seeds per kg was estimated as the mean of the total number in kg.

Animal management and feeding

The cattle used were six bulls of Gudali weighing 100 ± 1.42 kg, six rams (two year old) with live weight of 25 ± 0.6 kg and six goats of 15 months old buck weighing 18 ± 0.14 kg. The animals were respectively selected from the Teaching and Research Farm of the University of Ibadan, Nigeria, for the seed recovery trials. The bulls, rams and bucks were conditioned to confinement. The reason was to get rid of the possible seeds the animals were previously consumed during grazing. During the adaptation period, the bulls, rams and bucks were fed with Guinea grass and compounded ration of high plane of nutrition. While the animals were still getting used to the environment, their feces were properly examined for the presence of the any forage seed. The process of seed examination was continuous until none-feces-containing-seed were observed. Prior to commencement of the trial, the experimental animals were starved for 12 hours in order to increase consumption of the test pods to be served. Goats (buck), sheep (ram) and cattle (bull) were offered 1.5, 2.0 and 5 kg pods respectively only in the first day. In the second day, remnant was collected and weighed and was subtracted from the quantity served in order to determine the actual intake. All the animals were thereafter fed daily with formulated concentrate (2% body weight) in the morning and Guinea grass *ad libitum* in the evening.

Fecal collection and voided seeds

Each bull, ram and buck was worn with harness bag, which enables the total and separate collection of feces. Feces were collected for eight days both in the morning and in the evening. Feces obtained were collected in a glass container after which, the container was filled with water to make solution of the feces. The supernatant was decanted through a 2 cm sieve. This was repeated several times until seeds were clean and recovered as residue. The recovered seeds

were immediately counted and drained by spreading them on a thin sheet of polythene for air drying. Air dried seeds were weighed and counted manually five times. The amount of seed recovered was estimated as reported (Jolaosho *et al.*, 2006).

Silage preparation for dry matter digestibility

Guinea grass (Panicum maximum) was harvested at 6 week re-growth in October, 2007 from the Teaching and Research Farm, University of Ibadan, Nigeria. Grass was chopped into 2-5 cm sizes and air dried for two hours. Guinea grass (GG) and *Albizia saman* pods (ASP) were mixed together for silage in the following treatments: Treatment 1: 10% ASP + 90% GG; Treatment 2: 20% ASP + 80% GG; Treatment 3: 30% ASP + 70% GG; Treatment 4: 40% ASP + 60% GG. A forty capacity mini silo made of rubber plastic was used. The silo was lined with 20 mg thick polythene. Filling of material was done layer by layer until the container was filled up. Each lay was followed by manual compaction with a club, which was effective by standing on the material. 1 g of salt/kg of silage was added as the only additive. The polythene was sealed and heavy sand bag was placed on top of it to exclude air. There were five replicates and the material was left to ferment for 42 days.

Dry matter digestibility and seed recovery

In a complete randomized design, 12 rams (12-15 months old weighing 18 ± 0.54 kg) that were not used for seeds recovery were allocated to the four treatments for digestibility study. The three rams per treatment were kept each in metabolic cages specially made for separate collection of feces and urine. Rams were adapted to the cage condition for 7 days while the remaining 7 days were used for total collection of feces. Rams were fed 5% of their body weights with fresh water *ad-libitum*. In the following morning, orts were collected and deducted from the feed served to determine the feed intake. Total feces were collected and were divided equally into three parts. One part of the feces was oven dried at 105 °C for dry matter determination. The two-third was dissolved in cold water and washed several times to determine the presence of undigested seeds of the pods.

Statistical analysis

Descriptive statistics and analysis of variance using SAS (1995) were used. Significant means were separated using Duncan (1955) multiple range F-Test.

RESULTS AND DISCUSSION

Trial one

Seed recovery from the feces of cattle, goats and sheep

Table 1 presents analysis for estimation of *Albizia saman* pod. On the average, there were 1, 103 seeds of *A. saman* comprised 20.3% per kg of the whole pod weight. This lower percentage of the seeds is proportional in terms of protein availability and may not likely be indicative of low amount of crude protein contained in the pod. The present study did not analyze for protein content of either the seeds only or the pods. A separate analysis of the seeds showed that it

contained 29.3% (Babayemi *et al*. (2004a) while the whole pod comprised 24.7% CP (Otukoya, 2007), suggesting the protein potentials, which is the most limiting nutrient in the forages and roughages of feedstuffs for ruminants in the tropics. If the hard coat covering the seed is removed, the protein content may be improved and therefore could be comparable with those of conventional feedstuffs such as ground nut cake and soyabean that are becoming scarce and exorbitant.

Presented in Tables 2, 3 and 4 are the extent at which the ingested *Albizia saman* seeds were degraded by the ruminants. The cattle, sheep and goats were used in the present study as comparative for the utilization of hard-seeded pods due to their differential response to feeds and forages. Mean value (kg) of 4.2, 0.65 and 1.39 were ingested by cattle, goats and sheep respectively. The differences in the quantity consumed were likely due to their sizes which is a reflection in the rumen capacity. The amount consumed by the livestock was between 3-5% of their respective body weights. Such relatively high acceptability is expected since the pods of *Albizia saman* are palatable having sugar-like tegmen (Otukoya, 2007). These sets of animals are always seen scavenging around the trees and eaten the pods during the production period, which is always at the pick of dry season. Jolaosho *et al*. (2006) reported the consumption of *Albizia saman* pods by three breeds of cattle although at a restricted feeding for the monitoring of the breaking down of seeds.

There were large percentages of seeds unutilized such that 51%, 59.3% and 57.8% of the ingested seeds were voided through the feces by cattle, goats and sheep respectively. Result showed that cattle exhibited more potential to break hard seeds of the plant more than other ruminants. Among the small ruminants, sheep was more superior in breaking and digesting the hard seeds. High proportion of retained seeds of *Albizia saman* in the feces by different breeds of cattle was reported by Jolaosho *et al.* (2006). The crude protein essential for animal production is embedded in the seeds, and therefore those found undigested in the feces are wasteful.

Figure 1 shows the period of recovery and the longevity of the seeds in the gastrointestinal (GIT) system of the Bunaji cattle. Preponderance of the seeds was observed to be obtained from feces in the morning than in the evening hours of the day. This might be expected since animals were always fed in the morning, which took sometimes to be digested. It was noteworthy that seeds remained in the GIT for about eight days. This observation is of great concern as it may have nutritional consequences on the animal consuming the pods. For example, if the cattle were fed daily with the pods, there would probably be an incidence of accumulated seeds in the GIT, which might impair a free flow of digestives and may finally block the GIT orifice. Also, after a period of time, such cattle would consume less subsequent feed leading to low productivity.

Trial two

Dry matter digestibility of ensiled Albizia saman pods: Intake and digestibility of dry matter and organic matter

Table 5 presents the dry matter intake (DMI) and dry matter digestibility by sheep. The dry matter (DMI, g) ranged from 447.95 in 10% to 612.63 in 30% pods inclusions. Similar trends were observed for organic matter (OMI) (range 398.2-545.30 g). Ngwa *et al*. (2002) in a study in which leguminous pods silage were fed to sheep reported that hay intake increased linearly from 74.6 to 90.7 g/d as the proportion of pod in the silage increased. Mahgoub *et al*. (2005) reported

that feed intake of goats fed Meskit pod diet increased as the level of the Meskit pods increased up to 200g/kg but feed intake reduced when the level of the pods later increased to 300 g/kg in the diet.

Also shown in Table 5 are values for apparent digestibility of dry matter and organic matter (OM). The apparent digestibility for dry matter (ADDM) and organic matter (ADOM) respectively ranged from 65.5 in 40% to 70.2 in 20% pod and 65.0 in 40% to 69.4 in 20%. There were significant ($p<0.05$) variations ADDM and ADOM among the treatment means such that pod inclusion at 20% > 30% > 10% > 40%. Enhancement of digestibility in the present study with increasing inclusion of the pod in the silage fed the rams was normal. Reason for the increased digestibility in the high inclusion Albizia pods may be attributed to the high sugar content of the tegmen. This observation corroborates the reports of Meyer *et al.* (1986) that the high ruminal DM degradability of mesquite pods was due to the high soluble sugar content in it. Bruno-Soares and Abreu (2003) reported that significant differences were observed in dry matter digestibility from 56.3 to 60.2 and that of organic matter from 60.5 to 63.3 when *Gleditsia triacanthos* pods levels were increased in the diet fed to sheep. In a digestibility study with ram and pod silage, Ngwa *et al*. (2002) reported that addition of pod silage increased DM intake linearly.

Presence of seeds that probably escaped degradation by microorganisms in the rumen was not noticed as recovery seeds in the feces of the rams, suggesting ensiling with Guinea grass as a potential process of breaking the seedhardedness. When the pods were fed unprocessed to cattle of different breeds (Jolaosho *et al*., 2006), high percentage of the seeds were recovered from the feces after many days of ingestion. Otukoya (2007) reported abundant recovery of *Albizia saman* seeds when it was fed unprocessed to WAD goats.

CONCLUSION

The preponderance recovery of the seeds of *Albizia saman* even after 96 h from the faeces of cattle, sheep and goats showed hardiness of the seeds, suggesting incapability of the ruminants to digest it. The eventual zero recovery of the seeds after being subjected to silage treatment and fed to sheep for intake and digestibility indicated effectiveness of ensiling as potentials to soften the seeds of the plant. For optimum performance of ruminants, feeding on the pods of *Albizia saman* is recommended after ensiling with Guinea grass.

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Table 1: Estimation of the number of seeds in one kg (n = 3) pods of *Albizia saman*

Serial number	Weight of whole pods (kg)	Weight of pulp/kg (g)	Weight of seed/kg (g)	Number of seed/kg
1	1	829.0	171.0	1,120
2	1	839.7	160.3	1,050
3	1	826.0	174.0	1,140
Total	3	2,494.7	505.3	3,310
Mean	1	831.6	168.4	1,103

Table 2: Analysis of the seeds of *Albizia saman* ingested, degraded and voided by Gudali bull

Animal number	Pods (kg)		Seed ingested	Seed voided		Seed degraded	% seed degraded
	Served	Ingested		Morning	Evening		
Bull 1	5	4.4	4,840	1,218	1,205	2,417	49.9
Bull 2	5	4.1	4,510	1,479	821	2,210	49.0
Bull 3	5	4.0	4,400	1,468	773	2,159	49.1
Bull 4	5	4.0	4,400	1,200	885	2,315	52.6
Bull 5	5	4.3	4,730	1,499	1,142	2,089	44.2
Bull 6	5	4.5	4,950	1,514	997	2,439	49.3
Total	30	25.3	27,830	8,378	5,823	13,629	294.1
Mean	5	4.2	4,638.3	1,396.3	970.5	2,271.5	49.0

Table 3: Analysis of the seeds of *Albizia saman* ingested, degraded and voided by WAD bucks

Animal number	Pods (kg)		Seed ingested	Seed voided		Seed degraded	% seed degraded
	Served	Ingested		Morning	Evening		
Buck 1	1.5	1.1	1,210	528	314	368	30.4
Buck 2	1.5	0.8	880	261	112	373	42.4
Buck 3	1.5	0.9	990	313	128	441	44.6
Buck 4	1.5	1.0	1,100	329	135	464	42.2
Buck 5	1.5	1.3	1,430	410	237	647	45.2
Buck 6	1.5	0.9	990	271	119	390	39.4
Total	9.0	6.0	6,600	2,112	1,045	3,683	244.2
Mean	1.5	0.65	1,100	352	1,74.2	4,47.2	40.7

Table 4: Analysis of the seeds of *Albizia saman* ingested, degraded and voided by WAD rams

Animal number	Pods (kg)		Seed ingested	Seed voided		Seed degraded	% seed degraded
	Served	Ingested		Morning	Evening		
Ram 1	2.0	1.41	1,551	551	329	621	43.3
Ram 2	2.0	1.37	1,507	574	325	608	40.4
Ram 3	2.0	1.42	1,562	458	410	694	44.4
Ram 4	2.0	1.35	1,485	524	399	562	37.8
Ram 5	2.0	1.44	1,584	603	297	684	43.2
Ram 6	2.0	1.36	1,496	526	311	659	44.1
Total	12.0	8.36	9,185	3,236	2,071	3,828	253.2
Mean	2.0	1.39	1,531	539	345	638	42.2

Table 5: Dry matter and organic intakes (g) and digestibility (g/100 g DM) by WAD sheep fed ensiled *Albizia saman* pod and Guinea grass mixture

	Level of pod inclusion (%)				
Parameters	10	20	30	40	SEM
Feed intake					
Dry Matter	447.95^{d}	566.87^{b}	612.63^{a}	555.14^{c}	1.107
Organic matter	398.2^{c}	510.55^{b}	545.30^{a}	533.70^{a}	3.161
Apparent digestibility of nutrients					
Dry matter	66.92^{c}	70.16^{a}	68.13^{b}	65.54^{d}	0.156
Organic matter	67.42^{b}	69.40^{a}	65.51^{c}	65.03^{c}	0.281

a,b,c,d means on the same row with different superscripts differ significantly ($p<0.05$)

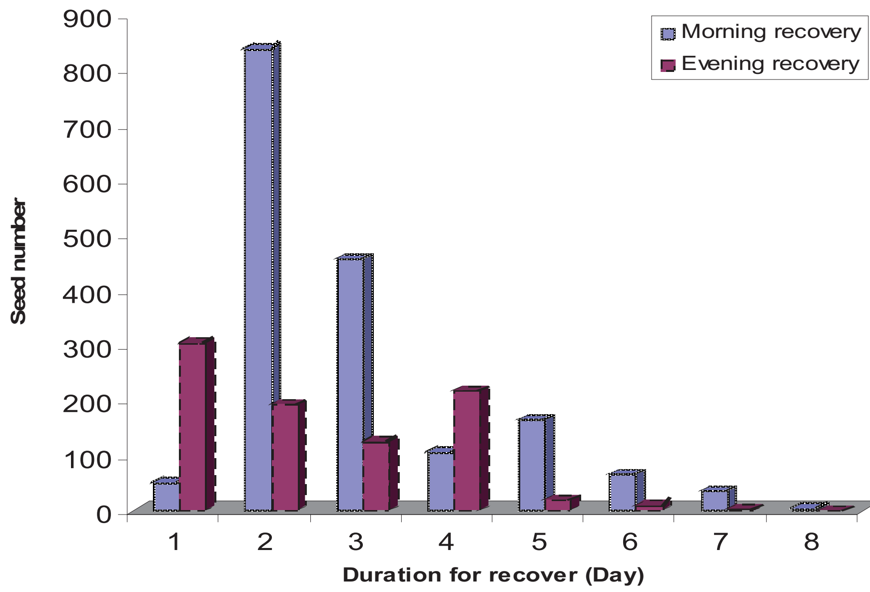

Figure 1. Pattern of the recovery of seed of Albizia saman from feces of Gudali bulls

Identification of GyrA mutations conferring fluoroquinolone resistance in *Salmonella* isolated from poultry and swine from Ogun and Oyo State, Nigeria

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ABSRACT

The quinolone-resistance determining-region of *gyrA* was PCR-amplified and sequenced in eight fluoroquinolone-resistant *Salmonella* (levofloxacin MICs ranging from 32-64 µg /mL) isolated from apparently healthy swine (n=2 isolates) and poultry (n=6 isolates) that died of septicaemic clinical diseases in Ogun and Oyo State, Nigeria. All of the eight isolates possessed the *gyrA* mutation encoding the histidine⇒tyrosine conversion at amino acid 150 (150His⇒Tyr). Additional substitutions included: 83Tyr⇒Ser in one *Salmonella enterica* serotype Give isolate (pig), 87Asp⇒Tyr in one *Salmonella enterica* serotype Kentucky isolate (pig); 83Tyr⇒Phe in one isolate characterized as 9,12:Nonmotile (poultry); and 87Asp⇒Gly in three poultry isolates including *Salmonella enterica* serotypes Kentucky (n=2), and Ituri (n=1). The 150His⇒Tyr is novel while the other mutations have been previously reported in *Salmonella* spp. This is the first study to associate *gyrA* mutations with fluoroquinolone resistance in Nigeria, where fluoroquinolone use in livestock is not tightly regulated.

INTRODUCTION

Considering the possibility of zoonotic transmission of infectious pathogens, transmission of drug resistant pathogens from animal to man could pose a great risk to public health in terms of treatment failure. For instance, 95% of the estimated 1.4 million cases of salmonellosis per year in United States of America were acquired through food-borne transmission (Mead *et al.*, 1999). Food animals are often incriminated as playing significant role in the development and spread of drug resistant pathogens (Threlfall *et al.*, 2000; Walker *et al.*, 2000). Development and transmission of genotypic traits encoding antimicrobial resistance is thought to be enhanced through agricultural and veterinary uses of antimicrobials as therapeutic and subtherapeutic agents (Allen and Poppe.2002).

Fluoroquinolones are the drug of choice against systemic salmonellosis (WHO 2010) but, unfortunately, high level fluoroquinolone resistance is increasingly prevalent in *Salmonella.* The unregulated use of these drugs can generate mutations at the quinolone-resistance determining-region (QRDR) of *gyrA* that encodes for subunit A of DNA gyrase, the enzyme targeted by

fluoroquinolones. These mutations eliminate the high affinity binding of fluoroquinolones and GyrA thus marginalizing the efficacy of these drugs.

The current study was carried out to characterize eight fluoroquinolone-resistant *Salmonella* strains isolated from swine and poultry in Nigeria. These isolates were subjected to serotype analysis and DNA sequencing of the QRDR in *gyrA*. Isolates were obtained from healthy swine and septicemic poultry.

MATERIALS AND METHODS

Isolation and identification of the isolates:

The two swine isolates were obtained from faecal samples collected from apparently healthy pigs in the University of Ibadan Teaching and Research farm. Samples were inoculated onto MacConkey and deoxycholate citrate agars and incubated aerobically at 37°C for 24-48hours. The yellowish glistening colonies were Gram-stained, and examined for motility and microscopic morphology. Tentative non-lactose fermenting *Salmonella* were further identified biochemically and serologically according to the Kauffman-White Scheme using polyvalent antiserum (Wellcome Research Laboratories, UK.) as *Salmonella species* (Edwards and Ewing, 1972; Barrow and Feltham, 1993). The other six *Salmonella* isolates were obtained from previous studies (Ogunleye *et al.*, 2010a,b).

Determination of the levofloxacin MIC values

Levofloxacin (Sigma Chemicals) MICs were determined using the two-fold micro-broth dilution method (CLSI. 2009). MICs were ascribed to the lowest concentration of levofloxacin that inhibited growth.

Serotyping of the isolate:

The two pig isolates and six poultry isolates were sub-cultured into TSA agar and submitted to National Veterinary Service Laboratories in Ames, Iowa, USA for serotyping. Serotyping was performed as per the Kauffman-White Scheme.

Amplification of the gyrA QRDR and DNA sequencing of the PCR product:

A 560 base-pair region of *gyrA* was amplified from chromosomal DNA obtained by heating the isolate at 99°C for 15 minutes. PCR was performed using the FailSafe™ System (EPICENTRE® Biotechnologies) in 50µl containing 1uM of forward and reverse oligonucleotides (F=5′ATGAGCGACCTTGCGAGAAATACACCG3′, R=5′TTCCAT-CAGCGCCCTTCAATGCTGATGTCTTC3′), 1.25 units of the FailSafe™ Enzyme, FailSafe™ PCR buffer B, and 1µl of crude DNA template. BIO-RAD MJ Mini personal Thermal cycler was used for the DNA amplification using the following PCR protocol: initial denaturation at 95°C for 2 minutes, followed by 40 cycles of 95°C for 1 minute, 53°C for 30 seconds, and 70°C for 45 seconds. Amplified DNA products were resolved using 1% (w/v) agarose gel electrophoresis. PCR products were purified and sequenced at Iowa State University DNA sequencing facilities (Ames, IA, USA).

RESULTS

Two *Salmonella* serotypes (Give and Kentucky) were isolated from the healthy pigs as shown in Table 1. The six poultry isolates belong to four different serotypes: Give, Kentucky, Ituri and 9,12:Nonmotile.

MIC values were essentially indistinguishable (32-64 μg/mL) given the nature of the assay. All eight isolates possessed gyrA mutations encoding the histidine to tyrosine substitution at amino acid 150 (150His⇒Tyr). One pig isolate (serotype Give) had an additional tyrosine to serine substitution at amino acid 83 (83Tyr⇒Ser), while the second pig isolate (serotype Kentucky) had contained the 87Asp⇒Tyr substitution. Three poultry isolates (two with the serotype Kentucky and one serotype Ituri) displayed the 87Asp⇒Gly alteration. The 9,12:Nonmotile poultry isolate bore a 83Tyr⇒Phe substitution. Two of the poultry isolates (9,12:Nonmotile and serotype Give) exhibited only the 150His⇒Tyr mutation.

DISCUSSION

The study herein describes the identification of gyrase mutations in fluoroquinolone-resistant isolates of *Salmonella* from Nigeria. *Salmonella enterica* serotypes Give and Kentucky were isolated from apparently healthy pigs while serotypes 9,12:Nonmotile, Give, Ituri, and Kentucky were isolated from septic poultry. All eight strains contained a novel histidine to tyrosine substitution at amino acid 150 while the 9,12:Nonmotile poultry isolate also expressed a novel tyrosine to phenylalanine substitution at amino acid 83. All other substitutions have been identified previously (Luque *et al.*, 2009; Kehrenberg *et al.*, 2007; Lee *et al.*, 2004; Ling *et al.*, 2003). The serotype Kentucky isolates from poultry may be clonal since they have identical genotypes and phenotypes in regards to the parameters studied herein.

The study also describes the novel isolation of *Salmonella enterica* serotype Ituri from septic chickens. This pathogen does, however, have a history as an avian pathogen since it was isolated from ducks in the Belgian Congo (Kauffman and Fain, 1953). Pathogenicity experiments will be conducted with this isolate using poultry as host in a future study.

The mutations described herein are stable and thus transferred to successive generations in the presence or absence of selective pressure. The observation in this study is therefore of public health concern, because of the possibility of transmission of drug resistance from broad host range serotypes from poultry and the apparently healthy pigs to man, thereby constituting a potential threat to treatment of *Salmonella* infections in humans and poultry.

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Table 1: Characterization of *Salmonella* isolates studied.

Animal Source	**Serotype**	**Levofloxacin MIC values (µg /mL)**	**Mutation(s) in the QRDR of *gyrA***
Pig	Give	32	150His⇒Tyr, 83Tyr⇒Ser
Pig	Kentucky	32	150His⇒Tyr, 87Asp⇒Tyr
Poultry	9,12:Nonmotile	32	150His⇒Tyr, 83Asp⇒Phe
Poultry	9,12:Nonmotile	32	150His⇒Tyr
Poultry	Give	32	150His⇒Tyr
Poultry	Kentucky	64	150His⇒Tyr, 87Asp⇒Gly
Poultry	Kentucky	64	150His⇒Tyr, 87Asp⇒Gly
Poultry	Ituri	32	150His⇒Tyr 87Asp⇒Gly

Investigation of Measles Vaccine Induced Immunity in Some Vaccinated Children in Ibadan, Nigeria.

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ABSTRACT

Measles account for more mortality than any other vaccine preventable diseases and responsible for over 10% deaths of children aged below 5 years worldwide. The use of measles vaccine over the last 40years has however reduced global measles morbidity and mortality. Five of the infants had protective levels of maternally derived IgG while 19 had lost it at the time of sample collection while the control group shows high IgG levels. Six weeks after vaccination all the infants tested (100%) had post vaccination protective levels of measles IgG (400 -21966 mlU/ml). However, we observed a drop in the IgG level of children with high maternal antibody level after vaccination, although the post-vaccination IgG levels were still higher than others. One of the infants had a dramatic rise in post vaccination IgG (from 367 to 21966 mlU/ml) this prompted the evaluation of pre and post vaccination measles IgM. Two of the children had detectable measles IgM before vaccination and 4 children after vaccination. It is concluded from this study that there is a high vaccine efficacy with measles vaccination at this particular, major and populously visited centre in Ibadan, Nigerian. The presence of measles IgM in some children reporting for vaccination shows possibility of recent exposure before vaccination or failure of the infant system to develop antibodies. Sustenance of measles vaccine coverage may therefore result to eradication of measles from Nigeria population.

Key Words: Measles, Vaccination, Seroconversion, IgG and IgM, Maternal Antibodies.

INTRODUCTION

Measles has been described as the greatest killer of children in human history (Fetter & Kessler, 1996; Burnett & Krusinski, 2005). Despite the availability of an effective vaccine that was developed more than 40 years ago, the measles virus still affects 50 million people and causes more than 1 million deaths annually, hence remains a leading cause of global death of young children (Measles Initiative, 2007). The highest incidence of measles and its associated morbidity and mortality has been observed in developing countries (Burnett & Krusinski, 2005).

Measles virus is a member of the Morbillivirus in the paramyxoviridae family. The paramyxoviruses are genetically stable viruses, primarily because they are composed of a single-strand of unsegmented, negative-sense ribonucleic acid (RNA) that apparently does not undergo recombination. Thus, the only mechanism that permits some degree of variation is mutation (Fennelly, 2006). Measles is highly contagious disease characterized by a prodromal illness of Fever, Coryza, Cough, and conjunctivitis followed by the appearance of a generalized

maculopapular rash (Wolfson et al, 2007). Measles virus infection recorded higher mortality and mobility than other known vaccine preventable diseases (VPD) as a result of complication (Meissner et al, 2005). In countries with weak measles vaccination programme the World Health Organization (WHO) estimated that in 2001, approximately 30 million cases of measles resulting in 745,000 deaths in children younger than 15 years were recorded (CDC, 1997). In other countries where measles immunization rate are less than 85% disease burden was reduced but still high (CDC, 2002).

