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IN VITRO AND *IN VIVO* ANTIMICROBIAL ACTIVITY OF PARTIALLY PURIFIED ENTEROCIN PRODUCED BY *ENTEROCOCCUS FAECALIS* AND ITS APPLICATION IN WOUND HEALING

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ABSTRACT

Background: The recent global upsurge in antibiotic resistance among bacteria associated with wounds has contributed to high treatment failures in wound management. Enterocin are produced by enterococci and has been reported to inhibit the growth of many bacteria including those associated with wound infections.
Objectives: In this study, antibacterial and physico-chemical properties of partially purified enterocin (PPE) from *Enterococcus faecalis* was determined. Also, the possible application of the enterocin in wound management was evaluated.
Materials and Methods: Eight different enterocin were tested and that with highest antibacterial (E3) was partially purified using standard methods. The molecular weight of the PPE was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis after which the *in vitro* anti-*Staphylococcus aureus* potential of the PPE was determined.
Results: Enterocin (E3) was effective against *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Enterobacter cloaca*, *Listeria monocytogenes* and *Proteus vulgaris*. The activity of the E3 was very prominent at pH of 4 and 8. The molecular size of the isolated enterocin was 5.5 KDa. The photomicrograph of the skin tissue of the skin treated with partially purified enterocin for day 7showed epidermis covered by atrophic stratified squamous epithelium. A synergistic interaction was noticed when Eusol was used with the enterocin.
Conclusions: From this study, enterocin from *E. faecalis* has a low molecular weight and inhibited bacteria isolates from wound and also aids physiological healing of wound.The antibacterial potency of this bacteriocin indicates that it is an alternative therapeutic agent that can be employed in wound care and management.

Key Words: Enterocin, *Enterococcus faecalis*, wounds, bacteriocin, *Staphylococcus aureus*, skin.

L'ACTIVITE ANTIMICROBIENNE IN VITRO ET IN VIVO DE L'ENTEROCINE PARTIELLEMENT PURIFIEE PRODUITE PAR L'ENTEROCOCCUS FEACALIS ET SON APPLICATION DANS LA GUERISON DES PLAIES.

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RESUME

CONTEXTE: La récente recrudescence mondiale de la résistance aux antibiotiques parmi les bactériesassociées à des plaies est responsable pour l'échec de traitement élevé dans la gestion des plaies. Les Enterocin sont produits par les enterocoques et on a rapporté qu'elles inhibent la croissance de nombreuses bactéries, y compris celles associées à l'infection de plaies.

But: Dans cette étude, les propriétésantibactériennes et physico – chimiques de l'enterocin partiellement purifié (PPE) de *l'Enterococcusfaecalis*ont été déterminées. On a également évalue l'application possible de l'enterocin dans la gestion des plaies.

Matériels et Méthodes: Huit enterocindifférents ont été testés et l'enterocin plus élevéantibactérien (E3)a été purifié partiellement en utilisant les méthodes standards. Le poids moléculaire du PPE a été déterminé par électrophorèse sur gel de polyacrylamide de dodécylsulfate de sodium après que le potentiel *in vitro* anti – *Staphylococcus aureus* du PPE a été déterminé.

Résultats: Enterocin (E3) a été efficace contre *Staphylococcus aureus, Klebdiella pneumoniae, Enterobactercloaca, Listeria monocytogenes* et *Proteusvulgaris*. L'activitéde E3 était trèséminente à pH de 4 et 8. La taillemoléculaire d'enterocin isolé était 5,5 KDa. La photomicrographie du tissu cutané de la peau traitée avec de l'enterocin partiellement purifié pour lr jour 7 montre l'épiderme recouvert d'un épithéliumpavimenteux stratifié atrophique. Une interaction synergique a été observéelorsqu'Eusol a été utilisé avec l'enterocin.

Conclusion: De cette étude, l'enterocinde *E. faecalis* isolé de chien a un faible poids moléculaire et inhibe les isolats bactériens de plaies et aide également la guérison physiologique de la plaie. La forceantibactérienne de cette bactériocine indique qu'il s'agit d'un agent thérapeutique alternatif qu'on peut êtreemployé dans le traitement et la gestion des plaies.

Mot clés: Enterocin, *Enterococcusfaecalis*, plaies, bactériocine, *Staphylococcus aureus*, la peau.

INTRODUCTION

During the course of bacterial growth, wide range of antimicrobial metabolites such as organic acids, ethanol, diacetyl, hydrogen peroxide, antibiotics, bacteriocins and other compounds have been recognized. Bacteriocins are antimicrobial peptides or precursor peptides synthesized ribosomally and are produced by bacteria. They have inhibitoryeffect on both closely and distantly related bacteria but not on the producers (1,2).

Bacteriocins are proteins or complexed proteins biologically active with antimicrobial action against other bacteria, especially closely related species (3,4). Bacteriocins differ from most therapeutic antibiotics in being proteinaceous agents that are rapidly digested by proteases in the human digestive tract. This creates the possibility of improving their characteristics to enhance their activity and spectra of action (5). Bacteriocinhave been extensively employed in the food industry since they inhibit the growth of most bacteria that spoil and contaminate food (1,6,7).

Bacteriocins are generally low molecular weight proteins that attack the target cells by binding to cell surface receptors. Their mechanisms of action vary and include pore formation of the cell wall or cytoplasmic membrane, degradation of cellular DNA, disruption through specific cleavage of 16S rRNA, and inhibition of peptidoglycan synthesis (8-10). Bacteriocins produce localized holes in cell wall and cellular membrane with the leakage of macromolecules such as proteins into external medium and cause death of pathogenic organisms. At lower concentration,bacteriocin modifies the ion permeability of the cells, dissipating both components of proton motive force (11,12). Enterocins are enterococcalbacteriocins which belong to class II bacteriocins, they are non-modified antimicrobial peptides (13,14).

Wound is a breach formed in the normal continuum of the cellular and molecular structure of the body, thereby creating a disruption in the anatomic and as well as in their functional continuity. Wound healing or wound repair is an intricate process in which the skin, organ or tissue repairs itself after injury (15,16).Wound is a serious public health problem globally. The management of people with wounds is a major challenge, in addition to their profound effect on the quality of life of patients with wounds. An understanding of the wound healing process is needed to successfully manage wounds and the identification and prevention of factors that may delay or interrupt wound healing. Early wound treatment will reduce public health expenditure and prevent impairment of the quality of life of affected patients (17).

Impaired skin integrity leads to the development of a wound that could involve different tissues, from the epidermis to deeper layers such as muscles. After injury, the blood clots form a scab that protects the injured area (18). A wound is a tissue damage which could be caused by mechanical force or chemical substances and it could involve more than one tissue or organ.

The cause of injury could be accidental, for example penetrating plants, animal bites or deliberate, like surgical interventions and gun shots (19). The discontinuity of the skin at such sites gives opportunity to microorganisms and other foreign bodies to explore the tissues (20) or the internal organ, therefore prompt attention to wounds is vital for timely healing.

Bacterial contamination, colonization and infection of wounds by single or mixed populations have been reported to lead to treatment failure. Pressure of antibiotics on bacterial associated with wounds has also been reported (18,21). Enterocin has been reported to be effective against both Gram-negative and Gram-positive bacteria. Most wound healing regimen performs two major roles: prevention of invasion of microorganism and/or elimination of colonized microbes and also facilitation or regeneration of damaged tissues (22). The possibility of application of enterocin in wound management has not been reported, hence the aims of this study.

MATERIALS AND METHODS

Sources of *Enterococcus faecalis*

Eight strains of *E. faecalis* were collected from the stock culture of the Department of Microbiology, Ekiti State University, Ado-Ekiti. The isolates were sub-cultured on Bile aeculin (Oxoid) and incubated at 37 °C for 24 h. All the isolates produced black halo on the agar. Gram staining, motility and oxidase tests were conducted on the isolates.

Preparation of Cell Free Supernatant (CFS)

The strains were cultured in Mann Rogosa Sharpe (MRS) broth and incubated for 24 h at 37 °C. The broth was centrifuged at 10,000 *g* for 10 min at 4°C. To ensure sterility, the supernatant was further filter through 0.45-µm pore size filters (Carl Schleicher &Schüll). The pH of the CFS was adjusted to 6.2 . A 1 N NaOH and 130 U/mL of catalase (Sigma Chemical Co., St. Louis, MO, USA) was also added to eliminate the activity of organic acids and hydrogen peroxides respectively in the supernatants.

Source of Test Bacteria and Determination of Antibacterial Activity of CFS

Nine different test bacteria comprises of 3 Gram positive and six Gram negative were collected from the stock culture of the Department of Microbiology Ekiti State University, Ado-Ekiti, Nigeria. The isolates were sub-cultured on different selective media and incubated at 37 °C for 24 h. The test bacteria include the following: *Bacillus subtilis*, *Enterobacter cloaca*, *Escherichia coli*, *Klebsiella pneumonia*, *Listeria monocytogenes*, *Proteus vulgaris*, *Salmonella* Typhi, *Serratia marcescens* and *Staphylococcus aureus***.** Each of the test organisms was grown at 37 °C in Mueller-Hilton broth (Oxoid) for 18 h and adjusted to an optical density of 0.5 McFarland Standard. The standardized culture was seeded on the surface of sterile Mueller-Hilton Agar (Oxoid). Agar well-diffusion test was used to determine the activity of the CFS on the isolates. The plates were incubated at 37 °C at 24h after which the zone of inhibition was measured to the nearest milliliter as described by Davoodabi *et al.* (23).

Determination of Rate of Kill of *Staphylococcus aureus*

The rate of killing of *S. aureus* by the crude enterocin was determined using spectrophotometric method. The crude enterocin was incorporated into 10 mL Nutrient broth in test tubes. Negative control tube has no enterocin. A 100 µL of standardized inoculums of *S. aureus* was inoculatedinto 10 mL of both test and control tubes. The tubes were incubated at 37 °C on an orbital shaker at 120 rpm. A aliquot was removed from the culture medium at 0, 4 and 8 h for the determination of the optical density at 560nm.

Partial Purification of Enterocin by Ammonium Sulphate Precipitation

Different concentrations (60%,70%, 80% and 90%) of ammonium sulphate was added to 50 ml of CSFto different levels of saturation with constant stirring and the solution was kept overnight at 4°C. The protein precipitate was pelleted by centrifugation at 10,000 × *g* for 20 min and dissolved in 500 ml of 20 mM sodium phosphate buffer (pH 5.0). The supernatant was transferred to a clean sterile container.The fraction that showed the highest inhibitory activity of the indicator organisms (*S. aureus*) activity was used for further analysis.

Determination of Protein Content of the Enterocin Produced

Bradford method of protein quantification was used to assay for protein content of the enterocin that has the best inhibitory activity on the isolates. A 0.4 ml Bradford reagent was added to 1.6 ml of the enterocin to make up to 2 ml total volume. Optical density (OD) of the resulting solution was thereafter taken at 595 nm after 5 min. The optical density of each of the samples was calculated from the equation of the bestfit linear regression line obtained from the graph of the bovine serum albumin (BSA) standard curve.

Determination of Molecular Weight of the Partially Purified Enterocin

The molecular size of the partially purified enterocin harvested was analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Coomasie blue (Sigma) was used for staining. For the estimation of enterocin size, the molecular weight marker for SDS-PAGE with six distinctly separated bands and known molecular weights.

Evaluation of *In vitro* Activity of Partially Purified Enterocin

Soft MRS agar (0.7%, w/v) containing indicator organisms (from the stock culture of Department of Microbiology, Ekiti State University, Ado-Ekiti), was overlaid onto Mueller Hinton plates. Wells were made in the lawn of hardened soft agars. Aliquots (50 µl) of supernatant of overnight cultures (16 h) were poured in the wells. The plates were incubated overnight at 37 °C. A clear zone of inhibition around the well was taken for enterocin production.

Determination of Stability of Enterocin

The sensitivity of the of partially purified enterocin from *E. faecalis* to different pH was estimated by adjusting the pH of the cell free supernatant to pH 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 with 1M NaOH or 1M

HCl and testing against the indicator strains after 30 minutes and 2 hours of incubation. The sensitivity to heat was also tested by heating cell free supernatant samples to 30, 40, 50, 60, 70, 80, 90 and 100 ^{o}C and assay the residual activity after 5, 10, 15, 20, 30 and 40 min of incubation.

The stability of the enterocin to some chemicals was tested on cell-free supernatant treated for 2 hours with 0.1 mg/ml and 1ml final concentration of the following chemical substance sodium chloride, magnesium chloride, sodium dodecyl sulphate (SDS), ethylene diamine tetra acetic acid (EDTA) and urea, while the untreated enterocin was used as control.After the treatment the activity of the enterocin was determined on *Staphylococcus aureus* using micro dilution method of CLSI (24), 200µl of the treated enterocin was introduced into each well of the microtitre plate and one loopful of the test organism *Staphylococcus aureus* was dispensed into each of the wells, covered and incubated for 37 ^{o}C for 24 hours. After incubation, the plates were examined for growth. One activity unit (AU) of enterocin was defined as the reciprocal of the last serial dilution demonstrating inhibitory activity.

Animal Experiment

Animal Care and Management

The study was carried out on thirty healthy Wistar rats weighing between 150-200 g. They were obtained from the Animal house of the College of Medicine, Ekiti State University, Ado-Ekiti and acclimatized therein for a week. The rats were housed under standard laboratory conditions of natural light/dark cycle at room temperature and humidity; fed on standard rat pellets (Ladokun Feeds, Ibadan, Nigeria) and given water *ad libitum*. The rats were assigned randomly into 5 groups of 6 rats each and housed in individual compartment of plastic cages. All animals were handled in accordance with the Guidelines for animal research as detailed in the National Research Council's Guide for the Care and Use of Laboratory Animals (25).

Induction of Experimental Skin Lesion and Animal Treatment

All animals were selected on the basis of non-presence of any pre-existing skin lesion and grouped as shown in Table 1. For surgical proceedings, the animals were weighed and anesthetized by intramuscular administration of 10% ketamine hydrochloride (Rotex Medica®, 0.1 ml/kg body weight) and diazepam (0.1 ml/kg body weight). Each animal was then shaved on the right dorso-lateral aspect of the thoracic wall by drawing an imaginary line caudally from the lower margin of the ear. Antisepsis of the area was performed with 4% alcohol based iodine soaked in gauze. In the center of the shaved area, a surgical skin lesion of 2cm by 2cm area of skin was measured and excised by exposing the dorsal muscle fascia with the aid of a surgical scalpel. Care was taken to remove the *Panniculus carnosus*. For pain control, animals received aspirin (100mg/kg weight) diluted in distilled water, until euthanasia.

The wounds were then dressed with gauze soaked with the appropriate agent for Groups A, B, C, D and E and then secured with gauze taped circumferentially round the animals. The wound of group A animals was treated with distilled water, Group B was treated with eusol, *Staphylococcus aureus* was inoculated on the wounds in Group D rats and then PPE was used for the treatment 24 hours after confirmation of inoculation of *Staphylococcus aureus*, PPE and Eusol was used to treat the wounds in Group E rats while Group C rats was treated with PPE alone. Wound dressing was done every two days. For the Group D rats, pus, swelling and redness of the skin were observed due to the treatment with *Staphylococcus aureus*.

Measurement of Wound Area and Percentage of Wound Closure

For biometric analysis, images of the skin wounds on the day of surgery and before each wound dressing was captured with a Digital camera (14.1 megapixels), and positioned at a distance of 15 cm. The 'Image J' program was applied to digital images to calculate the diameter of the wound area. Percentage of wound closure was calculated using the following formula: [(Area of 1 Day – Area of X Days)/Area of 1Day] × 100%.

Histological Examination

Under ether anaesthesia, 3 animals from each group were sacrificed at days 7 and 14. Specimens of the wound area was removed, fixed in 10% Neutral buffered formalin, for slide preparation according to routine procedures. Slices of 5µm were stained with Hematoxylin-Eosinfor demonstration of general skin architecture. Photomicrography of the tissue was carried out by examination under Leica DM750 research microscope with a digital camera attached. Digital photomicrographs of the tissue sections were taken at various magnifications.

Statistical Analysis

The values were analyzed using Statistical Package for the Social Science (SPSS) version 14. The results were subjected to Analyses of variance test and the post hoc (multiple comparisons) test was done by

Dunnett's test. The significance level was fixed at p=0.05.

RESULTS

All the strains of *Enterococcus faecalis* screened grew on Bile Aesculin Agar. And the spectrum of activity of the cell free supernatants of the isolates showed varying results. Only enterocin produced by *Enterococcus faecalis* E3 (Enterocin E3) was effective against *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Enterobacter cloaca*, *Listeria monocytogenes*, and *Proteus vulgaris* as shown in Table 2. The enterocin had the highest zone of inhibition on *Staphylococcus aureus* (176.79 mm^2) followed by *Enterobacter cloaca* and *Proteus vulgaris* with areas of inhibition of 165.20mm^2 and 154.00 mm^2.

TABLE 1: THE GROUPING OF ALBINO RATS IN WOUND HEALING EXPERIMENT

Group	Description	Treatment	Bacterial Challenge
A	Control	Not Eusol nor PPE treated	None
B	Eusol	Eusol treated	None
C	PPE	Partially purified enterocin treated	None
D	PPE + Staph	Partially purified enterocin treated	*Staphylococcus aureus*
E	PPE and Eusol	Partially purified enterocin and Eusol treated	None

TABLE 2: AREA OF INHIBITION OF ENTEROCINS PRODUCED BY *ENTEROCOCCUS FAECALIS* STRAINS AGAINST SELECTED BACTERIA (MM^2)

	Enterocins							
Organisms	E1	E2	E3	E4	E5	E6	E7	E8
S. aureus	0.79	0.00	176.79	3.14	3.14	78.57	0.79	3.14
K. pneumoniae	0.79	0.00	113.14	3.14	38.50	3.14	12.57	3.14
E. cloaca	3.14	0.79	165.20	19.64	7.07	19.64	113.14	3.14
E. coli	7.07	12.57	3.14	0.79	19.64	452.58	0.79	0.79
L. monocytogenes	0.79	38.50	113.14	0.00	0.79	12.57	50.29	19.64
P. vulgaris	19.64	3.14	154.00	0.00	113.14	132.79	63.64	7.07
S. marcescens	7.07	0.00	19.64	0.00	0.79	3.14	3.14	0.79
Salmonella Typhi	3.14	7.07	3.14	7.07	38.50	0.00	28.29	0.00
B. subtilis	0.79	0.79	7.07	3.14	63.64	0.00	0.00	19.64

The properties of partially purified enterocin E3 is shown in Table 3. The enterocin activity of the purified enterocin was 625AU/ml. The protein concentration of the purified enterocin is 14.18mg/ml, the total activity of the purified enterocin is 31250 AU/ml, total protein of the purified enterocin is 709 mg/ml, specific activity of the purified enterocin is 44.07AU/mg, yield of the purified enterocin is 10.01 and the fold of the purified enterocin is 12.68.As shown in Table 4, the activity of enterocin was tested over a temperature range of 40 to 100 ^{o}C. At each temperature the enterocin E3 was exposed for a period of 30 through 180 minutes. At temperature of 40 to 80 ^{o}C the enterocin was still active at 180 minutes of exposure. The evidence of activity was not noticed when the enterocin was exposed for 60 mins at 90 ^{o}C. At 100 ^{o}C there was no activity of the enterocin on the test organism (*Staphylococcus aureus*). As shown in Table 7, the *in vitro* activity of enterocin E3 on the test organism (*Staphylococcus aureus*).

With increased time of exposure to enterocin, the turbidity of the culture of the test organism decreased showing increased lysis (activity of enterocin). After 5 hours of exposure of the test organism to enterocin, the turbidity of the broth decreased by 34.88%. The inhibition of the enterocin was lower than the

gentamicin. At the probability level of 0.05, there was significant difference between the activity of enterocin and the control (normal saline) so also was gentamicin and the control. There was no significant difference between the activity of enterocin and gentamicin.

TABLE 3: PROPERTIES OF PURIFIED ENTEROCIN FROM *E. FAECALIS*PRIMARILY ISOLATED FROM DOGS

Properties	Crude extract	Ammonium sulphate precipitation
Volume (ml)	2000	50
Enterocin Activity (AU/ml)	156	625
Protein Concentration (mg/ml)	44.88	14.18
Total Activity (AU)	312000	31250
Total Protein (mg)	89760	709
Specific Activity (AU/mg)	3.47	44.07
Yield (%)	100	10.01
Fold	1	12.68

TABLE 5: EFFECT OF CHLOROFORM ON THE ACTIVITY OF PARTIALLY PURIFIED ENTERIOCIN E3

Concentration (%v/v)	Time (h)				
	1	2	3	4	5
25	+	+	+	+	+
50	+	+	+	-	-
75	+	-	-	-	-
98	-	-	-	-	-

+ = growth, - = no growth

TABLE 6: EFFECT OF METAL ION ON THE ACTIVITY OF PARTIALLY PURIFIED ENTERIOCIN E3

Metal ions	Concentration of metal ions (mM)			
	10	15	20	25
Na^{+}	+	+	+	+
Al^{3+}	+	+	-	-
Cu^{2+}	+	-	-	-
Zn^{2+}	+	+	+	-
Mn^{2+}	+	+	-	-
Fe^{2+}	+	-	-	-
Co^{2+}	-	-	-	-
Pb^{2+}	-	-	-	-

+ = growth, - = no growth

The macroscopic observation of wound healing in the skin of the experimental rats in different treatment groups is shown in Plate 1. The reactions of the six animals whose wounds were infected by *S. aureus* were shown in Table 8. Except the animal D1 all other animals developed pus with D2 and D6 shown copious pus formation. All the animals except D5 and D6 showed sign of swelling around the wound while all the animals showed sign of redness around the wound except D6.

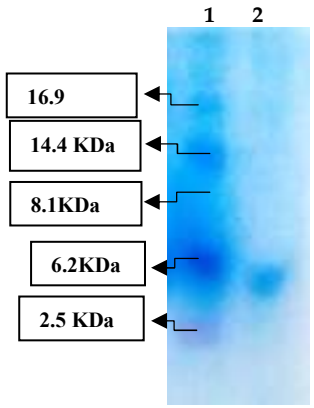


Figure 1: Coomassie blue-stained membrane enterocin E3 produced by *Enterococcus faecalis* E3 determined by SDS-PAGE gel electrophoresis. Lane 1, molecular weight markers; and lane 2, enteriocin (with molecular size of about 5 KDa).

As shown in Plate 2, the photomicrograph of the skin in group A for day 7 is showing tissue covered by stratified squamous epithelium. The sub-epithelial layer is made up of relatively normal looking skin adnexal structures.

