Oladejo et al. *Afr. J. Clin. Exper. Microbiol.* 2024; 25 (3): 303 - 311 https://www.afrjcem.org

African Journal of Clinical and Experimental Microbiology. ISSN 1595-689X Jul 2024; Vol.25 No.3
AJCEM/2341. https://www.ajol.info/index.php/ajcem

Original Article **Open Access**

Phenotypic and genotypic detection of antimicrobial-resistant bacterial pathogens from patients with infectious keratitis in selected hospitals in Ilorin, Nigeria

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Abstract:

Background: Infectious keratitis is a major cause of global blindness. Standard management approaches typically involve the collection of corneal cultures and initiation of broad-spectrum antimicrobials. However, conventional microbiological techniques, based on direct visualization or cultures of microorganisms, are limited by poor sensitivity and the prolonged time required to produce actionable results. Molecular methods based on nucleic acid amplification technique aim to circumvent the challenges of culture for hours or days. The objectives of this study are to detect the bacteria agents of infectious keratitis in Ilorin, Nigeria, using phenotypic and molecular methods, and to determine their resistance profiles to selected antimicrobials.
Methodology: This was a cross-sectional study of selected patients with clinical features of infectious keratitis attending the ophthalmology clinics of the University of Ilorin Teaching Hospital, Sobi specialist eye hospital, and the Civil Service clinic, in Ilorin, Kwara State, Nigeria, from July 2015 to July 2018. Corneal scraping samples were collected from the patients for conventional and molecular microbiological assessments. Antibiotic susceptibility testing to selected antibiotics was determined on each bacterial isolate by the Kirby-Bauer disc diffusion method. Methicillin-resistance among *Staphylococcus aureus* and extended spectrum beta-lactamases (ESBLs) among Gram-negative bacilli isolates, were detected by both phenotypic and genotypic testings. Data were analyzed by the Statistical Package for the Social Sciences (SPSS) version 20.0.
Results: A total of 81 patients with infectious keratitis were selected, with 59 (72.8%) males and 22 (27.2%) females. A total of 79 corneal scrapings yielded microbial isolates with 66 bacteria and 13 fungi. Out of the 66 bacterial isolates, Gram-positive bacteria (GPB) accounted for 28 (42.4%), with *S. aureus* 14 (21.2%) and coagulase negative staphylococci 10 (15.2%), while Gram-negative bacteria (GNB) accounted for 38 (57.6%). The most resistant isolates to the selected antibiotics are *S. aureus* (50.0%, 7/14), *Escherichia coli* (50.0%, 1/2), *Klebsiella pneumoniae* (25.0%, 1/4) and *Pseudomonas aeruginosa* (25.0%, 1/4). ESBL genes were harbored by 7 isolates of *K. pneumoniae*, *Klebsiella oxytoca, E. coli,* and *Citrobacter freundii*. Three of these harbored 3 ESBL (bla_{CTX-M}, bla_{SHV}, bla_{TEM}) genes each while 4 harbored 2 ESBL (bla_{CTX-M}, bla_{TEM}) genes each. Two (14.3%) of the 14 *S. aureus* isolates harbored *mecA* gene (MRSA).
Conclusion: *Staphylococcus aureus* was the predominant bacterial pathogen of infectious keratitis in Ilorin, Nigeria, a few of which harbored *mec*A gene. *Staphylococcus, Klebsiella* and other GNB were resistant to the commonly used antibiotics tested in the study.

Keywords: infectious keratitis, antimicrobial resistance, ESBL, MRSA

Received Feb 28, 2024; Revised May 11, 2024; Accepted May 12, 2024

Détection phénotypique et génotypique d'agents pathogènes bactériens résistants aux antimicrobiens de patients atteints de kératite infectieuse dans des hôpitaux sélectionnés à Ilorin, au Nigeria

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Résumé:

Contexte: La kératite infectieuse est une cause majeure de cécité mondiale. Les approches de gestion standard impliquent généralement la collecte de cultures cornéennes et l'initiation d'un traitement antimicrobien à large spectre. Cependant, les techniques microbiologiques conventionnelles, basées sur la visualisation directe ou sur des cultures de micro-organismes, sont limitées par une faible sensibilité et le temps prolongé nécessaire pour produire des résultats exploitables. Les méthodes moléculaires basées sur la technique d'amplification des acides nucléiques visent à contourner les défis de la culture pendant des heures ou des jours. Les objectifs de cette étude sont de détecter les agents bactériens de la kératite infectieuse à Ilorin, au Nigéria, à l'aide de méthodes phénotypiques et moléculaires, et de déterminer leurs profils de résistance à certains antimicrobiens.
Méthodologie: Il s'agissait d'une étude transversale portant sur des patients sélectionnés présentant des caractéristiques cliniques de kératite infectieuse fréquentant les cliniques d'ophtalmologie de l'hôpital universitaire d'Ilorin, de l'hôpital ophtalmologique spécialisé de Sobi et de la clinique de la fonction publique, à Ilorin, dans l'État de Kwara, au Nigeria, de De juillet 2015 à juillet 2018. Des échantillons de grattage cornéen ont été collectés auprès des patients pour des évaluations microbiologiques conventionnelles et moléculaires. Les tests de sensibilité aux antibiotiques sélectionnés ont été déterminés sur chaque isolat bactérien par la méthode de diffusion sur disque Kirby-Bauer. La résistance à la méthicilline chez *Staphylococcus aureus* et la bêta-lactamase à spectre étendu (BLSE) parmi les isolats de bacilles à Gram négatif ont été détectées par des tests phénotypiques et génotypiques. Les données ont été analysées par le progiciel statistique pour les sciences sociales (SPSS) version 20.0.
Résultats: Au total, 81 patients atteints de kératite infectieuse ont été sélectionnés, dont 59 (72,8%) hommes et 22 (27,2%) femmes. Un total de 79 raclages cornéens ont donné des isolats microbiens comprenant 66 bactéries et 13 champignons. Sur les 66 isolats bactériens, les bactéries à Gram positif (GPB) représentaient 28 (42,4%), avec *S. aureus* 14 (21,2%) et les staphylocoques à coagulase négative 10 (15,2%), tandis que les bactéries à Gram négatif (GNB) représentaient pour 38 (57,6%). Les isolats les plus résistants aux antibiotiques sélectionnés sont *S. aureus* (50,0%, 7/14), *Escherichia coli* (50,0%, 1/2), *Klebsiella pneumoniae* (25,0%, 1/4) et *Pseudomonas aeruginosa* (25,0%, 1/2). Les gènes de BLSE étaient hébergés par 7 isolats de *K. pneumoniae, Klebsiella oxytoca, E. coli* et *Citrobacter freundii*. Trois d'entre eux hébergeaient chacun 3 gènes de BLSE (*bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{TEM}$) tandis que 4 hébergeaient chacun 2 gènes de BLSE (*bla*$_{CTX-M}$, *bla*$_{TEM}$). Deux (14,3%) des 14 isolats de *S. aureus* hébergeaient le gène *mec*A (SARM).
Conclusion: *Staphylococcus aureus* était le pathogène bactérien prédominant de la kératite infectieuse à Ilorin, au Nigeria, dont quelques-uns abritaient le gène *mec*A. *Staphylococcus, Klebsiella* et autres GNB étaient résistants aux antibiotiques couramment utilisés testés dans l'étude.

Mots clés: kératite infectieuse, résistance aux antimicrobiens, BLSE, SARM

Introduction:

Infectious keratitis is also known as corneal infection and the fifth leading cause of vision impairment and blindness globally (1). The incidence of infectious keratitis is estimated at 2.5-799 per 100,000 population-year, with a significantly higher incidence reported in low-and-middle-income-countries (LMICs). It has resulted in about 5 million cases of blindness and/or significant vision impairment and is estimated to account for 1.5-2.0 million cases of monocular blindness per year (2). The predisposing factors of infectious keratitis vary with geographical location. The leading predisposing factors to infectious keratitis in Iraq include cornea abrasions and ocular surface disorders such as dry eye, trichiasis and old scars after a healed wound or ulcer (3). In Nigeria, there has been variation in the pattern of infectious keratitis as reported by the previous researchers (4,5).

Infectious keratitis is a painful and potentially sight-threatening condition that often requires medical intervention (2,6). A timely and accurate diagnosis is often the key to a successful clinical outcome. The cornea scrapings for laboratory diagnosis are obtained in minute quantity from the patient's eye and is usually insufficient for the conventional microbiological diagnosis of the disease. Therefore, there is need for a fast and accurate diagnostic method for infectious keratitis. Advances in molecular biology have produced culture independent diagnostic tests. Molecular methods such as nucleic acid amplification like polymerase chain reaction (PCR), and hybridization techniques, aim to circumvent the challenges of culturing for hours or days in the case of bacteria and weeks in the case of moulds (7).

