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RESEARCH ARTICLE

MICROBIOLOGICAL INVESTIGATION AND VIRULENCE FACTOR CHARACTERIZATION OF CHRONIC SUPPURATIVE OTITIS MEDIA IN A NIGERIAN TERTIARY HOSPITAL

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ABSTRACT

Background: Chronic suppurative otitis media (CSOM) is a common cause of hearing loss with the attendant poor academic performance and low productivity in workplaces.
Objectives: This study investigated the microbial aetiology and associated virulence mechanisms of CSOM in a Nigerian tertiary hospital.
Methods: This was a prospective study of 52 patients diagnosed with CSOM in either one or both ears who had not taken any antibiotics for at least the last seven (7) days. Ear swabs were taken by an otolaryngologist and sent to the Microbiology Department for processing. The specimens were cultured within 30 min of collection and the isolates identified using standard microbiological techniques. Selected virulence properties were characterized.
Results: The most common organism isolated was Staphylococcus aureus 18 (34.6%) followed by Pseudomonas aeruginosa 14 (26.9%). Fungal isolates were recovered from 6 11.5%) of specimens; in 11 (21.15%) of cases no microbe was detected. Age did not significantly affect incidence of CSOM of microbial origin ($p = 0.1742$) although most cases were in the age 31-40 years group. Male and female patients were almost equally affected (M: F = 1:1.08), DNase followed by biofilm formation were the predominant virulence phenotypes identified while coagulase, followed by haemolysin production, were least common.
Conclusion: Staphylococcus aureus and Pseudomonas aeruginosa were the most common organisms among CSOM cases and recovered from all age-groups. Early detection and infection control are key to reducing incidence of CSOM.

KEYWORDS: Microbiological, Virulence, Chronic, Suppurative, Otitis Media.

INTRODUCTION

Otitis media is the medical term for the infection of the middle ear with resultant inflammation. It occurs commonly as acute otitis media or as otitis media with effusion, although other forms, including chronic suppurative otitis media (CSOM) and adhesive otitis media, have also been identified.[1,2] CSOM has been identified as a major cause of acquired hearing impairment in children.[2] It is a recurrent inflammation localised in the middle ear or mastoid cavity[3] and results from infection with pathogens that proliferate when there is inflammation, trauma, high humidity or lacerations. CSOM manifests as persistent middle ear exudates due to a tympanic perforation with conductive hearing loss of varying degrees.[2,4,5]

Incidence of CSOM has been reported in developing[6,7] as well as developed countries.[7,8,9] When poorly managed, CSOM will result in hearing impairment, disability and poor performance in school, fatal intracranial infection and acute mastoiditis.[3] The disease is associated with

malnutrition, overcrowding, poor hygiene and frequent infections of the upper respiratory tract.[10,11]

Although more than 50% of the cases of CSOM and otitis media are caused by bacteria,[9] occasionally there are cases of CSOM of fungal[12,13] or viral origin.[14,15]

Studies conducted in specific areas give insights into possible circulating virulence factors and their consequences, as well as assist clinicians with gaining knowledge into the kind of pathogens implicated in CSOM. These will help in developing educational programmes towards improving ear health. The present study examined microbial aetiology and virulence mechanisms for cases of CSOM in Nnamdi Azikiwe University Teaching Hospital (NAUTH), Nnewi, Southeast, Nigeria.

RESEARCH METHODS AND DESIGN

Ethical Considerations

The study protocol was approved by the ethical board of Nnamdi Azikiwe University Teaching Hospital, Nnewi, Nigeria. Approval number: NAUTH/CS/66/VOL4/90.

Selection and Description of Participants

This study was a prospective study of all consecutive patients that presented to the Otorhinolaryngology clinic of Nnamdi Azikiwe University Teaching Hospital from June to December 2013. All new patients (no age limit and no gender preference) that presented with chronic middle ear discharge (discharge persisting for at least three months) and persistent tympanic membrane perforation, and who had not been on any antibiotic for at least 7 days were recruited into the study. Patients that had masses or aural polyps, middle ear bleeding or previous ear surgery were not recruited. Recruitment and sample collection were done by the otorhinolaryngologist who evaluated the subjects for age, gender, otomicroscopy, audiologic findings and duration of illness.

