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EDITORIAL
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INTERNAL AND EXTERNAL QUALITY CONTROL IN THE MEDICAL MICROBIOLOGY LABORATORY

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ABSTRACT

Culture media play a very important role in bacteriology as they are used in the isolation, identification and antimicrobial susceptibility testing. It is essential that the quality of media be safeguarded to have a successful microbiology laboratory. Microorganisms usually show typical morphological appearance and properties on solid media. Variations in the composition of the medium may alter this appearance and properties. There is therefore a need to ensure good quality media, which is capable of giving satisfactory results by ensuring a proper quality management system. Often times, majority of laboratories prepare their media for routine diagnostics and research purposes. Therefore, it is essential that certain parameters of media are checked thoroughly before they are considered suitable for laboratory use. Control methods are discussed in details in this report.

CONTRÔLE DE QUALITÉ INTERNE ET EXTERNE DANS LE LABORATOIRE DE MICROBIOLOGIE MÉDICALE

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ABSTRAIT

Les milieux de culture jouent un rôle très important en bactériologie, car ils sont utilisés dans les tests d'isolement, d'identification et de sensibilité aux antimicrobiens. Il est essentiel que la qualité des médias soit garantie pour avoir un laboratoire de microbiologie performant. Les micro-organismes présentent généralement une apparence morphologique et des propriétés typiques sur des milieux solides. Des variations dans la composition du support peuvent altérer cet aspect et ces propriétés. Il est donc nécessaire de garantir des supports de qualité, capables de donner des résultats satisfaisants en assurant un système de gestion de la qualité approprié. Souvent, la majorité des laboratoires préparent leurs médias à des fins de diagnostic et de recherche de routine. Par conséquent, il est essentiel que certains paramètres des supports soient soigneusement vérifiés avant de pouvoir être considérés comme pouvant être utilisés en laboratoire. Les méthodes de contrôle sont discutées en détail dans ce rapport.

BACTERIOLOGY LABORATORY

Source of Media

In the past, bacteriologic media were prepared from the basic chemical ingredients but this is no longer a common practice as many laboratories no longer do this. Rather, commercially-prepared dehydrated media which requires only the addition of water in for its reconstitution and use are now routinely used by most laboratories. Hence, the responsibility of quality control is that of the manufacturer however the quality of the media must be tested after its preparation due to changes which can occur following reconstitution and sterilization(1). For these dehydrated media, quantities ordered should be used up within 6 months or at most one year. It should be stored in a cool, dark, well ventilated place with the caps of all containers sealed tightly as dehydrated media are hygroscopic. Date of receipt and opening of media should be recorded. Darkened or caked dehydrated media must be discarded (2).

Often times, certain additives such as blood, serum, growth factors, specific vitamins, antibiotics are used in the preparation of media especially for fastidious organisms. The quality of blood used in the preparation of blood-containing media is essential to the performance of the media. The sterility, viscosity, homogeneity and colour of the blood should be carefully checked prior to its use for media preparation (3). In the case of other additives, the certificate of analysis and sterility conditions should be considered prior to usage while heat labile additives whose sterility is uncertain can be sterilized by membrane filtration (4). Commercially-prepared, ready to use media are also available and utilized by some laboratories. The quality control maintenance of such media is the responsibility of the manufacturer. A written assurance must be supplied by the manufacturer stating that Clinical and Laboratory Standards Institute (CLSI) standards were followed and this verification must be maintained along with the QC protocol of the laboratory for as long as the laboratory uses the specified media.4 In addition, a certificate of analysis stating the expiration date, storage conditions should accompany all commercially-prepared, ready to use media along with quality control organisms used in growth supporting ability and selectivity testing (5). However, the laboratories also need to inspect each shipment of media for its properties (1,4).

Quality and Quantity of Water

The quality of any media depends directly on the quality of raw materials used in its preparation with water being the most important raw material (6). Tap water should not be used in media preparation due to the presence of impurities rather distilled and/or deionized water should be used.1 The copper content, pH and conductivity of the water should be checked prior to its use in the preparation of culture media. Ideally, water used should have no copper in it because of its inhibitory effect on microorganisms. The pH of the water should be slightly acidic but not less than 5.5 while the conductivity should not be less than 15 µS (microsiemens) (3). The amount of water added while reconstituting media should be carefully measured in order to have adequate gel strength (1,3).

Weighing

Weighing balances used in the measurement of dry materials must be accurate as weighing errors significantly alter the composition of the final product (1).

Quality of Glassware

Glassware used in media preparation should be made of borosilicate glassware, as glassware made of soda can leach alkali into the media resulting in an alteration of the pH of the media.3 Glassware used should be carefully cleaned as residues on glass may be inhibitory in some fastidious organisms (1).

Quality of Sterilization

The process of sterilization also plays an important role in the quality of culture media. Generally, culture media is sterilized by autoclaving. But, certain parameters must be met in order to achieve adequate sterilization. These include the autoclaving time, temperature and pressure as well as close regulation of the quantity of media sterilized. Heat treatment of complex culture media may result in destruction of nutrients present in the media either by direct thermal destruction or reaction between its components. Sterilization of media at too high a temperature or for too long or both may result in the deterioration of some constituents of the media thereby rendering it unsuitable for use (1). Hence, it is necessary to minimize the heating damages by optimizing the heating process. The autoclave time to achieve sterility at a temperature of 121℃ (250°F) is 15 minutes while the autoclave pressure is 15 psi (100KPa).4 The volume of media in a sterilization batch should be small, ideally 2 Litres (3).

Regular checking of the sterilization process by the use of indicators is essential; the temperature and pressure should also be monitored constantly. Sterilization indicators are used to check the efficacy of the sterilization process. Biological sterilization indicators such as the spores of *Bacillus stearothermophilus* can be used to check the spore killing efficacy while the chemical indicator such as Bowie Dick test can be used to check the efficiency of the sterilization process (3).

The physical appearance of the media should be inspected after sterilization as there might be alteration in its appearance. The following may be observed: turbidity or a precipitate indicating that some constituent has come out of the solution; darker than normal colour indicating overcooking of sugar containing media, incorrect mixture of constituents or wrong pH; lighter than normal colour indicating incorrect mixture of constituents or wrong pH (1).

Dispensing

The quality of the Petri dish used in the preparation of the media is also important. Petri dishes are normally sterilized with ethylene oxide (Eto) or gamma irradiation. Eto sterilized Petri dishes should be checked for residual Eto toxicity which may inhibit the growth of microorganisms. The maximum

permissible limit for residual Eto is 1 µg/g. Residual Eto can be measured by standard gas chromatographic method (3). Aseptic technique is essential while dispensing media and accurate volume should be dispensed. About 25-30 ml of media should be dispensed into a 100 mm Petri dish. Inaccurate measurement of the amount of media may result in too shallow or too deep media rendering it unsuitable for use(1). Media should be dispensed into Petri dishes on a flat, level surface in order to have equal filling.

Physical Parameters of the Media

The gross physical appearance of media is an indicator of its quality. All media, either user-prepared or commercially-prepared should be screened for physical parameters such as colour, consistency, depth, smoothness, excessive bubbles or pits, unequal filling of Petri dishes, cracked medium in plate, clarity, freezing(seen as a crystalline pattern on the surface of the medium), presence of leakage and visible contamination. All these are checked visually by the naked eye. However, depth of the medium and unequal filling of Petri dishes can be checked at four points. The four points are the two ends of the two diameters of the plate which are at right angles to each other. The depths at the four points are noted, the mean thickness is calculated and reported as the mean thickness of the medium in the plate which must be 4.0±0.2 mm.3 Blood based media should be checked for signs of haemolysis. There should be no deviation from the normal colour of the media. It should be free of moisture but should have no signs of drying at the edges, Petri dishes should have not be broken or cracked.7 The pH of the medium must be checked while preparing the medium before and after autoclaving using a standard pH meter after calibration with standard buffers (3).

The gel strength which is an indication of the level of solidification of the agar within the medium is also measured. This is done with the aid of a tripod stand with a central rod that is used to impart pressure on the agar. The lower end of the rod has a spherical portion which rests on the surface of the medium while the upper end of the rod has a platform on which standard weights are placed. The spherical part of the central rod is placed on the medium and then weights are placed on the upper platform one after another and observed for a while. This process is continued until the agar breaks under the weight of the central rod. The gel strength can then calculated by deducting the weight of the central rod from the total weight. The force imparted by the rod on the agar surface is calculated by the formula: Wpr2 where W is the weight of on the platform, r is the radius of the spherical part of the lower end of the central rod and p is 3.14 (a constant). The acceptable gel strength is about 300-500 dynes/cm (2).

Contamination

This is a very important indicator of the media quality, it is also known as sterility testing. Each batch of media must be checked for contamination before it is declared fit for laboratory use. The common technique in sterility testing is randomly picking a small number of media (usually between 1-5% of the batch) at random and placing in an incubator at a selected temperature and time depending on the type of media though for general purpose 30-37℃ for 48-72 hours is typical. After which the plates are checked for microbial growth. If there is no growth the batch is declared fit for use, the samples used in the sterility testing should be discarded as they are unsuitable for inoculation due to the dehydration that occurs after up to 48 hours incubation. If there is growth, the process is repeated. If there is growth again, the batch is inferred to have been contaminated. It is recommended that a batch be discarded when there is more than 10% contamination (8). Alternatively, it has been suggested that contamination can be checked by leaving the whole batch of prepared media at room temperature for at least three days after which it is examined for visible growth (3).

Growth Supporting Ability

Growth supporting ability is the most important parameter when conducting quality control of a media. In order to demonstrate the growth promoting ability of media, a panel of microorganisms is required. Preferably, the recommended microorganisms should be traceable to a reputable culture collection such as the American Type Culture Collection. The media should be inoculated with overnight cultures of pure, well defined and appropriate test strains of the organism (9). When testing new lots of media, the results should be examined both qualitatively and quantitatively while using standard inoculating procedures. The previous batch and a new batch should be simultaneously inoculated. Results of previous batches of the same media should be compared with that of a new batch. During testing, some priorities must be established such as beginning the testing with media that are most likely to demonstrate deficiencies. Top priority should be accorded to blood, chocolate and Thayer Martin agars and secondary priority should be given to selective enteric media such as MacConkey, EMB, XLD and bile salt containing agars (1). Testing of selective media should include inoculation of organisms supported by the media and organisms inhibited by the media in order to demonstrate its

selective nature. To demonstrate the inhibitory effect, the medium can be challenged with a heavy inoculum of the organism, since if the medium can inhibit the growth of a large inoculums it will also prevent the growth of the small number of organisms present in specimens.1 When testing media used to identify a biochemical response such as hydrogen sulphide production or fermentation, species or strains of organisms which will produce the desired reaction should be used (1). All laboratory prepared media must be tested before use, using organisms expected to grow or not to grow or organisms expected to give a positive reaction or negative reaction. The microorganism selected should be the most fastidious for which the medium was designed. For primary plating media, testing should be done with dilute suspensions of the test organism while undiluted organisms should be used for biochemical media (7). CLSI has certain guidelines for the control organisms used for every medium, the inoculum concentration desired and the growth results expected. Table 1 shows some suggested control organisms, incubation conditions for some bacteriological media with their expected results.

For agar-based media, the qualitative, semi-quantitative or quantitative method can be used in testing for growth supporting9. Examples of the qualitative method is the spread plate, for the semiquantitative method econometric and spiral plate while that of the quantitative are pour plate, spread plate and the modified Miles-Misra also known as the drop count technique (1,10). Quantitative assessment is more useful in testing the performance of selective or inhibitory media such as Thayer Martin agar (1).

Quantitative assessment is more difficult to apply in liquid media, hence it is done using endpoint determinations and kinetic parameters. The end point determination focuses on the final result of growth which is seen as an increase in biomass. This can be indirectly determined by measuring the turbidity, changes in electrical properties such as the conductance of the broth, estimating the viable numbers within the broth, production of visible metabolic products such as gas, or reactive compounds such as indole.9,10 Examples of methods which utilize endpoint determinations include copious growth method, end point method and most probable number. Kinetic parameters involves comparison of the growth supporting ability of two batches of broth can be done by measuring the growth curves of identical inocula grown side-by-side. The growth rate of the challenge organism is then determined either by viable count or spectrophotometrically as a means of comparing the nutritive properties of the media. Alternatively, comparison can be made using the length of lag phases of the same inoculums on the test and control broths. This method is extremely labour intensive, subject to variability and difficult to implement (10).

Media Storage and Expiry

Media that are yet to be declared fit for use should be quarantined, tagged unqualified and ideally placed in a storage room separate from that where qualified media is stored. An alternative is tagging and placing them in a clearly identified area within the same room. All QC checks on the quarantined media must be completed before its release for use. Storage conditions of quarantined media should be similar to that of qualified media (10).

Prepared media should be stored away from sunlight and heat. Media containing blood, antibiotics and other organic additives should be stored in the refrigerator. When stored in a cool dry place, the shelf life of prepared media depends on the type of container used. The shelf lives typically are: 2 weeks for media in tubes with loose caps, 3 weeks for media in tubes with cotton wool plugs, 4 weeks for media in Petri dishes (if sealed in plastic bags) and 3 months for media in containers with screw-caps (2).

TABLE 1: PERFORMANCE TESTS ON COMMONLY USED MEDIA

Medium	Control Organism	Incubation condition and duration	Expected Result
Chocolate agar	*H. influenza*	**CO2, 24 hours**	**Growth**
Blood agar	*S. pyogenes*	**CO2, 24 hours**	**Growth and β-haemolysis**
	S. pneumonia	**CO2, 24 hours**	**Growth and α-haemolysis**
***Salmonella-Shigella* agar or Deoxycholate citrate agar**	*E. coli*	**24 hours**	**No growth**
	S. **Typhimurium**	**24 hours**	**Colourless colonies**
Selenite broth	*S.* **Typhimurium**	**24 hours**	**Growth after subculture**
	E. coli	**24 hours**	**No growth after subculture**
Bile-aesculin agar	*E. faecalis*	**24 hours**	**Growth and blackening**
	S. pyogenes	**24 hours**	**No growth**

Courtesy: WHO, Basic Laboratory Procedure in Clinical Bacteriology. 1991

Documentation of the performance and sterility of all prepared media must be done and records must be maintained for 2 years (2). For user prepared media, QC forms should contain the quantity prepared, source of the ingredient, lot number, sterilization date, preparation date, expiration date and the name of the person who prepared the media (4).

For commercially prepared, ready-to-use media, sample of the lot should be tested for sterility, usually 5% of any lot is tested in a batch of 100 units or less while a maximum of 10 units are tested in larger batches. Any one shipment of a product with the same lot number is considered a batch, it is considered a different batch if a separate shipment with the same lot number of a product is received and should be tested separately (4). Following the collection of data over several years by the CLSI Subcommittee on media quality control regarding the incidence of QC failures of commonly used microbiology media, a list of media which did not require retesting if purchased from a manufacturer who follows the CLSI guidelines. However, a written assurance stating that CLSI standards were followed must be supplied by the manufacturer and this verification must be maintained in the laboratory along with its QC protocol. This written assurance must be retained for as long as the laboratory uses the specified media. Some media however need to be retested by the laboratory due to the complexity or failure rate history of the media. Examples include chocolate agar and selective media for pathogenic *Neisseria* and *Campylobacter.* Each shipment must also be inspected for cracked media, unequal filling, excessive bubbles, clarity, freezing(seen as a crystalline pattern on the surface of the medium), presence of leakage and visible contamination.4 Blood based media should be checked for signs of haemolysis. There should be no deviation from the normal colour of the media. It should be free of moisture but should have no signs of drying at the edges, Petri dishes should have not be broken or cracked (7). The results of media observations should be recorded along with their lot numbers. When a medium does not meet the standards, corrective action must be taken and this should be documented on a separate record called media failures log.

In antimicrobial susceptibility testing, variability in the content of the media can affect the accuracy of the results obtained. Mueller-Hinton agar will be used in this article as it is best media for routine antimicrobial susceptibility testing of non-fastidious bacteria. Fastidious bacteria such as *Haemphilus influenza, Neisseria gonorrhoeae, Neisseria meningitides* and *Stroptococcus pneumoniae* do not grow well on un-supplemented Mueller-Hinton agar hence they require supplements or different media for their growth. Quality control of a batch or lot of Mueller Hinton is done by testing each batch or lot of agar plates or disks with the appropriate quality control strains to determine if the sizes of the zones of inhibition obtained within the batch fall within the expected range. The acceptable zone diameter quality control limits for a single quality control test (single test/single-organism combination) are listed in CLSI M100-S22, Tables 3A and 3B. For non-fastidious bacteria, significant variations might exist in the results obtained with some batches of Mueller-Hinton agar due to its inability to support adequate growth of the test organism. Factors which can result in such variations include:

1) **Cation content:** increase in Ca2+ and Mg2+ contents cause a false resistance of *P. aeruginosa* to the aminoglycosides and a false resistance of microorganisms to tetracyclines and vice versa. Ca2+ and/ or Mg2+ content can be verified by performing antimicrobial susceptibility test on *Pseudomonas aeruginosa* ATCC 27853 and obtaining correct zone diameters with the aminoglycosides. Variation in the Ca2+ also affects the results of daptomycin as low calcium content causes a false resistance to daptomycin and vice versa. The recommended Ca2+ content in Mueller-Hinton agar for optimal daptomycin activity is 50 mg/L which is similar to the physiological levels. Excess zinc ions in the medium may reduce the sizes of the zones of inhibition of carbapenems (11).
2) **pH:** The acceptable pH range of Mueller-Hinton agar is 7.2-7.4 at room temperature. The pH of each batch of the medium should be checked before and after gelling. A reduction in the pH of the media gives a false resistance of microorganisms to aminoglycosides, clindamycin, macrolides, quinolones and a false susceptibility of microorganisms to the penicillins and tetracyclines and vice versa. The pH of the media can be checked by macerating enough agar to submerge the tip of a pH electrode, by allowing a small amount of agar to solidify around the tip of a pH electrode in a beaker or by the use of a surface electrode. Incubation of the medium in CO2 should be avoided as it can result in a decrease in the pH (11).
3) **Thymidine and thymine content:** The thymidine content in Mueller-Hinton agar

should be as low as possible. Mueller-Hinton agar with excessive thymidine or thymine content can reverse the inhibitory effect of sulfonamides and trimethoprim thereby causing a false resistance to sulfonamides, trimethoprim, and trimethoprim-sulfamethoxazole. Evaluation of the thymidine or thymine content in Mueller-Hinton agar can be done by testing trimethoprim-sulfamethoxazole disks against *Enterococcus faecalis* ATCC 29212 or *E. faecalis* ATCC 33186. Clear, distinct zones of inhibition ≥ 20 mm is seen in a satisfactory medium while unsatisfactory medium will have no zone of inhibition, growth within the zone of inhibition or a zone of inhibition< 20 mm (11).

4) **Moisture (when using Bauer-Kirby test):** The surface of the medium should be moist but droplets of moisture should not be apparent on the surface of the medium or on the Petri dish covers during inoculation of the plates. Plates with excess moisture should be placed in an incubator at 35℃ or in a laminar flow hood at room temperature with lids ajar until excess moisture is removed by evaporation. This usually takes about 10 to 30 minutes (11,12).
5) **Agar depth (when using Bauer-Kirby test):** the media should have a uniform depth of about 4 mm. This corresponds to 60-70ml of medium for 150 mm diameter plates and 25 to 30 ml for 100 mm diameter plates (11,12).

QUALITY CONTROL OF VIROLOGICAL MEDIA

Isolation of viruses is still the gold standard in the diagnosis of infections due to viruses however, not all viruses are culturable. Viral cultures are expensive not only in terms of media, reagents and disposables but also in the time taken to propagate and prepare cell lines, extract the virus from the sample, apply the viral extract to the cell line and diagnose the outcome of the assay. Hence, it is essential to maintain strict quality control in order to maximize the performance of the cell line and limit negative effects of variables which can directly or indirectly affect the assay outcome. There are three methods used in the isolation of viruses in diagnostic virology namely: cell culture, animal inoculation and embryonated eggs. The most commonly used method in clinical virology is cell culture as animal inoculation is extremely costly and cumbersome while embryonated eggs are too expensive and inconvenient for use in the routine diagnostic laboratory though they are used for vaccine production (13). Cell culture is technically the culture of cells *in vitro.* Tissue or organ culture denotes the growth of tissues or organ is preserved. Clinical virologists usually use these terms interchangeably but cell culture is technically more correct. Previously, a lot of diagnostic virology laboratories initiated and propagated their own cell lines but now there are several commercially prepared cell lines which are more convenient and are commonly purchased. Quality is important in all aspects of cell culture as the quality of materials i.e. cell lines, media and other reagents affects the quality of the cultures and products derived from them. Hence, the main areas of concern in quality control of cell culture are: quality of reagents and material; provenance and integrity of the cell lines and avoidance of microbial contamination. The work/laboratory environment, equipment and personnel also affects the quality of virological media produced hence a brief mention on factors peculiar to the virology laboratory will be mentioned.

Facility Design

The virology laboratory space must be physically separate from the microbiology laboratory space with dedicated equipments and biosafety cabinets (minimum of level 2). Temperature must be maintained at 22-26℃ and relative humidity of 30-50%. There should be dedicated areas for reagent preparation, sample preparation and analysis. There should be dedicated hoods/biosafety cabinets for the preparation of cell lines, sample processing during viral analysis by tissue culture (14). Work surface areas must be made of material allowing regular decontamination. Waste should be segregated properly and disposed by following standard hospital infection control policies (15).

Reagents, Supplies and Equipment

Supplies of items such as glassware, plastic ware, reagents, commercially prepared culture media and laboratory equipment should be of high quality because they are crucial to the quality of results obtained. There should be consistent supply of all items and technological support of equipment. Quantity, lot number, source and date of receipt should be entered into a log book upon receipt. All supplies and consumables should be stored in appropriate conditions specified by the manufacturer.14 The reagents and materials are potential sources of contamination hence materials of good quality free from microbial contamination and breakage should be used. Materials and reagents for cell culture can be obtained from manufacturers who carry out a range of quality control tests screening for microbial contamination such as *Mycoplasma* and bovine viral diarrhoea virus (BVDV) (16).

Sterility testing should be carried out on cell culture flasks prior to their use by using a nonselective, antimicrobial free cell culture media. For prepared media, pyrogen-free water should be used, preferably Type 1 reagent grade water and this must be used as soon as it is produced because of leaching of metals or organic contaminants from the storage container into the water, bacterial contamination as well as a decrease in the resistivity which will occur (16).

Sanitization of pH probes, hoses, pumps and filter housings should be done using 0.525% solution of sodium hypochlorite which must be followed by neutralization using 1M solution of sodium thiosulfate. Fine instruments should be frequently serviced and calibrated by accredited service agencies (16).

Media: Every lot of culture media and supplements must be tested to confirm the support of the growth of the cell line and propagation of viruses are not affected negatively in the various assay formats. Media supplements such as serum or semi-synthethic serum surrogates which can affect the impact of cell growth and virus propagation must be evaluated prior to usage. A new lot of serum should be tested with all cell lines to evaluate the overall growth characteristics. Each lot of serum can be spiked with the various virus stock cultures in order to ensure it is not inhibitory to the virus infection, multiplication and it allows for production of sufficient virus titer. This evaluation should be done well in advance of exhaustion of the current lot of supplements and media. The entire process might take 4-6 weeks and consume laboratory resources hence sufficient time and careful planning is needed (14).

There may be significant variations in the sensitivity to virus isolation and this may depend on the cell sub-line or clone and the number of times passaged. Hence, information on a particular cell line including the source, type, number of times passaged, confluency and cell condition should be recorded. There should be provisions for back-up of routinely passaged cells in case of contamination or laboratory accident in order to avoid severe disruption of workflow. This back up can be done by freezing and storage of low passage cells at -70°C, use of aired stock flasks or carrying of a parallel set of stock flasks using a separate set of tissue culture reagents and glassware.

Most reagents and media used in cell culture can be sterilized either by autoclaving (for heat stable components e.g. water, salt solutions, amino acid hydrolysates) or membrane filtration (for heat labile components). The appropriate type of filter membrane should be selected for use. Cellulose acetate membranes are used for applications involving low protein binding while cellulose nitrate membranes are used for general purpose filtration. The pore size of the membrane should be 0.22µM in order to achieve sterilization. Thick cotton membranes or pads which have larger pore sizes can be used for pre-filtering.16 Sterility testing of the media should be done after filter sterilization. Aliquots of the media are obtained and examined for bacterial and fungal contamination. This should be done daily for 5 days and free from contamination before the lot is declared fit for use. Aliquots of all media components and supplements such as foetal calf serum and L-glutamine should also be checked for sterility (11).

Several organisms such as bacteria, yeasts and fungi may contaminate cell cultures. They are detected as turbidity or a change in the pH of the culture medium. However, organisms such as *Mycoplasma* and viruses do not cause turbidity or change in pH. They may or may not cause cytopathological effects (CPE) in the cell line. Thus, they are difficult to detect and may be passaged indefinitely without being detected. Cell lines which are notorious for adventitious virus contamination include primary rhesus monkey kidney cells, primary bovine kidney cells and primary African green monkey kidney cells. Hence cell lines must be tested for microbial contamination prior to use. This can be done by screening the cell lines can be screened for *Mycoplasma* contamination using DNA specific fluorescent staining and further confirmed by culture. Also available are commercial testing services for *Mycoplasma* and viruses such as MycoTect test (14).

Cell lines prepared by the laboratory should be subjected to daily growth rate and contamination checks, every month, Hoechst stain should be used to detect *Mycoplasma* contamination and the sensitivity of the cell line to viral isolation should be monitored by periodic TCID50 experiments with stocks of reference virus.15 *Mycoplasma* contamination can also result in altered growth rate, morphological changes, chromosome aberrations or alteration in amino acid and nucleic acid metabolism. Daily microscopic examination of the cultures ensures early detection of contamination and initiation of appropriate action. Close observation of the medium for colour changes is important as it indicates an increase or decrease in pH and rate of metabolism, this is essential as it determine if the cells need feeding or subculturing as epithelial cell lines such as HeLa and Hep2 have a rapid metabolism. Fibroblast cells such as MRC5 and WI-38 have a slower metabolism and therefore need feeding at least once a week and subculturing only once a week (16). Commercially prepared cells should be certified to be free from *Mycoplasma,* fungal and bacterial contamination and examined for contamination upon receipt.

Instruction sheets should accompany cells purchased from cell repositories as they contain recommendations on the appropriate medium, subculture procedures, correct seeding concentrations, feeding schedule and safety concerns. There should be daily checks of cells to ascertain growth rate and contamination. During use, cell lines should be handled separately and the cabinet decontaminated in between. Chances of contamination can be further minimized by the use of separate apparel, reagents and glassware for cell culture and exclusion of laboratory with infectious diseases from handling cell culture (14). Contaminated cell culture should be discarded upon detection and the work continued with earlier stocks known to be free of contaminants or obtain fresh stocks from a recognized source.

During cell culture, cells adherent to the cell culture flask can either be detached physically or chemically. In physical detachment, disposable cell scrapers should be used. This is done by removing the growth medium after which the cells are physically detached by scraping the cells from the surface of the flask. Chemical detachment involves the use of enzymes (such as trypsin, pronase or collagenase) and chelating agents (such as ethylene-diamine-tetra-acetic acid or versene) for the detachment process. Care must be taken when handling cell monolayers as the enzymes used in the detachment may be toxic to some cells. Some enzymes also remove important receptors and cell surface molecules which are important hence the effects on the enzymes on the chosen cell line must be known prior to its use (13).

Personnel and Environmental Monitoring

Individuals working with cell culture should wash hands upon entry into the laboratory in order to remove dry skin and loosely adherent microorganisms which are potential sources of contaminants. Surgical gloves and gowns must be worn and gloves must be frequently swabbed with 70% (v/v) sterile isopropanolol. Cabinets should be stocked with all materials needed in a clutter-free manner prior to the commencement of any procedure. All items that enter the biosafety cabinet must be sprayed with 70% (v/v) sterile isopropanolol to prevent dust and particles from entering into the cabinet. Avoid creating bubbles or generating aerosols in the medium or pipette as they can act as sources of contamination. Preferably, a pipette should be used only once and clean spills with 70% (v/v) sterile isopropanolol immediately they arise. Personnel should be with proven expertise. They should have training and experience in media and reagents preparation, preparation of dilutions, cell line manipulation, handling harvesting and distribution of cells, maintenance of cell cultures and the outcome of assays. They should be accurate in documentation. Poorly trained staff can also in core areas such as good laboratory practice and aseptic techniques are potential sources of contamination of the media especially during manipulation. They should undergo re-training. The training and experience of staff and laboratory activities documentation enhance quality of results obtained in the virology laboratory (14).

The importance of ensuring the quality of media medical microbiology and virology laboratory cannot be overemphasized as media of low quality will lead to problems with every aspect of the laboratory operation. For accurate and acceptable isolation of pathogens, QC testing of media using a standard protocol helps save time and resources. As the time and resources spent on ensuring production of quality media will be amply repaid in terms of reproducibility of data and reduction on the time spent on investigations of non-conforming results.

INTERNAL QUALITY CONTROL

The issue of quality control (QC) in the medical microbiology laboratory is complex1. Quality control is defined as all measures put in place to ensure the medical reliability of laboratory data1,2. Quality control is now recognized as being a part of a larger program referred to as 'Quality assurance' (2).

Quality control activities that take place must be recorded to prove their existence1,2. The responsibility for QC usually rest with one person, but in reality, everybody in the laboratory must participate if a program is to be successful1,2. QC programs for the medical microbiology laboratory should include procedures for control of media, temperature, reagents, equipment, susceptibility testing, and all personnel (1,2).

Temperature

Temperature checks should be done daily on all temperature dependent equipments like water bathes, incubators, refrigerators, heating blocks and freezers17,18

Thermometers in incubators and refrigerators are easier to read when they are permanently immersed in glycerol (17,18). Glycerol helps prevent temperature fluctuations that occurs when the door is opened to read the thermometer17,18. Each thermometer must be checked against a reference thermometer from the National Institute of Standards and Technology (NIST) before each use (17,18). A large batch of thermometers can be checked at the same time and at the temperature ranges likely to be used (17,18). Thermometers can also be calibrated by

batch on arrival to the laboratory and once the thermometer has passed calibration it is placed in use17,18. Repeat calibration of in-use thermometers is usually not necessary17,18. Certificates of calibration of the thermometers are kept for the life of the thermometer or until the expiration date on the certificate; after that date, the thermometer can be recalibrated or discarded17,18. For safety reasons, nonmercury thermometers are recommended17,18. Mineral spirits with nontoxic, red-dyed alcohol can be used in place of the mercury (17,18).

Equipment Quality Control

Equipment used in the clinical microbiology laboratory are usually tested for proper performance at intervals as appropriate17,18. The frequency of testing can vary from daily to yearly depending on the equipment in question17,18. A concept termed preventive maintenance is put in place to as an additional control measure17,18. It involves things like replacing filters, oiling and cleaning and instrument recalibration (17,18).