TABLE 7: *IN VITRO* ACTIVITY OF PARTIALLY PURIFIED ENTEROCIN ON *S. AUREUS*

Time	Enterocin	Normal Saline	Gentamicin
0	0.49±0.02	0.46±0.04	0.50±0.04
1	0.32±0.06	0.49±0.05	0.31±0.06
2	0.25±0.01	0.58±0.12	0.21±0.02
3	0.21±0.09	0.81±0.22	0.18±0.05
4	0.30±0.04	1.03±0.42	0.20±0.02
5	0.34±0.06	1.93±0.13	0.03±0.01

The values obtained from the measurement of the wound area and the percentage of the wound closure is in Table 9.

A fairly thickened fibro collagenous tissue (day 14)as shown in the photomicrograph of the skin tissue was covered by an atrophic stratified squamous keratinizing epithelium. Some skin adnexal structures are seen but randomly arranged in a loose connective tissue. The papillary dermis is not properly delineated hence the boundary between the epidermis and the dermis is not well defined.

TABLE 8: REACTIONS OF WOUNDS INFECTED WITH *STAPHYLOCOCCUS AUREUS*

Animal	Reactions of the animals		
	Pus	Swollen	Redness
D1	-	+	+
D2	++	+	+
D3	+	+	+
D4	+	+	+
D5	+	-	+
D6	++	-	-

Key: - = no reaction, + = mild reaction, ++ = Severe reaction

The photomicrograph of the skin in group B for day 7 is displaying marked acanthosis, hyperkeratosis, para keratosis and papillomatosis in areas. The epidermis shows undue epithelial proliferation and projection with prominent granular layer. There are some skin adnexal structures seen. For day 14, the photomicrograph is displaying acanthosis, hyperkeratosis and papillomatosis in areas. The epidermis shows undue epithelial proliferation and projection. The granular layer is prominent. There are some skin adnexal structures seen.

TABLE 9: CLOSURE IN INDUCED WOUND IN EXPERIMENTAL RATS TREATED WITH ENTEROCIN

Treatment group	Wound diameter (cm)					
	Days					
	1	7	14	1	7	14
Group A	6.462±1.096	3.984±1.048	2.138±0.017	0	38.35	66.91
Group B	4.953±0.799	1.988±0.712	0.023±0.004	0	59.84	99.54
Group C	6.440±1.154	1.968±0.523	0.076±0.011	0	69.44	98.82
Group D	5.990±1.428	1.350±0.465	0.067±0.009	0	77.46	98.88
Group E	5.305±0.755	1.865±0.538	0.068±0.009	0	64.84	98.72

The photomicrograph of the skin tissue in group C on day 7 is showing the epidermis covered by atrophic stratified squamous epithelium. A few skin adnexal structures and blood vessels are seen. There is evidence of healing. On day 14, the photomicrograph of the skin tissue is showing healed epidermis. The epidermis and the dermis are well delineated and the skin adnexal structures are conspicuously seen with blood vessels of various calibres.

The photomicrograph of the skin tissue in group D on day 7 is displaying mild papillomatosis and hypergranulosis. The sub-epithelial layer shows paucity of skin adnexal structures and marked clearance of inflammatory activity. However, there is mild infiltration of the stroma by chronic inflammatory cells with numerous proliferation of blood vessels of different calibres. For day 14, the photomicrograph is showing skin tissue with restoration of the architecture and scanty infiltration of the tissue by lymphocytes.

The photomicrograph of the skin tissue in group E on day 7 is showing skin tissue with the epidermis displaying elongation of the rete peaks and moderate band-like infiltration of the papillary dermis. There is also severe loss of the skin adnexal structures. On day 14, the photomicrograph is showing moderate presence of chronic inflammatory cells in the sub-epithelial layers.

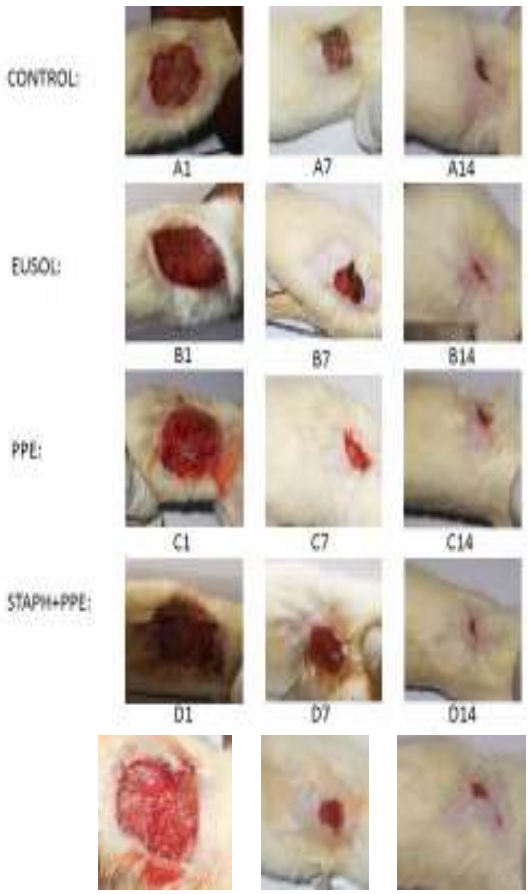
DISCUSSION

Enterocin-producing enterococci have been isolated from different sources and several strains of the species have been reported to produce this class of bacteriocins (26). Enterocins are heat stable and have low molecular weight (26). For this research the bacteriocin-producing enterococci used was isolated from rectal swab of dogs. It has been well established that some enterocins produced by enterococci have the ability to inhibit the growth of the some microorganisms including *Staphylococcus aureus* (27). This study confirms that enterocin E3 exhibited the inhibitory activity against some microorganisms including *Staphylococcus aureus*.

The purification of enterocin was done by using ammonium sulphate precipitation method since ammonium sulphate precipitation is the most commonly used method to purify proteins from culture broths (28). After the purification of the enterocin, the volume of the enterocin was reduced but the activity was increased; the yield of the purified enterocin was reduced but the fold was increased. This is similar to the report of Ohmomo *et al.* (29), David *et al.* (27).

Enterocins have been reported to be of low molecular weight (26). Majority of the enterocin that have been characterized so far, have molecular weight under 10 KDa (30). Enterocin E3 purified in this study also had this characteristic as it has a molecular weight of about 5 KDa. Jennes *et al*. (31) and Ohmomo *et al*. (29) reported enterocin with amolecular size of 3.4 KDa and 2.5 KDa respectively.From the result of the effect of temperature on the activity of the partially purified enterocin on Table 4, enterocin E3 is heat stable which complies with the report that enterocins are heat stable (26). The activity of enterocin E3 was very prominent at the pH range of 4-8. On exposure to lysozyme and lipase,its activity was not inhibited. This is similar to the report of Nemade and Musaddiq (10).

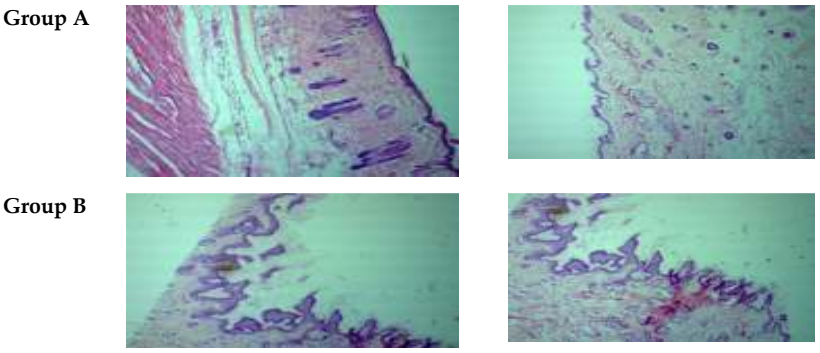
Histological observations showed that re-epithelialization tended to be greater in the PPE-treated wound than in the non-treated (control group). Many rete ridges were observed in the non-treated control group, but very few in the treated groups. Parkand Barbul (32) showed that histological analysis of a well-treated wound contained a large amount of fibroblast proliferation, collagen synthesis, and neovascularization, which resulted in an increased wound tensile strength and accelerated healing. Thus, PPE might be useful as an agent for wound dressing. Results obtained from our study agree with the work of Lancaster *et al.* (33) who reported that colistin was very effective in cancer management. They also suggested that probiotics reduced the period of inflammatory phase of wound healing as wound contraction and cell regeneration were compatible with each other.



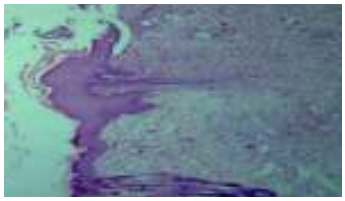
Also, in the non-treated group, the skin adnexal structures seen are randomly arranged in a loose connective tissue and the papillary dermis was not properly delineated, thus the boundary between the epidermis and the dermis were not well defined. These effects were not observed in the PPE treated group showing its efficiency in wound healing. In the Eusol treated group, on the 7th day of the experiment, marked acanthosis, hyperkeratosis, parakeratosis and papillomatosis was displayed in areas.The epidermis shows undue epithelial proliferation and projection with prominent granular layer. These features were still persistent on the 14th day of the experiment. The overall impression is consistent with pseudo-epitheliomatous hyperplasia. It is important to note that the histological features in the wound treated with eusol for 7 days are less prominent, when compared with the wound treated for 14 days. This implies that the longer the period of dressing with eusol the like hood of more undue proliferation of the epithelial cells in the epidermis.

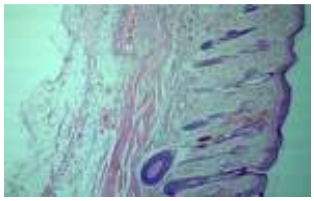
Plate 1: Macroscopic observations of wound healing in the skin of rats treated with Eusol, PPE, infected with *Staphylococcus aureus* and treated with PPE compared to control group at days 3 (A1, B1, C1, D1, E1), 7 (A7, B7, C7, D7,E2), 14 (A14, B14, C14, D14, E14) after excision.

Plate 2: Histological photomicrograph of the skin of the experimental animals. Group A=wounds without treatment, Group B=wounds with eusol, Group C= wounds treated with PPE, Group D= wounds colonized with *Staphylococcus aureus* and treated with PPE, Group E= wounds treated with eusol and PPE



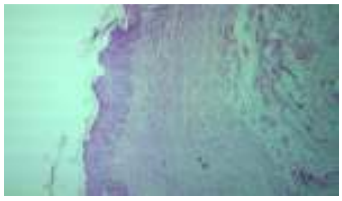
Group C





Group D

Group E



process and at day 14, there was a mild infiltration of the stroma by chronic inflammatory cells with numerous proliferation of blood vessels of different calibres. These histological features are suggestive of good therapeutic effect of PPE on the *Staphylococcus aureus*. This is in agreement with the report of Oscariz and Pisabarro (34) who reported that enterocins are active against Gram-positive food-borne pathogens such as *Staphylococcus aureus*.

In conclusion, the histological appearance seen in the PPE treated group may be suggestive of a good healing process and also a synergistic interaction was between eusol and partially purified enterocin.

Hence, the fewer the days eusol is used the better the outcome of the wound. Infections such as *Staphylococcus aureus* can dramatically slow the process of healing by prolonging the phases of wound healing.

In the *Stappylococcus aureus*-infected group, treated with PPE, the overall histologic changes on day 7 simulates granulation tissue which is a healing

Acknowledgments

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REVIEW ARTICLE

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A REVIEW OF THE VIRULENCE FACTORS OF PATHOGENIC FUNGI

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SUMMARY

Fungal infections are becoming more prevalent especially with increase in immunodeficiency disorders, immunosuppression following transplantation, cancers and cancer treatment. They are ubiquitous and cause infections which may be trivial or more deep seated and severe infections associated with mortality. The ability of some fungal species to cause disease is due to various virulence factors which help with fungal survival and persistence in the host resulting in tissue damage and disease. This review discusses these virulence factors. These factors include an ability to adhere to hosts' tissues, production of enzymes that cause tissue damage and direct interference with host defences. Pathogenic fungi produce catalases and Mannitol which protect against reactive oxygen species (ROS). Some fungi notably, dimorphic fungi and *C. albicans* have the ability to switch from one form to another. Thermotolerance, at least to 37^{0}C, is critical for survival in mammalian host and contributes to dissemination. Melanin is produced by a number of pathogenic fungi, and protects against harsh conditions such as UV radiation, increased temperature and ROS. The ability to obtain Iron (Fe) from the storage or transport forms in the host is also a virulence factor and calcineurin acts as a sensor for pathogenic fungi.

Key words: Fungi, virulence, pathogenic, infections, dimorphism, thermotolerance

UNE REVUE DES FACTEURS DE VIRULENCE DES CHAMPIGNONS PATHOGENES

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RESUME

Les infections fongiques sont de plus en plus fréquentes, en particulier avec l'augmentation des troubles de l'immunodéficience, l'immunosuppression après la transplantation, les cancers et le traitement du cancer. Ils sont ubiquitaires et provoquent des infections qui peuvent êtretriviales ou plus profondes et des infections graves associées à la mortalité. La capacité de certaines espèces fongiques à provoquer une maladie est due à divers facteurs de virulence qui aident à la survie des champignons et la persistance dans l'hôterésultant dans les dommages des tissus et la maladie. Cette revue traite ces facteurs de virulence. Ces facteurs comprennent une capacité à adhérer aux hôtes, la production d'enzymes qui causent des dommages des tissus et une interférence directe avec les défenses de l'hôte. Pathogènes produisent des catalases du Mannitol qui protègent contre les espècesréactives de l'oxygène. Certains champignons notamment les champignons dimorphes et C. albicans ont la capacité de passer d'une forme à l'autre. La thermo tolérance, au moins 370C, est essentielle pour la survie chez un hôtemammifère et contribue à la diffusion. La mélanine est produite par un certain nombre de champignons pathogènes, et protège contre les conditions difficiles telles que le rayonnement UV, la températureaugmentée et ROS. La capacité d'obtenir du Fe à partir des formes de stockage ou de transport dans l'hôte est également un facteur de virulence et la calcineurine agit en tant que capteur pour les champignons pathogènes.

Mots clés: Champignons, virulence, pathogène, infections, thermotolérance dimorphe.

1. INTRODUCTION

Fungal infections have become very prevalent with associated increase in mortality and morbidity. This is especially so for life-threatening invasive fungal infections as a result of increase in immunodeficiency disorders such acquired immune deficiency syndrome (AIDS), cancer and cancer treatment, and immunosuppressive therapy following transplantation. Fungi are ubiquitous, they cause infections when the spores are inhaled, e.g. *Aspergillus fumigatus*; by direct skin contact or implantation e.g. *Trichophytonrubrum*, or by commensals when there are changes in the host's normal flora or breech in mucosal barrier as seen with *Candida albicans*. However, for most immunocompetent

individuals, immune mechanisms are able to control and contain these fungal infections.

Fungi cause different disease types; the very common superficial infections, e.g. ring- worm or onychomycosis caused by the dermatophytes- *T.rubrum*, invasive or deep- seated severe infections such as meningitis or pneumonia caused by *C. immitis*or *C. neoformans*and allergic diseases in atopic hosts, e.g. allergic bronchopulmonary aspergillosis caused by *A. fumigatus*. While mucocutaneous infections may be seen in immunocompetent individuals, invasive opportunistic infections occur in the immunosuppressed.

The ability of some fungal species to cause disease is due to various virulence factors which help with fungal survival and persistence in the host resulting in tissue damage and disease. These factors include an ability to adhere to hosts' tissues, production of enzymes that cause tissue damage and direct interference with host defences. Some fungi, notably dimorphic fungi and *C. albicans* have the ability to switch from one form to another (1). Thermotolerance, at least to 37^0C, is critical for survival in mammalian host and contributes to dissemination (2).

2. PATHOGENIC FUNGI OF MEDICAL IMPORTANCE

Fungi are eukaryotes that propagate by the production of spores. Most fungi can reproduce both sexually and asexually, and are ubiquitous in the environment. There are three major phyla of fungi to which most of the human pathogenic fungi belong. These are the Ascomycota, Basidiomycota, and Zygomycota.

Ascomycota

The fungi that belong to this group are known as sac fungi (ascus), and are so named because they reproduce sexually by means of ascospores. Sexual reproduction involves the formation of new cells from the fusion of hyphae, this new cell divides to form the ascospores within the ascus. They also reproduce asexually by budding of their conidia which are asexual spores. Asexual reproduction occurs in favourable conditions. Examples of pathogenic fungi in this phyla are dermatophytes, (Microsporum, Trichophyton, and Epidermophyton) dimorphic fungi (*Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Candida spp*, *Paracocidioides brasiliensis*,and *Coccidioides immitis*) and septate filamentous fungi (*Aspergillus spp*).

Zygomycota

Zygomycetes reproduce sexually by the production of zygospores, and asexually by sporangiospores. They form broad aseptate hyphae with fast growing colonies. The fungi in this group are usually contaminants, but they are known to also cause invasive diseases. Examples include Mucorspp, Rhizopusoryzae, and Rhizomucor spp.

Basidiomycota

The fungi in this group are known as club fungi because they produce sexual spores with a club shaped structure. The sexual spores are known as basidiospores. They reproduce sexually and asexually. They are found in aquatic and terrestrial habitats, and also form ballistospores which are discharged forcefully into the air. Examples of pathogenic forms are *Cryptococcus spp*, *Malasseziaspp,* and Trichosporon.

3. VIRULENCE FACTORS OF PATHOGENIC FUNGI

There are thought to be about 1.5 million species of fungi on earth, but only about 600 are pathogenic to man, with about 30 commonly implicated in human disease. Fungal diseases are generally known as mycoses. The ability of fungi to cause disease and their virulence factors are borne out of strategies to overcome and survive in the harsh environment of the host. Primary pathogens cause disease in immunocompetent hosts; they are ubiquitous and on inhalation of their conidia in large doses, may convert to pathogenic forms causing disease. Examples are *C. immitis*, *H. capsulatum*, *B. dermatitidis*, and *P.* brasiliensis. Opportunistic pathogens may be commensals like C. albicans or saprophytes such as *A. fumigatus*and *C. neoformans*. They cause disease in immunocompromised persons. This distinction however, is not clear cut, as primary pathogens such as *C. immitis*may cause virulent disease in immunocompromised persons and *C. neoformans*may occasionally cause disease in immunocompetent persons.

Adhesins

Pathogenic fungi are able to cause disease by a number of virulence factors. These factors include structures that enable them to adhere to tissues so as to avoid being cleared or swept away by ciliary movement or mucous. *C. albicans*as an example is known to have a number of adhesion molecules. *C.albicans* is able to bind to medical devices forming a biofilm which enhances its pathogenicity (3). The adhesion molecules include Als proteins, Hwp1p, Eap1p, Cshlp and others (4). There are eight genes that code for Als proteins, these proteins mediate adhesion to collagen, laminin, endothelial cells, epithelial cells and cell-to-cell aggregation (5). Hwp1p mediates binding to epithelium while Int1p mediates adhesion to platelets (6). Abrogation of Als3 is the basis for the development of a vaccine to prevent invasive candidiasis (7).

Other examples of pathogenic fungi with adhesion molecules include *A. fumigatus*, *H. capsulatum*and *P. brasiliensis*. Conidia of *A.fumigatus* are covered with hydrophobic proteins known as rodlets. These rodlet proteins are encoded for by RODA and RODB genes and, mediate adhesion of the conidia to albumin and collagen. Receptors on the surface of hyphae include galactomannan and chitin of *A. fumigatus*which mediate adhesion to complement, fibrinogen, immunoglobulin, and surfactant A and D (8). Blastomyces adheres via BAD 1 which binds CR3 and CD14 on phagocytes and also modulates host immune responses (9). *H. capsulatum*uses HSP60 (10), while *P.*

*brasiliensis*uses glyceraldehydes 3-phosphate dehydrogenase (GAPDH) and polypeptides p19, p30, p32 (11), and Coccidioides uses its spherule outer wall (SOW) for adhesion (12).

Dimorphism and Thermotolerance

Another way pathogenic fungi become virulent is by morphogenesis. Most pathogenic fungi exhibit dimorphism, that is, they can switch from one form which is not pathogenic to a pathogenic form. While they exist as one morphotype in the environment or as commensals, they exist as another morphotype when they cause infection. Dimorphic fungi include *B. dermatitidis*, *C. immitis*, *H. capsulatum, P brasiliensis, and C. albicans*. They exist as yeast or moulds; yeasts are round or ovoid unicellular organisms. It reproduces by binary fission to yield a separate, independent daughter cell. Moulds on the other hand are filamentous, they grow by apical extension forming cellular units which are separated by septates but still attached to the mould. These branching cellular units are known as hyphae or mycelium. Some fungi may have other morphotypes, for example, *C. immitis*may form large endosporulatingspherules. Intermediate forms such as pseudohyphae may exist as is seen in *C. albicans*

Ability to grow at elevated temperature is another virulence factor. Fungi that cause systemic infections are able to grow at body temperature and even at febrile temperatures of 38-42^{0}C. *A. fumigatus*isis particularly thermophilic, and can grow at temperatures of up to 55-77^{0}C (13). HSP 70 is thought to be required by fungi to adapt to high temperatures (14). Pathogenic fungi also change from one form to the other at different temperatures, while most fungi exist as mould at ambient temperature; they become yeast at the mammalian temperature which is the pathogenic form. When the transition from mycelia to yeast is blocked in *H. capsulatum*, the organism continued to grow at 37^0 C but was avirulent (15). For *C. albicans*, both forms are pathogenic and, it changes its form in response to changes in the environment existing as the unicellular yeast at lower temperature and acidic pH, which is spread in the environment. The hyphal form is used for tissue invasion (4).

Capsules

Capsulated fungi are usually pathogenic. *C. neoformans*coats itself with capsule (glucoronoxymannan) with which it resists phagocytosis. The Polysaccharide capsules are usually prominent in isolates causing infections while environmental *C. neoformans*isare weakly encapsulated. Acapsular strains are not virulent as they are easily phagocytosed. The genes responsible for encapsulation are CAP 59 and CAP 64 (16). Capsules also deplete complement and cause a dysregulation of the cytokine network. The capsule also inhibits the mobilisation of leucocytes to the site of infection (17)

Production of enzymes

Pathogenic fungi release degradative enzymes which enable them to establish disease and disseminate, these enzymes cause tissue damage in the host and impair host immune defences. *C. albicans*secretes extracellular phospholipases, lipases and proteases. Pathogenic candida secrete much more phospholipase than commensal strains, and phospholipases A, B, C and D act by breaking the ester bonds. These enzymes are also important for nutrition and Fe acquisition (6,18). *C. albicans*also secretes SAP (secreted aspartyl proteinases) which hydrolyse extracellular matrix proteins, coagulation factors such as Hageman factor and factor X, host defence proteins e.g. mucin, IgA andlactoferrin and complements (19).

*A. fumigatus*secretes proteases (serine and aspartic protease, metalloprotease) and phospholipases which degrade elastin present in lung tissue. The serine proteases degrade collagen, fibrin and fibrinogen (2). *C. neoformans*also secretes proteases and phospholipases, lysophospholipase and lysophospholipase-transacylase (LPTA). These enzymes destroy lung surfactant and enhance adhesion (20,21). In addition, *C. neoformans*is thought to invade the CNS by the production of urease (22). Urease production is also utilised by *Coccidioides*, increasing alkalinity at sites of infection and urease deficient strains cannot disseminate (23).