In the joint strategic plan for measles mortality reduction, 2001- 2005, the World Health Organization (WHO) and the United Nations Children's Fund(UNICEF) targeted 45 priority countries (Africa inclusive)with high measles burden for implementation of a comprehensive strategy for accelerated and sustain measles mortality reduction (WHO, 2001). Despite the development of a successful live attenuated vaccine, measles remains a major cause of mortality of children in developing countries and cause of continuing outbreaks in industrialized nations. Measles remains a major cause of childhood morbidity and mortality in developing countries (Burnett & Krusinski, 2005). In developing countries measles is generally more severe than in developed countries and affect the younger children especially among the malnourished children. In the prevaccine era essentially everyone eventually acquired measles infection as a young child (de Quadros et al, 1996). However, measles includes lifelong immunity, but also causes immune suppression associated with increased susceptibility to other infections and in rare instances persistent infection of the nervous system (Auwaerter et al, 1996). Measles immunization improves the survival of children in developing countries. In areas where measles remain prevalent (developing countries) measles immunization is routinely performed at 9 months whereas in areas with little (developed countries) measles immunization is often done at 12 to 15 months of age (Shann, 1999). The administration of subpotent and\or impotent vaccine vis -a vis the status of immune response elicited in the vaccine may be one of the reasons for the occurrence of measles infection in vaccinated Nigerian (Omilabu et al, 1999).

Global vaccination coverage with live attenuated measles vaccine in children less than 1 year of age was in 1994 estimated to be 78. Immunization program worldwide now prevent greater than 1.5 million deaths from measles in developing countries. Yet approximately 1 million children continue to die each year from measles virus infection (Tarmin et al, 1994). Even though Meissner made it cleared that his recent experience demonstrated that prevention of endemic measles transmission is not possible in country with a single-dose immunization program, even when vaccination rate approach 100% (Meissner et al, 2005), the immunization coverage is still poorly distributed in some developing country especially in Nigeria where < 40% were covered (WHO/AFRO, 2006).

This study was designed to investigate the efficacy of vaccination being administered to children aged 9 months at the Institute of Child Health, University College Hospital, Ibadan Nigeria and determine the percentage of children with maternal antibodies at this age. It is also designed to determine whether the presence or absence of maternal antibody at the time of vaccination had any influence on the level of IgG produced after vaccination among these Nigerian children.

MATERIAL AND METHODS

Study population

The study was carried out at the Institute of Child Health, University College Hospital (UCH), Ibadan, for the period of 3months. Ibadan is the capital of Oyo State occupying the latitudes 40 2' E and 70 10'E and longitudes 70 3' N and 40 15' N. the study population comprised of the infants who were brought for vaccination at age of 9months by their parents and whose parents consented to the study. Information were obtained by structured questionnaire about the infants age, previous exposure to measles infection, the occupations of the parents, residential address, number of children and if any of such had been infected with measles virus before.

Specimen collection

Blood samples were obtained from the finger print of the infant into the EDTA bottles, this was spun and the serum separated immediately and kept frozen under -20^{o}C. Six weeks after vaccination post-vaccinated samples were obtained from the infants whose parents fully cooperated with the study. 6 cord blood samples were obtained from neonates in the maternity ward of University College Hospital, Ibadan as control group to show the level of maternal antibody at birth. All the sera were kept -20^{o}C until analysed.

Samples analysis

Using ELISA kits, the levels of maternally derived IgG was determined in the sera samples of these infants for prevaccination and seroconversion to measles virus vaccine administered-post vaccination IgG. Likewise measles IgM was also screened for on both pre-vaccinated and post-vaccinated samples.

RESULT

Measles IgG in infants sera and cord blood

Five of the nine months old children had protective levels of maternally derived IgG while 19 had lost maternally derived IgG at the time the samples was collected. (Fig.1). All cord blood samples tested were positive and had high IgG levels (Fig. 1).

Seroconversion to measles vaccine in vaccinated children

Seroconversion to measles vaccine in vaccinated children was found to be 100% of the children recruited at the time of vaccination who were asked to report at the clinic six weeks after vaccination to determine the level of measles IgG present after vaccination. All the 19 (100%) children out of the 24 recruited had protective levels of measles IgG (fig.2). The titres ranged from a minimum of 400mlU/ml to 21,965.72mIU/mI (Table 1). A summary of the IgG titres of all the children tested is shown in Table 1.

Comparison of influence of pre-vaccination IgG to seroconversion to vaccination

The IgG levels of the vaccinated children were compared to the pre vaccination levels to determine if the presence or absence of maternal antibody at the time of vaccination had any influence in the level of IgG produced after vaccination. Figure 3 shows a drop in the IgG level of children who had very high maternal antibody level at the time of vaccination, whereas this is not observed in children who had lost maternal antibodies at the time of vaccination. One of the children had a dramatic rise in titre post vaccination (Fig 3). This then prompted the investigation of measles IgM pre and post vaccination.

Protective measles IgG In pre and post vaccination sera

The protective level of IgG in the sera of the children was tested for. The children were grouped into three categories namely, Pre vaccination, Post vaccination and Cord blood as control. Only 21% of the pre vaccination sera had protective antibodies (Fig.4). All post vaccination sera (100%) had protective antibodies and all the cord blood samples (maternally derived antibodies) had protective antibodies also (Fig. 4).

Presence of measles specific IgM in the infants

All the samples previously tested for measles specific IgG were also tested for measles IgM to determine if there were any of the children who had been exposed prior to vaccination. We saw that only 2 children had detectable IgM titres before vaccination and 4 children had detectable measles IgM post vaccination (Fig.6).

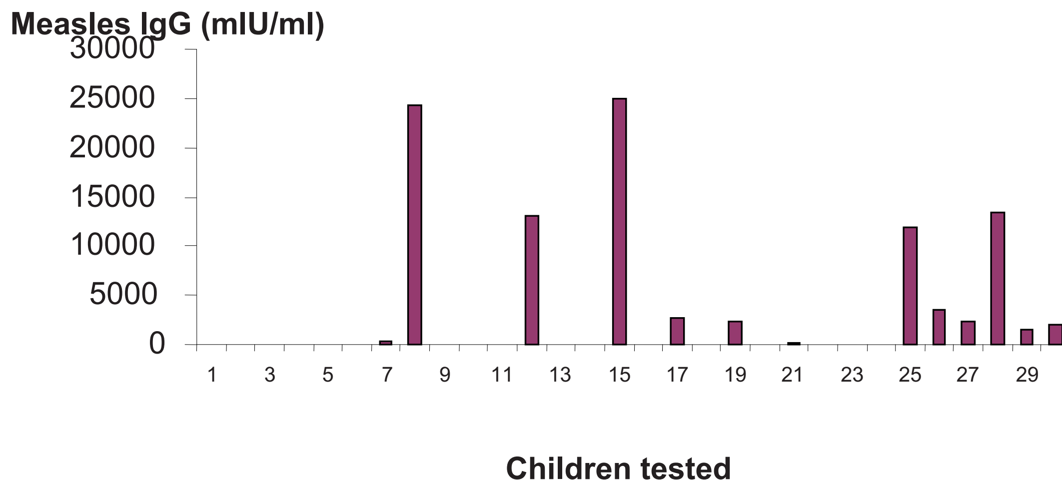

Fig 1: Measles IgG in infants sera and cord blood

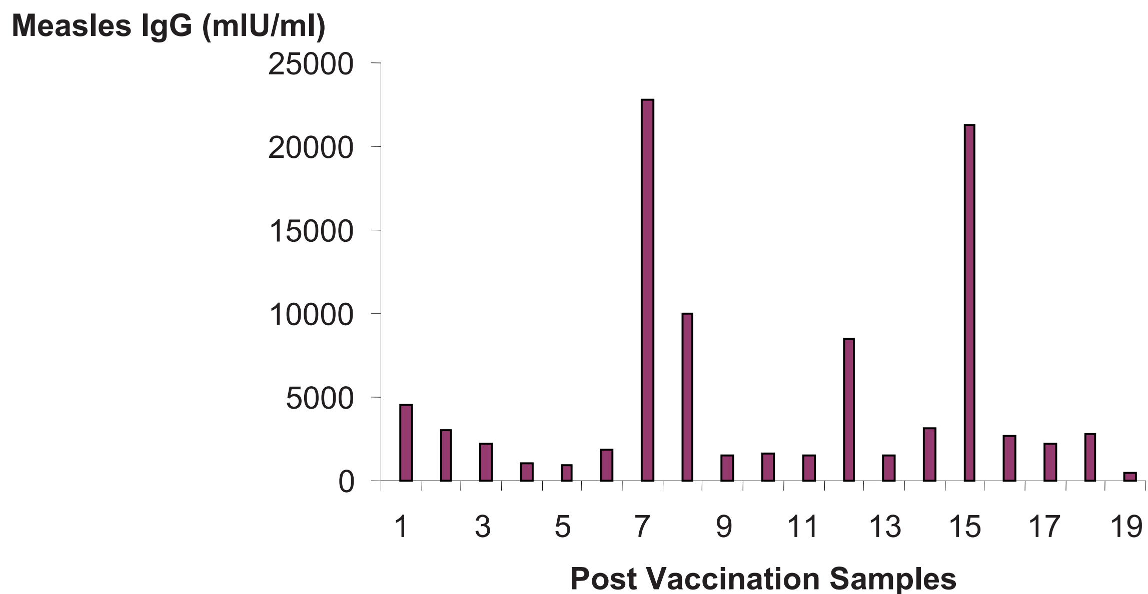

Fig 2: Post vaccination measles specific IgG in vaccinated children

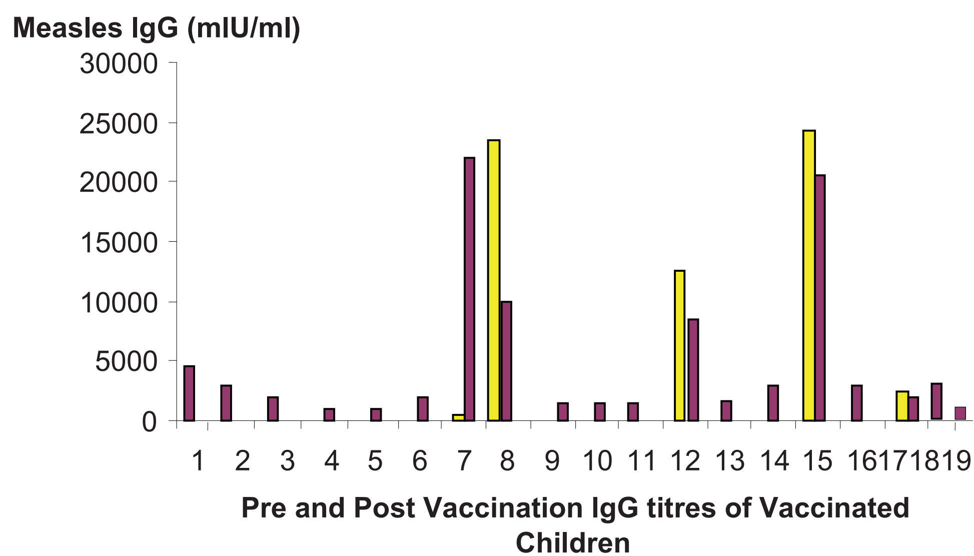

Fig 3: Comparison of Pre and Post Vaccination IgG titre of vaccinated children

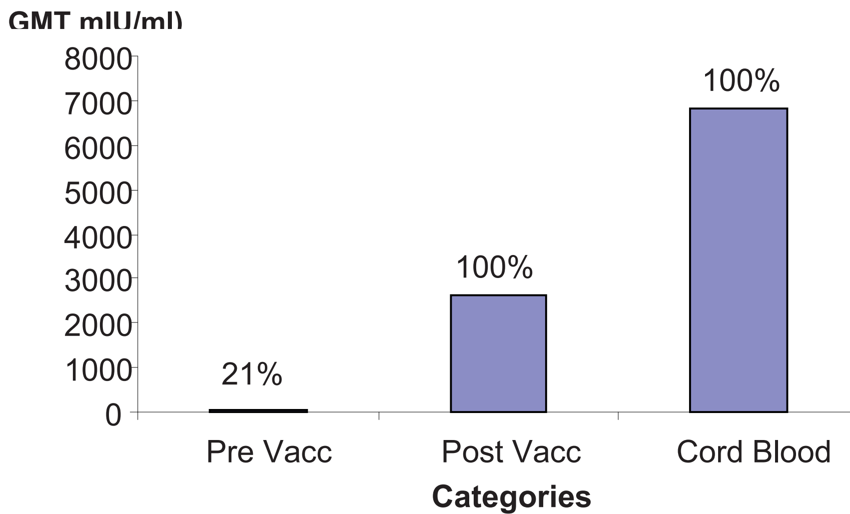

Fig 4: Protective measles IgG in pre and post vaccination sera

DISCUSSION

Since 1976, EPI has recommended vaccination against measles in the developing countries at age of 9months or as soon as possible. Program that has achieved and sustained high coverage with Schwarz at the age of 9months has substantially reduced measles incidence and mortality rates (Cutts et al, 1991). Before measles vaccination, immunity was acquired by maternal antibodies transferred in-utero (Englund, 2007). Our study reveals that some infants still carry maternal antibodies at the age of 9months even though majority had lost it at this age. All the children who reported back 6 weeks post vaccination had measles specific IgG showing a seroconversion rate of 100%. This shows a remarkable high vaccine efficacy in this particular centre used for the study. Previous workers have reported between 84% seroconversion to 92% in Haitian infants (Hasley et al, 1985), in Latin America a study recorded a seroconversion rate of 85%-95 % (Cutts & Markowitz, 1994).

All the samples were tested for IgM, 6 samples in total were positive for measles specific IgM, 2 prevaccination samples and 4 post vaccination samples. The IgG titres of the prevaccination samples was checked and it was discovered that both children had no measles specifics IgG prevaccination while a very high post vaccination IgG was recorded. These two children were most likely infected with measles shortly before vaccination; this can explain the presence of measles IgM in their sera prevaccination. Some children of the post vaccination sera with detectable IgM has been explained by Halls 1993 to be due to the fact that post- immunization antibody levels might be low and the detection of IgM after vaccination might not yield high results (Hall & Cutts, 1993).

Comparing the case of the infants who still possessed the maternal antibodies at the time of vaccination and those who had lost the maternal antibodies: studies have shown that maternal antibodies among different population had been found to correlate with socioeconomic status[18]

and that measles vaccine is most immunogenic if administered after the children have lost the maternal antibodies, but in our study, even though there was drop in the post vaccination IgG of those with maternal antibody, their IgG level were still higher than the rest that have lost maternal antibody. This correlates with Markowitz finding (Markowitz & Orenstein, 1990). The out come of this study also support the finding of WHO new data on global measles deaths which fell In Africa by 75 per cent, from an estimated 506,000 to 126,000 (WHO, 2007).

However, to obtain the optimal response to immunization, measles vaccine should be administered at an age when all children have lost maternal antibodies. The immunogenic response must also be balanced against the risk of measles at a young age (Hall & Cutts, 1993). From the result obtained in the study, it is cleared that if measles vaccine coverage is sustained, with a high vaccine efficacy the eradication of measles from Nigeria can become a reality. The presence of Measles IgM in some children reporting for vaccination shows that some children have been exposed shortly before vaccination. Effective surveillance of diseases of disease and vaccine coverage is essential to monitor changes in measles epidemiology, to identify high risk areas of group and to monitor progress toward program goals. For Nigerian, as the EPI enters the fourth decade it becomes more important to monitor our vaccine efficacy and seroconversion to vaccine in order to determine how well the vaccination programs are doing. It is therefore recommended that surveillance centres be set up to monitor measles outbreaks and effectiveness of the vaccination program.

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Rapid detection of DHFR resistance alleles in *Plasmodium falciparum* using Real time PCR with locked nucleic acid double-labeled probes

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ABSTRACT

Mutations in the *Plasmodium falciparum* gene encoding dihydrofolate reductase (*dhfr*) are associated with resistance to antifolates. *Plasmodium falciparum*, the most prevalent malaria parasite in sub-saharan Africa, has been reported to be resistance to almost all the available antimalarias including the antifolates. Functional polymorphisms in the *dhfr* gene at codon 51, 59 and 108 of *P. falciparum* were assessed by Real time PCR (RT-PCR) with double labeled Locked nucleic Acid (LNA) using blood samples taken from 133 children with uncomplicated *falciparum* malaria from osogbo Nigeria. LNA double labeled probes have been used in this work to detect and measure the frequency of *dhfr* resistance alleles in children with uncomplicated *P. falciparum*. Measurement of the frequency of resistant alleles in populations of *P. falciparum* was achieved by using the cycle threshold (*C*t) values and a standard curve derived from laboratory parasite with known allele frequencies. The prevalence of the mutant I51, R59 and N108 in the clinical isolates was 85%, 95% and 98.5% respectively. Double mutant genotypes of 51+59, 51+108 and 59+108 were found in 81.2%, 85.7% and 92.5% respectively. Triple genotype mutations 51+59+108 were found in 78.2%. None of the samples had the T108 mutation. The prevalence of the combination of triple mutant alleles was 78.2%. The LNA double labeled RT-PCR rapidly and simultaneously detected the I51 and R59 mutation in a single run while the N108 and T108 was also simultaneously detected in a single run. The lowest detection limit of the assay was about 1.5×10^{-3}ng/µl of DNA below 40 cycles, that is parasite density as low as 2 parasites/200 WBCs can be detected. In addition to its sensitivity and specificity the new assay has a low per test cost, fast, easily automated and well-suited for large scale epidemiological studies. The high *dhfr* alleles observed in this study is an indication of possible resistance to sulfadoxine pyrimethanine widely used antimalaria in the study area.

Key words: Malaria, dhfr, Real time PCR, genotyping

INTRODUCTION

The impact of drug resistant *Plasmodium falciparum* on public health in sub-Saharan African countries is enormous. This applies in particular to chloroquine (CQ) but also to the synergistic combination of pyrimethanine sulfadoxine (SP). SP is an antifolate class of drugs that has been widely used as first-line therapy for uncomplicated *P. falciparum* malaria throughout Sub-Saharan Africa, because of its affordability, its ease of administration, and, until recently, its

effectiveness (McCollum *et al.*, 2006). Drug pressure is considered to essentially promote the emergence of SP resistance, which is now widespread in East Africa, but also well recognised in West Africa (Marks *et al.*, 2005)

A good correlation has been reported between mutations in the dihydrofolate reductase (*dhfr*) and resistance to pyrimethamine and sulfadoxine, respectively (Kublin *et al.*, 1998; Talisuna *et al.*, 2004). Pyrimethamine is a selective, competitive inhibitor of *dhfr* (Brooks *et al.*, 1994; Bzik *et al.*, 1987). Several point mutations are connected to antifolate drug resistance but the quintuple mutation (triple *Pfdhfr***:** I51, R59, N108 and double *pfdhps*: G437, E540 is discussed as a the most relevant molecular marker of SP treatment failure (Happi *et al.*, 2005; Happi *et al.*, 2006; Kublin *et al.*, 2002). The S108N, C59R, and N51I mutations in the *Plasmodium falciparum* gene *Dhfr,* which encodes dihydrofolate reductase, confer resistance to pyrimethamine and are widespread in Africa (Lynch *et al.*, 2008). Recently additional non synonymous mutation at codon 50 (C50R) confers an increased level of resistance to pyrimethamine, and this mutation has been described only at sites in South America (Cortese and Plowe, 1998). Occurrence of a fourth mutation in the triple mutant at codon 164(I164L) confers a higher level of resistance to SP (Plowe *et al.*, 1998).

Resistance to antimalarial drugs can be assessed *in vivo* and also *in vitro* by parasite susceptibility assays or by the use of molecular techniques to detect genetic markers. A variety of different approaches to RT-PCR are used for SNP detection and measurement, including the use of several different chemistries in the synthesis of the probes (von Samson-Himmelstjerna and Blackhall, 2005). We have therefore investigated the prevalence of *dhfr* mutant genes using Locked Nucleic Acid (LNA) double labelled probe real-time PCR method for the detection of resistant alleles at positions 51, 59 and 108. LNA is a novel type of nucleic acid analogue that contains a 2'-O, 4'-C methylene bridge. This bridge, locked in 3'-endo conformation, restricts the flexibility of the ribofuranose ring and locks the structure into a rigid bicyclic formation (Karkare and Bhatnagar, 2006).

MATERIALS AND METHOD

Specimen Collection:

Dried-blood spots were prepared from the blood of *P. falciparum* infected children aged between 1–12 years old attending the Osun state Hospital and Ladoke Akintola University Teaching hospital Osogbo Nigeria between July 2004 and January 2005. Osogbo is the state capital of Osun state Nigeria and it represents a typical urban setting in Nigeria. Malaria is present throughout the year with a marked increase during the raining season (i.e. April – September). The children were recruited if the following criteria were met: presence of *P. falciparum*, without other *Plasmodium* species, parasitemia >2,000 asexual parasites per µL of peripheral blood. Two drops of blood were also blotted onto 3MM Whatman filter paper from the patients for molecular analysis. The study was approved by the the ethical review committee boards of the joint College of Health Sciences/Ladoke Akintola University Teaching Hospital and Osun State Hospitals Management Board.

For the evaluation of the LNA Taqman assay, Six *P. falciparum* laboratory clones were used. The laboratory clones used are 3D7, FCR, HB3, Dd2, S007 and K1. The parasites were maintained in culture and 5µl of the culture containing the parasite was blotted on 3MM Whatman filter paper for DNA extraction.

DNA Preparation

Parasite genomic DNA was extracted from the clinical samples and laboratory strains using a QIAamp DNA blood kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

Probes and Primers

The mutations at positions 51 and 59 were detected simultaneously in quadruplex assay while mutation at position 108 was detected in a triplex assay. A pair of LNA probes labelled with a florescent dye at the 5' position and BBQ (quencher) at the 3' position was designed to be complimentary to either the wild or the mutant allele at each position. The wild probe at position 51 was labelled with FAM dye while the mutant probe was labelled with JOE dye. For position 59 the wild probe was labelled with ROX dye while the mutant probe was labelled with CY5. For position 108 Two Mutant LNA probes labelled with Cy5 and Rox were designed to be complimentary for the two possible mutations (N108 and T108). The wild probe was labelled with Fam dye (table1).

The principle of the Taqman RT-PCR is based on DNA amplification and cleavage of the internal probe that is hybridized to the amplified DNA by the 5′-3′ exonuclease activity of the *Taq* DNA polymerase during PCR cycles. At the start of the reaction no fluorescence will be detected due to the proximity of the quencher and reporter dyes. However, during each cycle of the extension phase one molecule of reporter dye is released for each target molecule amplified. The threshold cycle (Ct) value is the number of cycles before the fluorescence emitted passes a fixed limit. The probes and primers shown in table 1 were designed and synthesized by TIB MOLBIOL DNA synthesis service, (Berlin, Germany).

Real Time PCR Amplification

All the real time experiments were performed on the Rotor Gene 3000 (Corbett, Sydney, Australia). For positions 51 and 59 Real-time PCR amplification was carried out in duplicate in 50µl final volume while for position 108 the final volume was 30µl. The PCR mix contains 18µl Multiplex PCR Master Mix (Qiagen, Hilden, Germany), 0.4µM forward and reverse primers, both mutant and wild-type probes were used at a final concentration of 0.2µM and 8µl of DNA was used. The PCR program started with an initial denaturing step at 95°C for 15 min and cycled 40 times, with each cycle consisting of 94°C for 20s and 60°C for 45s.

DNA Sequencing

The laboratory strains and some selected clinical sample were subjected to DNA sequencing in other confirm the real-time PCR result. In such cases amplicons from the real time PCR were purified using PeqGOLD Cycle pure kit (Classic-Line) (peQLab Biotechnologie GmbH, Erlangen, Germany) according to manufacturer's instructions. The resulting amplicon was re-amplified, purified with Sephadex TM G-50. The DNA sequence was determined using an ABI

PRISIM 3100 Genetic Analyser (Applied Biosystem). Sequence results were analysed using the freely available BioEdit Sequence Alignment Editor software. The resulting sequences were compared to the *Pfdhfr* portion of the *P. falciparum* clone 3D7 (accession number AF248537).

RESULTS

The potential for the simultaneous detection of point mutations in *dhfr* genes using LNA double labeled probes on RT- PCR instrument has been evaluated. The six *P. falciparum* laboratory strains used for the evaluation of the method showed the correct mutation profiles expected from the parasites (table 2). PCR product sequencing and the RT-PCR product were used as confirmation. The assay was able to detect both mutant (I51, R59) and wild (N51, C59) alleles at position 51 and 59 in a single run (figure 1) while the mutant (N108, T108) and wild (S108) alleles at position 108 were also detected simultaneously in a triplex assay (figure 2).

Sensitivity

Dilutions of both wild and mutant type DNA laboratory strains were tested to confirm the relative efficiency of both wild and mutant fluorescent reporters. A plot of the measured Ct for each dilution versus the amount of DNA tested showed that $1.5x10^{-3}$ng/µl of DNA is detectable at below 40 cycles (figure 3). The efficiency of the dyes have similar slope and the relationship is linear for the reporters with a correlation coefficient of 0.99.

Clinical isolate genotypes

LNA double labeled RT-PCR assay for the simultaneous detection of the novel *dhfr* mutations at positions 51, 59 and 108 in clinical isolates are summarized in figure 4. A total of 133 patients were screened. The result of the study showed that 96.2% of the isolates had the S108 mutation while no allele was detected for the T108 mutation. For positions 51 and 59 the prevalence was 85% and 95% respectively. The break down of the prevalence of each allele and mix alleles are shown in Table 3 and figure 4. The percentage of double mutations of N108+I51 and N108+ R59 was 85.7% and 92.5% respectively. The prevalence of the triple mutation N108+I51+R59 was 78.2%. The breakdown of the prevalence is shown in Table 4.

DISCUSSION

This paper describes a LNA RT-PCR assay for the detection of the most pivotal polymorphisms of the *P. falciparum dhfr* gene. The method successfully discriminates between wild, mutant and mixed alleles. This work also represents the first instance of a multiplexed allele specific LNA RT-PCR assay for the simultaneous detection of the *dhfr* gene mutations at the positions 51 and 59.

