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Microbiology and Pathology

Fungal and Yeast Involvement

Coagulase Negative Staphylococci

Highlights

Plastic Contaminants in Borehole

Immediate Detection of the Bacteria

Discovering Thoughts, Inventing Future

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Contents of the Issue

i. Copyright Notice
ii. Editorial Board Members
iii. Chief Author and Dean
iv. Contents of the Issue

1. Bioavailability and Health Effects of Plastic Contaminants in Borehole Water Stored in Plastic Containers. *1-10*
2. Electrochemical Biosensor based on Clay for the Immediate Detection of the Bacteria. *11-15*
3. Collective Memory and the Hologenome Concept. *17-24*
4. Detection of *Mycobacterial* Infections (MTB & NTM) by Different Molecular, Staining and Culture Techniques among Infertile Females. *25-32*
5. Linezolid and Methicillin-Resistant Coagulase Negative Staphylococci from Anterior Nares of Nigerian Tertiary School Students. *33-42*
6. Fungal and Yeast Involvement in Skin Diseases. *43-47*
7. Histopathological Studies of Chlorpyrifos Toxicity in Catfish. *49-54*

v. Fellows
vi. Auxiliary Memberships
vii. Process of Submission of Research Paper
viii. Preferred Author Guidelines
ix. Index

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Bioavailability and Health Effects of Plastic Contaminants in Borehole Water Stored in Plastic Containers

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Abstract- The aim of this study was to evaluate the bioavailability and health effects of plastic contaminants on borehole water stored in plastic containers. Three brands of plastic containers filled with borehole water were collected from homes in Ugbowo, Benin city. Physicochemical parameters were determined using standard methods. Total bacterial and coliform counts were determined using the pour plate technique. Conductivity, TDS, Chloride, Sulphate and Nitrate were within the recommended standards while turbidity and total iron were above recommended standards. The isolates identified include: *Klebsiella sp, Bacillus sp, E. coli, Pseudomonas aeruginosa, Aspergillus flavus, Saccharomyces* sp and *Aspergillus niger*. The total bacterial count in the water samples ranged from 1.4×10^3cfu/ml to1.8×10^3cfu/ml at week four while fungal counts was 1.3×10^2cfu/ml to 1.6×10^2cfu/ml. Bisphenol A was discovered to leach at detectable levels from the plastic containers as storage increased. The result of the BPA analysis revealed that BPA congeners ranged from 0.023mg/l within days of collection to 0.251ml/l at the fourth week of storage. This study has shown that storage of borehole water in plastic containers for prolonged period affects the bacteriological and chemical properties of the water, hence storage of borehole water in plastic for prolonged periods should be discouraged and discontinued.

Keywords: *plastic contaminant, bioavailability, borehole water, bisphenol A.*

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Bioavailability and Health Effects of Plastic Contaminants in Borehole Water Stored in Plastic Containers

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I. Introduction

Water is a transparent, colourles, odourless and tasteless liquid that makes up the sea, lakes, rivers, rainfall as well as the liquid that makes up living organisms (Michael, 2000). Water is a compound of two elements; hydrogen and oxygen atoms with a chemical formula H_2O and it is known to make up about 70 percent of the earth surface (Osei, 2005). Rivers, streams, wells and more recently boreholes, serve as the main source of drinking water and domestic use in developing countries like Nigeria, where most of the people reside in rural areas (Ibe and Okplenya, 2005). According to the World Health Organisation guidelines for drinking water underground water supplies are usually considered safe provided they are properly located, constructed and operated to WHO regulatory standards (WHO, 1971). Boreholes with hand pumps are commonly used by poor rural communities and this amounts to approximately 250,000 hand pumps in Africa. Studies have shown that water may become contaminated at any point between collection, storage and usage (Tambekar *et al*.,2006). Also, storing water in plastic containers and handling procedures of water at homes, hotels or restaurants causes water quality deterioration to such extent that it becomes potential risk of infection to consumers (Jagals *et al*., 1999).

Microorganisms associated with contaminated water includes *Salmonella* sp*, Escherichia coli and Vibro cholera* (Birmingham *et al*., 1997). Water borne diseases often arises when pathogenic microorganisms associated with contaminated water is consumed. Boreholes and wells are polluted either industrially, domestically and agriculturally. Industrial pollution may involve seepages of used water containing chemicals such as metals and radioactive compounds while domestic pollution may involve seepage from broken septic tanks, pit latrines and privies. Runoff water after rainfall carrying pesticides, fertilizers, herbicides and faecal matter may contribute to agricultural pollution. However the pollution sources, the quality of packaging plastic bottle cannot guarantee safety from contamination. In the natural environment there are compound that have the potential to disturb equilibrium in living organisms and are mistakenly recognised by oestrogen receptors, treated the same as those naturally present in the organism. Substances of this type are known as Endocrine Disrupting chemicals. Bisphenol A is one of the highest volume chemicals produced world wide with more than 6million pounds produced each year (Burridge, 2003). It serves as a base line in the manufacturing of plastics and a major compound in the production of epoxy resins, printers ink, powdered paints, dental sealants and composites (Vandenberg *et al*., 2009; Markey *et al.*, 2003). Bisphenol A can be released and leached into water from packaging materials. Hence, through consumption due to it use in packaging and storage containers, consumers are directly or chronically exposed to BPA (Brotons *et al.*, 1995). Several health cases have been

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attributed to bisphenol A and studies have been carried out on the chemical component at different scales. Some researches have focused on the detection and measurement of bisphenol A while others on the effects on humans and laboratory animals (Chang *et al*., 2009). For example a study in the United States have evaluated the presence of BPA in packaged water (Jin *et al*., 2004). In Iran, Raskari *et al*. (2011) and Jafari *et al*. (2006) investigated the presence of BPA in canned foods, surface water and waste water. Makinwa and Uadia (2015) carried out a survey on the levels of BPA in effluents, soil leachates, food samples, drinking water and consumer products in South Western Nigeria. However it seems that no study has been done on the measurement of BPA in borehole water filled into plastic containers in Nigeria. This study examined the bioavailability and health effects of plastic contaminants in borehole water filled into plastic containers.

II. Materials and Method

A total of three different brands of plastic containers filled with borehole water samples were collected from Ugbowo in Benin City. The samples were stored at room temperature for four weeks, thus mimicking typical conditions in retail outlets, supermarkets and in homes. Sub-samples were drawn from the stock samples on weekly basis and within days of being purchased for microbiological and physico-chemical analysis, using WHO analytical methods (WHO, 2011). Water samples for analysis of dissolved oxygen (DO) and biochemical oxygen demand (BOD) were collected in pre-sterilized brown bottle and fixed by adding 1.2ml of Winkler solution.

a) Microbiological Analysis of Water Samples

Total viable bacterial and fungal counts were determined by pour plate technique using standard methods (APHA, 1998). Nutrient agar medium was used for the enumeration of viable aerobic bacteria while Sabouraud dextrose agar was used for fungal count. MacConkey agar was used for coliform count while eosin methylene blue medium was used for faecal coliform and *E.coli* counts. The different brands of borehole water samples were serially diluted upto 10^{-3} dilution/ then 0.1ml of the appropriate dilutions were plated in Nutrient agar, Sabourand agar, MacConkey and Eosin methylene blue media. Nutrient and MacConkey agar plates were incubated at 37^0C for 24 hr, while Sabouraud and eosin methylene blue agar plates were incubated at room temperature for 72hr and at 44.5^0C respectively. After incubation, the number of discrete colonies were counted and recorded in colony forming unit per milliter (cfu/ml).

The isolates were sub-cultured to obtain pure cultures. The pure cultures so obtained were transferred to agar slants by streaking and further biochemical tests were carried-out to identify the isolates. Faecal coliform count that was determined using pour plate technique, was recorded by the organisms ability to appear as greenish metallic sheen. This was taken as positive for *E.coli*. However further confirmatory test was carried out by the ability of the organisms to ferment lactose at 44.4^0C.

b) Identification of Microbial Isolates

By streaking on their respective media plates, aseptically purified representatives of discrete colonies were obtained. They were further stored in agar slants for further characterization. All the bacterial and fungal isolates were initially examined microscopically for morphological characterization followed by appropriate biochemical test for bacterial isolates (Gram staining, indole, catalase, motility, citrate utilization, urea production, oxidase, congulase and oxidative/ fermentative utilization of lactose and glucose). The identification of bacterial isolates was done in accordance with criteria of Bergeys manual of Determining Bacteria (Holt *et al.,* 1994).

The fungal isolates were identified micro-scopically using lacto phenol cotton blue test. The identification was achieved by placing a drop of the stain on clean slide with the aid of a wire loop, where a small portion of the mycelium from the fungal cultures was removed and placed in a drop of lactophenol. The mycelium was spread on the slide with aid of wire loop. A cover slip was gently applied with little pressure to eliminate air bubbles. The slide was then mounted and observed with objective lens and identification done in accordance with Barneth and Hunter (1982) criteria.

c) Physico-Chemical Analysis of borehole Water Samples

Physico-chemical parameters determined included: pH, temperature, conductivity, total dissolved solid (TDS), total suspendend solid (TSS), turbidity, alkalinity, total hardness, total iron, chloride, sulphate, phosphate, nitrate and biochemical oxygen demand (BOD). In carrying out this analysis various sub-samples drawn from stock samples (stored) were taken to the laboratory in ice-packed coolers. Those that could not be analyzed the same day were stored in a refrigerator at a temperature of 4^0C. All the physico-chemical analysis were carried-out using standard method (APHA, 1998). Data collected were subjected to statistical analysis.

d) Sample Treatment and Analysis for Bisphenol a (BPA)

Bisphenol A was extracted from water samples using the modified procedure from Dean and Xion (2000). Fifty millitres (50ml) of water sample was measured into a separating funnel in which 100ml of dichloromethane (DCM) and shaked for 30min. The separating funnel was clamp and the mixture was allowed to separate–out. After separation, the DCM

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portion was collected. The process was repeated three times for complete extraction. Blanks were prepared following the same procedures without sample using di-ioned water. The standard sample used for quality control was prepared by adding the standard solution (Bisphenol A) to DCM. The extracts were separated, and activated copper was added to the combined extracts for desulphurization. After subsequent filtration over anhydrous sodium sulphate, the solution was concentrated to 1.0ml using a rotary evaporator, an internal standard mixture (Vinyl chloride) solution was run with the extract for quality control check using Hewlett Packard HP 5890 series II gas chromatograph with mass selective detection (GC-MS).

e) GC-MS Instrumentation and Conditions

Hewlett Packard HP 5890 series II Gas chromagraph equipped with an Agilent 7683B injector (Agilent Technologies Santa Clara, CA, USA), A 30m, 0.25mm i.d. HP-5MS capapillay column (Hewlett – Packard, Palo Alto, CA, USA) coated with 5% phenyl-methylsiloxane (film thickness 0.25μm) and an Agilent 5975 mass selective detector (MSD) was used to separate and quantify the BPA compounds. The samples were injected in the splitless mode at an injection temperature of 300^{0}C. The transfer line and ion source temperatures were 280^{0}C and 200^{0}C. The column temperature was initially held at 40^{0}C for 1min, raised to 120^{0}C at the rate of 25^{0}C/min, then to 160^{0}c at the rate of 10^{0}C/min and finally to 300^{0}C at 5^{0}C/min, held at final temperature for 15min. Detector temperature was kept at 280^{0}C. Helium was used as a carries gas at a constant flow rate of ml/min. Mass spectrometry was acquired using the electron ionization (EI) and selective ion monitoring (SIM) mode.

III. Results

Table 4 shows the mean values of the total viable bacterial counts, faecal coliform counts, *E. coli* counts and fungal counts of water samples collected at intervals of within few days, one week and after four weeks of storage at room temperature. Total viable bacterial counts of the borehole samples had a range from 1.4 $\times$ 10^3 cfu/ml within day of collection to 1.8$\times$ 10^3 cfu/ml at the fourth week. Coliform counts of the borehole water samples also ranged from (4.7 $\times$ 10^2 cfu/ml) week 0, (4.4 $\times$ 10^2 cfu/ml) week 1 and (4.9 $\times$ 10^2 cfu/ml) week 4. The range of coliform count is from (4.4 $\times$ 10^2 cfu/ml - 4.9 $\times$ 10^2 cfu/ml) with brand C having the highest counts. *E. coli* and fungal counts were; (6.0 $\times$ 10^1) week 0, to (7.0 $\times$ 10^1 cfu/ml) in week 4 and (1.3$\times$ 10^2 cfu/ml) week 0, to (1.6 $\times$ 10^{2}cfu/ml) week 4 respectively.

IV. Discussion

The result of this study has revealed the effect of prolonged storage of borehole water samples stored at room temperature on the total heterotrophic bacterial count. Increase in storage of the samples led to a gradual increase in the total heterotrophic bacteria count as shown in table 4. This result is in line with (Atuanya *et al*., 2014) who revealed the effect of storage on the physicochemical and bacteriological qualities of potable water in Benin City. Total coliform count recorded ranged from 4.7$\times$ 10^2 cfu/ml in week 0 to 4.9$\times$10^2 cfu/ml in week 4. This result is also in accordance with Rogbesan *et al*. (2002) who reported the presence of total coliform count above the range recommended by WHO. The observation from this result reveals that high heterotrophic bacteria count also reflected in total coliform count. *E.coli* and fungal counts recorded ranged from 6.0$\times$10^1 cfu/ml to 7.0$\times$10^{1}cfu/ml and 1.3$\times$10^{2}cfu/ml to 1.6$\times$10^{2}cfu/ml respectively. The presence of high heterotrophic bacteria count, total coliform count, *E.coli* and fungal count could be as a result of the proximity of the borehole to a pit latrine at a distance less than 30meters that is recommended by WHO or as a result of the nature of the pipes used for the distribution of the water. They may be rusty, thus allowing seepage of microbial contamination into the borehole. The bacterial and fungal isolate identified in this study showed that the water is not wholesome, therefore not fit for consumption without additional treatment.

The result of the physicochemical analysis of the borehole water showed an increase in the pH from 6.6 in week 0 to 5.6 in week 4 as shown in table 3. Akinde *et al.* (2011) and Agbaje *et al*. (2012) obtained similar results for stored sachet and borehole water samples. A low pH encourages corrosion of pipes while a pH above 7 requires more chlorine and contact time for proper disinfection. Turbidity values ranged from 5.30 in week 0 to 5.80 NTU in week4. These values were above the maximum acceptable limit of 5NTU recommended by WHO. This high levels of turbidity in plastic bottle filled with borehole water is a source of concern because the particles forming the turbidity could habour and shield pathogenic microorganisms and hence escape the action of disinfection (EPA, 2001). The total iron also increase from 1.031mg/l to 1.051mg/l in week 4 and exceeded the WHO recommended standard. This element is present in ground water in the soluble ferrous form (Fe^{2+}). It is easily oxidized to the insoluble ferric (Fe^{3+}) upon exposure to air.

Bisphenol A (BPA) is predominantly an intermediate to the production of other products. Its main use include binding, plasticizing and hardening functions in plastic products, paints/lacquers, binding materials and filling-in materials (Makinwa and Uadia 2015). However, exposure to BPA occurs primarily via hydrolysis of polycarbonate plastics and epoxy resins resulting in low concentration of free BPA in food and liquids thus making dietary consumption the major

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mode of human exposure (Wilson *et al*., 2007). As shown in table 5, the borehole water samples refilled into plastic containers contained high levels of BPA congeners ranging from 0.023mg/l to 0.251mg/l at week 4. This result is in tandem with Atuanya *et al*. (2016) who investigated the bioavailability of plastic contaminants and their effects on plastic bottled and sachet drinking water supplies. A progressive increase in the concentration of BPA congeners as storage increased was recorded. Although there was a significant higher levels, this result has shown that there was a gradual release of BPA congeners that increased with storage period.

Table 1: Cultural, Morphological and Biochemical Characteristics of the Bacterial Isolates from Refilled Borehole Water Samples

Isolates	Cultural	Gram staining	Motility	Spore staining	Catalase	Oxidase	Coagulase	Indole	Urease	Citrate	Glucose	Lactose	Organisms
1	Medium creamy colony, convex elevation and smooth margin	-ve rod in single	-	-	+	-	-	-	+	+	+	+	*Klebsiella* sp.
2	Small creamy colony, with rough surface	+ rod in chains	+	+	-	-	-	-	+	+	+	-	*Bacillus* sp.
3	Small creamy colony, convex elevation and smooth margin	-ve rod in single	+	-	+	-	-	+	-	-	+	+	*Escherichia coli*
5	Large creamy , translucent, flat elevation and entire margin	-ve rod in single	-	-	+	-	-	-	+	+	-	-	*Acinetobacter* sp.
8	Medium greenish colony, low convex and entire margin	-ve rod in single	+	-	+	+	-	-	-	+	+	-	*Pseudomonas aeruginosa*

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Table 2: Cultural and morphological characteristics of the fungal isolates

Characteristics	F1	F2	F3
Cultural characteristics	Greenish yellow colony with reverse side yellow	Medium creamy colony convex elevation and entire margin	Black fluffy colony with reverse side yellow
Microscopic characteristics			
Nature of hyphae	Septate	Pseudohyphae	Septate
Colour of spore	Yellow	Cream	Brown
Type of spore	Conidiophores	Chlamydospore	Conidiophores
Appearance of special structure	Foot cells	Budding	Foot cells
Possible isolates	*Aspergillus flavus*	*Saccharomyces* sp.	*Aspergillus niger*

Table 3: Physicochemical qualities of borehole water in plastic containers

Parameters	Week 0	Week 1	Week 4	WHO (2011) water standards
pH	6.6	5,6	5.6	6.5-S.5
Temperature (°C)	32.9	32.2	32,5	
Conductivity (us/cm)	40.6	29.0	29.5	900
TDS (mg/L)	15.5	7.8	9.5	1000
TSS (mg/L)	7.5	6.0	3.5	
Turbidity	5.30	5.80	5.50	5
Alkalinity (mg/L)	8.0	12.0	8.0	
Total hardness(mg/L)	42.90	44.60	43.10	
Total iron(mg/L)	1.031	1.051	1.001	0.30
Chloride (mg/L)	10.6	11.5	9.7	250
Sulphate (mg/L)	8^22	8.23	8.21	400
Nitrate (mg/L)	0.22	0.24	0.22	50
Phosphate(mg/L)	102.0	115.2	96.8	6.5
BOD(mg/L)	26.4	36.0	33.6	

Table 4 : Microbial Counts of Borehole Water

Plastic bottle filled with borehole water			
	Week 0	Week 1	Week 4
Total viable bacteria count	1.4×10^3	1.6×10^3	1.8×10^3
Total Coliform count	4.7×10^2	4.4×10^2	4.9×10^2
E. coli count	6.0×10^1	5.0×10^1	7.0×10^1
Fungal count	1.3×10^2	1.0×10^2	1.6×10^2

After subsequent cultural characteristics, morphology as well as biochemical tests, eight isolates were identified. Three of the isolates were fungal species. The isolates include: *Klesiella sp., Escherichia coli, Acinetobacter sp., Bacillus sp., Pseudomonas aeruginosa, Aspergillus niger, Aspergillus flavus* and *Saccharomyces cerevisiae* as shown in table 1 and 2

Table 5: Concentration of Bisphenol A in Plastic Bottles filled with Borehole water Samples

Parameter	Week 0	Week 1	Week 4	WHO Standard
Methylene chloride	0.015	0.031	0.065	1.0
Hexane	<0.001	<0.001	<0.001	1.0
Chloroform	<0.001	0.001	0.001	1.0
Toluene	<0.001	<0.001	<0.001	1.0
Benzene	<0.001	<0.001	<0.001	1.0
Vinyl chloride	0.008	0.067	0.164	5.0
Tetrachloroethylene	<0.001	0.015	0.021	1.0
Chlorobenzene	<0.001	<0.001	<0.001	1.0
Dichlorobenzene	<0.001	<0.001	<0.001	1.0
Total (mg/l)	**0.023**	**0.114**	**0.251**	