Defence against reactive oxygen and nitrogen species

Neutrophils and macrophages use oxidative mechanisms (ROS and RNS) to damage fungi by lipid peroxidation and nucleic acid breaks. Pathogenic fungi produce enzymes with which they can be protected from the effects of oxidation. They produce catalases for protection against ROS (24). *C. albicans* uses superoxide dismutase and HSP to protect against ROS (25), while*C. Neoformans* uses the production of copper, zinc and peroxidase to resist oxidation (26). *A. fumigatus*produces three catalases; Cat- A associated with conidia, and Cat 1p and Cat 2p associated with hyphae (8) as well as superoxide dismutases (containing Mn, Cu and Zn) that protects it from oxidative damage (27).

Melanin

Melanin is produced by a number of pathogenic fungi, it is hydrophobic and protects against harsh conditions such as UV radiation and increased temperature. It also protects against ROS (27). In *C. neoformans*, melanin has been shown to evade anti-fungal damage and inhibit antibody mediated phagocytosis (28). Melanin is also synthesised by *A. fumigatus*from acetate using a 6 genes pathway (8). *H. capsulatum, Blastomyces, P.brasiliensis* are other pathogenic fungi which produce melanin (23).

TABLE 1: VIRULENCE FACTORS OF SOME PATHOGENIC FUNGI

Fungal pathogen	Virulence factors	Role in pathogenicity	Ref
Aspergillus spp	Galactomannan,chitin,Rodlets	Adhesion.	8
	Proteases and phospholipases	Degradation of elastin in lung tissue and tissue damage.	2
	Catalases and SOD	Protection from oxidative damage.	8,27
	Melanin.	Protection from harsh conditions and oxidative damage.	8
	Fe-siderophores (Sid A gene)	Fe uptake for growth.	29
	Gliotoxin	Immunosuppression.	2
	Calcineurin CNAA gene	Growth of fungi and tissue invasion.	34
	Ability to grow at 37-420C	Survival in host tissues, ability to cause systemic infection.	13
Candida spp	Als, Hwp1p, Eap1p, Cshlp	Adhesion	4
	Dimorphism	Survival in host, tissue invasion and dissemination of disease.	4
	Phospholipases A, B, C, and D	Tissue damage and disease dissemination.	6,8,19
	Lipases and proteases (SAP SOD, HSP, catalases)	Protection from oxidative damage	25
	Siderophores, RBT5, Reductases	Fe uptake for growth and metabolism.	31,3
Cryptococcus spp	Capsule (CAP 59 and 64)	Resists phagocytosis.	16,1
	Lipases, phospholipases, lysophospholipase and LPTA.	Destroys lung surfactant and enhances adhesion.	20,2
	Urease		22
	Cu, Zn, peroxidase and mannitol	Invasion of the CNS. Protection from oxidative damage.	27,3
	Melanin	Protection from harsh condition inhibits phagocytosis and anti-fungal damage.	28 4
		Evasion of immune defences.	4
	Phenotypic switching	Survival and tissue invasion	4
	Calcineurin (CNA1 gene)	Fe acquisition	
	Reductases, cft1, cfo1		

Iron acquisition

Fe is needed by the fungi for growth, respiration and other metabolic processes, but is not available in the free form in the host. The ability therefore, to obtain Fe from the storage or transport forms in the host is a virulence factor. *A. fumigatus* uses three mechanisms of Fe uptake; reductase Fe uptake, siderophore-mediated Fe uptake and ferrous Fe uptake mechanisms (29).

Triacetylfusannine C (TAFC) and desferriferricrocin (DFFC) are two major siderophores identified for *A. nidulans* (30). *C.albicans* acquires iron by different mechanisms which include the use of siderophores, and by direct uptake from heme in red blood cells using haemoglobin receptors (RBT5 family) on their cell surface (31). *C. albicans* also employs a reductive mechanism using the reductases -Cfl1/Fre and Cfl95/Fre 10/Rbt 2 (32).

Toxins

A. fumigatus secretes a number of toxins such as aflatoxin and gliotoxin. Aflatoxin does not have any bearing on virulence of *A. fumigatus*, it is hepatotoxic and carcinogenic. Gliotoxin is immunosuppressive and inhibits phagocytosis by macrophages and T-cell activation (2). It also slows ciliary movement thus making it difficult for the fungal cells to be swept away, and causes damage to the epithelium (33). Most other fungi produce a number of secondary metabolites that have numerous cellular actions, some of which are probably important in pathogenesis.

The role of Calcineurin and Mannitol

Calcineurin acts as a sensor for pathogenic fungi. It is said to influence the expression of several virulence factors. Calcineurin CNAA gene is important for the growth of *A. fumigatus*, and contributes to tissue

invasion (34). Mannitol is especially used by *C. neoformans* in CNS infections where it protects the fungi by preventing oxidative damage. It is produced in large quantities and may contribute to brain oedema (35).

4. CONCLUSION

Fungal diseases as described are important causes of morbidity and mortality worldwide, affecting mostly the immunosuppressed and the immunocompetent as well. The incidences and associated mortalities of invasive fungal diseases have increased as a result of advancement in treatments, and cancers. The ability of some fungal species to cause disease is due to various virulence factors which help with fungal survival and persistence in the host resulting in tissue damage and disease. A knowledge of these virulence factors is important as research continues towards the development of drugs and vaccines effective in the prevention and treatment of fungal diseases.

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EPIDEMIOLOGIC CHARACTERISTICS OF *KLEBSIELLA PNEUMONIAE* ISOLATES IN VENTILATOR-ASSOCIATED PNEUMONIA IN INTENSIVE CARE UNITS

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ABSTRACT
***Klebsiella pneumoniae* is a common pathogen that causes ventilator associated pneumonia (VAP) in intensive care units (ICUs). Strain typing is a useful tool in tracking the spread of these infections. Primary objective was to study different strains causing VAP in Anesthesia ICUs. Secondary objective was to determine role of health-care workers (HCWs) and ICU environment in the transmission of these strains. Endotracheal aspirates of 60 VAP patients, surveillance samples from the HCWs (18)and the ICU environment (193)were collected. Antibiogram typing and enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) were used for comparison of the isolates from VAP patients and surveillance samples. Antibiogram showed 5 antibiotic susceptibility patterns that were designated A1-A5. ERIC-PCR yielded 1 to 5 amplification bands. All the isolates were typable by ERIC-PCR. Eight ERIC patterns were obtained ERIC(I)-ERIC(VIII). ERIC-PCR typing method gave higher discriminatory index (D) (0.7557) than antibiogram (0.6035). There was sharing of certain ERIC patterns among patient and HCWs or environmental sources. In Conclusion: *K.pneumoniae* is the most dominant pathogen in anesthesia ICUs. Throats and hands of HCWs are possible sources of pathogen transmission to patients. Surfaces with hand contact of the medical staff are often contaminated and may serve as vectors for cross transmission.**

Key words: Ventilator-associated pneumonia, ICU environment, health-care workers, *Klebsiella pneumoniae*, antibiogram typing, ERIC-PCR

LES CHARACTERISTIQUES EPIDEMIOLOGIQUES DES ISOLATS DE *KLEBSIELLA PNEUMONIAE* DANS LA PNEUMONIE ASSOCIEE AU VENTILATEUR EN UNITES DE SOINS INTENSIFS.

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RESUME

***Klebsiella pneumoniae* est un pathogène commun qui cause la pneumonie associée au ventilateur (VAP) dans les unités de soins intensifs (ICUs). Le typage de souche est un outil utile pour suivre la propagation de ces infections. L'objectif principal était d'étudier les différentes souches qui causent le VAP en anesthésieICUs. L'objectif secondaire était de déterminer le rôle des professionnels de la santé (HCWs) l'environnement des soins intensifs dans la transmission de ces souches. Aspiration endotrachéale de 60 patients de VAP, des échantillons de surveillance des travailleurs de la santé de l'environnement des soins intensifs ont été recueillis. Le typage antibiogramme et le consensus inter génique répétitifentérobactérieréaction en chaîne par polymérase (ERIC – PCR) ont été utilisés pour la comparaison des isolats des patients VAP et des échantillons de surveillance. L'antibiogramme a montré 5 modèles de susceptibilité aux antibiotiques qui ont été désignés A1 – A5. ERIC – PCR a donné 1 à 5 bandes d'amplification. Tous les isolats ont été typable par cette méthode. Huitmodèles ERIC ont été obtenusERIC(I)-ERIC(VIII). Le typage méthode d'ERIC – PCR a donné un indice discriminatoire plus élevé (D) (0,7557) que l'antibiogramme (0,6035). Il y avait le partage de certains schémas ERIC chez les patients et les travailleurs de la santé ou des sources environnementales. En conclusion, *K.pneumoniae* est le pathogène le plus dominant en anesthésie des unités de soins intensifs. Les gorges et les mains des travailleurs de la santé sont des sources possibles de transmission de pathogènes aux patients. Les surfaces à contact manuel du personnel médical sont souvent contaminées et peuvent servir de vecteurs pour la transmission transversale.**

Mots clés : Pneumonie associée au ventilateur, l'environnement de soins intensifs, les travailleurs de la santé, *Klebsiella pneumoniae*, typage antibiogramme, ERIC – PCR.

INTRODUCTION

Ventilator-associated Pneumonia (VAP) is defined as pneumonia occurring more than 48 hours after the initiation of endotracheal intubation and mechanical ventilation (MV) **(1).** Several studies have shown that critically ill patients are at high risk for getting such infection and so it continues to be a major cause of morbidity, mortality and increased financial burden in ICUs **(2)**. Health-care workers (HCWs), contaminated equipment, and the ICU environment have been implicated in health-care associated outbreaks. *K. pneumoniae* is very well adapted to the hospital environment since it exhibits higher survivability on hands and environmental surfaces than other *Enterobacteriaceae* **(3)**. Cross-transmission can also occur from patient to patient via hands of the HCWs **(4).** Strain typing by traditional phenotypic methods may lack discriminatory power and stability. Molecular techniques offer a considerable improvement, and can complement phenotypic data to obtain a better understanding of bacterial diversity **(5)**.

Enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) is a simple, high throughput, affordable, reproducible, and discriminatory molecular typing method. Furthermore, it has excellent sub typing results and does not require much skill to perform **(6).**
Because of the tremendous diversity of bacterial genomic DNA, sequences of ERIC-PCR bands are often unique to the genome of the strain used for amplification. Therefore, these sequences have been used to design primers for discriminating closely related bacterial strains **(7).**

METHODS

Study design: This prospective study was conducted from December 2012 to February 2014 in Medical Microbiology and Immunology Department and Anesthesia Intensive Care Units (ICUs), Zagazig University Hospitals. There are 15 beds separated by curtains in each of the two anesthesia ICUs with adequate space for movement of staff and equipment.
Ethical consideration: Approval for performing the study was obtained from the Institutional Review Board (IRB) and Medical Microbiology and Immunology Department and Anesthesiology and Intensive Care Department, faculty of medicine, Zagazig University.
Study population: This study included 60 patients who were suspected clinically to have ventilator associated pneumonia **(8)**. Demographic and procedure-related information were collected.
Collection of samples

A) Patients' samples: According to the method described by **Karen and co-workers (9)**; EA samples were collected from the patients early in the morning in screw-capped, sterile, wide mouthed plastic containers.

B) Health-care workers' samples:

Throat samples: They were collected from health-care workers (HCWs) who were requested not to take any antibiotic or mouth-washes eight hours before swabbing **(10).**

Hand impressions: They were requested to press their fingers onto blood agar plates. Sampling was performed at midday, by which time staff members had been in contact with patients for several hours **(3).**

C) Environmental samples: According to the results of patients samples, environmental samples were taken throughout the ICUs, concentrating on surfaces and areas with maximum potential for hand contact and cross-infection. A total of 175 samples; 25 were taken from the following; ventilator tube, ventilator screen, humidifier fluid, suction apparatus, bed rail, over bed and medicine trolley. The lumen of the ventilator tube and the humidifier fluid container were swabbed by rubbing sterile cotton swab sticks, against the inner wall of both of them in a horizontal, then vertical, and then diagonal direction several times then the swabs were rolled to expose unused sides. On the other hand, surfaces of the ventilator screen, suction apparatus, bed rail, over bed and medicine trolley were also swabbed with sterile cotton swab sticks, pre-moistened with peptone water **(11).**
Regarding air sampling, samples were collected from air in the ICUs starting from June 2013 during collecting the patients' samples, by agar settle plates method, where blood agar plates were left open to the air according to the 1/1/1 scheme (for one hour, at a height of one meter at least one meter from walls) **(12)** and compared to other plates left open for 24 hours **(13).**

Transport of samples: All samples were transported to the laboratory within one hour of sampling process. The environmental swabs were inoculated within one hour in enriched brain heart infusion (BHI) broth and incubated for 24 hours at 37°C **(9)**.

Samples processing: Endotracheal aspirates were examined microscopically by Gram's stain and 10µL were streaked on MacConkey

medium in four-quadrants consecutively, then incubated at 37°C for 24 hours. Interpretation was as the following; growth was classified as rare (1+), light (2+), moderate (3+), or heavy (4+), based on the number of colonies in each quadrant, (3+) grade was considered diagnostic for VAP **(14).** Microbiological confirmation of suspected VAP cases was based on a positive Gram stain (≥25 pus cells/low power field and ≥1 bacteria/oil immersion field) **(15)** and semi-quantitative endotracheal aspirate (EA) cultures of moderate (3+) or heavy growth (4+), where (3+) is equivalent to quantitative culture showing $\geq 10^5$ colony forming unit (CFU)/ml **(1).**

Throat swabs of health-care workers' were streaked out on blood agar and MacConkey agar plates. Then, they were incubated aerobically at 37°C for 24 hours **(16).** Blood agar plates of hands impression were also incubated at 37°C for 24 hours **(3).**

Environmental swabs were streaked out on blood agar and MacConkey agar plates. Then, they were incubated aerobically at 37°C for 24 hours **(16).** Blood agar plates of air samples were also incubated aerobically at 37°C for 24 hours **(9).**

Identification of *K. pneumonia* isolates: The isolates were identified according to the results of colonial morphology, microscopic examination of Gram-stained films, and conventional biochemical reactions including: oxidase test, action on triple sugar iron medium, indole test, methyl red test, Voges-Proskauer test, citrate utilization test, urease test and motility test **(17).** API 20 E strips **(Bio-Mérieux, USA)** were used for confirmation of some suspected isolates which were positive for indole-production.

Maintenance of the selected isolates:
The selected isolates that fulfilled the criteria of being *K. pneumoniae* were inoculated on nutrient agar slopes. After an overnight incubation at 37°C, the slopes were kept at 4°C. Subculturing of the isolates was done every 2-3 weeks. Also, before starting any experiment, subculture was done twice to allow the cells to restore its viability.

The study was conducted on 60 patients admitted to the ICUs and diagnosed as having VAP. They were 34 males and 26 females and their ages ranged from 18 to 75 years old (X±SD: 49.05±14.8). Out of the 60 patients' endotracheal aspirates, 9 (15%) showed no growth on MacConkey agar plates while 51 (85%) were Gram negative, of them; 9 (17.6%) were only colonized as they showed rare (1+)

Antibiotic susceptibility testing: Antibiogram typing was performed by the Kirby–Bauer disc diffusion method **(18).**The diameters were interpreted as Resistant, Intermediate, and Susceptible according to CLSI published diameters **(19).**

ERIC-PCR typing: For comparison of the isolates from the surveillance samples and VAP patients, ERIC-PCR was used. DNA extraction was done using G-spin™ Total DNA Extraction Mini Kit **(iNtRON Biotechnology, Korea)**. The supernatant containing DNA in the tubes were stored at - 20°C until being used.

ERIC-PCR was performed using PCR Premix **(iNtRON Biotechnology, Korea)**. Two primers were used (**Biolegio, Netherlands)**; ERIC1 and ERIC2 were designed according to **Versalovic and co-workers (20)** as; ERIC1: 5' ATG TAA GCT CCT GGG GAT TCA C 3'; ERIC2: 5′ AAG TAA GTG ACT GGG GTG AGC G 3′. ERIC-PCR was performed in a final volume of 20 µL containing 2 µL of the template DNA, 1 µL of primer ERIC1R (10pmol/µL), 1 µL of primer ERIC2 (10pmol/µL), 16 µL distilled Water. Each reaction mixture was amplified with a heated lid thermal cycler **(Biometra, UK)**.
Reaction conditions were as follows: 94°C for 1 minute, followed by 35 cycles at 94°C for 30 seconds, 25°C for 30 seconds, 72°C for 1.5 minutes, and a final extension at 72°C for 10 minutes **(21).** The amplified PCR products in parallel with a DNA molecular size marker that gave 11bands ranging from 100-1500 base pairs **(iNtRON Biotechnology, Korea)** were detected by agarose gel electrophoresis as described by **Viljoen and co-workers (22).** The gel was carefully removed and was viewed and photographed over the UV transilluminator **(Biometra, UK)**.

STATISTICAL ANALYSIS

The data were coded, entered and checked using the Statistical Package for Social Science (SPSS) software system (Version 11.0; Chicago, IL). The numerical discriminatory index (D) which is a measure of the discriminatory ability of the typing methods was calculated according to Hunter **(23)**.

RESULTS
and light (2+) growth by semi-quantitative culture, while 42 (82.4%) patients were infected and showed moderate (3+) and heavy (4+) growth. The infection was polymicrobial in 22 (52.3%) patients and monomicrobial in 20 (47.7%). Total number of isolated Gram negative organisms was 64 isolates.
The study showed that the frequency of *K. pneumoniae* isolation among Gram negative

bacilli isolates from VAP patients was 25/64 (39%) and that of HCWs throat and hand samples was 3/18 (16.7%) and 2/18 (11.1%); respectively. Regarding environmental and air samples, frequency of isolation was 44/ 175(25.2%) and 2/18 (25%); respectively. Highest frequencies of *K. pneumoniae* isolation from environmental samples were from ventilator tube 11/25(44%), humidifier fluid 11/25(44%) and ventilator screen 8/25(32%).
Results of antibiotic susceptibility testing of *K. pneumoniae* isolates were shown in (**Table 1).**

There were 5 antibiotic susceptibility patterns that were designated A1-A5. All the five patterns showed multidrug resistance (MDR) as strains were resistant to 5 or 6 antibiotics. The most alarming patterns were A4 and A5 as strains belonging to A4 were only sensitive to amoxicillin/clavulinic acid, imipenem and colistin. Also, A5 was the only pattern that showed resistance to amoxicillin/clavulinic acid, imipenem and colistin among all other patterns, in addition to its resistance to tobramycin, cefotaxime, ceftazidime and ampicillin **(Table 2).**

TABLE (1): RESULTS OF ANTIBIOTIC SUSCEPTIBILITY TESTING

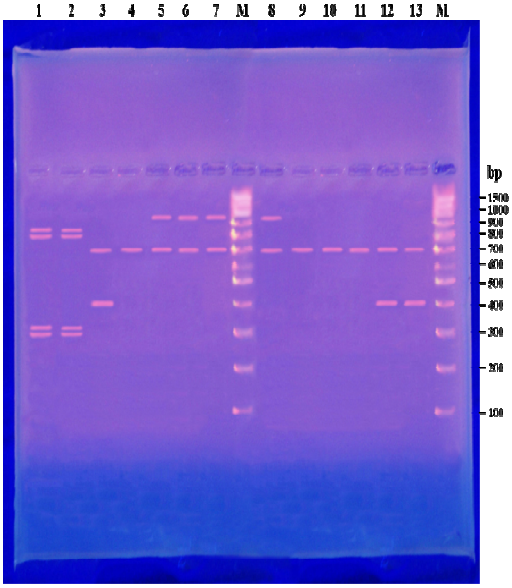
	Resistant *n* (%)	**Sensitive *n* (%)**
Amikacin	**22 (28.9)**	**54 (71.1)**
Gentamycin	**50 (65.8)**	**26 (34.2)**
Ampicillin	**76 (100)**	**0**
Piperacillin	**68 (89.5)**	**8 (10.5)**
Ceftazidime	**41 (53.9)**	**35 (46.1)**
Ceftriaxone	**76 (100)**	**0**
Amoxicillin/clavulinic acid	**8 (10.6)**	**68 (89.4)**
Cefotaxime	**76 (100)**	**0**
Ciprofloxacin	**39 (51.3)**	**37 (48.7)**
Tobramycin	**76 (100)**	**0**
Colistin	**8 (10.6)**	**68 (89.4)**
Imepinem	**8 (10.6)**	**68 (89.4)**
Total	**76 (100)**	

Figure 1 shows similarity between lane 1, for an over bed isolate and lane 2, for a ventilator tube isolate. Lanes 3, 12 and 13 for a patient isolate, an isolate from his ventilator tube, and an isolate from his humidifier fluid respectively show similarity. There is similarity among lanes 4, 9, 10 and 11 for a patient isolate, a ventilator screen isolate, hand of health-care worker isolate, and throat of health-care worker isolate respectively. Also, lanes 5, 6, 7 and 8 are for a patient isolate, an isolate from his ventilator tube, an isolate from his humidifier fluid and an isolate from his suction apparatus show similarity.
Figure 2 shows the eight ERIC-PCR patterns that were observed from the results. These patterns were designated ERIC(I)-ERIC(VIII). They yielded 1 to 5 amplification bands, where the size of amplified DNA bands ranged from 100 bp to 1000 bp.

TABLE (2): OBSERVED PATTERNS OF ANTIBIOTIC SUSCEPTIBILITY FOR ISOLATED *K. PNEUMONIAE* STRAINS.