As a surveillance tool, RT-PCR has certain advantages over classical PCR. With RT-PCR there is no need for gel and digestion with enzymes thereby reducing the risk of possible contamination. The LNA double labeled probe assay used in this study is rapid (72 samples can assayed in 2hours) and can reliably distinguish between four (2 wilds and 2 mutants) alleles in a

single run. LNA probes have been shown to help in improving the sensitivity and specificity compared with normal probes and, it also makes multiplexing easy (Petersen and Wengel, 2003; Walsh *et al.*, 2007). LNAs are modified nucleotides that increase the stability of the duplex when bound to the target (Braasch and Corey, 2001; Petersen and Wengel, 2003) and have previously been used in several allelic discrimination assays (Chieochansin *et al.*, 2006; Johnson *et al.*, 2004). This increased stability results in a higher melting temperature and stronger hybridization (Kumar *et al.*, 1998). Recent work has shown that when LNAs are incorporated into dual labeled probes (Taqman® probes) they provided increased specificity and sensitivity when compared to normal probes (Reynisson *et al.*, 2006). In this study we did not encounter any difficulty in multiplexing the assay as all the fluorophores used gave a similar and very good level of fluorescence. The sensitivity of the assay tends to be better when high concentration of DNA were used. The lowest detection limit of this assay which is about 1.5×10^{-3} ng/µl means that with clinical isolates, parasite density as low as 2 parasites/200 WBCs can be detected.

Molecular methods provide promising surveillance tools, and several genetic mutations have been identified and associated with resistance to antimalarials. The prevalence of these single-nucleotide polymorphisms (SNPs) in a population is often a good indicator of the level of clinical resistance. The prevalence of mutations in the *dhfr* and *dhps* genes in *P. falciparum* has been described as ‘extremely valuable for making predictions about the likely efficacy of antifolate drugs in many areas where malaria is endemic (Sibley *et al.*, 2001). The *dhfr* triple mutant Asn-108/Ile-51/Arg-59 or the *dhps* double mutants Gly-437/Glu-540 has been independently associated with *in vivo* SP resistance in Africa where malaria is endemic (Bates *et al.*, 2004; Cowman, 1997; Nzila *et al.*, 2000; Plowe *et al.*, 1997). The occurrence of high triple *dhfr* mutations (51, 59 and 108) in our study is thus similar to findings from other African countries where antifolate drugs have been used intensively owing to high level of resistance to chloroquine (Bates *et al.*, 2004; Falade *et al.*, 1997; Happi *et al.*, 2005). The prevalence of the triple-mutant alleles in our study area makes it clear that SP-driven selection for mutant alleles of *dhfr* is certainly in progress.

In conclusion, it has been shown that the use of LNA double labelled probes provides a basis for rapid, sensitive and reproducible assay for the simultaneous detection of *dhfr* mutations that has been associated with SP resistance. We anticipate that this method can be utilised to develop useful assays for detection of other malaria drug resistant mutations. We also report a high level of the triple *dhfr* mutation in *P. falciparum* isolates in our study area.

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Table 1: Oligonucleotide used in this study (listed 5' to 3')

Name	**Sequence**
*DHFR*51-Forward primer	TgAggTTTTTAATAACTACACATTTAgAggTCT
*DHFR*51-Reverse primer	TATCATTTACATTATCCACAgTTTCTTTgTT
*DHFR*51-Wild probe	FAM-AAATgTAATTCCCTAgA+TA+Tg—BBQ
*DHFR*51- Mutant probe	JOE-AAATgTATTTCCCTAgA+TA+Tg—BBQ
*DHFR*59-Wild probe	ROX-ATATTTT+Tg+TgCAg+T+TAC—BBQ
*DHFR*59-Mutant probe	Cy5-ATATTTTCg+TgCAg+T+TAC—BBQ
*DHFR*108-Forward primer	TggATAATgTAAATgATATgCCTAATTCTAA
*DHFR*108-Reverse primer	AATCTTCTTTTTTTAAggTTCTAgACAATATAACA
*DHFR*108-Wild probe	ROX-AgAAC+A+AgCTggg+A+A+A—BBQ
*DHFR*108-Mutant (N108) probe	Cy5-AgAACAAACTggg+A+A+Ag—BBQ
*DHFR*108-Mutant (T108) probe	FAM-AgAACAACCTgggAAA-BBQ

"+" Positions of the LNA modifications on the probes

Table 2: LNA TaqMan Real-time PCR genotype of laboratory parasites

Laboratory Strains	**51**	**59**	**108**
FCR	N51	C59	S108
3D7	N51	C59	T108
D10	N51	C59	N108
HB3	N51	C59	N108
Dd2	I51	R59	N108
K1	I51	R59	S108

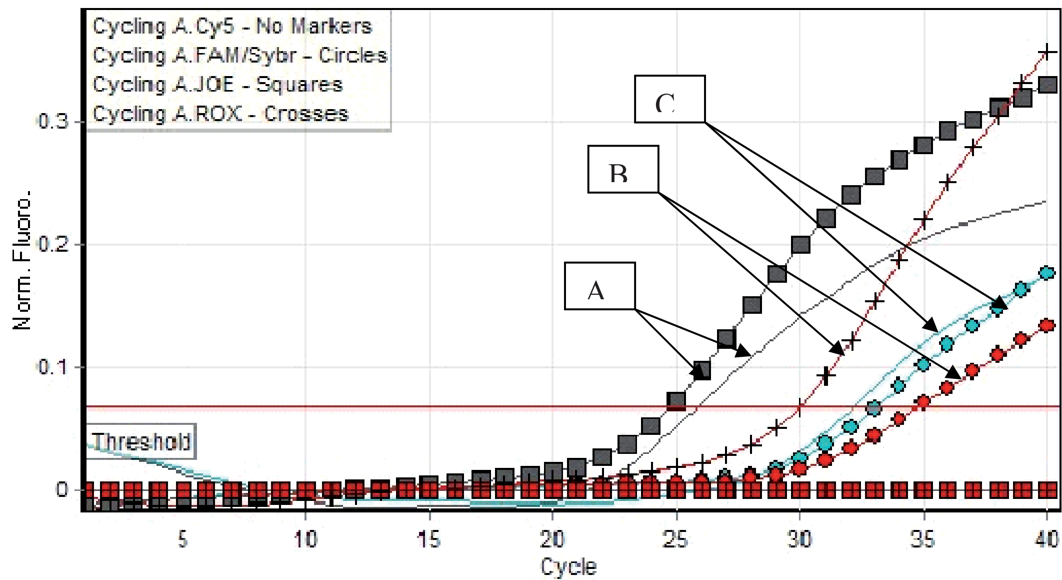
Table 3: Distribution of mutant and mix (wild and mutant) *Pfdhfr* alleles by age among children in Osogbo as determined by LNA real-time PCR

Age	**Freq**	**Sex**		**I51** Mut	**N51** Wild	**N51I** Mix	**R59** Mut	**C59** Wild	**C59R** mix	**N108** Mut	**S108** Wild	**S108N** Mix
		M	F									
0>4	79	43	36	65	13	1	74	4	1	78	0	1
4<8	37	21	16	31	5	1	36	1	0	33	2	2
8-12	17	9	8	13	2	2	15	1	1	17	0	0
Total	133	73	60	109 (82%)	20 (15%)	4 (3.0%)	125 (94%)	6 (4.5%)	2 (1.5%)	128 (96.2%)	2 (1.5%)	3 (2.3%)

Table 4: Distribution of the DHFR Alleles combination in *P. falciparum* isolates in Osogbo as determined by LNA real-time TaqMan assay

Allele Combinations	**Frequency**	**Percentage**
N = 133		
51 Mutant + 59 Mutant (I51+R59)	108	81.2%
51 Wild + 59 Wild (N51+C59)	4	3.0%
51 + 59 Mixed	21	15.8%
N = 133		
51 Mutant + 108 Mutant (I51+N108)	106	85.7%
51 Wild + 108 Wild (N51+S108)	0	0 %
51 + 108 Mix	27	21.8%
N = 133		
59 Mutant + 108 Mutant (R59+N108)	123	92.5%
59 Wild + 108 Wild (C59+S108)	0	0 %
59 + 108 Mix	10	7.5%
N= 133		
51Mutant + 59Mutant + 108Mutant	104	78.2%
51 Wild + 59 Wild + 108 Wild	0	0 %
51 + 59 + 108 Mixed	29	21.8%

Fig. 1: A typical Real-time PCR run for simultaneous detection of *P. falciparum* alleles of DHFR at positions 51 & 59

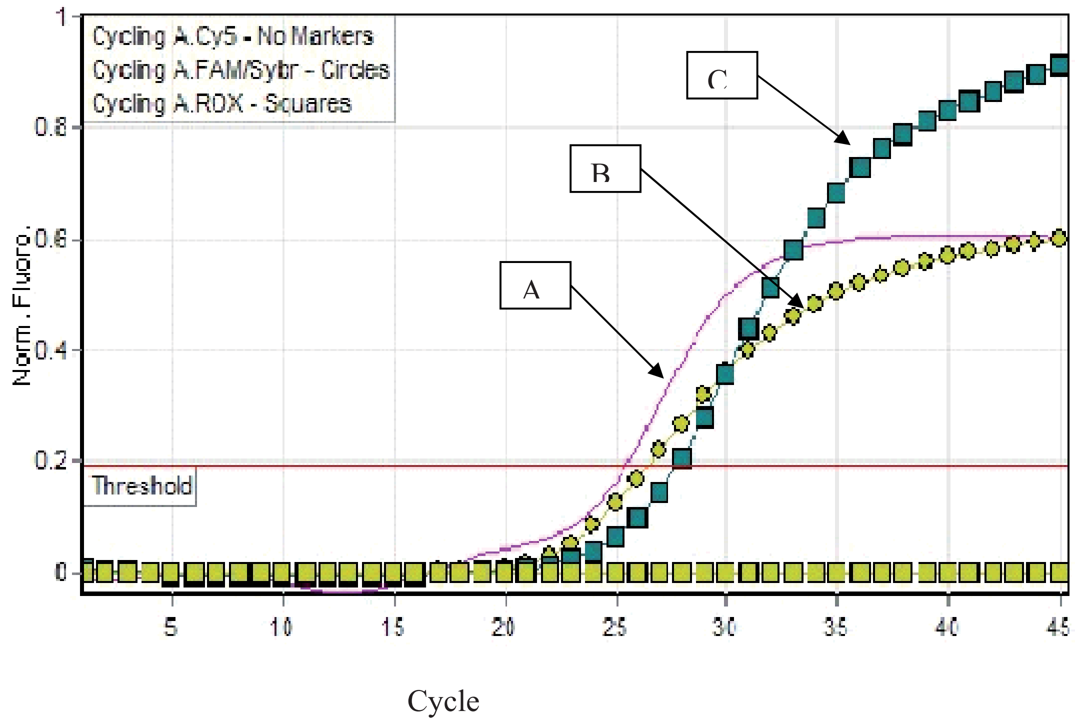


A. A sample that is mutant at both position 51(I51) and 59 (R59). The square is labelled with Joe probe that binds to the mutant alleles at position 51 while the line without a marker represents the cy5 probe which is labelled to bind to the mutant alleles at position 59

B. A sample that is wild at both positions 51(N51) and 59(C59). The circle represent FAM probe that binds to the wild alleles at position 51 while the crosses represent Rox probe that binds to the wild alleles at position 59

C. A sample with mixed allele (N51 and R59). Circle represent FAM probe that binds to the wild alleles at position 51 while the line without a marker represents the Cy5 probe which is labelled to bind to the mutant alleles at position 59.

Fig. 2: A typical Real-time PCR run for simultaneous detection of *P. falciparum* alleles of DHFR at position 108

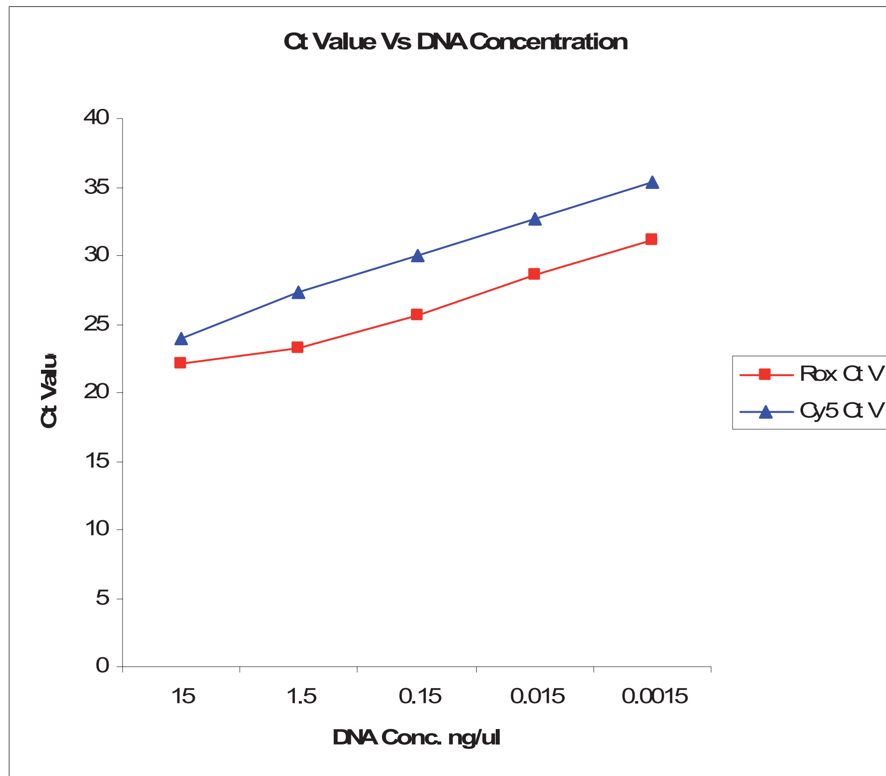


A. The line without marker is labelled with Cy5 probe which binds to the alleles that carries the S108 mutations

B. The line with circle is labelled with FAM probe which binds to the alleles that carries the T108 mutations.

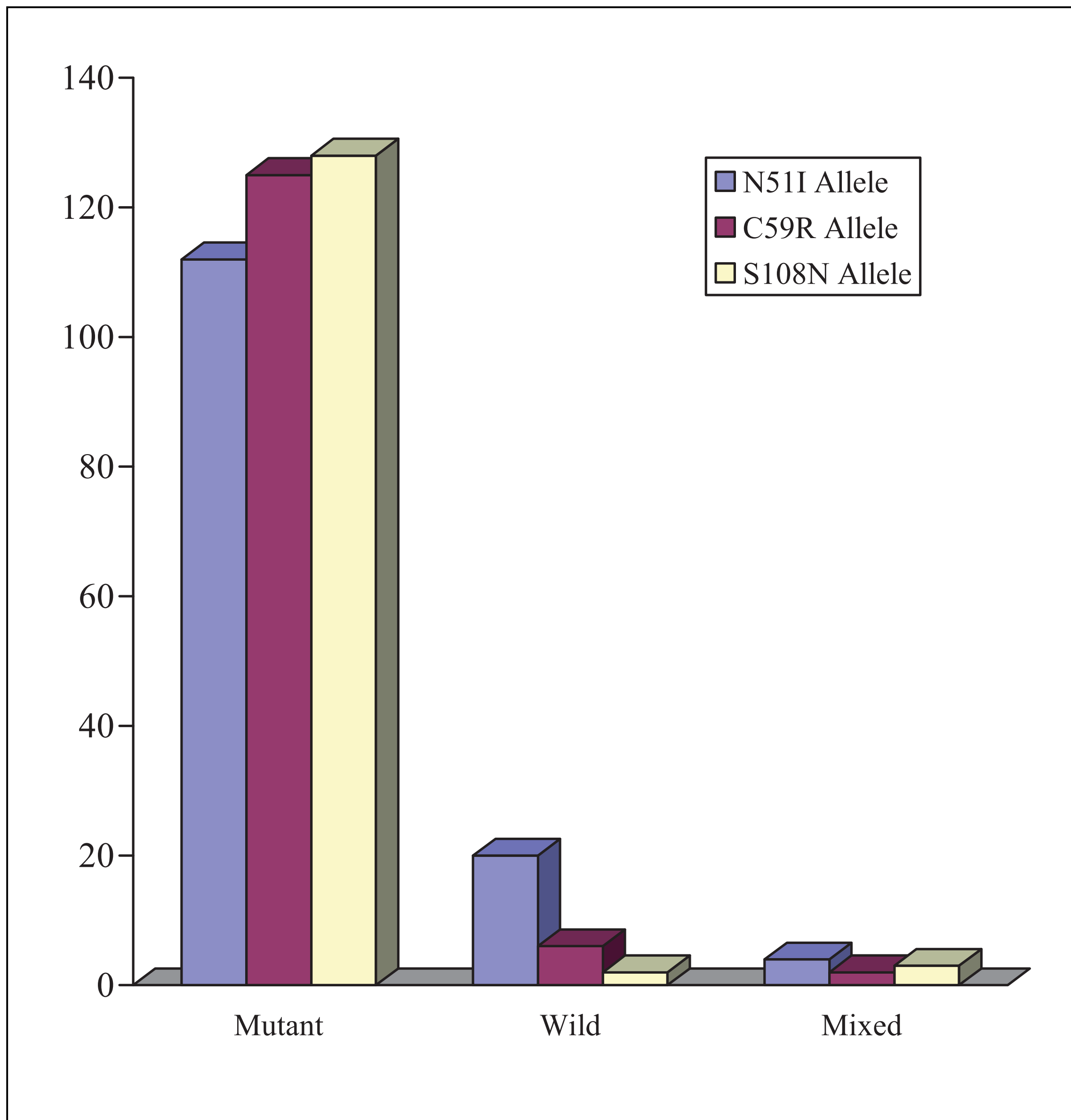
C. The line with square is labelled with Rox probe and it binds to the alleles that carries the N108 (wild alleles)

Fig. 3: A Cycle threshold (Ct's) response plot showing the relationship between the Rox and FAM Ct's and the amount of DNA tested.



15 ng/µl of DNA was detectable at about 22 cycles for Rox dye while for Cy5 it was at about 24 cycles. At 0.0015 ng/µl dilution of DNA, Rox dye was detectable at 29 cycles while Cy5 was at 34 cycles. The dyes have similar slope and the relationship is linear for both dyes

Fig. 4: Distribution of mutant and mix (wild and mutant) *Pfdhfr* alleles among children in Osogbo as determined by LNA real-time PCR



Isolates are classified as mutant if they posses the resistant allele and wild if they posses the sensitive allele and mixed if they contains both. Resistant alleles are highly prevalent in the population.

The Micronucleus Assay System: A Veritable Tool for Identification of Genomic Damage in Developing Countries.

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ABSTRACT

Increasing exposure to environmental pollutants is generally recognized as part of industrial development. Environmental exposure to toxicants is known to modify the genome, leading to diseases, including cancer and birth malformations. Genomic damage is probably the most important fundamental cause of developmental and degenerative diseases. It is expedient to have a reliable, relevant and affordable minimally invasive biomarker to aid biomonitoring, diagnosis, and treatment of disease caused by or associated with genome damage. The Micronuclei were first recognized by Howell in 1891 as distinct cellular inclusions in erythrocytes from anemic cats. Jolly in 1905 subsequently confirmed the existence of micronuclei during erythropoiesis in mouse and rat embryos. The micronucleus test system was then developed by Schmid and Heddle. It is an indirect measure of chromosome break (clastogenic effects). Micronuclei are believed to be formed as a response to chromosome damage, leading to the formation of small nuclei ('micronuclei').Micronuclei are round , membrane- bound cellular inclusion bodies varying from 1 -4 µm in size and contain DNA.They are distinct from the cell nucleus, but stain similarly owing to their DNA content. The micronucleus assay system has been applied widely as a short-term genotoxicity test for evaluating xenobiotics for mutagenic and carcinogenic potential. Substances which cause damage to genome may produce gene mutations, and/ or chromosomal aberrations which could be possible initiation events of the carcinogenic process or which could result in heritable disorders. The test though has a number of drawbacks compared to the comet assay, 8-OHdG, FPG in identification of DNA damage is an appealing method for determining potential clastogenicity in developing countries because the test is relatively rapid, inexpensive, and can be performed with minimal degree of instruction and experience 'though not designed for evaluation by fools.' The system is potentially an excellent candidate to serve as a biomarker especially given the number of modifications and improvements as well as the very recent international attention it has received. The Human Micronucleus (HUMN) project is a multicentre international collaborative effort aimed largely at addressing existing and perceived gaps in the micronucleus test system. This review is aimed at drawing attention to this veritable but poorly recognized tool to detect inevitable genome damage associated with the increasing exposure to ubiquitous environmental pollutants in rapidly industrializing developing countries including Nigeria.

Keywords: Biomarker, Clastogens, Developing countries, Environmental pollutants, Genome damage, Industrial development, Micronucleus assay

INTRODUCTION

As societies throughout the world are increasingly moving to greater levels of urbanization and industrial development, public concern is mounting over the state of the environment and much attention is now being given to improving the environment for future generations (Smith, 1996). In most developing countries, the situation is less encouraging where resources are limited and policies and regulatory measures are greatly inadequate. Smith (1996) has aptly observed that probably the most disturbing aspect of pollution is the increasing presence of toxic chemicals in the natural environment. The large- scale production and application of synthetic chemicals and their subsequent pollution of the environment is now a problem of serious concern because of the adverse effects on inhabitants of the environment. The US Environmental Protection Agency's list of high awareness pollutants (Alexander, 1981) includes: most pesticides, halogenated aliphatics, aromatics, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAH) and nitrosamine.

Many of these compounds are used directly by workers in agriculture and public health for undisputed beneficial results. Others are derived from a spectrum of industrial processes used to make a variety of useful products. Some particularly in oil producing countries like Nigeria are associated with the petroleum industry while others are solvents commonly employed in these rapidly industrializing countries. These toxic hazardous chemicals are insidiously entering a variety of environments. The synthetic compounds can be found at very high concentrations at the point of release (discharge), such as factory sites and industrial spillages where they can exert pronounced deleterious effects. Others may occur at low levels in the natural environment but owing to their potent toxicity gradually exert serious biological damage.

The threat of excessive exposures of humans to chemicals became evident as various occupational and therapeutic incidents later demonstrated. Genetic anomalies are implicated in human disease as suggested by: the presence of chromosomal abnormalities in individuals who are exposed with various clinical disorders (Brusick, 1980); findings that agents, including chemicals which are capable of inducing chromosomal or gene changes, also produce deleterious health effects in experimental models including mammals (Leber,1988). Thus increasing exposure to environmental pollutants is widely recognized as part of industrial development. Industrial development is also known to be at a rapid pace in many developing countries as an attempt to raise the economy of these countries. This inevitably involves the use of a lot of industrial materials (xenobiotics) many of which are chemicals and are released in to the environment wholly or as byproducts.

Environmental exposure to these toxicants modify the genome (Farmer and Singh, 2008), leading to diseases including cancer and birth malformations. Genomic damage is probably the most important fundamental cause of developmental and degenerative diseases. Owing to the seminal significance of DNA damage and genome instability there is great effort at understanding DNA damage and its repair mechanisms currently. It is expedient to have a reliable, relevant and affordable minimally invasive biomarker to aid biomonitoring, diagnosis, and treatment of diseases caused by or associated with genome damage. The micronucleus test system, originally developed as an indirect measure of chromosome break (clastogenic effects) has been applied widely as a short- term genotoxicity test for evaluating xenobiotics for mutagenic and carcinogenic potential. Substances which cause damage to genome may produce gene mutations, and / or chromosomal aberrations which could be possible initiation events of

the carcinogenic process or which could result in heritable disorders. In the advanced economies of the world risk assessment that leads to risk prevention is currently based on high technology driven processes that are capital intensive and outside the reach of these resource poor and or restructuring countries. Thus there is the need to search for a reliable inexpensive marker or genome damage index.

Current technology of genome damage detection

Current state of gnome damage detection follows advancement in technology (Farmer *et al.*, 2008) using such methods as: the COMET assay, 8-OHdG (using HPLC or ELISA), Fapygua, ^{32}P-labelling and mass spectrometry. These are all capital intensive techniques that are too expensive for most of these countries. The micronucleus assay system was used in the past for this purpose prior to current state of technology but largely perceived as obsolete by some (Slesinski and Guzzie, 1988) mainly from the economically advanced countries. Recent reports however, indicate a paradigm shift towards the micronucleus assay system as a genome damage index, judging by the international efforts aimed at improving the test system and current application. This appears an important step at tackling the genomic instability associated with industrialization (Fenech *et al.*, 2007).

What are micronuclei?

Micronuclei were first recognized by Howell (1891) as distinct cellular inclusions in erythrocytes from cats. Jolly (1905) subsequently confirmed the existence of micronuclei in experiments on erythropoiesis using mouse and rat embryo. Micronuclei are rounded cellular inclusion bodies and are usually membrane bound. They vary from 4µm in size and are composed of DNA (Schmid, 1975; Heddle and Carrano, 1977). Presence of micronuclei has been of diagnostic importance in haematologic pathology for the diagnosis of various anaemias. Since micronuclei were usually removed by the spleen, preponderance of micronuclei in circulation thus indicates spleen dysfunction. A break in chromosome or chromatids is the lesion primarily induced by most clastogens. Due to the fact that a break in chromatids material if not adequately repaired produces an accentric fragment, breakage can be detected and quantitated by counting micronuclei which form chromosomal fragments. Consequently, the major concern of the micronucleus assay system is whether clastogenic effects can be detected. Although a large population of cells are needed in this assay system usually about 1000 (≥1000) cells exposed to the toxicant of interest can be observed easily for micronuclei in a fraction of the time required to evaluated only 50 to 100 metaphase cells in classical metaphase test protocols(Slesinski and Guzzie, 1988).

Genesis of micronuclei and implications for the genome

Micronuclei are believed to arise as a result of damage to the chromosomes or to the mitotic apparatus as indicated above, culminating in the accentric chromosomal fragments or in aberrant mitotic segregation. Consequently, fragments of chromosomes or chromosomes with multiple centromeres coalesce into small nuclei ('micronuclei') which are distinct from cell nucleus but react similarly with stains because of their DNA content. The studies of Heddle and Carrano (1977) quantitated the DNA content of micronuclei from mouse bone marrow cells and demonstrated that the amount of DNA was consistent with the view that micronuclei are derivatives of chromosomes. Hayashi *et al.*, (1984a) taking this further also found that in

addition to the findings of Heddle and Carrano (1977) the distributions of DNA content of MN and length of accentric fragments in mouse bone marrow exhibited some homology.

Other lines of evidence suggest that the sizes of MN may indicate the mechanism of chromosome damage. Yamamoto and Kikuchi (1980) have reported that micronuclei produced by Clastogens were smaller than those produced by spindle inhibiting agents like vincristine. These findings suggest that the sizes of micronuclei can discriminate between agents operating by different cytogenetic mechanisms.

