Original Article

Prevalence and antibacterial resistance patterns of extended-spectrum beta-lactamase producing Gram-negative bacteria isolated from ocular infections

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Purpose: Extended-spectrum beta-lactamases (ESBLs) mediated resistance is more prevalent worldwide, especially among Gram-negative bacterial isolates, conferring resistance to the expanded spectrum cephalosporins. As limited data were available on the prevalence of ESBLs in this area, the current study was undertaken to determine the prevalence, antibacterial resistance patterns, and molecular detection and characterization of ESBL encoding resistance genes among ocular Gram-negative bacterial isolates from ocular infections. **Materials and Methods:** A prospective study was done on 252 ocular Gram-negative bacterial isolates recovered from ocular infections during a study period from February 2011 to January 2014. All isolates were subjected to detection of ESBLs by cephalosporin/clavulanate combination disc test and their antibacterial resistance pattern was studied. Molecular detection and characterization of ESBL encoding bla_{TEM}-, bla_{SHV}, bla_{OXA}-, and bla_{CTX-M} (phylogenetic groups 1, 2, 9, and 8/25) resistance genes by multiplex polymerase chain reaction and DNA sequence analysis. **Results:** Of all Gram-negative bacteria, *Pseudomonas aeruginosa* (44%) was the most common strain, followed by *Enterobacter agglomerans* and *Klebsiella pneumoniae* each (10%). Among the 252, 42 (17%) were ESBL producers. The major source of ESBL producers were corneal scraping specimens, highest ESBL production was observed in *P. aeruginosa* 16 (38%) and *Escherichia coli* 7 (16.6%). Among ESBL-producing genes, the prevalence of bla_{TEM}-gene was the highest (83%) followed by bla_{OXA}-gene (35%), bla_{SHV}-gene (18.5%), and $bla_{CTX-M-1}$-gene (18.5%) alone or together. **Conclusion:** The higher rate of prevalence of ESBLs-encoding genes among ocular Gram-negative bacteria is of great concern, as it causes limitation to therapeutic options. This regional knowledge will help in guiding appropriate antibiotic use which is highly warranted.

Key words: Antibiotic resistance, extended-spectrum beta-lactamases, multiplex polymerase chain reaction, ocular Gram-negative bacteria

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The eye is a unique organ that is almost impermeable to all external infectious agents,[1] though the eye surface invariably is exposed to a wide variety of microorganism.[2] Bacterial infection of the eye is mainly due to external sources or through the intraocular invasion of microorganism carried by the blood stream.[3] Alteration of the normal flora contributes to cause various internal and external ocular infections.[1,4-6] The most common Gram-negative bacteria causing ocular infections include *Pseudomonas aeruginosa*, *Escherichia coli*, and *Enterobacter* spp.[1,7] Despite the protection by the elements of a tear along with the blinking action of the eyelids, the resident bacteria of the conjunctival sac or environmental bacteria can establish infection, resulting in the need for antibiotic intervention to treat these infections. Understanding of the antibacterial resistance, especially to commonly used antibiotics, is very important in choosing the appropriate antibiotic to prevent sight-threatening complications. The factors contributing to the development of drug resistance among ocular isolates includes overuse of antibiotics for systemic infections as well as overuse of topical antibiotics in the eye.[5] As a consequence, routine antibacterial susceptibility testing accompanied by molecular biological techniques for the detection of the drug resistance among ocular isolates of Gram-negative bacteria is highly warranted to understand the prevalence of drug resistance to extended-spectrum of drugs, and also to confirm the availability of broad-spectrum antibacterial agents. The development of resistance to beta-lactam group antibiotics is an emerging problem and production of beta-lactamases is the most common drug resistance mechanism among Gram-negative bacteria, especially *Enterobacteriaceae* family.