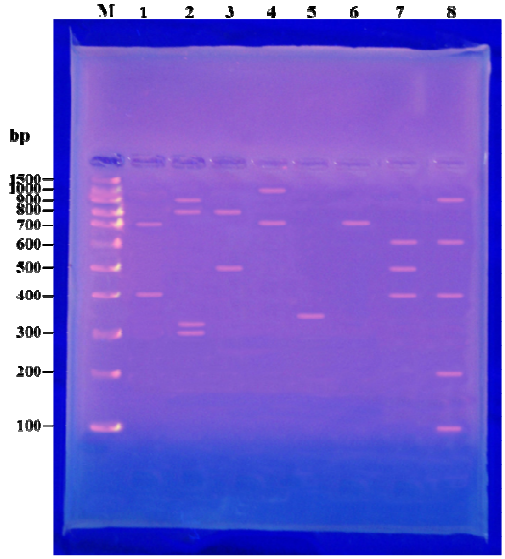
	A1	A2	A3	A4	A5
AK	S	S	S	**R**	S
CN	**R**	S	S	**R**	S
CIP	**R**	S	S	**R**	S
PRL	**R**	**R**	**R**	**R**	S
TOB	**R**	**R**	**R**	**R**	**R**
CTX	**R**	**R**	**R**	**R**	**R**
CAZ	S	**S**	**R**	**R**	**R**
AMP	**R**	**R**	**R**	**R**	**R**
IPM	**S**	S	**S**	**S**	**R**
CT	**S**	**S**	**S**	**S**	**R**
AMC	**S**	**S**	**S**	**S**	**R**
CRO	**R**	**R**	**R**	**R**	**R**
***n* (%)**	**18 (23.7)**	**18 (23.7)**	**10 (13.2)**	**23 (30.3)**	**7 (9.1)**

FIGURE 1: ETHIDIUM BROMIDE-STAINED AGAROSE GEL SHOWING RESULTS OF ERIC-PCR FOR ISOLATED *K.*



***PNEUMONIAE* STRAINS Lanes M: Molecular size marker which gave 11bands ranging from 100-1500bp. Lanes 1, 2: The size of amplified DNA bands is 300, 320, 800 and 900bp. Lanes 3, 12, 13: The size of amplified DNA bands is 400 and 700bp. Lanes 4, 9, 10, 11: The size of amplified DNA bands is 700bp. Lane 5, 6, 7, 8: The size of amplified DNA bands is 700 and 1000 bp.**

FIGURE 2: DIFFERENT OBSERVED ERIC-PCR PATTERNS FOR ISOLATED *K.PNEUMONIAE*



STRAINS Lanes M: Molecular size marker which gave 11bands ranging from 100-1500 bp. ; Lane 1: The size of amplified DNA bands is 400 and 700 bp. Lane 2: The size of amplified DNA bands is 300, 320, 800 and 900 bp. ; Lane 3:The size of amplified DNA bands is 500 and 800 bp. Lane 4: The size of amplified DNA bands is 700 and 1000 bp.; Lane 5: The size of amplified DNA bands is 340 bp. Lane 6:The size of amplified DNA bands is 700 bp.; Lane 7: The size of amplified DNA bands is 400, 500 and 600 bp. Lane 8: The size of amplified DNA bands is 100, 200, 400, 600 and 900 bp

ERIC-PCR typing method gave higher discriminatory index (D) (0.7557) than antibiogram (0.6035) (**Table 3**). By analyzing ERIC-PCR typing data, possible epidemiological linkages were proven **(Table 4)**.

TABLE (3): COMPARISON BETWEEN ANTIBIOGRAM AND ERIC-PCR

	No. of different patterns	No. of strains belonging to the most numerous pattern	Numerical discriminatory index
Antibiogram	5	23	0.6035
ERIC-PCR	8	18	0.7557

TABLE (4): EPIDEMIOLOGICAL ANALYSIS OF TYPING DATA

	Source	Antibiotic pattern	ERIC pattern
p1, p4, p5, p16	Patient	1	I
e1, e5, e23, e28	Ventilator tube	1	I
e2, e6, e7	Humidifier fluid	1	I
e22, e30	Ventilator screen	1	I
t3	Throat	1	I
a1	Air	1	I
e12	Ventilator tube	1	II
e18	Humidifier fluid	1	II
e42	Over bed	1	II
p2, p3, p8, p10, p11, p17, p18, p21	Patient	2	III
e3, e32	Ventilator tube	2	III
e4, e14	Bed rail	2	III
e13, e16	Ventilator screen	2	III
e15	Over bed	2	III
e17	Suction apparatus	2	III
e31	Humidifier fluid	2	III
e38	Medicine trolley	2	III
p6, p12, p15	Patient	3	IV
e20, e26	Ventilator tube	3	IV
e10, e21, e27	Humidifier fluid	3	IV
e8	Ventilator screen	3	IV
e9	Suction apparatus	3	IV
p7, p13, p22, p23	Patient	4	V
e11, e40	Bed rail	4	V
e24	Medicine trolley	4	V

	Source	Antibiotic pattern	ERIC pattern
e29, e35, e37	Humidifier fluid	4	V
e39	Suction apparatus	4	V
e41	Ventilator screen	4	V
p24, p25	Patient	4	VI
e43	Ventilator screen	4	VI
e44	Bed rail	4	VI
h1, h2	Hand	4	VI
a2	Air	4	VI
t1, t2	Throat	4	VI
e19	Over bed	4	VII
p9, p14, p19, p20	Patient	5	VIII
e25	Suction apparatus	5	VIII
e33	Ventilator screen	5	VIII
e34	Ventilator tube	5	VIII

KEY: p: patient endotracheal aspirate, e: environmental swab, t: throat swab of health-care worker, h: hand impression of health-care worker, a: air sample.

DISCUSSION

In spite of significant changes in the spectrum of organisms causing VAP, *K. pneumoniae* has held a nearly unchanged position as an important pathogen **(24).**

In the present study, we reported that the frequency of isolation of *K. pneumoniae* was the highest one; 25/64(39%). This is in accordance with that of a World Health Organization (WHO) cooperative study involving 55 hospitals in 14 countries where there was a predominance of Gram-negative pathogens causing VAP, *K. pneumoniae* was diagnosed in 40% of cases **(25).**

In addition, a relatively closer result was that of Set and co-workers **(26)** who isolated it from 33.3% of VAP patients from a tertiary care center in Mumbai. Also, in a Cairo University hospitals surveillance program by El-Kholy and co-workers **(27)** where it was 29.2% and by Krishnamurthy and co-workers **(15)** whose frequency was 24.78%.

Research into the frequency of contact of ICU patients with the medical staffs revealed that the medical staffs were in direct contact with patients 159 times per day and experienced indirect contact with patients 191 times per day **(28).**

In this study, we expected that one of the possible causes of transmission of infection with *K. pneumoniae* to the ICU patients was HCWs, as the organism was isolated from 3/18(16.7%) of their throat samples and 2/18(11.1%) of their hand samples. This might be due to inadequate application of standard precautions for infection control and hand hygiene measures.

Gupta co-workers **(29)** also found a dominant strain of *K. pneumoniae* on the hands of two medical staff in their investigations into the outbreak of *K. pneumoniae* in a neonatal intensive care unit (NICU).

In the present study the environmental sampling had shown that 44/175(25%) of the samples were positive for *K. pneumoniae* which is slightly higher than the result of Daef and co-workers **(30)** which was 16.4%. This figure reflected the fact that *K. pneumoniae* is ubiquitous in the hospital environment. These sites were ventilator tube 11/25(44%), humidifier fluid 11/25(44%), ventilator screen 8/25(32%), bed rail 5/25(20%), suction apparatus 4/25(16%), over bed 3/25(12%) and medicine trolley 2/25(8%).

In accordance with our results, Narciso and associates **(31)** isolated 2 strains from ventilator screen and suction device. *K. pneumoniae* was also isolated from 3.5% of suction apparatus samples and 5.6% of medicine trolley samples in NICU **(32)**.

Das and co-workers **(33)** pointed out that the presence of *K. pneumoniae* in air might be attributed to the bacterial aerosols generated due to coughing and sneezing. In the present study, no growth of *K. pneumoniae* obtained from agar plates after leaving them open for 1 hour, unlike obtaining 2 out of 8 *K. pneumoniae*

growth after leaving them open for 24 hours. This finding matched with that detected by Krishna and colleagues **(34)** who found that all air samples collected from NICU of Karnataka institute of Medical Sciences hospital in India were negative for *K. pneumoniae*, where the air sampling was done using settle plates exposed to the NICU air for only ½ an hour.

Calculating the numerical discriminatory (D) index for ERIC and antibiogram demonstrated that ERIC typing (0.7557) was more discriminatory than antibiogram (0.6035). This is in agreement with Freitas and Barth **(35)** who declared that the low discriminatory power of susceptibility tests was not surprising since the power of a method was determined by the number of types defined by it and the relative frequencies of these types.

In a study done by Mansour and colleagues **(36)**, ERIC typing gave a higher D index than antibiogram in Egypt and Saudi Arabia (0.801 and 0.785 respectively). By analyzing various typing data, we detected some possible epidemiological linkages; sharing of certain ERIC patterns among patient strains that may be explained by horizontal transmission from patient to another patient, probably from the hands of HCWs or environmental sources.

A direct link among two hand strains, two throat strains and two patients' strains, belonging to ERIC(VI) genotype was proven. In addition, a direct link among one throat strain and four patients' strains, belonging to ERIC(I) genotype was proven.

Ventilator tubes, humidifier fluid and ventilator screen had a central role in the spread of *K. pneumoniae* in the ICU. Epidemiological linkage was proven among patients and ventilator tubes by harboring strains belonging to ERIC(I), ERIC(III), ERIC(IV), ERIC(V) and ERIC(VIII) genotypes.

Regarding linkage among patients and humidifier fluid, both of them harbored strains belonging to ERIC(I), ERIC(III), ERIC(IV) and ERIC(V) genotypes. It may be explained by that fluid reservoir of the humidifier fluid may have been filled by non-sterilized water.

Epidemiological linkage was also proven among patients and suction apparatus by harboring strains belonging to ERIC(III), ERIC(IV), ERIC(V) and ERIC(VIII) genotypes. This might be explained by failure of sterilization of suction apparatus tubing and inadequate application of standard precautions for infection control.

Evacuation of suction apparatus fluid into drainage containers is a possible reason that could explain its linkage to bed rails and medicine trolley by harboring strains belonging to ERIC(III) and ERIC(V) genotypes, where any surface could has been contaminated by fluid spillage. Sharing of ERIC(I) and ERIC(VI) among ventilator screen, air, throats and hands of HCWs could be possibly explained by aerosols generated due to coughing or sneezing and hand contact where *K.pneumoniae* can survive on inanimate surfaces even for months.

The utilization of typing methods to draw possible epidemiological transmission linkage was done previously in other studies, for example, an outbreak caused by a multidrug-resistant *K. pneumoniae* (MRKP) strain occurred in a Tunisian neonatal ward, ERIC-PCR combined with other typing methods showed spread of at least two epidemic strains within the ward **(37)**.

An outbreak caused by a MRKP strain occurred also in the ICU of the St. Elisabeth Hospital in Tilburg, The Netherlands, using molecular typing, confirmed similarity of the isolates to those recovered from the roll boards **(38)**.

From the results obtained from this study, we recommend strict adherence to environmental infection control measures that is essential to prevent health-care-associated infections. Also, we recommend use of disposable suction tubes or properly disinfecting them every single suction for a patient. In addition, compliance of health-care workers to hand hygiene measures should be monitored. Use of face mask by health-care workers when manipulating patients or respiratory equipment to prevent droplet and bacterial particles transmission to patients must be applied. On the other hand, using (ERIC-PCR) typing method, which is proven to be superior to antibiotic typing in tracing source of infection, is recommended.

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REVIEW ARTICLE

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TRENDS IN PROFILES OF BACTERIA CAUSING NEONATAL SEPSIS IN CENTRAL NIGERIA HOSPITAL

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ABSTRACT

Developing countries suffer from a huge burden of neonatal sepsis. Neonatal mortality and long term sequelae or morbidity portends huge costs for the poor Nigerian economy. We identified trends in bacterial agents implicated in neonatal sepsis and their antibiotic susceptibility profiles at the National Hospital Abuja over two periods of three years each a decade apart. A retrospective study of bacterial agents of sepsis from 2013-2015 was carried out and this was compared to an already published study from the same hospital ten years earlier(2002-2004) to determine changing trends using standard statistical methods.

We identified a significant shift to predominance of gram positive organisms especially Staphylococcus aureus (59% vs 40%) as against the predominance of gram negative organisms especially *Klebsiella pneumoniae* (11% vs 44%) in the previous decade. Almost all antibiotics tested (92%) had reduced susceptibility in the later review compared to the former.

Surveillance of bacterial agents of neonatal sepsis is vital for the detection of trends in causative organisms and their susceptibilities. This is important to direct empiric therapy and also to encourage implementation and monitoring of antibiotic stewardship programs.

LES TENDANCES DES PROFILS DE BACTERIES CASANT LA SEPTICEMIE NEONATALE DANS L'HOPITAL AU CENTRE DU NIGERIA

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RESUME

Les pays en développement souffrent d'un énormefardeau de sepsisnéonatal. La mortaliténéonatale et les séquelles à long terme ou la morbidité annonce des coûts pour la pauvre économienigériane. Nous avons identifié les tendances des agents bactériens impliqués dans le sepsisnéonatal et leurs profils de sensibilité aux antibiotiques à l'hôpital national d'Abuja sur deux périodes de trois ans chaque décennie à part. Uneétuderétrospective des agents bactériens de sepsis à partir de 2013 – 2015 a été réalisée et ceci a été comparé à une étude déjà publiée du mêmehôpital dix ans plus tôt (2002 – 2004) pour déterminer les tendances changeantes en utilisant des méthodes statistiques standard.

Nous avons identifié un changement significatif vers la prédominance des organismes gram négative, en particulier le Staphylococcus aureus (59% vs 40%) au contraire de la prédominance des organismes gram – négatifs, en particulier la Klebsiella pneumoniae (11% vs 44%)au cours de la décennieprécédente.

La surveillance des agents bactériens du sepsisnéonatal est vitale pour la direction des tendances des organismes causatif set de leurs susceptibilités. Il est important de diriger la thérapie empirique et d'encourager la mise en œuvre et la surveillance des programmes d'intendance des antibiotiques.

INTRODUCTION

Neonatal sepsis causes significant morbidity and mortality worldwide especially in developing countries where the effects are often devastating for the baby, family and economy. It is estimated that sub-Saharan Africa had 2.6 million cases of serious bacterial infection in 2012, the highest for any WHO region with a case fatality of 14% (1). Mortality rates are high for neonatal sepsis and reports show that 17%- 41% of neonatal deaths in Nigeria are from sepsis (2-5).

Geographical differences in bacterial agents responsible for sepsis occur. Group B Streptococcus for example is not often reported in developing countries including Nigeria but is the most common cause of sepsis in the developed world (6). In Nigeria, the most common bacteria isolated from blood cultures of neonates include; *S. aureus, E.coli, K. pneumoniae, S. aureus* and *P. aeruginosa* (7-12).

Empiric therapy is usually based on international guidelines or local antibiograms that are rarely reviewed in Nigeria. Sometimes pressure from commercial pharmaceutical company representatives may influence the choice of empiric therapy. Because changes do occur over time in the types of bacteria causing sepsis and their susceptibilities to different antibiotics, it is of vital importance that to effectively manage neonatal sepsis, surveillance and tailored strategies to fit the local context are needed.

This study was aimed at assessing possible changes in the profile of organisms causing neonatal sepsis in NHA over a decade and also to detect changes in susceptibilities that have occurred over this time. The result would serve as a guide for modification of current empiric therapy choices and also provide a strong case for institution of efficient and effective infection control/antibiotic stewardship strategies.

METHODS

Neonatal blood cultures received at the medical microbiology laboratory of the National Hospital Abuja were reviewed for the periods 2002-2004 and 2013-2015. While in the 2002-2004 period the Oxoid signal system (Oxoid USA, Inc., Columbia, Md.) was used to process blood cultures, in the later period, the BACTEC system (Becton Dickinson, Ireland) was used. Otherwise, all protocols were the same for the two periods under review. The 2013-2015 data were compared with an already analysed and published data from 2002-2004 from the same laboratory. Paediatricians made the initial impression of sepsis based on clinical features in the neonate following which they collected 1-2mls of blood aseptically into culture bottles; Oxoid signal in 2002-2004 as previously described[9] and BACTEC Paeds-Plus in 2013-2015. Briefly, at the laboratory, culture bottles were incubated in aerobic conventional incubator for the oxoid signal system or in the BACTEC 9040 instrument following all manufacturer's instructions and incubated until growth was signaled or for a maximum of five days. Bottles that signal growth were removed and sub-cultured unto Blood agar, Chocolate agar and MacConkey agar. Isolates were identified according to standard methods and criteria. Isolates such as *K. pneumoniae, E. coli, S. aureus and P. aeruginosa* were regarded as pathogens while most coagulase negative Staphylococci and aerobic spore bearers were regarded as contaminants. Where there were doubts, the clinical features of the patients were factored into the decision making process as to whether an isolate was a pathogen or a contaminant. Antibiotic susceptibility test was performed using modified Kirby-Buer disc diffusion method and interpreted for all according to CLSI guidelines. *Escherichia coli* ATTC 25922, *Staphylococcus aureus* ATTC 25923, and *Pseudomonas aeruginosa* ATTC 27853 were used as controls.

RESULTS

A total of 1209 blood cultures were processed at the NHA medical microbiology laboratory during the 2013-2015 period. There were 260 blood cultures processed from neonates; 21.5% of the total. Bacteria were isolated in 85 of the 260 (32.7%) neonatal cultures while in the 2002 - 2004 report

, isolation rate was 22% (Table 1). Contamination rate in the present review was 1.5%. The study done 10 years ago did not evaluate for contamination.

Gram positive cocci accounted for 56 of 81 (69%) of isolates while it was 49.5% ten years ago. Gram negative bacteria were 31% and 51% in the two time periods respectively (Table 1).

TABLE 1: BACTERIAL ISOLATES IN NEONATAL SEPSIS

	2013-2015	2002-2004	p value
Total Blood cultures processed	1209	1555	
Neonatal blood cultures processed	290 (21.5%)	390 (25.0%)	0.6
Isolation rate	81 (32.7%)	85 (22%)	0.2
Contamination rate	4 (1.5%)	-	-
Bacterial Isolates during the two study periods			
S. aureus	48 (59.3)	34 (40.0)	0.01
K. pneumonia	9 (11.1)	37 (43.5)	0.001
P. aeruginosa	7 (8.6)	4 (4.7)	0.3
CoNS	5 (6.2)	2 (2.4)	0.3
E. coli	3 (3.7)	1 (1.2)	0.5
Enterobacter spp	3 (3.7)	-	-
Enterococcus spp	3 (3.7)	4 (4.7)	1.0
Alkaligenes spp	1 (1.2)	-	-
Salmonella spp	1 (1.2)	-	-
N. lactamica	1 (1.2)	-	-
Acinetobacter spp	-	1 (1.2)	-
S. pneumonia	-	2 (2.4)	-
Gram positive bacteria	56 (69.1)	42 (49.5)	0.01
Gram negative bacteria	25 (30.9)	43 (50.5)	0.3

Staphylococcus aureus accounted of 59% for isolates from newborn in this review but accounted for 40% in the prior review while Klebsiella pneumoniae constituted 11.1% as against the previous review where it accounted for 43.5% of neonatal isolates (table 1 below).

Decrease in susceptibility of *S.aureus* to various antibiotics was observed from 2002-2004 period to 2013-2015 period as follows; Amoxicillin-clavulanate from 85% to 76%, Cefuroxime from 45% to 0%, Ciprofloxacin from 71% to 67%; Erythromycin from 64% to 30%; Gentamicin from 40% to 29% and Ceftriaxone from 36% to 27%. See Figure 1.

For *K. pneumonia* isolates susceptibility decreased from 100% to 75% for Imipenem, from 15% to 0% for Ceftazidime, 100% to 63% for the fluoroquinolones for 2002-2004 and 2013-2015 respectively. An increase in susceptibility was observed for ceftriaxone from 12.5% to 66%.

FIGURE 1: SENSITIVITY PROFILE OF *STAPHYLOCOCCUS AUREUS* TO VARIOUS ANTIBIOTICS AT TWO TIME PERIODS

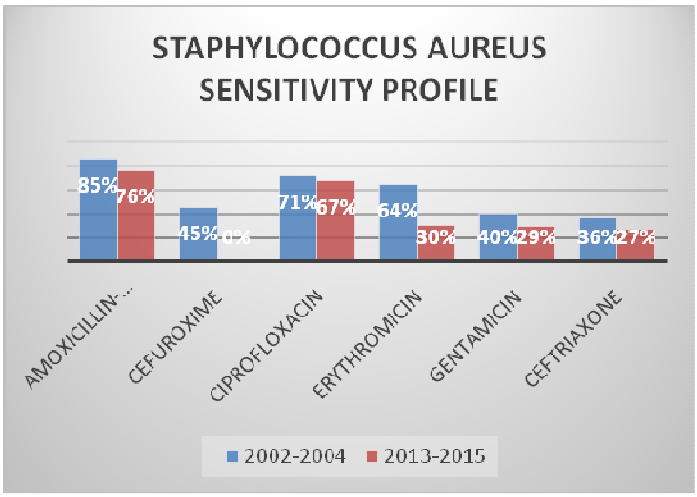
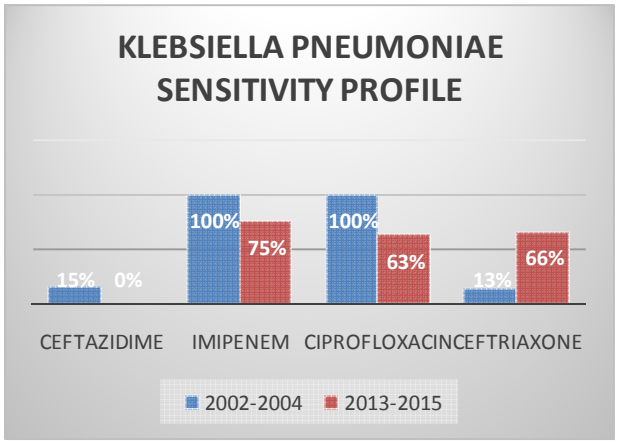


FIGURE 2: SENSITIVITY PROFILE OF KLEBSIELLA PNEUMONIAE TO VARIOUS ANTIBIOTICS AT TWO TIME PERIODS



DISCUSSION

The study showed that *S. aureus* and *K. pneumoniae* were the two dominant aetiologic agents of sepsis in the two periods evaluated. However, whereas the gram negative *K. pneumoniae* was the predominant agent in the 2002-2004 period, the gram positive, *S. aureus* was the predominant organism in the 2013-2015 period. Thus, over one decade the organisms causing neonatal sepsis had shifted from a predominance of gram negatives to gram positives. Previous studies have also established the predominance of gram positives in this decade (13-17). While bacteria are known to vary temporally, we postulate other reasons for this shift to more *S. aureus* in particular and consequently gram positive bacteria as a group; one postulate is that this may have resulted from a change in the type of bacteria colonizing the ano-genital region of the mother to mainly staphylococci and consequent newborn colonization with these organism; a future study assessing the maternal ano-genital colonization and newborn colonization with *S aureus* could answer this question. Another postulate is a changing antibiotic exposure of the population to groups active mainly against gram negative bacteria.

Similarly, the antibiotic susceptibility pattern changed remarkably. There was an alarming increase in resistance of major pathogens to most antibiotics commonly used in the management of neonatal sepsis. This will inadvertently lead to negative impact on the pattern of morbidity and mortality in the hospital, including resulting in longer hospital stay and increased cost of care. If the current trend continues, with the looming dryness of antibiotic pipeline, Nigeria will be faced with a situation similar to the pre-antibiotic era where even minor bacterial infections will be untreatable.