Proper treatment of infectious keratitis requires antimicrobials that target the organism responsible for a patient corneal ulcer (8). Antibacterial susceptibility testing provides a quantitative measurement of susceptibility, which can be used to monitor emergence and prevalence of antibacterial resistance in microbial population (9-12). The development of resistance to antibacterial agents by microorganisms could be due to production of enzymes such as beta-lactamases including the extended spectrum beta-lactamases (ESBLs), which are carried by genes on extra-chromosomal DNAs called resistant plasmids (R-plasmids) (13,14). Most ESBL genes belong to class A beta-lactamases which can be divided into genotypes; TEM, SHV, CTX-M and OXA (15). Resistance in methicillin-resistant *Staphyloco-*

ccus aureus (MRSA) is due to production of mutant penicillin-binding protein 2a (PBP2a or PBP2') encoded by *mec*A gene which is within the particular chromosomal region called Staphylococcus Cassette Chromosome (SCC*mec*) (16). Amplification of *mec*A gene can be done by PCR, which is the 'gold standard' method for detection of MRSA.

There is need to conduct research that will enable rapid detection of the increasingly emerging and re-emerging antibiotic-resistant bacterial infections, which have become great challenge and threat to public health in both the developed and developing nations of the world (8). The objectives of this study therefore are to detect the bacteria agents of infectious keratitis in Ilorin, Nigeria, using phenotypic and molecular methods, and to determine their antimicrobial resistance profiles to commonly used antimicrobial agents.

Materials and method:

Study setting:

This study was conducted at the Ophthalmology clinic of the University of Ilorin Teaching Hospital (UITH), Sobi Specialist Hospital, and Civil Service Clinic, which serve as referral hospitals in Kwara State, northcentral Nigeria, between July 2015 and July 2018.

Ethical consideration:

Ethical approval for the study was obtained from the Ministry of Health, Ilorin, Kwara State with approval code: MOH/KS/EC/777/88/24. Informed consent was also obtained from each participant.

Study participants and selection criteria:

A complete history was taken from each participant with regards to eye pain, photophobia, watering, and redness. Duration of symptoms and history of predisposing factors such as trauma, contact lens wear, dry eye, and surgery were noted. Ocular examination included visual acuity (VA) of both eyes, and slit lamp examination of the cornea for size, site, and depth of the ulcer, presence or absence of perforation. Fluorescein staining of the corneal ulcer for epithelial defect measurements and the presence or absence of hypopyon was also determined. The inclusion criteria was patient having corneal disease but with no immunosuppressive disease.

The exclusion criteria include patients with previous history of corneal disease, patients on antimicrobial medication prior to presentation, patients who declined to participate in the study and eyes with clinically suspected viral and parasitic corneal ulcers.

Cornea sample collection:

After explaining the procedure to the patient, corneal scrapings were collected from selected participants with infected cornea by the Ophthalmologist using 23G sterile needle under Slit Lamp Biomicroscope after instillation of non-preservative topical anaesthesia into the infected eye. During the corneal debridement, two corneal scrapings were directly inoculated into Brain Heart Infusion (BHI) broth and Tris EDTA buffer (which was stored at -80^{o}C).

Other corneal scrapings were smeared directly on two separate glass microscope slides for Gram staining and a final scraping was directly placed into a sterile centrifuge tube and immediately transported to the medical microbiology and parasitology laboratory of the University of Ilorin Teaching Hospital, where it was stored at -4^{o}C until transported to the molecular research laboratory of Obafemi Awolowo University, Ile-Ife, Nigeria, for analysis by PCR.

Culture isolation and identification of isolates:

The smears of the samples made on the slide was Gram stained for cellular morphology in accordance with Sagar (17), while the remaining sample was streaked directly onto Blood, Chocolate and MacConkey agar plates, which were incubated for 24 hours at 37^{o}C. Identification of microbial isolates was done by conventional biochemical test schemes.

Antibiotic susceptibility test of isolates:

Antibiotic susceptibility test (AST) was performed on each isolate against selected commonly used antibiotics by the Kirby-Bauer disc diffusion method (18) on Muller Hinton agar (Oxoid, UK). The antibiotic discs used include ceftazidime (30µg), ceftriaxone (30µg), cefuroxime (30µg), ciprofloxacin (5µg), amoxicillin-clavulanic acid (30µg), erythromycin (15µg), and gentamicin (10µg).

Inoculum of pure colonies of each isolate, standardized to 0.5 McFarland turbidity standards, was spread on Mueller-Hinton (MH) agar plates and allowed to air-dry at room temperature. The antibiotic discs were placed on the inoculated agar plates and incubated at 37^{o}C for 18 to 24 hours. The diameter of inhibition zone of each antibiotic tested against each isolate was measured using graduated meter rule and interpreted as sensitive, intermediate or resistant, according to the CLSI guidelines (19).