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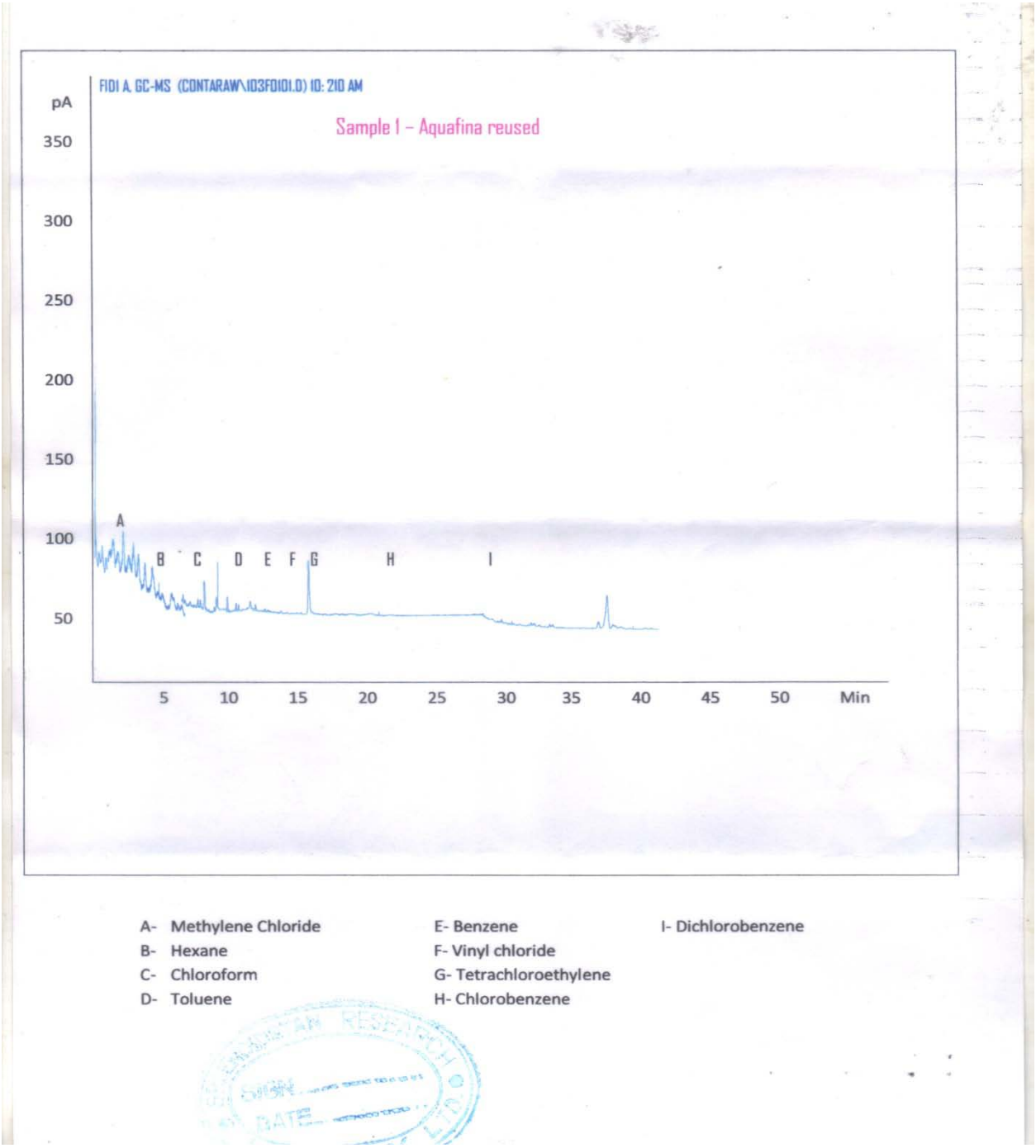


Figure 1: G.C.-MS peaks of plastic filled with borehole water at week 4

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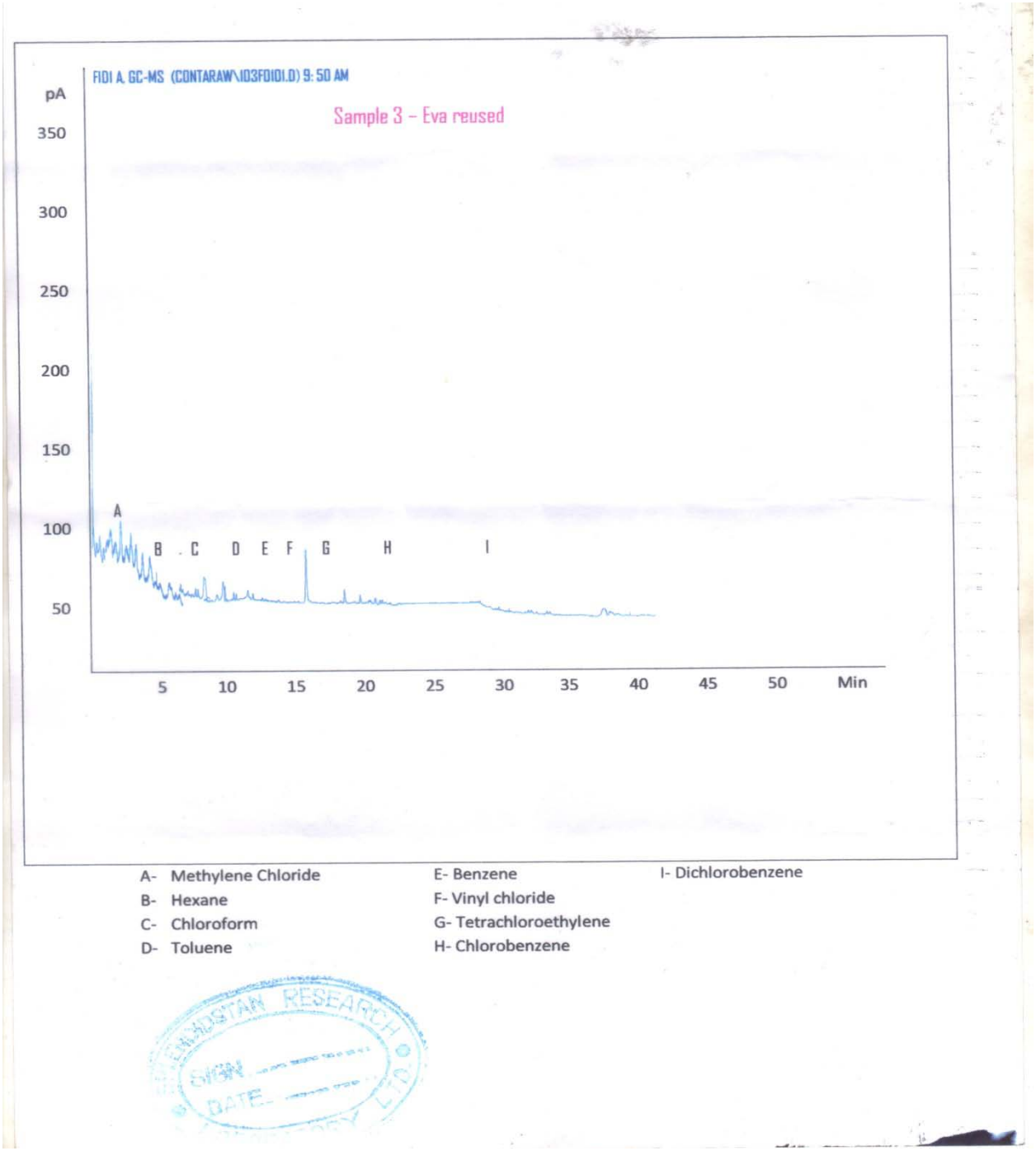


Figure 2: G.C.-MS peaks of plastic filled with borehole water at week 1

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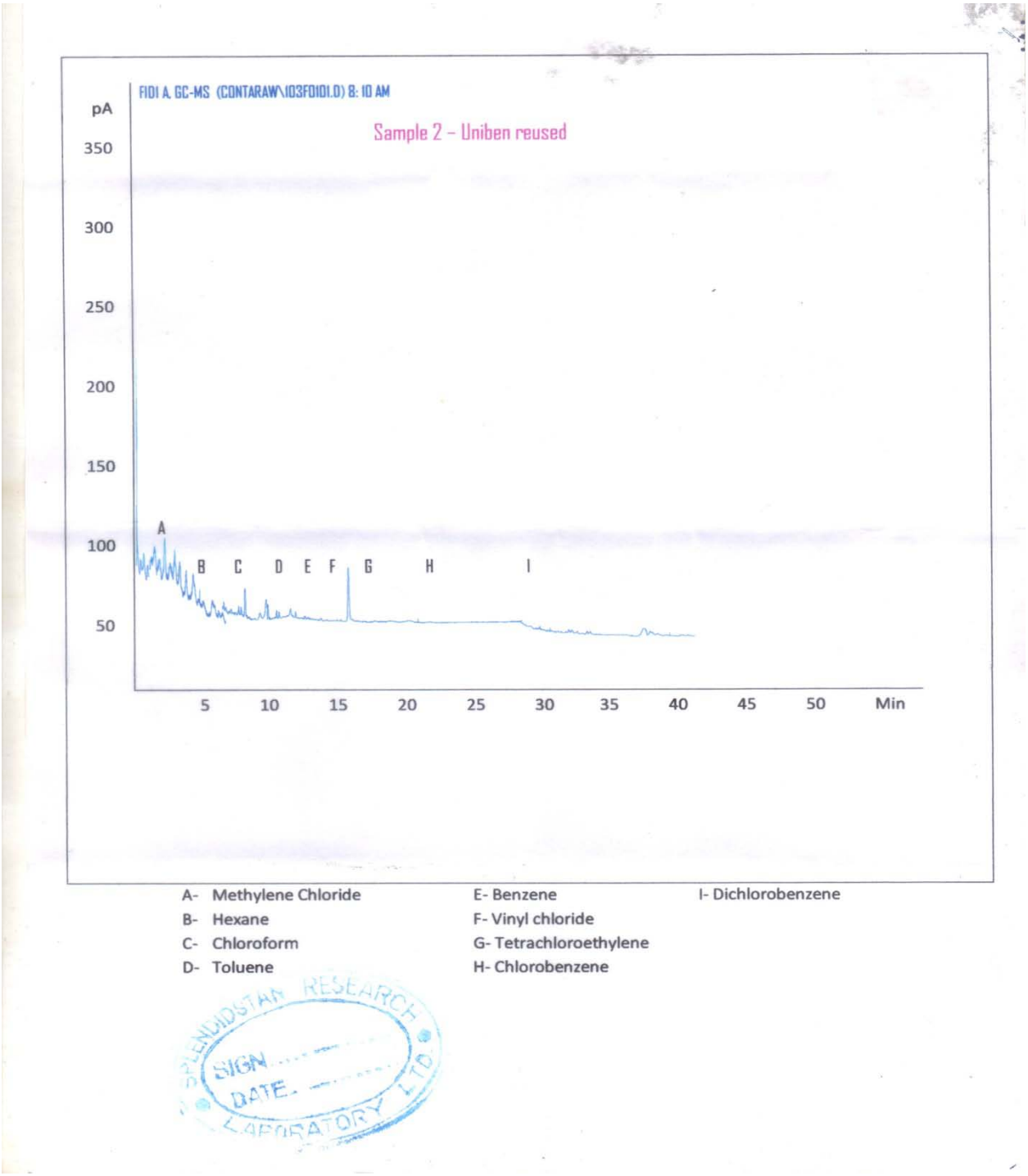


Figure 3: G.C.-MS peaks of plastic filled with borehole water at week 0

V. Conclusion

The result of this study has shown that the levels of bacterial population in borehole water stored at room temperature increased to maximum levels at the fourth week of water storage. It also showed that the bioavailability of bisphenol A components in borehole water, appeared to start manifesting at the fourth week of water storage. Storage temperature for long period plays a major role, creating impact on the acceptability of other organic constituents and enhancing the growth of microorganisms. Finally it is obvious from this study that BPA leaching from plastic containers into water can be affected by storage temperature and time, hence proper awareness should be created on the emergence of such an endocrine disrupting chemical.

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Electrochemical Biosensor based on Clay for the Immediate Detection of the Bacteria

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Abstract- The electrochemical detection of staphylococcus aureus by the electrode of clay paste modified by amoxicillin (AMX-Clay) is described. The electrodes of AMX-Clay were then used to detect the staphylococcus aureus with low optical densities by using the cyclic voltammetry (CV), the voltammetry with square waves (swv) and the spectroscopy of electrochemical impedance (EIS) in physiological mediums. Electrochemical parameters like the time of deposit and the concentration of the amoxicillin on the surface of the clay electrode were optimized. The elaborate electrode showed a good electroactivity, resulting in the packing of current, in the presence of the bacteria.

Keywords: *modified electrode; SWV; CV; EIS; bacteria; biosensor.*

GJMR-C Classification: *NLMC Code: QY 480, QZ 65*



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Electrochemical Biosensor based on Clay for the Immediate Detection of the Bacteria

Olivier François Aristide Bertrand Koffi [α], Bernadette Ehui Avo Bile [σ], Rachida Najih [ρ] & Abdelilah Chtaini [ω]

Abstract- The electrochemical detection of staphylococcus *aureus* by the electrode of clay paste modified by amoxicillin (AMX-Clay) is described. The electrodes of AMX-Clay were then used to detect the *staphylococcus aureus* with low optical densities by using the cyclic voltammetry (CV), the voltammetry with square waves (swv) and the spectroscopy of electrochemical impedance (EIS) in physiological mediums. Electrochemical parameters like the time of deposit and the concentration of the amoxicillin on the surface of the clay electrode were optimized. The elaborate electrode showed a good electroactivity, resulting in the packing of current, in the presence of the bacteria.

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I. Introduction

In the perpetual fight against the pathogenic bacteria, of research were carried out by certain researchers in order to work out new molecules likely to slow down the effects of those. One could quote amongst other things, the phenolic compounds and terpenes.

The phenolic compounds gather a great number of chemical substances which have at least an aromatic nucleus. This core carries one or more functions alcohol (grouping hydroxyl). The phenolic phytomolecules have structures going of simplest (acid gallic) to most complex (tanins). The phenolic compounds have many biological activities of which antimicrobic activities [1-5].

Terpenes are essentials oils of many plants. They are volatile and constitute the resin and the gasolines of the plants. It is the case of the spirits of turpentine isolated starting from the resin from pine. On the structural level, terpenes are derivatives of the isoprene (C_5H_8). Antimicrobic activities of the terpenoïdes were highlighted [6-8].

In this search for solutions to prevent the diseases caused by the bacteria, we have in this desired work to add our contribution to the building by working out an electrode based on clay able to detect *the staphylococcus aureus* in aqueous mediums.

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II. Experimental

a) Products

All the solutions were prepared in water having been the subject of a double distillation. The clay samples used in research were taken in the natural resources of Cameroun, more precisely in the town of Garoua, the area of the north of Cameroun [9]. The samples were washed with deionized water several times with constant agitation. After a calcination at 900°C of clay to the furnace during one hour, the samples were crushed, and finally filtered (125 μm). The chemical composition of the burnt clay is as follows [10]: SiO_2 (48.01%), Al_2O_3 (27.41%), Fe_2O_3 (7.34%), MnO (0.12%), CaO (0.06%), MgO (0.31%), K_2O (0.41%), Na_2O (0.02%), S (0.03%) and several metals in the proportions of the part per million.

The bacteria used in this study are *Staphylococcus aureus*. The bacteria were cultivated in medium LB (Luria Burtani) solid. After a sterilization in the autoclave of the culture medium, the bacteria were sown there and then incubation was done with 37 °C during 24 hours.

Provisions were taken for deoxygenation by splashing the solution with nitrogen gas during approximately 5 minutes. In order to obtain reliable and reproducible results, a new electrolyte was prepared for each handling.

b) Instrumental

The electrochemical methods used in this study are the cyclic voltammetry (VC), the voltammetry with square waves (SWV) and the spectroscopy of electrochemical impedance (EIS).

The equipment used for our measurements consists of an electrochemical cell with three electrodes (Calomel electrode saturated (*ER*), the platinum electrode (*EC*), the electrode with clay paste modified by amoxicillin (*EW*)) connected to a potentiostat of the type voltalab PGZ 100. The programming, the acquisition and the treatment of the results were carried out by the software voltamaster 4.

c) Electrodes

The clay powder was mixed with a binder (the paraffin oil) and a solvent (the absolute ethanol). The paste obtained was used to fill the cavity of the electrode of a surface of 0,1256 cm^2. Once the worked out electrode, it is dried at ambient temperature carefully

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during 24 hours. Dry once, a mechanical polishing of the surface of this electrode is carried out on smooth paper before its use to eliminate all the irregularities and to obtain a regular and more reproducible surface. The modification of the electrode was done by soaking the electrode of clay paste manufactured in the solution of amoxicillin (10g/L).

d) Analytical procedure

The modified electrode (AMX/Clay) was immersed in a cell containing the sample of bacteria and then characterized by the voltammetry cyclic, linear, with square waves and by the spectroscopy of electrochemical impedance. The electrolyte support used for our electrochemical measurements is the sodium chloride (NaCl) to a concentration of 0,1 M. All the experiments were carried out at the ambient temperature. The voltammogramms obtained were recorded in the window of going potential of -2V with 2V, with a scanning rate of 20 mV/s, an amplitude of 2 mV and a pulsation of 50 mV. The electrochemical spectroscopy of impedance was carried out in the frequency band going of 100 mHz with 100 kHz.

III. Result and Discussion

a) Determination of ideal amoxicillin accumulation time

In order to determine the time of optimal deposit of the amoxicillin on the surface of the electrode of clay paste, we soaked it in a solution of 10 g/L of amoxicillin at various times of preconcentration.

The ideal time of amoxicillin accumulation on the clay paste electrode, corresponds, in this study, to the minimum time which causes a significant change in the cyclic voltammogram recorded.

The cyclic voltammogramms of the electrode without modification and with modification were compared (figure 1). The time of identified optimal deposit is 10 min.

- To 10 min of preconcentration in the amoxicilline, the voltammogramm presents a considerable fall of current: There is formation of a film of amoxicillin on the surface of the electrode
- To 20 min, the density of current slightly increased, which indicates of a beginning of detachment of formed film.

The time of optimal contact is 10 min.

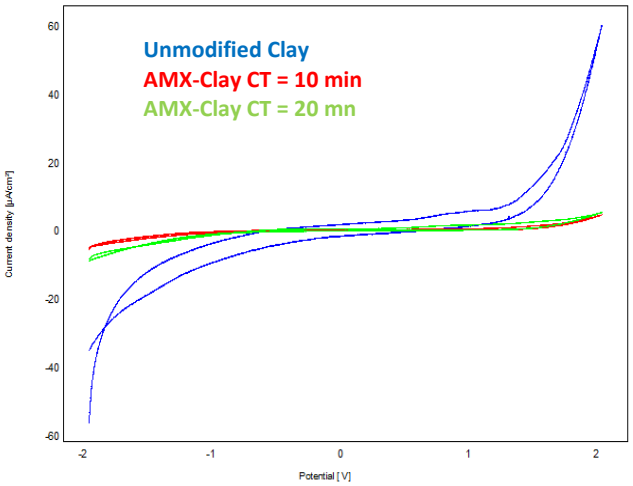


Figure 1: Superposition of the cyclic voltammogramms of Clay not modified and AMX-Clay at various times of contact, in NaCl to 0.1 M; v = 100mV/s, of -2V with 2V pH =

b) Determination of ideal amoxicillin concentration

The suitable concentration of amoxicillin deposited on the clay paste electrode surface, corresponds to the concentration which generates a significant response upon contact of the prepared electrode with the solution containing bacteria.

c) *Influences of accumulation time*

AMX-Clay was then characterized in the presence of the bacteria while varying the preconcentration time (contact of the modified electrode with bacteria solution), and the results obtained show a packing of current as the time of contact with the bacteria increases (figure 2 and table 1).

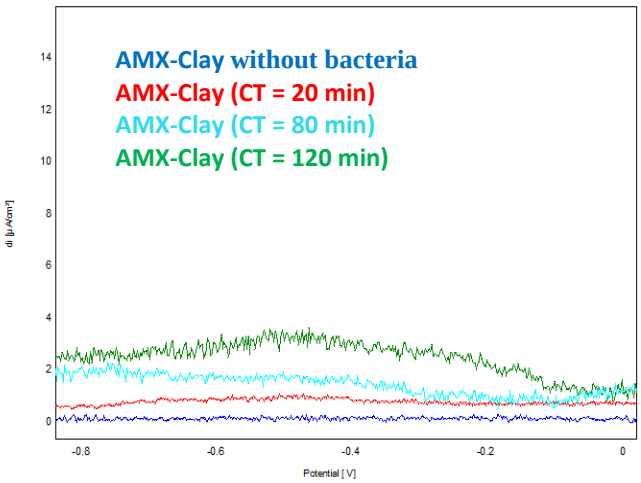


Figure 2: Superposition of the voltammogramms with square wave ofAMX-Clay without bacteria and AMX-Clay according to the time of contact with the bacteria, in NaCl to 0.1 M; v = 20mV/s, of -2V

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Table 1: Density of current according to the time of contact with the bacteria

Times (min)	0	20	40	60	80	100	120	140
di (µA/cm²)	0.072	0,912	1.528	1.338	2,014	2,396	3,282	2,913

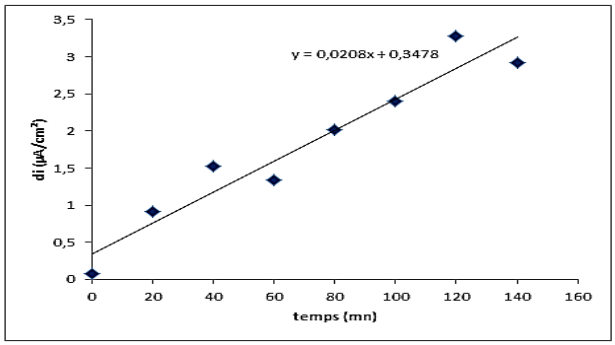


Figure 3: Density of current according to the time of contact

Figure 3 shows a linear increase in the density of current according to the time of contact of the electrode with the bacteria. This increase is represented by the line of equation:

$$di = 0.0208\ CT + 0.3478.$$

The EIS experiments were carried out in 0.1 mol L^{-1} NaCl in order to confirm the mechanisms suggested in the voltammetric part of this work regarding the AMX/Clay-bacteria interaction. Fig. 4 shows the Nyquist plot for bacteria-free AMX-modified electrode and AMX/Clay/bacteria system depending on precon-centration time. The Curves included a semicircle at higher frequencies corresponding to the electron transfer limited process and the linear part at lower frequencies corresponding to the diffusion process. The electrical parameters were calculated using Voltamaster 4.0 software. The results are summarized in Table 2. Cd is the double layer capacitance at the electrode/solution interface; the diameter corresponds to the difference between the electron transfer resistance Rt and the resistance of the electrolyte. It appears clearly from these data that the resistance became smaller in presence of bacteria and when preconcentration time increases.