Some mechanistic aspects of micronuclei

Nogstedt and Karlsson (1985) in an independent investigation of the findings of Yamamoto and Kikuchi demonstrated that agents which generate chromosome fragments such as X-rays and mitomycin C had MN of smaller sizes than those which affect the chromosomes and the mitotic spindle such as colcemid and vincristine. Thus, the micronucleus assay system can discriminate between different clastogenic mechanisms in addition to its role as a biomarker of potential chromosome damage (index of damage).

Some technical issues in micronucleus assay system

Due to the growing awareness of the functional and affordable nature of the micronucleus assay system a number of procedures have been developed and used as indicators of chromosome damage. These procedures have been employed in both studies involving fauna and flora. Ma (1979) used this test system to investigate genome damage in plants. The method has been used more in animals in studies involving animals or components of animals (Von Lebedur and Schimid, 1973, 1976, 1977). Currently the MN assay system has been used and is being extensively used for mammalian cell culture (Obe *et al.*, 1975, Countryman and Heddle, 1976, Lasne *et al.*, 1984, Holland *et al.*, 2008, Bakare and Utulu, 2009).

Identification of micronuclei

In animals studies in which micronuclei have been more extensively employed, micronuclei are most easily discernible in polychromatic erythrocytes (PCEs), which are anucleated cells whose relative age can be discerned by their staining differences. This is because PCEs contain residual RNA which gives juvenile red cells a pale-bluish staining reaction of the cytoplasm by the Romanowsky Giemsa stains (Schmid, 1973). Polychromatic cell erythrocytes are considered a relative late stage in erythropoiesis and they exist in the mouse model bone marrow for between 24 to 30 hours prior to full maturation to normochromatic erythrocytes. Schmid (1973) has suggested that restricting micronuclei studies to PCEs will enhance their sensitivity in detecting clastogenic effects as they can be readily distinguished from target cells. These investigators have also aptly suggested that PCEs provide a larger population of cells for examination, eliminating the need for cells at mitotic stage for the classic metaphase chromosome procedures. An important advantage of the MN assay system over the other high technology –based methods for detection of genome damage is that only moderate degree of experience is required for correct identification of micronuclei and to avoid technical artifacts.

However, Schmid (1975) has cautioned that though the micronucleus assay system is relatively simple but that it was not designed for evaluation by fools. The relative simplicity compared to other methods does not eschew the need to subject the assay system to the usual scientific

principles including quality control and common sense. An important technical issue to constantly bear in mind is the need to differentiate artifacts from true micronuclei. It should be noted that true micronuclei are membrane- bound and spherical (figure 1) and contain DNA.

Specific methods for the micronucleus assay system

A number of methods have been proposed for the demonstration of micronuclei. The fluorescent staining procedures that discriminate between RNA and DNA have been used extensively. Hayashi *et al.* (1983) have stained bone marrow smears using 0.24mM acridine orange in 1/15M Sorensen's buffer (pH 6.8) for 3 minutes followed by 3 rinses in the buffer. Under fluorescence illumination, micronuclei emit bright green fluorescence and easily distinguished from granules and artifacts or contaminants which fluoresce red.

MacGregor *et al.* (1983a) in their study used the double dye procedure employing Hoechst 33258, which binds to DNA and emits blue fluorescence and emits blue fluorescence and pyronin Y, which stains both DNA and RNA and emits red-orange fluorescence. The MacGregor *et al.* (1983a) method has a number of advantages worth enumerating. Some of the main advantages include: differentiation of micronuclei from basophilic granules, selective identification of DNA-containing objects and reduction of the number of staining steps. Apart from the highlighted advantages of the method of MacGregor et al (1983a) there are a number of other potentials; it has significantly advanced studies of micronuclei by opening up possibilities both for application of flow cytometry and image analysis methods (such as IPAP in immunochemistry) for quantitative studies in micronuclei studies in cells (MacGregor *et al.*, 1983b).

The haematoxylin and eosin (H &E) method

The haematoxylin and eosin (H and E) method deserves special consideration because of the traditional role it has played in conventional histopathology. The H and E method for micronuclei was developed by Pascoe and Gatehouse (1986). This method differentiates micronuclei from cellular inclusions and produces permanent staining as opposed to the fluorescence procedures discussed above. The H & E procedure stains micronuclei blue-black and does not stain micronuclei-like inclusions such as mast cell granules. However, other micronuclei-like artifacts produced by acridine dyes e.g. quiacrine are stained by H & E though less intensely (i.e. paler than micronuclei). Thus they can be excluded by this differential degree of staining between true micronuclei and artifacts.

Scoring and evaluation of micronuclei data

This is a very important aspect of the micronucleus assay system. The earlier publications of Stitch and Rosin and Stitch *et al.*, (1983, 1984, and 1985) have referred to the well established basic criteria for MN that were originally described by Heddle (1981). However, criteria identifying cells for inclusion into the MN frequency count were not defined. Consequently other authors refer to the Heddle criteria as such, or with minor modifications. The criteria developed by Tolbert *et al.* (1991, 1992) for scoring cells are the most widely used. They consist of the parameters shown below: intact cytoplasm and relatively flat cell position on the slide; little or no overlap with adjacent cells; little or no debris; nucleus normal and intact, nuclear perimeter smooth and distinct.

The suggested criteria recommended by these authors for identifying MN include the following: rounded smooth perimeter suggestive of a membrane, less than a third the diameter of the associated nucleus but large enough to discern shape and color, Feulgen positive i.e. pink in bright field illumination; staining intensity similar to that of the nucleus; texture similar to that of the nucleus; same focal plane as the nucleus; absence of overlap with, or bridge to the nucleus. Tolbert et al (1991) have outlined all the specific nuclear alteration that should be taken cognizance of when deciding inclusion or exclusion criteria for deciding the MN frequency in cells. Statistics is also an important consideration in this regard.

Statistical power is important for validity of data generated in the MN assay system. In typical experimental models a group size of 5 males and females per dose level and 1000 PCEs per animal have been recommended as the minimal sample sizes by various guidelines for this assay system (Heddle *et al.*, 1983, EPA, 1985; OECD, 1987). Schmid (1973) originally recommended that for a given animal a total of 2000 to 5000 erythrocytes are examined for presence of micronuclei. MacGregor et al (1980) were the first to demonstrate conclusively that Clastogens induced increases in the incidence of micronuclei in peripheral polychromatic erythrocytes from blood and bone marrow of mice that they showed a dose-effect relationship after administration of a number of toxicants such as cyclophosphamide (25mg/kg), nitrogen mustard (2.5mg/kg), 7,2- dimethylbenz[a]anthracene(40mg/kg). These investigators employed air dried blood smears which were fixed with absolute methanol, and the cells stained with Wright-Giemsa stain. To evaluate micronuclei in the PCEs, selected slides were stained with Feulgen and DNA- specific stains to assure correct identification of cellular inclusions from micronuclei.

Advantages of blood smear for micronuclei assessment

Evaluation of blood smears has a number of readily discernable advantages over bone marrow studies. Some of the advantages are that micronucleated PCEs in blood attain maximum values about 24 hours later than PCE in bone marrow. This gives ample time for a toxicant effect and appropriatiate response. Assessment of micronucleus in peripheral blood smear is advantageous because only a little drop of blood is needed for examination and the same animal(s) can be sampled repeatedly (Slesinski and Guzzie, 1988). The consecutive sampling from the same model(s) increases uniformity of data and reduces the number of subjects required and eliminates the variability encountered in typical group comparison studies. Importantly, peripheral blood smears permit a better estimation of the cellular kinetics of clastogenic effects because consecutive samples can be obtained using the same animal to which a given dose has been administered (Slesinski and Guzzie, 1988). MacGregor (1980) has observed that evaluation of peripheral blood PCEs provided comparable or greater sensitivity to micronucleus evaluations in bone marrow.

The observation of cumulative increases in the number of micronuclei is an exciting finding which further raises interest in this assay system. This is additional to its relative inexpensive nature thus making it of great promise in routine toxicology, particularly in developing countries. Taking advantage of the cumulative nature of MN has however been disappointing and fruitless owing to the painful realization that MN does not occur in rats. This is also expected in humans and related species with efficient spleens which eliminates these abnormal micronucleated red blood cells (Schlegel and MacGregor, 1984). Schelgel and MacGregor (1984) ingeniously devised a method to overcome this caveat by employing splenectomy of rats. This abolished the removal of micronucleated NCEs by the spleen, consequently permitting the application of the

peripheral blood micronucleus assay system to the animal. This however raises some questions about its application in humans who for ethical reasons cannot be splenectomized. This however, appeared to have been overcome by the buccal MN application (Holland *et al.* 2008).

Micronucleus assay system in occupational and environmental exposure to genotoxic agents

Due to intense industrial and restructuring activities going on in many developing countries the genome is also under constant assault and threat (Anetor, 2009). This may involve rapid DNA synthesis and repairs reminiscent of fetal life. Quantitative and qualitative lack of repair enzymes may also be prevalent owing to immaturity. This is analogous to the situation in developing countries where widespread deficiencies in micronutrients (Smith, 2000; Welch and Graham, 2000) involved in repair enzymes put population at greater risk of genomic damage and thus the need for an affordable means of early detection of the ensuing genomic injury. Quantitative and qualitative lack of DNA repair enzymes just as they contribute to the increased susceptibility of the fetus to damage produced by occupational and environmental exposures to genotoxic agents also holds true for industrial workers in developing countries. Deficient repair may arise from zinc deficiency and excretory mechanisms (schistosomiasis in the tropics) may raise the levels of genotoxins in such populations and accentuate the probability of genomic damage.

Oxidative DNA damage and human pathology: a role for micronucleus assay

DNA damage caused by reactive oxygen species (ROS) incorporates a large variety of lesions spanning from base and sugar damage to DNA –protein cross-links. Over the past several decades, accumulating evidence indicates an involvement of oxidative damage in human disease. Many common and life threatening human diseases, including neurodegenerative diseases, cancer and aging, have implicated free radical reactions as an underlying mechanism of injury. Oxidative base damage is highly mutagenic and if unrepaired might increase the risk of cancer development. Often repair is ineffective thus the need for a reliable system to detect the ensuing genomic damage. Alternatively, the high levels of oxidative stress and transcriptional demand, coupled with compromised capacity of the repair systems probably owing to micronutrient deficiency as is common in many developing countries (Sandstead, 1991; Gibson, 1994; Smith,2000; Welch and Graham, 2000;Anetor and Agbedana 2001;Anetor *et al.* 2009) may render populations from these countries particularly susceptible to defect in oxidative DNA damage repair systems and thus permissive to genome damage.

The implication for human health of altered functioning of the oxidative DNA damage repair are sufficiently serious, the more common being cancer of various sites and developmental disorders. Many investigators have proposed that tobacco smoke and occupational exposure cause DNA damage (Slesinski and Guzzie, 1988; Anetor *et al.*, 2008) and that a reduced DNA repair is not able to efficiently deal with the increased number of lesions, thus leading to increased mutagenesis and cancer risk. The contribution of the accumulation of endogenous damage to genomes and to clinical outcome of diseases is an emerging issue (D'Errico *et al.*, 2008). Thus the development of specific functional DNA-repair assay systems seems a promising tool to provide reliable data on the actual role of repair activities in cancer risk. The micronucleus assay system is sufficiently versatile to be modified to fulfill this role.

The micronucleus assay system in human populations

This assay system was used in the past largely for genetic screening. Recently it became evident that it can also be used to monitor individuals or populations exposed to xenobiotics. Micronuclei (Howell- Jolly bodies) have been used for decades for the diagnosis of dysfunctional spleen (Miale, 1982). Increases in MN demonstrated in hematological preparations provide a useful monitoring in the past that can confirm the conditions mentioned above. A good use to which the MN assay has been put to was in the monitoring of patients undergoing radio- or chemotherapy. Evaluation of micronuclei in bone marrow cells of these patients has been employed as an index of genetic effects of the treatments (Sorensen and Krough – Jensen, 1981).

Emergence of the micronucleus system in clastogenic studies

Stitch et al (1982a, b,c, 1983, 1984) were the pioneers in the use of the micronucleus assay system for assessing genomic damage arising from exposure to toxicants in humans including chemicals, food, and tobacco products. Hans Stitch and his colleagues demonstrated that chromosomal damage to epithelial cells could be detected and quantified in exfoliated cells from various mucosal tissues such as buccal or bronchial mucosal, urinary bladder, ureter, cervix, and esophagus. Thus these investigators laid the foundation for assessing the genotoxic effects of toxicants from the commonest routes of entry. This is also the origin of the micronucleus assay in human buccal cells that is becoming popular and reviewed recently by Holland et al (2008). An attraction for this assay system and thus for developing countries is that cells could be easily obtained for examination from centrifuged urine specimens, scrapings of the oral cavity, sputum or cervical smears. Land mark studies carried out by the pioneers (Stitch *et al.* 1982a, b, c, 1983, 1984) involved quantifying of micronuclei in human buccal mucosa cells exposed to suspected mutagens or carcinogens. In a study from a previously classified developing country now one of the emerging economies, India, 27 tobacco chewers exhibited significant increases in buccal cell micronuclei in all the subjects (Stich *et al.*1982a,c). Clastogenic agents were subsequently confirmed in vitro studies with both Chinese hamsters ovary and human cells (Slesinski and Guzzie, 1988). The India study appears to argue for the ease and relevance of the micronucleus assay system as a biomarker for human exposure to increasing environmental agents in developing countries. Indeed, Stitch et al (1984) have remarked that the MN assay system provides a high degree of ease and relevance unmatched by other available short-term clastogenicity test systems.

The micronucleus assay system in carcinogenesis

Though the major value of the MN assay system is in detection of clastogenic agents it is unquestionably relevant in the detection of carcinogenesis. This is reasonable because many if not all proven clastogens are also carcinogens (Radman *et al.*1982). The Gene-Tox program of USEPA has reported that there was a striking positive correlation of 94% (17 out of 18 cases) between carcinogenic and clastogenic activity (Preston *et al.*, 1981). Though this report has been criticized because of its small sample size, the data nevertheless are highly suggestive of a direct relationship between clastogenic and carcinogenic potential.

Derepression of oncogenes

At the molecular /genetic level, a growing body of evidence indicates that oncogenes present in the mammalian genome may be derepressed and activated by positional changes caused by chromosomal damage and rearrangement (Gilbert, 1983; Selsinski and Guzzie, 1988). Derepression of oncogenes is one of the more current and appealing hypotheses on the possible mechanisms for initiating cellular neoplasia. Evidence for a direct relationship between chromosomal changes and carcinogenesis increases the relevance of clastogenicity assay results for humans.

Despite the correlation between clastogenic and carcinogenic potentials, study reports have been mixed. The reports of Trzos *et al.* (1978) and Jensen and Remel (1980) have indicated that there is no relationship between clastogenic and carcinogenic effects. In contrast, and indirectly confirming the observation of Radman *et al.* (1982), Wild (1978) observed that the correlation between carcinogenic potential and positive micronucleus reactions suggested a useful role for the test. Again, extending the conclusion of Wild (1978) in a later report, Heddle *et al.* (1983) in the Gene-Tox program evaluated published micronucleus studies and found a 50% accuracy in predicting carcinogenic potential and speculated improved performance with modifications which are now commonly available. Thus some investigators have commented that it is unrealistic to expect the MN assay system to predict carcinogenic potentials for all carcinogens (Selsinski and Guzzie, 1988). This view is not far fetched in that some carcinogens cause gene mutation but not chromosomal damage or clastogenic effects (de Sorres and Ashby, 1981). Yet other carcinogens may act by extragenetic mechanisms such as the recently recognized epigenetic mechanisms (Jones and Baylin, 2007) these epigenomic alterations are not detectable by routine genotoxicity tests.

The micronucleus assay as a bioindicator of genomic damage

The micronucleus assay system has found growing acceptance as a biological indicator of genomic damage arising from the following situations: radiation exposure; monitor for genetic effects of various workplace chemicals and environmental pollutants (very common in developing countries as a reflection of the pace of development or progressive industrialization).

When employed for this role, the sensitivity of the test system to low level exposure and linearity of the dose-response curves are significant and have raised some controversial issues. Applegren *et al.* (1978) have reported that micronucleus assay demonstrated greater sensitivity than traditional methods.

Other reports have maintained the superior performance of the MN assay system. Diaz et al (1980) studied the clastogenic potential of benzene following subcutaneous injection, while Hite *et al.* in the same year performed the same experiment by oral intubation. Both groups observed significant dose related increases in the numbers of bone marrow micronuclei from this exposure. Siou *et al.* (1981) confirmed the clastogenic activity of benzene in both mice and Chinese hamsters and reported agreement of data from both metaphase and micronucleus evaluation. This appears to further support in retrospect the findings in blood smears from the National Toxicology program long-term carcinogenicity bioassay (Choy *et al.*, 1985). This later report indicated that the sensitivity of the MN procedure is sufficient to be used as a measure of genotoxicity. One clear advantage of this is that blood smears prepared for hematological studies

can be used to collect additional data on genotoxicity potential. This again should be attractive and of interest to resource poor nations.

The micronucleus assay system in environmental toxicology

Humans are exposed to a diverse array of environmental toxicants associated with the development of a number of diseases including cancer, acute organ toxicity and chronic inflammatory conditions. Many of these exposures are inadvertent while others may be due to life-style factors. Examples include carcinogenic and pro-inflammatory chemicals in tobacco smoke and air pollution, pharmaceuticals, dietary carcinogens, and occupational hazards (Osburn and Kensler, 2008).

Environmental toxicology is concerned with the harmful effects of exposure (often chronic) to agents present in the environment from natural or industrial sources as is currently the situation in many developing countries. Biological monitoring of environmental pollutants prevalent in these countries is thus an important area of practical application of the micronucleus procedure. Siboulet *et al.* (1984) used this method to study newt to evaluate or monitor clastogenic pollutants in aquatic environment. Significant dose-effect increases in the frequency of micronuclei were found. Watanabe who has been involved in numerous environmental toxicological investigations with his research team studied the clastogenic effects of a combination of chemical carcinogens and environmental pollutants employing bone marrow PCEs from strains of ddy mice (Watanabe *et al.*, 1982). They reported that the possibility of synergistic positive effects for some combinations of Clastogens was indicated. This appears to suggest the usefulness of this assay system in the investigation of exposure to chemical mixtures; the real life situation in environmental toxicology.

Cadmium, an important and pervasive environmental toxicant which was inactive initially when applied alone in producing increases in micronuclei, produced an enhanced positive response when combined with dimethylnitrosamine (DMN). Interestingly no similar synergistic effects were obtained when DMN and PCB were applied simultaneously, suggesting that synergy may be combination specific. Synergistic responses are serious concerns for complex environmental pollutants and the data of Watanabe *et al.* (1982) show the possible role of the MN method for detection of synergism for clastogenic chemicals.

Micronucleus assay system in biomonitoring of occupational and environmental exposures

Over the past one and a half to two decades the micronucleus assay system has been applied to assess clastogenicity in biomonitoring of human populations exposed to a variety of mutagenic and carcinogenic chemicals and other agents in occupational and environmental exposures. There is no definite pattern of variation among centers from different countries. Numerous studies report statistically significant increase of MN frequency in exposed individuals compared to referents. The observed effects though are relatively small, usually ranging from 1.1 to 4-fold (Machado-Santelli *et al.* 1994; Karahalil, Karakaya and Burgaz, 1999) and several others reported statistically non-significant findings (Pastor et al, 2001; Pastor *et al.*, 2001). A study in mortuary trainee workers involved in embalming using formaldehyde reported a 12-fold increase in MN frequency compared to baseline level (Suruda *et al.* 1993). Though this increase was unusually large, it has been confirmed independently in a study using a different staining procedure employing propidim iodide with pancentromeric FISH labeling (Titenko-Holand *et al.*

1996). Significantly elevated frequencies of MN have also been reported from people exposed to organic solvents, antineoplastic agents, diesel derivatives, PAH, lead-containing paints and solvents as well as potable water contaminated with arsenic (Gonsebatt *et al.* 1997; Manchado-Santelli *et al.* 1994; Celik *et al.* 2003; Aposhian, 1997; Basu *et al.* 2002; Tian *et al.* 2001; Goud *et al.* 2004; Sailaja *et al.* 2006 ; Pinto *et al.* 2000).

Studies on exposure to pesticides common in Nigeria due to increasing efforts to feed the teeming population of Nigerians have generally not shown increase except a study in a small population in Mexico in which a 2.6-fold increase in MN frequency was reported (Gomez-Arroyo *et al.* 2000) as well as a 3.9-fold increase observed in workers at the pesticide manufacturing company (Sailaja *et al.* 2006). The null findings or lack of effect reported by some investigators can be ascribed to the relatively weak genotoxicity of most modern pesticide manufacturing plant and the dermal rather than oral route of exposure (Garaj-Vrhovac *et al.* 2001; Bolognesi *et al.* 2002). Negative data have also been obtained in subjects exposed to the following agents: chromium, ethylene oxide (EO), nickel, benzene, and other environmental or occupationally encountered chemicals (Sarto *et al.* 1990; Suralles *et al.*1997; Gattas *et al.*2001; Torres-Burgarin, 1998; Heuser et al, 2005). More recent reports have also suggested genotoxicity and cytotoxicity of urban air pollution and ozone during summer especially in regions with elevated ambient levels (Chen *et al.* 2006; Huen *et al.*2006).

On the whole, though the association between toxicant exposure and increases in MN frequencies is strong for many chemical exposure conditions, additional studies appear desirable to build a reliable database on the effects of common exposures such as pesticide mixtures and air pollution particularly in developing countries where these are more common, on induction of MN and the application of the micronuclei assay system. Some investigators appear to already be heeding this clarion call though the number is sill small; Bakare and Utulu (2009) have in part employed the MN assay system in Nigeria to investigate DNA damage in the germ and bone marrow cells of mice and rats exposed to caffeine.

Micronutrients and the micronucleus assay system

Most cancers evolve from a single abnormal cell through several successive rounds of mutations and natural selection. The process commonly commences with a change in a cell's DNA sequence (mutation). DNA damage has to occur during its replication and there exist several phenotypic expressions of this alteration. The investigation of the biochemical and molecular alterations during cell transformation particularly after exposure to chemicals is one of the central themes of our research line. These events are often regulated by enzymes dependent on micronutrients or modulated by them. Micronutrients can modify the body's response to toxic agents such as toxic metals by altering their metabolism and transport.

A number of micronutrients including beta-carotene and other vitamins have been shown to significantly decrease MN levels several folds in healthy tobacco users, as well as in individual preneoplastic lesions (Stich *et al.* 1984a; Stich *et al.* 1984b; Prasad *et al.* 1995). The effect of chemoprevention on the remission of specific lesions such as leukoplakia, and on the prevention of new lesions, which are likely to be genomically unstable, may indicate a reversion to a more normal phenotype. But whether these changes are causal or chance events remains unclear. Some other micronutrients such as retinol, riboflavin, zinc, selenium have been reported to show failure to reduce MN frequency in an investigation conducted in an emerging economy like China in a

region with a high incidence of oesophageal cancer (Munoz *et al.* 1987).Reduced MN frequency, in contrast was associated with green tea consumption in patients with oral leukoplakia (Li *et al.* 1999). Similarly, declines in MN frequencies were reported in children and women who received controlled folate supplementation (Holland *et al.* 1998; Holland *et al.* 1998). It remains to be confirmed whether the observed decreases in MN frequencies in these investigations were attributed to a reduction in chromosomal instability contigent upon supplementation or to modification of basal cell proliferation that may induce alteration in the mode of expression of MN.

DNA repair pathways

It is perhaps appropriate to briefly comment on DNA repair pathways in a discussion like this for a number of reasons; DNA repair is intimately related to the micronutrients just discussed above, secondly defects in the DNA repair pathway will lead to genome damage and ultimately the degenerative diseases of which cancer is the most dreaded and finally the need for risk assessment in DNA repair pathway alterations.

It is very well known that abnormal regulation of or mutation in DNA genes can lead to diseases often associated with predisposition and high risk of cancer development. This is essentially due to the fact that cells are constantly subjected to many DNA damaging events arising from exogenous environmental insults such ionizing radiation, genotoxic chemicals, or endogenous processes such as ROS generated in mitochondria during bioenergetic processes also from environmental chemicals (Anetor *et al.*, 2009). Thus the absence of a competent DNA repair process will lead to accumulation of DNA lesions, to an increasing rate of mutagenesis and finally to tumor initiation and progression.

Four principal DNA repair pathways are recognized in all living cells: base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR) and recombination repair (RR). Base excision repair is responsible for repairing damaged bases or single-strand breaks caused by spontaneous chemical modifications (such as deamination, depurination, hydrolysis) or to exogenous agents (particularly ROS, UVA (Chan *et al.*, 2006; Sarasin and Kauffmann, 2008). Nucleotide excision repair is a multistep process able to repair bulky DNA damage produced by UV or chemicals for instance PCBs, PAHs (Sarasin and Kauffmann, 2008). In the absence of a full repair of bulky lesion before DNA synthesis occurs, replicative DNA polymerases are blocked by these lesions and ultimately distributive and error-prone translesion polymerases (TLS) are able to take over and replicate past the lesions. This pathway may be mutagenic depending on the type of lesions to bypass and the types of TLS polymerases employed (Sarasin and Kauffmann, 2008). Mismatch repair is a post-replicative repair process able to remove misrepaired bases and insertion/deletion that arise between microsatelite sequences during DNA replication.

It is estimated that the fidelity of normal DNA synthesis in human cells is around 10^{-6} before MMR and increase to 10^{-9} after MMR (Moderich and Lahue, 1996). Defective MMR results in a higher spontaneous mutation frequency and microsatelite instability (MSI). This is often caused by mutations in one of the MMR proteins involved in the recognition process such as MutS and the MutL, heterodimers. Germline mutations of these genes give rise to an inherited syndrome associated with disposition to colorectal cancers (HNPCC; hereditary non-polyposis colorectal cancer), endometrium or gstric cancers (Sarasin and Kauffmann, 2008). Germline or sporadic

MMR-deficient tumors show microsatellite instability that can be recognized and help clinicians give the best form of therapy for these tumors. MSI colorectal tumours are more sensitive than the others to some topoisomerase inhibitors such as camptothecin or etoposide (Jacob et. al.,2005). The prognosis is usually better than for the non-MSI tumours (Gryfe *et al.*,2000). Mismatch repair-deficient diseases are probably the most frequent syndrome with cancer predisposition among DNA-repair deficient diseases (Sarasin and Kauffmann, 2008).