Microbiological Investigation of The Samples

All patients with discharge from one or both ears had ear swabs taken by an otorhinolaryngologist for microscopy. The samples were appropriately cultured by streaking onto chocolate agar in a CO_2 enriched atmosphere, blood agar and MacConkey agar plates and incubated for 24 h at 37 °C. After the incubation period, the colonies were further sub-cultured and streaked onto a freshly prepared agar plates until pure colonies of the organisms were obtained. Microbial growth was further analysed using Gram staining, biochemical tests and analytical profile index for proper characterisation and identification of the organisms.

Virulence Factors Characterisation

The following tests were performed to characterize microbial virulence traits.

DNase Activity

The test for DNase activity is a traditional, easy and cost-effective test for the identification of some pathogenic organisms such as *Staphylococcus* spp. and some enterobacteriaceae. Modified Barrow and Feltham[16] and Narayana et al.[17] methods were used to test for DNase activity among *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans*, *Aspergillus fumigantus, and Streptococcus pneumonia*

isolates. Briefly, using DNase agar supplemented with 1% glucose, overnight pure culture of each isolate (except *Streptococcus pneumoniae)* was spotted onto DNase agar plates and incubated at 37 °C for 48 h. Also, DNase agar supplemented with salmon sperm DNA (2 μg) was spotted with a loop immersed in pure overnight cultures of *Streptococcus pneumoniae* (10^7) cells and incubated at 37 °C for 1 h followed by treatment with 0.5 M Ethylenediamine teyracetic acid (EDTA) to stop the reaction. The plates were then flooded with 0.1% 1 N Hydochloric acid (HCl. The development of a red colour or a zone of clearing around the spotting indicated secreted DNase activity.

Haemolysin Production

Haemolysin production was evaluated using the modified Manns et al.[18] method. Briefly, pure cultures of isolates grown overnight were streaked onto freshly prepared 5% defibrinated sheep blood agar in a trypticase soy agar base (Difco), supplemented with 2% glucose and incubated at 37 °C for 72 h. A clear halo around the inoculum spot indicated *β*-haemolysis, whereas a greenish zone indicated alpha (α)-haemolysis.

Bile Hydrolysis

Isolates were tested for ability to hydrolyse esculin as a carbon source by using an esculin hydrolysis test on a bile esculin agar slant (a nutrient agar-based medium containing 0.1% esculin and 10% bile salts) supplemented with 2% glucose. A pure culture of each isolate grown

overnight was streaked aseptically on the surface of a sterile bile esculin agar slant and incubated at 35 °C –37 °C for 24 h. When the isolates metabolise esculin in the nutrient medium, the dark brown compound esculetin is produced. Thus, blackening of the medium indicated a positive test for growth in the presence of bile.

Coagulase Activity

Coagulase is an enzyme produced by pathogenic *S. aureus*. The enzyme converts fibrinogen in plasma to the insoluble fibrin. The test for coagulase activity is used to differentiate *Staphylococcus aureus* (positive) from coagulase-negative *Staphylococcus*. The tube method was used in this study. One ml of a 1-in-6 dilution of rabbit plasma in 0.85% NaCl was placed in small tubes. Isolated bacterial colonies (5–6) of test organisms were emulsified in the 1 ml of the diluted plasma and incubated at 35 °C in a water bath for 4 h. At 1, 2 and 4 h intervals, the tubes were examined for clot formation. Negative tubes were left at room temperature overnight and re-eamimed. Tubes with no growth the following day were considered negative.