The more time of contact of AMX-Clay with

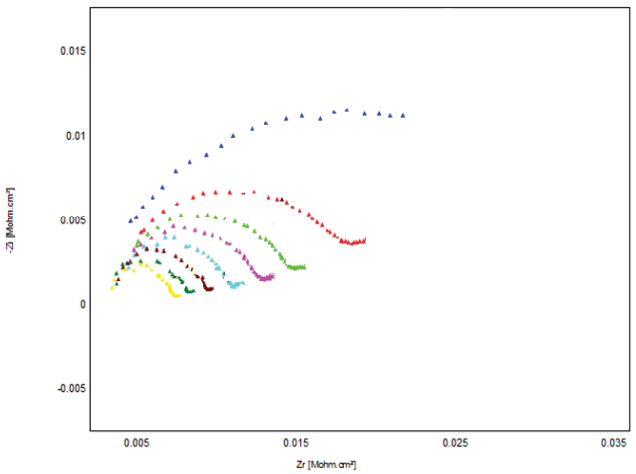


Figure 4: Superposition of the spectra of electrochemical impedance of AMX-Clay without bacteria and AMX - Clay according to the time of contact with the bacterium, in NaCl with 0.1 of 100 MHz with 100 Khz, pH = 7.42

Table 2: Parameters of the electrochemical spectra of impedance

	Diameters (kohm.cm²)	C (pF/cm²)
AMX-Clay withoutbacteria	27,78	291,2
AMX-Clay (CT = 20 mn)	17,68	232,1
AMX-Clay (CT = 40 mn)	12,48	289,3
AMX-Clay (CT = 60 mn)	9,925	322,6
AMX-Clay (CT = 80 mn)	7,771	410,1
AMX-Clay (CT = 100 mn)	6,327	447,8
AMX-Clay (CT = 120 mn)	5,393	525,9
AMX-Clay (CT = 140 mn)	4,279	662,5

In order to study the comportment electrochemical of AMX-Clay according to the time of contact with the staphylococcus aureus, we made characterizations electrochemical with intervals of 20 min. With these same intervals, taking away of the electrolyte containing the bacteria were made with an aim of measuring with a spectrophotometer the optical density of the sample.

Table 3: OD dependence of the contact time

Time (min)	0	20	40	60	80	100	120	140
OD	0,845	0,688	0.507	0.491	0.342	0.252	0.229	0.190

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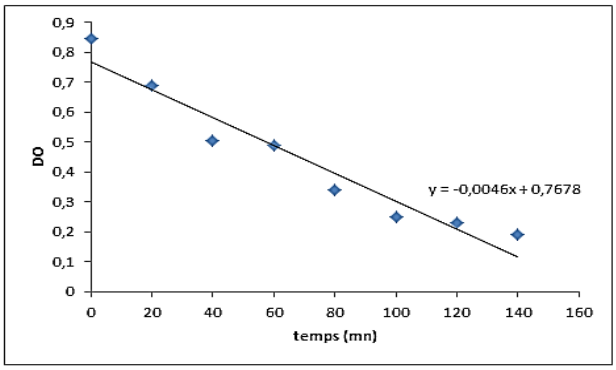


Figure 5: OD of the bacteria in function of the time of contact with AMX-Clay

Figure 5 and Table 3 showing a remarkable reduction in the optical density as the time of contact of AMX-Clay with the bacteria increases. This decrease results in the following line equation:

$$OD = -0.0046CT + 0.7678$$

Influences of amoxicillin concentration

The dependence of peak current on the amoxicillin concentration was also investigated (Fig. 6). The optical density decreases with the increasing in the amoxicillin concentration. The presence of a sufficient amount of amoxicillin on the electrode surface creates a significant number of active sites.

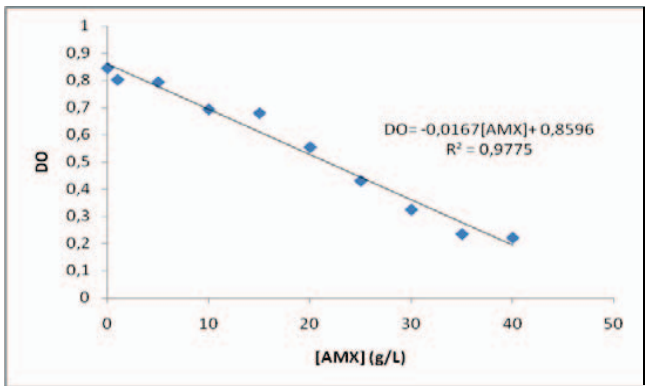


Figure 6: Evolution of bacteria OD wit amoxicillin concentration

The bêtalactamines inhibit the synthesis of the bacterial wall while being fixed on proteins binding penicillins (PLP). These proteins are carboxypeptidases and transpeptidases necessary to the connection between the side chains of the peptidoglycans. The inhibition of one or more of these enzymes makes accumulate precursors of peptidoglycans which activate the system autolytic of the vat tery and involve its lysis [11].

This result could be explained by the reaction mechanism which occurs on the surface of the electrode (Fig. 7).

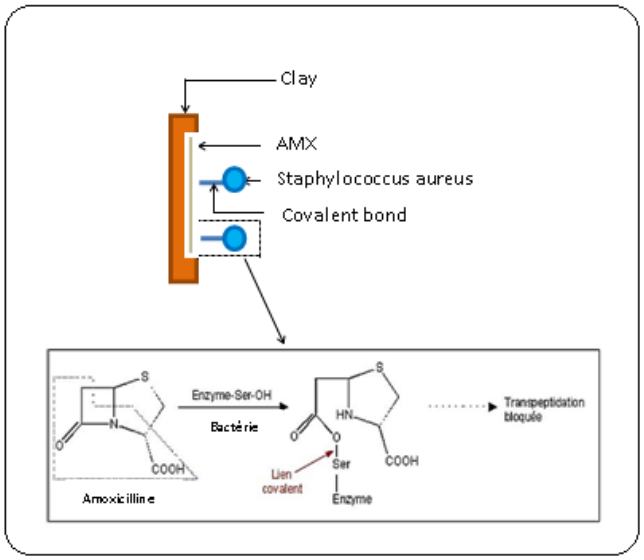


Figure 7: Mechanism illustrating the interruption of the reticulation of the bacterium

Surfaces of the electrode of clay paste without modification and with modification by the amoxicillin were observed using an optical microscope in reflexion. This microscope also enabled us to observe the surface of AMX-Clay after a time of contact of 120 min with the bacteria (figure 8).

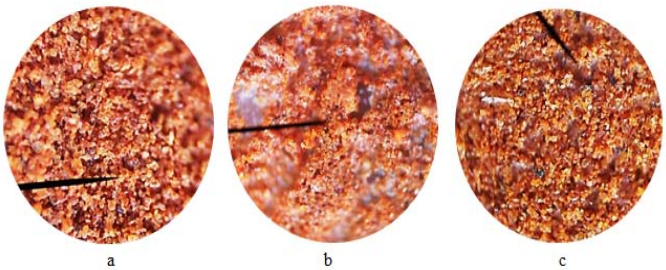


Figure 8: Clay unmodified (a), after modification by the amoxicillin (b) and 140 min of time of contact with the bacteria (c)

After 140 minutes of contact with the bacteria, the electrode of clay paste modified by the amoxicillin tends towards the initial state i.e. towards the not modified electrode. The bacterium after its lysis seems to involve the molecules of amoxicillin with it.

In the end, the combination of three electrochemical methods, CV, SWV and EIS allows the many more information and the CV has allows us to have information on the mechanism of the reactions taking place on the surface of the electrode, but this method does not establish the conditions of the analysis, since the generated electric current is the sum of the capacitive current and faradic current, hence the use of the SWV that can simply remove the capacitive term electric current, while the EIS allows marring time constants and to have information on the different stages of the reaction.

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IV. Conclusion

An electrode of clay paste modified by the amoxicillin was elaborate. It showed good results as for the spontaneous detection of the staphylococcus aureus. The study of the influence of the time of contact of AMX-Clay with this bacterium revealed that the more this time of passed, plus AMX-Clay posted an increased electroactivity translated by the packing of current. This electrochemical biosensor was characterized by stability, effectiveness and a good reproducibility of the results. In prospects, we plan to make an analytical application of this biosensor in the potato juice.

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Collective Memory and the Hologenome Concept

By Stephanie Rotem & Eugene Rosenberg
Tel Aviv University

Abstract- Collective memory requires a shared experience and the deposition of the experience in a manner that can be recalled at a later time. Collective memory can be transmitted orally, stored in writings, films, museums and other memorial sites, and also in our DNA. Recent studies have demonstrated that humans, like all animals, are themselves collectives, consisting of the host and abundant and diverse symbiotic microorganisms. The total DNA of a human, referred to as the human hologenome, consists of about 19,000 host genes and eight million microbial genes. It is now accepted that the microbial genetic information plays an important part in the fitness and evolution of animals and plants. We discuss here how the hologenome, especially the microbial component, interacts with cultural memory and contributes to collective memory. One of the novel points is that the microbial gene pool responds to changes in the environment on the basis of the principle of use and disuse. As such, the microbiome is particularly well-suited to serve as a vehicle for DNA-based collective memory.

Keywords: collective memory, collective unconscious, holobiont, hologenome, lamarckism, globalization, jung.

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Collective Memory and the Hologenome Concept

Stephanie Rotem [α] & Eugene Rosenberg [σ]

Abstract- Collective memory requires a shared experience and the deposition of the experience in a manner that can be recalled at a later time. Collective memory can be transmitted orally, stored in writings, films, museums and other memorial sites, and also in our DNA. Recent studies have demonstrated that humans, like all animals, are themselves collectives, consisting of the host and abundant and diverse symbiotic microorganisms. The total DNA of a human, referred to as the human hologenome, consists of about 19,000 host genes and eight million microbial genes. It is now accepted that the microbial genetic information plays an important part in the fitness and evolution of animals and plants. We discuss here how the hologenome, especially the microbial component, interacts with cultural memory and contributes to collective memory. One of the novel points is that the microbial gene pool responds to changes in the environment on the basis of the principle of use and disuse. As such, the microbiome is particularly well-suited to serve as a vehicle for DNA-based collective memory.

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I. Introduction

During the last few years, fundamental changes have taken place in our understanding of biology, which may be relevant to the concept of collective memory. In particular, it is now clear that all animals, including humans, contain abundant and diverse symbiotic microbes that play an important role in their adaptation, behavior and evolution. The fact that these microbial populations are dynamic and their vast genetic information can change as a function of the environment makes it possible for them particularly well-suited to acquire and store DNA-based memory. Furthermore, these changes in microbial DNA can be transferred horizontally to other members of the community and vertically to offspring. How these symbiotic microbes contribute to certain aspects of collective memory is the subject of this article.

The term "Collective Memory" is widely used in articles in history and sociology. Collective memory discourse began with the work of Emile Durkheim (1858–1917), a French philosopher, sociologist and social psychologist. Although never using the term "collective memory", Durkheim noted that societies require continuity and connection with the past to preserve social unity and cohesion. Maurice Halbwachs (1877–1945), a student of Durkheim, is the first sociologist to use the term "collective memory" and his work is considered the foundational framework for the study of societal remembrance (Halbwachs, 1980). Halbwachs suggested that all individual memory was constructed within social structures and institutions and claimed that individual private memory is understood only through a group context; these groups may include families, organizations, and nation-states. Cultural or social memory is the specific character that a person derives from belonging to a distinct society and culture as a result of socialization and customs (Assmann, 2003).

Carl Jung (1876-1961) used the term "collective unconscious" to describe the broad concept of inherited traits, intuitions and collective wisdom of the past. The collective unconscious, unlike the personal unconscious, is a type of genetic memory that can be shared by individuals with a common ancestor or history. According to Jung, the collective unconscious consists of implicit beliefs and thoughts held by our ancestors (Lu 2012). While we are not aware of the collective unconscious, it can influence how we act. What Jung termed the collective unconscious or genetic memory may now be referred to as DNA-based memory (Bullock andStallybrass1977).

During the last twenty years, new techniques of analyzing DNA have fundamentally changed our understanding of biology (Douglas, 2010). Animals, including humans, can no longer be considered individuals by the classical definitions of the term. All are holobionts, or collectives, consisting of the host and abundant and diverse symbiotic microorganisms (Zilber-Rosenberg and Rosenberg 2008; Rosenberg and Zilber-Rosenberg 2014). Symbiosis—once thought to be a peripheral phenomenon—is the hallmark of life on earth (Gordon, 2012). After reviewing our current understanding of the role of microorganisms in the fitness and evolution of multicellular organisms, we will examine the similarities and differences of collective memory as exhibited by the human genome, the human microbial DNA and cultures, as well as their interactions.

Since certain specialized terms are used throughout this article, we would like to define these terms before discussing the concepts. Symbiosis (from Greek σύν "together" and βίωσις "living") is the close and often long-term interaction between two or more

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different biological species. The term holobiont, introduced by Margulis (1991), describes a host animal or plant and all of its symbiotic microorganisms, including Bacteria, Archaea, fungi, algae and viruses. The term "host" is used here in the classical sense to denote the larger, multicellular organism in or on which the symbionts reside. Zilber-Rosenberg and Rosenberg (2008) introduced the term hologenome to describe the sum of the genetic information of the host and its symbiotic microor-ganisms. The aggregate of all microorganisms of a holobiont is known as the microbiota or microbiome, a term coined by Lederberg and McCray (2001).

II. The Hologenome Concept

We are in the midst of a paradigm change in biology. Numerous studies have demonstrated that all animals and plants contain abundant and diverse microbiota. The human body, for example, contains about the same number of microbial cells as human cells (Rosner, 2014).Because the microbial community is composed of several thousand different species of bacteria, the genetic information encoded in the microbiome (eight million unique genes)is more than 400times greater than the information in the human genome (19,000 genes) (Ezkurdia et al., 2014). The microbial symbionts contribute to the anatomy, physiology, development, innate and adaptive immunity, behavior, genetic variation and evolution of holobionts (Zilber-Rosenberg and Rosenberg 2008; Round et al., 2010; Gilbert et al., 2012; McFall-Ngai et al., 2013). As we shall reveal in this article, the DNA of the microbiota in addition to the human genome contributes to collective memory.

Microbial symbionts can be transmitted with fidelity from parent to offspring by a variety of methods, including cytoplasmic inheritance, coprophagy (consumption of feces), direct contact during and after birth, and via the environment (Rosenberg and Zilber-Rosenberg 2014). In humans, most of the colonization of the newborn gut occurs when the baby transits the birth canal via inoculation by maternal vaginal and fecal microbes. Furthermore, human breast milk has been shown to be a continuous source of bacteria to the infant gut (Fernández et al., 2013). The hologenome concept of evolution posits that the holobiont (host + symbionts) with its hologenome (host genome + microbiome) is an important unit of selection in evolution (Zilber-Rosenberg and Rosenberg 2008). Consideration of the holobiont as a unit of selection brings forth previously under-appreciated patterns of genetic variation (changes in the hologenome). In fact, acquisition of microbes and microbial genes is a powerful mechanism for driving the origin of species and evolution of complexity. In essence, holobionts are collectives and evolution proceeds both via cooperation and competition, going hand in hand.

In considering the role of DNA in collective memory, it is necessary to separate the hologenome into two parts: (i) the human genome, which consists of 19,000 genes located on the 23 pairs of chromosomes, and (ii) the human microbial genes, which consists of 8,000,000 genes and is present in the thousands of different species of microbial symbionts, mostly in our gut. Genetic variation in the human genome results from mutations, which are random events that occur rarely. Genetic variation in the human microbiome, however, can occur rapidly in response to changes in the environment (Rosenberg and Zilber-Rosenberg 2016). Accordingly, the microbiome is particularly well-suited to serve as a vehicle for DNA-based collective memory.

III. Acquisition of Collective Memory via Culture and dna

Both cultural and DNA-based memories can be gained or lost. Acquisition of collective memory requires a shared experience and the deposition of the experience in a manner that can be recalled at a later time (Gintis, 2011). An example of a recent cultural memory is the Holocaust, a genocide in which approximately six million Jews and five million non-Jews were killed by Adolf Hitler's Nazi regime and its collaborators.The Holocaust experience has been documented in personal accounts, historical writings, films and museums. In addition, an annual International Holocaust Remembrance Day is observed. As is often the case, different groups share divergent versions of the event, as is evident from the foci of various National Holocaust Museums (Rotem, 2013).

A classic example of host gene-culture coevolution is the consumption and digestion of milk. A major source of carbon and energy in milk is the disaccharide lactose. For lactose to be utilized, it must first be split into monosaccharides by the enzyme lactase. The enzyme is abundant in infants, but the activity of the enzyme is dramatically reduced after weaning (Swallow, 2003). When adult humans first began consuming milk and milk products from domesticated animals in central Europe approximately 10,000 years ago, they could not digest lactose. Genes that allowed for the digestion of lactose, referred to as lactase persistence genes (Gerbault, 2011), evolved and eventually spread among milk-drinking peoples. Current estimates for the age of lactase persistence-associated alleles bracket those for the origins of animal domestication and the culturally transmitted practice of dairying. Cultures that traditionally do not consume milk products, such as Australian Aborigines, Japanese, and Native Americans, have extremely high rates of lactose intolerance. There are many examples of cultural practice driving human evolution (Rowley-Conway and Layton 2011) but none are so well studied, clear-cut, widespread and well supported as the coevolution of

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lactase persistence and dairying (Holden and Mace 1997).

In European populations, a single mutation explains the distribution of the lactose persistence phenotype, whereas different point mutations are associated with it in Africa and the Middle East. It should be pointed out that the mutation does not result in a novel lactase but ratherin an enhancer region of the existing lactase gene (Harvey, 1995). Lactose persistence is readily explained by Neo-Darwinian variation by mutation followed by Darwinian selection. As we shall discuss below not all gene variation results from mutation of the human genome and not all cultural evolution involves individual selection.

As an example of collective memory that is DNA-based but did arise from mutation of the human genome consider the use of agar in Japanese cuisine. Agar is a complex polysaccharide found in seaweed, which forms the supporting structure in the cell walls of certain species of algae. Throughout history into modern times, agar has been used as a food ingredient in Japan and throughout Asia. Foods that contain agar include wagashi, a dessert made of small cubes of agar jelly, mizuyōkan, another popular Japanese food, and sushi. The techniques for preparing these foods have been passed down from generation to generation and constitute part of the Japanese cultural collective memory. Tax records from the eighth century list seaweed as payment to the Japanese government, showing that it had an important role in Japanese culture (Nisizawa et al., 1987).

Interestingly, the Japanese also have acquired and retained in their DNA the ability to digest agar, because they have a bacterium in their gut that contains a gene that codes for the enzyme agarase (an enzyme that breaks down agar). Westerners lack this bacterium and cannot digest agar. The question then arises of how the agarase gene was acquired by the Japanese gut bacteria. The source of the gene was traced to a marine bacterium that was present on the dietary seaweed. However, this marine bacterium cannot survive in the human gut. DNA analysis showed that the agarose gene was horizontally transferred from the marine bacterium to a resident gut bacterium and became part of the hologenome of the Japanese (Hehemann et al., 2010).Until recently it was accepted that biological (DNA) memory was altered only by the random process of mutation. However, when considering the microbiota, it is clear that biological memory can also be changed by experience. When a person eats a particular food, those specific bacteria which can multiply on that food will amplify. At some later time when the person is again exposed to that food, the bacteria will rapidly degrade the food.

Each person possesses their own personalized fingerprint of gut microbiota (Faith et al., 2013), which includes a core microbiota of ca. 100 species which are common to all humans (part of the collective memory of the human species), hundreds of microbial species that are common to a particular culture (Yatsunenko et al. 2012), and thousands of microbial species that are present in a combination unique to each individual. Some strains of symbiotic bacteria are so well conserved within cultural groups that they can be used as a window into human migration (Yamaoka et al., 2009). In particular, the stomach bacterium *Helicobacter pylori* has been used as a marker of ancestry and migration (Dominguez-Bello and Blaser 2011). For example, an American whose great-great- grandmother came from Japan still contains the Japanese strain of *H. pylori*. The reduction of genetic diversity among humans as distance from East Africa is mirrored by the genetic distances between *H. pylori* strains circulating among human populations. Such parallelism is consistent with co-evolution of bacteria and their human hosts since their exodus from Africa.

Mice experiments have demonstrated that gut microbiota not only is involved in digestion of food but also affects the brain and behavior (Heijtz et al., 2011). Germ-free mice (born and grown under sterile conditions) are more active and spend more time scurrying around their enclosures than conventional mice. They are also less anxious and more likely to take risks, such as spending long periods of time in bright light or open spaces, compared to the normal mice. Inoculating the gut microbiota from healthy mice into germ-free baby mice caused them to behave in the "normal" cautious way. If sterile adult mice were inoculated with the gut bacteria, their behavior did not change, suggesting that the microbiota affect the early development of the brain that subsequently influences adult behavior (Foster and Neufeld 2013). There appears to be a critical window during development when the microbiota influence the central nervous system wiring related to stress-related behaviors. The data suggest that during evolution, the colonization of gut microbiota has become integrated into the programming of brain development, affecting motor control, anxiety-like behavior and probably many other behaviors.

How do gut bacteria affect the brain? To begin with, the long branching vagus nerve transmits information about what happens in the gut to the brain. But the bacteria also signal the brain via changing levels of dietary metabolites and hormones (Shaw, 2010). Hormones, by definition, can affect parts of the body over long distances. For example, blood plasma levels of the neurotransmitter serotonin were 2.8-fold higher in conventional mice than germ-free animals (Bercik et al., 2011). With regard to physical and psychological stress, the interaction of gut bacteria with the brain is bidirectional. Stress can affect the composition of intestinal microbiota, and as was discussed above commensal microbes affect the neural network

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responsible for controlling stress responsiveness (Sudo et al., 2004).