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The extended-spectrum beta-lactamases (ESBLs) are typically mutant, plasmid-mediated, clavulanate-susceptible enzymes which are derived from the older, broad-spectrum beta-lactamases and that can hydrolyze penicillins, expanded-spectrum cephalosporins, and monobactams but are inactive against cephamycins and carbapenems. ESBLs are commonly found in Gram-negative bacteria isolates mainly in *Enterobacteriaceae* (*Klebsiella pneumoniae* and *E. coli* leap out as the significant ESBL producers) and have been reported worldwide.[8] In addition, ESBL-producing organisms frequently show the cross-resistance to other classes of non-β-lactam antibiotics including trimethoprim-sulfamethoxazole, aminoglycosides, and fluoroquinolones thus treatment of these infections is often a therapeutic challenge.[9] In the 1990s, bla_{TEM} and bla_{SHV} type ESBLs were more prevalent and recently rapid, and immense spread of bla_{CTX-M}-type ESBLs has been described.[8] Several reports and surveys have documented the prevalence and incidence of ESBL-mediated resistance among the Gram-negative bacterial isolates from community and hospital-acquired infections[10] and very few reports from ocular infections.[7] In India, SENTRY surveillance study reported, the prevalence of ESBLs ranges increased from 62% to 100% in *E. coli* and *Klebsiella* spp. isolated from respiratory infections, blood stream infections, skin and soft tissue infections,[11] and ocular infections caused by *K. pneumoniae* 58% and *E. coli* 42% have also been reported.[7] However, the prevalence studies on ESBLs producing ocular isolates from India are very few. Studies reported by Jayahar Bharathi *et al*.[7] and Sowmiya *et al*.[12] recorded the incidence of ESBL among ocular population to be 7% and 77%, respectively, and bla_{CTX-M}-gene (14%) was the common ESBL gene found among ocular *Enterobacteriaceae* family. As there is scanty data on the prevalence of ESBLs genes among ocular pathogens in India, the current study was undertaken to determine the prevalence of ESBL producing Gram-negative bacteria isolated from ocular infections and to detect and characterize the bla_{TEM}-, bla_{SHV}-, bla_{OXA}-, and bla_{CTX-M} genes by multiplex polymerase chain reaction (mPCR) and DNA sequencing.

Materials and Methods

Bacterial isolates and study subjects

A prospective analysis was done on 252 Gram-negative bacteria isolated from patients clinically diagnosed with bacterial ocular infections, such as blepharitis, conjunctivitis, internal and external hordeolum, scleritis, canaliculitis, keratitis, dacryocystitis, and internal infections such as endophthalmitis and panophthalmitis during the 3-year study period from February 2011 to January 2014, from a total of 4917 ocular specimens which were received at a tertiary eye care center in South India. Using standard techniques, culture and smears obtained from each ocular specimen were evaluated for significant microbiological features.[13,14] Bacterial isolates were identified up to species level using their cultural characteristics and standard biochemical tests.[15] *In vitro* susceptibility testing was performed by Kirby–Bauer disc diffusion method and interpreted using Clinical and Laboratory Standards Institute (CLSI) serum standards.[16] Pure, nonduplicate, single species of Gram-negative bacteria were isolated and subcultured onto MacConkey agar (M008; Hi-Media Laboratories Pvt. Ltd., India) for further analysis. The study was approved by the Institutional Ethical and Research Committee of the Aravind Eye Care system.

Antibacterial susceptibility testing

In vitro antibacterial susceptibility testing for each pure isolate was performed by Kirby–Bauer disc diffusion method against the third generation cephalosporins, (cefpodoxime, ceftazidime, cefotaxime, and ceftriaxone), monobactams (aztreonam), aminoglycosides (amikacin, tobramycin, and gentamicin), fluoroquinolones (norfloxacin, ciprofloxacin, levofloxacin, ofloxacin, and gatifloxacin), and phenolics (chloramphenicol) using Bio-Rad susceptibility discs and interpreted according to the CLSI standards guidelines.[16]