The NICU/SCBU at the National Hospital Abuja is a vital place to initiate antibiotic stewardship and infection control programs to mitigate this adverse antibiotic resistance trend. Intensive care units have been described as the factories where antibiotic resistance genes are created and spread to other parts of the hospital and community (18). This probably arises from the relatively large amounts of antibiotics consumed which in many instances are unnecessary or inappropriate. Regular antibiotic susceptibility surveillance is necessary to ensure evidence-based empiric treatment, while definitive treatment must be based on individualized testing. Notably, the antibiotics Cefuroxime and Gentamicin will be ineffective in the current decade as empiric choices for management of neonatal sepsis at National Hospital Abuja.

The isolation of coagulase negative staphylococci in pure culture warrants further investigation as to their role as agents of neonatal sepsis. They have hitherto been considered contaminants in developing countries. Perhaps there is a need to report susceptibilities for this group of isolates until it is proved that they are not a cause of sepsis in developing countries.

Conclusion

Continuous local surveillance of bacteria causing invasive disease in newborn units and their antibiotic susceptibilities is vital in ensuring improved outcomes for neonates. The implementation of standard infection control practices could also result in reduced incidence of neonatal sepsis at the National Hospital Abuja. Antibiotic stewardship as a vital part of infection control also needs implementation, and could result in decreased acceleration towards resistance or even reverse to susceptibility of particular drug classes.

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THE ETIOLOGY AND ANTIMICROBIAL SUSCEPTIBILITY PATTERNS OF URINARY TRACT INFECTIONS AT A PRIVATE NIGERIAN TEACHING HOSPITAL IN SOUTH WEST NIGERIA

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ABSTRACT

BACKGROUND: Urinary tract infections (UTI's) are among the commonest bacterial infectious disease in clinical practice with a wide range of etiologic agents. It frequently occurs in both the hospital and the community. AIMS/OBJECTIVES:: To determine the etiology of UTI at BUTH and obtain data on their susceptibility and resistance patterns. METHODS: This was a prospective analysis of data on patients with UTI obtained from in and outpatients over a six month period. Samples had been obtained by clean catch mid-stream urine or suprapubic aspiration. The organisms had been identified by biochemical methods with susceptibility and resistance testing performed. Data analysis was with EPI-INFO version 3.5.1
RESULTS: There were a total of 200 urine samples that had positive growth. Prevalent organisms were *Escherichia coli* (48%) and *Klebsiellaspp* (24%), followed by *Staphylococcus aureus* (10%) and Coagulase Negative *Staphylococci* (6.5%). The risk factors for UTI were female gender (p = 0.00), Diabetes mellitus (p = 0.03) and genitourinary surgery (p = 0.04). Effective antibiotics in-vitro to *Escherichia coli*were Nitrofurantoin and Cefepime at 84.8% and 92.3% respectively; while Cotrimoxazole performed poorly (32.5% susceptibility).
CONCLUSION: Urinary tract infections are an important cause of morbidity in our environment and inaccuracies in diagnosis will prolong morbidity and may lead to costly and unsafe treatments.The prevalent pathogens in our environment are the Gram negative bacilli, *Escherichia coli* and *Klebsiellapneumoneae*. Nitrofurantoin retains efficacy to both urinary pathogens.

KEY WORDS: Urinary Tract Infection, Catheterization, *Escherichia coli*, Risk factors, Nitrofurantoin

ETIOLOGIE ET LES MODELES DE SUSCEPTIBILITE ANTIMICROBIENNE DES INFECTIONS DES VOIESURINAIRES DANS UN HOPITAL PRIVE NIGERIAN D'ENSEIGNEMENT AU SUD OUEST DU NIGERIA

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RESUME
CONTEXTE : Les infections urinaires (UTI) sont parmi les maladies infectieuses bactériennes les plus courantes dans la pratique clinique avec une large gamme d'agents étiologiques. Il se produit fréquemment dans l'hôpital et la communauté. OBJECTIFS : Pour déterminer l'étiologie de UTI à BUTH et obtenir des données sur les modelés de leurs susceptibilités et résistances.
METHODES : Il s'agissait d'une analyse prospective des données sur les patients atteints de UTI obtenue des patients en hospitalisation et externes d'unpériode de six mois. Des échantillons ont été obtenus par prélèvement propre àl'urine moyenne ou par aspiration supra pubienne. Les organismes ont été identifiés par des méthodes biochimiques avec des tests de sensibilité et de résistance effectués. L'analyse des donnéesétait avec EPI – INFO version 3.5.1
RESULTATS : Il y avait un total de 200 échantillons d'urine qui avaient une croissance positive. Les organismes prédominantsétaient*Escherichia coli* (48%) et *Klebsiella spp* (24%), suivi de *Staphylococcus aureus* (10%) et Coagulase négative*Staphylococci* (6,5%). Les facteurs de risque de UTI étaient le sexe féminin (p=0,00), le diabète sucré (p= 0,03) et la

chirurgie génito – urinaire (p= 0,04). Des antibiotiques efficaces in vitro pour Escherichia coli étaient la Nitrofurantoïne et le cefépime à 84,8% et 92,3% respectivement ; tandis que le cotrimoxazole a présenté un mauvais rendement (susceptibilité de 32,5%).
CONCLUSION: Les infections des voies urinaires sont une cause importante de morbidité dans notre environnement et les inexactement dans le diagnostic plongera la morbidité et peut conduire à des traitements coûteux et dangereux. Les pathogènes prédominants dans notre environnement sont les bacilles Gram négatif, *Escherichiacoli* et *Klebsiellapneumoniae*. Le Nitrofurantoïne conserve son efficacité pour les deux pathogènes urinaires.

MOTS – CLÉS: L'infection des voies urinaires, Cathétérisme, *Escherichia coli*, Les facteurs de risque, Nitrofurantoïne.

INTRODUCTION

Urinary tract infections (UTI's) are among the commonest bacterial infectious disease in clinical practice with a high rate of morbidity. They are described as bacteriuria associated with or without urinary symptoms and are exceeded in frequency among ambulatory patients only by respiratory and gastrointestinal infections. It is the second most common infectious presentation in community practice and over 150 million people are diagnosed with UTI each year, with economic implications [1, 2, 3, 4].

The urinary tract is usually sterile, but bacteria may rise from the peri anal region, possibly leading to UTI in what is typically described as an ascending infection. Pathogens in the bladder may stay silent or can cause irritating symptoms like urinary frequency and urgency. UTI's are associated with sequelae such as renal scarring, pyelonephritis, renal and failure and as such need to be accurately diagnosed. In the light of antimicrobial resistance it is also vital that susceptibility results are reliable and accurate for the use of the clinician in the management of the patient.

Females bear the higher burden of UTI than males and are more likely to experience UTI than men. Nearly one in three women will have had at least an episode of UTI requiring antimicrobial therapy by the age of 24 years. In addition about fifty percent of all women will experience one UTI during their lifetime. Those at increased risk of UTI include infants, pregnant women, the elderly, patients with spinal cord injuries and/or those catheterized, patients with diabetes mellitus, patients who are immune-deficient, and patients with underlying urologic abnormalities[5].

Among women 18–30 years old, the incidence of acute uncomplicated urinary tract infections (UTIs) is estimated to exceed 0.5 episodes per annum. These infections are a major source of morbidity and health care costs in this population. Identified risk factors for such infections include sexual activity, spermicide-based contraception, and a history of previous UTIs[6].

The etiology of UTI is also affected byother underlying host factors that complicate UTI, such as age (the extremes of life), diabetes mellitus, spinal cord injury, or catheterization. Consequently, complicated UTI has a more diverse etiology than uncomplicated UTI, and organisms that often do not cause disease in healthy patients can cause significant disease in hosts with anatomic anomalies, metabolic derangements or immunologic compromise. The pathogens associated with UTI are changing the way they present in infections due to antimicrobial resistance [7].

There is the need to know the pattern of presentation of UTI's in our local practice as this would aid clinicians in the management of such patients. In addition few studies have been conducted in our local environment to understand the risk factors for the acquisition of UTI's as well as the picture as it pertains to resistance with first-line agents among patients with acute uncomplicated UTI.

The diagnosis may not always be straightforward as it may mimic other clinical conditions including an acute abdomen. Physicians therefore need accurate laboratory support in order to distinguish it from other diseases that have a similar clinical presentation as some UTIs are asymptomatic or present with atypical signs and symptoms.

AIM: To obtain data on etiology of UTI and their susceptibility patterns as well as the risk factors.

OBJECTIVES: To assess the in-vitro efficacy of antibiotics towards the pathogens responsible for UTI in BUTH and to determine the microbial pathogens causing UTI's at the Babcock University Teaching Hospital

MATERIALS AND METHODS

Study site/design/population: the study site was the Medical Microbiology Department of the Babcock University teaching Hospital, a 140 bed facility located in Ilisan South West Nigeria.; This is a retrospective study of data focusing on the frequency of uro-pathogens and their antibiotic susceptibility in different gender and age groups of patients and data from December 2015 – April 2016 were analyzed. The study population consisted of patients from ages One to Ninety Nine years, with suspected UTI being treated in the inpatient department or an outpatient clinic of a tertiary center in South West Nigeria. All

were referred because of urinary symptoms such as dysuria or unexplained acute febrile illness. Demographic data, epidemiological factors, and antibiotic susceptibility of pathogens were obtained from patient records.

Sample Collection: urine specimens had been previously obtained from adult patients via the clean-catch midstream technique.UTI was defined as the growth of a single pathogen of $>10^5$ colony forming units/ml by properly collected urine specimen (suprapubic aspiration, catheterization, or midstream specimen) in patients with urinary symptoms.

Sample Size: The average isolation rate of organisms from urine is approximately 15%, using the Kish formula this gives a sample size of 200 patients.

Antimicrobial Susceptibility Testing: This was done on Mueller- Hinton agar (Oxoid UK) using disk diffusion (Kirby Bauer's) technique. This method was done according to Clinical and Laboratory Standards Institute (CLSI) guidelines to determine susceptibility of UTIs agents [8].

Isolates with intermediate resistance were grouped with resistant isolates in the analysis. The antibiotic disks (Oxoid UK) comprised of ampicillin (10µg), ciprofloxacin (5µg), nitofurantoin (300µg), ceftriaxone (30µg), cefotaxime (10µg) and gentamicin (10µg), cefepimeand trimethoprim-sulfamethoxazole (25µg), cefepime (30µg), cefuroxime (30µg), cefpodoxime (30µg), ceftazidime (30µg), meropenem (30µg), ampicillin-sulbacatam (30µg), cefoxitin(30µg), co-amoxiclav (25µg).

Antimicrobial resistance for extended spectrum beta lactamases was determined by the double disk diffusion method where a Co-Amoxiclav disc was placed 20 mm centre to centre in between a Ceftazidime and Ceftriaxone disc. A dumb bell appearance or distortion on the side facing the Co-Amoxiclav was a phenotypic indicator of ESBL expression. MRSA – Methicillin Resistant *Staphylococcus aureus* were detected by using a Cefoxitin30µg disc as a surrogate marker, a zone size less than 20 mm was indicative of Methicillin resistance.

Data Analysis: EPI INFO version 3.5.1 was used to detect significant differences between the age groups and the prescribed treatment, the bacteriological culture results and the antimicrobial susceptibility of the isolates. A P value of <0.05 was considered statistically significant.Statistical analysis was performed with Pearson's χ^2 test as well as Fisher's exact test and odds ratio for categorical variables.Informed Consent: consent is implied to have been given during the course of specimen analysis. Ethical Issues: ethical approval was sought and obtained from the Babcock University Human Research Ethics Committee – BUHREC.

RESULTS

In all, there were 200 patients records recruited into the study. The mean age of the participants in the study was 38.2 years (Standard deviation 20.86) with the patients ranging from ages 1 – 99. The male to female ratio was 0.27:1. In the study 20% (n = 32) of the 158 females were pregnant. Also 17% (n=33) were catheterized, 35% of patients were hospitalized (n=70) (Table 1).

TABLE 1: SUMMARY STATISTICS

Variable		Frequency (N)	Percentage (%)
Gender	**Male**	**42**	**21**
	Female	**158**	**79**
Pregnant	**Yes**	**32**	**20**
	No	**126**	**80**
Pediatric	**Yes**	**21**	**10.5**
	No	**179**	**89.5**
Elderly	**Yes**	**33**	**16.5**
	No	**167**	**83.5**
Catheterized	**Yes**	**34**	**17**
	No	**166**	**84**
Admission Status	**Inpatient**	**70**	**35**
	Outpatient	**130**	**65**
Asymptomatic Bacteriuria	**Present**	**32**	**16**
	Absent	**168**	**84**

Variable		Frequency (N)	Percentage (%)
Post-operative patients	**Yes**	**10**	**5**
	No	**190**	**95**
Benign Prostatic Hypertrophy	**Yes**	**10**	**5**
	No	**190**	**95**
	No	**198**	**99**
ESBL production	**Positive**	**8**	**4**
	Negative	**192**	**96**
MRSA production	**Positive**	**4**	**2**
	Negative	**196**	**98**
Type of UTI	**Complicated**	**98**	**49**
	Uncomplicated	**102**	**51**
Pyelonephritis	**Present**	**6**	**3**
	Absent	**194**	**97**
Diabetes Mellitus	**Positive**	**7**	**3.5**
	Negative	**193**	**96.5**
Recurrent UTI	**Yes**	**5**	**2.5**
	No	**195**	**97.5**

ESBL = Extended Spectrum Beta Lactamases, UTI – Urinary Tract Infection Mean age = 38.32 years, Range 1 - 99 years, S.D = 20.86

Asymptomatic bacteriuria was seen in 16% (n=32). Post-operative patients accounted for 5% of patients (n=10), the same percentage of patients had Benign Prostatic hypertrophy with 2 male patients presenting with testicular swellings. About 4% of patients (n=8) had an ESBL elaborating organism and 2% (n =4) harbored an MRSA strain in their urine. In addition 49% (n=98) had a complicated UTI, while Pyelonephritis was recorded in 6% of patients (n=3). Diabetes mellitus was seen in 7 patients while 5 had a history of recurrent UTI, only one patient among the females presented with threatened abortion.

The predominant pathogen in the urine of our patients was *Escherichia coli*accounting for 48% (n=96) cases (Figure 1). This was followed by *Klebsiellaspp* with 24% (n=48) and *Staphylococcus aureus* 10.5% (n=21). Others isolated were *Staphylococcus epidermidis* 6.5%, (n=13), *Proteus spp* 4.5% (n=9), *Pseudomonas spp* 3.5% (n=7), *Staphylococcus saprophyticus* 2% (n=4) and *Salmonella spp* 1% (n=2).

Three risk factors were identified and these were: gender as females were 0.4 times more likely to have an organism isolated from their urine in our center [O.R=0.4, 95% CI=0.2-0.8, χ^2 = 6.64, p=0.00], diabetic patients who were 6.6 times more likely to have a UTI [O.R=6.6, 95% CI=0.8-55.7, χ^2 = 3.9, p=0.03] and Postoperative patients [O.R=4..44, 95% CI= 1.0-21.5, χ^2 = 4.05, p=0.04] (Table 2).

FIGURE 1: ORGANISMS ISOLATED FROM URINE

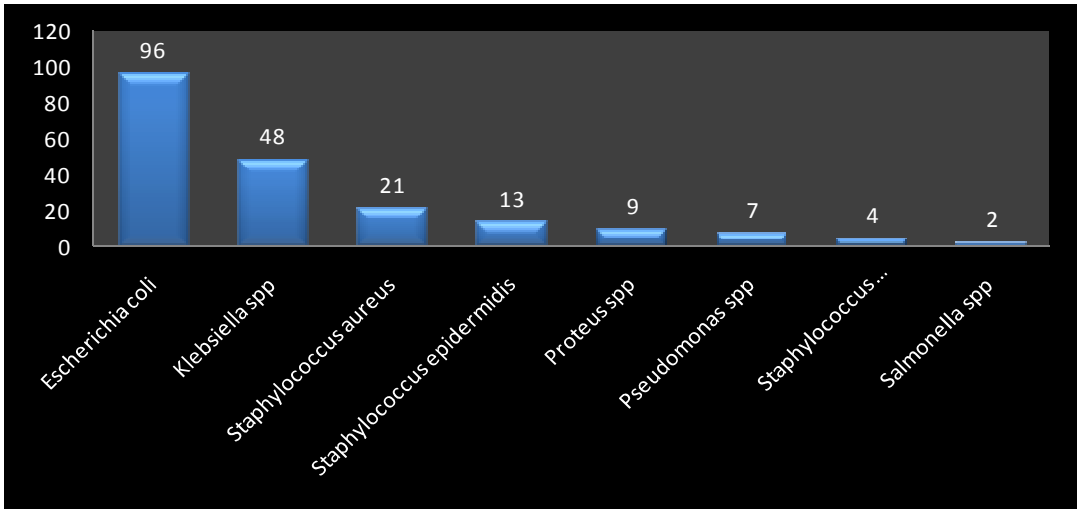

TABLE 2 RISK FACTORS FOR ACQUISITION OF *ESCHERICHIA COLI* UTI

Variable		*Escherichia coli*		Odds ratio	Confidence Interval	Chi Square	P value
		Positive	Negative				
Adults	Yes	68	77	0.74	0.4 – 1.4	0.9	0.33
	No	30	25				
Asymptomatic Bacteriuria	Positive	17	15	1.22	0.6 – 2.6	0.26	0.61
	Negative	81	87				
Gender	Female	70	88	0.4	0.2-0.8	6.64	0.00
	Male	28	14				
Type of UTI	Complicated	53	45	1.5	0.9-2.6	1.99	0.16
	Uncomplicated	45	57				
Diabetes Mellitus	Present	6	1	6.6	0.8 – 55.7	3.9	0.03*
	Absent	92	101				
Elderly	Yes	21	12	2.05	0.95 – 4.43	3.39	0.06
	No	77	99				
Admission status	In-patient	38	32	1.39	0.77 – 2.48	1.20	0.27
	Outpatient	60	70				
Postoperative	Yes	8	2	4.44	1.0 – 21.5	4.05	0.04*
	No	90	100				
Pregnant	Yes	11	21	0.49	0.22 – 1.07	3.26	0.07
	No	87	81				

* Fishers exact test

The rates of susceptibility by Escherichia was seen with Nitrofurantoin and Cefepime 84.8% and 92.3% highest respectively followed by Genticin, Ceftriaxone, Cefpdoxime and Co-Amoxiclav, 71.8%, 70,8%, 70.6% and 68.4% respectively. Ampicillin/Sulbactam and Cotrimoxazole displayed the lowest rates of susceptibility – 23.9% and 20.7% each (Table 3).

TABLE 3 ANTIMICROBIAL SUSCEPTIBILITY PATTERN OF *ESCHERICHIA COLI*

Antibiotic	Susceptible (%)	Resistant (%)	P value
Augmentin	**68.4**	**31.6**	**0.00**
Cefepime	**92.3**	**7.7**	**0.16**
Cefpodoxime	**70.6**	**29.4**	**0.79**
Ceftazidime	**69.5**	**30.5**	**0.78**
Ceftriaxone	**70.8**	**29.2**	**0.83**
Cefuroxime	**57**	**43**	**0.82**
Ciprofloxacin	**39.5**	**60.5**	**0.00**
Cotrimoxazole	**20.7**	**79.3**	**0.94**
Genticin	**71.8**	**28.2**	**0.40**
Nitrofurantoin	**84.8**	**15.2**	**0.00**
Ampicillin/Sulbactam	**23.9**	**76.1**	**0.23**
Piperacilin/Tazobactam	**52.0**	**48.0**	**0.20**

Linear regression analysis showed that with decreasing age there was a higher likelihood of isolating the following organisms *Klebsiellaspp. p*=0.01, *Proteus spp. p*=0.03, *Staphylococcus aureusp*=0.00 and *Staphylococcus saprophyticus.*Whereas with increasing age the following was likely to be isolated, *Pseudomonas sppp*=0.00 and *Escherichia colip*=0.04 (Table 4).

TABLE 4: LINEAR REGRESSION ANALYSIS OF AGE VERSUS ORGANISMS

Organism	Coefficient	Std Error	F-test	P-Value
Klebsiellaspp	**-8.53**	**3.43**	**6.20**	**0.01**
Proteus spp	**-15.05**	**6.76**	**4.96**	**0.03**
Pseudomonas spp	**21.85**	**7.59**	**8.29**	**0.00**
Salmonella spp	**-19.22**	**13.85**	**1.93**	**0.17**
Staphylococcus aureus	**-15.43**	**4.67**	**10.92**	**0.00**
Staphylococcus epidermidis	**-8.72**	**5.94**	**2.16**	**0.14**
Staphylococcus saprophyticus	**-16.47**	**9.89**	**2.77**	**0.01**
Escherichia coli	**8.40**	**2.89**	**8.40**	**0.04**

DISCUSSION

Urinary tract infections are prevalent in clinical practice both in in-patients and out patients. In our facility urine samples for microscopy culture and susceptibility are among the most frequently requested tests. Most physicians will attend to cases of UTI often in their routine health care practice. They are also one of the most frequent clinical bacterial infections in women, accounting for nearly 25% of all infections. Around 50–60% of women will experience an episode in their lifetime [9].

We report a rate of asymptomatic bacteriuria in 10% of our patients similar to those of some studies that reported 8% of patients having asymptomatic bacteriuria.We also report a higher frequency of UTI's in females,which is the norm in literature due to the anatomic differences of male and female genito-urinary tracts. Prior studies showed that one in three females will have at least one symptomatic UTI necessitating antibiotic treatment by the age of twenty four [10]. One of the possible reasons for repeated episodes in females could be due to errors in processing urine samples, thereby reporting commensals as pathogens. In addition urine samples may not be collected properly as a result of patients

not being educated on specimen collection by the physician. The young and sexually active tend to come down with it often, but it is also seen in elderly, postmenopausal women. The likelihood of recurrence is also high in patients who have had a previous episode of a UTI[11].

We found the following to be significant risk factors for UTI – female gender, the presence of diabetes mellitus and post-operative states. Clinicians in concert with the laboratory therefore need to have a heightened sense of awareness when dealing with urine samples from these patients.

The pathogens causing UTI are consistent across the globe. We report a preponderance of *Escherichia coli*, followed by *Klebsiellaspp* and *Staphylococcus aureus*. These findings also mirror those of other studies showing that the microbial ecology of urinary pathogens has largely remained the same. [12]

Enteric bacteria (in particular, *Escherichia coli*) have been and remain the most frequent cause of UTI, although there is some evidence in certain reports that the percentage of UTIs caused by *E. coli* is decreasing this does not appear to be the scenario in our hospital. A different study from ours showed the percentage of UTIs caused by *E. coli, Proteus* species, and *Pseudomonas species* decreasing, whereas the percentage of UTIs caused by yeasts, group B streptococci, and *Klebsiella pneumoneae* increased [13].