DNA-strand breaks (DSBs) are the most lethal forms of DNA damage as even a single DNA strand break is sufficient to kill a cell. Incorrectly repaired breaks can lead to gross chromosomal rearrangement, aneuploidy, and carcinogenesis and cell death. Double-strand breaks (DSB) are caused spontaneously during normal DNA synthesis and immunoglobulin diversification as well as following DNA insults due to ionizing radiation, ROS and antitumoral therapy. Double strand breaks as well as crosslinks (CLs) represent the most severe form of DNA damage because the genetic information is lost on the two strands of the helix at the same site. Efficient repair of these lesions is done by two specific recombination pathways: the homologous pathway (HR) and the non-homologous end joining (NHEJ).

The DSB is recognized by the MRN (Mre11, Rad50 and Nbs1 proteins) that allows the DNA end resection to yield 3′-ssDNA tails that will start strand exchange with the homologous duplex DNA catalyzed by RAD51, RAD52, and RAD54 complexes. While the HR is supposed to be error-free because the repair enzymes use the homologous sequences to repair the breaks, the NHEJ gives rise to mutations and particularly to deletions due to religation between the two ends in each side of the damage. The non-homologous end joining is a major DNA break repair pathway in both prokaryotes and eukaryotes. A core protein complex comprising ku and DNA ligase in addition to other repair factors assemble at the sites of DNA-strand breaks to mediate the repair of broken DNA ends. This pathway is often associated with deletions, loss of heterozygosity (LOH) and chromosomal translocations, which is involved in the multistep process of tumorigenesis (Valerie *et al.*, 2003; Petrini *et al.*, 2003). The genomic alterations arising from defects in these DNA repair pathways can be investigated indirectly by the MN assay system as the modified genomes may give rise to micronuclei.

CONCLUSION

Application of MN method in monitoring environmental toxicants and pollutants is likely to increase in more developing countries as new procedures are developed and verified. The relevance of available data for assessment of biological risks in developing countries requires studies so that they can be applied in a routine manner. The primary role of the MN assay system is in detecting Clastogens. This should not be forgotten in applications to environmental monitoring. A negative result in this system may only indicate absence of agents which damage chromosomes but does not imply absence of mutagens or carcinogens which act by different mechanisms. The MN test is gaining in extensive use to evaluate genome stability and damage, in many countries, but insufficiently so in many of the rapidly industrializing developing countries and increased MN frequency predicts the risk of cancer (Bonassi *et al.*, 2006), this is usually preceded by genome damage. Biomonitoring of the pathological changes that may lead to cancer and other illnesses (disease risk assessment) is now increasingly popular (though not in the majority of developing countries) and appears to be the most rapidly growing area of the MN

assay system application. Thus it is particularly suited for developing countries where greater incidences of cancer appear to also be reported. Therefore, the micronucleus assay system may be a veritable tool in environmental and biological measurements in the developing countries if the purpose and limitations of the assay that have been substantially remedied are kept in proper perspective.

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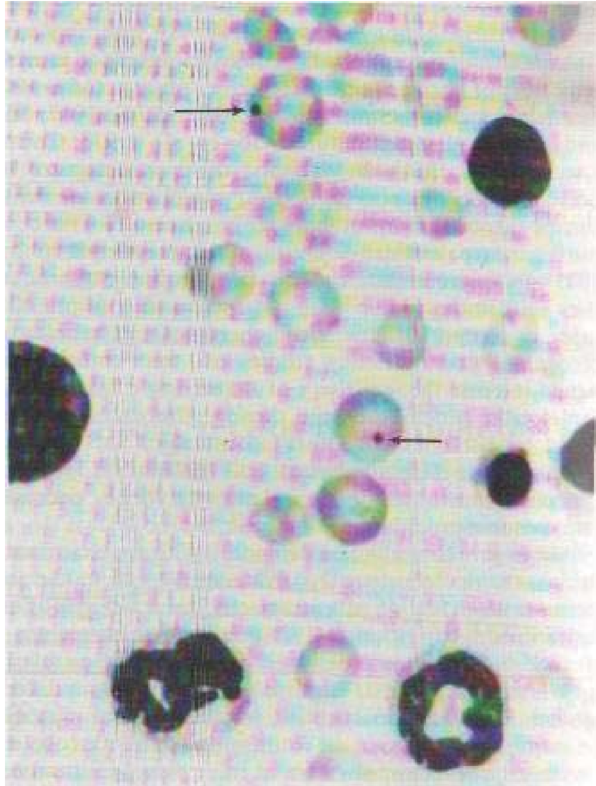

Fig.1: Micronuclei in red blood cells

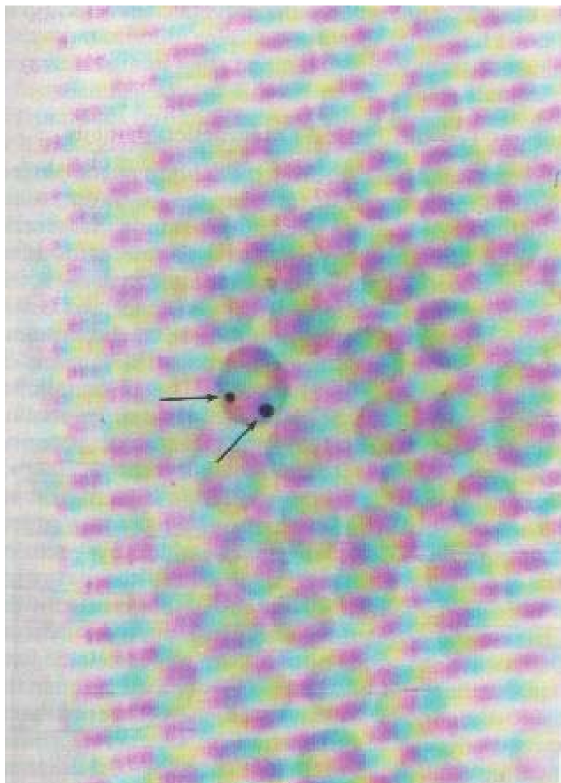

Fig.2: A red blood cell showing two micronuclei.

Evaluation of Heavy Metals Bioaccumultion Potentials of Plants Grown on Waste Contaminated Soils

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Introduction

Environmental pollution by heavy metals is a global issue that has generated a lot of concern in all parts of the world and which requires both local and universal attention to stem the growing threat it poses to survival on earth. Pollution generated in one part of the world can have far reaching and serious detrimental effects both locally and across the globe. Heavy metal pollutants reach and contaminate the environment from myriad of sources, the common ones being metallurgical processes, industrial activities, agricultural practices, waste disposal sites, sewage sludge and automobiles. These are potential sources of heavy metals that are known to cause distortions in human systems, in vegetations, in livestock and in wild animals. Heavy metals are chemical elements with a specific gravity of about 5.0g/dm^3 or greater (Bolan and Duraisamy, 2003). Examples of heavy metals include Arsenic (As), Cadmium (Cd), Chromium (Cr), Copper (Cu), Cobalt (Co), Lead (Pb), Manganese (Mn) Mercury (Hg), Nickel (Ni), Iron (Fe), Selenium (Se), Thallium (Th) and Zinc (Zn). Some well-known toxic heavy metals with a specific gravity that is greater than 5.0 g/dm^3 are arsenic, 5.7 g/dm^3 ; cadmium, 8.65 g/dm^3 ; iron, 7.9 g/dm^3 ; lead, 11.34 g/dm^3 ; and mercury, 13.5 g/dm^3.

Unlike organic contaminants, heavy metals once entered into soil, cannot be degraded but can be cleaned up from environmental media. This is termed remediation. Environmental remediation involves removal or reduction in heavy metal bioavailability. Two major approaches have been employed to decontaminate or ameliorate heavy metal contaminated soils: these include ex situ and in situ remediation techniques. Ex situ technique involves removal and transportation of contaminated soil to an authorized landfill site for cleaning, detoxification and physical or chemical destruction of contaminants while in situ technique involves cleaning on site. In-situ techniques whenever feasible are always preferred over ex-situ techniques due to low cost and less disruption on the ecosystem. Many methods have been used to decontaminate metals-contaminated soils thereby making the soils relatively safe for the environment and humans. The commonly employed methods include containment, chemical treatment, electrokinetics, biological treatments (bioremediation), mechanical and pyrometallurgical separation, solidification/stabilisation and immobilization. Many of these conventional technologies are expensive and not environment friendly but negatively impact ecosystem. Recently, phytoremediation, a type of biological treatment, has been identified as soil heavy metal decontamination technique. Low cost, little or no landscape disruption, wide applicability, preservation of ecosystem including soil fertility, generation of recyclable heavy-metal rich plant residues, applicability to a range of toxic heavy metals, public acceptance have been identified as advantages of phytoremediation (Ogundiran and Osibanjo, 2008).

Phytoremediation

Phytoremediation is a biotechnology that employs the use of plants and trees for in-situ removal, degradation or immobilisation of contaminants in organic (pesticides, polyaromatic hydrocarbons, crude oil) or inorganic (metals) contaminated tailings, soils and waters. Plant degrades organic pollutants and immobilises/stabilises metal contaminants. The phytoremediation process could be through the natural biological, chemical or physical activities of the plants and trees. The plants and the trees are grown on the contaminated media for a required period of time and thereafter could be harvested, processed and disposed off in designated landfills.

In phytoremediation of heavy metal contaminated soil or tailings/waste, the heavy metals contaminants are gradually removed from the soil by plant uptake and harvesting, thereby reducing the toxic effects of heavy metals on health of all living organisms and the environment. This remediation method has attracted attention in the recent years due to low cost of execution when compared to other remediation techniques and environmental friendliness. Out of Phytoremediation technologies available, phytostabilization and phytoextraction have been widely researched to mitigate metals-contaminated soils.

Phytostabilization

Phytostabilization technique involves the use of plants to contain or restrict the movement of heavy metals out of the polluting source thus preventing bioavailability of metals through leaching into the environment. Plant species that are used to stabilise heavy metals in polluted waste or soil are characterized with high tolerance of metals in surrounding soils, low accumulation of metals in the plant and dense root system (Ghosh and Singh, 2005). They function primarily on the contaminated soil by preventing soil erosion, percolation of rainwater through the soil, formation of heavy metal contaminated leachate and distribution through agents of rain and wind. However, this technique may only be used as an interim containment strategy until other remediation (Evanko and Dzombak, 1997). This is due to the fact that the amounts of the toxic metal contaminants remain relatively unchanged in soil. As hyperaccumulators have been applied in phytoextraction, excluders have been identified for phytostabilisation of heavy metal contaminants in soil. Excluders are plants that limit the levels of heavy metal translocation within them and maintain relatively low concentrations in their shoot in the presence of highly polluted soil/waste. They are employed in regenerating heavy metal contaminated soils (Baker, 1981). Many authors have measured heavy metals concentration in plants growing on heavy metal contaminated soils and mine tailings and have identified metal tolerant plants, excluders and accumulators (Franco-Hernandez *et al.*, 2010; Haque *et al.*, 2008; Del Rıo-Celestino *et al.*, 2006; Dahmani-Muller *et al.*, 2000; Wenzel and Jockwer, 1999).

Phytoextraction

Phytoextraction is based on the use of natural hyperaccumulator plants with exceptional metal-accumulating capacity to reduce the levels of bioavailable metal in heavy metal contaminated soils. These plants have several beneficial characteristics such as the ability to accumulate metals in their shoots and an exceptionally high tolerance to heavy metals (Kidd and Monterroso, 2005). Hyperaccumulators have been defined as plant species which contain more than 0.1% (1,000 mg/kg) of copper, lead, nickel chromium or cobalt, cadmium>100 mg/kg in their dried tissues. In

the case of zinc and Mn, a threshold of 1% (10,000 mg/kg) is proposed (Yanqun *et al.*, 2004; Boularbah *et al.*, 2006). In addition, a hyperaccumulator is regarded as plant which the concentrations of heavy metal in its above ground part are 10–500 times more than that in usual plant (Shen and Liu, 1998). In hyperaccumulator plants, the metal concentrations in shoots are greater than in roots, showing a special ability of the plant to accumulate and transport metals and store them in their above-ground part. The process of phytoextraction generally requires transportation of heavy metals to the shoots which can be easily harvested and disposed off. In some cases, root can be harvested as well. Several growth and harvesting of the plant is repeated until soil concentrations of heavy metals are reduced to environmentally acceptable levels. The metal-enriched plants can be harvested using standard agricultural methods, dried, ashed or composted plant residues highly enriched in heavy metals may be isolated as hazardous waste or recycled as metal ore (Kumar *et al.*, 1995).

Phytoextraction can be achieved via two means. Natural hyperaccumulation, in this case hyperaccumulators naturally accumulate heavy metal contaminants from the soil unassisted. Secondly induced hyperaccumulation, in which a chelator is added to soil to increase metal solubility which makes it easier for plants to absorb. To date, more than 450 plant species of metal hyperaccumulator plants have been reported in the literature and most of them (about 300) hyperaccumulate Ni (Boularbah *et al.*, 2006). Majority of the excluders for phytostabilisation and hyperaccumulators for phtoextraction have been identified through investigation into metal contaminated mine wastes (tailings) or soils.

Accumulators concentrate heavy metals in their shoots at both low and high soil metal concentrations and are utilized in extracting heavy metals from contaminated soils (Rotkittikhun *et al.*, 2006). Many field studies have investigated the accumulating capacity of plants that grew naturally on metalliferous wastes and on contaminated soils. It has been observed from such surveys that some plant species accumulate levels of heavy metals more than the normal levels encountered generally in plants (Boularbah *et al.*, 2006; Kidd and Monterroso, 2005; Yanqun *et al.*, 2004; Walter *et al.*, 2003; Bunzl *et al.*, 2001; Escarre *et al.*, 2000; Smith and Bradshaw, 1979) thereby proposing that such plants can be used as decontaminants of heavy metal polluted soils. In Nigeria, there is paucity of information about such metal tolerant plants locally and yet there are heavy metals contaminated sites all over the nation.

Hence in our study, we looked at the levels of heavy metals in soils and plants from four sites contaminated by abandoned secondary Pb slag dumpsites in Ibadan, Nigeria. Identification of plant species that accumulated exceptionally large concentration of Pb and Cd in shoots was the focus of the study. 26 plant samples of 20 species belonging to 12 families and 26 waste samples on which the plants were growing were collected. 12 out of the 26 plant samples demonstrated the features of Pb hyperaccumulators while 7 of the plants accumulated Cd. Enrichment coefficient of all the plant samples was lower than 1 for Pb except for *Andropogon tectonium(1.99)*. *Clome viscose, Gomphrena celosiodies, Elusine indica, andropogon, gayanus and urera lobata* are few of the plants that the results of this work identified as potential hyperaccumulators for Pb and Cd. These observed Pb and Cd accumulating plants could be used in bioremediation of lead and cadmium contaminated soils after further research into the accumulation mechanism. Field application of this technology will foster decrease in the risk of heavy metals to human, living organism and the environment as a whole.

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Daily Urinary Volume Model for Children and Adults in the South West of Nigeria

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ABSTRACT

The daily urinary volume excreted is very crucial in order to accurately determine the excretion rate of substance needed for bioassay monitoring purposes. The International Commission on Radiological Protection (ICRP) Publication 89 reported a worldwide reference value of daily urinary volume based on the data from the temperate environment. To the best of our knowledge the present value did not include contribution from the tropical Africa. Daily dietary habits and level of exercise are considered to contribute significantly to the daily urinary excretion in normal human subject. In addition, tropical environmental conditions also play major contributing role as indicated in a preliminary work conducted with a limited number of volunteers. In order to improve the statistical significance of the study, twenty four hours urine collection from large number (> 400) of subjects was conducted. The samples were collected from male and female adults (> 20 y), adolescence of ages 11 – 20 years, and children below 11 years. The results of the study revealed intra- and inter variability of urine excretion, the dependence on age, gender, and working habits. The influences of tropical environmental conditions on the daily urine volume were noticeable. The daily urinary volume for adult male subjects in Nigeria is lower than that predicted by the ICRP values. The value for the adult female subjects is however, higher than the recommended ICRP value for female. Values of urinary volume for adolescence and infant, which were not given by the ICRP, have been presented. The results suggest that the prevailing environmental factors play significant role in the urinary excretion of the subjects in the area. The Intra- and inter variability observed among subjects therefore, suggested dependence on age and gender. Mathematical models describing the relationship between the daily urinary volume and age for male and female subjects considered were proposed.

Keywords: urine, excretion, human subjects, tropical, model

INTRODUCTION

Internal exposure to ionizing radiations stem from the injection, ingestion and inhalation of substances containing radionuclides into the body. Evaluations of internal exposure involve bioassay measurements, which include in vivo and in vitro techniques. The in vitro method involves the determination of radionuclides in biological substances, such as blood, urine and faeces. The method involves daily collection of substance such as urine in order to determine the daily excretion of the desired radionuclide. The International Commission on Radiological Protection ICRP publication 30, (1979) formed the basis for interpreting bioassay measurements. The results of a thoroughly tested dosimetric model for a particular radionuclide could be correlated with that of the direct bioassay measurements. In order to achieve this, the excretion rate of the radionuclide must be reliably determined. In many parts of the globe, authors have been determining daily urinary excretion of radionuclides present in human urine (Beyer et al,

1993; Bagatti et al, 2003; Ting et al, 1999; Medley, et al, 1994; Dang et al, 1992; Galletti et al, 2003; Oeh et al, 2007).

The total daily urine volume excreted is very important for the accurate determination of the excretion rate. The amount of urine excreted per day depends on several factors. An adult may take in between 2000 ml and 3000 ml of water per day but daily consumption of water in fluids and food varies from person to person. In addition to this, metabolism of foodstuffs also leads to production of water (Johnson, 1998; ICRP, 2002). Therefore, the total daily urinary volume will depend on individual habits such as diet and exercise, and on environmental factors like air temperature and humidity. In order to maintain constant body water, an amount of these ingested and metabolised water needed to be eliminated. The main route of clearance from the body under sedentary conditions is the urine production in the bladder. The urinary bladder is considered as a separate pool that receives all material destined for urinary excretion (ICRP, 1995). The rates at which materials enter the bladder depend on their clearance rate from the body tissues and the renal urine production. Hence, the concentration of this material in urine at any given time depends largely on the total urinary volume. This makes the latter very crucial in interpreting bioassay measurements. The urine production rate has also been noted to be dependent on age, gender, diet, exercise, and other factors (ICRP, 2002). These factors include among others the environmental condition of the subject.

Apart from this notable water clearance route, water losses through the sweat pathway could be significant especially when environmental factor is taken into account. Large amounts of water may be lost in sweat production in a warm environment or during exercise, and as much as 3500 ml h-1 may be lost during vigorous exercise in a hot environment (Johnson, 1998). For a 70 kg male living in a temperate environment and engaged in light, indoor activities for 8 hours per day, an average sweat loss has been estimated as 500 ml d-1 (ICRP; 2002). Another form of water loss from the body is the invisible and insensible perspiration through the skin, even at room temperature. (Lamke et al, 1977). This kind of water loss increases with temperature and therefore, may be significant in warm, dry, windy conditions and at low pressure. Finally, loss through faeces is another form of water loss. Although a large volume of fluid is secreted into the gastrointestinal tract each day, most of it is re-absorbed before it reaches the end of the colon and rectum.

A central estimate for water balance in human adult male is presented in Table 1. The estimate of urinary volume of 1600 ml d-1 and 1200 ml d-1 as the worldwide reference value for adult male and female, respectively, by the ICRP (2002) was estimated based on the above mentioned entry and clearance of water in the body. However, in order to gain global acceptance, it is necessary to incorporate data from all parts of the world. The ICRP did not consider data from the tropical environment, and to the best of our knowledge, no data has been available from African countries for comparison until recently in a preliminary study involving nineteen (19) adult male subjects in Nigeria (Arogunjo et al, 2007). Nigeria falls on the tropical region of the world with considerable high temperature (> 45oC in some places) with warm and dry air. In this study, the result of the daily urinary volume conducted among male and female adults (> 20 y), adolescence of ages 11 – 20 years, and children below 11 years will be presented. The intra- and inter variability of urine excretion and the dependence on age, gender, working habits and the influences of tropical environmental conditions on the daily urine volume will be discussed.

Mathematical models describing the relationship between the daily urinary volume and age for male and female subjects considered will be presented.

MATERIALS AND METHODS

Sample collection and measurement

Twenty-four hour urine samples were collected from four hundred and thirty eight (438) different groups of male and female subjects in the south west of Nigeria. The male include 33, 43 and 257 infant, adolescence and adult, respectively, while the female include 19, 27, and 59 infant, adolescence and adult, respectively. The 24 hr urine was collected starting early in the morning. After wake-up, the subjects emptied their bladder in the toilet noting the time, and all urine thereafter was collected in a graduated 3000 ml container until the following morning, and for the last time at the exact time the bladder was emptied the previous morning. The first void collected at the start of the sampling was acidified with 0.5 ml HCL to prevent decomposition. The weight (using analytical balance) and the total volume of the 24 hr urine collected from each subject were noted and recorded. Personal effort was made to limit occurrence of all systematic errors in the determination of the urine volume.

RESULTS AND DISCUSSION

The mean daily volume and mass of the urine collected from female subjects alongside the mean age and mass of the donors are presented in Table 2. The infant female group with mean age 7.4 y and mean mass 25.9 kg has its urine volume ranged from 200 – 1680 ml d-1 with arithmetic mean ± SD value of 604.7 ± 385.0 ml d-1. The adolescence female group with mean age 16.8 y and mean mass 46.4 kg has its urine volume ranged from 600 – 2020 ml d-1 with arithmetic mean ± SD value of 1253.9 ± 459.4 ml d-1. The adult female group (up till age 60 y) with mean age 38.3 y and mean mass 61.1 kg has its urine volume ranged from 500 – 2770 ml d-1 with arithmetic mean ± SD value of 1245.7 ± 571.9 ml d-1.

The mean daily volume and mass of the urine collected from male subjects alongside the mean age and mass of the donors are presented in Table 3. The infant male group with mean age 7.8 y and mean mass 27.0 kg has its urine volume ranged from 300 – 1250 ml d-1 with arithmetic mean ± SD value of 626.1 ± 252.5 ml d-1. The adolescence male group with mean age 17.6 y and mean mass 56.0 kg has its urine volume ranged from 300 – 1850 ml d-1 with arithmetic mean ± SD value of 1031.6 ± 379.0 ml d-1. The adult male group (up till age 60 y) with mean age 44.1 y and mean mass 64.9 kg has its urine volume ranged from 450 – 2500 ml d-1 with arithmetic mean ± SD value of 1170.6 ± 546.8 ml d-1. The adult male group (up till age 84 y) with mean age 50.8 y and mean mass 64.3 kg has its urine volume ranged from 450 – 2550 ml d-1 with arithmetic mean ± SD value of 1241.0 ± 551.3 ml d-1. The adult male group (from age 61 - 84 y) with mean age 65.6 y and mean mass 63.1 kg has its urine volume ranged from 500 – 2550 ml d-1 with arithmetic mean ± SD value of 1396.8 ± 532.1 ml d-1.

The comparison between the mean daily urine volume in the different groups and the ICRP (2002) values for both sexes is presented in Figure 1. The figure clearly revealed that the mean

daily urine values for various groups of adult male fall below the reference values given by the ICRP. The percentage difference is about 27%, 22%, and 13%, respectively, for the adult male with age up till 60 y, adult male with age up till 84 y and adult male with age between 61 y and 84 y. The present result is in agreement with the preliminary work earlier conducted for adult male subjects in the tropical environment, which is about 20% lower the ICRP value. The lower values could be attributed to the prevailing weather condition in the studied area. The temperate environment where data were obtained in order to arrive at the reference values given by the ICRP is quite different from the tropical environmental conditions. According to Lamke et al (1977), insensible and invisible water loss from the body could range between 300 and 1000 ml per day especially in a warm and dry weather condition and at low barometric pressure. Apart from the imperceptive water loss, physical activities are expected to bring about appreciable water loss via sweat in such condition. However, the adult female value is within the ICRP value for female with slightly higher value of about 4%.

It can be observed from Figure 1 that the mean daily urine value for adult female (up till 60 y) within the retiring age limit of 60 years in Nigeria is higher than the male counterpart by about 6%. The Female adolescence also has its value more than the male counterpart by about 18%. These can be attributed to the active nature of men and are expected to produce more sweat during physical activities under the same environmental condition. The difference in the infant group is however, less than 4% for male although they are expected to be more active than the female counterpart. This can be attributed to the low number (< 20) of infant female subjects involved in this survey as against that of the male counterpart. It is also observed from the figure that the sedentary adult male group, when compared with the working adult male group, has higher mean daily urine volume. The mean daily urine volume for the adult male when the retiring age is included with the working age is about 6% higher than the working adult male. This value is about 16% more than the working class when the sedentary group (61 – 84 y) is considered alone. This is a clear indication that physical exercise play significant role in determining the amount of urine produced per day under normal environmental and health conditions. However, the impact of the degree of physical exercise can largely be influenced by factors like eating drinking habit (Arogunjo et al, 2007).

The error bars in Figure 1 are the standard deviations within the groups and an indication of the variation of urine volume collected within each group. Apart from this, large variations were also observed within subjects of the same age. In order to establish relationship between the daily urine volume and the age of the subjects considered, empirical models (figs 2 – 5) were fitted to predict the observed results. In Figure 2, the daily urine volume from infant to adolescence for female subjects was tested with three mathematical models as shown in the figure. The three models appear to describe the data; the power model best described it with the correlation coefficient of 0.68. However, the polynomial model appears to describe the data for the daily urine volume from infant to adult female (fig 3). Three models were also used to fit the data for the daily urine volume from infant to adolescence male as shown in Figure 4. The three models described the data well with correlation coefficient between 0.70 and 0.72. However, the models poorly described the data for the daily urine volume from infant to adult male as shown in Figure 5.