Because learning about situations that are necessary for survival of a species is probably saved as a kind of unconscious genetic memory, some of these fundamental human experiences could be somewhere in our DNA. Consider that one of your ancestors had a very bad experience with fire. Such an experience, resulting in knowledge useful for survival, could possibly be encoded in the hologenome and passed on to future generations. In the fields of human genetics and microbiota so much is not known, especially regarding the functions of non-coding DNA (Mercer, 2009) that for an open-minded person, theories about deep DNA memories cannot be ruled-out.

IV. Loss of Collective Memory

Cultural and DNA-based collective memories can be lost if they are not used. Many languages have completely disappeared because of processes associated with colonization. For example, of the 250 Aboriginal languages that existed in Australia, only 60 remain (Amano, 2014), and of the more than 300 different languages that were spoken in North America when the Europeans first arrived, only 91 are still spoken (Braun, 2008). When a language becomes extinct, it can take along with it much of the history and culture of the people who spoke it.

DNA information can be lost by two general mechanisms: mutation and loss of microbiota. Mutation is a low frequency random event. If the mutation leads to the loss of a function, the mutation will be selected for if it benefits the organism. In the example we discussed above, a mutation in the gene that codes for agarase in the Japanese gut microbiota will be selected for if the Japanese person does not eat food that contains agar because the bacterium does not bear the burden of producing a useless enzyme. This is a very slow and inefficient method of changing DNA information.

Unlike chromosomal DNA, the microbiome is flexible and able to be easily modified to respond to altered circumstances or conditions, such as lifestyle and dietary patterns (Mueller et al., 2006).Changes in the microbiota, driven by the environment, can result in rapid gain or loss of DNA memory. Consider again the agar-digesting microbe in the Japanese gut. If seaweeds were removed from their diet, the microbe could not compete with other microbes in the gut and would soon be depleted, resulting in loss of the DNA memory to consume agar. In general, sustained alteration in the diet leads to gain or loss of certain microbes from the gut.

In modern Western cultures, microbes are lost as a result of improved sanitation and living conditions, overzealous antimicrobial therapy, delivery by caesarean section, and formula-feeding infants. All of these practices prevent acquisition of beneficial symbionts, which have evolved to participate in the metabolism and health of human holobionts. Loss of these beneficial microbes predisposes individuals to metabolic diseases (Blaser and Falkow 2009), susceptibility to allergic and autoimmune diseases (Penders et al., 2006), and may help explain the rise in obesity and related syndromes (Musso et al., 2010).

V. Jung's Theory of Collective Unconscious is Compatible with the Hologenome Concept

Like Freud, Jung emphasized the importance of the unconscious in relation to personality. However, Jung proposed that the unconscious consists of two layers (McLeod, 2014).The first layer called the personal unconscious is essentially the same as Freud's version of the unconscious. The personal unconscious contains temporality forgotten information and well as repressed memories. The second layer and the most important difference between Jung and Freud is Jung's concept of the collective unconscious. This is a level of unconscious shared with other members of the human species comprising latent memories from our ancestral and evolutionary past(Jung, 1953).Jung called these ancestral memories and archetypes.

Jung drew an analogy between instinct and archetype. The fact that instinctive behavior is a genetic (DNA) property of animal species is well documented (Tinbergen, 1951). It follows that DNA has the potential of being the reservoir of the archetypal symbols of the collective unconscious. The contentious question is how instinctual behavior and collective memory is obtained. According to Jung it was obtained by common experiences according to Lamarckian principles:

i. Use and disuse – individuals lose characteristics they do not use and develop characteristics that are useful.
ii. Inheritance of acquired characteristics – individuals transmit acquired characteristics to offspring.

Jean-Baptiste Lamarck, are nowned French botanist, zoologist and philosopher of science, published in 1809 his book *Philosophie Zoologique* (discussed in Burkhardt, 1972), describing environmentally induced changes that were then passed on to future generations. Interestingly, Darwin believed, as did Lamarck and many others at the time, that an organism can transmit traits it acquired during its lifetime to its offspring. But with the advent of Neo-Darwinism at the beginning of the 20thcentury, Lamarckism and, by association, Jung's concept of collective unconscious were discredited and largely ignored. There were two major scientific arguments for rejecting Lamarckism. First, the evolutionary theorist August Weismann argued that inheritance only takes place by means of germ cells

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and that germ cells cannot be affected by anything somatic cells of the body acquire during their lifetime (Weismann, 1893). Second, Mendelian genetics considers that variation, the raw material for Darwinian evolution, occurs by random mutations in the population.

Since the 1980s, Lamarckism is being reconsidered with growing interest by mainstream evolution thought (Gould, 1999). It is now clear that environmental factors affect epigenetic inheritance systems that include DNA methylation, self-sustaining feedback loops, prions, chromatin-marking and RNA interference. Taken together these mechanisms include the inheritance of changes that are not DNA sequence based and therefore argue for withdrawal from the strict genotype–phenotype separation dogma of Neo-Darwinism (Jablonka and Lamb 2014).

Until recently it was accepted that biological (DNA) memory was altered by the random process of mutation of genes. However, consideration of the hologenome, namely the hostgenome combined with that of its microbiota, brings forth two additional modes of genetic variation which are specific to the holobiont and which conform to Lamarckism (Rosenberg et al., 2009). The first is microbial amplification, the increase of one group of microbial symbionts relative to others which can occur when conditions change. An increase in the number of a particular microbe is equivalent to gene amplification. Considering the large amount of genetic information encoded in the diverse microbial population of holobionts, microbial amplification can be a powerful mechanism for adapting to changing conditions. Examples of environmental factors that can lead to changes in the symbiont population and thereby tovariation in the hologenome are nutrient availability (Flint et al., 2007; Martens et al., 2008), temperature (Buddemeier et al., 2004; Koren and Rosenberg 2006), and antibiotics (de la Cruz and Davies 2005).

Another mechanism for introducing variation into holobionts is acquisition of new symbionts from the environment. Animals and plants come in contact with billions of microorganisms during their lifetime. It is reasonable to assume that occasionally, as a random event, some of these microbes will find a niche and become established in the host. Under the appropriate conditions, the novel symbionts may become more abundant and affect the phenotype of the holobiont. Unlike microbial amplification, acquiring new symbionts can introduce entirely new genes into the holobiont. Microbial amplification and acquisition of novel microbes into holobionts closely fit the Lamarckian first principle of 'use and disuse'. The holobiont loses characteristics (microbes) it does not use and gains characteristics (microbes) that are useful. These acquired microbes can be transmitted to off spring, thus satisfying the second principle of Lamarckism.

VI. Globalization and the Future of Collective Memory

Globalization refers to all those processes by which all the peoples of the world are incorporated into a single world society. Present media theorists sometimes link the notion of collective consciousness to signal the internet as a major intermediary in the creation of a truly global society. The Slovenian philosopher Slavoj Zizek described the consciousness of Internet culture as 'this neo-Jungian idea that we live in an age of mechanistic, false individualism and that we are now on the threshold of a new mutation. We all share a collective mind.'

Globalization is not limited to Internet usage, but takes many forms. Financial globalization is the integration of a country's local financial system with international financial markets and institutions. Large numbers of people are moving rapidly to distant locations, e.g., the recent mass migration of people from the Middle East and Africa to Europe. Food developed in one country soon becomes worldwide, e.g., coca cola and McDonald hamburgers. Similarly, sushi from the Far East is now consumed in the West. Globalization also has political, social, cultural and ideological aspects. It invades all aspects of our being, for better or for worse, in ways that were unimaginable only a few decades ago.

Collective memory is subject to both remembering and forgetting, suddenly and gradually (McBride, 2001).What we remember and what we forget is to a greater or lesser extent shaped by our social environment. The act of remembering goes on inside our heads but not independently of the social relations of which we are a part. Pieterse (2009) argues that globalization is a process of hybridization which gives rise to global mélange. For example, Pieterse explains how Turkish motifs were used in operas by Mozart, and American blues music reflects African Muslim origins. However, globalization is also a major contributing force in conflicts, such as the current violent confrontation between fundamental Islam and the West. In short, globalization results in numerous outcomes, including loss, gain and hybridization of collective memory and leads to both cooperation and competition.

Not only cultural memory but also DNA-based memory is affected by globalization. For example, the spread of Western diet and excessive hygienic practices has resulted in a loss of diversity in gut microbiota (Ley et al., 2008).The increasing role of industrial food in our alimentation is generating a globalization of our gut microbiota that may influence our health (Raoult, 2010). The increased movement of people and goods (part of globalization) has contributed to pandemics of infectious diseases caused by bacteria and viruses. It is also likely that there have also been pandemics of

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beneficial microbes; however, they generally go unnoticed.

One of the dangers of globalization is loss of diversity, both cultural and DNA based. Biology has taught us that genetic diversity has a direct relation to the fitness and survivability of species and populations; as genetic diversity decreases within a population, so does the fitness and survivability of that population. Genetic diversity in human holobionts involves variability in the human genome and microbiome. Genetic diversity is important because the more variability there is within the species, the higher the likelihood that at least some of the individuals will be able to survive a major disturbance, such as a highly virulent emerging disease (Tishkoff and Verrelli2003). The same arguments can be made for cultural diversity. A diversity of cultures, expressing different visions of the world, provides a powerful resource for innovation (Nathan and Lee 2013), collaborative problem-solving (Page, 2008) and adaptation to a changing environment (Crisp and Turner 2011). In conclusion, based on the hologenome concept, we present for the first time the potential of the microbiome to serve as a vehicle for collective memory. This hypothesis is supported by the fact that the microbiome responds to the environment, that changes in the microbiome are transmitted to offspring and that behavior is influenced by the microbiome. What particular parts of the DNA-based collective memory resides in the human genome and the microbiota remains to be determined.

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Detection of *Mycobacterial* Infections (MTB & NTM) by Different Molecular, Staining and Culture Techniques among Infertile Females

By P. S. Gangania, D. Bisht & V. A. Singh
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Abstract- Background: Mycobacterium Tuberculosis (TB) has been an important cause of mortality and morbidity all over the world but is specifically affecting developing countries like India where the disease is endemic. Inspite of being asymptomatic, female reproductive system is found to be very vulnerable to tubercular infections and by the time, Nontuberculous mycobacterial infections are also emerging and are found to cause serious genital infections in the females of child bearing age leading to infertility.

Objectives: Detection of Mycobacterial (MTB & NTM) infections among infertile females via different techniques like MRT-PCR, staining and their isolation by liquid media (using BACTEC 320).

Material and Methods: A total of 217 samples were processed involving the isolation of both (MTB and NTM). All three techniques were processed staining, culture and MRT-PCR to find the prevalence and efficacy of the techniques. For NTM and MTB differentiation SD MPT 64 card test was done.

Results: Out of 217, TB suspected infertile female's maximum lies under the age group of 26-30 i.e. 34.10% (approximately 74 females) and the lowest were under the age group of 15-20 years i.e. 2.3% (5 females). There were total 24 NTM MRT-PCR positives and 29 MTB positives.

Keywords: female genital tuberculosis (FGTB), NTM, ZN staining, MRT-PCR.

GJMR-C Classification: NLMC Code: QW 125.5.M9



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Conclusion: As Mycobacterium tuberculosis remains one of the leading causes of female infertility. According to a few studies, NTM's were found to be increasingly important pathogens causing male genital infections and infertility, but this study gave an insight that NTM's can even lead to female infertility and infections.

Keywords: female genital tuberculosis (FGTB), NTM, ZN staining, MRT-PCR.

I. Introduction

Tuberculosis remains to be the foremost Killer disease for Indians among all other infectious diseases. It is anticipated that 5-13% of pulmonary tuberculosis develops genital involvement [1, 2, 3].

This disease is a significant cause of infertility, menstrual irregularity, pregnancy loss, and in involvement with pregnancy, death rate to both the mother and child increases. HIV co-infection and resistant tuberculosis (MDR or XDR-TB) along with increased population immigration from developed to developing countries have now increased the tendency of the infection. Although new and finer diagnostic techniques for the detection of TB has been progressively available in the form of bacterial cultures and polymerase chain reaction (PCR) and other diagnostics, but still suspicion by clinician's continues to be the main tool for diagnosis of the disease.

Therefore, clinician's necessitate to be well trained to become "Tuberculosis Minded" [4]. Tuberculosis seems to be an important under diagnosed aspect in infertility. The possibility therefore exists that patients with genital tuberculosis can be classified as infertile.

Female Genital Tuberculosis (FGTB) which is commonly drawn in as a cause of infertility [5-10], is often asymptomatic, rare disease with non-specific, mild clinical pictures and low indicator of clinical suspicion. There are no consistent confirmatory analytical procedures to ascertain the cause of infertility [8, 9]. This is the most common form of extrapulmonary tuberculosis (TB), ranging about 27% (range, 14 to 41%) worldwide[10]. The prevalence of infertility in genital TB worldwide varies from 10-85% [8-11]; it is endemic in India, with an incidence of 58% [12] and common is in the reproductive age group (15-45 years)[13]. In 80-90% cases, it affects women with menstrual irregularities and other particular significant symptoms accounting for about 27% of manifestations of FGTB[14], even this rate can be higher among patients with tubal factor infertility (39-41%) [16].

Female genital tuberculosis is secondary to tuberculosis infection elsewhere in the whole body. Haematogenous or lymphatic spread is the most well-known method of spread. Infection may also spread from the adjacent and contagious intra abdominal sites. Patients may also have chief complaints like persistent lower abdominal or pelvic pain, or alterations in the menstrual cycles. Symptoms of tuberculosis toxaemia may not manifest and physical examination may be unexceptional [17-19].

On the other hand, Nontuberculous mycobacteria (NTMB) are saprophytic organisms capable of causing chronic disease in humans [20-26]. The

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Nontuberculous Mycobacteria (NTM) refers to all the species in the family of Mycobacteria that may cause human disease, but do not cause tuberculosis (TB), in other words it is also known as MOTT (Mycobacteria other than tuberculosis). There are about more than 120 identified Mycobacterial species recognized today to cause disease in human [26-30]. The prevalence of NTMB disease is gradually increasing and has emerged in formerly unrecognized populations. The diagnoses of Nontuberculous infections are often complicated or unconvincing. Treatment is also difficult and often controversial, requiring prolonged, inadequately tolerated courses of therapy that are unlikely to produce eradication.

II. Material and Methods

The present study was done at Santosh Medical College and Hospital (Ghaziabad Delhi NCR) in collaboration with Oncquest Laboratories Pvt. Ltd. (03 factory road, Safdarjung Delhi) in which a total of 217 samples were processed using different techniques like AFB smear microscopy, AFB liquid culture via BACTEC 320 and MRT-PCR.

a) Sample Type

- Menstrual blood
- Endometrial tissue biopsy
- Tubal tissue biopsy
- Product of conception

b) Inclusion Criteria

1. Females willing to participate with their consent were included.
2. Study involved infertile and TB suspected females with any of these symptoms like-

- Irregular menstrual cycle.
- Pelvic pain.
- Vaginal discharge that is stained with blood or which is persistent, heavy and discoloured.
- Bleeding after intercourse.
- Infertility
- Abdominal mass.
- Tubo-ovarian abscess.
- Pregnancy loss.
- Strong clinical suspicion of TB.

c) Exclusion Criteria

Eligible female patients not willing to participate and patients already on ATT were excluded for the study.

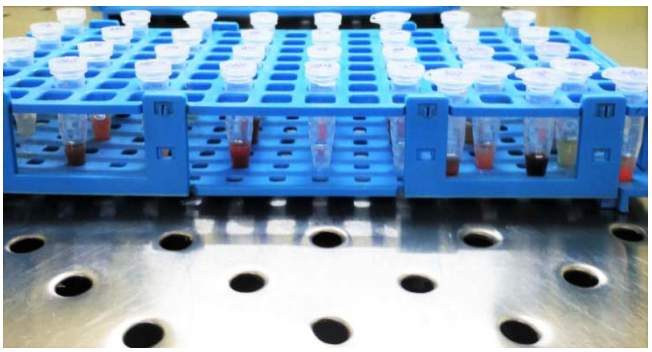
III. Methodology

a) Specimen Collection

Clinical specimens for female genital infections including endometrial and ovarian tissues along with menstrual blood were taken. Specimens were transported to the laboratory as soon as possible after collection. In case of delay, the specimens were refrigerated to inhibit the growth of unwanted micro-organisms.

b) Sample Processing

1. The sample was divided into three parts. First part was subjected for ZN staining, second was used for isolating the mycobacterial species by culturing and third was being used for molecular detection via MRT-PCR.
2. Samples were smeared with Ziehl-Neelsen (ZN) [31] staining to confirm Acid – fastness followed by Homogenization and decontamination by NAOH-NALC method.[31]
3. Isolation of Mycobacteria was carried out by culturing on liquid media by BACTEC 320 which works on the same principle of BACTEC 960 using MGIT tubes.
4. MPT 64 card test was performed to differentiate MTB and NTM from positive MGIT tubes.
5. DNA extraction was done to prepare the sample for doing PCR via AuPreP™ (Genomic DNA extraction Miniprep system) kit.



Picture-I: Samples for DNA extraction procedure

6. MRT-PCR was run to amplify the product using the proper gene (This test included) 2 targets for detection. IS6110 – specific primers for Mycobacterium tuberculosis complex and 16S rDNA –specific primers for Nontuberculous Mycobacteria (NTM) using lytestar™ PCR Kit 2.0.



Picture-II: showing the MRT-PCR

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Picture-III: Illustrating the results of NTM and MTB via MRT-PCR

7. Analysis of staining, molecular, and liquid culture diagnosis for MTB and NTM was done and the efficacy of the techniques was defined along with the prevalence rate of infected infertile females suspected to have *Mycobacterial* infections.

IV. Results

Out of 217, TB suspected infertile female's maximum lies under the age group of 26-30 i.e. 34.10% (approximately 74 females) and the lowest were under the age group of 15-20 years i.e. 2.3% (5 females).

By liquid culture there were 23 positives (12 NTM & 11 MTB).The maximum numbers of females were from the age group of 21-25 (18.18%) whereas the minimum number lied under the age group of 15-20 (0%).

Table 1: Depicting Age Wise Distribution of Infertility Due to *Mycobacterial* Infections diagnosed via BACTEC liquid culture technique

Age Range	Total Patients	(MTB + NTM) Culture Positive	Percentage
15-20	5	0	0%
21-25	33	6	18.18%
26-30	74	9	12.16%
31-35	70	4	5.71%
36-40	29	3	10.34%
41-45	6	1	16.66%
Total	217	23	10.59%

Out of total 23 culture positives 11 were found to be MTB strains (i.e. 5.06%) and 12 were NTM strains (i.e. 5.52%).

Table 2: Depicting AFB culture positives both for MTB and NTM

AFB Culture Positives (n = 217)		
	MTB	NTM
Total	11	12
Rate (%)	5.06 %	5.52%

By MRT-PCR technique there were 53 positives (24 NTM & 29 MTB). The maximum numbers of females were from the age group of 21-25 (30.30%) whereas the minimum number lied under the age group of 41-45(16.66%).

Table 3: Depicting Age Wise Distribution of Infertility Due to *Mycobacterial* Infections diagnosed via MRT-PCR

Age Range	Total Patients	(MTB + NTM) MRT-PCR Positive	Percentage
15-20	5	1	20%
21-25	33	10	30.30%
26-30	74	21	28.37%
31-35	70	12	17.14%
36-40	29	8	27.58%
41-45	6	1	16.66%
Total	217	53	24.42%

Out of total 53 PCR positives 29 were found to be MTB strains (i.e. 13.36%) and 24 were NTM strains (i.e. 11.05%).

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Table 4: Depicting MRT-PCR Positives both for MTB and NTM

MRT-PCR Positives (n = 217)		
	MTB	NTM
Total	29	24
Rate (%)	13.36%	11.05%

Total 217 cases of infertile TB suspected females were observed via MRT-PCR technique. Out of those 217, Endometrial Biopsy / Tissue (195), 24 were MTB positive whereas 18 were found to be NTM strains. Menstrual Blood (17), 4 MTB and 4 NTM were detected. POC (4), out of which there was no MTB strain but 2 NTMs were detected. Tubal Biopsy (1), which was MTB positive.

Table 5: Depicting Sample wise MRT-PCR Results (both Positives and Negatives) for *Mycobacterial* infections

MRT-PCR					
Type of Specimen	Total	MTB Positive	MTB Negative	NTM Positive	NTM Negative
Endometrial Biopsy / Tissue	195	24	171	18	177
Menstrual Blood	17	4	13	4	13
Product of Conception	4	0	4	2	2
Tubal Biopsy	1	1	0	0	1
Total	217	29	188	24	193

Out of total samples for liquid culture, Endometrial Biopsy / Tissue (195), 11 were MTB positive whereas 11 were found to be NTM strains. Menstrual Blood (17), only 1 NTM was detected. The other specimen didn't show any positivity for either of them.

Table 6: Depicting Sample wise AFB Liquid Culture Results (both positives and negative) for *Mycobacterial* infections

AFB Liquid Culture					
Type of Specimen	Total	MTB Positive	MTB Negative	NTM Positive	NTM Negative
Endometrial Biopsy / Tissue	195	11	184	11	184
Menstrual Blood	17	0	17	1	16
Product of Conception	4	0	4	0	4
Tubal Biopsy	1	0	1	0	1
Total	217	11	206	12	205

Out of total 217 samples, 10.59% were culture positive, 24.42% PCR positive and 1.84% was AFB smear positive by microscopy.