A retrospective analysis of UTI at Jos in Nigeria by Jombo et al, revealed that the commonest pathogens in outpatients was *Escherichia coli*, while in in-patients it was *Klebsiella spp*, with the Quinolones and Cefuroxime the most effective antibiotics in-vitro[14].

Other pathogens such as *Pseudomonas spp* and *Proteus spp* are often recovered from patients who are catheterized. *Pseudomonas aeruginosa* is an opportunistic human pathogen that is especially adept at forming surface-associated biofilms. It causes catheter-associated urinary tract infections (CAUTIs) through biofilm formation on the surface of indwelling catheters and it has high rates of therapeutic failure[15].

Catheter-associated UTI is the most common nosocomial infection, accounting for over a million cases in hospitals. The catheterization rate from our study was 34% which happens to be less than that reported by similar centers which report figures as high as 54%. The risk of UTI increases with increasing duration of catheterization. In non-institutionalized elderly populations, UTIs are the second most common form of infection, accounting for nearly 25% of all infections [5, 16].

Catheter associated UTI also extends hospital stay and adds to the direct cost of acute care hospitalization. It is associated with increased mortality. A Study on Catheterization rates suggested that nosocomial CAUTI are associated with substantially increased mortality rates. It is therefore important to accurately define the local epidemiology of CA-UTI [17].

Complications may arise from UTI's especiallyif bacteria enter the blood stream, they could cause severe complications, including septicemia, shock and, rarely, death. Conversely there may also be hematogenous spread of bacteria from the bloodstream into the kidney causing UTI's in patients who are hospitalized, catheterized or who have undergone genitourinary surgery[8, 18, 19]. In uncomplicated cases the infection is easily treated with a short course of an antibiotic, but in recent times there is increased resistance to many of these antibiotics resulting in treatment failure. Uncomplicated UTI should be distinguished from complicated UTI, which has a risk of severe illness and attendant effects such as renal scarring [20].

For the treatment of UTI's the Physician should be guided by the results of diagnostic tests and recent antimicrobial susceptibility of urinary pathogens also because the microbiologic characteristics of acute, uncomplicated UTI are highly predictable in women, antimicrobial therapy is usually empiric. As a result of this a short-course (3-day) therapy is commonly used, however practices may vary with fluoroquinolones in non-pregnant females preferred by[21]. The Infectious Diseases Society of America (IDSA) advocates trimethoprim-sulfamethoxazole (SXT) as initial therapy for females with acute uncomplicated bacterial cystitis in settings where the prevalence of SXT resistance does not exceed 10 to 20%.Our figure is 79.3% which is quite high. This guideline may not be suitable for Nigeria. Reports from elsewhere show that the resistance rates to SXT is unacceptably high and organisms such as *Enterococcus spp* are intrinsically resistant to it [22].

Other alternatives for short term therapy of UTI's include the use of Trimethoprim or Nitrofurantoin which were successful in over 80% of the cases. Nitrofurantoin appears to still retain high success rates even in this era of increasing antimicrobial resistance as evidenced from the high rates of susceptibility by pathogens. Cotrimoxazole on the other hand has disappointingly low rates of susceptibility and in some centers. Cotrimoxazole is not considered a first-choice drug for UTI [22].

Patients with frequent urinary tract infection ought to be placed on prophylactic antibiotics, which can be

patient-initiated, post-coital, or long-term low-dose therapy. Females with recurrent urinary tract infection in pregnancy should be considered for long-term antibiotic prophylaxis. In addition long term suppression of infection may include fiveday courses of β-lactams such as Co-Amoxiclav or Nitrofurantoin depending largely on local susceptibility patterns[22, 23].

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CONCLUSION

Urinary tract infections are important causes of morbidity in our environment; and care needs to be placed on the diagnosis and management of such infections. The prevalent pathogens in our environment are the Gram negative bacilli: *Escherichia coli* and *Klebsiella pneumoneae*. Nitrofurantoin however still retains efficacy to both Gram negative and positive organisms.

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COMPARING ANTIBODY RESPONSES TO *ONCHOCERCA VOLVULUS* AND NON-PARASITE ANTIGENS IN PLACEBO-CONTROLLED AND IVERMECTIN-TREATED ONCHOCERCIASIS PATIENTS

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ABSTRACT

Serum antibodies to parasite-specific and non-parasite antigens were evaluated using enzyme-linked immunosorbent assay (ELISA). Out of the 470 sera collected, 409 were from residents of an onchocerciasis hyper-endemic area, 55 non-endemic and 6 European normal sera served as control. The patients' age, sex, skin microfilaria densities, dermal and ocular clinical manifestations (colour of optic disc) have been well characterised. The study population had participated in a placebo-controlled (n=191) trial of ivermectin (Mectizan®) treatment (n=218). The parasite antigens are phosphate buffered saline crude extract of adult worms of *Onchocerca volvulus*, a recombinant antigen (Ov1.9) and a monoclonal antibody purified antigen (Cam 1). The non-parasite antigens are deoxycholate citrate extract of optic nerve (nerve-DOC) and commercially available IgA, IgM and IgG were used to assay for rheumatoid factor (Rh-F) auto-antibodies. Generally, antibodies to parasite antigens in onchocerciasis patients were remarkably higher than control group ($p<0.05$) using exact F-test. There was no significant difference ($p>0.05$) in antibodies to nerve-DOC and Rh-F in patients compared to control. Antibodies increased with increasing skin snip microfilaria load from 0.69±0.28 with 0mf/mg (n=54) as against 0.80±0.26 for those with 4-20mf/mg. Observed slight negative correlation in IgG antibody levels and severity of disc colour with mean OD values of 0.26±0.22 in those graded as having no optic nerve disease (OND) (disc 1, n=86) and 0.17±0.19 for those with severe changes (disc 3, n=49) was not statistically significant ($P>0.05$). An age dependent significant decrease ($P<0.05$) in antibodies were observed with 0.64±0.34 for 15-30yr old (n=48) compared to 0.48±0.35 for those 50yr (n=50) for PBS with a similar trend for IgG to Ov1.9 and Cam1. In conclusion, serum parasite-specific and non-parasite antibodies may not be responsible for the pathology of optic nerve disease. Onchocerciasis patients were apparently not at higher risk of developing rheumatoid arthritis than the control.

Key words: **Onchocerciasis; Antibodies; Antigens; Immune responses; Ivermectin.**

COMPARER LES REPONSES D'ANTICORPS AU *ONCHOCERCAVOLVULUS* ET AUX ANTIGENES NON PARASITAIRES DANS LES PATIENTS AVEC L'ONCHOCERCOSE CONTROLLES PAR PLACEBO ET TRAITES PAR L'IVERMECTINE.

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RESUME

Les anticorps sériques contre antigènes parasites spécifiques et non parasitaires ont été évalues en utilisant un dosage immunoenzymatique (ELISA). Sur les 470 sérums collectés, 409 provenaient de résidents d'une région hyper endémique de l'onchocercose, 55 sérums normaux non endermiques et 6 européens ont servi de témoins. L'âge des patients, le sexe, les densités de microfilaires, cutanées, les manifestations cutanées et oculaires (couleur du disque optique) ont été bien caractérisées. La population étudiée avait participé à un essai contrôlé par placebo (n=191)sur le traitement à l'ivermectine (Mectizan®) (n=218). Les antigènes parasites sont phosphate tamponné saline extrait brut de vers adultes de *l'Onchocerca volvulus*, un antigène recombinant (Ov1.9) et un antigène purifié par un anticorps monoclonal (Cam 1). Les antigènes non parasitaire sont l'extrait de citrate de desoxycholate du nerf optique(nerf - DOC) et on a utilisé des IgA, IgM et IgG disponibles dans le commerce pour doser les auto anticorps du facteur rhumatoïde (Rh – F). Généralement, les anticorps contre les antigènes parasitaires chez les patients atteints d'onchocercose étaient remarquablement plus élevés que le groupe témoin ($p>0,05$) en utilisant le test F – exact. Il n'y avait pas de différence significative entre les anticorps au nerf – DOC et les facteurs rhumatoïdes (Rh - F) chez les patients par rapport au témoin. Les anticorps augmentaient avec augmentation de la charge microfilaire de coupe de peau de 0,69±0,28 avec 0mf/mg (n=54) contre 0,80±0,26 pour ceux avec 4-20mf/mg. On a observé une légère corrélation négative dans les taux d'anticorps IgG et la gravité de la couleur du disque avec des valeursmoyennes de OD de 0,26±0,22 chez celles classées comme n'ayant pas de maladie du nerf optique (OND) (disque 1, n=86) et 0,17±0,19 pour les personnes ayant des changements sévères (disque 3, n=49) n'était pas statistiquement significative ($P>0,05$). Une diminution significative de de l'âge ($P<0,05$) dans les extraits d'anticorps a été observée avec 0,64±0,34 15 – 30 ans(n=48) compare à 50 ans (n=50) pour PBS avecune tendance similaire pour IgG à Ov1,9 et Cam1.En

conclusion, les parasites spécifiques du sérum et les anticorps non parasitaires ne peuvent pas être responsable pour la pathologie de la maladie du nerf optique. Les patients atteints d'onchocercose n'avaient apparemment pas un risque plus élevé de développer la polyarthrite rhumatoïde que le témoin.

Mots clés: L'onchocercose; Les anticorps; les antigènes; Réponses immunitaires; L'vermectine.

INTRODUCTION

Current control of onchocerciasis has relied on the mass drug administration of Ivermectin or Mectizan® (1). Lately, more emphasis is placed on operational research, drug screening and diagnostics development, while less attention is focused on basic research on the disease. Study into the immune responses of any infection is for a better understanding of the basis or mechanism of immunopathology including the role of autoimmune involvement, the diagnostic usefulness and to identify potential vaccine candidate immunogens. In onchocerciasis, humoral and cellular immune reactivity to parasite or the substance they release after death varies from one individual to another, and also show diverse clinical manifestations (2, 3). With time, the larva forms (microfilariae) and adult worm (macrofilaria) aged and die (4). Their fragments elicit host inflammatory responses with bystander effects believed to underlie dermal and ocular changes (5, 6). Immunological mechanisms are believed to play a major role in the broad range of dermal and ocular pathologies complicating *Onchocerca volvulus* infections. These have therefore prompted questions concerning involvement of antibodies to parasites-specific and autoantigens in the pathogenesis of onchocerciasis.

The parasite materials share a lot of biochemical and immunological homology with other parasites, related and unrelated species which are often co-endemic with onchocerciasis. This has posed a major problem in interpreting results of assays using undefined crude antigens, thus making the identification of diagnostic and clinical trends very difficult. The measure of antibody class and subclass response is thought would be able to reveal host-parasite immunological interactions that are involved in pathogenesis, and restricted to discrete clinical entity (7). Thus far, a positive association between skin microfilariae load and IgG3 to low molecular weight adult *Onchocerca volvulus* antigens and changes in immune responses following treatment have been reported while a possible cross-reactivity of parasite with host eye tissue component has been suggested. In this investigation, a measure of serum antibody against parasite crude and recombinant, and non-parasite rheumatoid factor (Rh-F) antigens using enzyme-linked immunosorbent assay (ELISA) were performed to know if there is any clinical trend based on age, sex, mf load and eye pathology (change in optic nerve disc colour). ELISA has been reported to show greater sensitivity and measuring IgG RF and IgA in addition to IgM RF by (8). Onchocerciasis was listed by (9) to be amongst diseases with possible features of autoantibodies to RA. Although there are formal classification criteria for RA according to the American College of Rheumatology, RFs are not a specific diagnostic tool for RA. However, the presence of high RF titers is predictive for developing RA in non-symptomatic subjects and titers are associated with a more aggressive and destructive course and with the occurrence of extra-articular manifestations in RA patients. Possible involvement of autoimmunity in the pathology of the skin and ocular lesions have been suspected. It has been suggested that autoimmunological reactions resulting from cross-reactivity between parasite antigens and components of eye tissues contribute to development of ocular pathology (10). Assessing the levels of antibodies in groups of clinical and parasitological defined onchocerciasis patients compared to control will show the cause and effect relation.

The aim of this research study is to determine the involvement of parasite-specific and non-parasite auto-antigens in the immunopathology of optic nerve disease, which is one of the major causes of irreversible blindness, skin clinical manifestations and correlation with status of microfilardermia. Secondly, the study will also determine the likely risk of developing rheumatoid arthritis in onchocerciasis patients compared to control.

MATERIALS AND METHODS

Experimental Design: A total of 470 sera, comparison 409 from onchocerciasis patients, 55 from Fatika, a non-endemic area in Kaduna State, Nigeria and 6 European normals as controls were screened for antibody responses to parasite and non-parasite antigens. The onchocerciasis patients comprised of 218 individuals receiving ivermectin and 191 receiving placebo. The individuals were previously characterized by age, sex, skin mf density and ocular pathology (colour of optic disc). All sera samples were stored at -70^{o}C in deep-freezer in 30µl aliquot and thawed once before use.

Antigens: Adult *O. volvulus,* phosphate buffered saline (PBS)-extracted crude antigen, and a monoclonal antibody purified antigen designated Cam 1 were prepared and provided by Dr. Engelbrecht. The Ov1.9 recombinant antigen described by (11) was supplied to the Immunology Research Laboratory (I.R.L), NITR, Kaduna, by Dr. McKennie of Cambridge University. A deoxy cholate citrate extract of human optic nerve tissue

(nerve-DOC) was kindly provided by the Institute McKennie. for Rh-F assay, commercially available human IgA, IgG and IgM (Calbiochem, U.K.) were used as auto-antigens as described by (8). The PBS extract was used at 1:1000 except for IgA at 1:500.

ELISA Protocol: Assays were performed following the protocol of Engelbrecht *et al*. (1992) with slight modification at the NITR Immunology Research laboratory. Wells of microtitre plates were coated with antigens and stored overnight in the fridge at 4^{o}C. Microplates were washed 3-5 times between each step. All other steps were performed at room temperature ranging from 28-32^{o}C. Sera, antigens, anti-human IgG and anti-mouse horseradish peroxidase conjugates were subjected to pre-titration experiment in checkerboard to obtain the optimal working concentration or dilution. Sera were used at 1:400 for IgG and IgG4, 1:250 for IgG1 and IgG3, and 1:50 for IgA. The PBS extract was used at 1:1000 for IgG, 1:2000 for IgG1, 1:8000 for IgG3 and IgG4 and 1:500 for IgA. Anti-mouse IgG hydrogen peroxidase was used at 1:800. The binding of antigen to antibody was demonstrated with hydrogen peroxide (H_20_2) in disodium hydrogen phosphate (Na_2HPO_4), citric acid and freshly prepared orthophenylene diamine (OPD) substrate solution at 15µl per well allowed to react for 15 minutes. The enzyme reaction was terminated with 2M H_2SO_4 at 30µl per well and plates were read after 5 minutes in a Dynatech MR4000 ELISA reader.

Data Analysis: The mean optical density (OD) values of cases and control, treated and non-treated, male or female, stratification by skin MFL and subjective grading of optic disc colour were computed using Microsoft Excel spreadsheet. Differences in mean±standard deviation (stdev) were subjected to exact F-test of unpaired data at 0.05 level of error.

RESULTS

Optical density (OD) values of the ELISA tests at 492nm of serum antibodies reaction with the parasite and non-parasite antigens were analysed. The mean and standard deviation of the OD-values were computed for each group and sub-groups. of Ophthalmology, Cambridge University. Dr. Antibody responses (IgA, IgG, IgG_1, IgG_3, and IgG_4) to PBS-crude extract, Ov1.9 and Cam 1 antigens in onchocerciasis patients were remarkably higher than those of control group (Table 1). Onchocercal sera (n=409) gave higher total anti-IgG titres of 0.75±0.24 compared to 0.62±0.3 and 0.37±0.18 for Ov1.9 and Cam 1, respectively. These values were comparatively higher than the mean values obtained for Fatika and European controls shown on Table 1. Similar trends were observed for the isotype (IgG1, IgG3 and IgG4) antibodies to the three antigens (PBS-extract, Cam1 and Ov1.9). The differences between the IgG4 responses to PBS-extract and Cam1 antigens were statistically significant ($p<0.05$). With cut-off points set as mean ± 3stdev of European control for PBS-extract (0.05) and Cam1 antigens (0.06) showed that 375 (91.7%) and 361 (88.3%) were found to be serological positive, respectively. Mean OD values for male were slightly higher than female ($p>05$). Only slight difference in anti-Rh-F IgG of 0.64±0.16, 0.59±0.15 and 0.41±0.22 for onchocerciasis sera, non-endemic and European controls, respectively were recorded. Therefore, there was no further analysis of the data on the basis of bioclinical and parasitological variables.

In isotype assays, anti IgG4 titres were prominently elevated in patients with a mean of 0.58±0.33 while absent in controls. IgG1 antibody titres were slightly high in patients with mean±stdev 0.24±0.23 compared with 0.11±0.15 OD-value for Fatika non-endemic control. European normals (n=6) were however negative. IgG3 assay was virtually negative irrespective of the test sample. Anti IgA titres were slightly higher in patients with a mean of 0.24±0.17 than controls, 0.19±0.14 for Fatika and 0.0.13±0.07 for European normals. Anti IgG2, IgE and IgM could not be developed due to high background reactions. Similar trend in antibody responses to PBS-extract were observed for Ov1.9 antigens, except that total IgG titres were comparatively lower- about half the OD-value (0.37±0. 2) as against 0.62±0.3. Overall, there were no significant differences between treated and non-treated groups for IgG responses.

TABLE 1: ANTIBODY RESPONSES TO PARASITE ANTIGENS COMPARED TO CONTROLS

(i) Antigen-Antibody Reaction	PBS IgG	PBS IgG1	PBS IgG3	IgG4	PBS IgA
Onchocerciasis sera (n=409)	0.75±0.24§	0.24±0.23	0.09±0.14	0.58±0.33φ	0.24±0.17
Non-endemic control (n=55)	0.38±0.26§	0.11±0.15	0.01±0.05	0.08±0.22φ	0.19±0.14
European negative (n=6)	0.11±0.05§	0.01±0.01	0.01±0.01	0.02±0.01φ	0.13±0.07
(ii) Antigen-Antibody Reaction	Ov1.9 IgG	Ov1.9 IgG1	Ov1.9 IgG3	Ov1.9 IgG4	Ov1.9 IgA
Onchocerciasis sera (n=409)	0.62±0.3ø	0.16±0.15	0.08±0.06	0.38±0.34¤	ND
Non-endemic control (n=55)	0.37±0.2ø	0.1±0.06	0.05±0.02	0.11±0.19¤	ND
European negative (n=6)	0.26±0.11ø	0.06±0.01	0.03±0.01	0.06±0.08¤	ND
(iii) Antigen-Antibody Reaction	Cam 1 IgG	Cam1 IgG1	Cam 1 IgG3	Cam 1 IgG4	IgA
Onchocerciasis sera (n=409)	0.37±0.18α	0.30±0.19	0.11±0.15	0.44±0.33⍰	ND
Non-endemic control (n=55)	0.23±0.15 α	0.19±0.16	0.08±0.08	0.05±0.19 ⍰	ND
European negative (n=6)	0.15±0.07 α	0.06±0.03	0.05±.04	0.00±0.02 ⍰	ND

The §, φ, ø, ¤, α, and ⍰ signs showed differences between onchocerciasis and non-endemic control were statistically significant (p<0.05) by exact F-test.

The OD values of non-treated group increases with increase in MFL for IgG anti-PBS-extract with mean±stdev of 0.69±0.28 for 0mf/mg (n=54) vs 0.8±0.26 for 4-20mf/mg (n=52). IgG_4 had corresponding values of 0.45±0.32 vs 0.66±0.34. A similar trend was observed in the treated group, where IgG_1 values were 0.19±0.23 (n=47) vs 0.32±0.20 (n=46) for the untreated group as shown on Table 2. Except for IgG response to 0vl.9 in treated group with 0.56±0.31 vs 0.7±0.3, there was no appreciable change with increase in mf density. Figure 2 shows there is age dependency with significant decrease (P<0.05) in mean OD values for IgG4 against PBS-extract from 0.64±0.34 for 15-30yr old (n=48) compared to 0.48±0.35 for those 50yr (n=50). Similarly, IgG4 reactivity to 0v1.9 were 0.47±0.37 vs 0.31±0.32 were comparably not different from those of Cam 1.

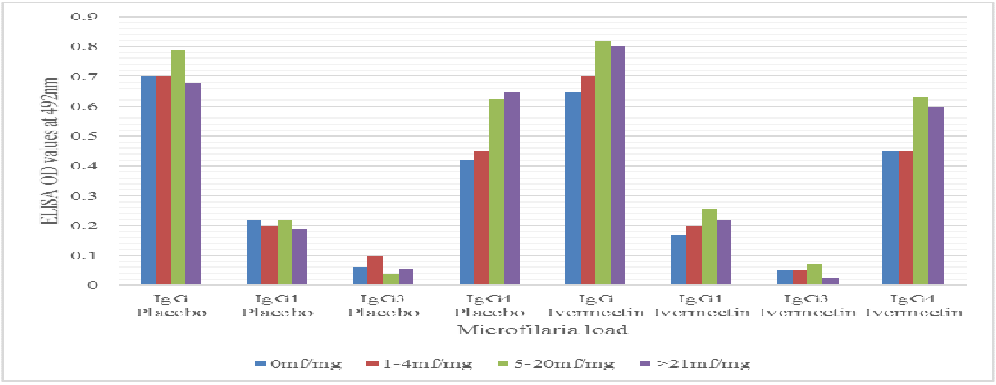

FIGURE 1: ANTIBODY RESPONSES TO MACROFILARIA LOAD IN TREATED AND NON-TREAT SUBGROUPS. ELSA=ENZYME LINKED IMMUNOSORBENT ASSAY, IG= IMMUNOGLOBULIN, MF= MICROFILARIA, NM-NANOMETRE, OD= OPTICAL DENSITY.

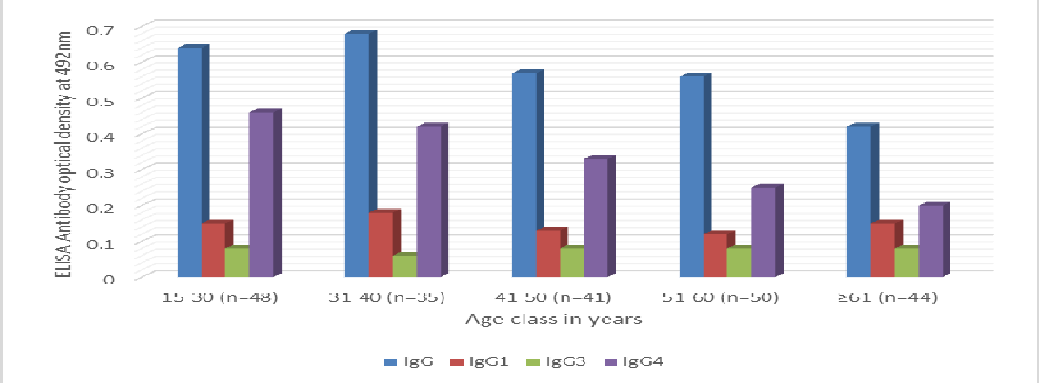

FIGURE 2: ANALYSIS OF ANTIGEN AND ANTIBODY REACTION IN TREATED ONCHOCERCIASIS PATIENTS BY AGE

In the treated group, a slight negative correlation in IgG antibody levels and severity of disc colour with mean OD values of 0.26±0.22 in those graded as having no OND (disc 1, n=86) and 0.17±0.19 for those with severe changes (disc 3, n=49). There was no significant difference ($p>0.05$) between cases and control for antibody response to optic nerve-DOC extract (only IgG and IgG4 were evaluated) neither between treated nor untreated groups in antibody levels.