Phospholipase Activity

Phospholipases are a ubiquitous group of enzymes that hydrolyse phospholipids thereby causing tissue injury and infection persistence. Phospholipase activity was determined using modified methods described by Kumar et al.[19] and Mobarak-Qamsari et al.[20] Briefly, fungal isolates were revived from stock cultures maintained on Sabouraud's dextrose agar slopes at 4 °C and later transferred on to fresh Sabouraud's dextrose agar plates and incubated at 37 °C for 24 h – 48 h. 50 μl of the fresh culture equivalent to 0.5 McFarland turbidity standard was aseptically inoculated by spotting (6 mm) onto Sabouraud's dextrose agar plates containing 1 M NaCl, 0.005 M $CaCl_2$ and 8% sterile egg yolk emulsion and incubated at 35 °C for 5 days. A positive phospholipase activity (checked daily) was taken as visible solid particles around the colony on the plate. Also, a loopful of 18 h pure bacteria colonies was streaked on to tributyrin agar plate and incubated at 37 °C for 24 h, then observed for zone of hydrolysis around the colony. The appearance of a clear halo around the inoculum spot was indicative of lipase production. In both methods, the diameter of colony and total diameter of colony and precipitation zone were measured and phospholipase activity index (defined as the ratio of the diameter of the colony to the total diameter of the colony plus the precipitation zone) was calculated. A phospholipase activity index of less than one indicated phospholipase production by the isolate. Low phospholipase activity index indicated stronger enzyme activity.

Protease Activity

Microorganisms that harbour protease enzymes are able to hydrolyse peptide bonds in protein molecules thereby destroying the protein structures of the host. The production of protease enzymes by the isolates was tested using modified methods described by Kumar et al.[19] and Mageswari et al.[21] Briefly, bovine serum albumin agar containing yeast carbon base (1.17%) were inoculated by spotting (6 mm) with 50 μl 0.5 McFarland standards of the fresh fungal culture and plates incubated at 37 °C for up to 5 days. The plates were then stained with 0.5% amido black and the zone of clearance around the colony was recorded as positive protease production. Scoring was carried out by determination of the proteinase zone value as for phospholipase activity; low protease activity index indicated stronger enzyme activity. Also, skim milk agar supplemented with 5% NaCl and 1% casein (HiMedia Laboratories) was inoculated with 18 h pure bacteria culture and incubated overnight at 37 °C for 24 hr. A clear zone, resulting from casein hydrolysis, seen around the inoculum spot was taken as a proof of enzyme production.

Biofilm Formation

The tube adherence test previously reported by Christensen et al.[22] and Yigit et al.[23] was used.

Acid Lability Test

The Edberg et al.[24] method was used.

DATA ANALYSIS

GraphPad Prism version 5.00 for Windows (GraphPad Software, Inc. San Diego California, United States, (www.graphpad.com) was used to analyse the data. One-way and two-way analysis of variance was used to check for mean differences in occurrence of the microbial isolates, as well as the effect of age on the incidence of CSOM of microbial origin. Student's *t*-test was used to check for mean differences in occurrence among both sexes, between the microbial and non-microbial CSOM and between bacterial and fungal CSOM. All *p* values reported are for a two-tailed test. The significance level was chosen at $\alpha = 0.05$.

RESULTS

Fifty-two patients (25 males and 27 females aged between 1 and 90 years) participated in the study (Table 1). Occurrence of infective organisms did not differ by sex in the study centre ($p = 0.8678$).

TABLE 1- Incidence of Microbial Isolates of Chronic Suppurative Otitis Media by Sex

Microbial Isolates	**Sex**		**Total**	***P***
	Male	**Female**		
Staphylococcus Aureus	10	8	18	0.8678
Pseudomonas Aeruginosa	7	7	14	
Streptococcus Pneumoniae	1	2	3	
Candida Albicans	1	3	4	
Aspergillus Fumigatus	1	1	2	
No Microbe Detected	5	6	11	
Total	**25**	**27**	**52**	**-**