Table 7: Depicting Positivity Rate via All Three Techniques

	Culture Positives	PCR Positive	AFB Smear Positive
Total Number (n = 217)	23	53	4
Positivity Rate	10.59%	24.42%	1.84%

While depicting sample wise details and positivity rate of MRT-PCR, culture and microscopy methods. Endometrial Biopsy / Tissue (195), 42 (21.53%) were PCR positive, 22 (11.28%) culture positive and 3 (1.5%) were found to be AFB smear positive. For Menstrual Blood (17), 8 (47.05%) were PCR positive and 1 (5.88%) culture positive whereas no AFB smear positive. For POC (4), 2 (50%) were PCR positive and in case of Tubal Biopsy (1), 1 (100%) was detected PCR positive.

Table 8: Depicting Sample Wise Details and Positivity Rate of MRT-PCR, Culture and Microscopy Methods

Type of Specimen	Number of Clinical Samples	MRT-PCR Positive	MRT-PCR Positivity Rate	Culture Positive	Culture Positivity Rate	AFB Smear Microscopy Positive	Smear Positivity Rate
Endometrial Biopsy / Tissue	195	42	21.53%	22	11.28%	3	1.5%
Menstrual Blood	17	8	47.05%	1	5.88%	0	0%
Product of Conception	4	2	50%	0	0%	1	25%
Tubal Biopsy	1	1	100%	0	0%	0	0%
Total	217	53	24.42%	23	10.59%	4	1.84%

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Out of total 217 samples, 4 (1.84%) were observed positive for all the three techniques (Culture, AFB Smear and MRT-PCR), 3 (1.38%) were positive for Culture and AFB, 76 (35.02%) were detected Culture and PCR positives whereas PCR and AFB were only 4 (1.84%).

Table 8: Depicting Positivity Rates of Different Method

	(NTM + MTB) Culture			
	Culture + AFB Smear + MRT-PCR	Culture + AFB	Culture + MRT-PCR	MRT- PCR + AFB
Total (n = 217)	4	3	76	4
Positivity Rate	1.84%	1.38%	35.02%	1.84%

V. Discussion

Genitourinary tuberculosis is frequent manifestation of extra pulmonary tuberculosis and claim 15% of all EPTB cases.

Fazal-ur-Rehman et al found AFB smear positive in 13 patients i.e. sensitivity of 51.5% from 50 symptomatic patients of genitourinary TB [32].The study of Warren D et.al, also found Acid-fast staining as the most reliable test, with a sensitivity of 22% to 81% [33]. A study by A. Webster and D J Wright showed only 0.2% positive results on ZN staining [34]. Khaled G et al reported positive genital TB of 0.66% on ZN staining [35]. However, the results of our study were just contradicting showing 1.84% of AFB smear positive to P Pranali et al where all the samples were negative for Acid fast bacilli by ZN staining technique [36].

S Rishi, in their study demonstrated that M960 system provided better isolation rate of *Mycobacteria* 98.06% from a variety of clinical samples than the LJ media 63.95% [14]. Various authors have reported similar findings ranging from 80 to 100% for M960 and from 59.7 to 87.2% for LJ [37-39]. According to the present study, isolation rate by M960 system was 10.58% in which 5.06% was MTB strains whereas 5.52% were NTM's. Besides higher isolation rate, even the time to detect *Mycobacteria* was shorter on M960 than on solid media, average being 9.66 days (2-39) with M960 and 28.81 days (7-48) with conventional solid media. Similar findings have been reported in the literature [40-45].

PCR positive patients in the study of P Pranali et al were 8.69% which is very less as compared to the study by Negi S et al 74.4%, Hemal A K et al (80.95%), and Bhanu et al. 53.3% [46-48].The study by Leonardo A. Sechi et al 6.3% showed low positivity as compared with the current study [49] which represented 24.41% for MRT-PCR positives constituting 13.36% MTB and 11.05% NTM's.

We compared the piece of various tests in different clinical samples for diagnosis of TB. PCR showed the uppermost sensitivity as compared to other tests as reported by others [50]. With the use of PCR test, we were able to detect *M.tuberculosis* in 97.87% smear negative samples which were positive by either of the culture methods. In a few samples, ZN smear examination and PCR results were positive but culture was negative; this could be due to the existence of nonviable mycobacteria in the samples [51]. The only drawback is that sometimes there may be false positive results by PCR test which could be due to the ability to detect very low number and even dead bacteria in a sample which can be present in a symptomatic individual [52].

The purpose of this investigation was to evaluate the usefulness of multiplex polymerase chain reaction (MRT-PCR) in detecting uterine tuberculosis in women with infertility.

According to a prospective study, the mean age of the women was 29.75 ± 4.66 years. A total of 25.48% women were diagnosed as having uterine tuberculosis by in any case one of the diagnostic methods. Smear for acid fast bacilli in 2.53%, and liquid culture in 15.18% patients. The in-house MRT-PCR was positive in 85.44% women. Of these, 95.55% samples were positive for *Mycobacterium tuberculosis*, while 4.44% were positive by MRT-PCR for Nontuberculous mycobacterial DNA. So, the diagnosis of uterine tuberculosis, MRT-PCR was found to be the most efficient diagnostic tool compared to the other methods in that study [53].

MRT-PCR test detected *M.tuberculosis* in less than one day, compared to average 24.03 days required for detection by liquid culture BACTEC 960 [54].

Molecular diagnosis of tuberculosis by MRT-PCR has a great potential to improve the clinician's ability to diagnose tuberculosis. This will make certain early treatment to patients and put a stop to further transmission of disease.

VI. Conclusion

As Mycobacterium tuberculosis remains one of the leading causes of female infertility. According to a study, NTM's are found to be increasingly important pathogens causing male genital infections and infertility, but the present study gave an insight that NTM's can even lead to female infertility and infections. NTM's are often misdiagnosed as tuberculosis and are considered to be TB MDR, XDR or even TDR now a days due to overburden of the disease, so the patients were diagnosed accurately and treated accordingly at the earliest. This study helped to highlight the current

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prevalence rate along with the diagnostic accuracy of Tuberculous and Nontuberculous Mycobacterial infections among infertile females.

Abbreviations

- MTB: Mycobacterium Tuberculosis
- FGTB: Female Genital Tuberculosis
- MRT-PCR: Multiplex Real Time Polymerase chain reaction
- AFB: Acid Fast Bacilli
- RIF: Rifampicin
- NTM: Nontuberculous Mycobacterium
- MGIT: Mycobacterium Growth Indicator Tube
- (ZN) staining: Ziehl-Neelsen staining

VII. Acknowledgement

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Linezolid and Methicillin-Resistant Coagulase Negative Staphylococci from Anterior Nares of Nigerian Tertiary School Students

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Abstract- *Background:* The carriage of Coagulase Negative Staphylococci in the anterior nares of subjects in the study environment has not been investigated before and this study is thus a reference study against which future studies can be compared. The isolates obtained were also tested against frequently used antibiotics as well as linezolid, an antibiotic which is of considerable importance in the treatment of Multidrug resistant staphylococci.

Methods: A total of 400 nasal swabs were collected from anterior nares of apparently healthy subjects aseptically using a sterile swab sticks. The antibiotic susceptibilities of isolates of S. aureus obtained against eight different antibiotics including Linezolid were determined using the disc-plate method.

Keywords: *coagulase-negative staphylococci; antibiotic susceptibility patterns; methicillin-resistant CoNS, linezolid-resistant CoNS, anterior nares, apparently healthy, reference study.*

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Linezolid and Methicillin-Resistant Coagulase Negative Staphylococci from Anterior Nares of Nigerian Tertiary School Students

Abdu Abdulrasheed α, Kemebradikumo Pondei σ & Lamikanra Adebayo ρ

Abstract- Background: The carriage of Coagulase Negative Staphylococci in the anterior nares of subjects in the study environment has not been investigated before and this study is thus a reference study against which future studies can be compared. The isolates obtained were also tested against frequently used antibiotics as well as linezolid, an antibiotic which is of considerable importance in the treatment of Multidrug resistant staphylococci.

Methods: A total of 400 nasal swabs were collected from anterior nares of apparently healthy subjects aseptically using a sterile swab sticks. The antibiotic susceptibilities of isolates of S. aureus obtained against eight different antibiotics including Linezolid were determined using the disc-plate method.

Results: The results showed that 136(34.03%) of the subjects tested are nasal carriers of CoNS: *S. epidermidis* 50(36.76%) which is the most prevalent. This is followed by *S. haemolyticus* 41(30.15%), *S. saprophyticus* 13(9.56%), *S. hominis* 10(7.35%), *S. cohnii* 8(5.88%), while *S. lugdunensis* and *S. xylosus* were 7(5.15%) each. The incidences of resistance observed with each of the antibiotics tested were as follow: Erythromycin 112(82.4%), Tetracycline 108(79.4%), Co-trimoxazole 94(69.1%), Cefoxitin 80(58.8%), Linezolid 75(55.1%), Ciprofloxacin 66(48.5%), Augmentin 43(31.6%) and Gentamycin 31(22.8%).

Conclusion: Treatment of CoNS infections within the study environment should be based on the results of *in vitro* susceptibility testing of the isolates. Gentamicin and Augmentin promises to be the best antibiotic for the treatment of disorders associated with Staphylococci in the study area. This is of special importance in an environment within which a very substantial proportion of the isolates are resistant to methicillin and linezolid.

Keywords: coagulase-negative staphylococci; antibiotic susceptibility patterns; methicillin-resistant CoNS, linezolid-resistant CoNS, anterior nares, apparently healthy, reference study.

I. Introduction

Coagulase-negative staphylococci (CoNS) are part of normal commensals of the skin, anterior nares, and ear canals of humans [1]. Because of their relatively low virulence, they have long been considered as nonpathogenetic, and were rarely reported to cause severe infections. However, as a result of the combination of increased use of intravascular devices and an increase in the number of hospitalised immunocompromised patients, CoNS have emerged and are increasingly recognised as a major agents of clinically significant infection of the bloodstream and other sites [2, 3, 4, 5, 6, 7, 8].

Given the frequency with which multiple antimicrobial resistance is encountered, treatment of CoNS infections can be challenging and oxazolidinone: Line zolid has been considered the drug of choice for the management of infections caused by gram-positive organisms, including resistant organisms, such as methicillin-resistant *Staphylococcus aureus*, methicillin-resistant coagulase-negative staphylococci (MRCoNS), vancomycin-resistant enterococci, and multidrug-resistant *Streptococcus pneumonia* [9, 10, 11, 12, 13, 14, 15]. However, widespread use of linezolid recently has led to the emergence of CoNS isolates with decreased susceptibility to these agents further limiting therapeutic options for treatment of infections caused by these organisms [16, 17, 18].

In Nigeria, to date, Linezolid-Resistant Coagulase Negative Staphylococcus (LRCoNS) have not been reported, although there are no indications of the use of linezolid within the study area, it is recognized as one of the few drugs that have been reported to be effective in the treatment of infections caused by MRCoNS. In the current study, we determined nasal carriage rate of CoNS and antimicrobial resistance profile of these coagulase-negative staphylococci isolates with linezolid resistance that were recovered from apparently healthy undergraduate students in Niger Delta University. This study will however serve as a reference point data for nasal carriage rate and Linezolid antimicrobial profile of CoNS for the region.

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II. Materials and Methods

a) Sampling Area

The study was carried out in Amassoma, a semi urban settlement in the Niger Delta and is home to the Niger Delta University with a student population of about 20,000. It is located on Latitude 4^0 59' 09'' N and longitude 6^0 06' 34'' E. Its land area is 2,682Km2 (1,036 sq miles) at an elevation/altitude of 9 metres. It is in an area of high humidity (mean: 300C) and temperature (average: 26.7°C with annual rainfall of about 1777mm.

The students sampled in this study were medical and nursing students of the university. They are of age: (range: 15-39, mean = 22), Sex: (Males: 124; Females: 276) and have stayed a period of 1 year minimum in the University

b) Sampling

Anterior nares swabs were collected in accord to protocols described by Rongpharpi *et al* [19]. A total of 400 nasal swabs were collected from anterior nares of apparently healthy subjects aseptically using a sterile swab sticks (Copan Diagnostics, Corona, CA, USA). Swabs were transported in Amies (Oxoid, England) transport medium to the Medical microbiology laboratory of the College of Health Sciences, Niger Delta University for bacteriological assay.

c) Isolation and Identification

In the laboratory, each swab was immediately inoculated onto Mannitol Salt Agar (MSA; Oxoid, England) plates and incubated at 37°C for 24 h. The characteristic isolates were aseptically isolated and characterized using established microbiological methods that include colonial morphology, Gram stain characteristics, haemolysin production catalase, coagulase tests as well as DNase production [20]. The various isolates were identified to species level by employing standard microbiological methods [20, 21]. Coagulase negative-Staphylococci isolates were confirmed through the use of the Staph identification 25 E (BioMeriux, France).

d) Antimicrobial Susceptibility Testing

The antimicrobial susceptibility pattern of all the isolates to Augmentin (30µg), Cefoxitin (30µg), Ciprofloxacin (5µg), Co-trimoxazole (25µg), Erythromycin (15µg), Gentamycin (30µg), Linezolid (30µg), and Tetracycline (30µg) all obtained from Oxoid (England) were determined using modified single disc diffusion technique in accordance to the guidelines of Clinical and Laboratory Standards Institute (CLSI, 2012) [22]. Briefly, standardized overnight culture of each isolate (containing approximately 106 cfu/ml) which was equivalent to 0.5 McFarland Standard was used to swab the surface of Mueller Hinton agar plates and excess drained off and dried while the Petri dish lid was in place. The standard antimicrobial discs were aseptically placed at reasonable equidistance on the inoculated plates and allowed to stand for I hr. The plates (prepared in duplicates) were then incubated at 37^0C for 18-24 h. The diameter of the zone of inhibition produced by each antimicrobial disc was measured with a ruler in millimeters. Breakpoints and interpretative for susceptibility/resistance was based on the CSLI criteria [22]. We used the agar dilution method to further confirm the Linezolid MIC's (lowest concentration at which growth was inhibited) values of the linezolid-nonsusceptible CoNS isolates. The MIC (µg/mL) interpretative standard for linezolid were those suggested by CLSI [22], (respectively:$\leq$ 4 susceptible, $\geq$ 8 resistant). The procedure was performed in duplicate on separate occasions, and the means of the duplicates were used. *Staphylococcus aureus* NCTC6571 was used as the quality control in each set of tests.

e) Statistical analysis

SPSS for Windows (version 20.0; SPSS) software was used for the analysis. Frequency distribution, mean, harmonic mean, standard deviation, analysis of variance (ANOVA) were determined. Categorical variables were compared by using Pearson's chi-squared test ($\chi 2$) or Fisher's exact probability tests. P-values were calculated and $P \leq 0.05$ was considered statistically significant

III. Results

As depicted in Table 1, 227(56.8%) of the 400 studied subjects yielded Staphylococci growths. The overall carriage rates of Coagulase Negative *Staphylococci* was 136(34.03%).

As shown in Figure 1, we identified and confirmed that the 136 CoNS strains belong to 7 species including *S. epidermidis* 50(36.76%) which is the most prevalent. This is followed by *S. haemolyticus* 41(30.15%), *S. saprophyticus* 13(9.56%), *S. hominis* 10(7.35%), *S. cohnii* 8(5.88%), while *Staphylococcus lugdunensis* and *S. xylosus* were 7(5.15%) each.

Figure 2, shows the antimicrobial susceptibility profile of the isolates. Overall 112(82.4%) of the 136 CoNS isolates showed resistance to Erythromycin, while resistance were 108(79.4), 94(69.1%), 80(58.8%), 75(55.1%), 66(48.5%), 43(31.6%) and 31(22.8%) to Tetracycline, Co-trimoxazole, Cefoxitin [MRCoNS], Ciprofloxacin, Linezolid, Augmentin, and Gentamycin respectively

The prevalence of multiple antibiotic resistance (MAR) of the isolates was investigated. One hundred and twelve (82.35%) of the isolates showed multiple resistance in varying degrees. Twenty-three (20.54%), 18 (16.07%), 26(23.21%), 22(19.64%), and 10 (8.93%) were resistant to 3, 4, 5, 6, and 7 antibiotics among the isolated strains respectively. Thirteen (11.61%) of the

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isolates were resistant to all the 8 antibiotics tested (Figure 3).

IV. Discussion

We conducted this study in order to determine the nasal carriage rate and antimicrobial resistance profile of CoNS strains isolated from the anterior nares of apparently healthy students of a tertiary institution in Wilberforce Island, Amassoma. The institution is situated in a semi urban area in Bayelsa-state in the Niger Delta. The result obtained from the present study will serve as a reference data for CoNS carriage rate. In addition, the study also gives an understanding into the patterns of antimicrobial resistance profile of these isolates in the locality.

The study revealed that 136 out of the 400 subjects examined were positive for CoNS in their anterior nares, indicating the nasal carriage rate of 34.03%. Earlier, report indicates the nasal carriage rate of CoNS to vary from 13% to 56% in different populations [13, 23, 24, 25]. Though we observed lower figure in the present study, our findings is in comparison with the carriage rates documented by Morgenstern et al. [26] and Lebeaux et al. [27] in Portugal and France respectively. Contrast with our findings, higher nasal carriage rates have however been reported by Koziol-Montewka *et al*., 2006 in Poland (55.8%) [28], Campeotto *et al*. 2004 in Brazil (66.1%) [29], Akhtar 2010 inPakistan (73.3%) [30] and Abadi et al. 2015 in Iran (77.7%) [31]. Shibabaw et al. [32] attributed these differences to various microbiological methods (sampling techniques to culture media) employed, the local infection control standards and the local prevalence rate. Aside from these, it has been suggested that carrier rates might also be influenced by poor personal hygiene, poor environmental sanitation [32] and age-related dynamics of the study participants [1]. The low recovery rate of CoNS observed in the present study might be due to the fact that our subjects being medical and nursing students may have been involved in good hygiene practices with hand washing inclusive. On the other hand, as documented by Onasoga, et al., 2015 [33], they may have also been involved in self-medication or predisposed to the misuse of antibiotics.

The results showed that seven species of CoNS were identified. The most common species isolated was *S. epidermidis* 50(36.76%). The similar results were recorded in many studies [34]. Various studies have indicated most CoNS isolates obtained in the present study as responsible for infections that are of endogenous origin particularly among immunocom-promised and individuals that are hospitalized [35, 36, 37, 38, 39, 40].

Over the years, studies have shown that antimicrobial therapy causes marked symptom improvement and shortens the duration of illness associated with *Staphylococci* infections. Before now, various types of antimicrobial agents have been efficacious in the management of *Staphylococci* infections, but options for treatment of these diseases are becoming restricted due to the appearance of multidrug-resistant strains of CoNS. There has been global concern about the emergence of antimicrobial resistance in common pathogens of community as well as nosocomial infections and CoNS have demonstrated a pattern of progressively increasing resistance to antibiotics worldwide [41, 42, 43, 44, 45, 46, 47, 48]. The results obtained from the present study indicates that 112(82.35%) of the 136 isolates from this environment are multiply resistant to antibiotics, (Figure 3). In comparison, the pattern of multidrug resistance demonstrated here has been described among CoNS isolates in different part of the world which includes Switzerland [26], India [49], Iran [31, 34], China [44] , USA [50] ,France [27], Pakistan [30], Italy [51] and Poland [28].

The antibiotic susceptibility pattern of the isolates shows that Gentamycin was the most effective among the CoNS, followed by augmentin, in that order (Figure 2). When compared with existing report, the 22.8% resistance of the CoNS isolates to Gentamycin in this finding corroborates the report of Ma *et* al. [48] and is different with report of Al-Muhanna et al. [34] that 32% of CoNS isolates were resistant to Gentamycin, while Roopa and Biradar [49] and Zhanel *et al*. [52] reported 0.0% and 78.8% resistance of these pathogens to Gentamycin respectively. So gentamycin is the only drug in this study that is proven to be effective for CoNS. One of the reason for this high susceptibility seen in this study may be that gentamycin appears to be infrequently used as it administered by injection, a dosage form which is far less amenable to self-medication than orally administered antibiotics in this locality[53].

On the other hand, the high susceptibility to augmentin observed in this study is in sharp contrast to existing reports (31.6% versus 70%; $P < 0.0001$) by Abdalla *et al*. [54] and Akinkunmi and Lamikanra [55] that 70% and 62.4% resistance of CoNS to augumentin respectively. Nonetheless, the present findings corroborates the report of Roopa and Biradar [49]. One of the reason that could be adduced to low resistance observed in this study may be that augumentin, though an orally administered antibiotics, seems to be rarely abused by individuals in the locality because of its exorbitant price (about 10USD) for a packet in a locality where people live below 1USD per day.

The antimicrobial resistance profile of CoNS isolated in this study indicated that 58.8% of the isolates were resistant to Cefoxitin [MR-CoNS]. This result is higher than earlier report [49, 55], and, lower than report made by Al-Muhanna *et al*., [34], Ma*et al*. [48] and

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Koksal *et al* [56]. However, it is similar to the report made by Lebeaux *et al*. [27] among the organisms isolated in their respective studies. Reports have documented that resistance to Cefoxitin by disc diffusion can be used for the detection of MRSA strains in routine testing [57] because Cefoxitin is regarded as a potential inducer of the system that regulates *mecA gene*[58]. For this reason, the resistant of our isolates which were found to be resistant to Cefoxitin are considered resistant to methicillin (58.8% MR-CoNS).