Media Quality Control

Media prepared in the medical microbiology department must be quality controlled with documentation on their performance and sterility17,18. Records of these documentation must be kept for at least 2 years17,18. Criteria used are generated by the Clinical and Laboratory Standards Institute (CLSI). Conditions such as moisture; to be sure that plates are moisture free before use and also, ensure that there are no signs of drying around the edges of the media17,18. Culture plates should be free of any form of contaminants17,18. Petri dishes should not be cracked or broken17,18. The appearance of media, blood-based plates for example, should not show signs of hemolysis, and also, other plate should not deviate from the normal color and should this happen, it should not be used (17,18).

Reagent Quality Control
Reagents like oxidase, kovacs, all stains, catalase, X and V strips, Voges-Proskauer and optochin should be checked regularly using negative and positive controls (17,18).

Antimicrobial Susceptibility Quality Control

Guidelines for the control of susceptibility testing is provided by CLSI, also provided, is a list of control strains from the American Type Culture Collection (ATCC) 17,18. The variables that need to be quality controlled to ensure release of quality reports include instrument failure, antibiotic potency, agar depth, pH, evaporation, inoculum concentration, temperature, moisture, difficulty in determining endpoints (17,18).

Personnel Competency

Proficiency testing is usually done to ascertain the level of competence of each personnel for each test he or she is expected to perform17,18. Review of work sheet, written examination and direct observation are also ways to determine personnel competence17,18. Tests done on patient samples must pass through proficiency testing at least twice a year17,18. Another form of quality control is the act of reviewing the work of a technologist by another, usually a senior technologist17,18. Encouraging this practice ensures that mistakes are easily caught prior to release of a result17,18. Documentation of test of competency for each employee must be done on employment and yearly from then on and a proof of competency must be kept in his or her personnel file(17,18).

Stock Cultures

These can be obtained from various sources including commercial sources, patient isolates, ATCC and proficiency testing isolates17,18. Stock cultures are best grown in large volume and divided into small quantities that can be stored for at least a year in order to achieve best results (17,18). This reduces the risk of organisms mutating during repeated sub-culturing. Stock cultures can also be stored using storage beads. This has the advantage of thawing a single bead and the only down side is its higher cost margin (17,18). All organisms stored in a freezer should be at -70^0C17,18. They can also be lyophilized or frozen in liquid nitrogen (17,18).

Quality Control Manual

This is a document that contains all rules and procedures for QC available in written form at the work stations for all employees (17,18). This document must be signed after review annually and subsequently revised by as supervisor as may be needed (17,18).

CONTROL OF IN-USE ANTISEPTICS

The importance of laboratory safety in the medical microbiology laboratory cannot be overemphasized19,20. The attendant risk of an individual being exposed to an infectious agent while working in the medical microbiology laboratory increases with the type of the organism, frequency of working on it and level of contact with the infectious agent19,20. This is why there must be appropriate systems in place to ensure antisepsis in th laboratory and also ensure the continuous control of these in-use antiseptics in the medical microbiology laboratory (19,20).

An antiseptics' effectiveness against the expected spectrum of pathogens is a prerequisite for an antiseptic agent (19-21).. Antiseptics used in the medical microbiology laboratory includes alcohols and iodine (19,20).

Ethyl alcohol and isopropyl alcohol are the two most effective alcohols used19-21. A solution of an alcohol that is to be used for antiseptic purposes is usually filtered through a 0.22-µm filter to remove any spores that may be present (19-21). Alcohols are not sporicidal, so, a sterile cotton ball should be used when they are used to saturate cotton balls to be used to prepare the skin for blood collection or inoculation (19-21). Alcohols should only be used in concentrations between 60% and 90% and, they must be allowed to evaporate from the surface to which they were applied for them to be effective19-21. The presence of organic materials easily inactivates alcohols (19-21).

Iodine

Iodine tinctures are alcohol and iodine solutions, used mainly as antiseptics and povidone-iodine is the best known iodophor used in the medical microbiology laboratory19,20. Povidone-iodine provides a slow and continuous release of free iodine19,20. They are commonly used as skin preparations from sites where blood is to be drawn19,20. It is important that there is proper amount of contact time when using it as it usually requires more than 30 seconds 19,20.. Iodine tinctures and iodophors must be completely removed from the skin to avoid irritation of the skin (19-21).

Quality Control Programs in the Medical Mycology Laboratory

The field of medical mycology has rapidly advanced over the last few decades, with tentacles twinning on many specialities of clinical medicine. The spectrum of diseases caused by fungi range from asymptomatic/mild mucocutaneous diseases e.g. dermatophytoses and vulvo-vaginal candidiasis, to chronic debilitating e.g. mycetoma and chronic pulmonary aspergillosis (CPA), to severe life-threatening invasive fungal disease (IFD) e.g. cryptococcal meningitis. In total, more than 1.5 million people die annually of fungal diseases worldwide22. *Aspergillus* spp., *Cryptococcus* spp., *Candida* spp., and *Pneumocystis jirovecii* are the main etiologic pathogens of IFD worldwide. Other clinically significant pathogens include the agents of mucormycosis, endemic mycoses, the dermatophytes, and other yeasts and moulds (23).

Timely and accurate diagnosis of IFD is essential but challenging because of the nonspecific clinical and radiographic findings, underlying co-morbid conditions that impedes potentially definitive diagnostic procedures24. Culture and culture-based methods of diagnosis allow species level identification and antifungal susceptibility testing, however, conventional cultures have low yield (24). Culture-independent serum antigen detection tests, such as the β-1,3-D-glucan (BDG) assay- a "panfungal" biomarker, *Aspergillus*-specific IgG – a key assay for the serological diagnosis of CPA, cryptococcal antigen (CrAg) tests – a lateral flow assay that has revolutionised the screening and diagnosis of cryptococcal disease, and galactomannan (GM) index-for the diagnosis of invasive pulmonary aspergillosis, may allow earlier diagnosis of IFD than is otherwise feasible with traditional methods (25-27).

Early initiation of antifungals is key to survival and recovery. Therapeutic drug monitoring (TDM) allows treatment optimisation by ensuring therapeutic antifungal serum levels. Available methods for antifungal TDM includes the bioassay, high performance liquid chromatography, liquid chromatography-mass spectrometry and tandem mass spectrometry, each with its own advantages and disadvantages28. Bioassay is the preferred method to measure flucytosine and itraconazole levels. Quality control assessment is a key requirement for triazole TDM (29).

The Global action Fund for Fungal Infections in its action plan aims at developing a more regional or continental EQA schemes for medical mycology, with a focus on assessing immunoassays (antibody and antigen tests), and molecular assays (30), these schemes should also include antifungal drug monitoring. Thus, the aim of an external assurance scheme in medical mycology would be focused at assessing (31,32);

1) Processing of clinical specimens for the diagnosis of fungal diseases
2) The actual identification of the pathogenic fungi (yeast or mould) isolated
3) Interpretation of results of identification (colonisation?, contaminant? or true infection?)
4) Serological and biomarker tests for invasive fungal diseases
5) Antifungal susceptibility testing of pathogenic fungi
6) Methodologies and techniques of therapeutic drug monitoring, and
7) Molecular and proteomic methods of identification of pathogenic fungi.

The ultimate goal of the exercise is to ensure that the laboratory results that are being reported are accurate, reliable and reproducible33. The internal control programme consists of various tests to check the reagents including the culture media and other consumable materials34. In the laboratory, reference strains have to be provided and, preparation of reagents are needed (34).

Competence of the technical staff in the laboratory can also be maintained through the identification process of unknown fungal strains34. The use of the international standards to identify competence in the laboratory results has improved the confidence and acceptance of laboratory results worldwide32. This can also be used in identifying the training needs of members of technical staff as well as evaluating and improving the performance of the laboratory31. However, issues have been raised considering the reliability of these testing procedures which only measure the overt proficiency testing (OPT) targeted at the optimal performance of the laboratory rather than being directed at the routine processes of the patients' samples (35). Another way of improving external quality assurance is to encourage the global registry which usually gives up to date information on rare and emerging fungal diseases (36).

Instructions on every routine procedure being performed must be readily available and it must have been those that have been properly evaluated in a competent manner33. A record of the dates when all media, reagents and stains to be used in medical mycology laboratory was first received, first opened and when to be discarded must be properly recorded (33). Supplies, equipment and the work area in the mycology laboratory must be maintained in an orderly manner to be able to maintain an efficient work flow (33). The equipment used must meet manufactures specifications. In the diagnostic medical mycology laboratory, preservation of the strains of fungi isolated should be kept alive for further evaluation for molecular identification, antifungal susceptibility testing and, epidemiological purposes34. The techniques for this, usually require serial sub-culturing as well as storage in liquid nitrogen, glycerol (10%) (at -20oC and -80oC) and lyophilisation. This also serves as the stock culture for the quality control organisms (31).

A new strain of fungus isolated in the diagnostic medical laboratory should be sent to the reference medical laboratory for further identification and final confirmation. A properly-labelled screw cap tubes with masking tape should be used35. There are different types of fungal culture media that are used for the isolation, identification and maintenance of the fungal strains. The performance of both laboratory and commercially prepared media must be monitored. A record containing the identification of the media, preparation, packaging evaluation as well as sterility check. The medium should be inoculated with the appropriate test organisms that produce negative and positive reactions (33).

On the African continent, a typical example of an EQA scheme is that of the National Health Laboratory Service (NHLS) Proficiency Testing Scheme (PTS) for Mycology, which operates across all NHLS laboratories in South Africa and laboratories in over 20 countries across Africa. NHLSPTS conducts three surveys per year, with each survey including, 4 mould and 1 yeast isolates for identification, slide preparation for microscopy, a simulated blood or cerebrospinal fluid samples for cryptococcal antigen testing, and 1 yeast isolate for identification and antifungal susceptibility testing (37). However, a real world experience on EQAS from seven countries on laboratory practices for diagnosis of fungal infection reported by the Asia Fungal Working Group initiative showed that only 56% of the labs participated in an EQAS, and only 43% conducted regular formal staff training (38). There is clearly a need to facilitate improved participation, as this will lead to improved laboratory performance in the mid-term.

Fungal diseases are an emerging threat to the public health worldwide, they are difficult to diagnose and treat. The true burden of fungal diseases in many countries across the world remains unknown; due both non-existence of mycologists and very limited availability of good, dedicated diagnostic mycology laboratories. EQA schemes allow specialist tests to be done outside of the reference laboratory in general laboratories reliably. Furthermore, EQA schemes enhances patient care and safety through improved laboratory practice.

There is need for programs to ensure the comparability of data among testing laboratories across different continents. In the past the differences in quality programmes operated in laboratories made this difficult however Laboratory accreditation programs on quality standards such as EN 45000 series and ISO Guide 25 has facilitated reproducibility in the data produced by different laboratories across the world thus ensuring wider acceptability and reproducibility of the data. The ISO 15189 for Medical Laboratories states that the laboratory shall participate in organised inter-laboratory comparisons, such as external quality assessment schemes, that encompass the extent and complexity of examination procedures used by the laboratory and the laboratory management shall monitor the results of external quality assessment and participate in the

implementation of corrective actions when control criteria are not fulfilled.

The objectives of an EQA are; to provide a measure for individual laboratory quality, to supplement internal quality control procedures, to provide a measure of the "state of the art" for a test, to obtain consensus values when true values are unknown, to investigate factors in performance (methods, staff etc) and to act as an educational stimulus to improvement in performance. In the case of microbiology EQA, the responses are evaluated against a predetermined intended response (e.g. the identity of the organism and its antimicrobial susceptibility pattern). The key steps towards a successful EQA as outlined by the Centre for Disease control involves the following steps occurring in a continuous cycle; defining the problems, planning for an EQA, implementing the EQA, follow up the EQA and finally to review and strategically plan the EQA.

EXTERNAL QUALITY CONTROL

With less restriction on trade throughout the world the comparability of data among testing laboratories becomes all the more important. In the past the differences in quality programmes operated in laboratories made this difficult. Laboratory accreditation to quality standards such as EN 45000 series and ISO Guide 25 [4] has given creditability to data produced by laboratories and has ensured wider acceptability of the data. Many European countries with national accreditation bodies now have multilateral recognition agreements

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ANTIBACTERIAL ACTIVITY OF *TERMINALIA GLAUCESCENS, MANGIFERA INDICA* AND *MITRACARPUS VILLOSUS* ON CARBAPENEM-RESISTANT ENTEROBACTERIACEAE

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ABSTRACT
The root of *Terminalia glaucescens*, stem-bark of *Mangifera indica* and leaves of *Mitracarpus villosus* were screened for antibacterial activities against 23 carbapenem-resistant Enterobacteriaceae (CRE) isolates. The phyto-constituents of the plants were extracted by cold maceration. Disc-diffusion and broth microdilution methods were used to determine the antibacterial activity and the minimum inhibitory concentration, respectively. The sensitivity of the isolates to the methanol extracts of the plant parts was between four to eight isolates (10 – 26.5 mm) including *Enterobacter aerogenes, Proteus mirabilis* and *Escherichia coli,* with the highest activity shown by *Mitracarpus villosus.* An overall higher activity was however observed with the ethanol extracts of the plant parts with potency on twelve to fifteen isolates (9 – 18.5 mm) including *Enterobacter aerogenes, Proteus mirabilis, Escherichia coli* and *Klebsiella pneumoniae*. Generally for all methanol extracts, a constant MIC value 100 mg/ml was observed for the susceptible isolates except two *Enterobacter aerogenes* isolates with MIC of 1 mg/ml while the MIC value of the ethanol extracts ranged from ≤ 0.1 – 100 mg/ml. Ethanol extracts of stem-bark of *Mangifera indica* and leaves of *Mitracarpus villosus* exhibited considerably higher activities compared to other extracts with low MIC values. The phytochemical screening showed that the extracts contained at least five bioactive metabolites with alkaloids, tannin and flavonoids present in all. The plants used in this study show promising antibacterial activity that can be explored in the treatment of multi-drug resistant Enterobacteriaceae infections.

ACTIVITÉ ANTIBACTÉRIENNE DE TERMINALIA GLAUCESCENS, MANGIFERA INDICA ET MITRACARPUS VILLOSUS SUR CARBAPENEM-RESISTANT ENTEROBACTERIACEAE

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ABSTRACT
La racine de Terminalia glaucescens, écorce de la tige de Mangifera indica et les feuilles de Mitracarpus villosus ont fait l'objet d'une activité antibactérienne contre 23 à l'épreuve des carbapénèmes Enterobacteriaceae (CRE) isolats. Les phyto-constituants de la plante ont été extraits par macération à froid. Disc-diffusion et de microdilution en méthodes ont été utilisées pour déterminer l'activité antibactérienne et la concentration minimale inhibitrice, respectivement. La sensibilité des isolats à l'usine de méthanol extraits des parties était de quatre à huit isolats (10 - 26,5 mm) y compris l'Enterobacter aerogenes, Escherichia coli et Proteus mirabilis, avec la plus forte activité affichée par Mitracarpus villosus. Une activité plus globale a toutefois observé avec l'éthanol extrait de la parties de plantes à l'activité sur 12 à 15 isolats (9 - 18,5 mm) y compris l'Enterobacter aerogenes, Proteus mirabilis, Escherichia coli et Klebsiella pneumoniae. En général pour tous les extraits au méthanol, une constante valeur MIC 100 mg/ml a été observée pour les isolats sensibles à l'exception de deux isolats Enterobacter aerogenes avec micro de 1 mg/ml alors que la valeur de la CMI d'extraits d'éthanol allaient de ≤ 0,1 - 100 mg/ml. Extraits de l'éthanol de l'écorce des tiges de Mangifera indica et les feuilles de Mitracarpus villosus présentaient des activités beaucoup plus élevé par rapport à d'autres extraits avec de faibles valeurs de CMI. La phytochemical dépistage préliminaire a montré que les contenus des extraits au moins cinq métabolites bioactifs alcaloïdes avec, de tanins et de flavonoïdes présents dans tous. Les plantes utilisées dans cette étude montrent une activité antibactérienne prometteuses qui peuvent être explorés dans le traitement des infections à entérobactéries résistantes aux médicaments

Keywords: Carbapenem – resistant Enterobacteriaceae (CRE), *Terminalia glaucescens, Mangifera indica, Mitracarpus villosus*, Antibacterial

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INTRODUCTION

The increase in the rate of antimicrobial resistance exhibited by bacteria, especially the Gram negative populace, and of major emphasis the Enterobacteriaceae family, is a threat to public health (1). Medicinal plants for centuries have been recognized for their use as remedies for infectious diseases because of the presence of biological components with therapeutic value (2). Carbapenem resistance in Enterobacteriaceae had been a negligible phenomenon before year 2000 (3). Carbapenem – resistant Enterobacteriaceae (CRE) are selected members of Enterobacteriaceae with hydrolytic activities on β-lactam drugs including carbapenems revered to possess the broadest antibacterial spectra over Gram negative bacteria (4, 5).

CRE cause serious infections in debilitated and immune-compromised patients, in association with prolonged hospitalization and increased fatality ranging from 24% to 70% (1). The CRE isolates used in this study - *Klebsiella pneumoniae, Escherichia coli, Enterobacter aerogenes* and *Proteus mirabilis* were isolated from urine and blood samples of in-patients attending three different selected tertiary hospitals in Ekiti, Osun and Oyo states, Nigeria.

Terminalia glaucescens is one of the about 100 species of the large flowering tree genus *Terminalia* belonging to the family Combretaceae. The plant is distributed in tropical, sub-tropical and savannah regions of the world. The root and stem of the plant have reportedly shown efficient bactericidal action against *Streptococcus mutans*, *Candida albicans* and *Staphylococcus saprophyticus* (6, 7, 8). *Mangifera indica* L, commonly called *mango* (English) is a large evergreen tropical tree in the family *Anacardiacea*. Mada et al. (8) have reported the use of the leaves, bark and root to treat oral candidiasis, malaria, skin infection, dysentery, diarrhea, thrush and shingles. *Mitracarpus villosus* is a member of the family Rubiaceae. In various parts of tropical Africa, it is traditionally used for treatment of sore throat, ringworm and eczema, fresh cuts, wounds and ulcer (9, 10, 11). Previous studies also revealed that the plant contains biologically active substances such as fatty acids, flavonoids and other phenolic compounds with potential antifungal, antimicrobial and anti-inflammatory activities (12, 13, 14, 15, 16, 17).

The anti-hemolytic, antibacterial and attenuation of quorum sensing and biofilm formation of few plants and essential oils against carbapenem-resistant isolates have been reported (16, 18); thus this study intends to provide information on plants with prospective efficacy against CRE. Based on this background, the susceptibility of 23 CRE isolates to ethanol and methanol extracts of *Terminalia glaucescens, Mangifera indica* and *Mitracarpus villosus* was evaluated and compared.

Materials and Methods

Source of the isolates

The details on the source of the isolates - *Escherichia coli, Enterobacter aerogenes, Klebsiella pneumoniae* and *Proteus mirabilis* are provided in Table 1.

Screening of herbal extracts

The medicinal plants used in this study are *Terminalia glaucescens, Mangifera indica* and *Mitracarpus villosus*.

Collection of plant materials: The root, stem-bark and leaves of *Terminalia glaucescens, Mangifera indica* and *Mitracarpus villosus* respectively were sourced from farms by herbal practitioners in Ondo town, Ondo state, Nigeria. Identification and authenticated at an herbarium in Ondo state, Nigeria.

***Preparation of plant extracts*:** The plant parts were air-dried and pulverized into fine powder using a milling machine, then extracted by cold maceration. One hundred grams of the powdered plant parts was soaked in 500 ml of ethanol 96% and methanol, each. These mixtures were kept on the rotator shaker for 72 hours for agitated extraction. The mixtures were then filtered using Whatman filter paper no 1. The filtrate was concentrated using a water bath at 60 ˚C until solvent was completely removed. The crude was stored in an air-tight container and kept in a refrigerator at 4 ˚C until use.

Disc preparation: Discs of 6-mm in diameter were cut from Whatman no. 5 filter paper. The discs were wrapped in aluminium foil and sterilized in hot-air oven for 15 minutes. Then 50 µl of the reconstituted extracts was impregnated into the discs accordingly, based on prior absorbance test.

Antibacterial screening of plant extracts: The standardized organisms (adjusted to 0.5 McFarland) were seeded onto solidified Mueller Hinton Agar (Rapid Labs, UK) plates by transferring 100 µl of the bacterial suspension to the agar surface. Then a glass spreader was used to evenly cover the agar surface with the inoculum. The plates were left on the work bench for 30 minutes, and then the discs were placed firmly on surface of the agar using sterile forceps. The plates were left for 1 hour for diffusion of the extracts and then incubated at 37 ˚C for 18 – 24 hours (18). After incubation, the zones of inhibition generated by the antibiotics were measured on three axis using a ruler; the mean and standard error mean (SEM) of the values were calculated and recorded in millimeter (mm).

Determination of Minimum Inhibitory concentration (MIC): The broth microdilution method as described by CLSI (19) was adopted for the MIC with slight modifications. Varying

concentrations (10 mg/ml, 1 mg/ml and 0.1 mg/l) of the extracts were prepared with Mueller Hinton broth (MHB) (Rapid Labs, UK) at 1:10 dilution from the stock concentration of 100 mg/ml and kept in tubes. The wells of the 96-well microtitre plate were filled with 100 µl of the plants extracts. Then, 100 µl of the standardized bacterial suspensions were inoculated into each well. Dimethyl sulfide was used as a control and MHB as a negative control. Imipenem was used as positive control for the isolates. The plate was incubated at 37 ˚C for 18 - 24 hours. The plate was read by optical density at 650 nm to observe microbial growth. The minimum concentration that showed no visible growth was taken as the MIC of the extract for each organism. This assay was carried out in duplicates.

Determination of Minimum Biocidal Concentration (MBC): The content of the wells that showed no microbial growth were subcultured on MHA and incubated at 37 ˚C for 18 – 24 hours. The least concentration that showed no visible growth on plate was taken as the MBC.

Phytochemical analysis of plant extracts: The extracts of *Terminalia glaucescens, Mangifera indica* and *Mitracarpus villosus* were analyzed for the presence of alkaloid, saponins, anthraquinone, steroids, tannin, flavonoid, and cardiac glycosides according to standard methods (19, 20).

RESULT

Antibacterial screening of plant materials

The root of *Terminalia glaucescens,* stem-bark of *Mangifera indica* and leaves of *Mitracarpus villosus* were screened for antibacterial activities against carbapenem-resistant Enterobacteriaceae isolates. Generally, the ethanolic extract of the plant parts exhibited more antibacterial activity than the methanol extract. The methanol extract of the root of *Terminalia glaucescens* showed potency on four (4) out of 23 isolates including *E. aerogenes* and *P. mirabilis* from urine and blood samples, with zone of inhibition ranging from 12 – 24 mm. The methanol extract of stem-bark of *Mangifera indica* was effective on five (5) of 23 isolates including *E. aerogenes* and *P. mirabilis* from urine and blood samples, with zone of inhibition ranging from 10 – 26.5 mm. The methanol extract of leaves of *Mitracarpus villosus* showed inhibitory effect on eight (8) isolates including *E. coli, E. aerogenes* and *P. mirabilis* from urine and blood samples, with zone of inhibition ranging from 9 – 26 mm (Figure 1). Overall, *E. coli* was highly resistant to the methanol extracts of the plant parts with just one of eight isolates showing susceptibility to *Mitracarpus villosus.* Also, *K. pneumoniae* showed no sensitivity to any of the extracts while a total of fifteen (15) isolates showed complete resistance to all methanol extracts of the plant parts.

The ethanolic extract of *Terminalia glaucescens* was effective on twelve (12) isolates including *E. coli, E. aerogenes, K. pneumoniae* and *P. mirabilis* from urine and blood samples, with zone of inhibition ranging from 9.5 – 20 mm. The ethanolic extract of *Mangifera indica* showed potency on fifteen (15) isolates including *E. coli, E. aerogenes, K. pneumoniae* and *P. mirabilis* from urine and blood samples, with zone of inhibition ranging from 9.5 – 18.5 mm. Lastly, the ethanolic extract of *Mitracarpus villosus* was effective on thirteen (13) isolates *E. coli, E. aerogenes, K. pneumoniae* and *P. mirabilis* from urine and blood samples, with zone of inhibition ranging from 10 – 19.5 mm. Six isolates showed complete resistance to all the three plant extracts (Figure 2).

Minimum Inhibitory Concentration/ Minimum Biocidal Concentration

For the methanol extract, lower concentrations of the extracts were less effective. A constant MIC value of 100 mg/ml was obtained for *Terminalia glaucescens* against four isolates including *E. aerogenes* and *P. mirabilis*. For *M. indica,* the MIC value of 10 (*E. coli, E. aerogenes, K. pneumoniae* and *P. mirabilis*) out of the 11 isolates that were inhibited was 100 mg/ml while one of the *E. aerogenes* isolates had MIC of 1 mg/ml. For *M. villosus,* seven isolates were inhibited at 100 mg/ml while two isolates had MIC of 1 mg/ml (Table 2).

Ethanol extract of *Terminalia glaucescens* had MIC value of 100 mg/ml on five isolates and then a lower value of ≤ 0.1 mg/ml on *Klebsiella pneumoniae*. The MIC value of ethanol extract of *M. indica* for fourteen isolates ranged from ≤ 0.1to 100 mg/ml while the MIC of ethanol extracts of *M. villosus* for thirteen isolates also ranged from ≤ 0.1to 100 mg/ml (Table 2). Succinctly, none of the extracts showed bactericidal properties on any of the isolates.

Qualitative Phytochemical analysis of the plant extracts

Table 3 shows the phytochemicals present in the extracts of the three plants. The ethanol extracts of both *Terminalia glaucescens* and *M. indica* contained all seven phytochemicals assayed for- alkaloid, saponin, tannin, anthraquinone, flavonoid, cardiac glycosides and steroids. The ethanol extract of *M. villosus* contained five of the seven phytochemicals except saponins and steroids.

The methanol extract of *Terminalia glaucescens* contained lesser phytochemicals than the ethanol extract. All phytochemicals were present except anthraquinones and cardiac glycosides. The methanol extract of *M. indica* contained all phytochemicals except steroids while *M. villosus* contained all except anthraquinones.

TABLE 1: SOURCE OF ISOLATES

S/N	Isolates	Sample	Age	Gender
U37	*Escherichia coli*	**Urine**	**70 +**	**M**
U50	*Escherichia coli*	**Urine**	**70 +**	**M**
B9	*Ent. Aerogenes*	**Blood**	**41 – 50**	**F**
B41	*Ent. Aerogenes*	**Blood**	**61 – 70**	**M**
U12	*Proteus mirabilis*	**Urine**	**51 – 60**	**M**
U31	*Proteus mirabilis*	**Urine**	**70 +**	**F**
U1	*Escherichia coli*	**Urine**	**21 – 30**	**M**
B16	*Escherichia coli*	**Blood**	**21 – 30**	**F**
B18	*Ent. Aerogenes*	**Blood**	**31 – 40**	**F**
B19	*Ent. Aerogenes*	**Blood**	**51 – 60**	**M**
B28	*Ent. Aerogenes*	**Blood**	**21 – 30**	**F**
U30	*Proteus mirabilis*	**Urine**	**41 – 50**	**F**
U42	*Proteus mirabilis*	**Urine**	**41 – 50**	**F**
B9	*Escherichia coli*	**Blood**	**51 – 60**	**M**
U18	*Escherichia coli*	**Urine**	**31 – 40**	**M**
U34	*Escherichia coli*	**Urine**	**41 – 50**	**F**
U50	*Escherichia coli*	**Urine**	**51 – 60**	**M**
U5	*Ent. Aerogenes*	**Urine**	**21 – 30**	**M**
U12	*Ent. Aerogenes*	**Urine**	**21 – 30**	**M**
U47	*Ent. Aerogenes*	**Urine**	**70 +**	**M**
B2	*Ent. Aerogenes*	**Blood**	**41 – 50**	**M**
B25	*Klebsiella pneumoniae*	**Blood**	**31 – 40**	**M**
U36	*Proteus mirabilis*	**Urine**	**51 – 60**	**F**

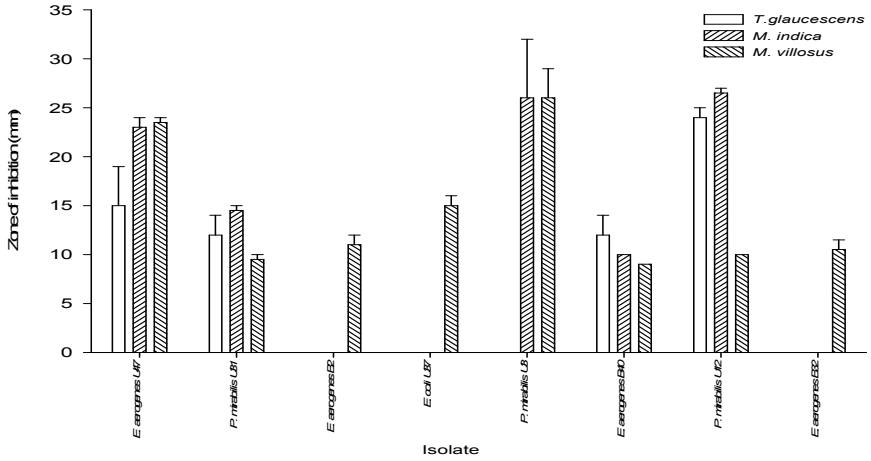

FIGURE 1: ANTIBACTERIAL ACTIVITY OF METHANOL EXTRACTS OF THE PLANTS

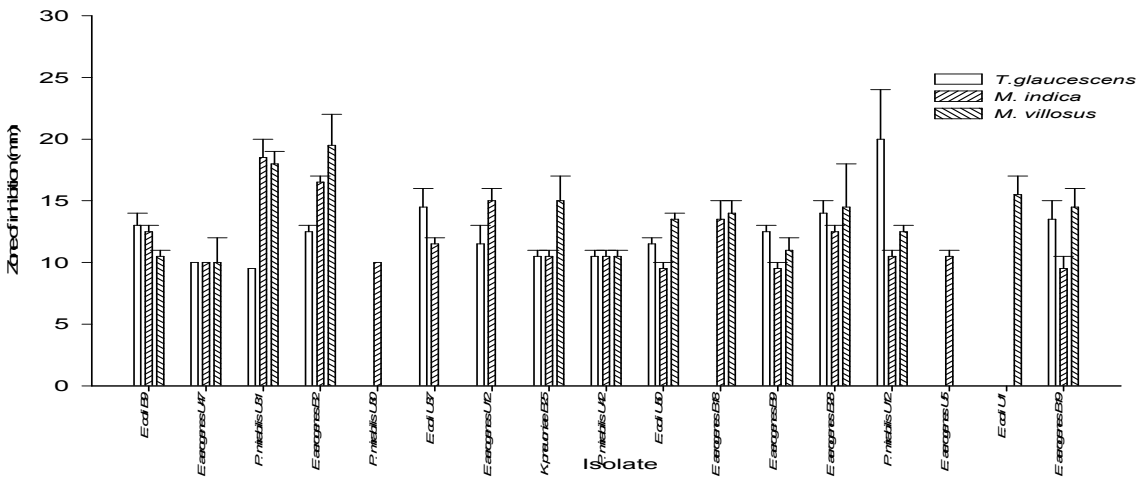

FIGURE 2: ANTIBACTERIAL ACTIVITY OF ETHANOL EXTRACTS OF THE PLANTS

TABLE 2: MINIMUM INHIBITORY CONCENTRATION OF THE METHANOL AND ETHANOL EXTRACTS PLANTS

Tag	Isolates	Methanol (× 10^3 µg/ml)			Ethanol (×10^3µg/ml)		
		T. glaucescens	*M. indica*	*M. villosus*	*T. glaucescens*	*M. indica*	*M. villosus*
U37	*Escherichia coli*	--	100	--	--	100	100
U50*	*Escherichia coli*	--	--	--	--	--	--
B9	*E. aerogenes*	--	100	100	--	0.1	0.1
B41	*E. aerogenes*	--	--	--	--	--	--
U12	*Proteus mirabilis*	100	100	100	100	100	100
U31	*Proteus mirabilis*	100	100	100	100	1	1
U1	*Escherichia coli*	--	--	--	--	--	100
B16	*Escherichia coli*	--	--	--	--	--	--
B18	*E. aerogenes*	--	--	--	--	0.1	100
B19	*E. aerogenes*	--	--	--	--	--	--
B28	*E. aerogenes*	100	100	100	100	100	100
U30	*Proteus mirabilis*	--	--	--	--	100	--
U42	*Proteus mirabilis*	--	100	100	100	0.1	100
B9	*Escherichia coli*	--	100	100	--	100	--
U18	*Escherichia coli*	--	--	--	--	--	--
U34	*Escherichia coli*	--	--	--	--	--	--
U50**	*Escherichia coli*	--	100	--	--	100	100
U5	*E. aerogenes*	--	--	--	--	0.1	1
U12	*E. aerogenes*	--	--	--	--	--	--
U47	*E. aerogenes*	100	100	1	100	100	0.1
B2	*E. aerogenes*	--	1	1	--	0.1	0.1
B25	*K. pneumoniae*	--	100	100	0.1	100	0.1
U36	*Proteus mirabilis*	--	--	--	--	--	--

- *Indicates 'no MIC' value*

TABLE 3: QUALITATIVE ANALYSIS OF PHYTOCHEMICALS IN THE PLANT EXTRACTS

Extracts	Phytochemicals						
	Alkaloids	**Saponins**	**Tannin**	**Anthraquinone**	**Flavonoid**	**Cardiac glycosides**	**Steroids**
Terminalia glaucescens [a]	+	+	+	+	+	+	+
Mangifera indica [a]	+	+	+	+	+	+	+
Mitracarpus villosus [a]	+	--	+	+	+	+	--
Terminalia glaucescens [b]	+	+	+	--	+	--	+
Mangifera indica [b]	+	+	+	+	+	+	--
Mitracarpus villosus [b]	+	+	+	--	+	+	+

'a' represents ethanol extract 'b' represent methanol extract '+' represents present '-' represent absent

DISCUSSION

This study reports significant antibacterial activities of ethanol and methanol extracts of root of *Terminalia glaucescens,* stem-bark of *Mangifera indica* and leaves of *Mitracarpus villosus* on CRE isolates recovered from urine and blood samples of humans. The methanol extract of *Mitracarpus villosus* was the most effective compared to the methanol extracts of *T. glaucescens* and *M. indica* by exerting antibacterial activity on 34.8% of the isolates including *E. coli, E. aerogenes* and *P. mirabilis*. The methanol extract of *T. glaucescens* was effective on 17.4% of the isolates including *K. aerogenes* and *P. mirabilis*; and *M. indica* was potent on 21.7% of the isolates *E. aerogenes* and *P. mirabilis*.