A total of 41 (78.9%) samples yielded microbial growth in the media used (Table 2). Three bacterial species (*Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Streptococcus pneumoniae*) and two fungal species (*Candida albicans* and *Aspergillus fumigatus*) were cultured; *Staphylococcus aureus* was most common (18 cases (34.6%)). One-way analysis of variance revealed that there was statistically significant difference in the occurrence of the different microorganisms ($p < 0.0001$) and that age did not significantly affect the pattern of microbial organism ($p = 0.1742$). However, the age 31–40 years group had the largest number of cases. Dunnett's multiple comparison test showed that in any particular age group, there was a statistically higher chance of isolating *Staphylococcus aureus* or *Pseudomonas aeruginosa* compared with other microorganisms in cases of CSOM in the study ($p < 0.05$). Student's *t*-test showed a significantly higher occurrence of bacterial CSOM compared with fungal CSOM ($p = 0.0007$) and higher occurrence of CSOM of microbial origin compared with CSOM of non-microbial origin ($p = 0.0038$).

TABLE 2- Frequency of Isolates by Patient Age Group

Age (Years)	**Bacteria**			**Fungi**		**Total Microbial Isolates (A)**		**Non-Microbial Origin (B)**	**Grand Total (A + B)**
	Staphylococcus aureus	***Pseudomonas aeruginosa***	***Streptococcus pneumoniae***	***Candida albicans***	***Aspergillus fumigatus***	**N**	**%**		
≤ 10	4	1	0	0	1	6	14.63	2	8
11–20	3	2	1	1	0	7	17.07	0	7
21–30	2	2	0	2	0	6	14.63	1	6
31–40	4	3	1	1	1	10	24.39	2	12
41–50	0	1	0	0	0	1	2.44	1	2
51–60	1	2	0	0	0	3	7.32	1	4
61–70	1	1	1	0	0	3	7.32	2	5
71–80	2	1	0	0	0	3	7.32	1	4
81–90	1	1	0	0	0	2	4.88	1	3
Total	**18**	**14**	**3**	**4**	**2**	**41**	**100**	**11**	**52**

DNase was the predominant virulence characteristic exhibited by the isolates, followed by biofilm formation, while coagulase followed by haemolysin production was the least common virulence property (Table 3). A two-way analysis of variance of the results showed that the isolates accounted for 85.55% of the total variance. The *p* value was less than 0.0001. The effect of the isolates in the infectivity properties (virulence) is considered

extremely significant showing that the isolates were highly (significantly) virulent. Also, the kind of the virulence factors possessed by the isolates accounted for 3.27% of the total variance, although the difference was not statistically significant (*p* value = 0.3499).

TABLE 3- Virulence Phenotypes by Microbe Type

Virulence Factors	Microbe										Total with Virulence Factor	
	S. Aureus		*P. Aeruginosa*		*S. Pneumoniae*		*C. Albicans*		*A. Fumigatus*			
	N	**%**	***N***	**%**	***N***	100.00	***N***	**%**	***N***	**%**	***N***	**%**
Haemolysin Production	15	83.00	9	64.00	3	100.00	0	0.00	0	0.00	27	65.85
DNase Activity	18	100.00	14	100.00	3	100.00	3	75.00	2	100.00	40	97.56
Bile Hydrolysis	12	67.00	14	100.00	3	0.00	4	100.00	2	100.00	35	85.37
Coagulase Activity	18	100.00	0	0.00	0	100.00	4	100.00	0	0.00	22	53.66
Phospholipase Activity	14	78.00	10	71.00	3	67.00	3	75.00	2	100.00	32	78.05
Biofilm Formation	17	94.00	13	93.00	2	100.00	4	100.00	2	100.00	38	92.68
Protease Test	14	78.00	11	79.00	3	100.00	1	25.00	0	0.00	29	70.73
Acid Lability Test	18	100.00	9	64.00	3	-	1	25.00	1	50.00	32	78.05
Total Number of Organisms Tested	**18**	**-**	**14**	**-**	**3**	**-**	**4**	**-**	**2**	**-**		