During the susceptibility test in the present study, one of our limitations was excluding Vancomycin, the drug considered efficacious for MRSA and MRCoNS, from the test because of unavailability of its commercial disc. Nonetheless, Delorme et al.[59] reported the exclusion of vancomycin from their study because vancomycin may produce erratic results in disc diffusion susceptibility test [59]. However, even with the absence of vancomycin susceptibility test, the result of this study can be compared with the findings of several outcomes including [60, 61, 62, 63] which established that linezolid is a drug that is as effective as vancomycin. Both antibiotics do not just have similar failure and success rates but adverse effects as well [61, 64].

Approximately, 69.1% of the CoNS isolates showed high resistance to trimethoprim/sulfamethoxazole in this study. This is similar to what has been reported by Koksal et al. [56] and Akinkunmi and Lamikanra [55] in Turkey and Ile-Ife, Nigeria respectively and many other researchers, a finding correlated to that by Ma et al [48] and Abadi *et al*. [31]. This could be due to the fact that this drug is very commonly available in our setting and is also indiscriminately used for prophylaxis by individuals with symptoms of Upper Respiratory Tract infections (URTI) and Urinary Tract Infections (UTI). The study by Paul et al.[65] showed zero resistance to trimethoprim/sulfamethoxazole to *Staphylococcus aureus* in Nigeria in 1985, while Gu *et al*.[10] showed 29.4% trimethoprim/sulfamethoxazole in Greece. This is worthy of mention and comparison. It shows that trimethoprim/sulfamethoxazole resistance has increased prodigiously over the prevailing years.

The majority of our CoNS isolates were highly resistant to erythromycin (82.4%), and the high rate (79.4%) of resistant to tetracycline and Ciprofloxacin (55.1%), found in this study is worrisome considering the ability of these organisms to spread easily by direct or indirect person-to-person contact with resultant therapeutic complications and considering that ciprofloxacin has been identified as the drug being the most efficaciousanti-infective drug in Nigeria [43, 66].

Combating the increase in mortality and morbidity due to therapeutic failures in the treatment of multidrug resistant Staphylococci infections, particularly those that are methicillin and vancomycin resistances, gave rise to the need for newer efficacious therapeutic options leads to the discovery and approval of oxazolidinone antibiotic: linezolid by FDA in 2000 as an attractive alternative to vancomycin and MRSA [60, 67]. It is tragic that barely one year of its introduction into treatment regime for multidrug resistant Gram-positive organisms, the first resistant among *Enterococcus faecium*, was reported [68] and Tsiodras *et al*. [69] reported the first Linezolid resistant *Staphylococcus aureus* in a US patient. Since then, linezolid-resistant *S. aureus* and CoNS have been detected in separate cases and outbreaks worldwide [10, 70, 71].

Currently, 48.5% of CoNS isolated from the present study were found to be linezolid resistant. Making this finding one of the highest resistance rate recovered among CoNS isolates in Nigeria and amongst those recorded globally. This study revealed that *S. epidermidis*, *S. haemolyticus*, *S. cohnii*, *S. saprophyticus*, S. *hominis*isolates, were resistant to linezolid in 52%, 50%, 48.78%, 46.15%, and 40% respectively, while *S. lugdunensis*and *S. xylosus* showed 42.86% resistance to linezolid each (Table 2). To confirm this resistivity, we decided to carryout Minimum Inhibitory Concentration tests on these isolates as suggested by CLSI 2012 [22], and the outcome showed that all our linezolid resistant isolates had MICs $>256\mu$g/mL.

Previous studies have shown various resistance profiles of CoNS to linezolid. For example, Morgenstern *et al*. [26] in Switzerland and [44] in China reported 0% resistance to linezolid respectively. However, globally, surveillance studies report <1% of CoNSas linezolid resistant. But, a study conducted by Potoski *et al*.[72] in the USA, observed Linezolid resistance in about 4.0% of MRCoNS isolates. Another study conducted in USA by Helio and colleagues reported LRCoNS in 0.1% [50]. Similarly, incidence of 0% was reported by Al-Muhanna *et al*. [34] from Iraq. Ugwu*et al*.[66] reported 0% LRCoNS in Southern Nigeria. The high incidence of linezolid resistance in the organisms isolated in this study is not expected since this antibiotic is not broadly used within the study environment, this is worrisome and is worthy of note. Particularly that linezolid is not routinely prescribed and administered in our locality. More so that the drug is very difficult, if available in our market, in other words, its availability for misuse or self-administration as reported among other antibiotics is not anticipated [73]. So, this high resistance recorded must be of concern to practitioners and public health, more so, that these organisms live in association with other organisms in their ecological niche and can disseminate these resistances to other organisms within the environment [73].This collaborates reports made by Garcia *et al*. [74] that horizontal transmission of linezolid resistance could pose a serious threat, because the *cfrgene* can also be transmitted between species, such as from *S. epidermidis*, which although not pathogenic, could become a reservoir for resistance genes and that

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this mode of transmission becomes more difficult to prevent and stop than those of nosocomial spread that are usually controlled with standard measures, such as isolation, barrier precautions, and antibiotic restriction On the other hand, these organisms and their antimicrobial resistancehave been documented to be associated with opportunistic infections and can be transferred from these individuals to the patients, hospital environments and the community [41, 75, 76] making it a life-threatening organism which may lead to increase mortality and morbidity, particularly among colonised individuals, immunocompromised and HIV patients.Staphylococcal resistance to linezolid (LZD) is said to be mediated through ribosomal mutations (23S rRNA or ribosomal proteins L3 and L4) or through methylation of 23S rRNA by the horizontally transferred *chloramphenicol-florfenicol resistance* (*Cfr*)plasmid-borne ribosomal methyltransferase that catalyzes methylation of A2503 in the 23S rRNA gene of the large 50S ribosomal subunit, conferring resistance to chloramphenicol, florfenicol, and clindamycin[9, 77, 78, 79, 80, 81, 82]. The first *cfr*-mediated, linezolid-resistant clinical isolate of MRSA was reported in 2007 by Toh*et al*. [83]. More recently, linezolid resistance has been identified due to acquisition of a natural resistance gene, *cfr*, so the high resistance of the present study CoNS isolates to linezolid might be due to acquisition of resistance to chloramphenicol, as chloramphenicol is one of the antibiotics that are readily available and most abuse, misuse and self-medicated in our locality. However, this assumption would be further investigated.

V. Conclusion

The study findings indicate the usefulness of investigation of CoNS colonisation of the nasal mucosa the primary ecological niche for these microorganisms in order to better understand the epidemiology of this phenomenon, but also to develop prevention measures and treatment strategies in case of established infections among predisposed individuals.

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Figures and Tables

Table 1: Age and Sex distribution of the subjects and the isolation (Carriage) Rate of Coagulase negative-Staphylococci colonising anterior nares

Age	Male	Female	No (%) Isolate
15-19	4	6	10(7.35)
20-24	30	49	79(58.09)
25-29	24	13	37(27.21)
30-34	4	3	7(5.15)
35-39	3	0	3(2.21)
Total	65	71	136(100)

Table 2: Antibiotics susceptibility of the isolated CONS species during the studied period

Antibiotics	No (%) CoNS of isolates resistant to antibiotics							Total
	S. epidermidis (n=50)	*S. haemolyticus* (n=41)	*S. saprophyticus* (n=13)	*S. hominis* (n=10)	*S. cohnii* (n=8)	*S. lugdunensis* (n=7)	*S. xylosus* (n=7)	
AMC	20(40)	16(34.02)	3(23.08)	1(10)	1(12.50)	1(14.29)	1(14.29)	43
CEF-MRCoNS	31(62)	25(60.98)	6(46.15)	5(50)	5(62.50)	4(57.14)	4(57.14)	80
CIP	25(50)	20(48.78)	10(76.92)	8(80)	4(50)	4(57.14)	4(57.14)	75
CN	20(40)	10(24.39)	1(7.69)	0(0)	0(0)	0(0)	0(0)	31
COT	45(90)	27(65.87)	7(53.85)	3(30)	4(50)	4(57.14)	4(57.14)	94
ERY	38(76)	41(100)	10(76.92)	7(70)	6(75)	5(71.43)	5(71.43)	112
LZD	26 (52)	20(48.78)	6(46.15)	4(40)	4(50)	3(42.86)	3(42.86)	66
TET	40(80)	36(87.80)	8(61.54)	8(80)	8(100)	4(57.14)	4(57.14)	108

KEY: AMC: Augmentin (30μg), CEF: Cefoxitin (30μg), CIP: Ciprofloxacin (5μg), COT: Co-trimoxazole (25μg), E: Erythromycin (15μg), CN: Gentamycin (30μg), LZD: Linezolid (30μg), and TE: Tetracycline (30μg)

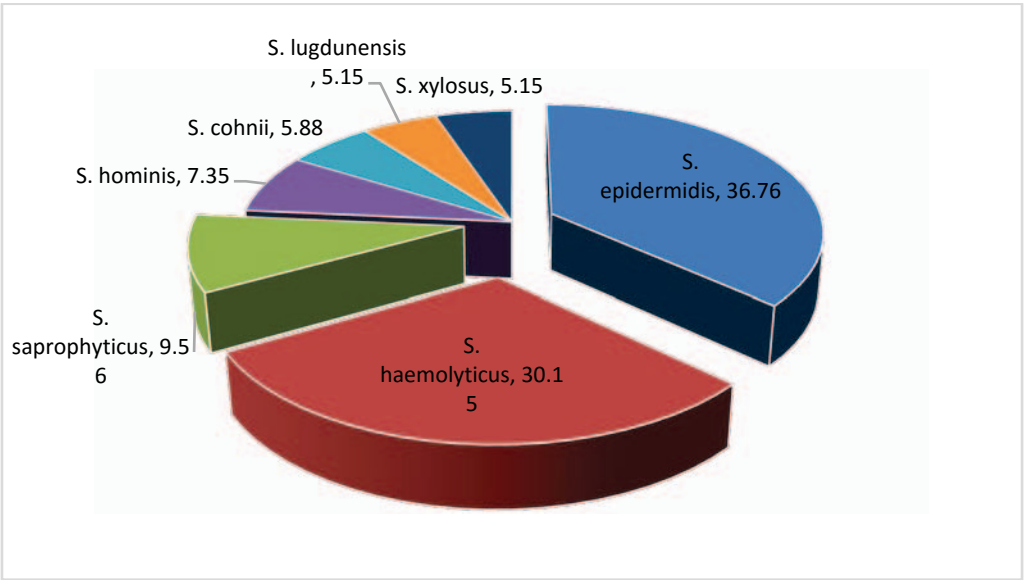


Figure 1: Coagulase negative staphylococci species identified.

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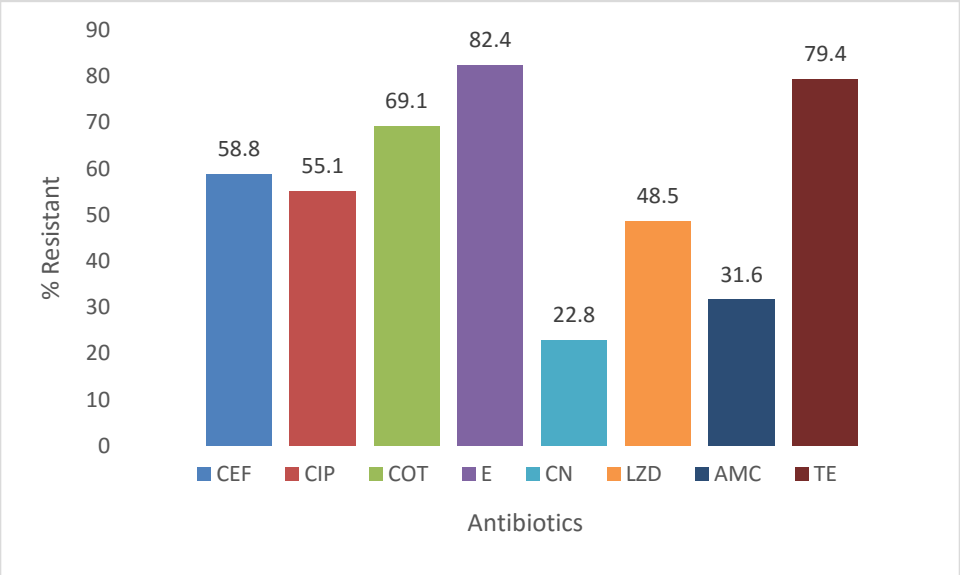


Figure 2: Antimicrobial susceptibility profile of CoNS isolates.

KEY: AMC: Augmentin (30μg), CEF: Cefoxitin (30μg), CIP: Ciprofloxacin (5μg), COT: Co-trimoxazole (25μg), E: Erythromycin (15μg), CN: Gentamycin (30μg), LZD: Linezolid (30μg), and TE: Tetracycline (30μg)

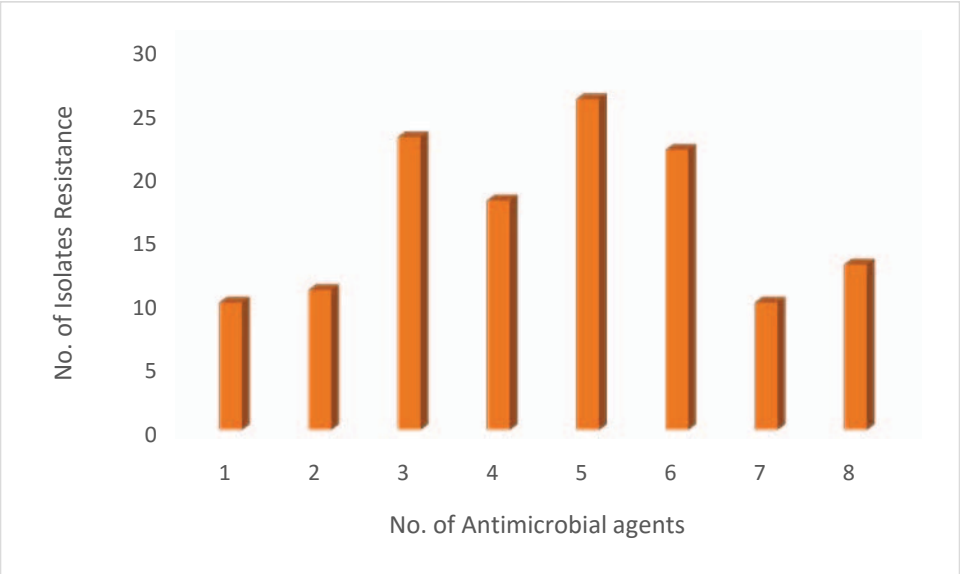


Figure 3: The prevalence of multiple antibiotic resistant of the CoNS Isolates

Key: Multiple Resistant ≥3 = 112

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Fungal and Yeast Involvement in Skin Diseases

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Abstract- A mycological investigation of skin scrappings, blood and aspirates from apparent clinical cases of cutaneous infection involving 100 patients was made. All samples were cultured on Sabouraud dextrose agar (SDA) for three weeks. A total of 27(43.5%) and 18(29.0%) fungi and yeasts respectively were isolated from the skin. Blood cultures yielded 8(9.9%) yeast species only from the patients while culture of aspirates showed more growths of yeasts (2 isolates) than filamentous fungi (1 isolate). The following dermatophytes and filamentous fungi were isolated from the skin: *Microsporum ferrugineum, Trichophyton mentagrophytes, T. rubrum, T. verrucosum and T. schoenleinii; A. fumigatus, A. niger, A. flavus and Penicillium species. Trichophyton mentagrophytes had the highest occurrence (50%) while Microsprum ferrugineum* and *Trichophyton verrucosum* were least in occurrence(9.1%) each. Five yeast species, *Candida albicans, C. tropicalis, C. krusei, Rhodorulla* species and *Torulopsis* species were also isolated from skin lesions. *Torulopsis* species was the most occurring yeast whereas *Candida krusei* and *Rhodotorulla* species showed the lowest occurrence (5.6%) each. Torulopsis species and *Candida tropicalis* were recovered from blood and aspirate of patients. Prevalence of fungi and yeast was similar in male and female patients. Fungi and yeast are involved in skin diseases.

Keywords: *fungi, yeast, skin disease.*

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Nwachukwu O.N [α], Onyeagba R.A [σ], Nwaugo V.O [ρ], Ugbogo O.C [ω] & Ulasi, A.E [¥]

Abstract- A mycological investigation of skin scrappings, blood and aspirates from apparent clinical cases of cutaneous infection involving 100 patients was made. All samples were cultured on Sabouraud dextrose agar (SDA) for three weeks. A total of 27(43.5%) and 18(29.0%) fungi and yeasts respectively were isolated from the skin. Blood cultures yielded 8(9.9%) yeast species only from the patients while culture of aspirates showed more growths of yeasts (2 isolates) than filamentous fungi (1 isolate). The following dermatophytes and filamentous fungi were isolated from the skin: *Microsporum ferrugineum, Trichophyton mentagrophytes, T. rubrum, T. verrucosum* and *T. schoenleinii; A. fumigatus* , *A. niger*, *A. flavus* and *Penicillium* species. *Trichophyton mentagrophytes* had the highest occurrence (50%) while *Microsprum ferrugineum* and *Trichophyton verrucosum* were least in occurrence(9.1%) each. Five yeast species, *Candida albicans, C. tropicalis, C. krusei, Rhodorulla* species and *Torulopsis* species were also isolated from skin lesions. *Torulopsis* species was the most occurring yeast whereas *Candida krusei* and *Rhodotorulla* species showed the lowest occurrence (5.6%) each. Torulopsis species and *Candida tropicalis* were recovered from blood and aspirate of patients. Prevalence of fungi and yeast was similar in male and female patients. Fungi and yeast are involved in skin diseases.

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I. Introduction

Skin is the most accessible organ of the body, the one most easily traumatized and therefore frequently subjected to infection. Normal human skin is colonized by large numbers of microorganisms that live harmlessly as commensals on its surface[1]. Skin diseases therefore is a complex subject involving diverse microorganisms that exhibit varying aetiological and pathogenic mechanisms.

Fungal diseases of the skin are a common public health problem worldwide. The prevalence of skin fungal diseases is expected to reach 20-25% of the world's population and its incidence continues to rise[2]. Fungal skin diseases constitute an important clinical and public health problem in tropical areas of the world where they are rarely managed[3].

There are many species of fungi that cause skin diseases in man. These are mainly Dermatophytes (Trichophyton species, Epidemophyton sp and Microsporon so), *Malassezia furfur* and Candida species and less commonly *Aspergillus species, Trichothecium roseum, Cladosporium sp* and *Fusarium sp*[4].

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The diseases caused by fungi (mycoses) can be clinically classified as superficial, deep or systematic mycoses[5]. Dermatophytes are the most important microorganism which cause superficial mycosis and the lesions are characterized by circular disposition, desquamation alopecia and erythema of the edges[6]. They invade and destroy the skin, hair and nails. These diseases have been reported in various studies from developing countries as the most common dermatozed[7,8]. They are also responsible for most of the skin infections among school children[9].

Fungal skin diseases constitute the majority of skin conditions seen by physicians in primary, secondary and tertiary health care centres in Nigeria[11-12].

They make the individual uncomfortable, unsightly and present a cosmetically poor appearance[13]. The situation is enhanced in a tropical country like Nigeria by warm humid weather, crowded living and poor sanitary conditions which are prevalent and support infection on human skin[14].

This study therefore seeks to investigate the rate of involvement of fungi and yeast in diverse kinds of skin diseases that present.

II. Materials and Methods

a) Study Area

This prospective study was carried out at Nnamdi Azikiwe University Teaching Hospital (NAUTH), Nnewi NAUTH is the largest tertiary hospital and referral centre in Anambra State Nigeria.

b) Subjects

Individuals aged 1 year and older with varied skin disease referred to the mycology section of microbiology laboratory for further assessment were invited to participate and enrolled at the time of presentation to the laboratory.

c) Sample Collection

Skin scrappings, venous blood and aspirates (where applicable) from 100 patients with clinical cases of cutaneous infection were collected after thorough physical examination. The clinically apparent lesions described as dry, scaly and matted or seranguinous were cleaned with 70% alcohol. Epidermal scales at the active edges of the lesions were scrapped using sterile surgical blades. The scrappings were collected in a piece paper, carefully folded and then placed in an envelope for storage in air-tight containers.

Aspirates were taken from pustular lesions or nodules where present. After cleaning the arm,

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tourniquet was applied to dilate the upper arm and 1ml of venous blood was withdrawn from the cubital region of the forearm. These samples were properly labeled and a brief history of the disease taken.

d) Processing of Samples

Each skin scrapping was inoculated onto Sabouraud Dextrose Agar (SDA) incorporated with 0.05mg/ml Chloramphenicol and Streptomycin 40mg/ml. Duplicate inoculations were made. They were incubated at room temperature (25-28^{0}C) and at 37^{0}C respectively for 21 days, examining daily for fungal and / or yeast growths.

Aspirates were also inoculated on SDA and incubated at 37^{0}C for 48hours.

One mililitre (ml) amount of blood specimens were introduced asceptically into 5ml of Sabouraud Dextrose Broth contained in MacCathney bottles. Duplicate inoculations were made. They were incubated at 37^{0}C for 48 hours before being subcultured onto solid medium, SDA. Incubation of blood cultures and subsequent subculture onto SDA continued for 21 days after which negative cultures were discarded.

e) Characterization and Identification of Isolates

The mycological identification was based on macroscopic and microscopic examination of culture isolates. Macroscopic examination of dermatophytes was characterized by duration of growth, surface morphology and pigment production on the reverse[15]. Microscopic examination of fungal growth was observed with Lactophenol cotton blue stain. Nature of mycelium and conidia formation help to differentiate various genera and species[16].

All cream to white-tan pasty colonies with characteristic yeast smell were stained by Gram's method examined microscopically. Budding yeast cells of Candida species were identified by germ tube formation, sugar fermentation and sugar assimilation[17].