The ethanol extract of *M. indica* showed the highest antibacterial activity compared to extracts of *T. glaucescens* and *Mitracarpus villosus* by exerting potency on 65.2% of the isolates. Both the ethanol and methanol extracts of the plants used in this study have been reported to show antibacterial efficacy against gram negative bacteria such as *Escherichia coli, Pseudomonas aeruginosa, Klebsiella* sp. and a range of gram positive bacteria (6, 7, 8, 9). *T. glaucescens* has been studied to show considerable levels of activity against *K. pneumoniae, E. coli* and *P. mirabilis* (22, 23, 24), which is also validated in this study.

Lower concentrations of the methanol extracts of all the plants were less effective against the test isolates compared to the ethanol extracts which could be attributed to the efficacy of solvents. Ethanol extracts of *Terminalia glaucescens* and *Mitracarpus villosus* showed remarkable activity on the carbapenem-resistant *Klebsiella pneumoniae* with MIC at ≤ 0.1 mg/ml. Gbadamosi and Ogunsuyi (24) reported that the ethanolic extract of the root of *Terminalia glaucescens* showed high potency and against multidrug resistant *Escherichia coli* at a concentration of 100 mg/ml which also conform with the findings of this study. The low MIC values recorded for plant extracts show their high efficacy as bacteriostatic agents. Despite the low MIC values recorded, none of the concentrations of the plants extracts showed bactericidal effect on any of the isolates.

There were variations in the phytochemical contents of the ethanol and methanol extracts of each plant which could be responsible for the different level of potency exhibited. The presence of phytochemicals has likewise been studied to vary depending on the

type of extraction and the solvent used. However, all the extracts contained alkaloids, flavonoids and tannins. Alkaloids are alkaline chemical substances with high ammonia content which act as stimulants; thus effective in the treatment of respiratory and gastrointestinal diseases (26). Also, flavonoids possess anti-oxidative and anti-inflammatory properties; thus effective in the protection of the blood capillaries (26). The acidic nature of tannins as well as the presence of gallic and epigallic acids has been studied to be effective as antiseptics. The antibacterial activities exhibited by the different extracts of the plants used in this study can therefore be attributed to the presence of these different phytochemicals.

Conclusion

The plants used in this study have shown explorable bacteriostatic efficacy against extensive/ pan-drug resistant Enterobacteriaceae isolates. Higher concentrations of the extracts may be required to exert bactericidal actions on the CRE isolates. The quantitative phytochemical analysis will also be necessary in determining the most abundant phytoconstituent that may be responsible for the inhibitory activity exhibited by the extracts.

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ETIOLOGIC AGENTS OF LOWER RESPIRATORY TRACT INFECTIONS AMONG PATIENTS ATTENDING TUBERCULOSIS CLINIC IN BENIN CITY, NIGERIA

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ABSTRACT

The emergence of HIV has reawakened the tuberculosis (TB) scourge and infected patients are prone to opportunistic infections, this study was carried out to determine the HIV status and etiologic agents of Lower respiratory tract infections (LRTI) among tuberculosis suspected patients in Benin City, Nigeria. The study was cross sectional. A total of 276 patients attending tuberculosis clinic were recruited. Questionnaires were filled for each patient. Blood and sputum specimens were collected in plain and sterile containers respectively and transported immediately to the laboratory. Blood samples were screened for the presence of HIV antibodies. The sputum specimens were then cultured following standard microbiological procedure, and thereafter processed using the GeneXpert MTB/RIF assay. Emergent bacterial colonies were identified and susceptibility testing was carried out following standard microbiological techniques. A total of 118 (42.8%) non-mycobacterial bacterial agents were recovered from the patients. Patients were more likely to be culture positive for LRTI in comparison with TB infection (p < 0.0001). There was no association between TB and HIV status (OR = 0.6161, 95%CI = 0.226, 1.648, p = 0.4474). HIV positive patients were more likely to be culture positive for bacterial agents and *Klebsiella pneumoniae* was more likely to be recovered (p = 0.0338). The fluoroquinolones, gentamicin and ceftriaxone-sulbactam were the most active antibacterial agents against bacterial isolates. The prevalence of LRTI in this study was 52.2%. The study draws attention on the need for physicians to request for bacteriological culture (non-mycobacterial) alongside the TB diagnostic algorithm in suspected TB cases.

Keywords: Tuberculosis, HIV, opportunistic infections, patients, bacteria.

AGENTS ETIOLOGIQUES DES INFECTIONS DU TRACTUS RESPIRATOIRE INFÉRIEUR CHEZ LES PATIENTS SOUFFRANT DE LA CLINIQUE DE TUBERCULOSE DANS LA VILLE DU BENIN, NIGERIA

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ABSTRAIT

L'émergence du VIH a réveillé le fléau de la tuberculose (TB) et les patients infectés sont sujets à des infections opportunistes, cette étude a été réalisée pour déterminer le statut VIH et les agents étiologiques des infections respiratoires basses chez les patients suspects de tuberculose à Benin City, Nigeria. L'étude était transversale. Un total de 276 patients fréquentant la clinique de la tuberculose ont été recrutés. Des questionnaires ont été remplis pour chaque patient. Les échantillons de sang et d'expectoration ont été recueillis dans des récipients simples et stériles respectivement et transportés immédiatement au laboratoire. Des échantillons de sang ont été examinés pour la présence d'anticorps anti-VIH. Les échantillons d'expectoration ont ensuite été cultivés suivant la procédure microbiologique standard, puis traités en utilisant le test GeneXpert MTB / RIF. Des colonies bactériennes émergentes ont été identifiées et des tests de sensibilité ont été effectués suivant des techniques microbiologiques standard. Un total de 118 (42,8%) agents bactériens non mycobactériens ont été récupérés chez les patients. Les patients étaient plus susceptibles d'être positifs à la culture pour le LRTI par rapport à l'infection tuberculeuse (p <0,0001). Il n'y avait pas d'association entre la tuberculose et le statut VIH (OR = 0,6161, IC 95% = 0,226, 1,648, p

Les patients séropositifs étaient plus susceptibles d'être positifs à la culture pour des agents bactériens et Klebsiella pneumoniae était plus susceptible d'être récupérée (p = 0,0338). Les fluoroquinolones, la gentamicine et le ceftriaxone-sulbactam étaient les agents antibactériens les plus actifs contre les isolats bactériens. La prévalence de LRTI dans cette étude était de 52,2%. L'étude attire l'attention sur la nécessité pour les médecins de demander une culture bactériologique (non mycobactérienne) parallèlement à l'algorithme de diagnostic de la TB dans les cas suspects de TB.

Mots-clés: Tuberculose, VIH, infections opportunistes, patients, bactéries.

INTRODUCTION

Acute lower respiratory tract infections (LRTIs) are a persistent and pervasive public health problem which causes a greater burden of disease worldwide than malaria, cancer, human immunodeficiency virus infection, or heart attacks (1). LRTIs affect all age groups and vary in severity from non-pneumonic LRTI in a young healthy adult to pneumonia or life threatening exacerbation in a patient with severe disabling chronic obstructive pulmonary disease (COPD) (2).

A variety of micro-organisms can cause LRTIs namely bacteria, viruses, parasites and fungi[3]. Several studies have outlined bacterial etiologic agents causing LRTIs to include the Gram positive cocci (*Staphylococcus* spp and *Streptococcus pneumoniae*), Enterobacteriaceae and oxidase positive rods (2, 3). The etiologic agent of tuberculosis (TB); *Mycobacterium tuberculosis* has also been implicated as playing a key role in the global burden of LRTI (4).

TB is the ninth leading cause of death worldwide and the leading cause from a single infectious agent, ranking above HIV/AIDS (5). In 2016, there were an estimated 1.3 million TB deaths among HIV-negative people and an additional 374,000 deaths among HIV-positive people[5]. Nigeria remains uniquely placed among the high burden countries where HIV, TB and drug-resistant TB are prevalent (5, 6).

The direct observed treatment short course (DOTS) program by WHO was instituted in the 1990's in developing countries to provide antibiotics for patients and have an effective TB control program (4). This has guaranteed patients' attending tuberculosis clinics early diagnosis of the disease, access to anti-TB treatment and an uninterrupted supply of anti-TB drugs (7).
However, the differential diagnosis of TB from bacterial pneumonia is not straightforward as it could present with similar signs and symptoms as LRTIs of other etiologies (8, 9). While preference is given to detection of TB owing to Nigeria's high burden of the disease (5), patients presenting with such signs and symptoms may have other etiologic agent(s). Moreover, HIV has been shown as a risk factor for opportunistic LRTI (6). It is against this background that this study was conducted to determine the HIV status and etiologic agents of LRTI among patients attending tuberculosis clinic in Benin City, Nigeria.

MATERIALS AND METHODS

Study Area:

The study was conducted at the University of Benin Teaching Hospital (UBTH) in Benin City, Nigeria. UBTH is a tertiary hospital with 700 beds and 20 wards, serving the specialist healthcare needs of Edo State and 6 to 10 other neighboring States.

Study Population

A total of 276 patients (non-repetitive) attending tuberculosis (DOTS) clinic were enrolled for the study. Structured questionnaires were given to patients. . The study was conducted between 3rd July 2017 and 28th February, 2018.

Ethical Consideration

Ethical approval was sought and obtained from the Ethical Committee of UBTH with protocol number: ADM/E 22/A/VOL. VII/1489.

HIV screening

Venous blood was obtained from all participants. Five milliliters of blood sample was collected into properly labeled plain containers, samples were sent to the laboratory and HIV serological tests were carried out on the centrifuged blood samples. For the detection of HIV-1 and HIV-2 antibodies in the blood, Determine® HIV-1/2 Test cards (Inverness Medical, Japan), Unigold™ Kit (Trinity Biotec, Ireland) and HIV - 1/2 Stat- Pak® Assay (Chembio Diagnostic Systems, USA) according to the national algorithm (10). These methods are immunochromatographic and detect the presence of antibodies to HIV-1 and HIV-2 in human blood and are read in-vitro having more than 99.9% sensitivity and 99.75% specificity.

Sputum collection and processing:

Sputum specimens were collected from these patients in sterile wide-mouth containers and sent to the Medical Microbiology Laboratory, University of Benin Teaching Hospital for microbiological analysis. Specimens were processed within two hours after collection. Specimens were cultured on blood, chocolate and MacConkey agar plates respectively. Films were made from the sputum specimens and

stained by Gram's method. Cultures with significant growth were identified following standard microbiological techniques (11).

TB Screening:

Sputum samples were thereafter processed for detection of TB using GeneXpert MTB/RIF automated system. Testing was performed according to the manufacturer's instructions. Sample reagent was added to untreated sputum at a ratio of 2:1. This was manually agitated and kept for 10 min at room temperature, then shaken again and kept for 5 min; 2 ml of the inactivated material was thereafter transferred to the test cartridge, inserted into the test platform and run (12). The system automatically interpreted all results from measured fluorescent signal into the following categories: invalid, if PCR inhibitors were detected with amplification failure, negative or positive. Positive results were scaled into 4 categories (very low, low, medium, high) depending on bacterial load and defined susceptible or resistant to rifampicin depending on detection of mutations in *rpoB* gene.

Disc susceptibility test

Disc susceptibility tests were thereafter performed on all bacterial isolates using the British Standard for Antimicrobial Chemotherapy (BSAC) method (13). The following antibacterial discs were used; Ciprofloxacin (5 µg), Ofloxacin (5 µg), Levofloxacin (5 µg), Sulfamethoxazole-trimethoprim (25 µg), Clindamycin (2 µg), Cefotaxime (30 µg), Cefuroxime (30 µg), Cefixime (5 µg), Ceftriaxone-sulbactam (30/15 µg) and Gentamicin (10 µg) (all from Axiom, India).

Statistical analysis

The data obtained were analyzed with statistical tools namely chi square as appropriate using the statistical software INSTAT® (GraphPad Software Inc, La Jolla, CA, USA).

RESULTS

Of this 276 patients that participated in this study, 26 (9.4%) patients were positive for TB only, 105 (38.0%) were culture positive while 13 (4.7%) patients were both culture and TB positive. The prevalence of LRTI (TB + non-TB) in this study was 52.2%. Patients were more likely to be culture positive (non-TB) for LRTI in comparison with tuberculosis infection ($p < 0.0001$) (Table 1).

TABLE 1: PREVALENCE OF TUBERCULOSIS AND OTHER BACTERIAL AGENTS AMONG 276 PATIENTS WITH SIGNS AND SYMPTOMS OF LRTI IN BENIN CITY, NIGERIA

Patients	Number positive
TB only	**26 (9.4)**
Culture only	**105 (38.0)**
TB + Culture	**13 (4.7)**

TB = tuberculosis, n = 276, p < 0.0001

A total of 51 (18.5%) patients were HIV positive, 46 of these patients were TB negative while 5 (12.8%) were infected with TB. There was no association between TB infection and HIV status among patients attending DOTS clinic (OR = 0.6161, 95%CI = 0.226, 1.648, p = 0.4474). (Table 2).

TABLE 2: PREVALENCE OF HIV AND TUBERCULOSIS AMONG PATIENTS ATTENDING TUBERCULOSIS CLINIC IN BENIN CITY, NIGERIA

Patients	Number Tested	HIV Positive (%)	OR	95%CI	p
TB positive	39	5 (12.8)	0.616	0.226, 1.648	0.4474
TB negative	237	46 (19.4)			

TB = uberculosis, n = 276.

A total of 118 (42.8%) non-mycobacterial bacterial agents were recovered from patients in this study. In relation to bacterial etiologic agents of LRTI among patients attending DOTS clinic and their HIV status, a total of 30 HIV positive patients (58.8%) were culture positive for bacterial agents while 88 patients (39.1%) that were HIV negative were culture positive. HIV positive patients were more likely to be culture positive for bacterial agents causing LRTI (p = 0.0158). *Klebsiella pneumoniae* showed high prevalence among HIV positive and HIV negative patients with 23.5% and 11.1% respectively but was more likely to be

recovered from HIV positive patients attending DOTS clinic (p = 0.0338) (Table 3).

TABLE 3: ETIOLOGIC AGENTS OF LRTI IN RELATION TO HIV SERO-STATUS

Organism	No of isolates	No HIV positive (n = 51)	No HIV negative (n = 225)	p
CoNS	5	0	5 (2.2)	0.6221
S. aureus	18	6 (11.8)	12 (5.3)	0.1721
S. pneumoniae	2	1 (2.0)	1 (0.4)	0.8155
E. coli	3	0	3 (1.3)	0.9352
Klebsiella pneumoniae	37	12 (23.5)	25 (11.1)	0.0338
***Enterobacter* spp**	21	4 (7.8)	17 (7.6)	0.9442
***Citrobacter* spp**	2	1 (2.0)	1 (0.4)	0.8155
***Acinetobacter* spp**	5	0	5 (2.2)	0.6221
***Providencia* spp**	5	0	5 (2.2)	0.6221
***Alkaligenes* spp**	2	1 (2.0)	2 (0.9)	0.5051
H. influenzae	12	4 (7.8)	8 (3.6)	0.3294
P. aeruginosa	5	1 (2.0)	4 (1.8)	0.9295
Total	118	30 (58.8)	88 (39.1)	0.0158

CoNS- Coagulase negative staphylococci, number in brackets = value in percentage

In relation to gender and infection rate, there was no statistically significant relationship (p = 0.6848). The age group 51-60 yrs showed the highest infection rate for LRTI with 67.5%. When compared with other age groups however, there was no statistical significance (p = 0.0717). Also, educational status, occupation and marital status of patients did not significantly affect the prevalence rate of LRTI due to bacterial agents (p >0.05). (Table 4)

TABLE 4: EFFECT OF SOCIO-DEMOGRAPHIC FACTORS ON BACTERIAL LRTI

Factor	Division	No of Patients (n = 276)	No Culture Positive	p
Gender	Male	122 (44.2)	50 (41.0)	0.6848
	Female	154 (55.8)	68 (44.2)	
Age	0-10	17 (6.2)	7 (41.2)	0.0717
	11-20	17 (6.2)	10 (58.8)	
	21-30	57 (20.7)	20 (35.1)	
	31-40	49 (17.8)	19 (38.8)	
	41-50	44 (15.9)	18 (40.9)	

	51-60	40 (14.5)	27 (67.5)	
	61-70	26 (9.4)	12 (46.2)	
	≥ 71	26 (9.4)	11 (38.9)	
Educational status	No Formal	24 (8.7)	10 (41.6)	0.9986
	Primary	53 (19.2)	22 (41.5)	
	Secondary	96 (34.8)	41 (42.7)	
	Tertiary	103 (37.3)	44 (42.7)	
Marital Status	Single	83 (30.1)	36 (43.4)	0.7864
	Married	161 (58.3)	66 (41.0)	
	Divorced	9 (3.3)	5 (55.6)	
	Widow(er)	23 (8.3)	11 (47.8)	
Occupation	Business/Trading	70 (25.4)	32 (45.7)	0.1952
	Artisan	30 (10.9)	19 (63.3)	
	Civil/Public Servant	30 (10.9)	10 (33.3)	
	Teacher/Lecturer	16 (5.8)	6 (37.5)	
	Unemployed	20 (7.2)	8 (40.0)	
	Student	47 (17.0)	22 (34.9)	
	Others	63 (22.8)	22 (34.9)	

Number in brackets = value in percentage

The distribution of bacterial agents implicated in patients that were TB positive is shown on Figure 1. Out of 39 TB positive patients, 13 (33.3%) yielded bacterial growth. *Klebsiella pneumoniae* showed the highest prevalence of bacterial agents co-infecting TB-positive patients.

The fluoroquinolones (ciprofloxacin, ofloxacin and levofloxacin), gentamicin and ceftriaxone-sulbactam showed high activity against bacterial agents isolated. Sulfamethoxazole-trimethoprim showed moderate activity while cefuroxime, cefotaxime, cefixime and clindamycin showed poor activity to bacterial agents isolated (Table 5).

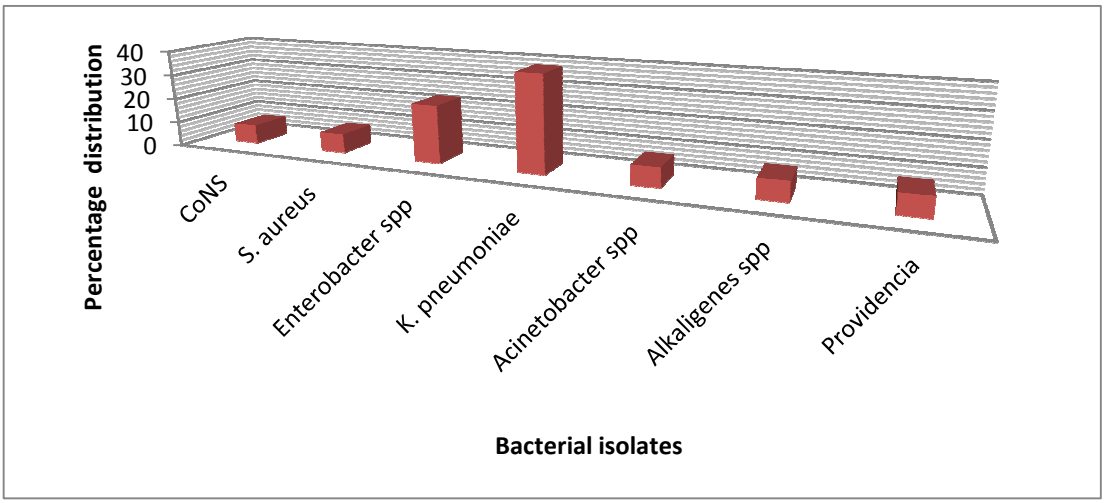

FIGURE 1: PERCENTAGE DISTRIBUTION OF BACTERIAL ISOLATES AMONG PATIENTS HAVING TUBERCULOSIS

TABLE 5: ANTIMICROBIAL SUSCEPTIBILITY PATTERN OF BACTERIAL AGENTS CAUSING LRTI IN BENIN, NIGERIA

Organism	No of isolates	CIP	OFX	LVX	SXT	CD	CTX	CXM	CFX	CRS	GEN
CoNS	5	3 (60.0)	3 (60.0)	3 (60.0)	3 (60.0)	1 (20.0)	1 (10.0)	1 (10.0)	0	3 (60.0)	4 (80.0)
S. aureus	18	14 (77.8)	13 (72.2)	12 (66.7)	10 (55.6)	5 (27.8)	5 (27.8)	1 (5.6)	3 (16.7)	16 (88.9)	18 (100)
Streptococcus pneumoniae	2	1 (50.0)	1 (50.0)	1 (50.0)	1 (50.0)	1 (50.0)	1 (50.0)	1 (50.0)	1 (50.0)	2 (100)	1 (50.0)
***Citrobacter* spp.**	2	2 (100)	2 (100)	2 (100)	0	ND	1 (50.0)	0	1 (50.0)	2 (100)	1 (50.0)
***Enterobacter* spp.**	21	18 (85.7)	17 (81.0)	17 (81.0)	13 (61.9)	ND	13 (61.9)	3 (14.3)	8 (38.1)	18 (85.7)	17 (81.0)
Escherichia coli	3	2 (66.7)	2 (66.7)	2 (66.7)	1 (33.3)	ND	1 (33.3)	1 (33.3)	0	2 (66.7)	2 (66.7)
Klebsiella pneumoniae	37	34 (91.9)	33 (89.2)	33 (89.2)	16 (43.2)	ND	18 (48.6)	6 (16.2)	12 (32.4)	27 (73.0)	26 (70.3)
***Acinetobacter* spp**	5	3 (60.0)	3 (60.0)	3 (60.0)	1 (20.0)	ND	1 (20.0)	1 (20.0)	1 (20.0)	4 (80.0)	3 (60.0)
***Providencia* spp.**	5	4 (80.0)	4 (80.0)	4 (80.0)	4 (80.0)	ND	2 (40.0)	1 (20.0)	2 (40.0)	4 (80.0)	4 (80.0)
***Alkaligenes* spp.**	3	3 (100)	3 (100)	3 (100)	1 (33.3)	ND	2 (66.7)	0	2 (66.7)	2 (66.7)	2 (66.7)
Haemophilus influenzae	12	3 (25.0)	4 (33.3)	3 (25.0)	1 (8.3)	ND	2 (16.7)	0	1 (8.3)	10 (83.3)	3 (25.0)
Pseudomonas aeruginosa	5	5 (100)	5 (100)	5 (100)	3 (60.0)	ND	0	0	0	2 (40.0)	4 (80.0)

Abbreviations: CIP-Ciprofloxacin, OFX-Ofloxacin, LVX-Levofloxacin, SXT-Sulfamethoxazole-trimethoprim, CD-Clindamycin, CTX-Cefotaxime, CXM-Cefuroxime, CFX-Cefixime, CRS-Ceftriaxone-sulbactam, GEN-Gentamicin. Number in brackets = value in percentages, ND= not done, CoNS = Coagulase negative *staphylococci.*

DISCUSSION

In this study, patients under the clinical suspicion of having TB and enrolled in the DOTS clinic were more likely to be infected with non-mycobacterial bacterial agents ($p < 0.0001$). Although no previous Nigerian study has considered etiologic agents in TB suspected patients, our finding is in tandem with a previous Cameroonian study in which majority of patients attending tuberculosis clinic were culture positive (8). TB could cause debilitating illness and present with similar signs and symptoms as LRTI of other etiologies (8, 9). The current algorithm in tuberculosis clinics in Nigeria excludes culture for non-mycobacterial bacterial agents; our study however suggests a need to review this.

HIV is a known risk factor for acquiring tuberculosis infection (14). Patients who are HIV positive and infected with TB have been previously shown to be 17 times more likely to develop active TB than people not infected with HIV (15). Among patients attending TB clinic in our study however, HIV was not a risk factor for TB although 12.8% of TB positive patients were co-infected with HIV. Our findings could be due to the fact that most patients were attending tuberculosis clinic for the first time and were being screened for TB unlike previous studies in which the pool of patients were from people living with HIV/AIDS (PLWHA) (15), or known TB patients (14). In these studies, a significant association was observed between HIV and TB. The prognosis for HIV/TB co-infection is however usually not favorable as globally, the recovery rate is about 73% with mortality rates being high (4). This study therefore justifies the screening of patients attending TB clinic for HIV as early detection would be helpful in tackling both infections.

The major causes of morbidity and mortality in HIV infected persons are opportunistic infections which vary from region to region (16). Among the

opportunistic infections associated with HIV, diseases like pneumonia of bacterial origin occur at a rate many times higher in HIV infected patients than in the general population (16). In this study, HIV positive patients were more likely to be co-infected with non-mycobacterial bacterial agents ($p = 0.0158$). This finding tallies with a similar study (8). Among patients showing signs of LRTI in Nepal, HIV positive patients were shown to be significantly more likely to be infected with bacterial pathogens (46.6%) than the HIV negative group (27%) (16).

Klebsiella pneumoniae was more likely to be co-infecting patients with HIV than any other bacteria in this study. This finding is in congruence with previous studies in Nigeria and Nepal (3, 16). It however differs from a similar study in Cameroon where *Streptococcus pneumoniae* showed high prevalence among HIV positive patients (8). Pathogenicity of *K. pneumoniae* is largely due to the production of a polysaccharide-rich cell surface that provides protection from the inflammatory response (17). In HIV infections where there is a defective immune response, these strains may colonize the lungs and worsen treatment outcomes if proper antibacterial therapy is not immediately instituted.

K. pneumoniae also showed high prevalence among bacteria co-infecting TB patients. *K. pneumoniae* has been known to mimic pulmonary reactivation tuberculosis because it presents with hemoptysis and cavitating lesions (9). It is equally difficult to treat because of the organism's thick capsule (17). In our setting, patients who are TB positive begin the treatment course in the TB clinic; little attention is accorded the possibility of a co-infecting bacterium. This may lead to inadequate care and poor treatment outcomes.

The role of gender in LRTI varies. Previous studies in Nigeria, Cameroon and Nepal observed no significant difference between male and females (3, 8, 16). Our finding was in tandem with these studies as there was no significant relationship between gender and LRTI. Our finding was however in contrast with an Indian study where males were more likely to have LRTI (2). In another Nigerian study, 54.7% of females in comparison with 45.3% of males had LRTI; this finding was however not subjected to statistical analysis (18).

Patients within the age group 51-60 yrs had a comparatively higher prevalence rate of LRTI with 67.5% in this study, although this finding was not statistically significant. This finding agrees with several other studies in which age did not significantly affect LRTI (8, 18). In the same vein, educational status, marital status and occupation type did not play a contributory role in LRTI prevalence although artisans had a comparatively higher prevalence rate of LRTI (63.3%). This may be due to occupational exposure among persons in this group namely carpenters, cement workers, stone cutters and so on. These occupations have been previously shown to predispose patients to LRTI (6).

The fluoroquinolones and gentamicin showed high efficacy against bacterial isolates in this study. This finding agrees with two Nigerian studies (3, 18). Ceftriaxone-sulbactam also showed remarkable efficacy. This drug is relatively new in our setting. Sulfamethoxazole-trimethoprim showed moderate activity against bacterial isolates. This finding strikingly differs from Egbe *et al*'s study which showed 0% efficacy (3). Owing to the local antibiotic rotation policy, the drug had been withdrawn for about 6 years and was recently re-introduced. This may explain the moderate potency of this drug. Also, cefuroxime, cefixime and cefotaxime showed poor activity especially to Gram negative bacterial isolates. The presence of extended spectrum β-lactamase- and AmpC β-lactamase- producing bacteria has been recently demonstrated among clinical specimens including sputum (33.9% and 9.7% respectively) in Benin, Nigeria (19). This may have played roles in the high level of resistance observed to these cephalosporins. Also, antibiotic abuse is rife in our setting and over-the-counter purchase is rampant (3, 19). Practices like this may create selective pressure and ensure the survival of resistant bacterial strains in the hospital and community settings.