DISCUSSION

The implicated microorganisms in CSOM aetiology are usually bacteria and fungi and at times, viruses.[14,15] In our study, infection occurred equally in patients of both sexes. *Staphylococcus aureus* 18 (34.62%) and *Pseudomonas aeruginosa* 14 (26.92%) were the most common pathogens implicated in the aetiology of CSOM in this study. There was no significant difference in the occurrence of *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Staphylococcus aureus though a remarkably versatile bacterium found in almost every kind of host and targets could equally be pathogenic in a human host.[25,26] Thus, its widespread presence in the sampled CSOM cases in this study comes as no surprise. Its presence raises issues of the ease and efficiency of propagation and the continuing challenge of the infection burden of *Staphylococcus aureus*, which if improperly treated may continue to develop and disseminate single or multiple-drug resistant strains.[27] Because it is found on skin, it can easily be transferred from the skin to the ear in this era of common use of ear-pieces for both making telephone calls and listening to music or radio. *Pseudomonas aeruginosa* is an opportunistic pathogen causing multifarious infections and is mostly associated with sepsis, bones and soft tissues where important organs could also be targeted.[28] Serious and widespread antimicrobial resistance issues have also been associated with *Pseudomonas aeruginosa*.[29] Although traditionally well known to be a very serious threat in wound infection and septicaemia, the consistent presence of *Pseudomonas aeruginosa* among the CSOM patients in this study constitutes a huge burden. High incidence of these two organisms in CSOM patients has been reported in several other independent studies inside and outside of Nigeria.[30,31,32,33]

Lysenko et al.[34] recovered also fungal organisms – *Candida albicans* and *Aspergillus fumigatus* – similar to our findings. Although the incidence and number of fungal isolates from our study was limited, the presence of fungal pathogens in middle-ear infections could still portend serious chemotherapeutic challenges, since it may present with chronicity and increased mobidity. In another related study carried out in Benin City, Nigeria, fungal isolates represented 21.2% of all the cases.[30] The absence of fungal and bacterial isolates in 11 out of the 52 cases of CSOM in our study supports the claim that there are other possible causes of CSOM including viruses, and less common bacteria, such as *Moraxella* catarrhalis and *Haemophilus species*.[14,15]

When we considered the age distribution of infected patients, we clearly observed a skew towards the the third- and fourth-decades category. Our findings that more patients in the age 31–40 year group suffered from microbial-induced CSOM (although this was not significantly different: $p = 0.1742$) compared with patients from other age groups differs to the outcomes of other studies previously conducted in Nigeria, which found infection more frequently among children aged 0–5 years,[30] young adults[35] and the age group 15 years and younger.[36] This disparity could be a result of differences in the study locality or environmental change-driven epidemiological demographics. In addition, none of the previous studies we reviewed included elderly patients (aged 81–90 years), as was the case our study.

Microbe-induced CSOM as a condition driven by microbial infection of the middle ear represents both present and future management challenges. Successful management is usually a combination of several measures, including correct and early diagnosis and the careful administration of effective antibiotics.[1] When the use of antibiotics is warranted, a wide range of antimicrobial agents such as co-amoxiclav, gentamicin, lincomycin, amoxicillin, ampicillin, erythromycin, chloramphenicol and tetracycline are normally prescribed in Nigeria for the treatment of CSOM of bacterial origin. Usually, appropriate selection of effective antimicrobial agents is preceded by culture and sensitivity laboratory test outcomes where the comparative efficacies of different antibiotics are assessed prior to clinical application and utility against the infecting microorganisms. Given that use and control of antibiotics in developing countries remain weak and often antibiotic abuse is common leading to resistance to previously effective antibiotics,[37] it is important to encourage proper screening and surveillance exercises, so as to continue to keep abreast of developments and changing microbiological dynamics surrounding any microbial-driven disease condition such as CSOM.

Possession of virulence factors by microbial agents has been reported as one of the causes of resistance to antimicrobial agents.[38,39,40] It is equally important that careful selection of antibiotics is done following diligent antibiotics susceptibility studies.