Phenotypic detection of extended spectrum beta-lactamase (ESBL):

Gram-negative bacteria isolate resistant to at least two third generation cephalosporin antibiotics in the AST were presumptively identified as ESBL-producers. Phenotypic confirmation of ESBL production was done by the combination disc diffusion method using the combination of cefotaxime (30µg) disc and cefotaxime/clavulanic acid (30/10µg) disc or the combination of ceftazidime (30µg) disc and ceftazidime/clavulanic acid (30/10µg)

disc (Mast, UK) according to the CLSI guidelines for non-fastidious bacteria (19).

A 0.5 McFarland standard suspension of the isolates was made in sterile saline and the bacterial suspension was evenly spread unto the surface of MH agar plate, using a sterile swab stick to rim the edge of the plate. Cefotaxime (30µg) disc alone and cefotaxime/clavulanic acid (30/10 µg) disc or ceftazidime (30µg) disc alone and ceftazidime/clavulanic acid (30/10µg) disc, were placed 25 mm apart on the MH agar plate and incubated aerobically overnight at 37^{o}C.

Zone diameters were manually measured manually with a graduated meter ruler to the nearest millimetre. A difference of greater or equal to 5mm between the inhibition zones of cefotaxime (30µg) and cefotaxime/clavulanic acid (30/10µg) discs or ceftazidime (30 µg) and ceftazidime/clavulanic acid (30/10µg) discs was confirmed to indicate ESBL production in line with CLSI recommendation (19). *Escherichia coli* ATCC 25922 and *K. pneumoniae* ATCC 70603, were used as controls strains.

Phenotypic detection of methicillin resistance in *Staphylococcus aureus*:

A 0.5 McFarland turbidity standard suspension of the bacteria was spread on MH agar and allowed to air-dry at room temperature. Cefoxitin disc (30µg) was placed on the inoculated agar and incubated at 37^{o}C for 18 to 24 hours. The inhibition zone was measured using graduated meter and interpreted as sensitive or resistant according to the CLSI guideline (20). The inhibition zone of *S. aureus* <22 mm for cefoxitin was taken to be methicillin-resistant *S. aureus* (MRSA) and *S. aureus* with the zone of inhibition greater than or equal to 22 mm was regarded as methicillin-sensitive *S. aureus* (MSSA).

Molecular detection of bacterial isolates and antibiotic resistance genes by PCR:

DNA extraction:

Bacterial DNA was extracted from all phenotypically identified bacterial isolates by the boiling method according to Grupta (20). Isolates were first grown on nutrient agar for 24 hours. A single colony growth was picked, transferred to 0.1 ml sterile water, and boiled for 10 minutes in a water bath, and then centrifuged for 5 minutes at 1000 rpm. About 5µL of the supernatant was used as the template DNA for PCR.

***PCR amplification of bacterial DNA*:**

PCR amplification of the extracted DNA was carried out in a T-3000 thermocycler (Biometra, Germany) using the universal bacterial primers and specific primers as shown in Table 1. All primers were prepared by Iqaba Biotechnology, West Africa Limited. They were used in 2 sets of PCR reactions as follows; the first set was standardized using the universal bacterial primers (27-F, 1525-R). The reaction mixture contained 12.5µL of 'go tag green' master mix, 1µM of each of upstream and downstream primers, 5µL of template DNA, and nuclease free water to complete the volume to 25µL. The PCR reaction involved 20 cycles of denaturation at 90^{o}C for 60 seconds, annealing at 50^{o}C for 30 seconds, and extension at 72^{o}C for 2 mins, and a final extension step at 72^{o}C for 5 minutes.

Table 1: Oligonucleotide primers used for amplification of bacteria DNA in the study

Primer name	Primer sequence	Uses	Product size
27-F 1525-R	5-AGCTAAATTCATAGCAGAAAGC-3 5-AAGGAGGTGATCCARCC-3	Universal bacteria	1,500
PA-GS-F PA-GS-R	GACGGGTGAGTAATGCCTA CACTGGTGTTCCTTCCTATA	*Pseudomonas* species	618
PA-SS-F PA-SS-R	GGGGGATCTTCGGACCTCA TCCTTAGAGTGCCCACCCG	*Pseudomonas aeruginosa*	956
SA-F SA-R	GACAACTAGAGATAGAGCCTTCC AGTCGAGTTGCAGACTAC	*Staphylococcus aureus*	324
91E-F 13E-R	GGAATTCAAATGAATTGACGGGGGC CGGGATCCCAGGCCCGGGAACGTATTCAC	16S rRNA *Staphylococcus epidermidis*	478

Table 2: Oligonucleotide primers used for amplification of ESBL genes and *mec*A gene in the study