DISCUSSION

Involvement of the parasite and non-parasite specific antibody responses in onchocerciasis has been studied. Active humoral immune response elicited by the antigenic stimulation of dead microfilariae has been demonstrated in this study. High level antibody responses against parasite antigens were more in onchocerciasis patients than in control, and the prominence of IgG than IgA is widely documented. The significant result obtained from the overall assay is observed preferential elevation of anti IgG4 titres in patients' sera. This finding is in agreement with those reported by (3, 12, 13, 14) Most of the IgG were accounted for by IgG_4 subclass followed by IgG_1 and IgG_3. Similar findings were reported in children by (15).

Changes in parasite specific IgE and IgG antibody responses were transiently enhanced at post-diethylcarbamzine (DEC) or Banocide® treatment, and after treatment, parasites possibly release antigens previously hidden from host immune response (16). On the contrary, there was no change in antibodies after ivermectin treatment is a confirmation that the difference may explain possible development of Mazzotti reaction peculiar to DEC treatment, which is very minimal or absent in ivermectin treatment.

It is very likely that the predominant subclass IgG_4 may act in blocking hypersensitivity reaction. The clinical status, age and mf density dependency of antibody responses have been documented by other investigators. Expectedly, reactivity to PBS-extract was more than those of Ov1.9 and Cam 1 antigens, since the formal contains immuno-dominant antigens (13, 17). This may be due to the presence of many epitopes, such as, phosphoryl choline (PC) and other carbohydrate determinants in crude PBS extract. The sensitivity and specificity of IgG_4 against PBS-extract was higher than those of Ov1.9, which is an indication that the later stimulates less antibody response (11). Measuring IgG_4 response to PBS extract hitherto has been documented to have potential diagnostic value (15). This subclass is non-reactive to phosphoryl-choline (PC), an immunodominant molecule responsible for the majority of cross-reactivity. Although an increase in both IgG and IgM reactivity with Rh-F has been established in loiasis patients with or without glomerulonephritis, the observed slight differences between onchocerciasis cases and controls, deserves further studies to validate if they play a protective role or are just epiphenomena. Onchocerciasis has been listed among the diseases with high risk of detecting Rh-factor by (18). Results obtained from this study did not support the rheumatoid arthritis playing any role in autoimmune disease involving any of the antibody classes (IgA, IgG and IgM). This is in accordance with the held belief that raised levels of IgM, IgG, and IgA RF have been reported in patients with Rheumatoid Arthritis (19). Several groups have reported that a high level of IgA RF is prognostic for a more severe disease outcome (20 and 21).

From all indication, it is obvious that the varied clinical manifestations of onchocerciasis are not due to direct parasite attrition (22). Moreover, it has been established that dead microfilaria elicit bystander response that attract cellular immune mechanism involving cyto-adherence in the process of clearing dead microfilariae with consequence dermal and ocular tissue damage have been documented by (6, 23). It has been shown by (24) that both IgM- and IgG-containing complexes were commonly involved but there was no correlation

between the levels of complexes containing these isotypes. One of the cardinal manifestations of autoimmune disorders is the presence of auto-antibodies and/or self-reacting cells (25), however, the detection of these autoimmune activities are not necessarily associated with clinical findings.

High level antibody responses against parasites antigens showed that IgG was more prominent than IgA. Most of the IgG were accounted for by IgG4 subclass followed by IgG1 and IgG3. This is a possible indication of the predominant subclass IgG4 may act in blocking hypersensitivity reaction (12). The clinical status, age, and mf density dependency of antibody responses have been documented by other investigators (11, 15). Expectedly, sensitivity and specificity of IgG4 against PBS-extract is higher than those of Ov1.9 and Cam 1, which is an indication that they stimulate less antibody responses. Measuring of IgG4 to PBS-extract has been reported to have potential diagnostic value.

Although an increase in both IgG and IgM Rh-F has been established in loiasis patients with or without glomerulonephritis, the observed slight differences between onchocerciasis cases and controls, deserve further studies to validate if they play a role protective role or not. More importantly, the study was carried out in savannah onchocerciasis endemic area, which may differ from what obtains with the forest species of the parasite.

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ORIGINAL ARTICLE

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PATTERN OF BACTERIAL PATHOGENS OF ACUTE OTITIS MEDIA IN A TERTIARY HOSPITAL, SOUTH WESTERN NIGERIA

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ABSTRACT
INTRODUCTION: Otitis media was reasonably prevalent prior to the use of antibiotics for treatment. In Nigeria, hospital incidence reports indicate that chronic suppurative Otitis media is the commonest. Complications that usually arise as a result of untreated Otitis media are meningitis, brain abscess, keratoma, otosclerosis, and hearing loss. The study aimed at providing information on the pattern of bacterial pathogens of acute Otitis media in LAUTECH Teaching hospital, Osogbo, Nigeria.
METHODS: It was a cross-sectional study involving patients with acute Otitis media attending ENT clinic at LAUTECH Teaching Hospital, Osogbo, Nigeria. Ear swabs were collected from the patients after informed consent. The samples were inoculated on general and selective laboratory media. Bacterial pathogens were isolated and identified. Antibiotic susceptibility testing was performed on each of the bacterial isolates using modified Kirby Bauer disk diffusion.
RESULTS: There were 115 isolates from 98 patients with acute Otitis media. Gram negative bacteria constituted 66.7% of the isolates. The most common organism was Pseudomonas *aeruginosa* (34.8%). Others were *Staphylococcus aureus* (30.4%), *Proteus* spp (15.7%), *Klebsiella spp* (11.3%), *Escherichia coli* (2.6%) and few Fungi (4.1%). Antibiotics sensitivity results of the isolates showed high resistance against most readily available antibiotics. The cumulative resistance of all the bacteria isolates to Augmentin was 89%, gentamicin 80%, ciprofloxacin 34% and ceftazidime 10%. About 88% of the Gram positive bacteria were resistant to penicillin G, amoxicillin, cotrimoxazole, and erythromycin. While 100% of the Gram negative bacteria were resistant to cotrimoxazole, tetracycline, and chloramphenicol. However, commonly isolated organisms were highly susceptible to few 3rd-generation cephalosporins especially ceftriaxone and ceftazidime.
CONCLUSION: Based on the result of this study, it is suggested that knowledge of antibiotic profile of etiological agents in Otitis media would be of great advantage in reducing the morbidity and mortality associated with Otitis media.

KEYWORDS: Otitis media, Bacterial agents, Antibiotic resistance.

PATHOLOGIE BACTERIENNE D'OTITE MOYENNE AIGUË DANS UN HOPITAL TERTIAIRE AU SUD – OUEST DU NIGERIA.

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RESUME
INTRODUCTION: L'Otite moyenne était prévalence avant l'utilisation des antibiotiques pour le traitement. Au Nigeria, le rapport d'incidence des hôpitaux indique que l'Otite moyenne suppurée est la plus courante. Les complications qui surviennent habituellement à la suite d'Otite moyenne non traitée sont la méningite, les abcèscérébraux, le kératome, l'otosclérose et la perte auditive. L'étude visait à fournir des informations sur le profil des pathogènesbactériens de l'Otite aiguë moyenne à l'hôpital d'enseignement LAUTECH, Osogbo, Nigeria.
METHODE: Il s'agissait d'une étude transversale impliquant des patients atteints d'Otite moyenne aiguë fréquentant la clinique ENT de l'hôpital d'enseignement LAUTECH, Osogbo, Nigeria. Des écouvillons auriculaires ont été prélevésauprès de patients après un consentement éclairé. Les échantillons ont été inoculés sur des milieux de laboratoire généraux et sélectifs. Pathogènesbactériens ont été isolés et identifiés. Des tests de sensibilité aux antibiotiques ont été effectués sur chacun des isolats bactériens en utilisant la diffusion de disque de Kirby Bauer modifiée.
RESULTATS: Il y avait 115 isolats de 98 patients atteints d'Otite moyenne aiguë. Gram négatif constituait 66,7% des isolats. Les organismes les plus communs étaient*Pseudomonasaeruginosa*(34,8%), *Staphylococcus aureus* (30,4%), *Proteus* spp (15,7%), *Klebsiellaspp* (11,3%), Escherichia *coli* (2,6%) et quelques champignons (4,1%). Les résultats de sensibilité aux antibiotiques des isolats ont montré une résistanceélevée contre les antibiotiques les plus facilement disponibles. La résistancecumulée de tous

les isolats de bactéries à Augmentin était 89%, la gentamicine 80%, la ciprofloxacine 34% et la ceftazidime 10%. Environ 88% des bactéries Gram positives étaientrésistantes à la pénicilline G, à l'amoxicilline, au cotrimoxazole et à l'érythromycine. Tandis que 100% des bactéries Gram négatifétaientrésistantes au cotrimoxazole, à la tétracycline et au chloramphénicol. Cependant, les organismes communément isolés étaienttrès sensibles à quelques céphalosporines de 3ème génération. CONCLUSION: Basé sur le résultat de cette étude, il est suggéré que la connaissance du profil antibiotique des agents étiologiques dans l'Otite moyenne serait un grand avantage pour réduire la morbidité et mortalité associées à l'Otite moyenne.

MOTS CLES: L'Otite moyenne, Agent bactériens, Résistance aux antibiotiques.

INTRODUCTION

Otitis media (OM) is an inflammatory disease of the mucosal lining of the middle ear that is frequently caused by the accumulation of fluid usually behind the blocked Eustachian tube (1, 2). It is one of the commonest reasons for the under-five children visitations to the general practitioners (3). Acute otitis media (AOM) is characterized by the rapid onset of symptoms like otalgia, fever, vomiting and accumulation of fluid in the middle ear cavity(4). Globally, over 700 million cases of AOM are diagnosed among the under-five group of children annually (5). A patient is said to have recurrent AOM (RAOM) after being diagnosed of three episodes of AOM within six months or over four episodes in 12 months (6), and this is usually noticed in about 65% of children in their first five years of life (6). Hearing loss is one of the commonest complications at a critical developmental stage of their lives (3). Otitis media related hearing impairment was found to be estimated at 30.82/10,000 annually (5). Otitis media with effusion (OME) usually resolves spontaneously within 3 months (7), however if this fails to happen few percentage of these children would experience persistent fluid in the middle ear for more than 3 months (7).

Some of the significant risk factors include poor living conditions, overcrowding, lack of access to medical care , environmental factors and exposure to cigarette smoke (8), Others include cultural , seasons of the year and family history of middle ear infections (4).

Initiation of infection in otitis media depends on the route of infection to the middle ear(9), and this could be caused by complications arising from diseases of nasopharyngeal areas, sinuses, oropharynx and tonsils (8, 9). OM is commonly prevalent in children because of their shortness and more horizontal Eustachian tube that contain flaccid cartilage than in adults (10). This usually impairs the opening of the tube in children (11) and facilitate the accumulation of fluids with the ultimate blockage (11).

The most common bacteria involved in the otitis media are *Haemophilus influenzae, Streptococcus pnuemoniae, Streptococcus pyogenes, Staphylococcus aureus, Moraxella catarrhalis* and *Pseudomonas aeruginosa* (12). Other important organisms that have been implicated in otitis media include *Bacillus* spp, *Escherichia coli* and *Proteus* spp (13).

In view of the well-known growing fact about microbial drug resistance all around the globe (14), an effective monitoring of the predominant bacterial and other microbial pathogens is important to inform new treatment strategies of this infection (3). The study aimed at providing information on the pattern of bacterial pathogens of acute Otitis media in LAUTECH Teaching hospital, Osogbo, Nigeria.

METHODS

It was a cross-sectional prospective study involving patients with features suggestive of acute Otitis media attending ENT clinic at the LAUTECH teaching hospital (LTH), Osogbo, Nigeria. Osogbo is situated in Olorunda Local Government and is the capital of Osun state, Nigeria. The city is about 500 kilometers from Abuja. The study was carried out between the periods of January –December 2005. An informed consent was obtained for each patient to collect the swab of an ear discharge by the physician and filled a short open-ended questionnaire. Ethical approval for the study was obtained from the ethical /research committee of Ladoke Akintola University Teaching Hospital.

Specimen collection and processing

Swabs of the ear discharge from each of the patients was collected aseptically at the ENT clinic after an informed consent and was transported to the microbiology laboratory of LTH, Osogbo for immediate processing. Ear discharge swabs were then inoculated on the laboratory media like blood, chocolate and MacConkey agars. Inoculated plates were then incubated aerobically at 37^{o}C for 18-24 hrs. Bacterial isolates from these specimens were identified by the standard bacteriological methods (15), and were then subjected to antibiotics susceptibility testing following the Clinical and Laboratory Standard Institute (CLSI) for the disc diffusion test(16). Disc susceptibility testings' were carried out on tetracycline (10ug), cefuroxime (30ug), ampicillin (10ug), erythromycin (15ug), ceftriaxone (30ug), ciprofloxacin (5ug), ofloxacin (5ug), gentamycin (10ug), ceftazidime (30ug), amoxicillin-clavulanic acid (10ug) and cotrimoxazole (25ug). The susceptibility patterns of the drugs were interpreted according to standard methods (16). The reference

strains of *Staphylococcus aureus* (NCTC 6751) and Escherichia coli (NCTC 10418) were used as quality control.

Data analysis Data were entered, cleaned manually and analysed using SPSS version 12.0. Frequency tables were generated and data were analysed using appropriate statistical methods. P value of < 0.05 was considered as statistically significant.

RESULTS

A total of ninety -eight patients with ear discharges were recruited into the study. Majority of the patients were males (53% vs 47%). The mean age of these patients was 15 yrs (+/- 16; Range 9 months – 78 years). Most of the study participants were under the age of 10 (55.2%) (Table 1).

TABLE 1: DISTRIBUTION OF THE PATIENTS BY AGE AND SEX

	Characteristics	Frequency (N=98)	Percentage (%)
Age (Yrs)	< 10 11-20 21-30 31-40 41-50 >50	54 8 20 4 3 9	55.2 8.1 20.4 4.1 3.1 9.1
Sex	Male Female	52 46	53.0 47.0

Ear discharge was the commonest clinical finding observed in all the patients examined (100%). Chronic nasal discharge was the most prominent predisposing factor noticed among the patients as 40.0% of them had a long standing antecedent history of nasal discharge. Trauma mostly due to head injuries accounted for 7.1% of the cases. Other predisposing factors at presentations were as indicated in Table 2.

There were 115 microbial isolates from 98 patients. The overall prevalence of bacterial isolates was 96.5% and 74 (66.7%) were gram negative bacteria (GNB). *Pseudomonas aeruginosa* was the most prominent GNB isolated (34.8%) while Staphylococcus *aureus* (30.4%) was the commonest gram positive bacteria isolated (Table 3).

TABLE 2: DISTRIBUTION OF THE PATIENTS BY THE CLINICAL FEATURES AND RISK FACTORS

Predisposing factors	Frequency (N=98)	Percentage (%)
1. Trauma	7	7.1
2. Chronic Nasal discharge	39	40.0
3. Ear affected		
a. Unilateral	64	65
b. Bilateral	34	35
4. Earache	48	49
5. Ear discharge	98	100
6. Hearing loss	36	37

TABLE 3: DISTRIBUTIONS OF THE BACTERIAL ISOLATES IN ACUTE OTITIS MEDIA

Bacterial isolates	Frequency (N=115)	Percentage (%)
Pseudomonas aeruginosa	40	34.8
Klebsiella spp	13	11.3
Proteus spp	18	15.7
Escherichia coli	3	2.6
Staphylococcus aureus	35	30.4
Streptococcus spp	2	1.7
Fungi	4	3.5

The antibiotic susceptibility profiles of the bacterial isolates are shown in Table 4. Ceftazidime has the highest susceptibility rate for *P. aeruginosa* (97.5%), while it was 100% for the *Proteus* spp and *E. coli* respectively. The quinolones (Ciprofloxacin, Ofloxacin and Perfloxacin) demonstrated 50% resistance to *P. aeruginosa*. 97.5-100.0% of *P. aeruginosa* were resistant to Ampicillin, amoxicillin, Augmentin and Penicillin respectively. *Staphylococcus aureus* demonstrated high level of resistance against all the common antibiotics (Table 4).

TABLE 4: ANTIBIOTIC SUSCEPTIBILITY PATTERN OF BACTERIAL ISOLATES TO ANTIMICROBIAL AGENTS

Antibiotics	Susceptibility pattern of bacterial isolates to antimicrobial agents					
	Pseudomonas aeruginosa N=40	*Klebsiella spp* N=13	*Proteus spp* N=18	*Escherichia coli* N= 3	*Staphylococcus aureus* N= 35	*Streptococcus sp* N= 2
	S (%)	S (%)	S (%)	S (%)	S (%)	S (%)
Penicillin	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	2(5.7%)	0(0.0%)
Ampicillin	3 (7.5%)	1(7.7%)	0(0.0%)	0(0.0%)	4(11.4%)	0(0.0%)
Amoxicillin	1(2.5%)	1(7.7%)	5(27.8%)	3(100.0%)	3(8.6%)	0(0.0%)
Augmentin	1(2.5%)	1(7.7%)	5(27.8%)	0(0.0%)	3(8.6%)	1(50.0%)
Erythromycin	5(12.5%)	1(7.7%)	0(0.0%)	0(0.0%)	5(14.3%)	1(50.0%)
Gentamycin	8(20.0%)	11(84.6%)	6(33.3%)	2(66.7%)	16(45.7%)	1(50.0%)
Ciprofloxacin	19(47.5%)	8(61.5%)	13(72.2%)	2(66.7%)	21(60.0%)	2(100.0%)
Ofloxacin	20(50.0%)	9(69.2%)	8(44.4%)	2(66.7%)	22(62.9%)	2(100.0%)
Perfloxacin	20(50.0%)	6(46.1%)	8(44.4%)	3(100.0%)	20(57.1%)	1(50.0%)
Ceftriaxone	38(95.0%)	12(92.3%)	17(94.4%)	3(100.0%)	33(94.3%)	0(0.00%)
Ceftazidime	39(97.5%)	10(76.9%)	18(100.0%)	3(100.0%)	34(97.1%)	1(50.0%)
Cephalexin	30(75.0%)	12(92.3%)	16(88.9%)	2(66.7%)	30(85.7%)	2(100%)
Cotrimoxazle	0(0.0%)	2(15.4%)	3(%)	0(0.0%)	0(0.0%)	0(0.0%)

DISCUSSION

The burden of bacteria as aetiological agents associated with otitis media was found to be very high in this study. The prevalence of bacterial isolates from the ear discharge samples was 95.6%. Otitis media is one of the commonest diseases of infants and young children and its sequelae persists in some individuals into adult years. In this study, most of the bacterial isolates were found among the under 10 years old children (55.2%), 34 (34.7%) of which belong to under-five years old children. This was in agreement with previous studies (11, 17-20). The increased incidence of AOM in children has been attributed to the shortness and horizontal Eustachian tube that contain more flaccid cartilage than in adults (10), and this facilitates the accumulation of fluids with the ultimate blockage in children (11).

Male gender has been reported as an important risk factor for the development and acquisition of otitis media (20, 21).

This study demonstrated increased prevalence of otitis media among male infants and young children than in female (53% vs 47%) and this is similar to the findings in other studies (18, 22). However, it contradicts the work of other researchers that believed there was no significance difference (20).

Gram negative bacteria (66.7%) were the commonest isolates compared with 33.3% of gram positive organisms. This is in agreement with other previous studies from Nigeria (17, 23, 24) and other countries (11, 21, 22). *P. aeruginosa* was the predominant bacterial isolated as it was recovered in 34.8% of the cases. The organism has been found to be the commonest aetiologic agent of otitis media in Nigeria (9, 18, 25) and other African countries (11, 19). Other important gram negative bacterial isolates recovered in this study were *Proteus* spp (15.7%), *Klebsiella* spp (11.3%), and *E. coli* (2.6%). *Staphylococcus aureus* (30.4%) was the second most common organism, but the predominant gram positive bacteria isolated. This

is similar to the bacteriological profile discovered in another study as shown in the review by Verhoeff et al, 2006 (26). However, in another study, Saunders *et al* found that *S. epidermidis* was the predominant gram positive organisms (27).

The clinical symptoms and the predisposing factors observed in the study showed that ear ache and hearing loss to be most common symptoms observed at the presentation. This is also similar with findings in other study (11).

Susceptibility profile of the bacterial isolates showed high resistance level against the commonly prescribed antibiotics. Our results have shown that penicillin, ampicillin, amoxicillin, erythromycin, cotrimoxazole and amoxicillin –clavulanic acid in general are resistant to both gram negative and positive bacteria isolated from the ear discharge samples. The resistance rate of the antibiotics ranges from 50-100%. This finding contradicts the earlier study that showed excellent sensitivity to the above listed antibiotics (11). The only exception observed was the 100% susceptibility of *E. coli* to amoxicillin compared to the previous study that found high resistance level (19).

The most effective antimicrobial agent against *P. aeruginosa* was ceftazidime (97.5%), and this was closely followed by ceftriaxone (95.0%) and cephalexin (75.0%) respectively. This is contrary to 67.7% susceptibility to ceftazidime observed in another study in Nigeria(18) .The high susceptibility rate demonstrated by the third generation cephalosporin to *P. aeruginosa* was similar to what was obtained against *Proteus* spp and *Klebsiella* spp. *P. aeruginosa* also showed moderate level of resistance to all the available quinolones in study , however, this contradicts the finding of another study that showed 100% susceptibility to ciprofloxacin (18).

Highly level of resistance noticed against the commonly prescribed antibiotics was due to indiscriminate use antibiotics in Nigeria by patronizing many unlicensed community pharmacy shops. Judicious use of antimicrobial therapy may prevent the development of antimicrobial resistance. Based on the results of this study, it is suggested that knowledge of antibiotic profile of etiological agents in Otitis media would be of great advantage in reducing the morbidity and mortality associated with Otitis media. We are encouraging many hospitals to adopt the principle of antibiotics stewardship to reduce this inappropriately usage of antibiotics.