Selection of the extended-spectrum beta-lactamase producing strains

According to the CLSI guidelines, laboratories using disc diffusion method for antibiotic susceptibility testing can screen for ESBL producing isolates showing inhibition zone size of ≤17 mm with 10 μg cefpodoxime, ≤22 mm with 30 μg ceftazidime, ≤25 mm with 30 μg ceftriaxone, ≤27 mm with 30 μg cefotaxime, and ≤27 mm with 30 μg aztreonam discs which can be identified as potential ESBL producers and shortlisted for the phenotypic confirmation of ESBL production.[16]

Phenotypic confirmatory test for extended-spectrum beta-lactamase production

For the confirmation of ESBL-production, as per the guidelines of CLSI, the disc test was done with confluent growth of the test isolates (0.5 McFarland standard) on Mueller-Hinton agar plates, with a distance of 25 mm of disc containing cefotaxime disc (30 μg/disc) and ceftazidime disc (30 μg/disc) with the combination of cefotaxime with clavulanate (30 μg + 10/μg/disc) and ceftazidime with clavulanate (30 μg + 10/μg/disc). Plates were incubated overnight at 37°C, and after incubation a zone of inhibition that showed ≥5 mm increased in the cephalosporin discs and their respective cephalosporin/clavulanate discs was taken to be a phenotypic confirmation of ESBL production.[16]

Quality control

The antibacterial agents used were tested for their efficacy against the standard American Type Culture Collection (ATCC) non-ESBL-producing organism (*E. coli* ATCC 25922) and an ESBL-producing organism (*K. pneumoniae* ATCC 700603) for reference and quality.

Molecular detection and characterization

Preparation of template DNA from isolated Gram-negative bacteria

A single colony of each nonduplicate Gram-negative isolate was inoculated from MacConkey agar into 5 ml of Luria-Bertani broth (M1245; Hi-Media Laboratories Pvt. Ltd., Mumbai, India) and incubated for 20 h at 37°C. Cells from 1.5 ml of the overnight culture were harvested by centrifugation at 12,000 rpm for 5 min. The DNA was extracted from each bacterial isolate by alkaline lysis method using QIAamp DNA Mini Kit (Code No. 51304) according to the manufacturer's instruction (Qiagen Gmbh, Hilden, Germany). Along with the test isolates, DNA of bla_{CTX-M}-gene carrying *E. coli* 4712 (positive control for $bla_{CTX-M-15}$; GenBank accession number KC200080), DNA of bla_{SHV}-gene carrying *K. pneumoniae* (ATCC 700603) (positive control for bla_{SHV}-gene),[17] DNA of bla_{TEM}-gene carrying *E. coli* (ATCC 35218) (positive control for bla_{TEM}-gene),[18] DNA of bla_{OXA}-gene laboratory isolate of *K. pneumoniae* 7888 (positive control for bla_{OXA}-gene GenBank accession number JN565742),

and DNA of non-ESBL-producing organism, *E. coli* ATCC 25922 were also extracted for performing positive and negative control during each mPCR. All DNA extraction procedures were carried out in a Class II biological safety cabinet (Clean Air, India) placed in a room (room B) physically separated from that used to prepare nucleic acid amplification mixes (room A) and that used for post-PCR analysis (room C).

Multiplex polymerase chain reaction

Total DNA (2 µL) was subjected to each mPCR in a 50 µL reaction mixture containing 1X PCR buffer (10 mM Tris-HCl, pH 8.3, and 1.5 mM $MgCl_2$) 200 µM each deoxynucleotide triphosphate, 1U of Taq DNA polymerase (Bangalore Genei, Bengaluru, India), and variable concentration of gene-specific primers [Table 1].[19] Amplification was carried out as follows: 94°C for 10 min, 30 cycles of 94°C for 40s, 60°C for 40s, and 72°C for 1 min and final extension step at 72°C for 8 min for the amplification of ESBL genes bla_{TEM}, bla_{SHV}, bla_{OXA}, and bla_{CTX-M} (phylogenetic groups 1, 2, 9, and 8/25). Amplicons were visualized under ultraviolet transilluminator and documented by gel document system (Vilber Lourmat, France) after running at 120V for 45 min on a 2% agarose gel containing ethidium bromide. A 100 bp DNA ladder (Bangalore Genei, Bengaluru, India) was used as a marker.