Gene	Primer	Sequence (5'-3')	Expected size (bp)	Annealing Temperature (°C)
SHV	SHV-F SHV-R	CGCCTGTGTATTATCTCCCT CGAGTAGTCCACCAGATCCT	293	60
TEM	TEM-F TEM-R	TTTCGTGTCGCCCTTATTCC ATCGTTGTCAGAAGTAAGTTGG	403	60
CTX-M	CTX-M-F CTX-M-R	CGCTGTTGTTAGGAAGTGTG GGCTGGGTGAAGTAAGTGAC	874	60
*mec*A	*mec*A-F *mec*A-R	AAAATCGATGGTAAAGGTTGGC AGTTCTGGAGTACCGGATTTGC	533	53

The second set of PCR amplification was carried out using species-specific primers [*Pseudomonas aeruginosa* (PA-SS-F, PA-SS-R), *Staphylococcus aureus* (SA-F, SA-R) and *Staphylococcus epidermidis* (91E-F, 13E-R)] on the samples that gave positive result in the first set of PCR reactions. The reaction mixture was of the same composition but conditions of each set of primer were different. For *P. aeruginosa,* after an initial denaturation for 2 min at 95°C, 25 cycles were completed, each consisting of 20 seconds at 94°C, 20 seconds at 58°C, and 40 seconds at 72°C. A final extension of 1-minute at 72°C was applied. For *S. aureus*, 35 cycles of amplification consisted of 94°C for 15 seconds, 54°C for 1-minute, 72°C for 2 minutes, and a final extension step at 72°C for 7 minutes. For *S. epidermidis*, an initial denaturation at 94°C for 2 minutes, followed by 30 cycles of amplification (denaturation at 94°C for 1-minute, annealing at 55°C for 1-minute and extension at 72°C for 2 min), and a final extension at 72°C for 5 minutes.

PCR amplification of ESBL and mecA genes:

Primers used for PCR amplification detection of resistance ESBL genes and *mec*A gene are shown in Table 2. After a hot start at 94°C for 3 minutes, amplification of the ESBL genes followed 30 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 1-minute and a final extension at 72°C for 7 minutes. For *mec*A gene amplification, the thermal cycling protocol comprised 95°C for 3 minutes, followed by 33 cycles of 94°C for 1-minute, 53°C for 30 seconds and 72°C for 1-minute, with a final extension at 72°C for 6 minutes.

Gel electrophoresis of DNA amplicons:

The PCR amplicons were examined following electrophoresis at 120 volts for 30 minutes on 2% agarose gel stained with ethidium bromide and the images of the amplicon band sizes were visualized under ultraviolet illumination in a Gel Documentation System.

Statistical analysis:

The data were presented as frequency tables, charts and figures. Data analysis was done using the Statistical Package for the Social Sciences (SPSS) version 20.0 software (Chicago, USA). Numerical variables were described by percentages, mean and standard deviation. The means of two or more than two independent variables were compared by the Student '*t*' test and analysis of variance (ANOVA) respectively. Independent categorical variables were compared using the Chi-square test. Confidence interval was set at 95% and for all statistical test, $p<0.05$ was considered significant.

Results:

A total of 81 patients with infectious keratitis were recruited with 59 (72.8%) males and 22 (27.2%) females. A total of 79 corneal scrapings yielded microbial isolates, with 66 bacteria and 13 fungi. Out of the 66 bacterial isolates, Gram-positive bacteria (GPB) accounted for 28 (42.4%), with *S. aureus* 14 (21.2%) and coagulase negative staphylococci 10 (15.2%), while Gram-negative bacteria (GNB) accounted for 38 (57.6%) (Table 3).

Staphylococcus aureus isolates exhibited high resistance to amoxicillin-clavulanic acid (52.4%), cefuroxime (51.7%), ceftriaxone (47.6%) and ciprofloxacin (40.0%), but low resistance rate to gentamicin (1.0%). Coagulase negative staphylococci exhibited low resistance to amoxicillin-clavulanic acid (14.2%) and cefuroxime (14.1%) while other isolates exhibited lower resistance (<10.6%) to all the tested antibiotics. *Klebsiella* spp exhibited low resistance to ceftazidime (15.9%), ceftriaxone (14.2%) and amoxicillin-clavulanic acid (14.2%) and much lower resistance rate to ceftazidime (<10.0%).