Conclusion: The prevalence of LRTI in this study was 52.2%. Patients attending tuberculosis clinic with signs and symptoms of LRTI were more likely to be infected with bacteria (non-mycobacterial). Some of these patients had co-infection of TB and HIV, HIV and opportunistic bacteria, and TB and other opportunistic bacteria. *K. pneumoniae* was more likely to be the bacterial agent in these instances. The study draws attention on the need for physicians to request for bacteriological culture (non-mycobacterial) alongside TB algorithm for diagnosis in suspected TB cases.

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THE PREVALENCE OF NON-TUBERCULOUS RECURRENT COUGH IN-PATIENTS WITHIN CROSS RIVER STATE, NIGERIA

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ABSTRACT

Introduction: Nontuberculous recurrent cough is becoming rampant among hospitalized patients.

Aim: Hospitalized patients and out-patients in some hospitals in Cross River State were studied for the prevalence of non-tuberculous recurrent cough in the state

Methods: This was carried out using standard procedures in Microbiology.

Results: The bacterial isolates from the sputum samples were *Staphylococcus aureus, Streptococcus pneumoniae, Streptococcus pyogenes, Haemophilus influenzae, Escherichia coli, Klebsiella pneumoniae,* and *Corynebacterium diphtheriae.* The patients' age group 20 – 39 years had the highest prevalence of non – tuberculous recurrent cough (46.7%) while the least prevalence of 1.0% was among the age group ≥ 80 years. The prevalence rate of 33.7% was the highest among the social class and businessmen, while the least prevalence rate of 8.2% was found among students. Out of a total of 1466 samples studied, 93.6% was non-tuberculous cases.

Conclusion: *Mycobacterium tuberculosis* is therefore not the predominant cause of cough in the study area as at the time of this study

Keywords: non-tuberculosis, recurrent cough, Ziehl-Neelsen, *Mycobacterium tuberculosis*

LA PRÉVALENCE DE LA TOUX RÉCURRENTE NON À DES PATIENTS AU SEIN DE L'ÉTAT DE CROSS RIVER, AU NIGÉRIA

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RÉSUMÉ

Introduction: La toux récurrente non devient monnaie courante chez les patients hospitalisés. Objectif : les patients hospitalisés et les patients externes dans certains hôpitaux dans l'État de Cross River ont été étudiés pour la prévalence de la toux récurrente non tuberculeuse dans l'état

Méthodes: Il a été réalisé à l'aide de procédures standard en microbiologie.

Résultats: Les isolats bactériens de l'échantillons d'expectoration étaient Staphylococcus aureus, Streptococcus pneumoniae, Streptococcus pyogenes, Haemophilus influenzae, Escherichia coli, Klebsiella pneumoniae, et de Corynebacterium diphtheriae. Le patients' le groupe d'âge 20 - 39 ans ont la plus forte prévalence de non - toux récurrentes tuberculeuse (46,7 %) tandis que la prévalence de moins de 1,0 % a été parmi le groupe d'âge de moins de 80 ans. .

Le taux de prévalence de 33,7 % a été le plus élevé parmi les classes sociales et d'affaires, alors que les taux de prévalence de 8,2 % a été constaté chez les élèves. Sur un total de 1466 échantillons étudiés, 93,6 % des cas non tuberculeuse -.

Conclusion: Mycobacterium tuberculosis n'est donc pas la principale cause de la toux dans la zone d'étude au moment de l'étude

Mots clés: non-tuberculose, toux récurrentes, Mycobacterium tuberculosis, Ziehl-Neelsen

INTRODUCTION

Some reflex actions are evidence that pathogens or pollen had gained entrance into the body and such action (s) is/are meant to eliminate the non-self component and excessive mucus. Cough is an example of such sudden mechanical action. It is controlled by same contraction of muscles that control breathing [1,2] and it expels deoxygenated contaminated air from the lungs. Coughing, a reflex response of the body caused by an irritation in the throat or windpipe, participate in protecting the delicate lung tissue from pathogens and other substances [2-4]. Apart from the need to eliminate infections, other causes of cough include allergies and cancer.

Cough can be acute, sub-acute or chronic[5]. Acute cough lasts for a short period of time not exceeding 21days. It is usually due to viral infection or bacterial infection of the upper respiratory tract infections (URTI) among others [2]. As soon as the infection being cleared is eliminated, acute cough seizes. So, it is usually transient. Chronic cough may be caused by a variety of underlying diseases including asthma, cystic fibrosis, allergies, gastroesophageal reflux disease (GERD), and chronic postnasal drip [6,7]. A cough may be productive or dry. A productive cough brings up mucus while a dry cough does not. Dry cough may result from a viral illness and may last up to several weeks. Children with pneumonia can appear sick with fever and a mild cough or be very ill with high fever and laboured breathing [8]. Pneumonia is caused either by viruses or bacteria. For proper diagnosis of cough, information regarding the duration of cough, other symptoms that may accompany it and the environmental factors that may influence it are very useful. The treatment of cough, like other symptoms depends highly on the severity and underlying cause of the cough. Acute cough like pneumonia may require antibiotics. Anti-tussives suppress a cough. Cough suppressants may lessen cough while dextromethorphan depresses the brain's cough centre.

Non – tuberculous Mycobacteria (NTM) have emerged as important opportunistic pathogen of the human being in recent years. They are ubiquitous as environmental organisms that sometimes cause respiratory disease usually in patients with pre-existing pulmonary damage [9]. Mycobacterial studies have been conducted on the citizens in South Africa [10, 11]. On one of such studies, the sputum from 1,196 Zulus in Natal, South Africa, the presence of mycobacteria was assessed[10] without recourse to clinical information. A NTM prevalence rates of colonization/infection was 1,400/100,000.

A similar research among patients with lower respiratory infection in Chest Clinic in Lagos, Nigeria showed the screening of six hundred and sixty eight ramdomly for pulmonary tuberculosis [12]. An observation of 11% NTM prevalence was made.

Information on NTM or non tuberculous recurrent cough in the study location was rare to the best of our knowledge. This study therefore was aimed at assessing the prevalence of non tuberculous recurrent cough in patients with recurrent cough in Cross River State, Nigeria.

MATERIAL AND METHODS

Sources and Collection of Samples

One thousand four hundred and sixty six sputa were collected from both in – patients and out – patients in hospitals with recurrent and productive cough in Cross River State. The study was carried out from January to October 2009 (10 months). Sputum from each patient was collected into a sterile universal bottle in the hospital and transported to the laboratory for analysis within 2 hours of collection. The hospitals studied represent different community/locations. The referral hospitals include Infectious Disease Hospital, Ogoja (IDHO), General Hospital, Ugep (GHU), General Hospital, Calabar (GHC), General Hospital, Obudu (GHB), General Hospital, Ikom, (GHI), General Hospital, Akamkpa (GHA), and General Hospital, Ogoja (GHO). The research was done without violating the ethical standards and the patients' right.

Processing Of Samples for Microscopic Examination

Ziehl Neelsen staining technique was used to stain each of the sputum samples to isolate samples which contained acid – fast bacilli (AFB) [13-17].

Sputum culture and Identification of bacteria

A sterile wire loop was used to aseptically inoculate each of the sputum samples on Blood agar, MacConkey agar and Chocolate agar for microbial morphology and identification. The seeded plates were then incubated. Blood agar and McConkey agar plates were incubated aerobically at 37^{o}C for 24 hours while chocolate agar plates were incubated in a carbon (IV) oxide enriched atmosphere at 37^{o}C for 24 hours. All the isolates were purified by sub-culturing. The pure culture of the isolate were characterized and identified using colonial morphology, Gram's staining, microscopic examination, biochemical tests and sugar ferment action profile. Biochemical tests carried out were coagulase, oxidase, catalase, citrate, urease, bile solubility, indole, Voges-Proskauer, methyl red, nitrate reduction, motility, and spore tests [18-20].

Statistical Analysis: The results were statistically analyzed using ANOVA with the $p>0.05$

RESULTS AND DISCUSSION

Out of the 1466 samples studied, five hundred and forty eight (37.4%) samples were collected from Infectious Disease Hospital, Ogoja; 169 (11.2%) were collected from General Hospital, Calabar, 120 (8.2%) from General Hospital, Ogoja; 126 (8.6%) from General Hospital, Ugep; 250 (17.1%) from General Hospital, Ikom; 189 (12.9%) from General Hospital, Obudu and 64 (4.4%) of the samples were collected from General Hospital, Akamkpa. 1372 (93.6%) were AFB-negative while 94 (6.4%) were AFB positive. The hospital which had the highest no of TB patients was infectious Disease Hospital, Ogoja with the prevalence of 532 (97.1%) out of 548 tested; followed by General Hospital, Ugep with the rate of 122 (96.8%) out of 126 screened, then General Hospital, Calabar 158 (93.5%) out of 169 screened, General Hospital, Obudu 178 (93.1%) out of 189 studied, General Hospital, Ogoja 110 (91.7%) out of 120 sampled, General Hospital, Akamkpa 56 (87.5%) out of 64 screened, while General Hospital, Ikom had the least prevalence 218 (87.2%) (Table1). Results of social class prevalence revealed that businessmen were the most affected group with the prevalence of 33.7%, followed by farmers with the rate of 28.5%; public/civil servants had the prevalence of 19.8% while dependents had 9.9% prevalence. However, students recorded the least prevalence of 8.2% (Table 2).

The age prevalence results revealed that adults aged 20 – 39 years had the prevalence of 46.7% which happened to be the highest. Adults aged 40 – 59 years recorded the prevalence of 25.2%, the age group between 60 – 79 years recorded 11.3% prevalence while children and adolescents between the age of 0 – 19 years old had the prevalence 9.5%; adults 80 years old and above recorded the least prevalence of 1.0% (Table 3) . The sex prevalence results revealed that male had the highest prevalence of 93.2% out of 755 samples studied (i.e 704 positive samples) while female had 94.0% prevalence of the 668 samples studied (Table 4) (i.e. 628 positive samples). The increase on the male prevalence might be due to active lifestyles leading to exposure to the aetiologies. There was statistical significant difference ($p>0.05$) in the prevalence among male and female subjects studied. Different bacteria encountered in this research include *Staphylococcus aureus, Streptococcus pneumonia, Streptococcus pyogenes, Haemophilus influenzae, Escherichia coli, Klebsiella pneumoniae, Corynebacterium diphtheriae*. The adults between the ages of 20 – 39 years had the highest prevalence with the rate of 46.7%, This high prevalence may be due to exposure to recreational activities. The highest prevalence of 33.7% recorded by businessmen in the social class may be as result of exposure to over crowded places during business. The prevalence of 93.6% in this study is comparatively higher than 6.7% prevalence of non - tuberculous mycobacteria in bronchiectatic population reported by Guang – Suo *et al*. [9]. Many researchers have undertaken some studies on detailed analysis of non – tuberculous mycobacteria (NTM). Wickremasinghe *et al*. [21] reported 2% of NTM patients in the context of bronchiectasis. Fowler *et al.* [22] reported 10% of prevalence of NTM out of 98 patients in a cohort of patients with adult onset of bronchiectasis. The result obtained from this study is in agreement with earlier report by Pasteur *et al*. [23] who found low prevalence in their studies. Serious emphasis should be laid on non-tuberculous Mycobacteria.

TABLE 1: DISTRIBUTION OF NON – TUBERCULOUS COUGH AMONG THE HOSPITAL STUDIED

Hospitals	Total Number of Samples	AFB-Negative	AFB-Positive
IDHO	548(37.4)	532(97.1)	16(2.9)
GHC	169(11.5)	158(93.5)	11(6.5)
GHO	120(8.2)	110(91.7)	108.3)
GHO	126(8.6)	122(96.8)	4(3.2)
GHI	250(17.1)	218(87.2)	32(12.8)
GHB	189(12.9)	178(93.1)	13(6.9)
GHA	64(4.4)	56(87.5)	8(12.5)
Total	1466(100)	1372(93.6)	94(6-4)

TABLE 2: SOCIAL CLASS PREVALENCE OF AFB-NEGATIVE CASES IN CROSS RIVER STATE

Location	Total AFB - Negative cases	Students	Business	Farmers	Public/Civil servant	Dependent
IDHO	532	30(5.6)	116(30.3)	156(29.3)	95(17.9)	90(16.9)
GHC	158	20(12.7)	59(37.3)	16(10.1)	55(34.8)	8(5.1)
GHO	110	20(18.2)	36(32.7)	28(25.5)	20(18.2)	6(5.5)
GHU	122	5(4.1)	46(37.7)	50(41.0)	16(13.1)	5(4.1)
GHI	218	18(8.3)	79(36.2)	74(33.9)	30(13.8)	17(7.8)
GHB	176	15(8.5)	71(40.3)	44(25.0)	36(20.5)	10(5.7)
GHA	56	4(7.1)	10(17.9)	23(41.1)	19(33.9)	0(0)
TOTAL	1372	112(8.2)	462(33.7)	391(28.5)	271(19.8)	136(9.9)

TABLE 3: AGE RELATED PREVALENCE OF NON-TUBERCULOSIS COUGH IN CROSS RIVER STATE

Location	Total Number of Sample	Age in Years				
		0-19	20-39	40-59	60-79	80 and above
IDHO	548	46(8.4)	226(41.2)	155(28.3)	97(17.7)	8(1.5)
GHC	169	19(11.2)	103(60.9)	28(16.6)	5(3.0)	3(1.8)
GHO	120	22(18.3)	43(35.8)	31(25.8)	12(10.0)	2(1.7)
GHU	126	7(5.6)	81(64.3)	26(20.6)	8(6.3)	0(0)
GHI	250	20(8.6)	119(47.6)	58(23.2)	21(8.4)	0(0)
GHB	189	18(19.5)	85(45.0)	52(27.5)	19(10.1)	2(1.1)
GHA	64	7(10.9)	27(42.2)	19(29.7)	3(4.7)	0(0)
TOTAL	146	139(9.5)	684(46.7)	369(25.2)	165(11.3)	15(1.0)

TABLE 4: PREVALENCE OF NON-TUBERCULOSIS RECURRENT COUGH IN CROSS RIVER STATE BASED ON SEX

Hospitals	Total No. of Samples	No. of Samples (%)		AFB-Negative Case	
		Male	Female	Male (%)	Female (%)
IDHO	548	296(54.0)	252(46.0)	287(97.0)	245(97.2)
GHC	169	72(42.6)	97(57.4)	65(90.3)	93(95.9)
GHO	120	69(57.5)	51(42.5)	65(94.2)	45(88.2)
GHU	126	49(38.9)	77(61.1)	46(93.9)	76(98.7)
GHI	250	140(56.0)	110(44.0)	123(87.9)	95(90.9)
GHB	189	99(52.4)	90(47.6)	91(91.9)	85(94.4)
GHA	64	30(46.9)	34(53.1)	27(90.0)	29(85.3)
Total	1466	755(51.5)	711(48.5)	704(93.2)	668(94.0)

CONCLUSION

Cough in the study area originated as a reaction from non non – tuberculous infection. The patients' age group 20 – 39 years had the highest prevalence of non – tuberculous recurrent cough while the least prevalence was among the age group ≥ 80 years. The prevalence rate of 33.7% was the highest among the social class and businessmen, while the least prevalence rate was found among students. There was statistical significant difference ($p>0.05$) in the prevalence among male and female subjects studied. Non – tuberculous prevalence rate of 93.6% was observed. *Mycobacterium tuberculosis* is therefore not the predominant cause of cough in the study area as at the time of this study.

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MOLECULAR STUDY OF *HELICOBACTER PYLORI* VIRULENCE GENES *CagA, Hpa* AND *BabA2* IN EGYPTIAN PATIENTS

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ABSTRACT
Objective: The objective of this study was to detect virulence genes of *Helicobacter pylori (H.pylori) cagA, babA2* and *hpa* in gastric biopsies from patients with different stages of gastritis by polymerase chain reaction to correlate the presence of genes with the severity of the diseases.
Method: A total of 80 non repetitive gastric biopsies from antrum of the stomach were obtained from the patients and subjected to study for histological examination, unease activity, culture for *H.pylori*, and polymerase chain reaction studies of virulence genes *cagA, babA2 and hpa*.
Results: The most frequent detected gene by PCR was hpa (66.7%) and followed by cagA and babA2 (61.6%) for each. There was significant association between the three genes (P=0.0001). The study of the association between the virulence gene of *H.pylori* and different clinical symptoms revealed significant association of dyspepsia with c*agA*(P=0.001) *babA2* and *hpa* (P=0.0001), regurgitation with *cagA* and *babA2*(P=0.002),vomiting with *cagA* and *babA2* (P=0.01, P=0.002, respectively) and nausea with *cagA* and *babA2* (P=0.0001, P=0.03, respectively). The virulence genes were detected in gastric ulcer. The degree of inflammation in histopathological examination was also statistically significant associated with the presence of virulences genes *cagA* (P=0.01), *babA2* (p=0.0001) and *hpa* (P=0.0001)
The present study highlights the presence of virulence genes in H.pylori associated with gastric ulcer. The genes cagA, babA2 and hpa are prevalent among the strains affecting the patients. Moreover, these genes are associated with marked clinical and pathological severity. The genes are significantly associated with each other. Further studies are recommended to validate these findings.

Keywords: Gastritis, Genotypes, H.pylori, cagA, babA2, hpa, PCR

ÉTUDE MOLÉCULAIRE DES GÈNES DE VIRULENCE HELICOBACTER PYLORI CagA, Hpa Et BabA2 DANS LES PATIENTS

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Résumé
Objectif: L'objectif de cette étude était de détecter des gènes de virulence de l'Helicobacter pylori (H. pylori cagA), babA2 et hpa dans les biopsies gastriques de patients atteints de différentes étapes de la gastrite la réaction en chaîne par polymérase à corréler la présence de gènes avec la gravité des maladies.
Méthode: un total de 80 biopsies gastriques répétitifs non d'antre de l'estomac ont été obtenus de patients et l'objet d'étude pour l'examen histologique, l'activité, la culture malaise pour H. pylori, et des études de réaction en chaîne de la polymérase de gènes de virulence, cagA babA2 et hpa.
Résultats: Le plus fréquemment détecté par PCR des gènes a été hpa (66,7 %) et suivie par babA et cagA2 (61,6 %) pour chacun. Il y avait une association significative entre les trois gènes (P =0,0001). L'étude de l'association entre les gènes de virulence de H. pylori et différents symptômes cliniques ont révélé une association significative de la dyspepsie cagA avec(P = 0,001) babA2 et hpa (P = 0,0001), régurgitation avec babA et cagA2(P =0,002), avec des vomissements et cagA babA2 (P = 0,01, P = 0,002, respectivement) et des nausées avec babA et cagA2 (P =0,0001, P =0,03, respectivement). Les gènes de virulence ont été détectés dans l'ulcère gastrique. Le degré d'inflammation dans l'examen histopathologique statistique était associé à la présence de gènes de virulence (cagA P =0,01), babA2 (p =0,0001) et hpa (P =0,0001)
La présente étude met en évidence la présence de gènes de virulence associée à H. pylori dans l'ulcère gastrique. Les gènes, cagA babA2 et hpa est très répandue chez les souches affectant les patients.

De plus, ces gènes sont associés à la mention de la gravité clinique et pathologique. Les gènes sont associés de façon significative avec l'autre. D'autres études sont recommandées pour valider ces résultats.

Mots-clés: gastrite, génotypes, H.pylori cagA,, babA2, hpa, PCR

INTRODUCTION

Helicobacter pylori (*H.pylori*) is spiral shaped gram negative bacilli that is associated with varieties of gastrointestinal disorders that range from mild gastritis up to gastric cancer. This bacterium is acquired during early adulthood, transmitted to family members and in the absence of adequate treatment leads to prolonged life time colonization (1). There is a strong association between the *H.pylori* and gastric cancer that leads to its classification as type 1 definite carcinogen by WHO (2).

H.pylori has several virulence factors that is associated with the severity of the clinical symptoms related to the infection by this pathogen (3). Among the known virulence factors are *ureA, cagA. VacA, dupA, bab* and *SabA. CagA* gene encodes a high immunogenic protein that has been used to identify *H.pylori*. *CagA* interacts with intracellular components of gastric epithelium and leads to its disruption with proinflammatory cytokines secreted due to the infection (4). *VacA* is another virulent gene that induce virulent factor leading to the host cells vaculation. This virulence factor is known to be a multi-receptor protein that leads to membrane depolarization, mitochondrial dysfunction, autophagy, activation of mitogen-activated protein kinases, inhibition of T cell function, and induction of apoptosis. These functions contribute to the persistent colonization of *H. pylori* and its pathogenesis in several upper digestive tract diseases(5) .

CagA gene is another virulent gene that has been studied extensively *as H.pylori* virulent gene (6). *CagA* gene encodes a highly immunogenic protein (7). In Western countries, it has been reported that individuals infected with *cagA*-positive *H.pylori* is at high risk for severe gastrointestinal disorders (8). *CagA* gene is located at one end of the cag pathogenicity island(PAI), that is inserted into *H.pylori* genome from an unknown source (9). *CagA* molecules are directly translocated into gastric epithelial cells by a bacterial type-IV secretion system(T4SS). cagA (10).

Another virulent gene factor is *H.pylori* agglutinin (HPa) that leads to formation of HPa that is a flagellar protein that binds to the surface of gastric mucosal cells. (11). *Hpa* genes code proteins that facilitate bacterial virulence by increasing the production of cytotoxin and cell adhesion to the host cell. The presence of these genes have severe clinical consequences in gastroduodenal patients associated with dyspepsia (11).

Outer membrane proteins (OMPs) are another important virulence proteins. There are several OMPs such as *BabA, SabA, AlpAB,* and *OipA* that have been predicted to play a vital role in adhesion of *H.pylori* to gastric mucosal cells. BabA2 protein is binding factor in *H.pylori*, that is identified by its ability to bind to B-blood type Lewis antigen on the epithelial cells of the stomach (12). The gene that codes BabA2 protein has two alleles babA1 inactive gene and babA2 is the active gene form . (12).

There are evidence that certain combinations of virulence factors such as presence of cagPAI with OMPs leads to virulent strains with severe clinical manifestations (13).

The objective of this study was to detect virulence genes of *Helicobacter pylori cagA, babA2* and *hpa* in gastric biopsies from patients with different stages of gastritis by polymerase chain reaction to correlate the presence of genes with the severity of the diseases.

Methods

The study was carried out in Mansoura University Hospital laboratory. Patients with various types of dyspepsia and subjected to diagnostic endoscopy were recruited from Gastroenterology Center, Mansoura University from January 2017 till January 2018. The study was performed according to the principles of Declaration of Helsinki and approval consents were obtained from each patients.

The inclusion criteria were patients above 18 years, eligible for endoscopy complaining of dyspepsia more than 2 weeks. Patients with history of antibiotics and acute dyspepsia due to drugs intake were excluded from the study.

Samples

A total of 80 non repetitive gastric biopsies from the antrum of the stomach were obtained from the patients and divided into two containers one container with 10% formalin for histopathological examination and the other with sterile normal saline in sterile container for microbiological studies. Samples were rapidly transported to the laboratory.

Histologic Evaluation

Hematoxylin and esosin staining was performed for slides according to the standard techniques of the examination and the grade of the inflammation was reported according to score from 0-3. Geimsa stain was used for detection of *H.pylori* (14).

Rapid Tube Urease Test

The gastric biopsies for microbiological studies were dissected and part of it was inoculated to agar tube to perform rapid unease test as described previously (15). The positive result was indicated by turn of the media to pink color after one hour

incubation at 35° C.

Culture for *H.pylori*

Part of the biopsies in sterile saline was homogenized by sterile glass road and inoculated to Columbia blood agar supplied by 5% of blood and incubated for 10 days under microaerophilic conditions supplied by gas packs (Campy Pak; Becton Dickinson) at 37°C. The colonies were identified by gram stain, positive urease, oxidase and catalase biochemical reactions (16).

Positive samples for H.pylori was interpreted according to positive results of any laboratory tests positive urease, culture and/ or histopathology (17).

Polymerase Chain Reaction for Virulence Genes (PCR)

DNA Extraction

DNA was extracted from part of gastric sample transported in sterile saline that was positive for *H.pylori*. DNA was extracted by mini kit of Qiagen used for tissue DNA extraction according to the manufacturer recommendations (Qiagen extraction kit). The extracted DNA was kept at -20^{o}C till amplification procedures.

Multiplex PCR for amplification of *babA* and *hpa* Genes

The primers sequences used for amplification of *babA* and *hpa* genes were summarized in table 1 (20).

The total volume of the amplification reaction was 50 μL with 5μL DNA template and 5 μL 10X PCR buffer, 2.5 mM Mgcl2, 0.2 mM of each dNTP, 1 μL forward and reverse primer, and 2U Taq polymerase enzyme (Qiagen).

The PCR cycling situations were as follow: original denaturation stage at 95 for 5 minute, 35 cycles of 95°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute, and last extension phase at 72°C for 5 minutes. PCR outcomes were analyzed by 1.5% agarose gel electrophoresis premixed with ethidium bromide and visualized under a UV transilluminator (18).

PCR for *cagA*

The total amount of the amplification mixture was 25 µl with 3 µ of the extracted DNA and 10 pmol of primers of cag5c-F and cag3c-R added to ready to use amplification mixture supplied by Qiagen. Products were amplified using Perkin-Elmer 9700 thermal cycler with the following program denaturation for 3 min at 94°C, 35 cycles of sequential 1 min at 94°C-1 min at 55°C- 1 min at 72°C, and finally 10 min at 72°C. Detection was performed by gel electrophoresis 2% for 20 minutes (19).

Statistical Analysis

Data were collected, revised, coded and entered to the statistical package for social science (SPSS) version 20. The quantitative data were presented as mean, standard deviations and ranges. The comparison between the studied groups were done by using One Way Analysis of Variance (ANOVA). P was considered significant>0.05.

RESULTS

The present study included 80 patients, 42 females (52.5%) and 38 males (47.5%) with mean± age SD 56.1± 10.5 years. The most frequent symptoms were regurgitation (90.0%), abdominal pain (87.5%), vomiting (86.3%) and dyspepsia (83.8%). The most frequent findings of the endoscopic examination was gastric ulcer (50%). The histopathological examination of the gastric biopsies revealed moderate inflammation in 25% of the samples and severe inflammation in 18.8%. *H.pylori* was positive by culture in 45%, by histopathological examination in 45% and by rapid urease test in 41.3%. The total positive samples by any of the used methods was 45%, table 2.

TABLE (1): THE PRIMERS SEQUENCES AND BP OF THE AMPLIFICATION PRODUCTS

Gene	Primer Sequences	bp
bab A2	**5′-AATCCAAAAAGGAGAAAAAGTATGAAA-3′** **5′-TGTTAGTGTGATTTCGGTGTAGGACA-3′**	**832**
hpa	**5′-ATAAAGCTTTCGGTG GTGGTGGAACGATG-3′** **5′-TATCTCGAGTTGTCGGTTTCTTTGC-3′**	**850**
cagA '	**5′-GTTGATAACGCTGTCGCTTC-3′** **5′-GGGTTGTATGATATTTTCCATAA-3′**	**350**

1TABLE (2): DEMOGRAPHIC, CLINICAL AND LABORATORY FINDINGS OF PATIENTS (N=80)

Gender **Male (N0.-%)** **Female (N0.-%)**	 **38 47.5** **42 52.5**
Age (mean SD-years)	**56.1± 10.5**
Dyspepsia(N0.-%)	**67 83.8%**
nausea(N0.-%)	**60 75%**
Abdominal pain (N0.-%)	**70 87.5%**
Vomiting (N0.-%)	**69 86.3%**
Regurgitation (N0.-%)	**72 90.0%**
Floating (N0.-%)	**8 10.0%**
Dyspepsia (N0.-%)	**20 25%**
Gastric ulcer (N0.-%)	**40 50%**
Duodenal ulcer (N0.-%)	**20 25%**
Grade of inflammation **Mild (N0.-%)** **Moderate(N0.-%)** **severe(N0.-%)**	 **15 18.8%** **20 25.0%** **15 18.8%**
Culture (N0.-%)	**36 45%**
Histopathology (N0.-%)	**36 45%**
Rapid urease tube test (N0.-%)	**33 41.3%**
Positive for H.pylori (N0.-%)	**36 45%**

The comparison of the clinical and pathological findings of the patients with *H.pylori* and those without, revealed significant association of dyspepsia (P=0.0001), regurgitation (P=0.001), vomiting (P=0.001), abdominal pain (P=0.0001) and nausea (P=0/003) and presence of *H.pylori*. Moreover, the degree of the inflammation by pathological examination revealed significant association of moderate (44.4%) and severe inflammation in patients with *H.pylor*i compared to those without *H.pylori* (P=0.0001), table 3.

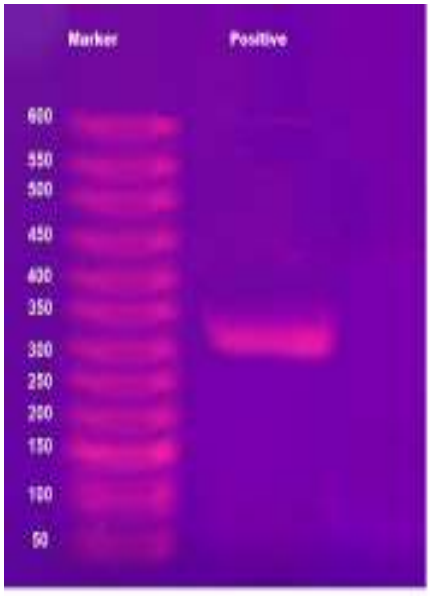

FIGURE (1): PCR POSITIVE FOR CagA GENE COMPARED TO MARKER

TABLE (3): THE COMPARISON OF THE CLINICAL AND PATHOLOGICAL FINDING BETWEEN THE PATIENTS WITH H.PYLORI AND PATIENTS WITHOUT H.PYLORI

	Positive (n=36) No. %	**Negative (n=54) No. %**	**P**
Gender **Male** **Female**	 **20 55.6%** **16 44.4%**	 **18 33.3%** **36 66.7%**	**P=0.2**
Dyspepsia	**24 66.7%**	**5 9.2%**	**P=0.0001**
Regurgitation	**8 22.2%**	**0 0%**	**P=0.001**
vomiting	**10 27.8%**	**1 1.9%**	**P=0.001**
nausea	**9 25%**	**1 1.9%**	**P=0.003**
Abdominal pain	**16 44.4%**	**2 3.7%**	**P=0.0001**
Floating	**6 16.7%**	**2 3.7%**	**P=0.1**
Gastric ulcer	**36 100%**	**0 0%**	**P=0.001**
Grade of inflammation **No** **Mild** **Moderate** **severe**	 **0 0%** **7 19.4%** **16 44.4%** **13 36.1%**	 **28 51.9%** **8 14.8%** **6 11.1%** **2 3.7%**	**P=0.0001**

TABLE (4): THE FREQUENCY OF THE VIRULENCE GENES IN H.PYLORI

Gene	No. %
cagA	**22 61.15%**
babA2	**22 61.6%**
hpa	**24 66.7%**

The most frequent detected gene by PCR was *hpa* (66.7%) and followed by *cagA* and *babA2* (61.6%) for each, Table 4. There was significant association between the three genes (P=0.0001), data not shown.