Sequencing analysis of polymerase chain reaction amplicons

In order to study the molecular characterization of the gene (s) that were identified in the mPCR which code for enzymes, bla_{TEM}, bla_{SHV}, bla_{OXA}, and bla_{CTX-M} a total of six mPCR amplicons were performed. After gel electrophoresis, targeted gene of PCR product was eluted from the gel and purified with the EZ-10 Spin Column DNA Gel Extraction Kit (Bio Basic Inc., Canada). Purified products were sequenced at MWG AG Biotech, Bengaluru, by using an ABI 3730 XI automated sequencer (PE Applied Biosystems, Foster City, CA, USA). The sequences were analyzed and identified using the MegaBLAST search program of GenBank databases. The percentage similarity was determined using the sequence alignment of the test isolate with the GenBank sequence of the type strain by BLAST program.

Results

Bacterial isolates and study subjects

During the 3 years study period, a total of 252 Gram-negative bacterial isolates were recovered from 221 ocular specimens. From ocular specimens of 199 patients, single species were isolated and while from samples of 22 patients; two or three species were isolated. Among the 252 isolates, 162 (64%) showed non-*Enterobacteriaceae* family, of which 56% were from male and 44% from female patients. The average age of male patients was 48.3 years (4 months to 90 years) and that of female patients 47.9 years (1 month to 85 years). Whereas, 90 (36%) showed *Enterobacteriaceae* family, of which 50% were from male and 50% from female patients. The average age of male patients was 36.5 years (2 months to 71 years) and that of female patients 46.3 years (2 months to 95 years). The predominant Gram-negative bacterial species isolated were *P. aeruginosa* (112 of 252; 44.4%) followed by *Enterobacter agglomerans* (25 of 252; 10%), *K. pneumoniae* (25 of 252; 10%), *Alcaligenes denitrificans* (20 of 252; 8%), and *E. coli* (13 of 252; 5%). *P. aeruginosa* was more prevalent in keratitis cases (61 of 112; 54.5%), while *E. agglomerans* dominated in contact lens-associated keratitis (7 of 25; 28%), *K. pneumoniae* in dacryocystitis (7 of 25; 28%), *A. denitrificans* in contact lens-associated keratitis (9 of 20; 45%), and *E. coli* in conjunctivitis (4 of 13; 31%). Distribution of Gram-negative bacterial isolates recovered from various ocular infections is presented in Table 2.

Phenotypic detection of extended-spectrum beta-lactamase producers

In the present study, of the total of 252 isolates of ocular Gram-negative bacteria tested for the production of ESBLs by the cephalosporin combination disc test, 42 (17%) were detected as ESBL producers, and 210 (83%) were detected as non-ESBL producers. Among the 42, the highest percentage of ESBL production was seen in *P. aeruginosa* 16 (38%), followed by *E. coli* 7 (17%) and *A. denitrificans* 6 (14%). Ocular Gram-negative bacterial isolates of non-*Enterobacteriaceae* family showed 69% positivity, whereas in *Enterobacteriaceae* family showed 31% positivity for ESBL production. The highest rate of isolation

Table 1: Sequences of the primer pairs utilized in the multiplex polymerase chain reaction for amplification of extended-spectrum β-lactamase encoding resistance genes