Pathogenic organisms usually express multiple virulence factors that can damage the host tissues and thus contribute to their pathogenesis. They can modify their virulence mechanisms to escape host defence systems. Human pathogenic *Staphylococcus aureus*, for instance, secretes coagulase, which converts fibrinogen to fibrin, thereby promoting blood or plasma clotting.[41] This is an important virulence characteristic necessary for *Staphylococcus aureus* infection pathogenesis generally and in CSOM especially. This is the basis for the formation of purulent exudates and bacterial persistence in host tissues.[42] Also, haemolysin production is common in *Staphylococcus aureus*, *P. aeruginosa*, *S. pneumoniae* and other bacteria. This helps the bacteria in degrading the haem component of erythrocytes. Haemolysin production is mainly common in CSOM as opposed to acute otitis media[43] and CSOM patients are more susceptible to anaemia than acute otitis media patients.[44] Once pathogens infect the middle ear or mastoid cavity and are able to express DNase, progression to CSOM is easy, because the DNase will enable the pathogen escape destruction by neutrophils, thereby enhancing microbial pathogenesis. Other established roles of microbial DNases in infection pathogenesis include hydrolysis of host nucleic acids to yield oligonucleotides needed for microbial growth and biofilm maturation.[45,46] As much as 98% of the isolates in our study possessed this virulence factor.

Bile salts also control microbial pathogenesis. Several pathogenic organisms carry out bile salt hydrolysis and hydroxy group dehydrogenation reactions.[47] Hydrolysis plays an essential role in the metabolism of fats and oil. Pathogens that colonise the middle ear or mastoid cavity are able to metabolise the oily wax of the ear by secreting bile salt hydrolase proteins. In this way, they can establish infection and cause CSOM. Cerumen impaction may hide these organisms and later lead to disease progression.

Phospholipase and proteinase activities are important virulence mechanisms in a number of pathogens including *Candida* species.[48] They aid in the digestion of the phospholipid bilayers and protein component of the host cell thereby enhancing microbial penetration into the host. Biofilm formation is a major virulence mechanism in microbial pathogenicity and represents a serious threat to antibiotic therapy of bacterial and fungal infections.[49] The isolates in our study demonstrated the ability to form biofilms. Their colonisation of the middle ear and consequent formation of biofilms could enhance antibiotic resistance and progression to CSOM. Hyaluronic acid capsule, lactic acid, capsular sialic acid and fatty acids produced by microbes act as antiphagocytic factors in

several microbial species.[50,51,52] In some cases, sialic acid produced by the host cell may be copied (molecular mimicry), used as carbon, energy and nitrogen sources and precursors of microbial cell wall synthesis or used for cell signalling by mammalian pathogenic microbes.[53]

Infection prevention is the key to reducing incidence of microbe-associated CSOM and microbial infections generally.[54] In many Nigerian villages, people still bathe in contaminated ponds and rivers, practise unsterile ear piercing and clean their ears with unsterile cotton buds. Although CSOM is a multifactorial persistent inflammatory infection of the middle ear, it has similar origin in adults and children. Behavioural change is therefore recommended and educational programmes need to be developed and mounted, especially in rural communities and primary schools with the goal of infection prevention and control. If these are done, the prevalence of ear diseases and other infections will reduce drastically.

LIMITATIONS

A major limitation to our study is the low population studied. Further studies with even larger population would be required for validity.

CONCLUSION

Staphylococcus aureus and *Pseudomonas aeruginosa* were the most common organisms detected in CSOM cases in our study, regardless of age group. DNase followed by biofilm formation were the most common virulence properties exhibited by the isolates. Early detection and infection control are key to reducing the incidence of CSOM. It is therefore recommended to prevent contamination by these organisms through the adoption of hygienic practices, mass education through all available public and social media and regular ear check-ups.

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Competing Interests

The authors declare that they have no financial or personal relationships that may have inappropriately influenced them in writing this paper.

Authors' Contributions

I.M.E. designed and conceptualised the study, E.E.A. did diagnosis and sample collection, O.I.G. did the laboratory work and results collation and interpretation, EEA, IME and OIG drafted and finalised the manuscript. All authors read and agreed to its content. The paper has not already been published in any journal and is not under consideration by any other journal.

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