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PUBLIC HEALTH PRACTICES AT MEAT PIE RETAIL POINTS IN MAKURDI, BENUE STATE AND ITS POTENTIAL EFFECT ON CONSUMER'S HEALTH

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ABSTRACT

Observance of public health best practices at point of sales by meat pie retailers in Makurdi, Benue State was evaluated by studying three (3) retail sources namely eateries, supermarkets and street hawkers. Observations were carried out ninety (90) times between March to July 2013. The neatness of the vendor, sales environment, and state of the product storage containers were assessed. Microbiological analysis revealed the presence of *Staphylococcus aureus*, *Escherichia coli*, *Staphylococcus* spp, *Enterobacter* spp, *Proteus* spp, *Pseudomonas* spp, *Citrobacter* spp, *Edwardsiella* spp, *Bacillus* spp, *Klebsiella* spp and *Shigella* spp. There was a positive relationship between multiple bacterial contamination and the constituents of the meat pie fillings. A total of 64 (35.6%) fillings had between 3 – 7 bacterial contaminants, 57 (31.7%) had at least 2 bacterial contaminants, 58 (32.2%) had at least 1 contaminant while only 1 (0.6%) was without any bacterial contaminant. Only 5(5.6 %) of the vendors and 10(11.1%) of the sales environment were very neat, while 23(25.6%) and 22(24.4%) of the storage containers were observed to be partially accessible to air/dust and insects respectively. None of the vendors (90:100%) used hand gloves, none (90:100%) used an apron, 89 (98.9%) used no cutlery and 89 (98%) had uncovered hair while serving the product. The paper submits that the health of consumers is endangered by this negligence. Hence, the need for regulatory authorities to create awareness on, as well as enforce the observance of established point of sales practices for the sake of the public health.

Keywords: Public, health, meat pie, fillings, negligence, contamination.

LES PRATIQUES DE SANTE PUBLIQUE AUX POINTS DE VENTE DE TARTES A LA VIANDE A MAKURDI, ETAT DE BENUE ET SON EFFET POTENTIEL SUR LA SANTE DES CONSOMMATEURS.

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RESUME

L'observance des meilleures pratiques en matière de santé publique par les détaillants de tartes à la viande à Makurdi, état de Benue a été évalué en observant trois (3) sources de détail, à savoir les entrées, les supermarchés et les colporteurs. Les observations ont été effectuées quatre – vingt – dix – neuf (90) fois entre mars et juillet 2013. La propreté du vendeur, l'environnement des ventes et l'état des conteneurs de stockage du produit ont été évalué. L'analyse microbiologique a révélé la présence de *Staphylococcus aureus*, *Escherichia coli*, *Staphylococcus* spp, *Enterobacter*spp, *Proteus*spp, *Pseudomonas* spp, *Citrobacter*spp, *Edwardsiella*spp *Bacillus* spp, *Klebsiella* spp *et Shigella*spp. Il y avaitune relation positive entre la contamination bactérienne multiple et les constituants des garnitures de tartes de viande. Un totale de 64 (35,6%) garnitures ont eu entre 3 – 7 des contaminants bactériens, 57 (31,7%) ont eu au moins 2 contaminants bactériens, 58 (32,2%) ont eu au moins 1 contaminant tandis qu'une (0,65) seule était dépourvue de contaminants bactériens. Seulement 5 (5,6%) des vendeurs et 10 (11,1%) de l'environnement de vente étaienttrès propres, tandis que 23 (25,6%) et 22 (24,4%) de conteneurs de stockage ont été observés d'être partiellement accessibles respectivement à l'air / a la poussière et aux insectes. Aucun des vendeurs (90 :100%)a utilisé les gants, aucun (90 :100%) a utilisé un tablier, 89 (98,9%) n'utilisaient pas de couverts et 89 (98%) ont eu les cheveux découverts tout en servant le produit. L'article soutient que la santé de consommateurs est menacée par cette négligence. Il est nécessaire, donc, pour les autorités de réglementation de sensibiliser et de faire respecter des pratiques établies de point de vente pour le bien de la santé publique.

Mots clés: Publique, santé, tartes é la viande, garnitures, négligence, contamination.

INTRODUCTION

Meat pies and other pies are popular the world over and belong to the class of ready-to-eat foods known as pastries (1). A pie refers to any food or dish that consists of a crust with a filling. Examples are fruit pies, cream pies, custard pies and meat pies (2). Their popularity owes to the fact that they are convenient to stock, sell and consume (3). Meat pie like other ready to eat foods, is common and can be found on many streets, supermarkets, motor parks and food outlets, thereby providing a ready source of nutrition for the teaming populace who now have little or no time to make their own food.

Studies have indicated that producers and retailers of ready-to-eat foods are either ignorant of accepted hygienic and sanitary practices or overlook them (4, 5, 6, 7, 8). Individuals and families are thus, left with no other option than to eat just what they buy. From recent findings, food mixtures such as pastries, salads, sauces and soups have been frequently incriminated in food poison outbreaks (10, 11, 12, 13). Food-borne infections have caused the death of many children in the developing world and have also affected their growth, as well as physical and cognitive development (4). Bacteria such as *Pseudomonas* spp, *Enterococcus* spp, *Klebsiella* spp, *Clostridium perfringens*, *Salmonella* spp, *Escherichia coli*, *Staphylococcus aureus* and *Proteus* spp have been isolated from meat pie and other locally prepared ready-to-eat foods (8, 14, 15).

Microorganisms enter foods from both internal and external sources. Their number and type depend on the care used during production, processing and storage (16). Some sources of food contamination have been identified as unhygienic practices by food handlers, untidy preparation or service environment, contaminated water, utensils and ingredients used for preparation of the food (4, 8, 17, 18). Point of sale practices by vendors has also been reported as a major source of food contamination. It is required that food safe for human consumption should be displayed in a clean environment and in containers that are insect and dust proof. Serving of food at retail points should also be done with sanitized utensils (19). Unfortunately, vendors of meat pie often use bare hands to serve, wear no aprons and leave their hair uncovered. As a result, the same hands used for collecting money are used to package the food for buyers (17, 20). Bacteria are also introduced into food through aerosols released during vendor–client communication at the point of sale (18). Bacteria in the released aerosol from saliva settle on the food and are carried away by the buyer unknowingly. Sometimes, flies and insects are not totally prevented from making contact with the food. This could lead to cross contamination (8). This study was therefore aimed at investigating observance or otherwise, of point of sale best practices by vendors of meat pie in Makurdi and to highlight their potential effect on consumer health. Information generated from this study will reveal the extent of adherence to accepted point of sales best practices. It will also aid in laying emphasis on the need for health, environmental and regulatory agencies to further strengthen the implementation of existing food hygiene laws and enforce acceptable point of sale practices.

METHODOLOGY

Study area

The research was carried out in Makurdi Benue State. Makurdi is the capital of Benue State and is located on latitude 7°38' and 7°50′ N and longitude 8°24' and 8°38′ E (21). Makurdi is situated in the middle-belt region of Nigeria, thereby serving as a link town between the North and the East, as well as other local government areas of the state, and hence has a large number of parks and fast food outlets which witness a high number of travelers on a daily basis.

Study design

A descriptive cross-sectional study design was used for this study and was carried out between March and July 2013. Inclusion and exclusion criteria were also adopted in the selection of the study sites. The inclusion criteria were outlets that either prepared meat pie themselves or were first level retailers who receive stock directly from a producer. Second level retailers and outlets were excluded.

Data collection

The three major sources of retail meat pie in Makurdi identified as eateries, supermarkets and hawkers were sampled. Sample sites were randomly selected from across the five major areas of the town namely Ankpa ward, Wadata, High Level, Wurukum and North Bank. A total of ninety (90) observations in all, were made at the outlets. Structured questionnaire and observational checklist designed to obtain information about the vendors' personal hygiene and behavior during food vending were used for the study. Outlets at major business and work areas with high human activity and patronage were carefully noted and selected. Point of sale practices were assessed based on the use of hand gloves, use of apron, use of service cutlery, use of hair covering and talking while serving. The neatness of the service environment and vendor was made by visual observation. The show glasses were also assessed based on their accessibility to dust, air and insects judging from the presence and size of openings found on them. Show glasses that were wholly enclosed were judge to be inaccessible to dust and insects.

Sample collection

Meat pie samples were obtained from the three main sources of meat pie sold in Makurdi identified as

eateries, supermarkets and street hawkers spread across the five major areas of the town namely Ankpa ward, Wadata, High Level, Wurukum and North Bank. Thirty (30) samples were obtained from each source, making a total of ninety (90) meat pie samples. The filling and crust of each sample were analyzed separately owing to the difference in their composition and preparation.

Isolation and identification of isolates

Ten (10) grams of the sample (filling or crust) were weighed into 90 ml of sterile normal saline and homogenized in a sterilized electric blender. A loop full of the stock preparation was streaked on the surface of Nutrient agar (Titan Biotech Ltd.), Mannitol salt agar (Oxoid), Eosin methylene blue agar (Titan Biotech Ltd.) and MacConkey agar (Titan Biotech Ltd.) and incubated at 37^{o}C for at least 18 hours. The morphological and cultural characteristics of colonies on the various media used were recorded. Colonies with similar morphological characteristics where selected, sub-cultured, and discrete colonies obtained were used for identification tests. The Gram reaction of each isolate was determined. Biochemical identification was achieved by performing catalase, coagulase, indole, citrate utilization, oxidase, lysine decarboxylase, sugar fermentation, motility and hydrogen sulfide production tests on the various isolates.

Statistical analysis

Statistical analyses were done with Statistical Package for the Social Sciences (SPSS 17, 2008) software, using descriptive statistics such as frequencies and percentages. Chi-square test was used to determine associations.

RESULT

Eleven different bacterial genera were isolated from the meat pie samples collected (Table 1). *Staphylococcus aureus*, *Bacillus* spp, *Pseudomonas* spp, *Klebsiella* spp, *Escherichia coli*, *Enterobacter* spp and *Proteus* spp were found as contaminants in both fillings and crusts of all samples collected from all the locations. *Staphylococcus* spp was isolated from samples obtained from hawkers and supermarket, while *Edwardsiella* spp was isolated from eateries samples only. *Shigella* spp was not isolated from the fillings of hawked samples and those collected from eateries. *Citrobacter* spp was isolated from all sample sources except those from supermarket.

TABLE 1: PRESENCE OF BACTERIAL CONTAMINANTS IN FRESH MEAT PIE SAMPLES FROM HAWKERS, SUPERMARKETS AND EATERIES

	Hawkers		Supermarket		Eateries	
Bacterial isolates	**Filling**	**Crust**	**Filling**	**Crust**	**Filling**	**Crust**
Staphylococcus aureus	+	+	+	+	+	+
Bacillus Spp	+	+	+	+	+	+
Staphylococcus Spp	+	+	+	+	-	-
Pseudomonas Spp	+	+	+	+	+	+
Citrobacter Spp	+	+	-	-	+	-
Klebsiella Spp	+	+	+	+	+	+
Shigella Spp	-	+	+	+	-	+
Escherichia coli	+	+	+	+	+	+
Enterobacter Spp	+	+	+	+	+	+
Proteus Spp	+	+	+	+	+	+
Edwardsiella Spp	-	-	-	-	+	+

+ = Present, - = Absent

TABLE 2: MULTIPLE BACTERIAL CONTAMINATION IN NINETY MEAT PIE SAMPLES COLLECTED FROM HAWKERS, SUPERMARKET AND EATERIES

No. of Contaminants	Eatery (%)	Supermarket (%)	Hawkers (%)	Total (%)
0	0 (0)	0 (0.0)	0 (0.0)	0 (0.0)
1	0 (0)	1 (3.3)	0 (0)	1 (1.1)
2	10 (33.3)	9 (30.0)	6 (20.0)	25 (27.8)
3	1 (3.3)	3 (10.0)	3 (10.0)	7 (7.8)
4	8 (26.7)	5 (16.7)	8 (26.7)	21 (23.3)
5	11 (36.7)	12 (40.0)	13 (43.3)	36 (40)
Total (%)	30 (33.3)	30 (33.3)	30 (33.3)	90 (100)

$\chi^2 = 5.207$, $p = 0.735$ ($p<0.05$)

Bacteriological analysis (Table 2) showed that only one sample obtained from the supermarket was contaminated with only one bacterial genera (1: 1.1%). Multiple bacterial contamination with two different

bacterial genera was observed in 25 (27.8%) of the samples, three genera in 7 (7.8%) and four genera in 21 (23.5%) respectively. Multiple contamination of five bacterial genera and above was observed in 36 (40.0%) samples with the highest in this category coming from hawked samples (43.3%). All the samples were contaminated by at least one bacterial genera. The differences in multiple bacterial contamination between the three retail outlets were not statistically significant (p>.05).

TABLE 3: LEVEL OF MULTIPLE BACTERIAL CONTAMINATION IN CONSTITUENTS OF MEAT PIE FILLINGS FROM EATERIES, SUPERMARKETS AND HAWKERS

	Number of contaminants (%)			
Constituents of fillings	**Zero**	**1**	**2**	**3 – 7**
Carrot, meat and irish potato	0 (0)	17 (9.4)	15 (8.3)	16 (8.9)
Meat, onion and pepper	0 (0)	4 (2.2)	4 (2.2)	4 (2.2)
Meat and irish potato	1 (0.6)	22 (12.2)	17 (9.4)	20 (11.1)
Irish potato only	0 (0)	15 (8.3)	21 (11.7)	24 (13.3)
Total (%)	**1 (0.6)**	**58 (32.2)**	**57 (31.7)**	**64 (35.6)**

$\chi^2 = 4.430$, p = 0.881 (p>0.05)

The level of contamination in the various constituents of meat pie sample fillings was as shown in Table 3. Fillings of meat pie samples containing meat, onion and pepper were the least contaminated, whereas fillings made up of only irish potato where the most contaminated. Multiple contaminations involving three to seven (3 - 7) contaminants was the most observed. Chi-square results showed no statistically significant difference in the contamination levels in relation to the various filling constituents examined.

Most of the meat pie vendors at the sampled outlets ignored accepted point of sale practices. None of the vendors (90: 100%) used any covering such as hand gloves while selling the products and only 1 (1.11%) vendor used a cutlery and hair covering while serving. No vendor (0: 0%) used a service apron while serving. The least compliance with the accepted point of sale practices assessed was observed with the hawkers (Table 4).

TABLE 4: ASSESSMENT OF OBSERVANCE OF POINT OF SALE HYGIENIC PRACTICES BY MEAT PIE VENDORS

Point of sale practice assessed	**Hawkers (n = 30)**	**Supermarkets (n = 30)**	**Eateries (n = 30)**	**Total (N = 90)**
No hand gloves	**30 (100)**	**30 (100)**	**30 (100)**	**90 (100)**
No apron	**30 (100)**	**30 (100)**	**30 (100)**	**90 (100)**
Used cutlery	**0 (0.0)**	**1 (3.3)**	**0 (0.0)**	**1 (1.11)**
No cutlery	**30 (100)**	**29(96.7)**	**30 (100)**	**89 (98.9)**
Covered hair	**0 (0.0)**	**0(0.0)**	**1 (3.3)**	**1 (1.11)**
Uncovered hair	**30 (100)**	**30 (100)**	**29(96.7)**	**89 (98.9)**
Not talking while serving	**30 (100)**	**30 (100)**	**30 (100)**	**90 (100)**

Following assessment of some indicators of environmental hygiene, it was observed that only 5.6% (N = 90) of the vendors were assessed to be very neat, while only 11.1% (N = 90) of the vending sites were very neat. Altogether, 25.6% (N = 90) of the containers used for display of the product were observed to be partially open to air and dust while 24.4% (N = 90) were observed to be partially accessible to insects.

DISCUSSION

The results of this study demonstrate that both fillings and crusts of meat pie sold in Makurdi are contaminated with different bacterial types. Investigations revealed that addition of the fillings into the pastry before baking is a manual process and this practice may have contributed to bacterial contamination. Similar studies (8, 14, 15, 22, 23) have implicated most of the bacteria isolated in this study in meat pie and other ready-to-eat foods. *Bacillus* spp in meat pie raises an issue of concern since some species are known to cause food poisoning by preformed toxins in food or by the production of enterotoxins in the small intestine (24). The presence of *Escherichia coli* in food poses a threat to the health of consumers since it has been associated with traveler's diarrheoa and hemorrhagic colitis. Its presence is an indication of gross contamination and could be due to faecal contamination of the water sources and raw materials used during the production (8, 15, 18, 25). *Staphylococcus aureus* occurring in meat pie also requires attention because

of its enterotoxigenic ability even at a toxin dose of less than 1 microgram (26). The incidence of *Staphylococcus aureus* suggests excessive human handling, since the bacteria occurs as a normal flora of the human and animal skin (15).

The presence of bacterial contamination in all samples analyzed is indicative of high contamination rate in meat pie. Samples collected from hawkers registered the highest rate of multiple bacterial contamination. This is not surprising because of the sort of treatment they receive. In the process of vending, the hawkers expose the product to contaminated air and dust through the continuous opening of their show glasses in both clean and dirty environments. Some of the hawkers are found stationed around and within motor parks where the environment is often times, not clean and the air significantly contaminated.

TABLE 5: ASSESSMENT OF CONDITIONS AT POINTS OF SALE OF MEAT PIE

Indicators	Assessment	Hawkers (n = 30)	Supermarkets (n = 30)	Eateries (n = 30)	Total (N = 90)
Vendor assessment:	Very Neat	0(0)	0(0)	5(16.7)	5(5.6)
	Neat	5(16.7)	27(90.0)	25(83.3)	57(63.3)
	Fairly neat	25(83.3)	3(10.0)	0(0.0)	25(27.8)
	Dirty	0(0.0)	0(0.0)	0(0.0)	0(0.0)
	Very dirty	0(0.0)	0(0.0)	0(0.0)	0(0.0)
Site assessment:					
	Very Neat	0(0.0)	0(0.0)	10(33.3)	10(11.1)
	Neat	21(70.0)	28(93.3)	20(66.7)	69(76.7)
	Fairly neat	9(30.0)	1(3.3)	0(0.0)	10(11.1)
	Dirty	0(0.0)	1(3.3)	0(0.0)	1(1.1)
	Very dirty	0(0.0)	0(0.0)	0(0.0)	0(0.0)
Container state:	Not open to air	19(63.3)	18(60.0)	30(100)	67(70.3)
	Partially open to air	11(36.7)	12(40.0)	0(0.0)	23(25.6)
	Not open to dust	19(63.3)	18(60.0)	30(100)	67(70.3)
	Partially open to dust	11(36.7)	12(40.0)	0(0.0)	23(25.6)
	Not accessible to insects	19(63.3)	19(63.3)	30(100)	68(75.6)
	Partially accessible to insects	11(36.7)	11(36.7)	0(0.0)	22(24.4)

Findings of this study also show that fillings of meat pie sold in Makurdi are made of different materials, depending on the source. It was observed that fillings of the samples from eateries were made of carrot, Irish potato and minced meat. Some other samples obtained from eateries were made of onion and minced meat with plenty of pepper while samples from supermarkets contained only minced meat and irish potato; hawked samples were made up of Irish potato only. Although the level of contamination in relation to the constituents of the fillings did not differ statistically (χ^2 = 4.430, p = 0.881), it was observed that fillings made of Irish potato only, had a higher multiple bacterial contamination rate of 2 genera (57: 31.7%) and between 3 – 7 genera (64: 35.6%) respectively, compared to the other constituent types, making it the highest contaminated constituent type. Fillings made of minced meat, onion and pepper recorded fewer bacteria than others. Methanol, ethanol and acetone extracts of pepper and onion have been reported to inhibit growth of pathogenic bacteria of clinical origin such as *Escherichia coli, Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Proteus vulgaris* (27); *Escherichia coli* and *Pseudomonas florescence* (28); *Staphylococcus aureus* and *Klebsiella pneumoniae* (29); *Staphylococcus aureus* (30); and *Vibrio cholera* (31). Hence, the reduction in contamination observed with the peppered fillings may not be totally unlinked to the use of onion and pepper in their preparation.

Some vital point-of-sale best practices were observed to be neglected during sales of the product. It was observed that most of the vendors did not use any cutlery, hand covering, or hair cover while serving. The neglect of serving utensils does not conform to

the WHO requirements for point of sale operations. A higher rate of compliance with the use of serving utensils was reported in a study of ready-to-eat salad vegetables where only 31% (n = 1,985) of staff used bare hands to prepare or handle salad vegetables (32). No vendor in all the three retail outlets wore an apron. This finding is similar to the report of (33), though a higher usage of apron (30.8%) and hair covering (82.2%, n = 185) was reported to have been observed among food vendors in Ilorin, Nigeria. Another similar study by (34) reported that only 9(15%) out of 60 food vendors covered their hair, and only 31 (52.0%) wore an apron while serving food. Although wearing of hair covering and apron has more to do with food aesthetics and inspiring consumer confidence than food safety (19), it is the opinion of the researchers that this should be strongly encouraged among food vendors. Poor personal hygiene such as not washing hands and serving of the product without any cutlery by food vendors can lead to contamination of food (32). For instance, *Shigella* spp picked from infected diarrheal stool can be deposited unto food substances which when ingested, could cause the disease. Sweat and aerosols produced by handlers during talking or sneezing can introduce bacteria to the raw materials and may even re-contaminate the finished product (18).

Some of the show glasses used for display of meat pie by supermarkets and hawkers were observed to be accessible to air, dust and insects such as ants and flies. Regrettably, some of these outlets are situated in areas with high human and vehicular activities thereby exposing the product to contaminated air and dust from the environment. These could possibly have served as routes of contamination. Some of the show glasses assessed had openings large enough for insects to freely go through. This could lead to the depositing of bacteria from other sources in the environment onto the internal surfaces of the show glass or directly on meat pie as they are displayed for sale. Exposure of some vended food to flies has also been reported (33, 35). A study by (34) reported that about 55.0% of vendors adequately protected their food from flies and dust whilst 45.0% had no protection, thus exposing their food to flies and dust. The WHO recommends as a requirement at the point of sale, that food should be sold in a clean environment protected from sun, dust, rain, wind and insects (19). Appropriate regulatory authorities should hence step up monitoring of food retail outlets to ensure compliance to accepted point of sale practices. Periodic food safety awareness and education for food vendors will also go a long way to increase observation of acceptable point of sale and good food handling practices.

An assessment of both the vendors and the vending environment showed that vendors and vending environments of eateries were generally neater, followed by those of supermarkets. In a similar study by (35), 259 (90.5%) of the food vending environment assessed were judge to be clean. The high level of compliance with neatness observed in this study is commendable. Only hawkers were observed not to be satisfactorily clean. A deliberate effort at sensitizing meat pie hawkers on the need to dress neatly while vending meat pie could go a long way to encourage neatness among this group.

CONCLUSION

The study has observed that some important and recommended point of sale practices were neglected by some of the vendors at the retail outlets studied and these could contribute to the contamination of the product and endanger the health of consumers. Meat pie vendors and retail outlets should continue to maintain neatness as it is not only suggestive of health consciousness, but also has the ability to boost the confidence of their customers, thereby increasing patronage. Periodic food safety awareness and education for food vendors is also advocated as a possible way to increase observation of acceptable point of sale and good food handling practices.

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