The study of the association between the virulence gene of *H.pylori* and different clinical symptoms revealed significant association of dyspepsia with *cagA*(P=0.001) *babA2* and *hpa* (P=0.0001), regurgitation with *cagA* and *babA2*(P=0.002),vomiting with *cagA* and *babA2* (P=0.01, P=0.002, respectively) and nausea with *cagA* and *babA2* (P=0.0001, P=0.03, respectively). The virulence genes were detected in gastric ulcer. The degree of inflammation in histopathological examination was also statistically significant associated with the presence of virulences genes *cagA* (P=0.01), *babA2* (p=0.0001) and *hpa* (P=0.0001), table 5.

TABLE (5): THE ASSOCIATION OF DETECTED GENES WITH CLINICAL AND PATHOLOGICAL FINDINGS.

	CagA	BabA2	hpa
Gender			
Positive	**13**	**14**	**12**
Male	**9**	**8**	**12**
Female	**25**	**24**	**27**
Negative	**33**	**34**	**29**
P	**0.2**	**0.1**	**0.5**
Age			
Positive	**55.01 7.6**	**55.6 8.9**	**54.7 7.5**
Negative	**56.2 7.1**	**56.3 6.4**	**56.6 6..9**
P	**P=0.9**	**P=0.7**	**P=0.3**
Dyspepsia	**14**	**15**	**15**
P	**P=0.001**	**P=0.0001**	**P=0.0001**
Regurgitation	**6**	**6**	**5**
P	**P=0.002**	**P=0.002**	**P=0.05**
Vomiting	**7**	**6**	**5**
P	**P=0.01**	**P=0.002**	**P=0.05**
Abdominal pain	**5**	**6**	**5**
P	**P=0.1**	**P=0.02**	**P=0.2**
Nausea	**12**	**9**	**9**
P	**P=0.0001**	**P=0.03**	**P=0.2**
Floating			
	3	**4**	**4**
P	**P=0.5**	**P=0.2**	**P=0.2**
Gastric ulcer	**22**	**22**	**24**
Grade of inflammation			
No	**2**	**1**	**0**
Mild	**5**	**5**	**5**
Moderate	**7**	**6**	**9**
severe	**8**	**10**	**10**
	P=0.01	**P=0.0001**	**P=0.0001**

DISCUSSION

The endoscopic examination for patients complaining of upper gastrointestinal disorders such as dyspepsia is an essential tool for appropriate diagnosis for differentiation between functional and organic disorders (20).

In the present study endoscopic examination revealed that 50% of the patients had gastric ulcer and 25% had duodenal ulcer and 25% had simple dyspepsia. Previous study reported that simple dyspepsia can be detected in up to 30% of patients upon endoscopic examination (20). Therefore

endoscopic examination and biopsies of patients with dyspepsia is recommend for appropriate diagnosis.
In the present study all gastric ulcer were due to presence of *H.pylori* similar to previous reports (20). On contrary , there were various reports about the decline of *H.pylori* infections in developed countries due to improved sanitation (21, 22). However, in patients with age above 50 years like those in the present study still more affected by *H.pylori* due to earlier exposure.

The virulence genes associated with *H.pylori* also affect the outcome of the infection. The adhesion of *H.pylori* to gastric epithelium leads to symptomatic infections and persistence of infection (4). Among the virulence factors is *babA2* which attach to the blood group antigen Lewis-b present in the gastric epithelium cells. BabA2 was a frequent virulence gene detected in the present study in 61.6% of the positive samples for *H.pylori*. This prevalence was similar to previous reports with range for detection of this gene from 71.6% up t0 82.3% (23, 24) and higher than other report (18).
BabA2 gene is associated with severe inflammatory reactions in gastrointestinal epithelium and it was reported to be a marker to identify patients who will develop severe forms of *H.pylori* associated disease (25). This statement was on line with our findings of the association of babA2 with severe clinical symptoms and pathological findings of moderate to severe pathological scores.

The most prevalent virulence gene detected in the present study was *hpa* gene which was detected in 66.7% of *H.pylori*. This prevalence is similar to that reported by previous study of Heider et al., 2017 (18). *Hpa* gene produce adhesion protein A which is a conserved protein of the surface lipoprotein of *H.pylori* that is essential for adhesion of *H.pylori* and induction of the inflammatory immune response with specific productions of antibodies to it. This ability to produce specific humoral response is being studied and it gives the appeal for *hpa* to be candidate to develop a vaccine for *H.pylori* (26).

Hpa gene was associated also in the present study with severe clinical symptoms, association with the presence of gastric ulcer and with moderate to severe inflammatory scores in the histopathological examination. In previous study *hpa* was the most frequent detected in gastric ulcer, however there was no significant association was reported with the presence of this gene and the severity of the clinical presentation (27). This difference between the findings in the present study and previous might attributed to the finding of the significant association of the different virulence gens present in the present study. So, virulence genes association enhance the pathogenic effects of each other.
The other virulence gene studied in the present study was *cagA* gene. The prevalence of *cagA* gene, another virulence gene of *H.pylori*, was 61.6%. Previous studies reported that the prevalence of cagA gene van reach up to 100% among *H.pylori* strains by different methods of studies either serological or by PCR (28, 29). However, other studies reported a lower prevalence rates (30,31). The prevalence rates of cagA depends upon the geographical location of the isolates.

There was association with gastric ulcer and the presence of *cagA* gene and also significant association of severe clinical symptoms and moderate to severe degree of the inflammation of the histopathology scores. This findings were similar to previous studies (31,32). *CagA* factor is known to be a strong inducer to the pro inflammatory cytokines such interleukin 8 associated with severe inflammatory response in the gastric mucosa (33). CagA also is associated to the risk of gastric cancer development due to H.pylori infection (34).
The present study highlights the presence of virulence genes in H.pylori associated with gastric ulcer. The genes cagA, babA2 and hpa are prevalent among the strains affecting the patients. Moreover, these genes are associated with marked clinical and pathological severity. The genes are significantly associated with each other. Further studies are recommended to validate these findings.

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ASYMPTOMATIC BACTERIURIA IN AN APPARENTLY HEALTHY POPULATION AND ITS RELATION TO HYPERTENSION

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ABSTRACT
Background
Hypertension is a major health problem in sub-Sahara Africa. Several studies have suggested a role of asymptomatic bacteriuria (ASB) in the aetiology of hypertension, but there is a dearth of information on this association in Africa where the burden of hypertension is high. We investigated the prevalence of asymptomatic bacteriuria, its association with hypertension and determined the antibiotic resistance patterns of implicated bacterial isolates in an urban community of Ile-Ife.
Methods
One hundred and seventy-four apparently healthy individuals were investigated for ASB. Relevant information was obtained from them with standard proforma. Their Blood pressure was measured with a standard mercury sphygmomanometer. All samples were processed on cysteine lactose electrolyte deficient medium and chocolate agar. Antimicrobial susceptibility testing was done using Kirby-Bauer disk diffusion technique.
Results
Fifty (28.7%) individuals were positive for ASB. ASB was commonly detected among the female subjects (χ^2=5.619; p-value = 0.01777), and among individuals in the age group of 50-59 years. Those that were hypertensive were two and a half times more likely to have ASB (Odd ratio=2.5; p-value=0.01369; CI=1.19-5.35). The highest percentage of hypertensive female participants with ASB was found in the age group of 30-39 years (33.3%) while among the male participants, the highest percentage was found in the age group of 60-69 years (9.5%). *Escherichia coli* (n=13; 26%) and *Staphylococcus aureus* (n=13; 26%) were the commonest organisms implicated in ASB. The majority of the isolates (>90%) were multidrug resistant. Isolates of *Escherichia coli* were commonly resistant to ampicillin (83.3%), ceftriaxone (72.7%) and cefepime (66.7%). Isolates of *Staphylococcus aureus* were all (100%) resistant to erythromycin, cloxacillin and streptomycin. All isolates were least resistant to cotrimoxazole (<8%).
Conclusion
Women as well as men in the age group of 50-59 years were more likely to develop ASB. ASB could be contributing to the rising incidence of hypertension in this environment. Co-occurrence of hypertension and ASB portends a grave problem for apparently healthy individuals in this environment.

Keywords: Asymptomatic bacteriuria, Hypertension, *Escherichia coli*, healthy individuals, urban community

LA BACTÉRIURIE ASYMPTOMATIQUE DANS UNE POPULATION APPAREMMENT EN BONNE SANTÉ ET DE SON RAPPORT À L'HYPERTENSION

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Résumé

Contexte: L'hypertension est un problème de santé majeur en Afrique subsaharienne. Plusieurs études ont suggéré un rôle de la bactériurie asymptomatique (ASB) dans l'étiologie de l'hypertension, mais il y a une pénurie d'information sur cette association en Afrique où la charge de l'hypertension est élevée.

Nous avons étudié la prévalence de la bactériurie asymptomatique, son association avec l'hypertension et déterminé les tendances de la résistance aux antibiotiques des isolats bactériens impliqués dans une collectivité urbaine d'Ile-Ife.

Les methods: 1 cent soixante-quatre personnes apparemment en bonne santé ont été examinés pour l'ASB. Les informations pertinentes ont été obtenues à partir de celles-ci avec une proforma standard. Leur pression artérielle a été mesurée à l'aide d'un sphygmomanomètre au mercure standard. Tous les échantillons ont été traités sur un milieu déficient en cystéine d'électrolyte de lactose et de chocolat. Les tests de sensibilité aux antimicrobiens a été effectuée à l'aide technique de diffusion disque Kirby-Bauer.

Résultats: Cinquante (28,7 %) personnes ont été positifs pour l'ASB. ASB est souvent détecté chez les sujets de sexe féminin ($\chi 2$ = 5,619 ; p = 0,01777), et parmi les individus dans le groupe d'âge 50-59 ans. Ceux qui n'ont de l'hypertension étaient deux fois et demie plus susceptibles d'avoir ASB (Odd ratio =2,5 ; valeur p =0,01369 ; IC = 1.19-5.35). Le plus haut pourcentage de participants féminins hypertendus avec ASB a été trouvé dans le groupe d'âge 30-39 ans (33,3 %) alors que chez les hommes, la proportion la plus forte a été observée dans le groupe d'âge 60-69 ans (9,5 %). L'Escherichia coli (n =13 ; 26 %) et Staphylococcus aureus (n =13 ; 26 %) ont été les plus fréquemment impliqués dans des organismes ASB. La majorité des isolats (>90 %) étaient multirésistantes. Les isolats d'Escherichia coli ont été fréquemment résistantes à l'ampicilline (83,3 %), à la ceftriaxone (72,7 %) et cefepime (66,7 %). Les isolats de Staphylococcus aureus ont été tous (100 %) résistantes à l'érythromycine, la cloxacilline et la streptomycine. Tous les isolats étaient résistants à au moins le cotrimoxazole (<8 %).

Conclusion: Les femmes ainsi qu'aux sujets dans le groupe d'âge 50-59 ans sont plus susceptibles de développer l'ASB. ASB pourraient contribuer à l'augmentation de l'incidence de l'hypertension dans cet environnement. La co-occurrence de l'hypertension et de l'ASB laisse présager un problème grave pour les personnes apparemment en bonne santé dans cet environnement.

Mots clés: La bactériurie asymptomatique, l'hypertension, l'Escherichia coli, les individus en bonne santé, communauté urbaine.

INTRODUCTION

Asymptomatic bacteriuria (ASB) is the presence of a positive urine culture with at least 10^5 cfu/ml collected from a patient with no symptoms or signs of urinary infection (1). ASB is common in people with abnormal genitourinary tract condition, and its prevalence varies among diverse populations, and depends on sex, age and conditions like diabetes mellitus or spinal cord injury and the presence of functional or structural genitourinary abnormalities (2). The prevalence of ASB increases with age in both men and women. In young women, the prevalence of ASB is 1–5%, and it increases to 6–16% in women over the age of 65 years (3). In healthy women above 80 years that reside in the community, the prevalence of ASB is about 20%. Asymptomatic bacteriuria is uncommon in healthy men before 60 years of age, but for ageing men in the community the prevalence rates of ASB is 3.6%–19% (3). Complications associated with asymptomatic bacteriuria include urolithiasis, genitourinary cancers, renal failure, hypertension and even death (4).

Hypertension is one of the major health problems in sub-Sahara Africa. It is the major cause of 50% of heart disease, stroke and heart failure. Due to significant improvements in the control of infectious diseases, and increased risk of cardiovascular disease (CVD) and kidney disease, attention has shifted to the control of non-invasive diseases and high blood pressure. However, unwillingness of patients with high blood pressure to seek for medical care, asymptomatic nature of the disease as well as poverty, increasing urbanization and bad eating habits have made the disease difficult to control, hence its rising incidence in Africa (5).

The role of asymptomatic bacteriuria (ASB) in the aetiology of hypertension has been reported by several authors in the past (4, 6–8). Those studies have shown that patients with renal scarring caused by pyelonephritis are more likely to develop hypertension and chronic kidney disease. For instance, Kass observed small differences in blood pressure between 444 bacteriuric and non-bacteriuric women who were watched for the development of high blood pressure in relation to *E. coli* bacteriuria at baseline. *E. coli* bacteriuria was associated with the development of hypertension during follow-up (1). In another study, Sinha and Postlethwaite reported that, a proportion of children who have suffered from a urinary tract infection (UTI) will go on to develop renal scarring-which could lead to systemic arterial hypertension (8). A more recent study by Meiland *et al.* also suggested that bacteriuria may increase the risk of future hypertension in a population of asymptomatic women that were followed up (9). In spite of these reports, there is a dearth of information on the role of ASB in the aetiology of hypertension in Africa where the burden of hypertension is high, and its pathogenesis remains unclear. Considering these facts, we decided to screen apparently healthy urban community dwellers (men and women) between the age of 20 and 70 years for ASB, determine its association with hypertension and the susceptibility patterns of implicated pathogens.

METHODS

Study population

The study was conducted in Ile-Ife, an urban community in South Western Nigeria. One hundred and seventy-four apparently healthy individuals within the age group of 20-70 years were recruited into the study from January to December, 2015. Informed consent was obtained from each participant prior to sample collection.

Collection and processing of samples

Midstream clean voided urine samples were collected from participants and sent to the laboratory for investigations. Asymptomatic bacteriuria was defined as the presence of 10^5 bacteria in 1mL of urine. Plating of the samples was performed using a calibrated loop of 1 microliter on CLED and chocolate media (Oxoid, England), and incubated for 24h in aerobic conditions at 35°C. Bacterial identification was performed using conventional biochemical tests (10).

Blood pressure

Blood pressure was measured with a standard mercury sphygmomanometer (Accoson, England, United Kingdom) after the subject had been seated for five minutes. In this study, hypertension was defined as the previous use of antihypertensive medication (assessed at follow-up by the question: "Have you ever been treated with drugs for high blood pressure?") and/or a measured systolic blood pressure of at least 140 mm Hg or a diastolic blood pressure of 90 mm Hg or higher (11).

Antimicrobial susceptibility testing

Antibiotic susceptibility testing was done according to Clinical and Laboratory Standard Institute (CLSI) modified Kirby-Bauer method. A sterile cotton swab was dipped into each of the standardized solution of bacterial cultures and used for even inoculation of Mueller-Hinton plates (Himedia, Mumbai) and allowed to dry. Thereafter, antibiotic discs with the following drug contents: Amoxicillin (25µg), Ceftriaxone (30µg), Chloramphenicol (10 µg), Co-trimoxazole (25 µg), Erythromycin (10 µg), Gentamicin (10 µg), Ofloxacin (30µg), Perfloxacin (30µg), Tetracycline (30 µg), Amoxicillin/clavulanic acid (30 µg), and Cloxacillin (10 µg), Nitrofurantoin (300 µg), Cefuroxime (30 µg), Ceftaxidime (30µg), Nalidixic acid (30 µg), Penicillin (10 units), Streptomycin (10 µg), Cefepime (30µg) were placed on the plates, spacing them well to prevent the overlapping of inhibition zones. The plates were incubated at 37°C for 24 h, and the diameters were measured. The results were read and interpreted as recommended by the CLSI (10).

Statistical analysis

All data were analyzed with R Statistical package (12). Chi square ($\chi 2$) test and t-test were used to test for statistical comparisons between the groups and a $p < 0.05$ was considered as statistically significant.

RESULTS

Fifty (28.7%) of 174 individuals that were investigated for ASB were positive, while 124 (71.3%) were negative. Of the 50 individuals that were positive for ASB, 34 (37%) were female and 16 (19.5%) were male. There was no statistical significant difference between the mean age of those with ASB (52.6800) and the mean age of those without ASB (50.1129) (t=-1.9653; p-value= 0.05167). Furthermore, as shown in table 1, the prevalence of ASB is significantly higher among the female subjects (χ^2= 5.619; p-value = 0.01777).

The distribution of ASB among the various age groups revealed that the frequency of ASB increases with age and it is relatively higher among the female counterparts (Table 2).

TABLE 1: PREVALENCE OF ASB IN RELATION TO GENDER

Sex	Asymptomatic bacteriuria (%)		P value
	No	Yes	
Female	**58 (63.0)**	**34 (37.0)**	**χ^2 = 5.619; p-value = 0.01777**
Male	**66 (80.5)**	**16 (19.5)**	
Total	**124 (71.3)**	**50 (28.7)**	
Mean age	**50.1129**	**52.6800**	**t= -1.9653; p-value = 0.05167**

TABLE 2: PREVALENCE OF ASB IN RELATION TO AGE OF PATIENTS

Age (years)	Sex	No of subjects	No.with Asymptomatic bacteriuria (%)	ASB Total
20-29	M	2	0 (0)	0((0)
	F	1	0(0)	
30-39	M	5	0 (0)	3 (20)
	F	10	3 (30)	
40-49	M	22	3 (13.6)	9 (17.3)
	F	30	6 (20)	
50-59	M	38	10 (26.3)	33 (40.7)
	F	43	23 (53.5)	
60-69	M	13	3 (23.1)	5 (23.8)
	F	8	2 (25)	
≥70	M	2	0 (0)	0(0)
	F	0	0 (0)	

ASB and Blood Pressure

Of 174 individuals whose blood pressure was measured, 91 were hypertensive, while 83 were normotensive. Thirty-four (68%) individuals with high blood pressure had ASB while 16 (32%)

individuals that were normotensive had ASB. Twenty-three (67.6%) of the 34 individuals with high blood pressure were females while 11(32.3%) were males. As shown in table 3, there was an association between asymptomatic bacteriuria and high blood pressure in this study (χ^2= 6.0783; p-value = 0.01369), and the hypertensive individuals were two and a half times likely to have ASB compared with those that are normotensive (Odd ratio=2.5; p-value=0.01369; CI=1.19-5.35).

Figure 1 shows the age categories of healthy individuals with hypertension and bacteriuria. The highest percentage of hypertensive female participants with ASB was found in the age group of 30-39 years (33.3%) followed by the age group of 50-59 years (18.5%). However, among the male participants, the percentage was highest in the age group of 60-69 years (9.5%) followed by the age group of 50-59 years (7.4%). No ASB was detected among the hypertensive female participants in the age groups of 20-29 years and 70 years and above. Among the hypertensive male participants, no ASB was detected in the age groups of 30-39 and 70 years and above.

TABLE 3: PREVALENCE OF ASB IN RELATION TO PATIENTS' BLOOD PRESSURE

Blood Pressure	Asymptomatic Bacteriuria							Statistics		
	No (n=124)			Yes (n=50)			Total (n=174)	Chi-squared ; p-value	Odd ratio	Confidence Interval
	Male	Female	Total	Male	Female	Total				
High	35 (28.2)	22 (17.7)	57 (46)	11 (22)	23 (46)	34 (68)	91(52.3)	χ^2= 6.0783; p-value = 0.01369	2.50	1.19-5.35
Normal	31 (25)	36 (29)	67 (54)	5 (10)	11 (22)	16 (32)	83(47.7)			

*Pearson's Chi-squared test with Yates' continuity correction

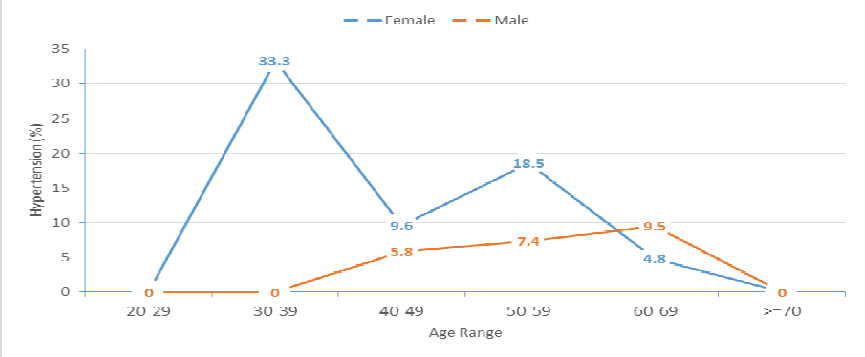

FIGURE 1: AGE CATEGORIES OF HYPERTENSIVE INDIVIDUALS WITH BACTERIURIA

Frequency of Bacterial Isolates

Among all the bacterial species isolated from the urine samples of those with ASB, *Escherichia coli* (n=13; 26%) and *Staphylococcus aureus* (n=13; 26%) were the commonest, followed by *Klebsiella* spp (n=11; 22%) and *Pseudomonas aeruginosa* (n=10; 20%). *Pseudomonas aeruginosa* predominated (13) among the female subjects while *Staphylococcus aureus* (6) predominated in their male counterparts. *Morganella morgani* and *Proteus mirabilis* were isolated only from the female subjects.

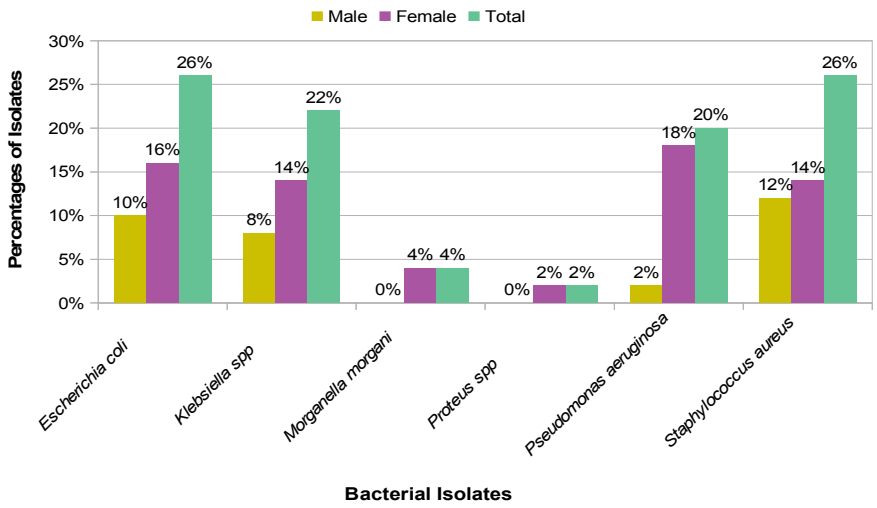

FIGURE 2: PERCENTAGES OF BACTERIAL ISOLATES IN RELATION TO SEX

Antimicrobial resistance patterns of the isolates

The majority of the Gram negative isolates were resistant to most of the antibiotics tested. Many isolates (>65%) of *Klebsiella*, *Pseudomonas* and *Morganella* were resistant to ampicillin, gentamicin, ceftriaxone and augmentin. Isolates of *Escherichia coli* exhibited high percentages of resistance to ampicillin (83.3%), erythromycin (83.3%) and ceftriaxone (72.7%). *Staphylococcus aureus* isolates were commonly resistant to erythromycin (100%), tetracycline (92.3%), ampicillin (92.3%), cloxacillin (100%), penicillin (100%), and streptomycin (100%). All isolates were least resistant to cotrimoxazole (<30%).

TABLE 3: ANTIMICROBIAL RESISTANCE PATTERNS OF THE ISOLATES

Organisms	ERY	GEN	NIT	CEFTR	CEFTA	OFLO	AUG	TET	COT	AMP	CLOX	PEN	STR	CEFP
Escherichia coli (n=13)	5/6 (83.3)	8 (62.5)	4/9 (44.4)	8/11 (72.7)	9 (69.2)	6/12 (50)	8 (62.5)	0 (0)	1 (7.7)	5/6 (83.3)	NT	NT	NT	4/6 (66.7)
Klebsiella spp. (n=11)	1/1 (100)	9 (81.8)	7/10 (70)	9 (81.8)	9 (81.8)	7(63.6)	7(63.6)	0 (0)	0 (0)	10 (90.9)	NT	NT	NT	6 (54.5)
Morganella morgani (n=2)	0 (0)	2 (100)	0 (0)	2 (100)	1 (50)	0 (0)	2 (100)	0 (0)	0 (0)	2 (100)	NT	NT	NT	0 (0)
Proteus spp (n=1)	1 (100)	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	NT	NT	NT	0 (0)
Pseudomonas aeruginosa (n=10)	1/1 (100)	8 (80)	4 (40)	6/8 (75)	4/7 (57.1)	5 (50)	7 (70)	3 (30)	3 (30)	7 (70)	NT	NT	NT	4/7(57.1)
Staphylococcus aureus (n=13)	13 (100)	9 (69.2)	NT	NT	0 (0)	NT	NT	12 (92.3)	1 (7.7)	12 (92.3)	13 (100)	13 (100)	13 (100)	0 (0)

NT= Not tested; ERY=Erythromycin; GEN= Gentamicin; NIT=Nitrofurantoin; CEFTR=Cetfriaxone; OFLO=Ofloxacin; AUG=Augmentin; TET=Tetracycline; COT=Cotrimoxazole; AMP=Ampicillin; CLOX=Cloxacillin; PEN=Penicillin; STR=Streptomycin; CEFP=Cefepime

Multi drug resistance

Multi-drug resistance in this study was defined as resistance of an isolate to three or more classes of antibiotics (Magiorakos *et al.*, 2012). Majority of the isolates (>90%) were multidrug resistant. 92.3% and 90.9% of *Escherichia coli* and *Pseudomonas aeruginosa* isolates were resistant to three or more classes of antibiotics respectively. All isolates (100%) of *Klebsiella* spp, *Morganella morgani, Proteus* spp., and *Staphylococcus aureus* were resistant to three or more classes of antibiotics

.

TABLE 5: MULTIDRUG RESISTANCE PATTERNS OF THE ISOLATES

Organisms	No of isolates resistant to classes of antibiotics (%)		
	1	2	≥3
Escherichia coli (13)	0 (0)	1(7.7)	12 (92.3)
Klebsiella spp (11)	0 (0)	0 (0)	11 (100)
Morganella morgani (2)	0 (0)	0 (0)	2 (100)
Proteus spp (1)	0 (0)	0 (0)	1 (100)
Pseudomonas aeruginosa (11)	0 (0)	1 (9.1)	10 (90.9)
Staphylococcus aureus (13)	0 (0)	0 (0)	13 (100)

DISCUSSION

Asymptomatic bacteriuria (ASB) in healthy people is a minor problem that requires no treatment or clinical follow up, but it is considered relevant when there are underlying conditions; such as pregnancy, renal transplantation, severe neutropenia, urologic disorders (13).

The prevalence of ASB in the studied population was 28.7%, and ASB was commonly detected among the female subjects (χ^2 = 5.8098; p-value = 0.01594). Our finding is comparable to the reports of previous investigators in Nigeria (14, 15). This might be due to the fact that women possess shorter urethra, which gives bacteria from the urethral meatus and the perineum a shorter distance to the bladder (16).

The prevalence of ASB was highest in the age group of 50-59 years. Among the male subjects, the prevalence of ASB increased with age and peaked at the age group of 50-59 years. Likewise, among the females, the prevalence of ASB peaked at age group 50-59 years. This finding could be attributed to changes in postmenopausal status or presence of comorbidities. This is likely true of the later as 25.9% of participants with ASB and hypertension were found in the age group 50-59 years old.

The role of ASB in the aetiology of hypertension has been reported by several investigators in the past but the pathogenesis is not understood (4, 6–8). For example, a cohort study of 444 women who were followed for the development of hypertension in relation to *E. coli* bacteriuria at baseline suggests that bacteriuria increase the chance to develop hypertension, and that those who were hypertensive were more likely to develop ASB than those who were normotensive . Considering the fact that the incidence of hypertension is increasing in Africa due to poverty, urbanization, bad eating habit, etc, and the possibility of ASB is adding to this increase. Thus, of the 174 individuals that were studied, thirty-four (68%) of 91 hypertensive individuals had ASB, while 16 (32%) of 83 normotensive individuals had ASB which suggested an association between asymptomatic bacteriuria and hypertension (χ^2= 6.0783; p-value = 0.01369), and those who were hypertensive were two times more likely to have ASB (Odd ratio=2.5; p-value=0.01369; CI=1.19-5.35) when compared with those that were normotensive. Our finding agrees with the observations of previous investigators that reported an association between bacteriuria and hypertension (4, 7, 8). Although, we did not determine whether bacteriuria preceded hypertension or vice versa, neither did we prove that a causal relationship exists. A plausible explanation for our findings would be that hypertension arises secondary to renal scarring caused by the uropathogens, even though the participants were asymptomatic at the time the study was conducted. This finding suggests that ASB may have a role to play in the aetiology of hypertension in this environment. Co-occurrence of hypertension and ASB may portend a grave problem for individuals in this environment. Hence, the nature of this association needs to be investigated in future studies because of the grave effect of hypertension in our society.

The commonest pathogens implicated in ASB were *Escherichia coli* (26%), *Staphylococcus aureus* (26%), *Klebsiella* spp (22%) and *Pseudomonas aeruginosa* (20%). The aetiology of ASB varies from one geographical location to another and with patients' conditions. Globally, *E. coli* is the commonest uropathogens implicated in ASB (17–20). A previous study in the study environment also reported it as the commonest pathogen implicated in ASB (21). Therefore, its preponderance in this study agrees with the reports of previous investigators.

Isolation of *S. aureus* as uropathogen is not exclusive to our study. A previous study in Ile-Ife by Odetoyin *et al*. (22) and across the globe similarly reported it as a commonly isolated organism from patients with ASB (18, 23, 24). *Staphylococcus aureus* has in recent times been implicated in complicated UTI (25). The other isolates in this study included *Klebsiella* spp and *Pseudomonas aeruginosa* which have also been implicated in UTI (26).