CONCLUSION

Twenty four hours urine collection from more than 400 male and female subjects in the south western Nigeria has been conducted. The samples were collected from male and female adults (> 20 y), adolescence of ages 11 – 20 years, and children below 11 years. The results of the study revealed intra- and inter variability of urine excretion, the dependence on age, gender, and working habits. The influences of tropical environmental conditions on the daily urine volume were noticeable. The daily urinary volume for adult male subjects in Nigeria is lower than that predicted by the ICRP value while the female value is slightly higher by 4%. Values of urinary volume for adolescence and infant, which were not given by the ICRP, have been presented. The results suggest that the prevailing environmental factors play significant role in the urinary excretion of the subjects in the area. The Intra- and inter variability observed among subjects therefore, suggested dependence on age and gender. Mathematical models describing the relationship between the daily urinary volume and age for male and female subjects considered were proposed.

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Table1: Central estimate for water balance in human adult male and female (ICRP, 2002)

	Male	Female
Water intake in food and fluids (ml/day)	2600	1960
Oxidation of food (ml/day)	300	225
Losses (ml/day)		
Urine	1600	1200
Insensible loss[a]	690	515
Sweat	500	375
Faeces	110	95

[a] assumed to be divided equally between the lung and skin.

Table 2: Mean values of age, frequency and urine volume for female subjects

Age (y)	Frequency	Urine volume (ml)
3	2	405.0
5	3	243.3
6	3	490.0
8	2	515.0
9	4	522.5
10	5	1072.0
11	2	995.0
12	2	1300.0
13	2	1225.0
14	2	1882.5
15	1	2020.0
16	1	1260.0
17	2	1350.0
18	3	1116.7
19	6	1233.3
20	6	1053.3
21	7	1348.9
22	5	898.0
23	7	1357.1
24	1	1180.0
25	3	1416.7
26	3	1224.1
41	1	850.0
42	2	1385.0
43	2	1050.0
44	2	1400.0
45	4	952.5
46	1	1650.0
47	1	1780.0
48	1	2000.0
50	2	1460.0
51	1	1980.0
52	3	1266.7
53	1	840.0
54	2	1275.0
55	2	850.0
56	1	1000.0
57	3	1180.0
58	1	780.0
60	3	1363.3

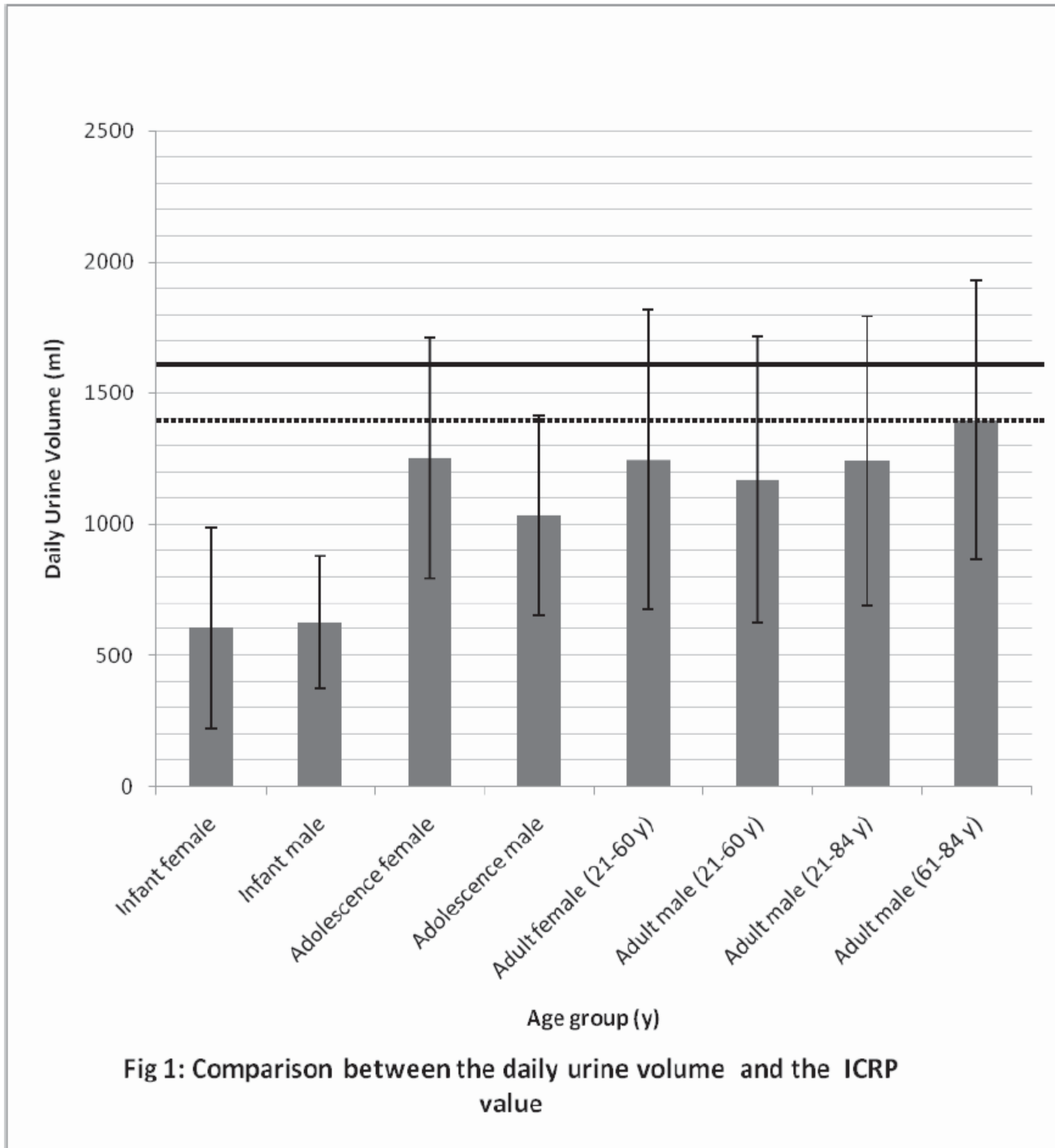

Fig 1: Comparison between the daily urine volume and the ICRP value

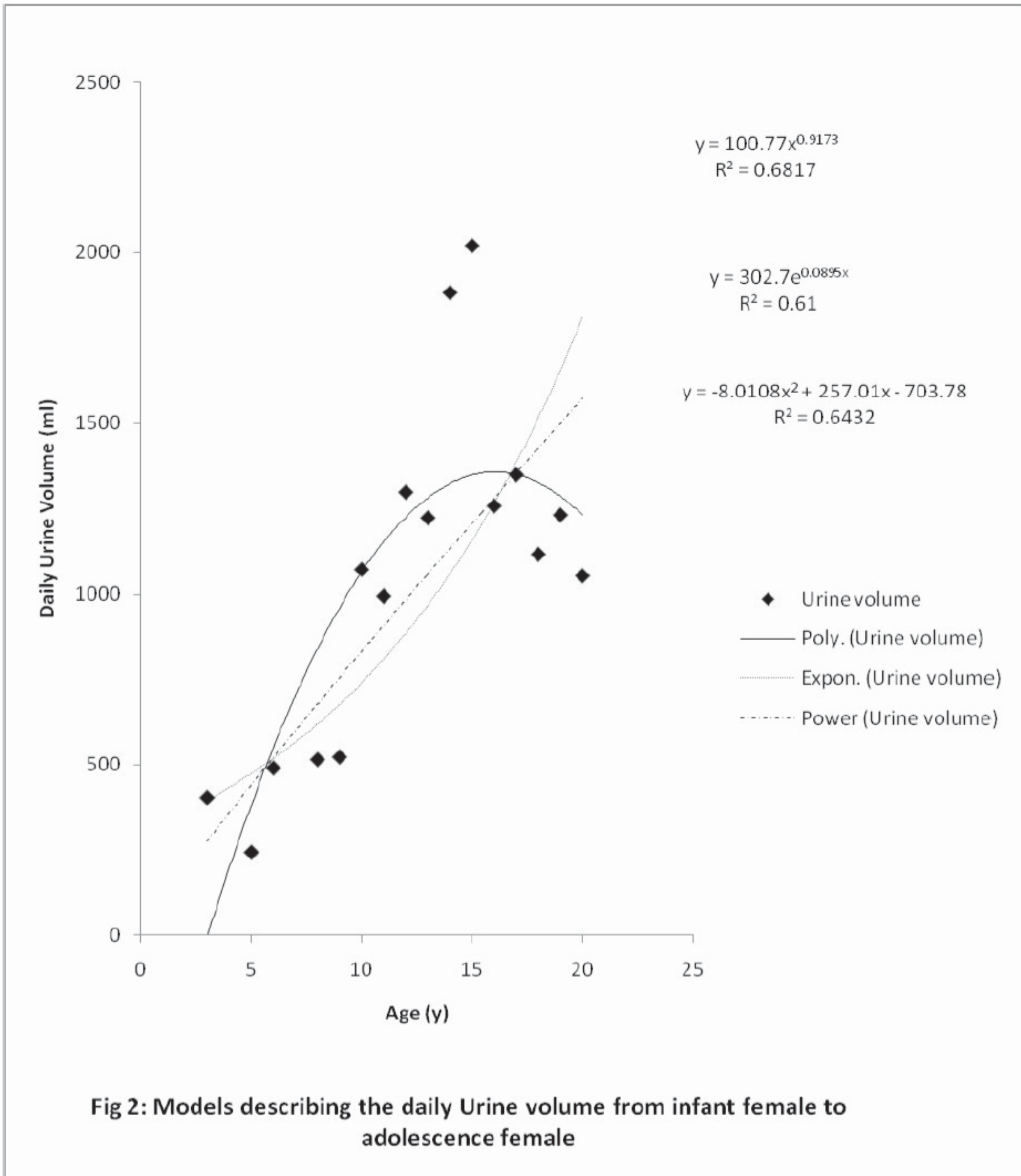

Fig 2: Models describing the daily Urine volume from infant female to adolescence female

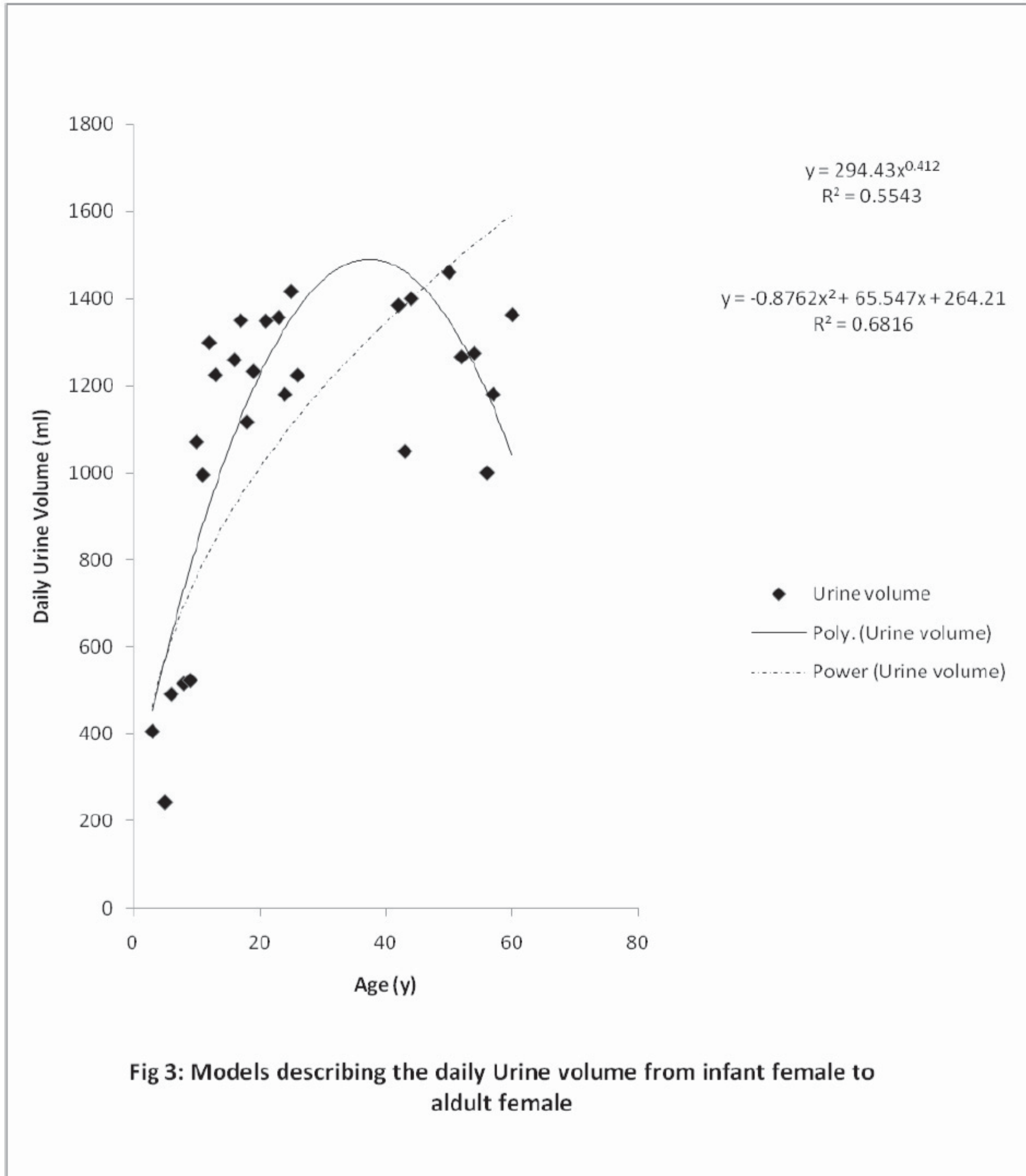

Fig 3: Models describing the daily Urine volume from infant female to aldult female

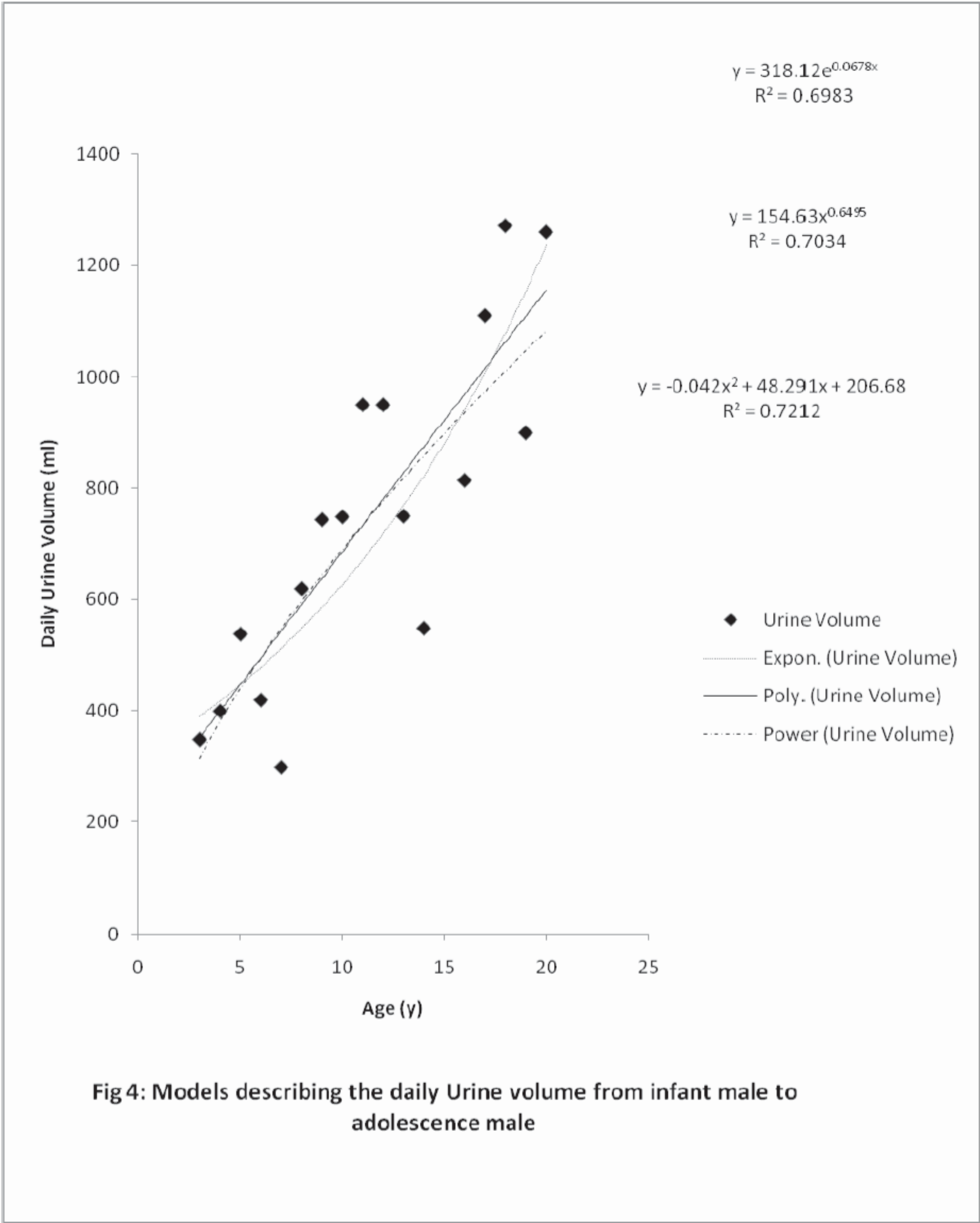

Fig 4: Models describing the daily Urine volume from infant male to adolescence male

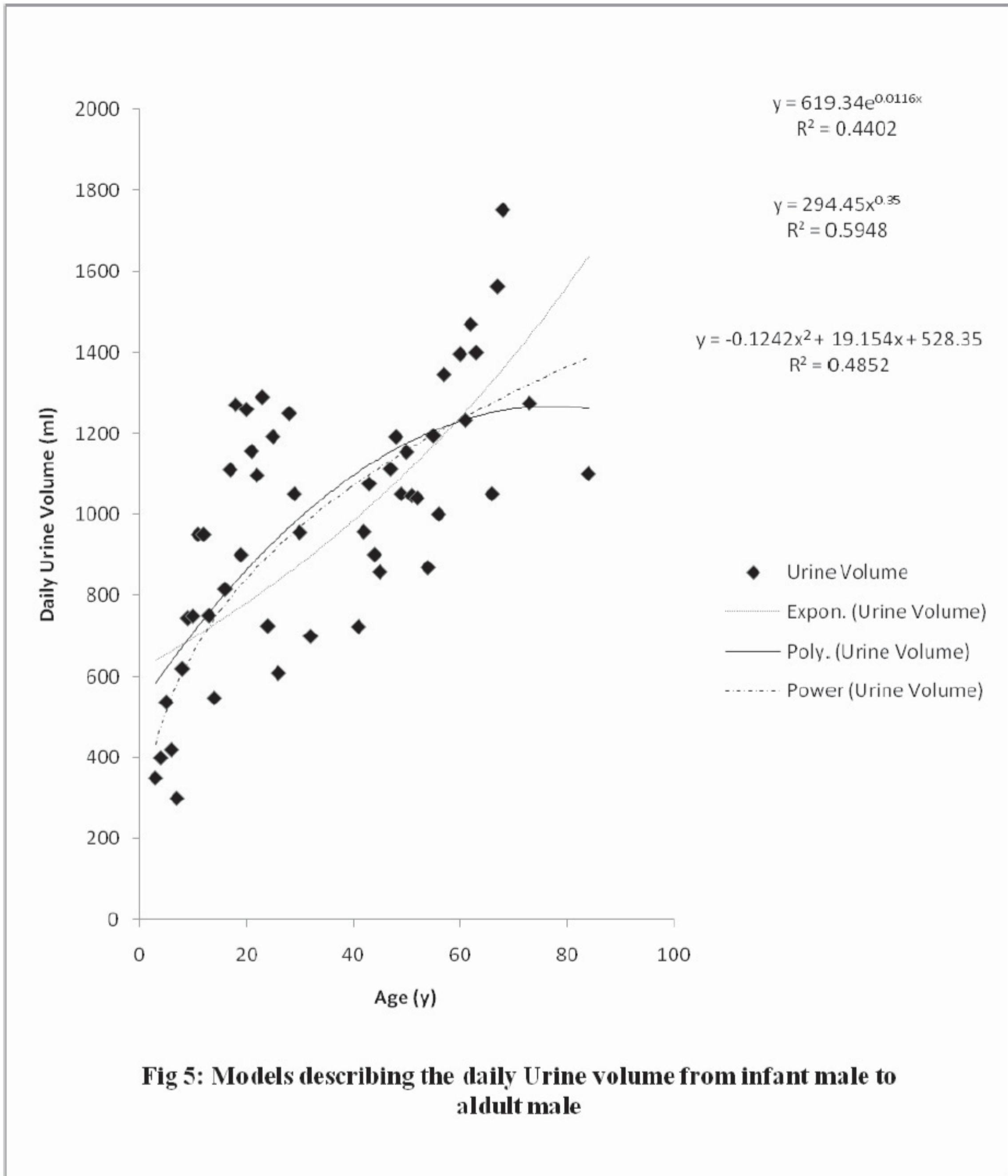

Fig 5: Models describing the daily Urine volume from infant male to aldult male

Exploring the Genetic Causes of Male Infertility in Nigeria: The Role of Gene Mutations, Deletions and Polymorphisms in Spermatogenetic Failure

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ABSTRACT

Abnormal semen quality is often associated with male infertility. In most cases semen analysis remains the only test for the evaluation of male infertility in Nigeria. Though the percentage of male factor contribution to couple infertility is estimated to be as high as 54%, the possible causes of this spermatogenic defect remain uncertain. Although, the process of spermatogenesis is relatively efficient and resistant to damages, ours and others previous studies have implicated environmental factors such as exposure to toxic agents as possible cause of spermatogenic defects. Recent studies have focused on examining the prevalence of certain genetic defects in infertile males and analyzing the molecular basis of infertility. Infertile men with severe spermatogenic defects and low or no sperm count have significantly higher rate of genetic abnormality than fertile males but the molecular basis of most human male infertility arising from spermatogenesis disruption is poorly understood, especially in developing countries including Nigeria. This is partly because of the lack of useful investigating systems and the lack of expertise by the investigators. Accumulated evidence however, suggests that genetic component may be responsible for about 15% of severe cases of male infertility. Such genetic factors include chromosomal aberration, Y-chromosome long arm microdeletions involving one or more azoospermic factors (AZF) and mutations in the androgen-receptor (AR). All this conditions are suspected causes of male infertility directly affecting spermatogenesis. However, only scant data is available from Africa. Using established conventional biotechnology tools, we investigated the possible contribution of selected genetic factors (deletions, mutations and polymorphisms) to male infertility due to spermatogenetic failure (oligozoospermia and azoospermia) in Nigerian men. Our studies reveal that both genetic investigations on genes located on both Y and X chromosome could provide diagnostic value for management of male infertility. Although, we demonstrated variation in the sequence of the transcriptional gene - TAF7L, these mutations do not appear to contribute to causes of male infertility in Nigeria. Of more interest is the androgen receptor polymorphism, which shows unique distribution of GGN allele in our Nigeria population significantly difference from Caucasian population. Nigerian population then shows a different CAG-GGN haplotype compared to Caucasian. However, the significant and relevant of this different to fertility status is not clear. Even though, it appear as if Y-chromosomal deletion is less common in our population compared with Caucasians, a routine screening may be useful in genetic counseling of infertile males. Furthermore, our study suggested that association of gr/gr (risk factor) deletion with other genetic factors may precipitate infertility. Molecular diagnosis or genetic screening of infertile Nigeria males may bring a positive revolution to the management of infertility in Nigeria.

INTRODUCTION

Involuntary infertility is a world-wide problem the frequency of which varies from one area to another. Despite the high fertility rate in some African countries, infertility consumes a large percentage of limited health care resources in developing countries, including Nigeria (Ikechebelu et al, 2003 and Overstreet et al, 1993). About one third of gynaecological consultation is related to complain of infertility in Nigeria (Okonofua, 1996). Available evidences suggested that male infertility is an important but neglected reproductive health issue in Nigeria. Published studies indicated that the male factor accounts for as high as 50% of the causes of infertility (Chukwudebulu et al, 1978 and Esimal et al, 2002). Until recently, little attention has been placed on male contribution to infertility due to general erroneous believe that infertility is only a female problem. Hence the woman is more aggressive than her male partner in seeking medical assistance and many male partners of infertile couple are still reluctant to be investigated ((Nwofor and Ugezu, 2003). The male factor is now recognized to constitute a major factor in couple infertility (Frazer, 1999), reported to be responsible for up to 33.3% of cases of infertility (WHO, 1999). Male factor is estimated to contribute up to 40% of couple infertility in Nigeria, with 54% of males in infertile couples in Nigeria found to have abnormal semen analysis (Belsey, 1976; Chukwudebelu *et al.*, 1978; Nwofor and Ugezu, 2003; Ikechebelu *et al.*, 2003).

Spermatogenesis is critically dependent on the hormonal milieu (Behre *et al.*, 2000) and the hypothalamic-pituitary-gonadal (HPG) axis is quite sensitive to disruption by endocrine disorders (Jarrow et al, 2003). Analysis of hormonal profiles is helpful in the investigation of couple infertility to identify hypogonadism. Previous own studies have shown that male infertility in Nigeria is characterized by hypergonadotropism, hyperprolactinaemia and increase seminal plasma testosterone (Arowojolu *et al.*, 2003, Arowojolu *et al.*, 2004 and Akinloye *et al.*, 2007). Although, luteinizing hormone (LH), follicle stimulating hormone (FSH) and testosterone are commonly analyzed in the investigation of male infertility, the sensitivity of this approach is limited, since serious disturbances in spermatogenesis are often observed in the presence of normal hormone levels (Server and Hessol., 1985). Serum testosterone can be slightly reduced in male idiopathic infertility (Andersson *et al.*, 2004) but hormone measurement is rarely of diagnostic value concerning the cause of infertility.