Ethical approval for the study was obtained from the ethical committee of the hospital. All patients consented to participate in the study.

III. Results

A total of one hundred and fifty-seven samples consisting of 62 skin scrapings, 81 blood and 14 aspirates, collected from 100 patients were cultured. Culture of the skin scrapings revealed that 27(43.5%) isolates yielded fungi whereas 18(29.0%) of the isolates were yeast-like organisms. Eight (9.9%) of the eight-one blood samples yielded yeast-like organisms. Similarly, 1 (7.1%) of 14 aspirate samples cultured yielded a fungus and 2 (14.3%) yeast-like organisms(Table 1).

Table 1: Proportion of Fungi and Yeasts in Skin, Blood and Aspirates of Patients

Nature of sample	No examined	No positive for fungi (%)	No positive for yeasts (%)
Skin scrapings	62	27(43.5)	18(29.0)
Blood	81	0(0)	8(9.9)
Aspirate	14	1(7.1)	2(14.3)
Total	157	28(17.8)	28(17.8)

Five species of dermatophytes were isolated from 100 patients who had superficial infections: *Microsporum ferrugineum, Trichophyton mentagrophytes, T. rubrum, T. verrucosum* and *T. schoenleinn. T. mentagrophytes* had the highest occurrence (50%) followed by *T. rubrum* (18.2%). Five yeast species including *Candida albicans. C.tropicals, C.krusei, Rhodotorulla sp* and *Torulopsis, sp* were also recovered from skin lesions. *Torulopsis sp* was the most occurring yeast (44.4%) while *C. krusei* was the least occurring (5.6%) *Aspergillus niger* was the most common filamentous fungi (44.4%) isolated from the skin whereas *A. flavus* was the least recovered (11.1%). *Torulopsis sp* (50%) and *Candida tropicals* (50%) were isolated from the blood as presented in Table 2,

Table 2: Prevalence of Fungi and Yeast Isolates in Skin, Blood and Aspirates of Patients

Fungal species	Skin	Blood	Aspirates
Dermatophyte			
Microsporum ferrugineum	2(9.1)	-	-
Trichophyton mentagrophytes	11(50)	-	-
T. rubrum	4(18.2)	-	-
T. verrucosum	2(9.1)	-	-
T. schoenleinii	3(13.6)	-	-
Total	**22**	**0**	**0**
Filamentous fungi			
Aspergillus niger	4(44.4)	-	-
A. fumigates	2(22.2)	-	-
A. flavus	1(11.1)	-	-
Penicillium sp	2(22.2)	-	1(100)

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Total	9	0	0
Yeast species			
Candida albicans	5(27.8)	-	-
C. tropicalis	3(16.7)	3(37.5)	1(50)
C. krusei	1(5.6)	-	-
Rhodotorulla sp	1(5.6)	-	-
Torulopsis sp	8(44.4)	5(62.5)	1(50)
Total	18	18	2

Fungi were isolated more from males (57.6%) than from females (42.4%). Similarly, yeasts were more frequently isolated from males (60.7%) than females (39.3%) Table 3.

Table 3: Gender distribution of Fungi and Yeast among Patients

Gender	No of patients examined	No positive for yeast	No positive fungi (%)
Males	54	17(31.5)	19(35.2)
Females	46	11(23.9)	14(30.4)
Total	100	28(28.0)	33(33.0)

Different fungi and yeast species were isolated from various body sites (Table 4).

Table 4: Common Skin Diseases, Body Sites and Microorganisms Involved

Skin disease	Body site affected	Fungi isolated	Yeast isolated
Tinea capitis	Scalp	*Microsporum ferrugineum* *Trichophyton mentaphytes* *T. rubrum* *Penicillium sp*	*Candida tropicalis* *Rhodotorulla sp*
Tinea corporis	Hand, trunk face, groin leg, buttocks	*T. mentagrophytes* *T. verrucosum* *Penicillium sp* *A. fumigatus ,A.niger*	*Candida krusei* *C. albicans* *Torulopsis sp*
Tinea imbricate	Breast	*T. rubrum* *T. mentagrophytes*	-
Paronychia (Tinea unguium)	Toe web Toe nail	*T. schoenleinii* *Penicillium sp* *T. mentagrophytes* *A. niger*	- *C. albicans* *C. tropicalis*

IV. Discussion

This study has revealed the skin as most susceptible to infections by fungi (43.5%) and yeast (29.0%) as compared to blood (0%) and (9.9%) respectively, lending credence to the work of Yahya *et al.*[18] that skin is the most accessible organ to infection. The result of the species of dermatophytes isolated from skin diseases which include *Microsporum ferrugineum, Trichophyton mentagrophytes, T. rubrum, T. verrucosum* and *T. schoenlleinii* is in agreement with previous studies in Korea, Iran, India and Jos, Plateau State, Nigeria.[19-23]

An earlier study carried out about 30 years ago in Eastern Nigeria[24] also isolated these dermatophytes. This implies that fungal infections are still highly prevalent in these areas. *Trichophyton mentagrophytes,* one of the several dermatophytes that cause cutaneous mycoses and *T. rubrum* were the most common dermatophytes isolated from skin lesions. This finding is in consonance with the work of Ta'ama *et al*[23] who recovered *T. mentagrophytes* constantly and that of Kannan *et al*[22] where *T. rubrum* was the most prevalent causative agent implicated in skin fungal infections.

Majority of dermatophytes were isolated from scalp (Tinea capitis) of patients. Among these were two members of a family. It had been reported.[2,14,25] that frequent interchange, poor sanitary conditions, sharing of hair brushes, combs and hats have played some role in the spread of the disease. These conditions may be responsible for the observation made in the case of the two family members.

Non-isolation of *Trichophyton tonsurans* from any of the clinically observed lesions in this study is a deviation from earlier reports.[24,26] in Eastern and Northern parts of Nigeria where this fungus was frequently encountered. The relative small sample size of this study may have accounted for it.

In the screening for systemic involvements in patients with long standing skin diseases, *Candida*

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tropicalis (50% of the total yeast, isolates made), Rhodotorula species (37.5%) and Torulopsis species (12.5%) were isolated. Previous studies[5,27] had shown that *Torulopsis* species and *Candida tropicals,* both opportunistic pathogens, were capable of establishing themselves in the blood. The patient's defenses may have been weakened by some other processes for these pathogens to be recorded from blood. Petmy *et al*[28] reported that it may have been as a result of frequent usage of antibiotics, immunosuppressive drugs and various conditions like organ transplantations, lymphoma, leukemia and human immunodeficiency virus (HIV) infection. This study did not however, attempt to establish if there had been any previous but continuing disease, clinically or through laboratory diagnosis. However, some of the patients had been on antibiotics for long periods, a situation that may promote human infection by the yeasts.[17] In one of the cases studied, Rhodotorula species, was isolated from the skin of an infant (1 year old). This condition may be the result of certain complications such as napkin dermatitis or due to no clearly defined pre-disposing factor.

The pathogenic status of *Candida tropicals* and *Torulopsis* species was further highlighted in this study as these were isolated from aspirates.

Prevalence of fungal skin infections in males and females were similar (33.0% vs 28.0%). Similar studies[19,23] supports our finding showing that large numbers of people are often affected by fungal skin diseases irrespective of their gender.

V. Conclusion

Fungi and yeasts are involved in skin diseases. *Trichophyton mentagrophytes* was the most common dermatophyte while *Torulopsis* species was the highest occurring yeast involved in skin diseases. Prevalence in males and females were similar.

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Histopathological Studies of Chlorpyrifos Toxicity in Catfish

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Keywords: *chlorpyrifos, histopathology, sub-lethal, toxicity, necrosis.*

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HISTOPATHOLOGICALSTUDIESOFCHLORPYRIFOSTOXICITYINCATFISH

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Histopathological Studies of Chlorpyrifos Toxicity in Catfish

Nazia Khatun [α], Taibur Rahman [σ] & Rita Mahanta [ρ]

Abstract- Histopathological changes in different tissues induced by sub-lethal concentrations of chlorpyrifos in *Heteropneustes fossilis* was highly reflected in the present study. Fishes were exposed to both low (1/50th of LC_{50}) and high concentration (1/10th of LC_{50}) doses of chlorpyrifos for duration of 30 days. Several histopathological alternations were observed in the tissue section. Gradual degenerative changes in different organs observed were disorganization of hepatic cords, necrosis with complete loss of hepatocytes at many places, swollen gill lamellae, pyknosis of nuclear structures in many necrotic cells, vacuolation and loss of glomerular structures in kidneys and atrophy on the villi structure in the treated fish. The histopathological alterations were found to be dose dependent as the cellular alterations were more pronounced with the higher concentration of the chemicals.

Keywords: chlorpyrifos, histopathology, sub-lethal, toxicity, necrosis.

I. Introduction

Pesticides have large impact on the histopathological changes in many fresh water fishes. Large scale production and overall uses of pesticides worldwide has change the bioconcentration of these chemicals which shows its effect in different organs of the fish species. The use of pesticides rises exponentially with the industrial development and agricultural growth. Side by side these pesticides create serious threat to the non-target organisms both in terrestrial as well aquatic ecosystems. Hazardous chemicals from industrial waste water and agricultural runoff are the main cause of water pollution. Aquatic organisms mainly fishes accumulate many contaminants and toxicants directly through their gills and skin and indirectly via their food chain, which may causes diverse alternations in histopathology.

Histopathology showed to be a suitable biomarker in the evaluation of the health of organism exposed to pollutants and can be used as biomonitoring tools for toxicity studies (Meyers and Hendricks, 1985). One of the great advantages of using histopathological biomarkers in environmental monitoring is that this study allows examining specific target organs, including gills, kidney and liver, that are responsible for vital functions, such as respiration, excretion and the accumulation and biotransformation of xenobiotics in the fish (Gernhofer et al., 2001).

Chlorpyrifos (O, O – diethyl O-3, 5, 6 trichloro-2-pyridyl-phosphorothioate) is used as broad-spectrum chlorinated organophosphate insecticide. Chlorpyrifos (CPF) are widely used in agriculture and against pests. It is used on fruit, grain, nuts, vegetables, livestock, ornamentals, golf courses, buildings, and for treating wood products. It is formulated as liquid, granular, and flowable concentrates, baits, wettable powders and dusts. Different workers at different times reported that exposure of chlorpyrifos to fishes and other aquatic organisms has caused severe damage to liver, kidneys, gills, intestine, etc. Chronic exposure causes significant histopathological changes in liver (Barbhuiya and Dey, 2014)

Hence the present study was undertaken to examine the effect of different sub lethal dose of Chlorpyrifos at different concentrations on histopathological changes in different organs of catfish, *Heteropneutes fossilis* (Bloch).

II. Materials and Method

Healthy live fish *Heteropneustes fossilis* were purchased from the local fish market of Dhubri, Assam. The fishes were acclimatized for one week in the laboratory condition in a glass tank prior to the start of the experiment. The fishes of length 12 ± 1 cm and weight 10 ± 1 gm were selected from the tank and transferred to glass aquaria for experimental procedure.

a) *Experimental design*

Three (3) different groups (each with 5 fishes of both sexes) were used for the experiment. Group-I represent normal control group, Group-II represent sub lethal concentration (1.42 ppm) Chlorpyrifos treated group and Group-III comprises of sub lethal concentration (0.28 ppm) Chlorpyrifos treated group. The experiments were conducted in aerated glass aquaria. The fishes were exposed to two different concentrations of chlorpyrifos for a period of 30 days and one aquarium was left untreated as control group.

b) *Histological procedure*

At the end of exposure period of 30 days, fishes from each group were collected randomly and blotted

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dry with soft absorbent paper. Each fish was then sacrificed and dissected to collect the pieces of liver, kidney, intestine and gills. These tissues were then kept in normal saline to remove traces of blood and fixed in 10% formalin for about 24 hours. The specimen were processed for dehydration in graded alcohol, cleared in xylene and finally embedded in molten paraffin wax. Tissues were then sectioned at 4μm thickness using rotary microtome. The tissue sections were stained with haematoxylin and eosin (H & E) and mounted in DPX. Finally the prepared slides were observed under light microscope for histopathological interpretation and microphotographs were taken.

III. Results and Discussion

a) Histopathological changes

i. *Liver*

The liver of the control fish (Group-I) exhibited a normal architecture with continuous mass of hepatic cells forming a cord like structure (fig.1.A). These cords of hepatocytes were arranged around the central vein. The hepatocytes were large sized, polygonal cells with centrally located nuclei. The sinusoids were seen as communicating channels occupied by blood cells and Kupffer cells. The liver treated with high dose of Chlorpyrifos (Group-II) showed centrilobular necrosis characterized by necrosis of hepatocytes around the congested central vein. Hepatocytes showed increased granularity of cytoplasm with nuclear pyknosis leading to necrosis and complete loss of hepatic parenchyma (fig.1.C). The liver of Group –III fishes treated with low concentration of chlorpyrifos was reflected by disorganization of hepatic cords, nuclear pyknosis, necrosis with complete loss of hepatocytes at many places (fig.1.D). Fine fibrillar structures were present at many places with loss of hepatic cytoplasm, hemorrhage and congestion.

Similar observations were made by Pandey and Dubey, (2015) which showed degeneration and disintegration in most cytoplasmic contents, necrosis along with pyknosis and rupture of hepatocytes on exposure of pentachlorophenol (PCP) to *H.fossilis* for 21 days. Pyknotic nuclei in liver of malathion treated *H.fossilis* were also observed by Deka and Mahanta (2012). Cytoplasmic vacuolation, cellular degeneration, congestion in blood sinusoids has also been reported in the earlier studies after exposure of aluminium in Tilapia zilli for 96 hours (Hadi and Alwan, 2012). This finding is in agreement with Barbhuiya and Dey (2014), Sakr and Lail (2005).

ii. *Kidney*

There was no histological change in the kidney tissue of fish in control group. Histological structure of kidney of control *H.fossilis* showed numerous nephrons, which was composed of renal corpuscles with a well developed glomerulus and renal tubules. Glomerular tuft consists of blood capillaries surrounded by Bowman's capsule (fig.2.A). The tubules were lined with single epithelial cell layer having basal nuclei at the proximal segment while the distal part showed nuclei in central position. The interstices of the tubules were enriched with haematopoietic tissue. Results of the present study demonstrated that sublethal concentration of Chlorpyrifos produces large histopathological alternations in the kidney of treated fishes. Changes observed in Chlorpyrifos group (Group-II) fishes include extensive damage to the renal tubular epithelium with necrosis and complete loss of hematopoietic tissues with presence of some golden brown haemosiderin pigments indicating haemolysis (fig.2.C). The kidneys of Group III chlorpyrifos treated *H.fossilis* exhibited tubular degeneration and the glomerular tufts showed severe damage with vacuolation and loss of glomerular structures (fig.2.D).

Disintegration of glomeruli, increase in Bowman's space, elongation of tubules and damage of haematopoitic tissue in kidney was observed at the end of 21 days treatment of pentachlorophenol (PCP) by Pandey and Dubey, (2015). Similar findings were observed in dieldrin and BHC treated fish *Cyprinus carpio* (Satyanarayan *et al*., 2012). Shrunken glomerulus and conjested to severe degeneration of tubules, vacuolization and dialation of tubules were reported in the study of Tripathi and Srivastava, (2010) with chlorpyrifos in Wister rats. Shrinkage of glomeruli and widening of nephritic tubules was also reported in catfish, *H.fossilis* exposed to chlorpyrifos at a concentration of 2mg per litre (Srivastava *et al.,* 1990).

iii. *Gills*

Normal architecture of gill was observed with intact primary and secondary lamellae, gill arches and gill rays (fig.3.A). Comparing with the control set, it was found that the treated fish with higher concentration of sublethal dose showed swollen gill lamellae with mild congestion. All structures are greatly enlarged as compared to the control group. There was also increase in number of infiltrating cells in both the filaments as well as the lamellar structures (fig.3.C). Degenerative changes were also distinctly observed in experimental fishes with lower concentration of sublethal dose though it is of lower intensity (fig.3.D).

Literature review shows that when Tilapia zilli treated with aluminium, histopathological changes such as cellular hypertrophy or hyperplasia of primary filaments and fusion of secondary lamellae occurred (Hadi and Alwan, 2012). Degenerative epithelium of gill filaments and secondary lamellae accompanied by separation of their epithelium from the lamellar supporting cells was also demonstrated in the experiment of Bhuvaneshwari et al., (2015) on Zebra fish exposed to organo chlorine pesticide and heavy metals. Dilation of blood capillaries, abnormal swellings

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epithelium was also observed by Banee et al., (2013) in Rainbow trout exposed to diazinon.

Architectural distortion of the gill tissue to the chlorpyrifos exposed tadpole larvae of Asian common toad, *Duttaphrynus melanostictus* was reported in the study of Bandara et al., (2012). Hypertrophy of lamellar epithelium, destruction of gill lamellae and blood congestion was reported in cadmium chloride treated fish *Ophiocephalus (Channa) striatus* (Bais and Lokhande, 2012).

iv. *Intestine*

The intestine of normal control fish *H.fossilis* showed lymphoid aggregation at the base of villi. Glandular epithelial layer having cuboidal and longitudinal tissue at the base of villi structure was also observed. The sublethal dose (1.42 ppm) of chlorpyrifos exposure results showed significant changes in the intestine of *H.fossilis*. There was atrophy on the villi structure, degeneration and necrosis of mucosal epithelium of intestine and depletion of lymphoid follicles (fig.4.C).

0.28 ppm of chlorpyrifos exposure for 30 days also showed atrophy and complete disappearance of villi structures in most of the areas. Some of the villi showed focal areas of necrosis (fig. 4.D). The mucosal epithelium was found to be flattened and shrunken.

Various studies at different times reveal that intestinal mucosa and villi structure degenerate upon exposure to different harmful chemicals which ultimately hamper the absorption process. The histopathological alternations observed in the intestinal tissue of the experimental fish are in agreement with those of Mandal and Kulshrestra, (1980) who carried the experiment on *Clarius batracus* treated with submithion; Yildririm *et al*., (2006) showed deleterious effects on tissues on exposure to deltamethrin. Disintegration of the intestinal tissue was observed in the study of cypermethrin administered fish *Oreochromis mossambicus* by Karthigayani *et al*., (2014). Ulceration alongwith erosion of the mucosa, distortion of papillae was demonstrated in flying barb *Esomus danricus* upon exposure to 0.179 μg/l and 1.79 μg/l of malathion respectively for 28 days (Das and Gupta, 2013). Necrosis, degenerative change of mucosal epithelium as cloudy swelling was observed in the intestine of cadmium chloride treated fish, *O.striatus* by Bais and Lokhande, (2012). Flattening of the intestinal folds was well defined in aldrin exposed *Cyprinus carpio* (Satyanarayan et al., 2012).

IV. Conclusion

The present study showed that chlorpyrifos at different sublethal concentration causes significant histomorphological changes in the liver, kidney, gill and intestine of *H.fossilis*. The histopathological changes seen in all the tissues were more pronounced in sublethal concentration at higher dose of chlorpyrifos than lower dose level. Hence the results of the present study are indicative of the related changes in different tissues induced by chlorpyrifos toxicity which was found to be significant at high dose level and chlorpyrifos in sublethal dose of minimum 0.28ppm is found to be effective in causing degenerative changes in tissue architecture.