Contrary to earlier studies (27–30), this study has demonstrated that *in vitro*, co-trimoxazole is the single most efficacious antibiotic against all the strains of uropathogens isolated, with sensitivity rate as high as 100% against *Proteus, Morganella, Klebsiella*, 93% against *E. coli* and *Staphylococcus aureus* but lower (70%) for *Pseudomonas aeruginosa*. In Africa, there are reports of increasing resistance to this drug due to its availability over the counter and its indiscriminate use for unrelated conditions (14, 22). Likewise, the second most effective antibiotic in this study is tetracycline, with 100% sensitivity against *E. coli, Klebsiella, Morganella, Proteus*, 70% against *Pseudomonas aeruginosa*, a pattern which is dissimilar to other studies in Africa (14, 27, 31). The efficacies of co-trimoxazole and tetracycline in this study may be due to the fact that people have shifted to newer drugs like the cephalosporins and quinolones for treatment which favours resistance to them due frequent use and the older drugs like tetracycline and co-trimoxazole which have been neglected are now becoming effective due to lack of frequent use. Hence, frequent use of an antibiotic is a risk factor for its resistance.

Surprisingly, Nitrofurantoin an old drug and ofloxacin a relatively new drug demonstrated a rather low *in vitro* sensitivity of less than 50% for *E. coli*, *Klebsiella* spp, *Pseudomonas aeruginosa*. Fluoroquinolone resistance (FQR) in UTI pathogens has been increasing globally. The Study for Monitoring Antimicrobial Resistance Trends (SMART) collected 1,116 FQR gram-negative urinary pathogens from hospitalized patients in 33 countries during 2009-2010. FQR rates varied widely from country to country with a range of 6% to 75%. Regional FQR rates were 23.5% in North America, 29.4% in Europe, 33.2% in Asia, 38.7% in Latin America, and 25.5% in the South Pacific (32). Studies across Nigeria also reported various degree of FQR up to 50% which is almost comparable to the present study (33–35). Over the counter use of these drugs in Nigeria has probably led to such low degree of sensitivity of uropathogens to this drug. Resting such antibiotic from use by making it unavailable on the market and/or restricting its use may allow it to recover its potency.

Sensitivities to gentamicin and ampicillin which are also commonly prescribed for treatment of UTI are also relatively low, with overall sensitivities of only 61.4% and 40% respectively. Studies done in Nigeria showed similar trends as well (5, 31, 36–39).

Conclusion: The prevalence of significant bacteriuria among healthy people was at 27.8%. The most commonly implicated pathogens were *Escherichia coli* and *Staphylococcus aureus.* Cotrimoxazole was the most efficacious antibiotic to all the uropathogens isolated. The high rate of resistance to ofloxacin, augmentin, ampicillin and gentamicin may prevent the use of these antibiotics for empiric treatment of UTI in Nigeria. The association of ASB and hypertension was established in this study and the co-occurrence of them could portend a grave problem for apparently healthy individuals in this environment.

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COMPARATIVE STUDY OF MICROBIAL, PROXIMATE AND HEAVY METAL COMPOSITIONS OF SOME GASTROPODS, BIVALVE AND CRUSTACEAN SEAFOOD

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ABSTRACT
Background: Seafood are known to be nutritionally rich, however, water bodies from where these foods are harvested are often burdened with wastes from industries, household and run-offs. Thus seafood can harbor pathogenic microorganisms and heavy metals which can pose a serious health hazard to consumers. **Materials and Methods:** In this study, 5kg each of *Littorina littorea, Achatina fulica,Tympanotonos fuscatus, Dorsanum miran*, *Egeria radiate* and *Penaeus notialis* were investigated for microbial, proximate and heavy metal qualities using standard microbiological and chemical methods of analysis. **Results:** The gastropod mollusks *Tympanotonos fuscatus, Littorina littorea and Achatina fulica* had significantly ($p \leq 0.05$) higher microbial loads compared to other samples. All the samples had significantly ($p \leq 0.05$) higher coliform counts compared to standard specifications. The microbial isolates from the samples included species of *Bacillus, Klebsiellae, Citrobacter, Providencia, Pseudomonas, Staphylococcus aureus, Escherichia coli* and *Aspergillus niger, Aspergillus flavus,* yeasts, Alternaria, Absidia, Rhizopus, and Mucor. Antibiogram of the isolates revealed multiple resistances with 79.55% and 66.67% resistance to antibacterial and antifungal agents respectively. The seafood was rich in protein (8.0±0.03 to 46.0±0.03%) and fat (0.6±0.01 to 8.8±1.00%) but low in carbohydrate (1.18±0.84 to 3.81±0.86%).The heavy metal compositions of the seafood were generally low except for manganese 6.36±0.03ppm in *Dorsanum miran* and nickel 0.81±0.50 ppm in *Penaeus notialis*. **Conclusion:** The microbial loads of the seafood are significantly ($p \leq 0.05$) higher in gastropods and more than acceptable standard, effective protection of water bodies for food cultivation and constant monitoring of seafood is necessary. Adequate processing and employing good manufacturing practices can reduce the microbial loads to an acceptable level and prevent food borne hazards that could be associated with seafood consumption.
Keywords: Seafood; Heavy metals; Microbial profile; proximate compositions

ÉTUDE COMPARATIVE DE LA PROLIFÉRATION MICROBIENNE, PROCHAINE ET HEAVY METAL COMPOSITIONS DE CERTAINS GASTÉROPODES, MOLLUSQUES ET CRUSTACÉS LES FRUITS DE MER

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Résumé
Contexte: les fruits de mer sont connus pour être nutritionnellement riches, cependant, les étendues d'eau d'où ces aliments sont récoltés sont souvent surchargés de déchets provenant des industries, de ruissellement et des ménages. Ainsi, les fruits de mer peuvent héberger des micro-organismes pathogènes et les métaux lourds qui peuvent constituer un grave danger pour la santé des consommateurs. Matériel et méthodes : Dans cette étude, 5kg chacun de Littorina littorea, Achatina fulica, Tympanotonos fuscatus, Dorsanum miran, Penaeus notialis Egeria rayonner et ont été étudiés pour les microorganismes, les causes immédiates et qualités de métaux lourds en utilisant des méthodes microbiologiques et chimiques de l'analyse. Résultats: L'amphibien Tympanotonos fuscatus, Littorina littorea et Achatina fulica avaient significativement ($p \leq 0,05$) les charges microbiennes comparativement à d'autres échantillons. Tous les échantillons avaient significativement ($p \leq 0,05$) plus élevée de coliformes fécaux par rapport aux spécifications standard. Les isolats microbiens provenant des échantillons des espèces de Bacillus, Klebsiellae, Citrobacter, Providencia, Pseudomonas, Staphylococcus aureus, Escherichia coli et de l'Aspergillus niger, Aspergillus flavus, levures, Alternaria, Absidia, Rhizopus, Mucor et. Antibiogramme des isolats ont révélé de multiples résistances avec 79,55 % et 66,67 % résistance à propriétés antibactériennes et antifongiques, respectivement. Les fruits de mer sont riches en protéines (8,0 ± 0,03 à 46,0 ± 0,03 %) et de matières grasses (0,6 ± 0,01 à 8,8 ± 1,5 %) mais faible en glucides (1,18 ± 0,84 à 3,81 ± 0,86 %). Le heavy metal les compositions des fruits de mer ont été généralement faibles, sauf pour le manganèse 6,36± ± 0,03 ppm dans Dorsanum miran et nickel 0,81 ± 0,50 ppm dans Penaeus notialis. Conclusion : Les charges microbiennes de la mer sont significativement ($p \leq 0,05$) plus élevée chez les gastropodes et plus de norme acceptable, une protection efficace des plans d'eau pour la culture des aliments et d'une surveillance constante de la mer est nécessaire. Le traitement adéquat et une bonne pratiques de fabrication peuvent réduire la charge microbienne à un niveau acceptable et de prévenir les risques d'origine alimentaire qui pourraient être associés à la consommation de fruits de mer.

Mots-clés: Fruits de mer ; métaux lourds ; profil microbien ; compositions immédiate

INTRODUCTION

Seafood is any fish or shellfish from the sea used for food (1). It includes all fresh or saltwater finfish, mollusks, shellfish, crustaceans, and other forms of edible aquatic animal life (2). Seafood is consumed all over the world (3). It is generally accepted that seafood is important in a healthy, safe, nutritious, and balanced diet. A well-balanced diet that includes a variety of fish and shellfish can contribute to heart health and children's growth and development (4, 5). Over one billion people rely on seafood as their primary source of animal protein; it provides the world's prime source of high-quality protein, 14–16% of the animal protein consumed world-wide (3). Also, seafood is an important source of valuable nutrients, like minerals (e.g., calcium, phosphorus, magnesium, iodine, zinc, copper etc.), vitamins specifically fat soluble vitamins A, D, E, K, fatty acids (e.g., long chain n-3 polyunsaturated fatty acids, EPA-Eicosapentaenoic Acid, DHA-Docosahexaenoic Acid, and is low in saturated fats (4, 5, 6, 7, 8). Polyunsaturated n -3 fatty acids in seafood are known to decrease the risks of coronary heart disease and cancer and to improve the response to inflammatory diseases, like psoriasis, and rheumatoid arthritis, increase heart rate variability, decrease the risk of stroke and heart attack, reduce levels of triacylglycerides in serum, reduce blood pressure, reduce insulin resistance and modulate glucose (sugar) metabolism thus help prevent development of diabetes, beneficial effect on patients with attention deficit/hyperactivity disorder (ADHD) and schizophrenia; they are also known to have a beneficial effect on managing depression in adult patients (9, 10, 11, 12). Seafood products harvested from contaminated waters or which have been improperly preserved after harvesting are known to play an important role in infections, especially by *Vibrio* spp (13). Consumption of raw or undercooked seafood, particularly shellfish, contaminated with *V. parahaemolyticus* may lead to the development of acute gastroenteritis characterized by diarrhoea, headache, vomiting, nausea, abdominal cramps, and low fever (14). This bacterium is recognized as an important seafood-borne pathogen throughout the world (15, 16).

Heavy metals are natural trace components of the aquatic environment, but their levels have increased due to industrial, agricultural and mining activities (17). As a result, aquatic animals are exposed to elevated levels of heavy metals. The levels of metals in upper members of the food web like fish can reach values many times higher than those found in the aquatic environment or in sediments. Thus contamination in the region is an important issue regarding the health of the aquatic animals and, in turn, that of the seafood consumers (17).

Seafood has a natural tendency to concentrate mercury and other heavy metals in their bodies, often in the form of methyl mercury, a highly toxic organic compound of mercury (18, 19). Heavy metals pose a great concern as they cause environmental contamination, cannot be degraded further, and their toxic effects can be long lasting, unlike the organic contaminants that decompose into other chemicals with time (20). Heavy metals are known to have toxic effects even at low concentration, and their concentration in biota can be increased through bio-accumulations (21, 22, 23).

The purpose of this study was to determine and compare the microbial quality, proximate and heavy metal composition of some selected seafood (gastropod mollusks, bivalve mollusk, and crustacean) with a view to contributing microbiological and nutritional information and proffering necessary measures to prevent food borne hazards that could be associated with seafood consumption.

METHODS

Collection of samples

Samples of gastropod mollusks: Littorina littorea (common periwinkle), *Achatina fulica* (Giant African Land Snail), *Tympanotonos fuscatus, Dorsanum miran* (sea snail); bivalve mollusk: *Egeria radiata* (hard clam) and crustacean *Penaeus notialis* (shrimp), were randomly purchased from ten fishermen and ten seafood vendors in Itu, Akwa Ibom State, Nigeria. Itu is located at 5°10′0″N7°59′0″E (fig. 1 (24). Two hundred and fifty (250) g of samples of the seafood from the different sources (fishermen and seafood vendors) were pooled based on types to obtain 5kg of each sample type. Samples for further analysis for each of the seafood were from the pool. Samples were washed with distilled water to remove any adhering contamination and were transported in cold chain 4°C to the Department of Biological Sciences laboratories of Covenant University for analysis. The samples were identified at the Biology unit of Department of Biological Sciences, Covenant University, Ota, Nigeria.

Sample preparation

The outer shell of *Littorina littorea,Achatina fulica*, *Tympanotonos fuscatus,, Dorsanum miran*and *Egeria radiata* were washed in running tap water using a nail brush and rinsed in several changes of distilled water. The shells were then disinfected using swabs moistened in 70% alcohol. After wards, they were de-shelled aseptically. Similarly, the carapace of *Penaeus notialis* was also removed. Sterile forceps and dissecting kits were employed to remove the edible portions after shells and carapaces were removed for further analyses.

Microbiological analysis

Twenty-five gram (25g) portion of samples was pulverized in sterile Stomacher lab blender and

MAP OF STUDY AREA

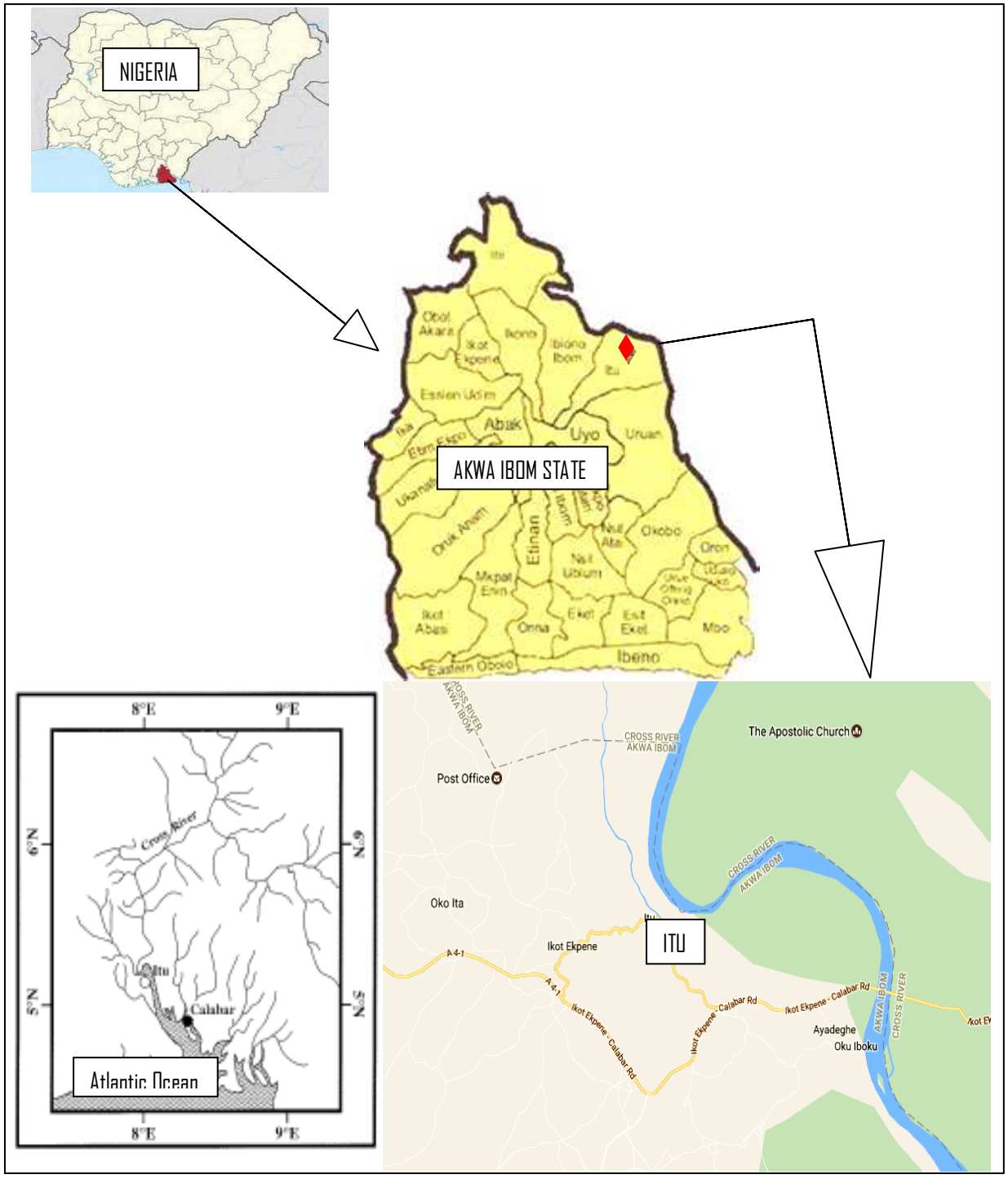

FIGURE 1: MAP OF AKWA IBOM STATE SHOWING STUDY AREA AND ITS LINKS TO THE WATERWAYS. ADAPTED FROM GOOGLE IMAGE, 2017 AND ETIM AND BREY, (1994)(24)

homogenized in 225 mL distilled water for 1:10 dilution. Further tenfold dilutions of the sample homogenate to 10^{-6} were achieved. Aliquot 0.1 mL of appropriate dilutions were spread plated in triplicate onto Nutrient agar (NA) for total aerobic plate count (TAPC), MacConkey agar (MA) for coliform count and Potato

dextrose agar (PDA) for the fungal count. One gram (1 g) samples were inoculated into Eosin Methylene blue (EMB) broth with inverted Durham tube in Macartheny bottles and subsequent plating out on Eosin Methylene blue agar after incubation for the coliform test. Cultures on NA, MA, and EMB broth were incubated for 24 to 48 h at 37°C. PDA was incubated at 28±2°C laboratory room temperature for 3 to 7 days.

Enumeration and identification of microbial isolates from seafood

Culture plates were examined at the expiration of incubation period, and colonies were enumerated using Gallenkamp digital colony counter (Gallenkamp, England). Means of total and specific microbial population from the triplicate plates were expressed as colony forming units per ml (cfu/mL). Colonial morphology and other cultural characteristics were observed and recorded and pure cultures of microbial isolates were obtained by repeated sub-culturing on appropriate media. Preliminary identification of bacterial isolates was based on cultural, morphological and basic biochemical characteristics; Gram staining, catalase activity, indole, methyl red, Voges Proskaur test, motility, citrate utilization, urease production, oxidase, starch hydrolysis, gelatin liquefaction, coagulase and fermentation of sugars. Further identification of bacterial isolates was based on standard bacteriological procedures (25) and employing the Biomerieux® sa API system. Confirmation for coliform organisms was based on presumptive, confirmatory and completed tests following the description of Speck (26, 27).

Fungal isolates were identified based on cultural and morphological characteristics, pigmentation on media, and microscopic characteristics, sporulation, mycelia arrangement, and sugar assimilation tests and with reference to standard identification key and atlas (28).

Sensitivity test of isolates to commonly used antibiotics and antifungal agents

Disc diffusion technique as described by Lalitha, (29) and CLSI, (30) was used to carry out the susceptibility testing of the isolates. Three to five colonies of pure isolates were transferred into test tubes containing 5ml of peptone water and incubated for six hours. The turbidity of the broth culture was adjusted to that of the 0.5 McFarland standards- approximately 1 to 2 x 10^8 cfu/ml (for bacteria). Turbidity for fungal cultures was adjusted to 1 to 5 x 10^6 cells /ml. A sterile cotton swab was dipped into the adjusted suspension, rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab. The entire dried surface of Müeller-Hinton agar plate (Oxoid) prepared based on manufacturer's instruction, was evenly streak inoculated. Müeller-Hinton agar for fungal sensitivity was, however, supplemented with 2% glucose + 0.5 µg /ml Methylene Blue Dye. The plates were allowed to stand for 5 minutes to allow for any excess inoculum to diffuse before introducing the discs. The following antimicrobial agents were employed; Cefuroxime (30µg), Ceftriaxone (30µg), Erythromycin(5µg), Amoxycillin (25µg), Cotrimetrazole(25µg), Nitrofurantoin (50µg), Gentamycin (10µg), Nalidixic acid (15µg), Ofloxacin (5µg), Tetracycline (10µg), Streptomycin (10µg), Chloramphenicol (30µg) and Amphotericin B (20µg),Ketoconazole (15µg), Fluconazole (25µg), Griseofulvin (10µg) and Nystatin(100 unit).The plates were incubated at 37°C for 18-24 hours for bacteria and the fungi culture was kept at room temperature for 5 days after which the zones of inhibition were measured.

Proximate analyses

The samples were pulverized and the method of the Association of Official Analytical Chemists (AOAC) (31) was adopted for determination of proximate compositions (moisture, protein, lipid, ash, and fibre). The percentage moisture content was measured from 5 g sample oven dried at 100 to 110°C to constant weight. The loss in weight multiplied by a hundred was regarded as a measure of moisture. The Nitrogen content in the sample was estimated by the micro Kjeldahl method and crude protein was calculated by multiplying the evaluated nitrogen by a factor of 6.25 (N % × 6.25). Total lipid was extracted from the sample with petroleum ether (60 to 80°C) in a Soxhlet apparatus for about 6 to 8 hours. The residual solvent was evaporated in a pre-weighed beaker and an increase in the weight of the beaker gave total lipid. Ash was determined from sample heated in a furnace at 550°C until white or grayish white ash was obtained. The Weight of the ash was noted directly. Crude fiber was determined by treating the sample with 1.25% Tetraoxosulphate vi acid (H_2SO_4), 1.25% Sodium hydroxide (NaOH) and then 1% Nitric acid (HNO_3), filtered and washed with hot water after each step. The residue obtained was dried in an oven at 130°C and ashed at 550°C in a furnace. The loss in weight on ignition was expressed as content of crude fiber. The value of total Carbohydrate was determined by the Phenol-Sulfuric Acid Method following the description of Nielsen (32).

Determination of heavy metals

The heavy metal contents of the samples: lead (Pb), copper (Cu), cadmium (Cd), iron (Fe), zinc (Zn), nickel (Ni), manganese (Mn), and arsenic (As) were determined using the atomic absorption spectrophotomete (G105 UV-VIS, Thermo Fisher Scientific, GeneSys, Madison, USA), as described in the methods of the Association of Official Analytical Chemists (31).

Statistics

Results are presented as mean and standard deviations, antibiograms were presented as percentages of susceptibility. One way analysis of variance was employed to compare mean microbial and chemical compositions and correlation analysis and test of significance for the microbial, proximate, heavy metal compositions at p=0.05.

RESULTS

Table 1 reveals that the gastropod mollusks *Tympanotonos fuscatus, Littorina littorea and Achatina fulica* had significantly ($p \leq 0.05$) higher microbial loads compared to other samples. The table also reveals that all the samples had significantly ($p \leq 0.05$) higher coliform counts compared to standard specifications.

Table 2 reveals the predominant bacterial and fungal species isolated from each of the seafood samples. It shows that *Bacillus, Klebsiella* and *E. coli* were the most predominant bacteria ~~spp~~, while *Aspergillus, yeasts* and *Mucor* were the predominant fungi isolated from the samples.

TABLE 1 MEAN MICROBIAL COUNT (cfu/g) OF THE SEAFOOD SAMPLES

Sample	TAPC	TCC	TFC
Littorina littorea	$4.3 \times 10^7 \pm 2.06^b$	$3.9 \times 10^5 \pm 1.01^f$	$3.0 \times 10^6 \pm 1.02^a$
Achatina fulica	$2.0 \times 10^6 \pm 1.50^c$	$1.0 \times 10^6 \pm 2.04^e$	$1.0 \times 10^3 \pm 0.02^d$
Tympanotonos fuscatus	$3.0 \times 10^8 \pm 2.20^a$	$2.0 \times 10^5 \pm 1.64^f$	$2.0 \times 10^6 \pm 1.84^a$
Dorsanum miran	$1.3 \times 10^7 \pm 2.64^b$	$1.5 \times 10^3 \pm 1.00^h$	$1.1 \times 10^5 \pm 0.84^b$
Egeria radiate	$4.0 \times 10^4 \pm 0.05^e$	$1.0 \times 10^4 \pm 0.54^g$	$2.0 \times 10^4 \pm 0.03^c$
Penaeus notialis	$9.2 \times 10^5 \pm 1.04^d$	$2.5 \times 10^3 \pm 0.06^h$	$1.4 \times 10^5 \pm 0.34^b$

KEY: TAPC= Total Aerobic plate count, TCC= Total coliform count, TFC=Total fungal count. Super script abcdefgh= counts with different superscript for the same count (within the same column) are significantly different

TABLE 2 PREDOMINANT BACTERIAL AND FUNGAL SPECIES ISOLATED FROM SEAFOOD

Sample	Bacteria spp isolated	Fungi spp isolated
Littorina littorea	***Escherichia coli; Bacillus spp; Acinetobacter spp***	***Aspergillus niger, Aspergillus flavus,* yeast**
Achatina fulica	***Klebsiella spp; Staphylococcus; Citrobacter spp; Bacillus spp***	***Aspergillus niger,* mucor, streptomyces spp**
Tympanotonos fuscatus	***Escherichia coli; Pseudomonas spp; Bacillus spp***	**Yeast, mucor, *Aspergillusspp*, *Aspergillusniger***
Dorsanum miran	***Bacillus sp;Staphylococcus sp; Escherichia coli;Aeromonas spp***	***Saccharomyces cerevisiae; Mucor* sp; *Aspergillus niger***
Egeria radiate	***Klebsiella sp; Bacillus sp, Citrobacter spp***	***Mucor* sp Alternaria, Absidia, Rhizopus**
Penaeus notialis	***Bacillus sp; Pseudomonas spp, Aeromonas spp, Klebsiella spp***	***Penicillium spp, Aspergillus spp; Saccharomyces cerevisiae***

Table 3 and 4 reveals that the bacterial isolates were multi-resistant to the antimicrobial agents. *Pseudomonas* gave 90.91% resistance and *Klebsiella* showed 81.82% resistance. These organisms were 100% resistant to Cotrimoxazole, Tetracycline, and Chloramphenicol and all the bacterial isolates were 79.55% resistant to all the antibacterial agents and 20.45% were sensitive. Table 4 shows that all the fungal isolates were 60% and above resistant to the antifungal agents, except for *Alternaria* spp. Species of *Penicillium and Absidia* were 100% resistant to the antifungal agents tested. All the fungal isolates were 66.67% resistant to all the antifungal agents and 33.33% sensitive.

The proximate composition of the seafood samples is shown in Table 5. It explains that seafood is rich in protein, lipid, and ash and have high moisture, total solid and organic matter contents. The proximate analysis shows that the crude protein contents ranged from 8.0 to 46.0%, total lipid and carbohydrate contents ranged from 0.6 to 8.8 % and 1.18 to 3.81% respectively.

Penaeus notialis (crustacean) and bivalve mollusk (*Egeria radiata*) has significantly ($p \leq 0.05$) higher protein content than the gastropods except for *Dorsanum miran.* The lipid contents of the samples were not significantly different, except however, for *Dorsanum miran* which has significantly ($p \leq 0.05$) higher lipid content and *Penaeus notialis* with significantly ($p \leq 0.05$) lower lipid content compared to all other samples

TABLE 3 PERCENTAGE (%) SUSCEPTIBILITY OF BACTERIAL ISOLATES TO COMMONLY USED ANTIBACTERIAL AGENTS

ORGANISM	OFL	GEN	COT	AMX	TET	AUG	STR	CHL	ERY	CTR	NAL	CRX	CAZ	NIT	CLX	%S	%R
Bacillus sp.	S	S	R	R	R	R	R	R	R	-	-	-	-	S	R	27.27	72.73
Klebsiella sp.	R	S	R	-	R	-	R	R	-	R	S	R	R	R	-	18.18	81.82
Staph. sp.	S	S	R	R	R	R	S	R	R	-	-	-	-	R	R	27.27	72.73
Pseudo. sp.	S	R	R	-	R	-	R	R	-	R	R	R	R	R	-	9.09	90.91
% S	75.0	75.0	0.0	0.0	0.0	0.0	25.0	0.0	0.0	0.0	50.0	0.0	0.0	25.0	0.0	20.45	
% R	25.0	25.0	100	100	100	100	75.0	100	100	100	50.0	100	100	75.0	100		79.55

Key: OFL=Oflaxacin, GEN=Gentamycin, COT=Cotrimoxazole, AMX=Amoxicillin, TET=Tetracycline, AUG=Augmentin, STR=Streptomycin, CHL=Chloramphenicol, ERY=Erythromycin, CTR=Ceftriaxone, NAL=Nalidixic acid, CRX=Cefuroxime, CAZ=Ceftrazidime, NIT=Nitrofurantoin, CLX=Cloxacillin, R=Resistant, S=Sensitive, % S= %Susceptible, % R= % Resistant, - Not applicable

TABLE 4 PERCENTAGE (%) SUSCEPTIBILITY OF FUNGAL ISOLATES TO COMMONLY USED ANTIFUNGAL AGENTS

GANISM	AMC.B	KTZ	FLZ	GRV	NYT	%S	%R
Aspergillus niger	S	S	R	R	R	40	60
Aspergillus flavus	R	S	R	S	R	40	60
Yeast.	S	S	R	R	R	40	60
Mucor spp	S	R	R	S	R	40	60
Streptomyces spp	S	R	S	R	R	40	60
Penicillium spp	R	R	R	R	R	00	100
Alternaria *spp*	R	S	S	S	R	60	40
Absidia *spp*	R	R	R	R	R	00	100
Rhizopus *spp*	R	S	R	R	S	40	60
% S	44.44	55.56	22.22	33.33	11.11	33.33	
% R	55.56	44.44	77.78	66.67	88.89		66.67

KEY: AMC=Amphotericin B, KTZ=Ketoconazole, FLZ=Fluconazole, GRV=Griseofulvin NYT=Nystatin

TABLE 5 PERCENTAGE (%) PROXIMATE COMPOSITION OF SEAFOOD

SAMPLE	Moisture	Protein	Lipid	Ash	Fibre	Total carbohy Drate	Total solid (dry matter)	Organic matter
Littorina littorea	51.80±2.04^{a}	8.0±0.03 ^{a}	4.18±0.05^{a}	19.80±0.22^{a}	8.48±2.05^{a}	1.94±1.05^{a}	48.20±0.05^{a}	80.20±2.34^{a}
Achatina fulica	55.10±3.05^{a}	32.0±0.01^{b}	4.77±0.23^{a}	4.21±0.68 ^{b}	7.08±1.01^{a}	2.52±1.04^{a}	44.90±1.44^{a}	95.79±1.02^{b}
Tympanotonos fuscatus	74.80±2.02^{b}	27.0±0.02^{b}	5.20±0.08^{a}	4.16±0.60 ^{b}	2.88±0.05^{b}	2.67±1.03^{a}	25.20±0.98^{b}	95.84±1.00^{b}
Dorsanum miran	68.80±1.22^{ab}	43.0±0.05^{c}	8.80±1.00^{c}	12.71±1.05^{c}	4.00±0.05 ^{b}	1.32±0.23^{c}	31.20±0.08^{c}	87.29±1.66^{a}
Egeria radiata	75.50±2.04^{b}	46.0±0.03^{c}	4.57±0.08^{a}	4.79±1.01 ^{b}	9.96±0.66^{ca}	1.18±0.84^{c}	24.50±0.52^{b}	95.21±0.10^{b}
Penaeus notialis	74.50±1.01^{b}	46.0±0.02^{c}	0.60±0.01^{b}	5.36±0.54 ^{b}	3.83±1.01^{b}	3.81±0.86^{b}	25.50±0.06^{b}	94.64±2.02^{b}

Super script abc= values with different superscript for the same parameter (within the same column) are significantly different

Table 6 reveals the heavy metal composition of the seafood samples. It shows that lead and Arsenic were not detected in the samples except for trace amount in some samples. *Dorsanum miran* had significantly ($p \leq 0.05$) higher levels of Mn, Zn and Cu compared to other samples except for Cu in *Littorina littorea. The correlation among microbial load (0.331), proximate composition (0.223) and heavy metal composition (0.192) was weak.*

TABLE 6 HEAVY METAL COMPOSITIONS (PPM) OF SEAFOOD

SAMPLE	Mn	Fe	Zn	Ni	Cd	Cu	Pb	As
Littorina littorea	0.722±0.01[a]	5.28±0.05 [b]	17.2±0.05 [a]	0.16±0.00 [a]	0.12±0.02 [a]	7.56±0.31 [c]	trace	Nil
Achatina fulica	Trace	2.32±0.02 [a]	2.6±0.01 [b]	0.12±0.10 [a]	0.14±0.01 [a]	2.83±0.04 [a]	Nil	Nil
Tympanotonos fuscatus	2.190±0.04[a]	1.98±0.01 [a]	3.5±0.02 [b]	0.15±0.01 [a]	0.12±0.00 [a]	1.50±0.01 [a]	Nil	Trace
Dorsanum miran	6.36±0.03 [b]	2.90±0.05 [a]	23.1±0.02 [a]	0.11±0.00 [a]	0.16±0.00 [a]	4.55±0.20 [b]	Nil	Trace
Egeria radiate	1.02±0.01 [a]	2.14±0.02 [a]	5.3±0.01 [b]	0.23±0.01 [a]	0.15±0.01 [a]	1.64±0.02 [a]	trace	Nil
Penaeus notialis	Trace	1.81±0.04 [a]	5.5±0.01 [b]	0.81±0.50 [a]	0.13±0.00 [a]	2.71±0.03 [a]	trace	Nil
Maximum limit (ppm) (FAO, 1983; WHO, 1985; FAO/WHO, 1984	50	-	30-50	80	0.3-1.0	20-30	1.5	-

Key: Mn = Manganese, Fe=Iron, Zn= Zinc, Ni=Nickel, Cd= Cadmium, Cu= Copper, Pb= Lead, As= Arsenic, - = Not provided
Super script abc= values with different superscript for same parameter (within same column) are significantly different

DISCUSSION

The total microbial count obtained in this study shows that the microbial quality of the seafood samples was poor compared to the specified standard limit. According to the International Commission on Microbiological Specifications for Food (ICMSF) (33), a total aerobic plate count (TAPC) of not more than 5×10^5 cfu/g is recommended for fresh bivalve mollusks and 10^7 cfu/g for raw crustaceans. It means that *Littorina littorea,Tympanotonos fuscatus, Achatina fulica* and *Dorsanum miran* had total aerobic plate counts significantly ($p \leq 0.05$) higher than the recommended microbial limits for fresh mollusks. Similarly, maximum total coliform count recommended by ICMSF[33] is 11 cfu/g for raw crustaceans of good quality products and 500 cfu/g for marginally acceptable quality products. Maximum coliform level recommended for fresh bivalve mollusk is 16 cfu/g. Table 1 reveals that all the samples had coliform counts significantly ($p \leq 0.05$) well above the recommended microbial limits. The higher microbial counts in the gastropod mollusks compared to bivalves could be associated with the univalve anatomy that tends to trap and incubate more materials that sieve into the mollusks.