Abnormal semen quality is often associated with male infertility (Kitilla, 2002). In most cases semen analysis remains the only test for the evaluation of male infertility in Nigeria (Chukwudebelu et al 1979). Though, the percentage of male factor contribution to couple infertility is estimated to be as high as 54% (Chukwudebelu *et al.*, 1979), the possible causes of this spermogenic defect remain uncertain. Although, the process of spermatogenesis is relatively efficient and resistant to damages (Cozzolino *et al.*,2000), previous studies have implicated environmental factors such as exposure to toxic agents as possible cause of spermatogenic defects (Akinloye *et al.*, 2005 and Akinloye *et al.*, 2006). Recent studies have focused on examining the prevalence of certain genetic defects in infertile males in analyzing the molecular basis of infertility (Foresta *et al.*, 2001 and 2004, Gromoll et al, 2006, Akinloye et al, 2007 and 2009). Infertile men with severe spermatogenic defects and low or no sperm count have significantly higher rate of genetic abnormality than fertile males (Nudell and Turek, 2002; Foresta *et al.*, 2004) but the molecular basis of most human male infertility arising from spermatogenesis disruption is poorly understood, especially in developing countries including

Nigeria, because of the lack of useful investigation systems and the lack of expertise by the investigators. Sertoli cells dysfunction has been implicated in most cases of male infertility in Nigeria (Arowojolu *et al.*, 2003, Arowojolu *et al.*, 2004 Akinloye *et al.*, 2007). The role of this supporting Sertoli cell in mammalian spermatogenesis and the molecular basis for its role in spermatogenesis needs to be fully understood for future advancement in this field. Accumulated evidence however, suggests that genetic component may be responsible for about 15% of severe cases of male infertility (Foresta *et al.*, 2002; Foresta *et al.*, 2004). Such genetic factors include, chromosomal aberration (Lanfranco *et al.*, 2004), Y-chromosome long arm microdeletions involving one or more azoospermic factors (AZF) (Simoni *et al.*, 1997; Foresta *et al.*, 2001), and mutations in the androgen-receptor (AR) (Hiort *et al.*, 2000). All this conditions are suspected causes of male infertility directly affecting spermatogenesis (Ferlin et al, 2004). However, only scant data is available from Africa including Nigerian.

METHODOLOGY

Study population

The studies described in this publication were based on a case-controlled prospective study design. The study group consists of infertile patients attending the Urology Clinic of the Department of Surgery, semen donors for heterologous insemination and male partners of women attending the Antenatal Clinic of the Department of Obstetrics and Gynaecology, both of the University College Hospital, Ibadan, Nigeria. Out of over 200 subjects recruited for the study, only 102 gave informed consent and submitted blood for analysis. The low participation is due to erroneous custom, traditional falsehood and taboo attached to donation of semen and blood for analysis in our environment. Patients were included in the cohort if they had a minimum of 1-yr history of infertility and sperm counts <20×106/ml as determined by 2 consecutive semen analyses. All patients were examined by a clinician to exclude known causes of testicular damage and other possible causes of infertility other than genetic causes. Patients with obstructive syndromes of the genital tract, genitourinary operations, occupational exposure to radiation or chemicals, systemic illness and those under treatment with spermatogenesis-impairing medication were excluded from the study.

All samples were analyzed for spermogram, hormonal assay, androgen receptor polymorphisms (CAG and GGN repeats) and Y-chromosome microdeletion and gr/gr deletion. However, for TAF7L gene study, patients with Y-chromosome microdeletion were excluded from the study, leaving a final study population consisting of 60 infertile patients comprising 20 azoospermic men, 14 severely oligozoospermic (sperm concentration <5×106/ml) and 26 oligozoospermic (sperm concentration 5-20×106/ml). The remaining 38 subjects are aged-matched, normozoospermic men.

Semen analysis

Assessment of sperm concentration was performed according to World Health Organization (WHO, 1999) guidelines.

Hormone assays

Serum FSH and LH concentrations were measured by fluoroimmunoassay using AutoDELFIA® (PerkinElmer Life and Analytical Sciences Wallac Oy, Turku, Finland). Testosterone (T) was measured by enzyme-linked immunosorbent assay (ELISA) (DRG Instruments, Marburg, Germany) Laboratory total assay variation for FSH was 2.6% at 2.4 IU/l and 3.3% at 26 IU/l, for LH it was 2.7% at 6.9 IU/l and 3.6% at 42IU/l and for serum T was 4.0% at 3.4 nmol/l and 5.4% at 9 nmol/l.

Genetic analyses

Genomic DNA was extracted from EDTA whole blood using the FlexiGene DNA kit (QIAGEN GmbH Hilden, Germany) according to the manufacturer's instruction.

Analysis of TAF7L gene sequence

Using single strand conformation polymorphism (SSCP), conformational difference of single stranded nucleotide sequences in all the samples were distinguished by means of gel electrophoresis, which separates the different conformations. This serve as screening test to identify samples with conformational different in any of the exons. Relevant exons were then amplify and compared with the NCBI sequence.

TAF7L gene sequence (BC043391) on NCBI website was used as reference sequence (http://www.ncbi.nlm.nih.gov/). The TAF7L gene has a total gene size of 24805 bp with 13 exons (NM_024885.2) translating to 463 amino acid sequences (NP_079161.2). Primers were designed to amplify and sequence the entire coding region and parts of the flanking introns of the TAF7L gene (Table 1) by direct sequencing using ABI 3730 genetic analyzer (Applied Biosystem, Foster City, CA, USA). Primers details and methods are described in our previous paper (Akinloye et al, 2008)

Determination of the AR CAG and GGN repeat numbers

The CAG and GGN repeat polymorphisms in the AR gene were analyzed as described in our previous publication using an ABI 310 genetic analyzer (Applied Biosystem, Foster City, CA, USA) Akinloye et al, 2009.

Determination of Y-chromosomal microdeletions

Y-chromosomal microdeletions were analyzed according to the standard method recommended by the European Academy of Andrology (Simoni *et al.*, 2004). Gr/gr deletions was analyzed by the well established method previously described (Hucklenbroich *et al.*, 2004). All these methods are base on PCR.

RESULTS AND DISCUSSION

Mutation in X chromosome linked gonad specific gene; TAF2Q/ TAF7L in azoospermic (spermatogenic failure) Nigerian male

Spermatogenesis is a complex process requiring the specialised function of multiple cell types including the somatic and germ cell that collectively result in the continuous production of functional sperm in adult males. Because of the hemizygote exposure of men to sex chromosome, mutations in genes located on the X or Y chromosome cannot be compensated for by a second normal gene as in the case of autosomal gene. Hence, the phenotypical effect of mutation in the sex chromosome genes in males could be more severe and pronounced. A recent systemic search for genes expressed in mouse spermatogonia but not in somatic tissues identified 25 genes, 19 of which were novel, expressed only in male germ cells, 3 of these genes are Y-linked and 10 are X-linked. This indicates that the X-chromosome has a predominant role in pre-meiotic stages of mammalian spermatogenesis. One of this novel X-linked genes identified is the RNA polymerase II TBA associated factor II (taf2q), which encode a protein with testis specific expression (Wang et al, 2001).

Wang et al, 2001 showed that in mammal, X chromosome has a role in the meiotic stage of spermatogenesis. This is possibly because the X and Y chromosome evolved from an ordinary pair of autosomes, adaptative forces caused the X chromosome, as it differentiated from Y chromosome to accumulate so many genes expressed in early stages of spermatogenesis. Mutation of these genes may generate adaptive pressure to limit gene expression to males. Based on this theoretical scenario, it was postulated that X chromosomes should evolve to carry disproportionate share of male specific gene functioning in male differentiation (Rice et al, 1984).

The precise temporal and partial expressions of specific transcription factors have long been described to be essential for proper execution of spermatogenesis (Sassone-Corsi, 1997). Emerging evidences now suggest that specialized component of the basal RNA polymerase II machinery are critical for the execution of gonad-specific program of gene expression (Hocheimer and Tjian, 2003). The RNA polymerase II general transcription factor (TFIID) complex contain the TATA binding protein (TBP) and 14TBP –associated factors (TAFs) that function in core promoter recognition and polymerase II recruitment (Verrijzer and Tjian, 1996).

Inactivation of yeast and mammalian TAF genes have been shown to lead to cell cycle arrest. Falender et al (2005) have shown that the autosomal TAF4b gene is required in mouse spermatogenesis. Wang et al (2001) identify TAF gene expressed specifically in mouse testis; Taf2q and their human orthologos TAF2Q. In Drosophila melanogaster mutation in the dTAF5L gene have been reported to cause male sterility. This autosomal gene is also a testis specific homologue of dTAF5 (Hiller et al, 2004). Sex chromosome linked TAF genes (TAF7) have been isolated in mouse spermatogonia. In mice as well as human, TAF7/TAF7L is located on the X chromosome (Wang et al, 2001). From mouse studies, it is known that this gene is expressed during spermatogenesis, from the stage of spermatogonia till the stage of round spermatids. In mammals, TFIID component are expressed at high level at specific stages of spermatogenesis (Schmidt and Schibler, 1995). Knockout mice appear to be healthy and showed no apparent abnormalities at the gross and histological level. However, testis from the adult knocks out mice showed size and weight reduction of -50%. Semen samples extracted from the vas deferens and

epidedymis lacked spermatozoa (Zhang et al, 2001). The human testis is now established to contain a unique paralog of TAF7/ TAF7L (Pointud et al, 2003).

A current attempt to investigate the role of hTAF7L gene in the aetiology of male infertility observed differences in the sequence of hTAF7L gene in 12 infertile male with spermatogenic failure compared to published hTAF7L sequence (Genbank-BC043391). This study reported an alteration in exons 1 (c181 G>A), 9 (c922 A>G), 13 (c1373 G>A) and a deletion of six base pairs in exons 10 (GGATGA) at position 1047-1052, causing an absence of glutamic acid and aspartic acid at position 350 and 351. However, some of these changes, except the alteration in exon 13, were also find in some of the controls, though in a reduced frequency (Stouffs et al, in press). These observations further strengthen the need for more studies on the possible role of this gene in infertile males. In more recent study of carefully selected non obstructive azoospermic German population, we showed that TAF7L gene mutations are rare in normogonadotrophic nonobstructive azoospermia. Moreover, the TAF7L gene is a highly polymorphic gene and most of these polymorphisms are not significantly associated with gonadal dysfunction (Akinloye et al, 2007). A screening of 21 azospermia and 14 severe oligozoospermia from infertile Nigeria males also found SNP intron 8 nucleotide exchange in flaking intron of exon 8 (15061 A > G) of one of the azoospermic patient. This nucleotide exchange was similar to the one reported in our German population (Akinloye et al, 2007).

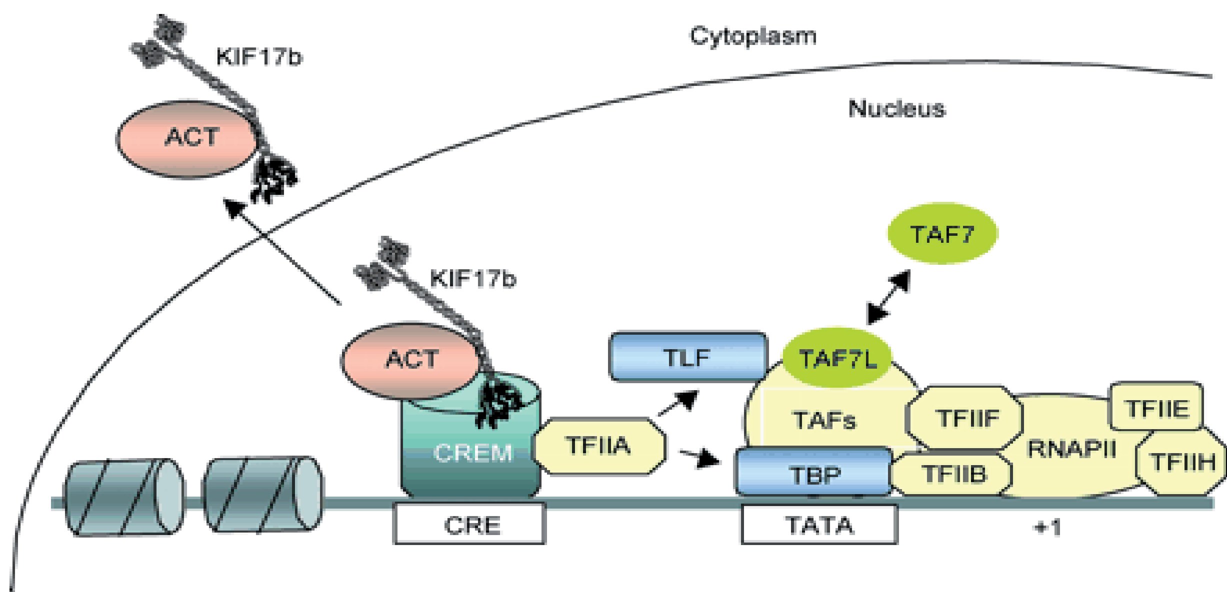

Fig 1: TAF7, a factor of the TFIID complex, is exchanged at a critical stage in germ cell development for the testis-specific paralogue TAF7L (Kimmins et al, 2004)**.**

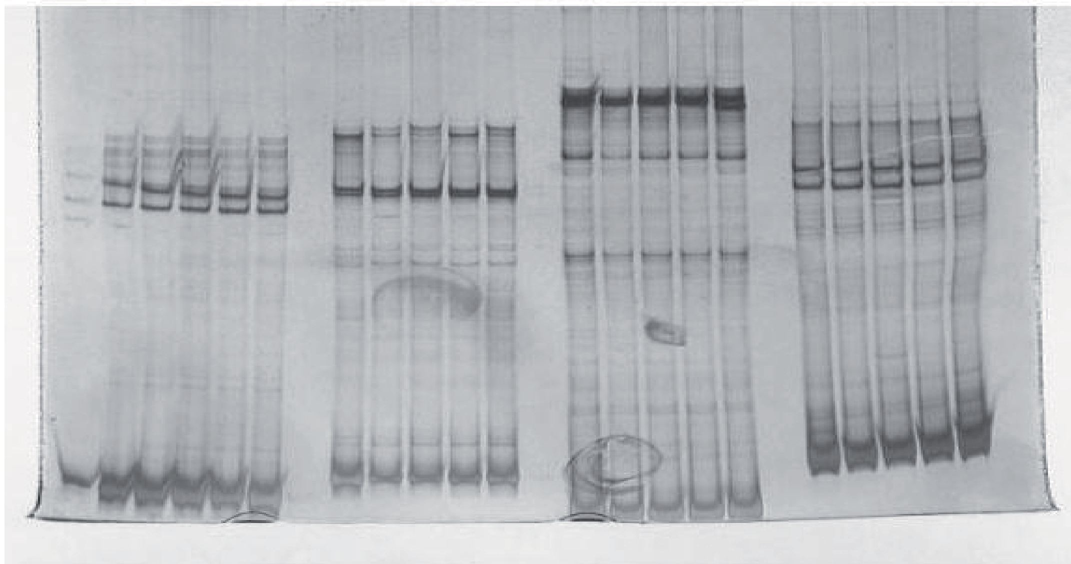

Fig. 2: SSCP gel of TAF7L gene in five infertile Nigerian males

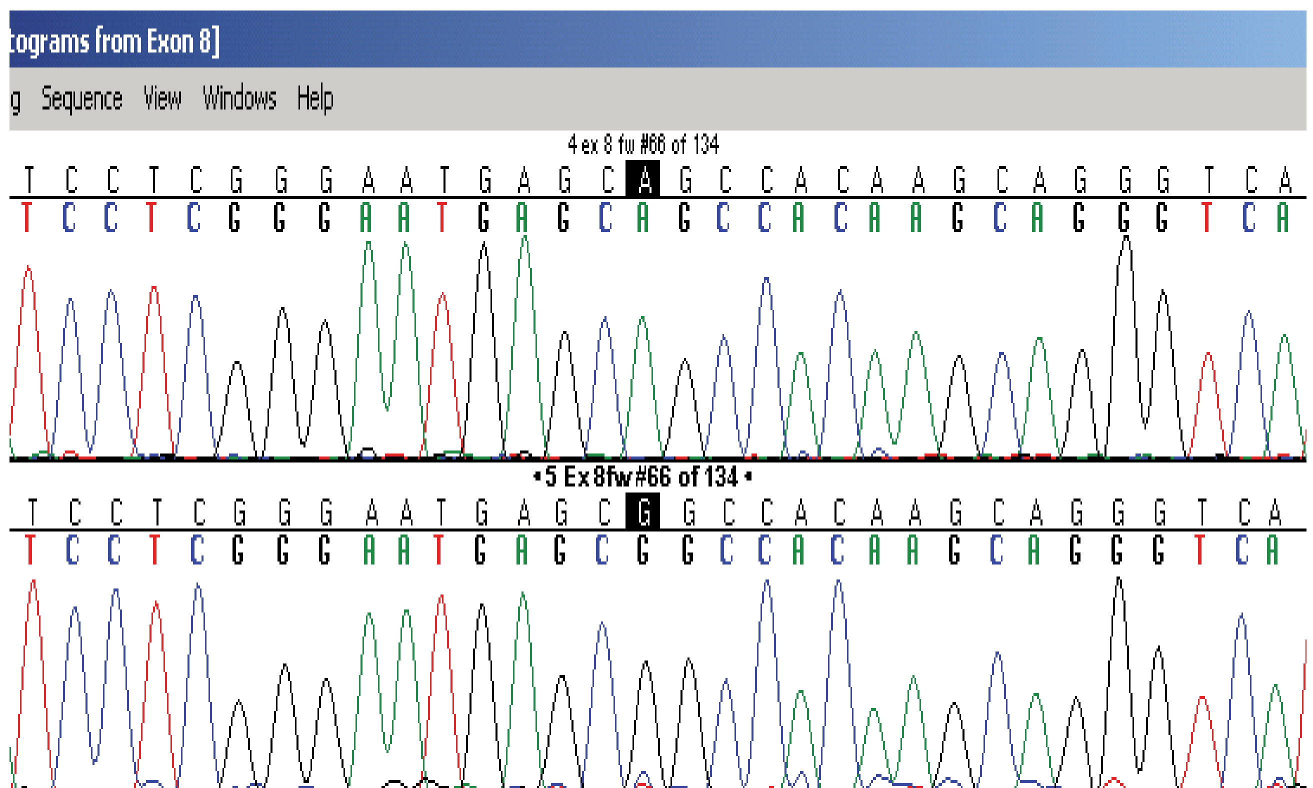

Fig 3: Electrophetograms of nucleotide sequence of the flaking intron of exon 8 of TAF7L gene

Androgen Receptor (AR) gene polymorphisms in infertile Nigerian Male

Male sexual differentiation, testicular descent, and spermatogenesis require androgen. Androgen and a functional AR are essential for development and maintenance of the male phenotype and spermatogenesis (Ferlin *et al.*, 2004). The action of androgen is mediated through the AR, which binds to the androgen responsive element on DNA and regulates the gene transcription. Consistent with this, mutations in the AR gene cause a variety of defects related to androgen insensitivity with the less severe phenotype represented by isolated cases of male infertility (Aiman *et al.*, 1979; Quigley *et al.*, 1995; Hiort etal.,2002). Action of AR is expressed in the testis by Leydig, peritubular and Sertoli cell. The fact that sperm concentration ranges from 20-300 million/ml in normal men without any indication of changed endocrine parameters led to assumption that genetic variation in the transduction of androgen signaling could be important in male infertility (von Eckardtstein *et al.*, 2001). The AR has two polymorphic sites in exon 1, characterized by different numbers of CAG and GGN repeats resulting in variable length of polyglutamic and polyglycine stretches. Longer CAG repeat result in a reduced AR transcription activity, whereas the role of GGN triplets is less clear. A relationship between decreased spermatogenesis and moderate expansion in the CAG tracts has been found to be significantly different between fertile and infertile patients (Ferlin et al, 2004). Androgen receptor gene GGN and CAG polymorphisms have been found to be correlated to infertility in severely oligozoospermic and azoospermic Swedish men (Ruhayel *et al.*, 2004). CAG repeat length is shorter in black African men, but its relationship to spermatogenesis was first reported in our previous study (Akinloye et al, 2009).

Although, our study, did not found direct relationship between CAG and GGN male infertility, we observed a longer CAG repeats length in our infertile patients with (GGN)n 22 alleles. The pharmacophysiologic implication of this to male fertility needs to be elucidated. Data from this study shows an averagely equal distribution of CAG repeats in fertile and infertile Nigeria population within normal range (19-21), implying that CAG repeats polymorphism may not be a critical index of male infertility in Nigeria. However, we have more fertile population with lower CAG repeats (39%; <19) although, not significantly different from controls. Lower CAG repeats have been implicated in high risk of prostate cancer. Our recent study on prostate cancer in Nigeria population shows a significantly lower repeats compared with control. This scenario post a dilemma of which is a reality in Nigeria population, higher risk of prostate cancer or increased fertility potential. An extensive population screening of CAG repeats polymorphisms in Nigeria population will be helpful to answer this important question and help to understand these two common plagues in Nigeria population. Whichever, it is interesting to observed that our Oligozoospermics subject has an highest frequency of longer CAG repeats (37%; >21). Suggesting that CAG > 21 appears to be a prediction of depressed spermatogenesis rather than complete failure.

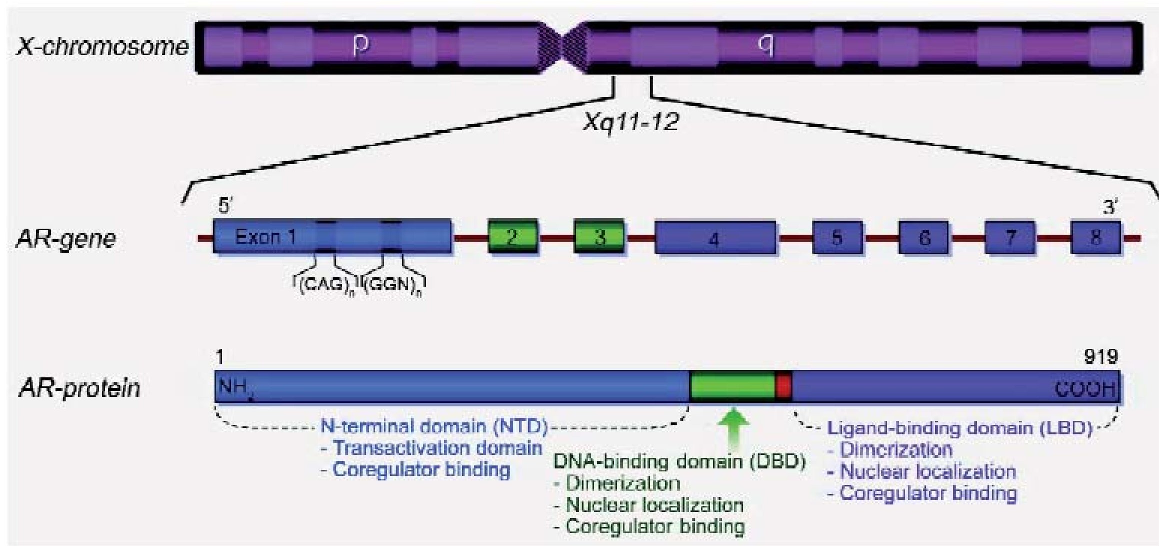

Fig. 4 Genetic organization of the *AR* gene on the X-chromosome and major functional domains of the encoded protein (Rajender et al, 2007).

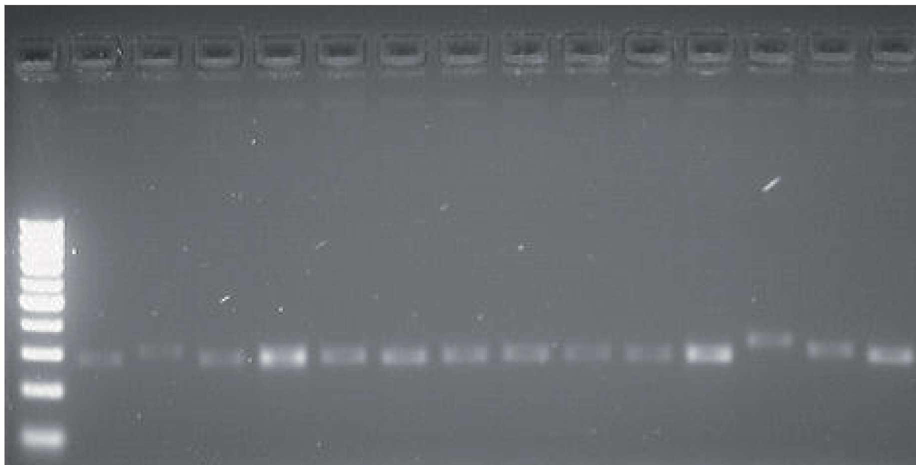

Fig. 5 Androgen Receptor - Exon 1, CAG repeat Gel.

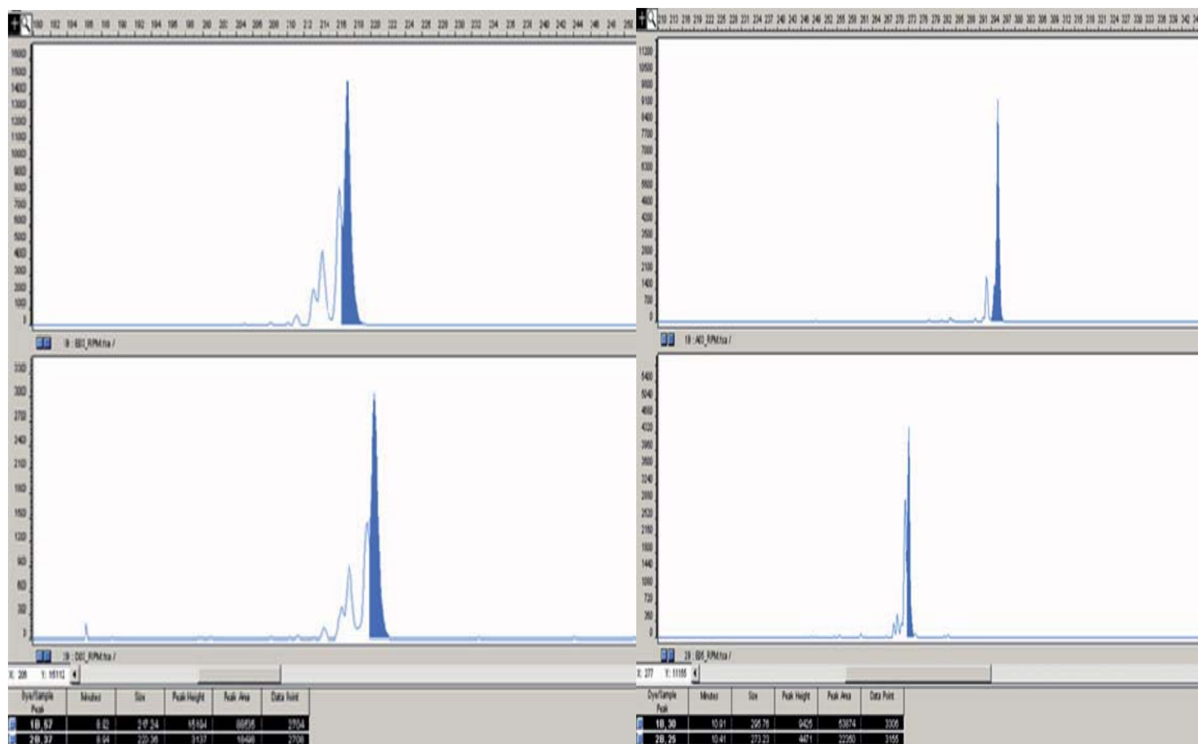

Fig. 6 GeneScan electropherograms showing GGN of 23 and 24, CAG repeat lengths of 26 and 18, respectively.