Target	Name of the primer pairs	Sequences (5'-3' direction)	Annealing position	Amplicons size in bp
bla_{TEM}	bla_{TEM}-F	CATTTCCGTGTCGCCCTTATTC	13-34	800
	bla_{TEM}-R	CGTTCATCCATAGTTGCCTGAC	812-791	
bla_{SHV}	bla_{SHV}-F	AGCCGCTTGAGCAAATTAAAC	71-91	713
	bla_{SHV}-R	ATCCCGCAGATAAATCACCAC	783-763	
bla_{OXA}	bla_{OXA}-F	GGCACCAGATTCAACTTTCAAG	201-222	564
	bla_{OXA}-R	GACCCCAAGTTTCCTGTAAGTG	764-743	
$bla_{CTX-M-1}$	$bla_{CTX-M-1}$-F	GCGTGATACCACTTCACCTC	540-559	260
	$bla_{CTX-M-1}$-R	TGAAGTAAGTGACCAGAATC	780-779	
$bla_{CTX-M-2}$	$bla_{CTX-M-2}$-F	CGTTAACGGCACGATGAC	345-362	404
	$bla_{CTX-M-2}$-R	CGATATCGTTGGTGGTRCCAT	748-728	
$bla_{CTX-M-8/25}$	$bla_{CTX-M-8/25}$-F	AACRCRCAGACGCTCTAC	172-189	326
	$bla_{CTX-M-8/25}$-R	TCGAGCCGGAASGTGTYAT	497-479	
$bla_{CTX-M-9}$	$bla_{CTX-M-9}$-F	TCAAGCCTGCCGATCTGGT	299-317	561
	$bla_{CTX-M-9}$-R	TGATTCTCGCCGCTGAAG	859-842	

F: Forward, R: Reverse

Table 2: Distribution of Gram-negative bacteria (*n*=252) isolated from patients with ocular infections

Name of the bacterial isolates	Total number of isolates	Number of the Gram-negative bacterial isolates recovered from ocular infections (%)							
		Keratitis	Contact lens-associated keratitis	Contact lens-associated conjunctivitis	Dacryocystitis	Conjunctivitis	Postoperative endophthalmitis	Bandage contact lens associated corneal infection	Other ocular infections[#]
Ocular non-*Enterobacteriaceae*	162	75	24	13	11	9	16	7	7
Pseudomonas aeruginosa	112	61	11	4	7	6	13	4	6
Alcaligenes denitrificans	20	3	9	3	1		2	2	
Pseudomonas alcaligenes	12	5	1	1	2	1	1		1
Alcaligenes faecalis	9	2	3	2		1		1	
Aeromonas hydrophila	5	1		3		1			
Acinetobacter lwoffii	4	3			1				
Ocular *Enterobacteriaceae*	90	12	17	16	16	16	0	1	12
Enterobacter agglomerans	25	3	7	4	2	5			4
Klebsiella pneumoniae	25	4	3	5	7	4			2
Escherichia coli	13	2		1	3	4		1	2
Serratia marcescens	12	1	5	4		1			1
Enterobacter aerogenes	4				2				2
Enterobacter cloacae	2		1						1
Klebsiella oxytoca	2	1			1				
Morganella morganii	2	1		1					
Proteus mirabilis	2				1	1			
Citrobacter diversus	1			1					
Citrobacter freundii	1					1			
Citrobacter koseri	1		1						
Total number of isolates (%)	252	87 (34.5)	41 (16.3)	29 (11.5)	27 (10.7)	25 (9.9)	16 (6.3)	8 (3.2)	19 (7.6)

[#]Other ocular infections include scleritis (*n*=4), blepharitis (*n*=3), hordeolum (*n*=2), infection after scleral buckling surgery (*n*=2), scleral suturing infection (*n*=2), canaliculitis (*n*=1), endophthalmitis due to corneal ulcer (*n*=1), post-PKP endophthalmitis (*n*=1), posttraumatic endophthalmitis (*n*=1), traumatic endophthalmitis (*n*=1), and panophthalmitis (*n*=1). PKP: Penetrating keratoplasty

of ESBL producing non-*Enterobacteriaceae* was from contact lens-associated keratitis cases 27.5% (8 of 29) followed by bacterial keratitis and postoperative endophthalmitis 24% (7 of 29), whereas among *Enterobacteriaceae* ESBL producing isolates were obtained from dacryocystitis case 31% (4 of 13) and contact lens-associated conjunctivitis case 23% (3 of 13). The phenotypic test results of ESBL producing Gram-negative bacteria isolated from various ocular infections are presented in Table 3 and Fig. 1.

