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Research Article
Prevalence, Biofilm Formation and Anti-microbial Susceptibility Testing of Bacterial and Fungal Etiological Agents Associated with External Ocular Infections in Nigeria

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Abstract

Background and Objective: Infections of the cornea, eyelids, conjunctiva and other external parts of the eye can infrequently spread to adjacent tissues where they result in severe complications, blurred vision or blindness if left untreated. There is a paucity of data on the epidemiological profile of ocular infections in Nigeria. To the best of our knowledge, this present study represents the first which tries to survey both bacterial and fungal agents responsible for external ocular infections in Enugu and Ebonyi states, Nigeria. An important virulence factor of organisms which cause external eye infections is their ability to form biofilm, especially on contact lenses. The aim of this present study was therefore to isolate and identify bacterial and fungal isolates causing external ocular infections in some parts of Enugu and Ebonyi states, check for their antibiotic susceptibility profile and ability to form biofilms on catheter discs. **Materials and Methods:** Well-organised questionnaire was used in collecting relevant demographic data of the study participants. Corneal and lid margin samples, in addition to conjunctival scrapings, were collected and plated on blood, nutrient, MacConkey, Eosin methylene blue and Sabouraud dextrose agar, respectively. The isolated colonies, were subjected to standard microbiological procedures for identification. The susceptibility profile of isolates to different antimicrobial drugs was determined using the disk-diffusion method. The biofilm quantification was determined using the crystal violet binding method. **Results:** Out of a total of 133 eye infected cases considered in this study, 97 were conjunctivitis while blepharitis and keratitis were found in 23 and 13 cases, respectively. A demographic study of the eye patients showed a large number to be rural dwellers (72.18%), with farming forming the major occupation (60.90%). Culture-processing of the collected ocular samples led to the recovery of 122 microbial isolates, with *Staphylococcus aureus* being the predominant isolate (37.7%), followed by *Pseudomonas aeruginosa* (31.2%), *Escherichia coli* (13.9%), *Bacillus cereus* (9.8%) and *Aspergillus fumigatus* (7.4%). While most of the bacterial isolates were susceptible to the tested antibiotics, *P. aeruginosa* showed high resistance (100%) against augmentin, amoxicillin and chloramphenicol. *A. fumigatus*, the only fungal isolate in this study was resistant to all the tested antifungal drugs. Biofilm quantification showed only 28.57% of the tested *P. aeruginosa* isolates to be moderately adherent, while the tested *S. aureus* and *A. fumigatus* strains were classified as non-adherent. **Conclusion:** *Staphylococcus aureus* is the predominant cause of external eye infections in this part of Nigeria. Agricultural occupation can be considered a risk factor for these infections. Due to the rise of antibiotic resistance, the susceptibility profile of implicated isolates should be considered before administering relevant drugs. More work should be done to determine the ability of ocular pathogens to form biofilms on contact lenses.

Key words: Eye infection, Nigeria, bacteria, fungi, biofilm

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Though usually localised, external ocular infections-infections of the cornea, eyelids, conjunctiva and other external parts of the eye can infrequently spread to adjacent tissues where they result in severe complications, blurred vision or blindness if left untreated[1-3]. Though viruses and parasites have been implicated in their aetiology, bacteria and fungi represent the major causative agents of these infections. They cause inflammation and loss of important epithelial cells in the eye leading to keratitis, cataract, glaucoma, blepharitis and conjunctivitis. Predisposing factors to these infections include but are not limited to socio-economic status, personal cleanliness, nutrition, age, trauma, genetic constitution and general living conditions[1,3-5].

The severity of the infection has also been heightened by the escalating cases of antimicrobial resistance, important "emergency" in the medical world. This increases the risk of treatment failure with the attendant serious health implications. Antimicrobial resistance keeps on evolving; thus, there is a need for updated information on antimicrobial therapy for ocular infections[3,4].

There is a paucity of data on the epidemiological profile of ocular infections in Nigeria. To the best of our knowledge, this present study represents the first which tries to survey both bacterial and fungal agents responsible for external ocular infections in Enugu and Ebonyi states, Nigeria. An important virulence factor of organisms which cause external eye infections is their ability to form biofilms, especially on contact lenses. In this sessile state, they are able to persist in the lenses with high stability to several disinfectant systems. There are few works on the biofilm-forming ability of ocular pathogens. In this study, we isolated different bacterial and fungal pathogens responsible for external ocular infections in these areas of Nigeria and investigated their ability to resist common anti-microbial agents and form biofilms on catheter discs.