Salmonella and *vibrio* species were not isolated from examined seafood thus this report is not in tandem to the report of Adebayo-Tayo *et al* (34), who reported the isolation in a high number of *salmonella* and *vibrio* species from seafood. Coliforms (*E. coli*, *Klebsiella, Citrobacter*) and *Staphylococcus aureus* were, isolated in significantly high counts and from a large number of the seafood samples examined. This is in conformity with the reports of Nwiyi and Okonkwo (35), Zaky and Ibrahim (36); Although Nwiyi and Okonkwo (35) isolated high number of salmonella which is at variance with this report. Coliforms are indicators of faecal contamination, and this is corroborated by isolation of faecal *E. coli* from the samples analyzed, their presence in these samples is of public health significance. The presence of *S. aureus* calls for concerns, as this organism has been implicated in foodborne diseases[37] and is associated with various human diseases (38). *S. aureus* is a normal human flora and can easily contaminate processed food product, control of its presence in raw foods will help minimize the presence in finished products. Contamination by these microorganisms of water bodies from where the seafood were harvested could be from human activities such as bathing, disposal of faecal matters, sewage discharge by municipal authorities, runoff, unhygienic handling and improper packaging/transportation and storage by the fishermen and seafood vendors (39, 40, 41). Fish and other free-swimming marine animals do not usually carry those organisms generally considered to be typical of the mammalian microflora, including *Escherichia coli,* the 'faecal coliforms', and enterococci. The presence of human enteric organisms on marine food products is clear evidence of contamination from a terrigenous

source (36). Dabadé *et al* (42) evaluated the bacterial concentration and diversity in fresh tropical shrimps (*Penaeus notialis*) and the surrounding brackish waters and sediment and observed that total mesophilic aerobic counts in shrimp samples were significantly higher than in water or sediment samples. In contrast, bacterial diversity was higher in sediment or water than in shrimps. They concluded that the bacterial community of tropical brackish water shrimps cannot be predicted from the microbiota of their aquatic environment. The results of this work corroborate some reports on the bacteriology of seafood (43, 44, 45).

Fungal species are spore bearers and common environmental contaminants of food and food products. The isolation of these organisms in seafood is in tandem with the reports of (36, 46, 47, 48, 49). These fungi are observed as pathogens or opportunistic pathogens in fresh and salt water fishes and have also been implicated in veterinary and human diseases (27, 50, 51). The presence of these fungi in seafood could be associated with contamination from the water environment, the fishermen and vendors, and instrument of fishing (52, 53, 54, 55).

The antibiogram revealed that most of the microbial isolates were multi-resistant to the common antimicrobial agents; this report is in tandem with the observations of Zaky and Ibrahim, [36] that screened bacterial and fungal biota associated with *Oreochromis niloticus,* it, however, differs from the report of Shimaa *et al* (56) who reported high sensitivity of bacterial isolates from ponds cultured Indian shrimps. That most organisms in this study are multi-resistant to the common antimicrobial agents, could be explained that theseorganisms may have been subjected to several harsh environmental conditions and exposed to sub-lethal doses of antimicrobial substances, causing them to adapt and mutate into resistant strains. Inappropriate use of antimicrobials, lack of health care personnel with continual health education on antimicrobials and poor quality drugs have also been reported as a major cause of antimicrobial agents resistance specifically in developing countries (57, 58).Several mechanical, epidemiological and genetic factors may lead to the development of drug resistance (57, 59, 60, 61). The occurrence of drug resistance genes in microbial isolates will result in microorganisms that are multi-resistant to virtually all antimicrobial agent. This poses a serious public health concern because should such a multi-drug resistant organism cause infection, empirical treatment will be difficult (57, 58, 62).

The nutritional composition of the samples revealed high protein and lipid content for some of the seafood samples, this compares favourably with similar works reported by other researchers (8, 34, 63, 64, 65, 66). The proximate values, however, differ significantly with the reports of some commercially important seafood presented by Mohammad and Yusuf[67]. Mean levels of heavy metals in the sea food samples were generally found to be below the acceptable limits of heavy metal pollution of fishes and shell fishes (68, 69, 70, 71). A level of 0.81 ppm was reported for nickel in *Penaeus notialis,* Nwabueze (72) reported a higher level of nickel in Egeria radiate harvested from creeks in Delta state, Nigeria. The mineral levels reported for shellfishes by Davies and Jamabo (73) is higher than the level presented in this work. Manganese was detected in the sea snail sample at 6.36 ppm. This level is significantly $p \leq 0.05$ higher than the adequate intake (AI) levels stipulated for infants (0.003mg/day), for adult male (2.3mk/day) and 2.6 mg/day in lactation. The level reported is however within the range stipulated for tolerable upper intake level (UL) 2mg/day to 11mg/day for infants and adults respectively. Manganese is both nutritionally essential and potentially toxic; it plays an important role in a number of physiologic processes as a constituent of multiple enzymes and an activator of other enzymes (74). There is little risk of excessive exposure to manganese through ingestion of fish or shellfish emanating from contaminated waters unless the manganese levels in the fish are extremely high and/or the fish are eaten as subsistence. The inhalation of air contaminated with particulate matter containing manganese is the primary source of excess manganese exposure for the general population (74, 75, 76). Manganese toxicity can result in early symptoms such as irritability, aggressiveness, and hallucinations this will be followed by a permanent neurological disorder known as manganism with symptoms that include tremors, difficulty walking, facial muscle spasms, adverse cognitive effects, including difficulty with concentration and memory problems (74, 75, 76). Although the levels of heavy metals presented in this work are low, some researchers (22, 23, 77, 78)however, observed that heavy metals have relatively high density and are toxic or poisonous even at low concentrations. In view of this, there is a need for constant monitoring of the levels of heavy metals to forestall any significant rise in their levels resulting in ecological and health concerns such as biochemical variations, mutations, deformities and sometimes death in the organism, bioaccumulation in the food chain and toxicity to man (79, 80, 81).

Conclusion

The present study revealed that samples of the selected seafood from Itu, Akwa Ibom state Nigeria contain an unacceptable level of microorganisms, the presence of organisms such as feacal *Escherichia coli,* other coliforms, and *Staphylococcus species* implies a poor sanitary condition of the water bodies from where the samples were harvested. The gastropod mollusks had

significantly higher microbial loads than the bivalve mollusk and crustacean. The isolated organisms prove to be multi-resistant to commonly used antibacterial and antifungal agents, this presents serious public health concerns. The seafood is nutritionally rich and with low heavy metal concentrations. This study did not establish a significant difference in the proximate and heavy metal compositions of the gastropod mollusks, bivalve mollusk, and crustacean. Effective policing of water bodies for seafood cultivation by all concerned is necessary, public enlightenment campaign on the need to keep the water bodies free of pollutants and the health benefits of seafood consumption is advocated.

Conflict of interest

Authors declare no conflict of interest and have approved the submission of this article to this journal

Significance Statement

This study discovers that gastropod mollusks are often more contaminated than bivalve mollusk and crustacean from the same water environment. The isolated organisms were multi-resistant with 79.55% resistance to all the antibacterial agents and above 60% resistance to the antifungal agents. This work also observed that there was no significant difference in the heavy metal compositions of the gastropod mollusks, bivalve mollusk, and crustacean from the same environment. This study will help the researcher to uncover the critical area of aquaculture and seafood processing and preservation

Authors Contributions

Author Oranusi, S. designed, supervised and wrote the manuscript

Authors Effiong, E.D. and Duru, N. U. Performed the laboratory analysis and wrote the draft

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PREVALENCE OF HOOKWORM SPECIES AMONG BUTCHERS IN AKURE METROPOLIS

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ABSTRACT
The prevalence of hookworm species among butchers in Akure metropolis was carried out by collecting 200 feacal specimens from four different abattoirs within Akure Metropolis. The specimen was examined by iodine preparations for a clearer picture of the eggs and formol-ether technique. The result obtained from this study showed that there was significant difference between (P<0.05) hookworm infection and gender distribution with masculine gender accounting for an infectivity rare of 71.4% while the female gender shows infectivity rate of 28.6%. it was equally observed the hookworm infection usually existed solely or alongside with other helminthes with infectivity rate of 5.5%.
Keywords: Hookworm, Butchers, Akure

PRÉVALENCE DE L'ANKYLOSTOMIASE CHEZ LES ESPÈCES EN METROPOLIS BOUCHERS AKURE

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Résumé
La prévalence de l'ankylostomiase chez les espèces de bouchers Segou Metropolis a été effectuée par la collecte de spécimens de feacal 200 quatre abattoirs différents au sein de Segou Metropolis. Le spécimen a été examiné par l'iode les préparatifs d'une image plus claire de l'oeufs et le formol-éther technique. Le résultat de cette étude montre qu'il y a une différence significative entre (P <0,05) et de l'ankylostomiase avec répartition par sexe masculin de la comptabilisation d'une infectiosité rare de 71,4 % tandis que le taux d'infectivité genre montre féminine de 28,6 %. Il a également pu être observé les ankylostomiases existait habituellement uniquement ou avec d'autres taux d'infectivité helminthes avec 5,5 %.

Mots-clés: l'ankylostome, bouchers, Segou

INTRODUCTION

The synonymous of hookworm infections and trade of animal husbandry and butchery dates back to the early days of civilization, the correlation between the two was not clear enough until the 1880's. Butchers are people who slaughters or dresses the flesh of edible animals for sale and for food. Most of their activities in the tropics are carried out in an unwholesome manner, thus, predisposing them to numerous microbiological and parasitic infections paramount of which is hookworm infection (1).

Hookworm is a parasitic nematode worm that lives in the small intestine of its host, which may be a mammal such as a dog, cat or human. Two species of hookworm commonly infects humans, *Ancylostoma duodenale* and *Necator americanus.* Hookworms are bilateral, meaning that it can cut in half, the worm would be the exact same on each side. *Necator americanus* predominates in the Americas, Sub-Saharan Africa, Southeast Asia, China and Indonesia, while *A. duodenale* predominates in the Middle East, North Africa, India and (formerly) in Southern Europe (2, 3).

MATERIALS AND METHODS

Sample Collection: Fresh faecal samples were collected from four abattoirs within Akure metropolis. The sex and age of the patients were collected along side the samples. The specimens were examined at the Parasitology Laboratory unit of the Department of Microbiology, OAUTHC, Ile-Ife and where delay is anticipated, they were preserved and fixed with warm formalin.

Ethical Consideration: informed verbal consent of the recruited individual was obtained

Sample Analysis: Examination of the Faecal Sample: The samples were analyzed according to methods described by Arora (4)

Data Analysis: SPSS statistical analysis used were ANOVA, Cumulative frequency.

RESULTS

Out of 200 butchers examined in all the four abattoirs, 42(21%) were tested positive to hookworm infection only as shown in table 3 out of 30 (71.4%) were male and 12 (28.6%) were female. The statistical analysis of these result showed that there was significant difference ($P<0.05$) between hookworm infection and gender distribution as shown in table 2. This shows that hookworm infection is male dominated.

TABLE I: GENDER DISTRIBUTION AMONG BUTCHERS IN AKURE METROPOLIS

Gender	Abattoirs A	Abattoirs B	Abattoirs C	Abattoirs D	Total	%
Male	39	38	50	50	177	88.5
Female	11	12	--	--	23	11.5
Total	50	50	50	50	200	100

TABLE 2: DISTRIBUTION/INCIDENCE OF HOOKWORM INFECTIONS

	Positivity/Negativity percentage infectivity %	Gender	
	42+ve	30 male	71.4
		12 female	28.6
	158-ve	145 male	91.8
		13 female	82.2
Summation	200	200	

The result also revealed that 26(13%) patient tested positive to helminthes only while 11(5.5%) tested positive to both hookworm and helminthes. The remaining patients were negative to both infections 121(60.5%) as shown in table 3.

This study also showed that age group 28-37 years old were mostly infected with hookworm with infectivity rate of 30.0% followed by age group 18-27 years and 38-47 years with infectivity rate of 26.5% and 25.5% respectively. Age 48-57 years showed the least infectivity rate of 18.0% as shown in table 4.

The statistical analysis of the result showed that there was no significant difference ($P<0.05$) between age and hookworm infection.

TABLE 3: DISTRIBUTION OF HOOKWORM AND OTHER HELMINTHES INFECTIONS

Tested patient	Abattoir A	Abattoir B	Abattoir C	Abattoir D	Total	%infectivity
Positive to hookworm only	18	13	5	6	42	21
Positive to helminthes only	9	11	3	3	26	13
Positive to both hookworm and Helminthes	3	5	1	2	11	5.5
Positive to both hookworm and Helmminthes	20	21	41	39	121	60.5
Total	50	50	50	50	200	100

TABLE 4: INFECTIVITY RATE OF HOOKWORM BY AGE DISTRIBUTION

Age	Abattoir A	Abattoir B	Abattoir C	Abattoir D	Total	%
18-27	11	18	15	9	53	26.5
28-37	19	12	13	16	60	30.0
38-47	13	13	13	12	51	25.5
48-57	7	7	9	13	36	18.0
Total	50	50	50	50	200	100

DISCUSSION

This study provides a data on prevalence of hookworm among butchers within Akure metropolis. The overall prevalence rate of hookworm infection among all the butchers was 21%. This infectivity agrees with previous report by other researchers (5, 6) that the prevalence is usually connected with poor sanitation, poor personal and environmental hygiene.

As evidenced in my study, there was a significant difference between hookworm infection and gender distribution because the trade is mainly male dominated as it required physical strength and long working hours. Moreover, based on the result obtained from the study, hookworm infection is common to all ages represented in this trade. Age group 28-37 years and 18-27 years with prevalence of 30.0% and 26.8% respectively were mostly infected with hookworm. This is so because these are active age of these groups of people that are usually involve in the trade and also due to the nature of the work in which majority of them walk barefooted on unhygienic and dirty environment of the abattoir. With an estimated 740 million individual infections worldwide, hookworm is a major public health concern in our world today. While hookworm infection may not directly lead to mortality, its effects on morbidity demand immediate attention as evidenced in my case study. As can be seen, 42 positive cases (21%) were encountered which numerically might be insignificant but looking at the overall infection rate is very significant.

Most of public health concerns have focused on children who are infected with hookworm. This focus on children is largely due to the large body of evidence that has demonstrated strong associations between hookworm infection and impaired learning, increased absences from school, and decreased future economic productivity. However, as evidenced during the course of this study, downturn of economic productivity among adult cannot be overemphasized as almost all butchers in my study has had occasionally visited the hospital for illnesses that end up been treated with anti-helminthes.

Conclusion: This study not only buttresses, but also advocates for mass de-worming of both young and

old to reduce hookworm burden which is against the resolutions of the 54th World Health Assembly of 2001 demanding member states to attain a minimum target of regular de-worming of at least 75% of all at risk school children only, by 2010 (7). Hookworm infection is generally considered to be asymptomatic and this is attested to in this study, but as Normal Stoll described in 1962, hookworm is an extremely dangerous infection because its damage is silent and insidious. There are general symptoms that an individual may experience soon after infection such as ground-itch, which is an allergic reaction at the site of parasitic penetration and entry, is common in patients infected with *N. americanus*. This is also evidenced during the course of any interaction with the understudied butchers. The prevalence of the infection among masculine gender as seen in this study is worthy of note and this may be accounted for based on the fact that the trade is mainly male dominated as it requires physical strength and long working hours. It is also of importance to stress the point that though hookworm is prevalent among butchers due to the mode of infection of hookworm coupled with unwholesome manner the trade is practice, other intestinal helminthes infections are often associated with the butchers, this is also evidenced in this study.

Recommendations

Since the infective larvae develop and survive in an environment of damp dirty, particularly sandy and loamy soil. They cannot survive in clay or muck. The main lines of precaution are those dictated by sanitary science:

I. Do not defecate outside latrines, toilets etc
II. Do not use human excrement or raw sewage as manure/fertilizer in agriculture
III. Deworm pet dogs-canine and feline hookworms rarely develop to adulthood in humans (*Ancylostoma caninum*, the common dog hookworm, occasionally develops into adult to cause eosiophilic enteritis in people). But their invasive larvae can cause an itchy rash called cutaneous larva migrans.
IV. Avoid trekking barefooted in and around abattoir
V. Butchers should ply their trade with utmost sanitary
VI. Mass deworming exercise should be extended to adults
VII. Butchers should desist from eating raw animal flesh and consumers should eat only properly beef
VIII. In endemic areas, people should avoid eating raw fruits and vegetables that cannot be peeled. Only bottled water, filtered water, or water that has been boiled for atleast one minute should be taken.
IX. Early detection and treatment of hookworm is essential for all human mostly to prevent anaemia in pregnant women and children
X. Good hygiene and hand washing after using toilet will prevent self infection in a person already infected with tapeworms
XI. Improvement of living conditions, especially safe treatment of sewage used for farming should be aimed at.
XII. Health education programs often stressing the importance of preventive techniques such as: always wearing shoes, washing your hands before eating and staying away from water/area contaminated by human and animal faeces.

Therefore, it is strongly advised that butchers and animal farmers should adhere strictly to the following recommendations to avoid been predisposed to hookworm infection with resultant effect of illnesses, frequent hospital visitation/admissions and loss of gross domestic products.

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RE-EMERGENCE OF MONKEYPOX IN NIGERIA: A CAUSE FOR CONCERN AND PUBLIC ENLIGHTENMENT

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ABSTRACT
Monkey pox infection is a zoonotic infection transmitted by direct or indirect contact with blood, body fluids and lesions of an infected animal. Human to human spread of Monkey pox has been described and infection is usually self-limiting, with an incubation period of 6-16 days. In Nigeria, the last case of monkey pox infection was recorded over 46 years ago. The recent emergence in Nigeria occurred in the year 2017 and was reported to have spread to 24 states with 228 suspected individuals affected. Laboratory diagnosis, as well as management and prevention of monkey pox infection in Nigeria, remain challenging as Nigeria is a resource-poor country with limited infrastructure, technical skill and training which is required in making a diagnosis. The ability of the monkey pox virus to evolve, its potential bioterrorism potential, as well as its recent emergence in Nigeria further justifies the need for improving the understanding of the presentation and prevention of monkey pox infection.

Keywords: monkey pox, emerging virus, Nigeria, rash, zoonosis, orthopox

RÉ-ÉMERGENCE DU VIRUS AU NIGERIA: UNE SOURCE D'INQUIÉTUDE ET L'INFORMATION DU PUBLIC

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Résumé
Infection au virus de la variole du singe est une infection zoonotique transmise par contact direct ou indirect avec du sang, des liquides organiques et des lésions d'un animal infecté. Homme à homme propagation de la variole du singe a été décrit et l'infection est habituellement auto-limitation, avec une période d'incubation de 6 à 16 jours. Au Nigeria, le dernier cas d'infection au virus de la variole du singe a été enregistré plus de 46 ans. L'apparition récente dans le Nigeria s'est produite dans l'année 2017 et se serait étendue à 24 membres avec 228 individus présumés affectés. Le diagnostic de laboratoire, ainsi que la gestion et la prévention de l'infection au virus de la variole du singe au Nigeria, sont ambitieux que le Nigeria est un pays pauvre en ressources avec une infrastructure limitée, la compétence technique et de la formation qui est nécessaire pour faire un diagnostic. La capacité de la variole du singe Virus à évoluer, son potentiel potentiel bioterrorisme, ainsi que sa récente apparition au Nigeria est une raison supplémentaire de la nécessité d'améliorer la compréhension de la présentation et la prévention de l'infection au virus de la variole du singe.

Mots-clés : virus, virus émergents, le Nigeria, l'éruption, zoonose, orthopoxvirus

ABSTRACT
Monkeypox infection is a zoonotic infection transmitted by direct or indirect contact with blood, body fluids and lesions of an infected animal. Human to human spread of Monkeypox has been described and infection is usually self-limiting, with an incubation period of 6-16 days. In Nigeria, the last case of monkeypox infection was recorded over 46 years ago. The recent emergence in Nigeria occurred in the year 2017 and was reported to have spread to 24 states with 228 suspected individuals affected. Laboratory diagnosis, as well as management and prevention of monkeypox infection in Nigeria, remain challenging as Nigeria is a resource-poor country with limited infrastructure, technical skill and training which is required in making a diagnosis. The ability of the monkeypox virus to evolve, its potential bioterrorism potential, as well as its recent emergence in Nigeria further justifies the need for improving the understanding of the presentation and prevention of monkeypox infection
Keywords: monkeypox, emerging virus, Nigeria, rash, zoonosis, orthopox

INTRODUCTION

Monkeypox is a relatively rare viral zoonotic infection which occurs primarily in remote parts of Central and West Africa, especially in the rural rainforest regions of Congo Basin and Democratic Republic of Congo, where it is considered to be endemic. It was first identified in 1958 during an investigation of a pox-like disease among monkeys in the State Serum Institute in Copenhagen, Denmark hence its name (1). The first case of human monkeypox infection was first described in a 9-year-old boy from Equateur province of Zaire, Democratic Republic of Congo, Central Africa, who developed a smallpox-like illness which was later confirmed as human monkeypox by World Health Organization (2).

Monkeypox virus is an oval brick-shaped virus with double-stranded DNA, which belongs to the Poxviridaefamily,thesubfamily; Chordopoxvirinae and genus *Orthopoxvirus*. Other members of the family include Variola virus, Vaccinia virus (the virus used in smallpox vaccine), Cowpox virus, Camelpox virus and Ectromelia virus[3]. Since the eradication of smallpox, monkeypox is the foremost *Orthopoxvirus* affecting man (4). There are two distinct clades of Monkeypox virus namely the Congo Basin and the West African clades; The Congo Basin clade is more virulent and has a higher rate of human-to-human transmission[4–6].
The recent outbreak of monkeypox in Nigeria which spanned October 2017 to February 2018 was a source of public health concern. There was an initial diagnostic dilemma as many individuals; physicians inclusive had no knowledge of the aetiology of the disease condition. The Centre for Disease Prevention and Control (CDC) played a vital role in diagnosis, as well as implementation of treatment plan, prevention and control.

Monkeypox virus is a potential agent of bioterrorism as it is second to Variola virus, the agent of smallpox in terms of orthopoxvirus virulence with a mortality rate of 10% (3,5). Hence, there is an urgent need to educate physicians and the general public on the diagnosis, management and control of this disease.

Biology of Monkeypox virus

Monkeypox virus is an enveloped zoonotic virus and a species of the genus Orthopoxvirus in the family Poxviridae. It has a similar morphology, size, and genome to Variola virus; however, it has a very broad host range, making it capable of infecting many species such as rodents, monkeys, and humans. This has allowed the virus to persist in wild host reservoir, causing sporadic human diseases thus avoiding global eradication by vaccination. The monkeypox virus measures about 200-250nm in size, it appears brick shaped, with characteristic surface tubules and a dumb bell shaped core. Its genome is approximately 199 kb of double stranded linear DNA and contains approximately 190 non-overlapping Open reading frame (ORFs) >180 nt long (1,7-9,26). Like all orthopoxviruses, the central coding region sequence (CRS) at monkeypox virus nucleotide positions 56000–120000 is highly conserved and flanked by variable ends that contain inverted terminal repeats (ITRs). At present, there are virtually no studies of the biology of the monkeypox virus as there have been no animal models in which to study monkeypox.

EPIDEMIOLOGY

Monkeypox is indigenous to the rainforests of Central and West Africa. Between 1970 and 1986, 10 cases of human monkeypox infection were reported in Sierra Leone, Nigeria, Liberia and Côte d'Ivoire all (5). A total of 338 cases were reported from surveillance reports from 1981 to 1986 in the Democratic Republic of Congo. Another outbreak occurred from 1996-1997 in the Democratic Republic of Congo, with an attack rate of 22 cases per 1000 population, sporadic occurrences in neighbouring countries have also been reported (5). The first report of human monkeypox infection outside Africa occurred in 2003 in the United States following shipment of rodents imported from Ghana to Texas. Eighty-one (41% laboratory confirmed) cases of monkeypox were reported in this outbreak (6). In 2003, 11 cases of monkeypox and 1 death were reported from the Democratic Republic of Congo while in 2005, 10 cases were reported in Sudan.

Between September and December 2017, Nigeria reported 89 confirmed and 228 suspected cases of monkeypox from 24 out of the 36 states in the country (10). Studies of monkeypox virus have identified at least 2 different genetic types (clades) of the virus, both of which segregate based upon geographic separation, with one type being found in West Africa and the other in Central Africa (11). The West African clade was implicated in this outbreak. Most of the confirmed cases were among adults whose ages ranged from 21- 40 years with male to female ratio of 2.5:1. Six deaths were reported among which four reportedly had background immunosuppressive illness (10,12.) Prior to this, only 3 cases had been previously reported in Nigeria. The first reported case occurred in 1971, in a 4-year-old boy from the South Eastern part of the country while the last reported case occurred in 1978 (13). Prior to the recent outbreak, there had been no reported case of monkeypox in Nigeria since 1978. Another West African country, Liberia has also reported 2 confirmed cases of monkeypox in November 2017 14).

In endemic countries, transmission occurs all through the year without peak month or season

(15). Incidence of monkeypox infection is equal in males and females with no racial predilection. In epidemics seen in Africa, children less than 15 years of age were the most affected and this might be linked to the cessation of smallpox vaccination (16).

Pathogenesis

Transmission to humans occurs via handling of infected animals, direct contact with the blood, body fluids, inoculation from mucocutaneous lesions on an infected animal, especially when the skin barrier is lost due to scratches, bites or other trauma or ingestion of inadequately cooked meat of infected animals (5,15). Transmission has been documented through handling of infected monkeys, Gambian giant squirrels, rats, rabbits, dormice, porcupines, gazelles and prairie dogs (5). Outbreaks typically occur among inhabitants of small villages involved in hunting and gathering with close physical contact being the most significant risk factor for monkeypox infection. No animal has been identified as its exact natural reservoir however, rodents are being considered. Person to person transmission can occur via large respiratory droplets during prolonged face-to-face contact. Congenital monkeypox infection can also occur via placental transmission (5) .

Monkeypox infection begins with infection of the dermis or respiratory epithelium following transmission from infected animals or person respectively. Dissemination of the virus occurs through the lymphatics to the blood with resultant primary viraemia and systemic infection. Secondary viraemia occurs and results in infection of the epithelium with resultant skin and mucosal lesions. Replication of the virus in mucosal surfaces can result in its transmission via oropharyngeal secretions to close contacts (15). In spite of the host immune response, the density of virus in oropharyngeal secretions, proximity and duration of contact and virus survival likely affects the risk of transmission of the virus as the monkeypox virus has evolved mechanisms of evading the host immune response (15).

Clinical manifestations

Monkeypox has an incubation period which ranges from 4 to 21 days with an average of 6 to 16 days. This is followed by a prodromal illness which lasts for 1 to 5 days. Typical characteristics of the prodromal illness include fever (38.5-40.5℃), malaise, intense headache, lymph node enlargement, back pain, myalgia and intense asthenia (4,16). The prodromal phase is also known as the pre-eruptive phase and an individual may be infectious during this phase. Rashes appear within 1 to 10 days of development of fever, this is known as the eruptive or exanthema phase. The rash usually begins on the face and then spread to other parts of the body, the palms and the soles may also be affected. Affectation of the face is seen in 95% of cases (17). The lesions seen in monkeypox are extremely similar to that of smallpox: they appear monomorphic, hard and pea-sized on an erythematous base; often described as "dew drops on a rose petal"[18]. However, unlike smallpox, the lesions may appear in crops and do not have a strong centrifugal spread (5).