Antibacterial susceptibility testing

The susceptibility of the ESBL producing ocular *Enterobacteriaceae* and non-*Enterobacteriaceae* family isolates to commonly used antibiotics is depicted in Table 4. Out

Table 3: Determination of extended-spectrum β-lactamase producing Gram-negative isolates using cephalosporin/clavulanate combination disc test

Name of the bacterial isolates	Number of isolates tested	Number of ESBL producers	Clinical diagnosis						
			BK	CL-BK	DCT	POE	CON	CL-CON	Others[†]
Ocular non-*Enterobacteriaceae*	162	29	7	8	3	7	2	0	2
Pseudomonas aeruginosa	112	16	5	2	1	5	1		2
Alcaligenes denitrificans	20	6		5		1			
Pseudomonas alcaligenes	12	3			1	1	1		
Alcaligenes faecalis	9	2	1	1					
Aeromonas hydrophila	5	0							
Acinetobacter lwoffii	4	2	1		1				
Ocular *Enterobacteriaceae*	90	13	1	0	4	0	2	3	3
Enterobacter agglomerans	25	0							
Klebsiella pneumoniae	25	2			1			1	
Escherichia coli	13	7	1		2		2		2
Serratia marcescens	12	1						1	
Enterobacter aerogenes	4	1			1				
Enterobacter cloacae	2	1							1
Klebsiella oxytoca	2	0							
Morganella morganii	2	1						1	
Proteus mirabilis	2	0							
Citrobacter diversus	1	0							
Citrobacter freundii	1	0							
Citrobacter koseri	1	0							
Total in numbers (%)	252	42 (17)	8 (19)	8 (19)	7 (17)	7 (17)	4 (9.5)	3 (7)	5 (12)

[†]Others include ocular non-*Enterobacteriaceae* panophthalmitis (*n*=1) and scleritis (*n*=1) and ocular *Enterobacteriaceae* endophthalmitis due to corneal ulcer (*n*=1), hordeolum (*n*=1), and canaliculitis (*n*=1). BKL Bacterial keratitis, CL-BK: Contact lens-associated bacterial keratitis, DCT: Dacryocystitis, POE: Postoperative endophthalmitis, CON: Conjunctivitis, CL-CON: Contact lens-associated conjunctivitis, ESBL: Extended-spectrum β-lactamase

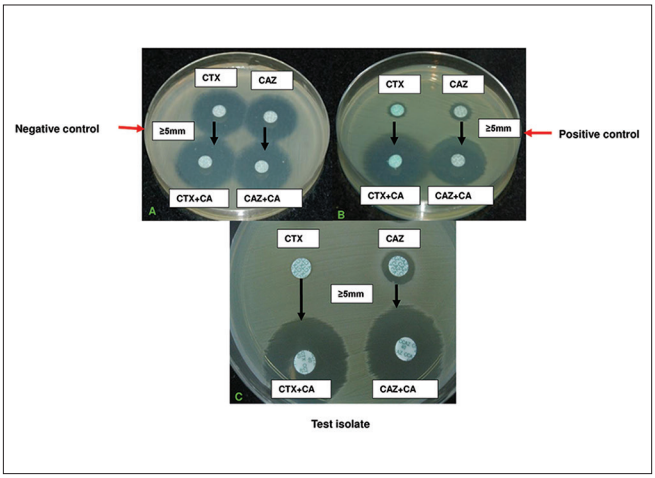

Figure 1: Phenotypic confirmatory test results of cephalosporin/clavulanate combination disc test. (a) Negative control *E. coli* ATCC 25922; (b) positive control *K. pneumoniae* ATCC 700603; (c) test isolate *E. coli* 3923 from canaliculus pus specimen. CTX: Cefotaxime (30 μg), CAZ: Ceftazidime (30 μg), CTX/CA: Cefotaxime/clavulanic acid (30 μg/10 μg), CAZ/CA: Ceftazidime/clavulanic acid (30 μg/10 μg). *E. coli*: *Escherichia coli*, *K. pneumonia*: *Klebsiella pneumoniae*