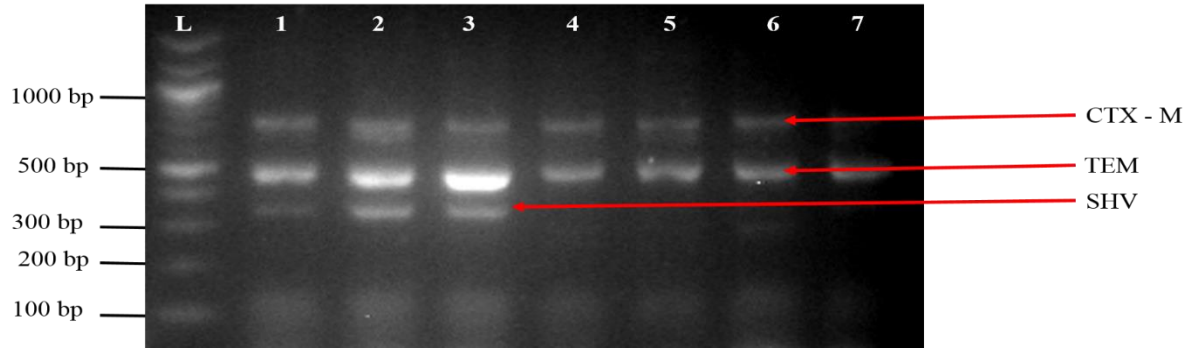
Molecular analysis of the ESBL producing GNB by PCR shows that *K. pneumoniae, K. oxytoca, E. coli* and *C. freundii* harbored ESBL genes (Fig 1). Three isolates harbored 3 ESBL

genes (*bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{TEM}$) each while 4 isolates harbored 2 genes (*bla*$_{CTX-M}$, *bla*$_{TEM}$) each as indicated in Table 4. Only 2 *S. aureus* isolates harbored *mec*A gene (Fig 2 & Table 5)

Table 3: Antibiotic resistance of bacteria isolates from patients with infectious keratitis from selected hospitals in Ilorin, Nigeria

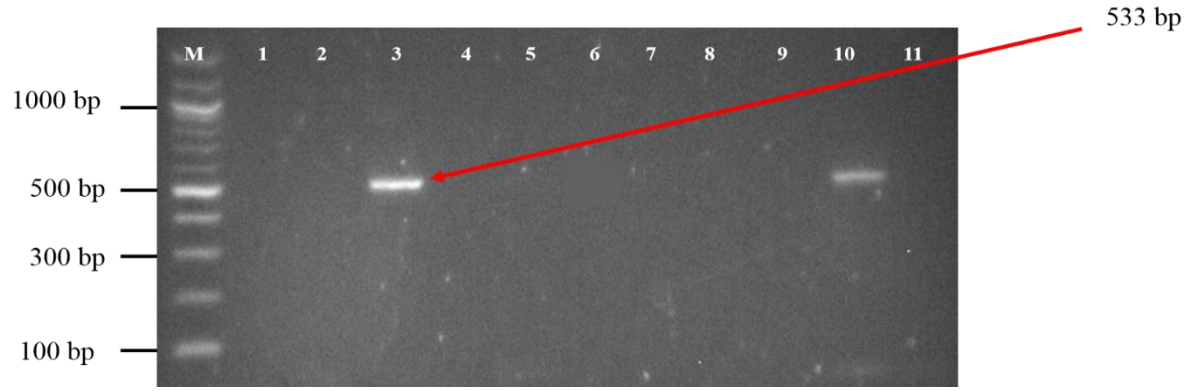
Bacteria isolates	Number of bacteria isolates	Number of resistant isolates	% of resistant strains	Antibiotic resistance in %					
				GN	CIP	CAZ	CRO	CXM	AUG
Gram positive bacteria									
Staphylococcus aureus	14	7	50.0	16.0	40.0	-	47.6	51.7	52.4
Staphylococcus auricularis	5	1	20.0	4.0	0	-	9.8	14.1	14.2
Staphylococcus xylosus	3	0	0	4.0	4.0	-	4.8	14.1	14.2
Staphylococcus epidermidis	2	0	0	0	0	-	0	4.7	0
Micrococcus spp	1	0	0	4.0	0	-	0	9.5	0
Enterococcus faecalis	1	0	0	0	0	0	0	0	0
Aeromonas salmonicida	1	0	0	8.0	4.0	5.3	4.8	4.7	0
Sub total	**28**	**8**	**28.6**						
Gram negative bacteria									
Klebsiella pneumoniae	4	1	25.0	8.0	4.0	14.2	15.9	9.5	14.2
Klebsiella oxytoca	1	0	0	0	4.0	4.8	5.3	4.7	4.8
Pseudomonas aeruginosa	4	1	25.0	8	8.0	-	15.9	-	-
Pseudomonas luteola	5	0	0	4.0	0	-	0	-	-
Pseudomonas oryzihabitus	1	0	0	0	0	-	5.3	-	-
Pseudomonas fluorescein	1	0	0	4.0	0	-	5.3	-	-
Escherichia coli	2	1	50.0	0	8.0	9.5	10.6	9.5	9.5
Citrobacter freundii	2	0	0	0	0	0	0	0	4.8
Proteus mirabilis	1	0	0	4.0	0	4.8	5.3	4.7	4.8
Enterobacter cloacae	1	0	0	8.0	4.0	0	0	4.7	9.5
Serratia marcescen	1	0	0	4.0	0	4.8	5.3	4.7	4.8
Other GNB	8	0	0	0	0	0	0	-	0
Subtotal	**38**	**3**	**7.9**						