MATERIALS AND METHODS

Study population and sample collection: Samples were collected from designated eye clinics in Enugu and Ebonyi states, all in the south-eastern part of Nigeria. A total of 133 eye patients were included in this study. Patients who had antibiotics treatment within the last 5 days or who had undergone previous ocular surgery within the last 7 days of recruitment of study subjects were excluded from the study. The clinical features of the eye infections were first determined by the ophthalmologists in charge of the eye centres, while moistened sterile swab sticks were used in collecting samples from the purulent conjunctivitis, eyelids and cornea of patients suffering from external ocular infections. The samples were immediately transported to the laboratory of the Department of Microbiology, University of Nigeria, Nsukka for analysis. Ethical approval was gotten from the management of clinical centres while informed written consent was gotten from patients or parents/guardians (for children). The study participants were also asked to fill a well-designed questionnaire in order to get a reflection of their demographic profile.

Isolation and characterization of bacterial and fungi pathogens: The collected specimens were inoculated onto the following media: MacConkey agar, blood agar, mannitol salt agar, nutrient agar and sabouraud dextrose agar and examined for growth after 24-48 h incubation at 37°C. Specimens were considered culture-positive using the threshold criteria for the diagnosis of ocular infections[6]. Standard microbiological procedures were used in identifying bacterial and fungi isolates from the culture-positive specimens.

Antimicrobial susceptibility test: The susceptibility of the bacterial and fungi pathogens to different anti-microbial agents were determined using the disk diffusion method[3].

Biofilm quantification assay: A modified method of Adetunji and Isola[7] was used in the preparation of the isolates for biofilm assay. Briefly described, sterile catheter discs were put in a 50 mL broth containing either grown cultures of the bacterial or fungi isolates. The negative control contained everything in the test except the isolates. The media was then incubated at room temperature (26-28°C) for 24-72 h. At 24 and 72 h intervals, the discs were sampled for biofilm formation using the crystal violet binding method of Stepanovic *et al.*[8] with slight modifications. Here, the catheter discs removed from both the bacterial and fungi broth were washed thrice to remove any cell which have not adhered. While the discs from the bacterial broths were fixed with 2.5 mL of methanol per disk and stained with 0.5% (w/v) of crystal violet for 15 min, the discs from the fungal broth were directly stained with the crystal violet for 30 min. After the staining process, the discs were washed under running tap to remove excess stain and allowed to air-dry. The dye bound to the adherent cells in each discs was solubilised using 2.5 mL of 33% glacial acetic acid. The optical density of each re-solubilised liquid was determined at 620 nm using a spectrophotometer (722s, B. Bran Scientific and Instrument Company, United Kingdom). The re-solubilised liquid from the negative control was used as the blank.

Statistical analysis: Microsoft Office Excel and Statistical Package for the Social Sciences (SPSS v20) were used in data analysis. The assay to check for biofilm formation was repeated two times separately for each isolate, with the results being represented as mean±standard deviation. Differences in the degree of biofilm formation were examined by the Friedman test. The $p<0.05$ were considered significant.

RESULTS

Out of the 133 patients included in this study, 51.88% (n = 69) were females while the remaining (48.12%) were males. Majority of the study participants were rural dwellers (72.18%), with farming forming the major occupation (60.90%). Other demographic data of the study participants are shown in Table 1. After processing the 133 collected ocular samples, 108 were found to be culture-positive with a total of 122 bacterial and fungal isolates recovered. The clinical characterization of the external eye infections showed a majority of the study participants suffer from conjunctivitis (n = 97), followed by blepharitis (n = 23) and keratitis (n = 13). As shown in Table 2, *S. aureus* and *P. aeruginosa* represent the most implicated species in the study, with a prevalence of 37.7 and 31.2%, respectively. Other recovered species in this study include *E. coli* (13.9%), *B. cereus* (9.8%) and *Aspergillus fumigatus* (7.4%). More males (56.70%) than females (43.30%) suffered from conjunctivitis while age 46-65 years represents the most affected by external ocular infections (Table 3). When tested against a variety of drugs normally active against Gram-positive bacteria, most of the *S. aureus* isolates (61.36 and 59.10%) were, respectively resistant to amoxicillin and ampiclox, but were highly susceptible to other drugs used in the study (Table 4). *Aspergillus fumigatus* was resistant to all antifungal drugs which include clotrimazole, amphotericin B, fluconazole and miconazole.