The sequence of appearance of the rash is as follows; macules, papules, vesicles and pustules involving both covered and uncovered areas. The rash evolves synchronously over 14 to 28 days, usually between 1-10 days after the onset of fever (3,5). Some of the lesions may have a central umbilication. The lesions of chickenpox occur in successive crops with various stages of development being visible at any time hence aiding its differentiation from that of monkeypox, as these are usually in one stage of development and slowly progress to the next stage with each stage lasting 1-2 days (4,17). Lesions may be few to several thousands in number, with affectation of the oral mucosa, genitalia and conjunctiva in 70%, 30% and 20% of cases respectively. Corneal affectation has also been described. Pruritus may be seen as well as petechiae and ulceration, pain is unusual. Crusting of the rash is seen in about 10 days after its onset and complete disappearance observed within 2-4 weeks of onset (17). An individual is infectious from the onset of exanthem through to the crusting of the rash. Fever subsides within 1-3 days after the onset of the rash, however, a second febrile period has been described when the lesions become pustular and this has been linked with deterioration in the general condition of the patient (4.)

Other symptoms seen include chills and/or drenching sweats, severe headache, backache, pharyngitis, myalgia, cough and shortness of breath. Enlargement of the submental, submandibular and cervical or inguinal lymph nodes is a common feature. The lymph nodes appear firm and sometimes tender. The presence of lymph node enlargement is a key feature in differentiation of monkeypox from smallpox and chickenpox, as it is a rare feature in smallpox while it is seen in 90% of unvaccinated patients with monkeypox infection[4,5,17]. Pitted scars and/or areas of hypo/hyperpigmentation may persist after crusts have fallen (12,19).

Typically, human monkeypox infection is a self-limiting illness which lasts for 14-21 days. Severe cases are seen in children and may require intensive care and are usually related to the extent of virus exposure, health status, vaccination status, comorbidities and severity of complications (5,17). Individuals previously vaccinated against smallpox have a milder form of the disease as they have fewer and smaller lesions, less lymphadenopathy

and generally less severe disease (4,16). Conditions which suppress cell-mediated immunity such as HIV may alter the natural history of the disease and may result in more severe infections (15). The lesions seen in them are also noticed to have regional monomorphism and a centrifugal distribution. Lesions in children may appear as nonspecific 1-5 mm erythematous papules, resembling an arthropod bite reaction. Mortality rates range from 1 to 10% with most cases occurring in children and in unvaccinated persons (16). Indirect or low-level exposure may result in asymptomatic or subclinical infections especially in persons living in or beside forests. Complications of monkeypox infection include pitted scars, deforming scars, secondary bacterial infection, keratitis, corneal ulceration, blindness, bronchopneumonia, septicaemia and encephalitis (3,5,17).

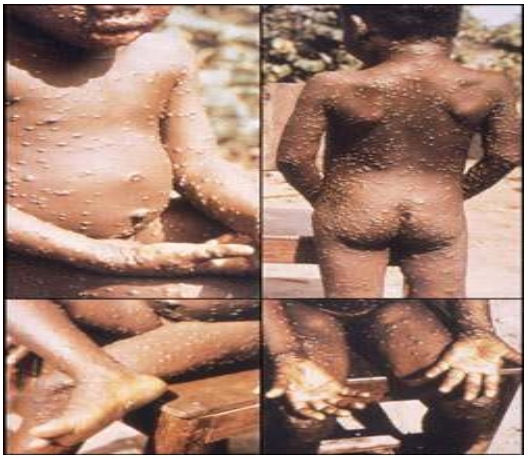

Figure 1: A child affected by monkeypox, showing generalized vesiculo-pustular rashes involving the trunk, limbs, face and limbs, extending to the palms and soles of the feet. Photo was reproduced from Centres for Disease Control and Prevention (https://www.cdc.gov/poxvirus/monkeypox/index.html)

Laboratory diagnosis

Diagnosis of monkeypox infection is made by cell culture, polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA) or Western blotting with PCR being used for definitive diagnosis[3–5,18]. Standard, contact and droplet precautions must be applied during specimen collection and all samples potentially infected with monkeypox virus should be handled in Biosafety level 2 facilities. Specimen that may be obtained to aid diagnosis include tonsillar tissue, oropharyngeal tissue or nasopharyngeal tissue swab, lesion biopsy using the punch biopsy kit, lesion fluid, lesion roof, scab/crust, whole blood, acute and convalescent phase sera (4–6,16). Oropharyngeal or nasopharyngeal swab can be obtained for viral culture using MK2 cells, LLC-MK2 cells and Vero E6 cells. Growth is detected by the presence of cytopathic effects which is seen as multinucleated syncytial keratinocytes. However, this cytopathic effect is also seen with Vaccinia and Cowpox viruses thereby causing a difficulty in differentiating these viruses via cell culture. The virus can be identified from the culture by DNA restriction analysis (5).

Exudates of the lesion on a swab, scabs of the lesion, skin biopsy of the vesiculopustular rash or a sample of the roof of an intact vesiculopustule can also be used for diagnosis. These can be analysed using PCR for DNA sequence-specific for monkeypox virus such as the monkeypox extracellular-envelope virus protein gene and the conserved segment of the DNA polymerase gene (E9L) (19,20). Maintenance of a cold chain upon specimen collection is preferable but if cold chain is unavailable, the viral DNA in the specimen is stable for a long time if kept in a cool, dark environment. Detection of antibodies; immunoglobin M (IgM) or immunoglobin G (IgG) in serum or plasma using ELISA is an effective means of diagnosis of monkeypox infection (21). IgM is detected in serum about 5 days after the onset of rash while IgG is detected after more than 8 days of the onset of rash. Paired sera for IgG and IgM titers may be analyzed for increasing titre values to detect seroconversion which is used as an indicator of recent monkeypox infection (21). Tzanck smear can be used to differentiate monkeypox infection from other non-viral disorders but it does not differentiate monkeypox infection from smallpox or herpetic infections. Antibody Immuno Column for Analytical Processes (ABICAP), an immune-filtration technique which works based on gravity-driven flow-through antigen capture ELISA has been developed. It is an on-site laboratory diagnostic test which can be used in both humans and animals (22).

Biopsy specimen from lymph nodes or scab material; vesicular fluid, blood specimen or viral culture can be examined using electron microscopy with negative staining revealing a large brick-shaped particle with rounded corners which is characteristic of a poxvirus (18). Round-to-oval intracytoplasmic inclusions with sausage-shaped structures centrally measuring about 200-300 µm may also be seen on electron microscopic examination. These inclusions are commonly found with Orthopox viruses thereby aiding differentiation from Herpes and Parapox viruses (5).

Histological examination of papules may reveal acanthosis, individual keratinocyte necrosis and basal vacuolation along with a superficial and deep perivascular, lymphohistiocytic infiltrate in the dermis ([5,20]). Histology of the vesicles may reveal spongiosis with ballooning and reticular

degeneration. Pustules may reveal necrosis of the epidermis with numerous eosinophils and neutrophils, with many displaying karyorrhexis. The necrosis may extend through the entire thickness of the epidermis with a sharp lateral demarcation from adjacent intact epidermis. An associated perivascular infiltrate consisting of eosinophils and neutrophils in addition to lymphocytes and histiocytes may indicate secondary vasculitis. Presence of viral inclusions may be suggested by the presence of amphophilic intranuclear structures within the keratinocytes[5]. Eosinophilic Guarnieri-type intracytoplasmic inclusions are also seen within the keratinocytes (20). Orthopox viral antigens can be assessed using immunohistochemistry staining but its availability is limited to select reference laboratories.

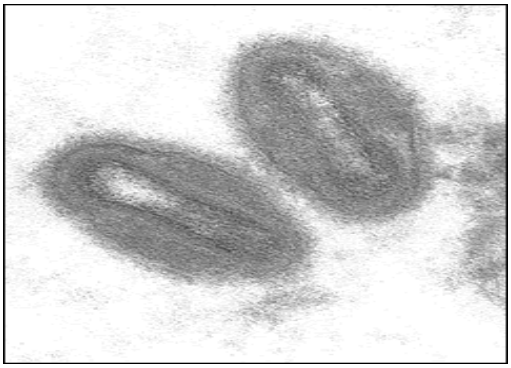

Figure 2: Electron micrograph of monkeypox virus. Reproduced from: http://www.uwlax.edu/clinmicro/clinicalmicro.htm University of Wisconsin-La Crosse, Microbiology program (No Author Specified).

Case Management

Monkeypox is self-limiting hence treatment is mainly by bed rest and supportive care. However, hospitalization and possible intensive care may be necessary in severe cases. Preferably, nursing should be done in a negative pressure room while applying airborne and contact precautions to limits its spread. Isolation of infected individuals is also a necessary precaution in preventing the spread of the infection and this must be continued until the last crust is shed as direct contact with skin lesions and fomites are considered infectious (6).

There is no proven treatment for human infection at the moment but cidofovir and brincidofovir (CMX-001) have been shown to have anti-monkeypox viral activity *in vitro* and in animal studies (3,6). A reduction in mortality was observed with the use of cidofovir than therapeutic use of smallpox vaccine following intratracheal infection of cynomolgus monkeys (23). However, CDC recommends consideration of cidofovir in individuals with severe monkeypox infection; brincidofovir has an improved safety profile over cidofovir as it has less renal toxicity during its use in the treatment of cytomegalovirus infection. Tecovirimat (previously known as ST-246) is an oral antiviral agent with activity against orthopoxviruses including monkeypox *in vitro* and in animal studies but its effectiveness in humans is unknown (24).

Vaccinia Immune Globulin (VIG) is a blood product which is rich in antibodies against Vaccinia virus; it is obtained from pooled blood of individuals who have been inoculated with the smallpox vaccine. There is no available data on the effectiveness of VIG on the prevention and treatment of complications from monkeypox infection, however its use may be considered in patients with severe infection[15]. CDC recommends the prophylactic use of VIG in persons who have been exposed to the virus but have severe cellular immunodeficiency with contraindication to smallpox vaccination (6).

Prevention and Control

Restriction of movement of monkeys and small African mammals may limit the spread of monkeypox virus outside Africa[6,25]. Animals with suspected monkeypox infection which may be demonstrated as rhinorrhea, respiratory distress, mucocutaneous lesions, ocular discharge and/or lymphadenopathy should be quarantined while avoiding contact especially bites and scratches and exposure to body fluids and secretions. Animals that might have come in contact with an infected animal must be quarantined and observed for symptoms of monkeypox for 30 days. Use of gloves and wearing of protective clothing while handling sick animals or during slaughtering procedures, thorough cooking of all animal products before eating also limits the risk of infection[6,25]. There is need to avoid contact with any material that has been in contact with infected animal, practicing good hand hygiene after contact with infected animals or persons also limits the risk of infection and its spread (5).

There is currently no commercially available vaccine specific for monkeypox. Immunization of healthcare workers and those exposed to their samples with the smallpox vaccine such as ACAM2000, which contains live Vaccinia virus confers 85% protection from monkeypox infection[4]. Subsequent monkeypox infection may be milder even several years post vaccination with a reduction in the incidence of complications (16). The investigational vaccines Aventis Pasteur Smallpox Vaccine (APSV) which contains a replication-competent Vaccinia virus and Imvamune (MVA-BN): a replication-deficient smallpox vaccine which contains an attenuated live virus are also available but have restricted use[6]. Imvamune may be used in individuals with certain immune deficiencies. The vaccine, LC16m8 which also contains attenuated vaccinia virus and has less adverse effects than ACAM2000 has been licensed

for used in Japan (4.) Prior vaccination with vaccinia virus is known to provide 85% protection and reduce the severity of the infection but caution is needed in population with high HIV prevalence[3,4]. The discontinuation of the general smallpox vaccination and the ability of the virus to evolve may have contributed to increased susceptibility of humans to monkeypox infection (4,15).

In the case of significant unprotected exposure to an infected animal or person, CDC recommends pre-exposure smallpox vaccination to field investigators, laboratory personnel, veterinarians, and healthcare workers investigating or caring for patients with suspected monkeypox. Additionally, post-exposure smallpox vaccination within two weeks of exposure preferably within 4 days of exposure is preferable (6). Due to the relative rarity of monkeypox infection, vaccination of an entire population is uncommon and caution must be applied when considering vaccination in populations with high prevalence of HIV infection due to the risk of complications (5).

Challenges with Diagnosis, Management and Prevention of Monkeypox in Nigeria

It is over 46 years since the first case report of monkeypox in Nigeria, however there is still limited public awareness of the disease. Laboratory diagnosis, as well as management and prevention of monkeypox infection in Nigeria remain challenging. Nigeria is a resource-poor country with limited infrastructure, technical skill and training which is required in making a diagnosis. This might be attributed to the relative rarity of the disease as well as poor political willpower in health-related matters. There is a need to increase public awareness among the populace, physicians inclusive by dissemination of accurate information through authentic platforms on the risk factors, symptoms, signs, prevention and control of the disease. Emphasis should be made on contact precautions while handling animals and thorough cooking of animals before ingestion. There is also a need to clarify myths about monkeypox disease in the various communities and states. Furthermore, access to basic health care should be provided for all through health insurance schemes in order to aid early diagnosis, treatment and control of the infection. Improvement of political willpower in health-related matters via policy making, provision of funds for infrastructure, personnel training and research is also necessary. Development of a field-deployable point-of-care test further aiding the diagnosis is important.

CONCLUSION

In conclusion, monkeypox virus infection is relatively rare and usually self-limiting. In the era of globalization, with consequent increased mobility of man and cross-border transport of animals, there is a potential for the spread of monkeypox virus to several parts of the world. The ability of the virus to evolve, the potential threat of its use in bioterrorism, as well as its re-emergence in Nigeria further justify the need for better understanding of monkeypox infection.

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OPTIMIZATION OF PECTINASE PRODUCTION BY *ASPERGILLUS NIGER* USING CENTRAL COMPOSITE DESIGN

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ABSTRACT

Pectinases are a group of enzymes that catalyze the breakdown of pectin. Pectinase producing *Aspergillus niger* was obtained from a five-day old Eba (Cassava flakes). Response surface methodology was used for optimizing the process of the pectinase produced. Four independent variables which are, temperature, pH, substrate concentration and time of Heating at 70^{o}C were used to optimize the significant correlation between the effects of the variables on pectinase production. A second-order polynomial was fitted to data and validated by ANOVA. The results revealed maximum pectinase production at pH 6.0, 50^{o}C Temperature, 0.02% substrate concentration and the enzyme lost all its activity within 7 min of heating at 70^{o}C. The study revealed that optimization of pectinase through RSM could improve the enzymatic characteristics and yield of the enzyme. The models used were highly significant with a correlation coefficient (R^2) of 0.901

Keywords: Pectinase; *Aspergillus niger*; Response Surface Methodology; Central Composite Design

OPTIMISATION DE LA PRODUCTION DE LA PECTINASE PAR ASPERGILLUS NIGER AIDE PLAN COMPOSITE CENTRAL

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Résumé

Pectinases sont un groupe d'enzymes qui catalysent la décomposition de la pectine. La production de la pectinase Aspergillus niger a été obtenu à partir d'un vieux de cinq jours (EBA) flocons de manioc. La méthodologie de surface de réponse a été utilisée pour optimiser le processus de la pectinase produite. Quatre variables indépendantes qui sont, de la température, du pH, de la concentration du substrat et le temps de chauffage à 70o C ont été utilisées pour optimiser la corrélation significative entre les effets des variables sur la production de la pectinase. Un polynôme de deuxième degré a été monté aux données et validées par ANOVA. Les résultats ont révélé la production maximale de la pectinase à pH 6,0, 50 °C Température ambiante et à un 0,02 % de la concentration du substrat et l'enzyme a perdu toute son activité dans les 7 min de chauffage à 70oC. L'étude a révélé que l'optimisation de la pectinase par RSM pourrait améliorer le rendement et les caractéristiques enzymatiques de l'enzyme. Les modèles utilisés étaient très significatives avec un coefficient de corrélation (R2) de 0,901

Mots-clés: la pectinase, Aspergillus niger ; la méthodologie de surface de réponse ; plan composite centra

INTRODUCTION

Pectinases are industrially important enzymes (1). They are a group of enzymes that catalyze the breakdown of pectin (2). The two major sources of pectinase are from plants and microorganisms but microbial source of the enzyme is becoming increasingly important (3).The role of pectinase in the clarification of green, yellow and red apple juice had been reported (4). *Rhizopus* sp. isolated from deteriorated grapes produce pectinase (5). A total of 44 fungal strains isolated from different agricultural and non agricultural soils in Chittoor district, Andhra Pradesh in South India revealed only four trains identified as *Aspergillus niger, Aspergillus flavus, Aspergillus japonica*s and *Chaetomium globosum* as pectinase producing microorganisms under submerged fermentation (6). Pectinases have the ability to break down a variety of polysaccharides found within fruit juice extract to release soluble sugars which can clarify the juice producing a clearer, sweeter product (7). The immense potential of pectinase from microbial source especially from *Aspergillus niger*, a fungi which has been given the Generally Regarded as Safe (GRAS) status for clarification of fruit juice has necessitated the optimization of the production process of pectinase to achieve maximum yields. Response surface Methodology had been identified and widely used as an efficient tool for the optimization of different physiochemical parameters with the advantage of determining the influence of variables on enzyme units and to optimize the variables in order to achieve maximum yields under the best possible economic conditions (8). This investigation therefore reports the optimization of the production process of pectinase by *A.niger* by submerged fermentation.

MATERIALS AND METHODS

Isolation of *Aspergillus niger*

Aspergillus niger was obtained from a five-day-old Eba (Cassava flakes) and cultured on potato dextrose agar (PDA) for 4 days at room temperature. Pure cultures were obtained by sub-culturing and further subculturing on potato dextrose agar (PDA). *A. niger* was identified by morphological characterization and the results compared with the "Atlas (9). Pure cultures were maintained on potato dextrose agar (PDA) slants and sub cultured periodically throughout the duration of this research work.

Enzyme Production

This was carried out by submerged fermentation (SMF) according to the method described (10). The fermentation medium was made up of 0.1% NH_4NO_3, 0.1% $MgSO_4.7H_2O$, 0.1% $NH_4H_2PO_4$ and 1% pectin autoclaved at 121^{o}C for 20min. One disc of actively growing *A. niger* from a 96-h-old culture medium was inoculated each into seven different flask containing 50ml fermentation medium using a cork borer of 10mm diameter and separated from a single flask by filtration using muslin cloth and the filtrate was analyzed daily for pectinase activity.

Pectinase Assay

Pectinase assay was carried out according to the method of (11). 0.5g of the pineapple pectin (substrate) will be weighed and dissolved in 100ml of sodium acetate buffer at a pH of 5.0. The protein content will be determined according to (12).

Partial purification of Pectinase

This was carried out using activated charcoal according to the method (13). Three percent (w/v) of activated charcoal was added to the crude pectinase (pH 4.5) and incubated at 30^{o}C for 30min with occasional stirring. The mixture was centrifuged at 2500rpm in a bench centrifuge for 10min. Pectinase activity and the protein content were determined according to the methods described earlier.

Pectinase Production Optimization

Optimization of the physical parameters for maximum pectinase production was carried out using the response Surface Methodology which is a powerful and efficient mathematical approach widely applied in the optimization of enzymes for industrial processes. Central Composite Factorial Design was used in the optimization of the conditions for pectinase production. A total of 30 experiments were used in the study. The independent variables studied were pH (X_1), temperature (X_2, ^{o}C), Substrate Concentration (X_3, mg/ml) and Time of heating (X_4, min). The response (dependent variable) was pectinase activity (Units/ml). Each independent variable was studied at three coded levels (-1, 0, +1). The minimum and maximum levels of each independent variable and the experimental design with respect to their coded and uncoded levels (Table 1). The minimum and maximum ranges of variables and full experimental plan with respect to their values in actual and coded forms (Table 2). The relationship between the coded values and actual values are as described (Eq.1)

TABLE 1: LEVELS OF THE FOUR INDEPENDENT VARIABLES (FACTORS) USED IN RSM

Variables	Unit	Low (- 1)	Actual (0)	High (+ 1)
pH (x_1)	-	3.0	4.5	6.0
Temp. (x_2)	^0C	30	45	60
Substrate Conc. (x_3)	*mg/ml*	0.2	1.6	3.0
Time of Heating (x_4)	*min.*	0	15	30

First Degree Polynomial (First-Order Model)

$$y=\beta_0+\beta_1x_1+\beta_2x_2+\beta_3x_3+e\equiv\beta_0+\sum_{i=1}^{p}\beta_ix_i+e \quad (1)$$

Second Degree Polynomial (Second-Order Model)

$$y=\beta_0+\sum_{i=1}^{p}\beta_{ii}x_i^2+\sum_{i}\sum_{j>1}\beta_{ij}x_ix_j+e \quad (2)$$

Where i, j are linear, quadratic coefficients, respectively, while β is regression coefficient, y is response variable (pectinase activity), *k* is the number of factors studied and optimized in the experiment, *e* is the random effect and β_0 is the intercept. The second order model used to fit the response to the independent variables is as described (Eq. 2). A second-order regression analysis of the data was carried out to get empirical model that defines response in terms of the independent variables. Analysis of variance (ANOVA) was performed in coded levels of variables to study the effects of the independent variables. The 2D graphs were generated to understand the effect of selected variables individually and in combination to determine their optimum level (Fig. 1a – f))

Coding Method

$$X_j=\frac{ActualLevel-(HighLevel+LowLevel/2)}{(HighLevel-LowLevel/2)}$$

RESULTS

The results of this study revealed that the Response Surface Methodology (RSM) used to optimize the parameters (pH, temperature, substrate concentration, and time of heating) and the Central Composite Design with a total of thirty experiments produced experimental and predicted values of yields of pectinase are in good agreement with the observed values (Table 2). The competence of the model and fitness evaluated by using ANOVA (Analysis of variance) and regression coefficients for the experimental design used (Tables 3, 4 & 5).

TABLE 2: CENTRAL COMPOSITE ROTATABLE DESIGN OF THE VARIABLES WITH ENZYME ACTIVITY AS RESPONSE

Run	pH (x_1)	Temp. (x_2)	Substrate Conc. (x_3)	Time of Heating (x_4)	Actual Value	Predicted Value
1	- 1	+ 1	- 1	- 1	0.238	0.227
2	0	0	0	0	0.300	0.308
3	+ 1	+ 1	- 1	- 1	0.315	0.329
4	0	0	0	0	0.491	0.451
5	- 1	+ 1	+ 1	+ 1	0.128	0.134
6	+ 1	- 1	+ 1	- 1	0.100	0.109
7	0	0	0	0	0.089	0.067
8	0	0	0	0	0.001	0.002
9	0	0	0	0	0.015	0.019
10	0	0	0	0	0.051	0.061
11	- 1	- 1	+ 1	+ 1	0.003	0.005
12	- 1	- 1	- 1	+ 1	0.001	0.002
13	+ 1	+ 1	+ 1	- 1	0.001	0.007
14	0	0	0	0	0.001	0.003

TABLE 2 (CTD)

Run	pH (x_1)	Temp. (x_2)	Substrate Conc. (x_3)	Time of Heating (x_4)	Actual Value	Predicted Value
15	**0**	**0**	**0**	**0**	**0.002**	**0.005**
16	**- 1**	**- 1**	**+ 1**	**+ 1**	**0.506**	**0.511**
17	**+ 1**	**- 1**	**+ 1**	**+ 1**	**0.023**	**0.039**
18	**+ 1**	**+ 1**	**- 1**	**+ 1**	**0.009**	**0.011**
19	**0**	**0**	**0**	**0**	**0.006**	**0.008**
20	**- 1**	**+ 1**	**+ 1**	**- 1**	**0.003**	**0.002**
21	**+ 1**	**- 1**	**- 1**	**+ 1**	**0.001**	**0.004**
22	**0**	**0**	**0**	**0**	**0.070**	**0.086**
23	**+ 1**	**+ 1**	**+ 1**	**+ 1**	**0.015**	**0.018**
24	**- 1**	**+ 1**	**- 1**	**+ 1**	**0.009**	**0.008**
25	**0**	**0**	**0**	**0**	**0.043**	**0.048**
26	**+ 1**	**- 1**	**- 1**	**- 1**	**0.214**	**0.300**
27	**0**	**0**	**0**	**0**	**0.161**	**0.182**
28	**- 1**	**- 1**	**- 1**	**- 1**	**0.101**	**0.126**
29	**0**	**0**	**0**	**0**	**0.005**	**0.008**
30	**0**	**0**	**0**	**0**	**0.031**	**0.044**

TABLE 3: ESTIMATED REGRESSION COEFFICIENTS FOR THE SECOND-ORDER MODEL

Term	Coefficient	Standard Error of Coefficients	T_{-value}	P_{-value}
Constant	.102787	.086512	1.1881	.0022
x_1 (pH)	.235401	.342109	.6881	.0001
x_2 (Temperature)	.541989	.210006	2.5808	.0003
x_3 (Substrate Conc.)	1.000452	.419412	2.3854	.0012
x_4 (Time of Heating)	.000231	.994188	.00002	.05904
$x_1 x_2$	2.985412	1.004518	2.9719	.0004
$x_1 x_3$	2.000341	3.783198	.5287	.0701
$x_1 x_4$	1.620954	3.033128	.5344	.06713
$x_2 x_3$	.011299	.457899	.0247	.0804
$x_2 x_4$	1.446698	2.459163	.5883	.0576
$x_3 x_4$	2.094219	2.859674	.7323	.0159
x_1^2	.851953	1.009412	.8440	.0009
x_2^2	-.528971	.124976	-4.2326	.0901
x_3^2	1.095518	1.459731	2.8996	.0000
x_4^2	1.951987	.179346	16.1674	.0002

TABLE 4: ANALYSIS OF VARIANCE (ANOVA) FOR RESPONSE SURFACE SECOND-ORDER MODEL

Source of Variation	*Df*	Sum of Squares	Mean Square	F_{-value}	P_{-value}	Remarks
FO	4	.043345	.010836	.1802	.1198	Not Significant
TWI	6	.137678	.022946	.3815	.0009	At least one i s significant
PQ	4	.094199	.023549	.3915	.0104	At least one is significant
Residuals	15	.902111	.060140			
Lack of Fit	12	.356319	.029693	.4937	.0211	Not Significant

TABLE 5: STATISTICAL ANALYSIS OF PECTINASE PRODUCTION

Model Term	Values
Multiple *R*-squared	0.9008
Adjusted *R*-squared	0.8992
Std. Dev.	0.0190
PRESS	0.0309

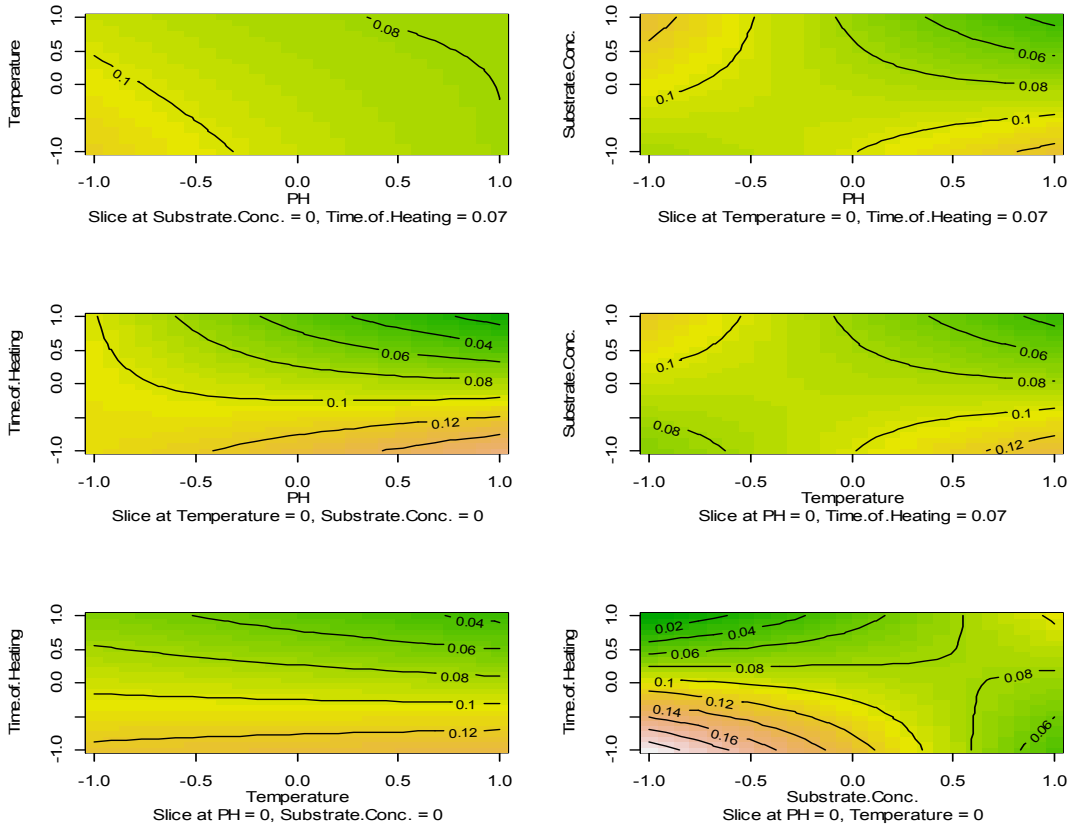

Fig.1. (a) Effect of temperature and pH on the production of pectinase keeping substrate concentration and time of heating at zero level (coded)
(b) Effect of substrate concentration and pH on the production of pectinase. Temperature and time of heating were held at zero level (coded)
(c) Effect of Time of heating and pH on the production of pectinase. Substrate concentration and temperature were kept at zero level (coded)
(d) Effect of substrate concentration and temperature on the production of pectinase with pH and time of heating kept at zero level (coded)
(e) Effect of time of heating and temperature on the production of pectinase. Other variables pH and substrate concentration were held at zero level (coded)
(f) Effect of time of heating and substrate concentration on the production of pectinase. Other variables, pH and temperature were held at zero level (coded).

DISCUSSION

The results of this study revealed that the Response Surface Methodology (RSM) used to optimize the parameters (pH, temperature, substrate concentration and time of heating) and the Central Composite Design with a total of thirty experiments was successful for the production process of pectinase produced by *A.niger*. Ibrahim and Elkhidir (14) reported that Response Surface Methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching for the optimum conditions. It has successfully been used in the optimisation of bioprocesses. They reported their data using RSM for maximization of enzyme production and minimization of the cost for cellulase, xylanase and phytase by different researchers**.** The Production optimization of a heat-tolerant alkaline pectinase from *Bacillus subtilis* ZGL14 with optimal temperature and pH of 50°C and 8.6 respectively was reported (15). Handa *et al*. (16) reported a novel strain *Rhizopus* sp. C4 isolated from compost for the production of pectinase and Response surface methodology (RSM) was employed to optimize the various environmental parameters (temperature, moisture and incubation days) were studied statistically for a total of 20 runs using central composite design for pectinase production. They obtained the highest yield of the enzyme, i.e., 11.63 IU/mL from 1:3.5 moisture ratios in 7 days at 30°C.

Conclusion

The different parameters of temperature, pH, substrate concentration and time of heating ha d a great effect on the production of pectinase by *A. niger*. These relationships can be explained by the second-order polynomial. The two dimensional contour plots were used to set the optimum values of the variables and significant improvement in the production of pectinase by *A. niger* was observed.

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