GN- Gentamycin (10µg), CAZ- Ceftazidime (30µg), CRO- Ceftriaxone (30µg), AUG- Augmentin/Amoxicillin-clavulanic acid (30µg), CIP- Ciprofloxacin (5µg), CXM- Cefuroxime (30µg); GNB-Gram negative bacteria



Lane L: 100bp ladder; Lane 1: Isolate 1 (CTX-M, TEM, SHV); Lane 2: Isolate 2 (CTX-M, TEM, SHV); Lane 3: Isolate 3 (CTX-M, TEM, SHV); Lane 4: Isolate 4 (CTX-M, TEM); Lane 5: Isolate 5 (CTX-M, TEM): Lane 6: Isolate 6 (CTX-M, TEM); Lane 7: Isolate 7(CTX-M, TEM)

Fig 1: Gel electrophoresis of ESBL gene PCR amplicons



Lane M: 100bp ladder; Lane 1: CS 1: Lane 2: CS 8; Lane 3: CS 7; Lane 4: CS 2; Lane 5: CS 3; Lane 6: CS 4; Lane 7: CS 5; Lane 8: CS 6; Lane 9: CS 9; Lane 10: CS 10

Fig 2: Gel electrophoresis of the PCR amplification of *mecA* gene (533bp)

Table 4: ESBL genes detected among the GNB isolates

Isolates	ESBL genes detected
Klebsiella pneumoniae	*bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{TEM}$
Klebsiella oxytoca	*bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{TEM}$
Escherichia coli	*bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{TEM}$
Klebsiella pneumoniae	*bla*$_{CTX-M}$, *bla*$_{TEM}$
Citrobacter freundii	*bla*$_{CTX-M}$, *bla*$_{TEM}$
Escherichia coli	*bla*$_{CTX-M}$, *bla*$_{TEM}$
Klebsiella pneumoniae	*bla*$_{CTX-M}$, *bla*$_{TEM}$

GNB = Gram-negative bacteria, ESBL = extended-spectrum beta-lactamase

Table 5: *Staphylococcus aureus* isolates with *mec*A gene

Isolates	*mec*A gene (MRSA)
CS1	-
CS2	-
CS3	+
CS4	-
CS5	-
CS6	-
CS7	-
CS8	+

+ = Detected, - = Not detected, CS = Cornea scrapings

Discussion:

In this study, resistance of bacteria isolates of infectious keratitis to commonly used antibiotics was evaluated. There was relatively high resistance rate of *S. aureus* to commonly used antibiotics such as amoxicillin-clavulanic acid (augmentin) (52.4%), cefuroxime (51.7%), ceftriaxone (47.6%), ciprofloxacin (40.0%), and *K. pneumoniae* to ceftazidime (15.9%), ceftriaxone (14,2%), and amoxicillin-clavulanic acid (14.2%). However, the coagulase negative staphylococci showed low resistance to amoxicillin-clavulanic acid (14.2 %) and cefuroxime (14.1%) while other bacterial isolates showed much lower resistance (<10.6%) to the tested antibiotics. These findings indicate that *S. aureus*, *Klebsiella* spp and other Gram-negative bacilli were resistant to commonly used antibiotics and are similar with the findings of a retrospective study conducted on patients with suspected ocular infections in Harbin, China (21). There are reports of increasing resistance rates in bacteria keratitis as noted in our study. This could be caused by mis-use of antibiotic eye drop, use of counterfeit or expired drugs, and non-compliance with drug regimen for eye care, leading to poor treatment outcome.