The biofilm quantification of only multi-resistant strains was considered. Spectrophotometric measurement of optical densities (OD) of adherent cells was used in classifying the clinical isolates into four categories: non adherent ($OD \leq 0.2$), weakly ($0.2 < OD \leq 0.4$), moderately ($0.4 < OD \leq 0.8$) and strongly ($OD > 0.8$) adherent strains. Of the 14 muti-resistant *P. aeruginosa* isolates examined, 8 (57.14%) were designated as non-adherent, 2 (14.28%) as weakly and 4 (28.57%) as moderately adherent. On the other hand, all the *S. aureus* (11) and *A. fumigatus* (9) isolates tested yielded values below 0.2, hence were classified as non-adherent.

Table 1: Socio-demographic profile of participants in the present study

Demographic particulars	Number	Percentage
Age (years)		
≤18	5	3.76
19-45	47	35.34
46-65	61	45.86
> 66	20	15.04
Locality		
Rural	96	72.18
Urban	37	27.82
Gender		
Male	64	48.12
Female	69	51.88
Occupation		
Government employee	19	14.29
Farmer	81	60.90
Housewives	10	7.52
Retired	7	5.26
Unemployed	16	12.03

Table 2: Prevalence of isolates across different clinical spectrum of external eye infections

	Recovered in different external ocular infections (%)		
Implicated species (%)	Blepharitis	Conjunctivitis	Keratitis
Staphylococcus aureus (37.7)	30.4	40.2	0.0
Pseudomonas aeruginosa (31.2)	13.0*	35.1	7.7*
Escherichia coli (13.9)	8.7*	15.5	0.0
Bacillus cereus (9.8)	26.1	5.2	7.7*
Aspergillus fumigatus (7.4)	0.0	4.1	38.5

*Mixed infections

Table 3: Distribution of external ocular infections among different gender and age groups

	Sex (n)		Age (n)			
Clinical infection	Male	Female	<18	19-45	46-65	66
Conjunctivitis (n = 97)	55	42	5	32	48	12
Keratitis (n = 13)	5	8	0	3	4	6
Blepharitis (n = 23)	4	19	0	12	9	2
Total (n = 133)	64	69	5	47	61	20

n: Number of occurrence

Table 4: Antimicrobial resistance patterns of the bacterial isolates

	Number of isolates (%) resistant to Gram-positive drugs									
Isolates	CN	APX	RD	AMC	S	NB	C	CIP	E	LEV
Staphylococcus aureus	11.36	59.10	11.36	61.36	2.30	2.30	2.30	2.30	0.00	0
Bacillus cereus	0	38.46	23.08	69.23	7.69	7.69	7.69	7.69	7.69	0
	Number of isolates (%) resistant to Gram-negative drugs									
Isolates	SP	CIP	AMC	AU	CN	PEF	OFX	S	C	
Pseudomonas aeruginosa	12.82	12.82	100	100	13.33	0	7.69	100	100	
Escherichia coli	0	0	100	64.70	0	0	0	0	0	

%: Percentage frequency of occurrence, C: Chloramphenicol, CIP: Ciprofloxacin, AMC: Amoxicillin, CN: Gentamicin, E: Erythromycin, LEV: Levofloxacin, APX: Ampiclox, NB: Norfloxacin, RD: Rifampicin, S: Streptomycin, SP: Sparfloxacin, AU: Augmentin, PEF: Pefloxacin, OFX: Ofloxacin

DISCUSSION

External ocular infections remain a major health concern, especially among rural dwellers[9,10].In this study, majority of the patients (60.90%) were farmers who reside in the rural areas. This high prevalence may not be unconnected to a high risk of corneal injury during agrarian activities, which ultimately serve as a predisposing factor for external eye infections[3,6]. The high occurrence of external eye infections among adults (19-45 and 46-65 years) considered in this study is not surprising since the study participants are coming from a working background where they are exposed to different hazards that can cause ocular infections[1]. Out of the three external eye infections considered in this study, conjunctivitis had the highest prevalence (72.93%) and more males than females were diagnosed with external ocular infections. Though with varying frequencies, reports exist in literature on the predominance of conjunctivitis as an external eye infection affecting both male and females[1,2]. The gender variations of external ocular infections observed in this study may be due to the tendencies of males to have more outdoor activities than women, thereby having more chances of getting eye infections[3,11].