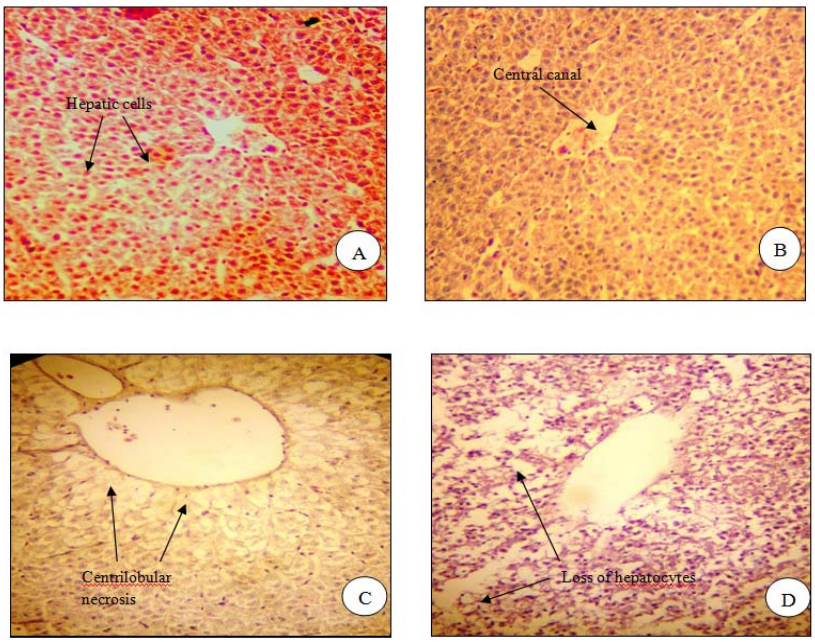


Fig.1: (A) Liver of normal control fish. (Magnification X100)
(B) Liver of normal control fish showing the central canal. (Magnification X100)
(C) 1.42 ppm (1/10th of LC_{50}) Chlorpyrifos treated Liver. (Magnification X400)
(D) 0.28 ppm (1/50th of LC_{50}) Chlorpyrifos treated Liver. (Magnification X100)

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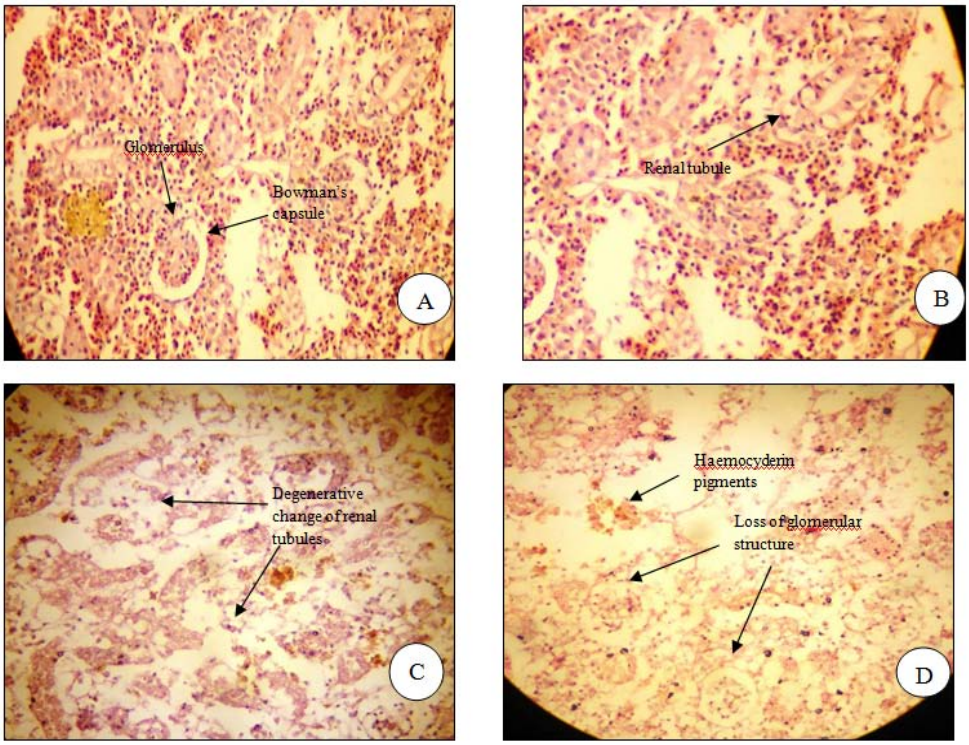


Fig. 2: (A) Kidney of normal control fish. (Magnification X100)
(B) Kidney of normal control fish showing the renal tubules. (Magnification X100)
(C) 1.42 ppm (1/10th of LC_{50}) Chlorpyrifos treated Kidney. (Magnification X100)
(D) 0.28 ppm (1/50th of LC_{50}) Chlorpyrifos treated Kidney. (Magnification X100)

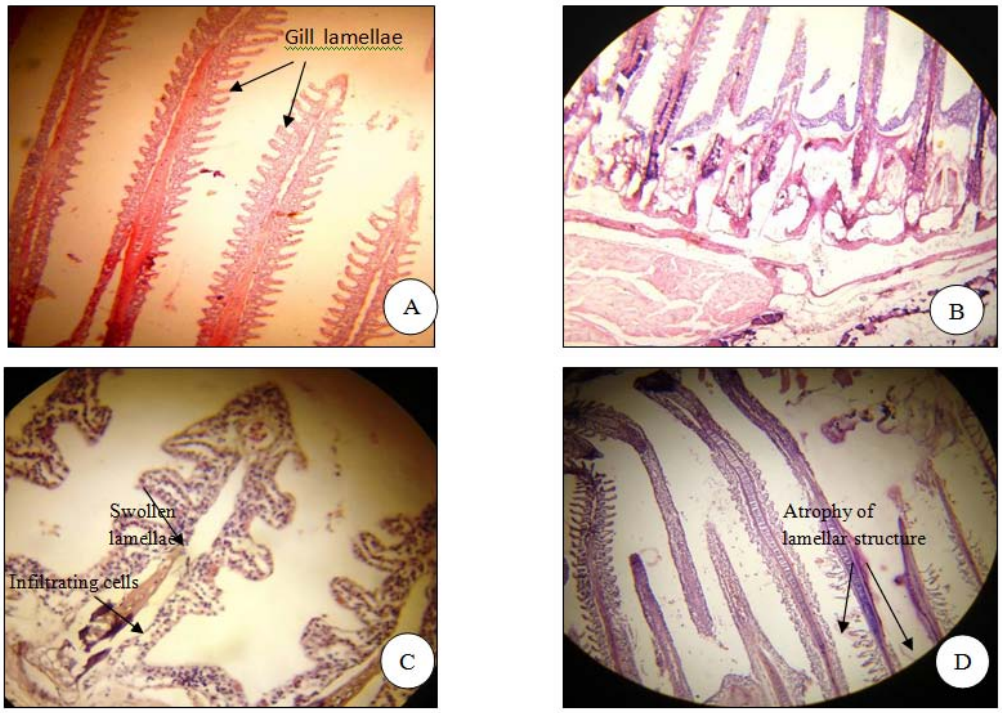


Fig. 3: (A) Gill structure of normal control fish showing the gill lamellae. (Magnification X400)
(B) Gill structure of normal control fish. (Magnification X100)
(C) 1.42 ppm (1/10th of LC_{50}) Chlorpyrifos treated gill structure. (Magnification X400)
(D) 0.28 ppm (1/50th of LC_{50}) Chlorpyrifos treated gill structure. (Magnification X100)

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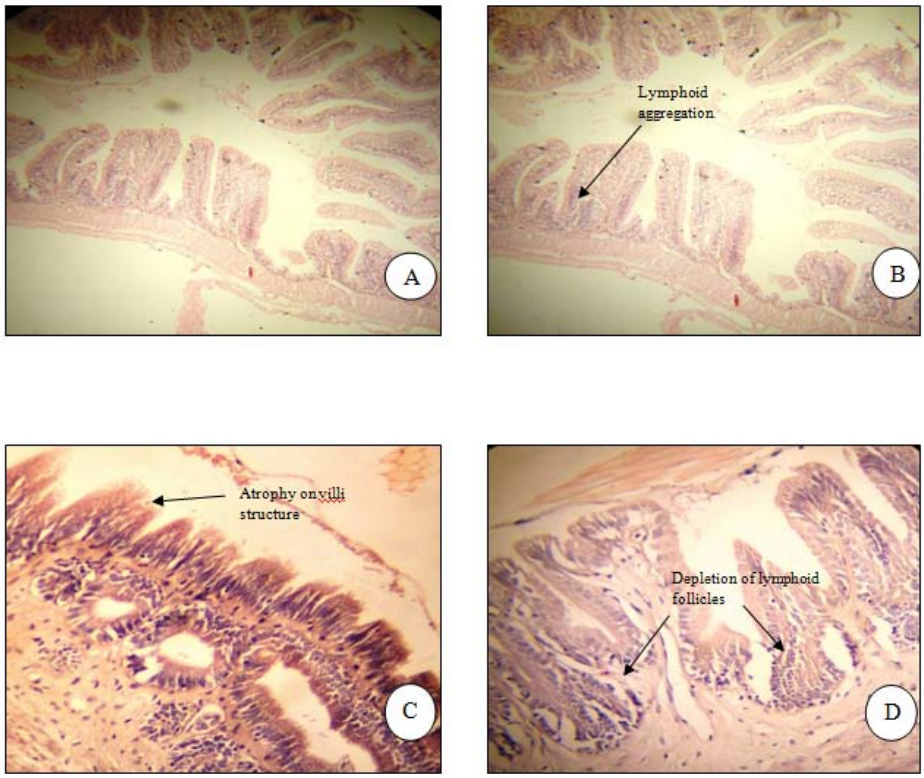


Fig. 4: (A) Intestine of normal control fish. (Magnification X100)
(B) Intestine of normal control fish showing the lymphoid aggregation. (Magnification X100)
(C) 1.42 ppm (1/10th of LC_{50}) Chlorpyrifos treated Intestine. (Magnification X100)
(D) 0.28 ppm (1/50th of LC_{50}) Chlorpyrifos treated Intestine. (Magnification X100)

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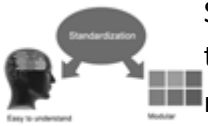


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21. Arrangement of information: Each section of the main body should start with an opening sentence and there should be a changeover at the end of the section. Give only valid and powerful arguments to your topic. You may also maintain your arguments with records.

22. Never start in last minute: Always start at right time and give enough time to research work. Leaving everything to the last minute will degrade your paper and spoil your work.

23. Multitasking in research is not good: Doing several things at the same time proves bad habit in case of research activity. Research is an area, where everything has a particular time slot. Divide your research work in parts and do particular part in particular time slot.

24. Never copy others' work: Never copy others' work and give it your name because if evaluator has seen it anywhere you will be in trouble.

25. Take proper rest and food: No matter how many hours you spend for your research activity, if you are not taking care of your health then all your efforts will be in vain. For a quality research, study is must, and this can be done by taking proper rest and food.

26. Go for seminars: Attend seminars if the topic is relevant to your research area. Utilize all your resources.



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27. Refresh your mind after intervals: Try to give rest to your mind by listening to soft music or by sleeping in intervals. This will also improve your memory.

28. Make colleagues: Always try to make colleagues. No matter how sharper or intelligent you are, if you make colleagues you can have several ideas, which will be helpful for your research.

29. Think technically: Always think technically. If anything happens, then search its reasons, its benefits, and demerits.

30. Think and then print: When you will go to print your paper, notice that tables are not be split, headings are not detached from their descriptions, and page sequence is maintained.

31. Adding unnecessary information: Do not add unnecessary information, like, I have used MS Excel to draw graph. Do not add irrelevant and inappropriate material. These all will create superfluous. Foreign terminology and phrases are not apropos. One should NEVER take a broad view. Analogy in script is like feathers on a snake. Not at all use a large word when a very small one would be sufficient. Use words properly, regardless of how others use them. Remove quotations. Puns are for kids, not grunt readers. Amplification is a billion times of inferior quality than sarcasm.

32. Never oversimplify everything: To add material in your research paper, never go for oversimplification. This will definitely irritate the evaluator. Be more or less specific. Also too, by no means, ever use rhythmic redundancies. Contractions aren't essential and shouldn't be there used. Comparisons are as terrible as clichés. Give up ampersands and abbreviations, and so on. Remove commas, that are, not necessary. Parenthetical words however should be together with this in commas. Understatement is all the time the complete best way to put onward earth-shaking thoughts. Give a detailed literary review.

33. Report concluded results: Use concluded results. From raw data, filter the results and then conclude your studies based on measurements and observations taken. Significant figures and appropriate number of decimal places should be used. Parenthetical remarks are prohibitive. Proofread carefully at final stage. In the end give outline to your arguments. Spot out perspectives of further study of this subject. Justify your conclusion by at the bottom of them with sufficient justifications and examples.

34. After conclusion: Once you have concluded your research, the next most important step is to present your findings. Presentation is extremely important as it is the definite medium though which your research is going to be in print to the rest of the crowd. Care should be taken to categorize your thoughts well and present them in a logical and neat manner. A good quality research paper format is essential because it serves to highlight your research paper and bring to light all necessary aspects in your research.

Informal Guidelines of Research Paper Writing

Key points to remember:

- Submit all work in its final form.
- Write your paper in the form, which is presented in the guidelines using the template.
- Please note the criterion for grading the final paper by peer-reviewers.

Final Points:

A purpose of organizing a research paper is to let people to interpret your effort selectively. The journal requires the following sections, submitted in the order listed, each section to start on a new page.

The introduction will be compiled from reference matter and will reflect the design processes or outline of basis that direct you to make study. As you will carry out the process of study, the method and process section will be constructed as like that. The result segment will show related statistics in nearly sequential order and will direct the reviewers next to the similar intellectual paths throughout the data that you took to carry out your study. The discussion section will provide understanding of the data and projections as to the implication of the results. The use of good quality references all through the paper will give the effort trustworthiness by representing an alertness of prior workings.

Writing a research paper is not an easy job no matter how trouble-free the actual research or concept. Practice, excellent preparation, and controlled record keeping are the only means to make straightforward the progression.

General style:

Specific editorial column necessities for compliance of a manuscript will always take over from directions in these general guidelines.

To make a paper clear

· Adhere to recommended page limits

Mistakes to evade

- Insertion a title at the foot of a page with the subsequent text on the next page
- Separating a table/chart or figure - impound each figure/table to a single page
- Submitting a manuscript with pages out of sequence

In every sections of your document

· Use standard writing style including articles ("a", "the," etc.)

· Keep on paying attention on the research topic of the paper

· Use paragraphs to split each significant point (excluding for the abstract)

· Align the primary line of each section

· Present your points in sound order

· Use present tense to report well accepted

· Use past tense to describe specific results

· Shun familiar wording, don't address the reviewer directly, and don't use slang, slang language, or superlatives

· Shun use of extra pictures - include only those figures essential to presenting results

Title Page:

Choose a revealing title. It should be short. It should not have non-standard acronyms or abbreviations. It should not exceed two printed lines. It should include the name(s) and address (es) of all authors.



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Abstract:

The summary should be two hundred words or less. It should briefly and clearly explain the key findings reported in the manuscript--must have precise statistics. It should not have abnormal acronyms or abbreviations. It should be logical in itself. Shun citing references at this point.

An abstract is a brief distinct paragraph summary of finished work or work in development. In a minute or less a reviewer can be taught the foundation behind the study, common approach to the problem, relevant results, and significant conclusions or new questions.

Write your summary when your paper is completed because how can you write the summary of anything which is not yet written? Wealth of terminology is very essential in abstract. Yet, use comprehensive sentences and do not let go readability for briefness. You can maintain it succinct by phrasing sentences so that they provide more than lone rationale. The author can at this moment go straight to shortening the outcome. Sum up the study, with the subsequent elements in any summary. Try to maintain the initial two items to no more than one ruling each.

- Reason of the study - theory, overall issue, purpose
- Fundamental goal
- To the point depiction of the research
- Consequences, including definite statistics - if the consequences are quantitative in nature, account quantitative data; results of any numerical analysis should be reported
- Significant conclusions or questions that track from the research(es)

Approach:

- Single section, and succinct
- As a outline of job done, it is always written in past tense
- A conceptual should situate on its own, and not submit to any other part of the paper such as a form or table
- Center on shortening results - bound background information to a verdict or two, if completely necessary
- What you account in an conceptual must be regular with what you reported in the manuscript
- Exact spelling, clearness of sentences and phrases, and appropriate reporting of quantities (proper units, important statistics) are just as significant in an abstract as they are anywhere else

Introduction:

The **Introduction** should "introduce" the manuscript. The reviewer should be presented with sufficient background information to be capable to comprehend and calculate the purpose of your study without having to submit to other works. The basis for the study should be offered. Give most important references but shun difficult to make a comprehensive appraisal of the topic. In the introduction, describe the problem visibly. If the problem is not acknowledged in a logical, reasonable way, the reviewer will have no attention in your result. Speak in common terms about techniques used to explain the problem, if needed, but do not present any particulars about the protocols here. Following approach can create a valuable beginning:

- Explain the value (significance) of the study
- Shield the model - why did you employ this particular system or method? What is its compensation? You strength remark on its appropriateness from a abstract point of vision as well as point out sensible reasons for using it.
- Present a justification. Status your particular theory (es) or aim(s), and describe the logic that led you to choose them.
- Very for a short time explain the tentative propose and how it skilled the declared objectives.

Approach:

- Use past tense except for when referring to recognized facts. After all, the manuscript will be submitted after the entire job is done.
- Sort out your thoughts; manufacture one key point with every section. If you make the four points listed above, you will need a least of four paragraphs.

- Present surroundings information only as desirable in order hold up a situation. The reviewer does not desire to read the whole thing you know about a topic.
- Shape the theory/purpose specifically - do not take a broad view.
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This part is supposed to be the easiest to carve if you have good skills. A sound written Procedures segment allows a capable scientist to replacement your results. Present precise information about your supplies. The suppliers and clarity of reagents can be helpful bits of information. Present methods in sequential order but linked methodologies can be grouped as a segment. Be concise when relating the protocols. Attempt for the least amount of information that would permit another capable scientist to spare your outcome but be cautious that vital information is integrated. The use of subheadings is suggested and ought to be synchronized with the results section. When a technique is used that has been well described in another object, mention the specific item describing a way but draw the basic principle while stating the situation. The purpose is to text all particular resources and broad procedures, so that another person may use some or all of the methods in one more study or referee the scientific value of your work. It is not to be a step by step report of the whole thing you did, nor is a methods section a set of orders.

Materials:

- Explain materials individually only if the study is so complex that it saves liberty this way.
- Embrace particular materials, and any tools or provisions that are not frequently found in laboratories.
- Do not take in frequently found.
- If use of a definite type of tools.
- Materials may be reported in a part section or else they may be recognized along with your measures.

Methods:

- Report the method (not particulars of each process that engaged the same methodology)
- Describe the method entirely
- To be succinct, present methods under headings dedicated to specific dealings or groups of measures
- Simplify - details how procedures were completed not how they were exclusively performed on a particular day.
- If well known procedures were used, account the procedure by name, possibly with reference, and that's all.

Approach:

- It is embarrassed or not possible to use vigorous voice when documenting methods with no using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result when script up the methods most authors use third person passive voice.
- Use standard style in this and in every other part of the paper - avoid familiar lists, and use full sentences.

What to keep away from

- Resources and methods are not a set of information.
- Skip all descriptive information and surroundings - save it for the argument.
- Leave out information that is immaterial to a third party.

Results:

The principle of a results segment is to present and demonstrate your conclusion. Create this part a entirely objective details of the outcome, and save all understanding for the discussion.

The page length of this segment is set by the sum and types of data to be reported. Carry on to be to the point, by means of statistics and tables, if suitable, to present consequences most efficiently.You must obviously differentiate material that would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matter should not be submitted at all except requested by the instructor.



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Content

- Sum up your conclusion in text and demonstrate them, if suitable, with figures and tables.
- In manuscript, explain each of your consequences, point the reader to remarks that are most appropriate.
- Present a background, such as by describing the question that was addressed by creation an exacting study.
- Explain results of control experiments and comprise remarks that are not accessible in a prescribed figure or table, if appropriate.
- Examine your data, then prepare the analyzed (transformed) data in the form of a figure (graph), table, or in manuscript form.

What to stay away from

- Do not discuss or infer your outcome, report surroundings information, or try to explain anything.
- Not at all, take in raw data or intermediate calculations in a research manuscript.
- Do not present the similar data more than once.
- Manuscript should complement any figures or tables, not duplicate the identical information.
- Never confuse figures with tables - there is a difference.

Approach

- As forever, use past tense when you submit to your results, and put the whole thing in a reasonable order.
- Put figures and tables, appropriately numbered, in order at the end of the report
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Figures and tables

- If you put figures and tables at the end of the details, make certain that they are visibly distinguished from any attach appendix materials, such as raw facts
- Despite of position, each figure must be numbered one after the other and complete with subtitle
- In spite of position, each table must be titled, numbered one after the other and complete with heading
- All figure and table must be adequately complete that it could situate on its own, divide from text

Discussion:

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- Research papers are not acknowledged if the work is imperfect. Draw what conclusions you can based upon the results that you have, and take care of the study as a finished work
- You may propose future guidelines, such as how the experiment might be personalized to accomplish a new idea.
- Give details all of your remarks as much as possible, focus on mechanisms.
- Make a decision if the tentative design sufficiently addressed the theory, and whether or not it was correctly restricted.
- Try to present substitute explanations if sensible alternatives be present.
- One research will not counter an overall question, so maintain the large picture in mind, where do you go next? The best studies unlock new avenues of study. What questions remain?
- Recommendations for detailed papers will offer supplementary suggestions.

Approach:

- When you refer to information, differentiate data generated by your own studies from available information
- Submit to work done by specific persons (including you) in past tense.
- Submit to generally acknowledged facts and main beliefs in present tense.

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Topics	Grades		
	A-B	**C-D**	**E-F**
Abstract	Clear and concise with appropriate content, Correct format. 200 words or below	Unclear summary and no specific data, Incorrect form Above 200 words	No specific data with ambiguous information Above 250 words
Introduction	Containing all background details with clear goal and appropriate details, flow specification, no grammar and spelling mistake, well organized sentence and paragraph, reference cited	Unclear and confusing data, appropriate format, grammar and spelling errors with unorganized matter	Out of place depth and content, hazy format
Methods and Procedures	Clear and to the point with well arranged paragraph, precision and accuracy of facts and figures, well organized subheads	Difficult to comprehend with embarrassed text, too much explanation but completed	Incorrect and unorganized structure with hazy meaning
Result	Well organized, Clear and specific, Correct units with precision, correct data, well structuring of paragraph, no grammar and spelling mistake	Complete and embarrassed text, difficult to comprehend	Irregular format with wrong facts and figures
Discussion	Well organized, meaningful specification, sound conclusion, logical and concise explanation, highly structured paragraph reference cited	Wordy, unclear conclusion, spurious	Conclusion is not cited, unorganized, difficult to comprehend
References	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring

INDEX

B

Biosensor · 12
Bisphenol · 1, 2, 9, 10, 11
Borehole · 1, 4, 5

C

Chloramphenicol · 37, 41
Ciprofloxacin · 33, 34, 35, 36, 42, 43

E

Epidermidis · 33, 34, 35, 36, 37, 38, 39, 40, 41, 42

F

Fumigatus · 45, 47

G

Gentamycin · 35

H

Hamburgers · 21
Hologenome · 17, 18, 20, 24

M

Methicillin · 33, 38, 39, 41
Mizuyōkan · 19

N

Nontuberculous · 25, 26, 30, 31

P

Parallelism · 19
Phenotype · 19, 21

S

Staphylococci · 33, 34, 35, 36, 37, 38, 39, 40, 41
Symbionts · 18, 20, 21

T

Turbidity · 3, 5





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