In a study by Egrilmez and Yildirim-Theveny (14) among the coagulase-negative staphylococci (n=1475 isolates), moxifloxacin resistance was observed in 31.1% overall and in 51.5% among methicillin-resistant strains. The study emphasized that the high *in vitro* resistance rate in coagulase-negative staphylococci should be considered when treating patients with ocular infections. In a 20-year follow-up study (1991-2012) conducted in the USA, Chang et al., (13) reported an MRSA rate of 30.7% and increasing resistance to fourth-generation fluoroquinolones. This is similar to the finding of our study that showed high resistance to the commonly used antibiotics tested, with more than 40.0% resistance to amoxicillin-clavulanic acid, cefuroxime, ceftriaxone and ciprofloxacin among some GNB isolates. The study by Peng et al., (16) in the United States between 1996 and 2001 reported an increase in MRSA rate from bacterial keratitis. Similarly, in a 20-year follow-up study conducted in Taiwan, Liu et al., (22) compared susceptibility data from the years 1992-2001 with those from 2007 to 2016 and reported rising rates of antibiotic resistance among Gram-positive bacteria as well as significant increase in oxacillin resistance. Although MRSA is frequently detected in ocular infections worldwide, it has been reported that methicillin-resistant *Staphylococcus epidermidis* can also cause ophthalmic infections and blindness (23, 24). It is therefore important to have good knowledge of the antimicrobial susceptibility pattern of the microbial pathogens in a given locality, which would guide the choice of appropriate antibiotics in the management of infectious keratitis.

In this study, the predominant organism was *S. aureus* (n=14, 21.2%) and Gram-positive bacteria constituted 42.4% (n=28). In the study by Sarkar et al., (24), Gram-positive bacteria were reported in 78.5%. The lower rate of Gram-positive bacteria isolates in our study may be due to the low sample size. Getahun et al., (25) and Nithya et al., (26) reported significant variation in the prevalence of MRSA ocular infections geographically at different times. The absence of the gene-coding methicillin-resistance (*mec*A) in *Staphylococcus* species is a reliable predictor of phenotypic oxacillin susceptibility in clinical isolates and may be used to narrow therapy (27). In a study of *mec*A-positive isolates (n=234), 27 (11.5%) were phenotypically oxacillin susceptible, 7 of which had oxacillin resistant co-pathogen that explained the detection of *mec*A gene (28,29). Methicillin resistance was also reported by Das et al., (30) in 26 of 173 *S. aureus* isolates, given 15.0% of MRSA by disc testing with cefoxitin. Keratitis was the most common ocular diagnosis in Taiwan, with MR SA accounting for 36.1% (31). These studies reported higher rates than the 20.0% (2 of 10 phenotypic MRSA isolates) reported in our study. The low rate in our study may be due to small sample size as well as geographical variations in the incidence of methicillin-resistant *S. aureus*.

The ESBLs of clinical significance are the CTX-M and TEM β-lactamases families. In our study, 7 Enterobacteriacaeae isolates harbored ESBL genes, with 3 isolates harboring 3 genes (*bla*$_{CTX-M}$, *bla*$_{SHV}$ and *bla*$_{TEM}$) each and 4 isolates harboring 2 genes (*bla*$_{CTX-M}$ and *bla*$_{TEM}$) each. The CTX-M-type ESBL enzymes have become the most prevalent among clinical isola-

tes (mostly *E. coli*) in Asia, Europe and South America (32). Since the first recognition of the CTX-M enzyme in the clinical settings in the 1990s, over 130 variants have been identified and genetically classified based on amino acid differences into 5 major groups; CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25, mostly identified in *E. coli* and *K. pneumoniae* isolates from different geographical locations (32). ESBLs have predominantly been detected among *K. pneumoniae* and *E. coli* in almost all parts of the world. In Europe and North America, the class A ESBL gene, $bla_{\text{CTX-M-15}}$, was the most prevalent (reported in >90% of *E. coli* isolates) and in 35-65.5% of *K. pneumoniae* isolates, although bla_{SHV} and bla_{TEM}-type genes were also common (33). Available data on ESBLs show considerable geographical differences in prevalence.

Conclusion:

Our study showed that *S. aureus, Klebsiella* spp and other GNB isolated from cases of infectious keratitis were resistant to the commonly used antibiotics in Ilorin, Nigeria. *Staphylococcus aureus* was the single most common bacterial pathogen, some of which harbored the *mecA* gene that confer resistance to methicillin and other beta-lactamase resistant penicillins. The absence of *mecA* gene is a reliable predictor of phenotypic susceptibility in *S. aureus* and may be used to narrow therapy of both *S. aureus* and coagulase-negative staphylococcal ocular infections.

The fact that 7 Enterobacteriaceae isolates of infectious keratitis in our study harbored ESBL genes, support the reports of increasing antimicrobial resistance to the commonly used antibiotics for therapy of ocular infections in low-income resource countries like Nigeria.

Contributions of authors:

OOJ and OJM were involved in study conceptualization; OOJ, OJM, AA, OB, OV and TT were involved in the study methodology; OOJ, OJM, OB and OP were involved in software use and formal analysis of data; OOJ, OJM, and OB were involved in writing and review of the manuscript; OOJ was involved in project administration. All authors approved the manuscript submitted for publication.

Source of funding:

No funding was received for the study

Conflict of interest:

No conflict of interest is declared

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