In the aetiology of these eye infections, *S. aureus* played a leading role (37.7%), followed closely by *P. aeruginosa* (31.1%), with *A. fumigatus* having the least prevalence (7.4%). This finding is slighting consistent with the study of Ubani[1], who reported *S. aureus* (22.7%) as the predominant cause of external eye infections in Aba, Nigeria. Examining the bacterial profile of external ocular infections in Ethiopia, Tesfaye *et al.*[4] reported *S. aureus* as the major etiologic agent (28.4%) with *P. aeruginosa* being the most implicated Gram-negative isolate (20.9%). Also, while implicating *S. aureus* (19.1%) as the predominant bacterial isolate in eye infections in New Delhi, India, Sherwal and Verma[2] noted that keratitis was majorly caused by *Aspergillus* sp. This agrees with our findings where *A. fumigatus* had the highest occurring frequency (38.5%) in keratitis cases. In contrast, Gugnani *et al.*[12] and Bharathi *et al.*[6] respectively reported *Fusarium* sp. as a major cause of keratitis in Nigeria and India. This differences confirm the widely-held notion that the causative agents of ocular infections may vary with time, climate and geographical area. Similarly, Wojtyczka *et al.*[13] evaluated the prevalence of the biofilm forming coagulase-negative Staphylococci (CoNS) in a hospital environment as a risk factor for nosocomial infections in Poland. Of the 122 isolated and tested strains of CoNS the most frequent were: *S. epidermidis*-32 strains, *S. haemolyticus*-31 strains, *S. capitis* subsp. capitis-21 strains, *S. hominis*-11 strains, *S. cohnii* subsp. *cohnii*-9 strains. Though this study had a different focus, it is interesting to know that *S. epidermidis* and *S. haemolyticus* are the two most common etiological agents of nosocomial infections in the hospital environment. The ability of these species to form biofilms makes them more likely to resist antibiotic treatment.

Moreover, other authors in India[14] found that the Gram-positive cocci viz., streptococci and staphylococci were the predominan aetiologic agent of ocular infections in the country. This suggested that there are variations in the etiological agents affecting individuals in different countries. It will be necessary to do a molecular epidemiological study to understand the relationship among strains of the same species causing similar infections in different countries.

The susceptibility of the isolates to major drugs groups was also considered. While the bacterial isolates were susceptible to some of the tested antibiotics, *S. aureus* and *B. cereus* showed a relatively moderate resistance to amoxicillin (61.36 and 69.23%) and ampiclox (69.10 and 38.46%), while amoxicillin, augmentin, streptomycin and chloramphenicol had little or no effect on *P. aeruginosa*. This is slightly inconsistent with the antimicrobial susceptibility pattern of external ocular pathogens described by Shiferaw *et al.*[3], where majority of the Gram-positive isolates showed high resistance to amoxicillin, while their Gram-negative isolates counterparts resisted tetracycline, norfloxacylin, ceftriaxone and ciprofloxacin. The reasons for this variation are not so clear but may be due to the inherent antimicrobial properties of strains isolated in the various studies.

For ocular pathogens, biofilm-enclosed microbial population in an extracellular matrix can be considered as a virulence factor since it enables the organism to colonise the eye lens and subsequently infect the eye[8]. Using the relatively simple crystal violet binding method for biofilm quantification, only 28.74% (n = 4) of the tested 14 *P. aeruginosa* isolates were found to exhibit moderate adherence capacity. On the other hand, all the tested *S. aureus* and *A. fumigatus* isolates were classified as non-adherent since they had an optical density value that is less than 0.2. Among other factors, variations in the biofilm quantification of different isolates may be due to the surface properties of the solid object used during the simulation of biofilm formation[7]. In this study, we used catheter disk, a rubber-based hydrophobic material, which tends to favour the colonization and adherence of the hydrophobic *P. aeruginosa* isolates. More studies need to be done using other materials such as plastics, glass and wood for biofilm simulation exercises.

CONCLUSION

This study found *S. aureus* as the major cause of external eye infections in south-eastern Nigeria. Agricultural occupation can be considered a risk factor for these infections. Due to the rise of antibiotic resistance, the susceptibility profile of implicated isolates should be considered before administering relevant drugs. More work should be done to determine the ability of ocular pathogens to form biofilms on contact lenses.

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