Y chromosome microdeletions in infertile Nigeria male

Studies in the mechanism underlying spermatogenesis have identified a number of genetic determinants of male infertility. One of the most important genetic factors implicated in male infertility is microdeletion within the region of the Y chromosome containing candidate gene for spermatogenesis (Huynh *et al.*, 2002). For over a decade, Y- chromosome has been the subject of interest to geneticists. Until now its role has been proven in male sex determination and development, but the functions of the remaining gene located on it is still under investigation. Studies on identification and location of sequences responsible for normal spermatogenesis have been performed since 1970s and the complete sequence of the Y chromososme is now known (Skatetsky *et al.*, 2003). Several pattern of microdeletion of the so-called azoospermia factor (AZF) region of the Y chromosome are now known (Repping *et al.*, 2002). It is now known that 10-15% of cases of azoospermia and about 5-10% of cases of severe oligospermia are caused by Y- chromosome microdeletion (Foresta *et al.*, 2002; Slezak and Sasiadek., 2002). Relevant data are however not available in Nigeria. Several studies involving infertile men with idiopathic oligozoospermia from many countries of the world established Y-chromosomal microdeletions to contribute significantly to male infertility (Simoni *et al.*, 1998; Maurer *et al.*, 2001; Foresta et al, 2002, Madgar 2002 and Lnvshyts *et al.* 2002). Therefore the screening for Y-chromosomal microdeletions has become fundamental part in the workup of infertile men as this will

determine if genetic counseling is needed prior to starting infertility treatment (Krausz *et al.*, 2000) and also improve the knowledge about nature of genes responsible for spermatogenesis thus forming a platform for future research.

More recently, partial deletions within the distal part of the AZF region, called AZFc have been identified (Repping *et al.*, 2003). These even smaller microdeletions, called gr/gr deletions, are situated at the cutting edge between a common polymorphism and a real causal factor for spermatogenetic failure. Some very recent studies suggest that gr/gr deletions are more frequently found in men with spermatogenetic failure than in men with normal spermatogenesis (Repping *et al.*, 2003; Ferlin *et al.*, 2004; deLlanos *et al.*, 2004), while others could not find this correlation (Hucklenbroich *et al.*, 2004; Machev *et al.*, 2004). It appears that gr/gr deletions are particularly frequent in certain Y-chromosomal hyplogroups and can be well compatible with normal fertility. No data on African men are available at the moment.

We screened for Y- chromosome deletions in 20 azoospermia, 40oligozoospermia and and 40 controls and found no microdeletion in any of the samples. However, we detected gr/gr deletion in one of our azoospermic subject. Ironically, same patient with the grgr deletion has SNP intron 8 nucleotide exchange in intron 8 (15061 A > G) of TAF7L gene. This patient also has a slight increase in CAG repeats length (25) and average GGN repeats (20) with hyplotype CAG24/GGN20.

CONCLUSION

Only one nucleotide variance in the flaking intron of exon 8 of TAF7L gene was observed in one Nigeria male with spermatogenetic failure. It is not know if this variance will decrease the functional annotation of the gene. However, it is known that loss of TAF7L results in reduced fertility but not sterility (Cheng et al, 2007). From our published work, we reported that TAF7L gene is a highly polymorphic gene. Most of these polymorphisms are not significantly associated with gonadal dysfunction. Although, our data showed that common sequence variants of TAF7L gene do not play a major role in spermatogenic failure, inactivating mutations might exist, not identified in our study (Akinloye et al, 2007). Also, our data show that CAG and GGN repeats polymorphisms are not critical index of male infertility. While we do not find a relationship with CAG and GGN repeats haplotypes and male infertility, we report for the first time a unique and wider distribution of GGN allele in our Nigerian population which is significantly different from Caucasian population. We observed a longer CAG repeats length in our infertile patients with (GGN)n 22 alleles. The functional advantage or relevant of this variance to male fertility warrants in-depth elucidation (Akinloye et al, 2009). The important of Y- chromosome microdeletion in male infertility have been documented in most developed world. Our study from 60 infertile and 40 controls Nigeria population did not find microdeletion in any of the subject but gr/gr deletion in one azoospermic subjects. This same subject has a slightly long CAG repeats and nucleotide variance in TAF7L gene. This suggests that multiple or combined genetic factors; gene mutation, polymorphisms and deletion may play a role in spermatogenic failure.

Male infertility is a multifactorial disease process with a number of potential contributing causes. The knowledge of the roles of genetic factors, though in small population of patients have long

been established in developed countries. Such knowledge are since been use in management and counseling of infertile males. This knowledge is still very preliminary in Africa due to lack of evidence base research. Our experiences are first line evidence of the possible contribution of genetic factors to male infertility in Nigeria. Our data shows that collective genetic factors may play important role in male infertility in Nigeria. Evidences from the studies discussed in this presentation should stimulate a more elaborative and active research in this field.

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Higher Education Institution Management and E-Learning

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ABSTRACT

Human development comes through education and higher education (HE) in particular. Higher education institutions (HEI) in the developing countries are at a crossroad. Do they join the world and employ best practices utilized the world over or remain as external departments of the government of the day in their respective countries? All the tertiary institutions in Nigeria suffer from a terrible lack of funds due to poor funding by their proprietors, improper management of available financial resources and lack of attention to the development of new resources. HEI managers in Nigeria, due either to lack of experience or time, operate within a box defined by the (lack of) policies, rules and regulations from the proprietors. These managers need assistance to learn to think beyond the box, develop innovative financial resources and think strategically and competitively about quality, efficiency and productivity. This paper examines the possible textures of collaboration between HEIs in Nigeria, Africa and globally. The specific opportunities for Humboldtians who have HEI management experience or responsibility is explored. The most efficient route back to school, for HE policy makers and managers and Humboldtians is traced to e-learning.

INTRODUCTION

Nigeria has 94 approved universities: 27 federal, 32 state and 35 private (FME, 2009). It should be noted that no notice is taken of the 33 universities identified by the National Universities Commission (NUC) as operating illegally in Nigeria. There are 86 colleges of education and 115 polytechnics/monotechnics. Then there are 62 innovative enterprise institutions. All these make up the tertiary or higher education institutions (HEI) which admit students based on their performance in the examinations of the Joint Admission and Matriculation Board (JAMB). In 2008/2009 academic session, about 1 million students applied for admission into the universities, 233,000 into polytechnics and 59,000 into colleges of education. The admission capacity in all the universities was 150,000, 160,000 in the polytechnics and 120,000 in the colleges of education. NUC has projected that the proportion of eligible candidates to be admitted will continue to fall until it reaches 2.4% in 2012. The Open University now has reached a capacity for 30,000 and is planned to expand at 8% per year. For specific professional courses like medicine, law, pharmacy, engineering, computer science, international relations, economics, etc, the chance for admission (in 2009) is less than 1 in 6. Many of these courses have subject requirements for SSS and JAMB; have widely varying cut-off marks; and different time periods to complete the academic programme. In addition, there are quotas for merit, catchment areas, educationally disadvantaged areas and discretionary admission. In short, the admission process, as well as the entire Nigerian university system (NUS), is a complex system deserving of astute management techniques which are learnt at expensive management courses. But where are the funds?

The need for capacity development in HEI management

The quality and relevance of research, teaching and services has declined in universities in Nigeria. Many universities operate with overcrowded and deteriorating physical facilities, limited and obsolete library resources, insufficient equipment and instructional materials, outdated curricula, unqualified teaching staff, poorly prepared newly admitted students, and an absence of academic rigour and systematic evaluation of performance. All the public tertiary institutions suffer from a terrible lack of funds due to poor funding by their proprietors, improper management of available financial resources and lack of attention to the development of new resources. The subvention of the federal government to the federal universities has risen from N11 billion in 1999 to over N120 billion in 2009. This low level of funding has posed a great handicap to management of the NUS. But there is another limitation to building the management capacity of HEI. There is a deficiency in quantum and quality of HEI.

While Nigeria has only 94 universities for a population of 140 million, Russia has 608, all public, Japan has 708 of which 3/4ths are private, and the United States has 4,800 of which only one – the military academy at West Point - is federal for a population of 250 million. In spite of the very low number of universities in Nigeria, there is inadequate number and quality of academic staff. Of the 50,000 academics required in the NUS, only 27,394 were available in 2006. The shortfall cannot be made up from other HEIs as they also have an even greater shortfall. In the same 2006, the polytechnics had only 12,938 academics out of the required 22,702; and the colleges of education had 11,256 out of the required 26,114. In addition to the frank shortage of all academics, there is also a shortage of senior academics. In the NUS over 60% of academic staff are in the category of Lecturer I and below while less than 40% of academic staff in the polytechnics have higher degrees. Hence, HEI managers in Nigeria are forced to operate within a box defined by (the lack of) available quantity and quality of staff and severe funds restrictions. There is absence of deliberate policy strategies and programmes for recruitment and continual training of academic and non-academic staff. These HEI managers need assistance to learn to think beyond the box, develop innovative financial resources and think strategically and competitively about the quantity and quality, efficiency and productivity of human resource.

A grave challenge facing HEI managers is in the poor standards and lack of quality assurance in their institutions. The quality challenge is often related to "instability of the academic calendar, infrastructural decay and obsolescence of equipment in the face of the population explosion and academic staff shortages" as stated above. An overlooked factor is the very low management capacity of the HEI managers themselves and the dearth of local opportunities to build their capacity. Of course , there are Masters and Doctoral programmes in business management and even in HEI management and public administration. However, existing curricula in many of these programmes are out-dated, not globally competitive nor relevant to the specific needs of the HEI. They do not cover critical and relevant aspects of science and technology and the approach is not based on solving problems.

Some existing opportunities within Nigeria and abroad

All is not lost as there are some Humboldtians with experience in HEI management – from Vice Chancellors to Departmental Coordinators. I am personally acquainted with three Vice-Chancellors, four Deputy Vice-Chancellors, two Provosts (Deans) of postgraduate colleges,

several Deans of Faculties, Heads of Departments and Directors who are Humboldtians. Indeed, there are very few successful Humboldtians who have never held some administrative positions in the NUS. The real question is: how many feel sufficiently proficient in administration to consider themselves as expert in HEI management? They are very few indeed. Ironically, the management experts of industry and commerce will be the first to disqualify themselves from managing HEI. The last option is to look abroad for help. The USA, in particular and to some extent, Great Britain, have a growth industry in the field of training which continues to be strong even in the midst of global recession. A two-weeks training in the USA for top HEI managers can easily cost up to $30,000 at the current esta code rate. Nigerian HEIs, that are so underfunded that all the staff unions have been on strike for close to 8 weeks as this paper is being written, are hardly the right candidates for such training programmes.

The management of linkages and knowledge flows

In view of this situation, the opportunity for building capacity in HEI management lies in the management of linkages and establishment of channels for the flow of knowledge from sources, wherever they may be, to the HEI managers in Nigeria. Within each institution and Nigeria, there is need to enquire about what information is available on the role and activity of the institution and the entire HEIs relating to: (a) Evaluating, acquiring and diffusing best-practices in HEI management; (b) Linking academic outputs to training and consultancy services; (c) Improving access by smaller and younger HEIs to needed training and advice; (d) Linking the institutions to programmes and activities within the country; (e) Linking the institutions to international activities; (f) The management of joint projects involving private and public sectors; (g) The management of networks or consortia for the implementation of programmes; (h) The management of joint ventures or consortia for the provision of services. E. Human Resources Development and Capacity Building in HEI management.

The information available can be enriched by attempting the following questions. Within your HEI, how available are: (a) programmes and facilities for the education and training in institutional management for all levels of personnel? (b) programmes to promote international training of personnel? (c) programmes to promote improved management? (d) mechanisms to maintain the vitality of the institutional management community? (e) mechanisms to stimulate public interest in and support of national initiatives in HEI management? Answers to these questions in the various HEIs will reveal opportunities for training-of-trainers and institutional networking across great distances – i.e. via distance learning (DL) or electronic learning (e-Learning). It will be discovered that there are valuable resource personnel scattered throughout the NUS. Bringing them together as a faculty of HEI management may not be too difficult, but taking them across the country so they can impact on the 94 Nigerian universities will be nigh impossible.

In November, 2008, after a DAAD/Humboldt Foundation-sponsored workshop on "Network Dialoguing and the Strengthening of Academic Exchange between German and Nigerian Universities" at Abuja , some Humboldtians who attended the workshop decided to float a training programme similar to the "Deans' Course" that some participants had attended while in Germany. Careful plans were made about selection of host institutions, participants, resource persons and course content. However, it has not been possible to mount this programme due to conflicts in dates, problems in resource persons' availability, budgeting challenge and lack of clear financial sponsorship. It is, therefore, proposed that the AvH-Club and DAAD alumni in

Nigeria develop training opportunities in HEI management for their members first, and others as well. The pool of resource persons, Humboldtian as well as non-Humboldtian, who is located within the NUS can be supplemented with management experts from the management consultancy industry in Nigeria. The "Faculty of HEI Management" can be strengthened by linkages with the701 Humboldtians in Africa, the Humboldt Foundation in Bonn and the capacity building initiatives in Europe, Asia and America. The thesis is that building capacity in HEI management is just as important, if not more so than building scientific and research capacity of Humboldtians who then have to suffer in institutions that are so poorly managed that the research and scientific work of the Humboldtians suffer a great deal, if they are not completely jeopardized. It is recommended that this management training be treated as an equivalent to the Mandatory Continuing Professional Education (MCPE) but the Faculty as well as the training be "virtual", "electronic" and at a distance. F. The General Need of HEI for e-Learning

In order to meet the first challenge of the FME's Roadmap for the Nigerian Education Sector (2009); i.e. the challenge of Access and Equity, there is a need for HEI managers to strengthen and expand Open and Distance Learning (ODL) systems and improve ODL programmes. To meet the second challenge of the Roadmap; i.e. that of Standards and Quality Assurance, HEI managers must strengthen "linkages with experts and academics in the Diaspora (LEAD), and resuscitate the Nigeria Expatriate Supplementation Scheme (NESS). To address the third Roadmap challenge: of Teacher Development, Motivation and Retention, HEI managers must, among other things, increase availability of e-learning resources to staff. This will support aggressive capacity-building, staff training and development and improvement of conditions of service as well as the availability of a competitive research grant scheme. Increased partnership and articulation with foreign institutions and with organized private sector will require increased ICT infrastructures and services. Indeed, to implement government directive on ICT initiatives for Tertiary 5

Institutions, HEI managers must establish Student Resource Centres and campus-wide wireless connectivity, computer acquisition scheme for staff (and students), upgrade of websites, pooling of bandwidth to drive down cost, development of video-driven lectures, and promotion of the development of instructional materials in electronic format. When all academic staff attain computer literacy, and all own their own computers, it will be much easier to strengthen and expand e-learning so as to expand access to quality education. This essentially will restructure the teaching-learning environment to become ICT-driven. The restructuring will move well when it is possible to provide alternative power supply such as solar panels to support ICT development and deployment. With this, it becomes quite possible to create virtual fora and community-based ICT facilities that strengthen accessibility to information and networking among tertiary institutions within and outside the country. The sooner this stage is achieved, the sooner will HEI managers be able to build their own capacity while ensuring that HEI students receive a world class education that is globally relevant and highly marketable.

Ethical Challenges and Conduct of Life Science Research in the Twentieth Century

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According to Belmont Report, research is any activity that is designed to test a hypothesis, permit conclusions to be drawn, and there from develop generalizable knowledge. It is a systematic process of search to produce new knowledge or authenticate existing information. The generated knowledge is eventually converted to breakthroughs which ultimately impact on developments and societal benefits. The past two centuries have witnessed a plethora of research activities in many parts of the world. This blossoming of the research enterprise has been witnessed in all branches of human learning including engineering, the life sciences, social sciences and the humanities.

The life sciences consist of a vast array of scientific disciplines that involve the study of man and other living things within his environment. Specifically and for the purpose of this discussion, they are areas of learning and research that include but not limited to medicine, biology, biochemistry, molecular biology and biotechnology. Life science research is the lifeline of breakthroughs and advancements in medicine. The depth and magnitude of such research have increased tremendously in the past century in many nations including the developing world. More recently, there has been an explosion in genomics research as a result of improvements in cutting edge scientific tools and the expanded knowledge in molecular biology and genomic technology.

All aspects of human life are touched directly or indirectly by research. The progress that human medicine has witnessed today in all its branches came about as a result of questions being asked and answers being sought through research activities. However this progress has not been without cost, both to the system and to research participants. The basis of biomedical research is the generation of new knowledge and its application in the care of present and future patients. In spite of this altruistic foundation, the history of medical research is replete with episodes of harms that were suffered by human participants directly or indirectly as a result of their participation in the research process. This knowledge, and the increasing necessity to respect human values and wishes in research, make the conduct of present and future biomedical research subject to ethical scrutiny in addition to scientific justification.

Ethical issues in life science research

In the world today, the need to conduct life sciences research in conformity with existing international and local ethical guidelines engages the attention of researchers, funders, government regulatory agencies, medical humanists, research participants, the lay public and others. This came about partly as a fall out from the scandals that have been recorded in the history of biomedical research in the past. Moreover, new frontiers of research and medical practices such as clinical trials, tissue banking, genetic typing and molecular biotechnology,

among others create diverse moral and ethical concerns. These concerns and similar moral issues arising from medicine and biomedical sciences and research are addressed in a new body of knowledge called *bioethics*. One of the goals of bioethics is to increase our awareness of the moral dimensions of health care and biological research and to help us in incorporating those ideals into everyday practice (1).

Research Ethics

Research ethics is a subset of applied ethics (bioethics) and is concerned with responsible conduct of biomedical research in conformity with standard norms and practices. These norms were written partly in response to unpleasant events in the history of biomedical research and have developed into codes and guidelines that are now widely accepted and applicable in many national and international jurisdictions. Asides from national codes that are enforceable within countries where they exist, those with international relevance are especially applicable to international research. Ethical issues in conduct of life sciences research revolve around the choice and use of humans as research participants, conduct of research to follow standard scientific protocols and processes and protection of vulnerable research participants from exploitation and harms. It is also concerned with ensuring accurate and objective analysis, interpretation and dissemination of research findings.

Ethical challenges in life science research in developing countries

Life science research in developing countries faces diverse ethical challenges. These challenges occur at different levels including the selection and involvement of research participants, the conduct of the research process and the handling of research products. The most important resources of human subjects' research are human beings whose interests form the basic driving force behind all such endeavours. The indirect use of human biological specimens in research creates the potential for ethical concerns as much as the direct involvement of human persons. Moreover, the humane use laboratory animals in biomedical research engages the fancy of ethicists and animal rights groups. The conduct of biomedical research in poor resource settings, whether basic or translational, descriptive clinical observations or clinical trials, has raised many ethical conundrums which continue to be discussed in many academic fora and scientific publications. According to Perkins, the mission of research is the acquisition of knowledge and this knowledge must be disseminated for it to be of any good to the public (2). Data and material acquisition, ownership of research information and materials, data analysis and interpretation, information dissemination in scientific meetings and through journal publications - all raise ethical issues which become profound given the rather inadequate regulatory processes in many developing countries.

There are myriads of ethical issues that emanate from life sciences research in developing countries. Though most of these challenges cut across all types of research, the magnitude may vary from one type to another. For example, clinician scientists who are recruiting patients into clinical trials among groups of patients face some ethical concerns that may be different in nature and magnitude compared to those that non-clinicians or clinicians who work on biological samples encounter. The examples in the following paragraphs illustrate some of those ethical issues that are germane for consideration among non-clinical biomedical researchers.

Funding of Research

A recent report in the Lancet showed that except for sub-Saharan Africa, many developing countries of the world now devote more funding to health unlike in the past (3). For a long time, poor funding from indigenous governments has been a major clog in the wheel of progress of biomedical research in many African countries. In-country spending on health research by government, pharmaceutical industries and philanthropic sponsors in Nigeria has been very minimal. Most funding comes from external agencies or through collaborative research with colleagues from the developed world. The ethical challenge in depending solely on external funding sources includes the fact that the motives of external sponsors and local collaborators for conducting a particular research may not be congruent. There may be little or no applicability of research benefits to the country or local community (4, 5). Moreover, both the local researcher and his/her institution may be vulnerable to funding pressures and such imbalance may unwittingly diminish their bargaining power.

International Collaborations

North-South and South-South collaborations in research should result in strengthening the scientific capacity and creating sustainable science infrastructure and technology in the South (6). Apart from technology transfer, such collaborations also encourage cross fertilization of ideas, enrich interdisciplinary discourse and provide mutual academic and professional development for the collaborating parties. In the recent past, many clinical trials of the West are being "outsourced" into developing countries. It is estimated that about half of clinical trials in the world take place in the developing countries where trials are cheaper to conduct, participants are readily available and easier to recruit and research regulatory processes are less stringent. Many ethical issues present in the setting of collaborative research. First, the collaborators are from different ethical and cultural settings and standards. While ethical standards may be similar, the emphasis and focus may differ. The differences in culture and ethical emphasis may affect the initiation, progress and perhaps the outcome of research. Another potential challenge in collaborative work is the issue of data management, manuscript development and authorship. Conflicts can arise if there is no explicit documentation about access to data and assignment of responsibilities for joint manuscript development and authorship at the onset. Some elements of collaboration which need to be formalized in written agreement at the beginning of joint research include line of communication, responsibilities of each party, accountability, authorship, data sharing, custody and ownership (7)

Ownership of tissue samples

One potentially contentious issue in research, especially in both national (inter-departmental or inter-institutional) and international collaborations involves tissue collection and ownership. Akin to this is the development of innovations or new findings that lead to intellectual property rights where applicable. Most young and inexperienced researchers or faculty staff in the developing world may not scrutinize and understand the terms of agreement before signing on for research partnership. They are more likely to fall for the dashing prospects of opportunities to do research (any research), be a co-author on papers with colleagues from outside their domain, and travel abroad for research related activities. Apart from the institutional oversight function of guiding and protecting local researchers, the ethics review committees have the responsibility to educate and midwife such contracts as contained in materials transfer agreements (MTAs) and

clinical trial agreements (CTAs). According to the regulations of the National Research Ethics Committee (NREC) of Nigeria, each local Human Research Ethics Committee should "protect researchers from exploitation regarding ownership of and rights of access to data, resources, intellectual property and infrastructure generated in the course of the research" and also provide "oversight and review MTAs and CTAs" (8).

Regulatory oversight of research

In this context, regulatory oversight of research falls into two categories, scientific and ethical. Scientific oversight is provided by the scientific committee which is made up of colleagues and contemporaries, usually within the establishment, who perform peer review of the science of the research protocol. Thereafter, the protocol is sent to the institution's ethics committee that looks into the ethical issues involved in the research. Research ethics committees in many jurisdictions perform both functions. However, in many developing countries, the capacity for ethical regulatory oversight is either lacking or still very rudimentary. Many institutions do not have properly constituted committees with trained ethicists. In some, the expertise to review some complex protocols that involve highly invasive procedures or those with far reaching family or community implications is grossly inadequate. In addition, in many places, the protective role of the ethics committee over the local researcher is obviously underperformed. The good news is that in Nigeria, research ethics awareness is on the increase and research ethics committees are springing up where none existed before. This is because of the increasing recognition of the necessity of this committee locally and partly in fulfillment of requirement for receiving funding for collaborative research from international agencies like the National Institute of Health of the US. Furthermore, there is a functional NREC of Nigeria which oversees the establishment, training, functioning and administration of institutional research ethics committees in Nigeria.

Research integrity

Integrity in research involves the researcher's commitment to intellectual honesty and personal responsibility. It also includes the institution's responsibility to provide an environment for researchers to embrace standards of excellence, trustworthiness and lawfulness in conducting research (7, 9). The basic principle that underlies research is *trust*. The society finances research, directly or indirectly, and reposes trust in the scientist. It is expected that researchers propose, carry out, interpret and report research findings with honesty, accuracy, efficiency and objectivity (7). Anything short of these calls to question the character of the researcher. Albert Einstein once said, "most people say that it is the intellect that makes a great scientist. They are wrong; it is character" (10). Responsible conduct of research requires that each scientist demonstrate a high level of integrity in all aspects of research activities, in protecting the rights and dignity of human participants, in giving due credit to others where necessary, in avoiding all activities that constitute scientific fraud and misconduct, and in complying with local and international guidelines. Integrity in research is important to the scientist, the institution he/she represents, the public and the overall scientific enterprise.

Conclusion

There has been an increase in the quantity and quality of research that is taking place in many developing countries including Nigeria. Life science research is a necessary tool for advancing the public good. The improvement in research infrastructure and the increase in research

activities in developing countries raise many ethical concerns and challenges. Research ethics addresses these issues, some of which have been highlighted in this discussion. It is also concerned with responsible conduct of biomedical research in conformity with standard local norms and international standards. Individual researcher should task himself/herself to upholding high ethical standards and principles in spite of the social, cultural, economic, and political milieu in which he/she is working. According to Steneck, "responsible conduct in research is simply good citizenship applied to professional life" (11). In addition to academic qualifications and scientific expertise, researchers in the developing world should enhance their skills and output by availing themselves of the opportunities that are available locally and online to acquire requisite ethical training.

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