of *Enterobacteriaceae* (36% [90 of 252]) isolates 14% (13 of 90) were ESBL producers, and the remaining 86% (77 of 90) were non-ESBL producers. Among the 13 ESBL producing *Enterobacteriaceae* family isolates, the highest rate of susceptibility observed to chloramphenicol 12 (92%), followed by amikacin 11 (85%), gatifloxacin 9 (69%), and tobramycin 7 (54%) and the highest percentage of resistance was observed in the following order: Cefazolin 11 (85%), cefotaxime 10 (77%), ceftazidime 10 (77%), ciprofloxacin 10 (77%), norfloxacin 9 (69%), and cefpodoxime 9 (69%). Among the 77 non-ESBL producing *Enterobacteriaceae* family isolates the rate of susceptibility to various antibacterials was as follows: Tobramycin 76 (99%), amikacin 73 (95%), levofloxacin 72 (94%), and the highest rate of resistance was observed with respect to cefazolin 47 (61%) and cefpodoxime 23 (30%).

Among the ocular non-*Enterobacteriaceae* 64% (162 of 252) isolates tested, 18% (29 of 162) were ESBL producers and the remaining 82% (133 of 162) were non-ESBL producers. The 29 ESBL producing non-*Enterobacteriaceae* family isolates showed the highest rate of susceptibility to gatifloxacin 19 (66%), ofloxacin and gentamicin each 16 (55%), and the highest percentage of resistance was observed to cefazolin 7 (87.5%) (34 isolates tested) and ceftazidime 8 (80%) (38 isolates tested). Among the 133 non-ESBL producing non-*Enterobacteriaceae* family isolates showed the highest rate of susceptibility was observed to tobramycin 125 (94%), gatifloxacin 122 (92%), levofloxacin 121 (91%), and ciprofloxacin 118 (89%) and the highest rate of resistance was observed with cefazolin 20 (77%) (34 isolates tested) and cefpodoxime 15 (54%) (38 isolates tested).

Table 4: *In vitro* antibacterial susceptibility profile of extended-spectrum β-lactamase and nonextended-spectrum β-lactamase producing *Enterobacteriaceae* (*n*=90) and non-*Enterobacteriaceae* (*n*=162) isolates recovered from ocular infections

Name of the antibacterial agents (concentration)	*Enterobacteriaceae* (*n*=90)						Non-*Enterobacteriaceae* (*n*=162)					
	ESBL producers (*n*=13) (14%)			Non-ESBL producers (*n*=77) (85%)			ESBL producers (*n*=29) (18%)			Non-ESBL producers (*n*=133) (82%)		
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
Amikacin (30 mcg)	11 (85)	2 (15)	0	73 (95)	2 (2.5)	2 (2.5)	15 (52)	2 (7)	12 (41)	113 (85)	4 (3)	16 (12)
Gentamicin (10 mcg)	4 (31)	1 (8)	8 (61)	67 (87)	3 (4)	7 (9)	16 (55)	0	13 (45)	106 (80)	1 (1)	26 (19)
Tobramycin (10 mcg)	7 (54)	1 (8)	5 (38)	76 (99)	1 (1)	0	14 (48)	2 (7)	13 (45)	125 (94)	0	8 (6)
Cefazolin (30 mcg)*	2 (15)	0	11 (85)	30 (39)	0	47 (61)	1 (12.5)	0	7 (87.5)	6 (23)	0	20 (77)
Cefotaxime (30 mcg)	1 (8)	2 (15)	10 (77)	62 (80.5)	13 (17)	2 (2.5)	7 (24)	12 (41)	10 (35)	65 (49)	56 (42)	12 (9)
Cepfodoxime (10 mcg)#	3 (23)	1 (8)	9 (69)	29 (38)	25 (32)	23 (30)	0	2 (20)	8 (80)	7 (25)	6 (21)	15 (54)
Ceftazidime (30 mcg)	1 (8)	2 (15)	10 (77)	67 (87)	9 (12)	1 (1)	8 (27)	6 (21)	15 (52)	97 (73)	15 (11)	21 (16)
Ceftriaxone (30 mcg)	5 (39)	2 (15)	6 (46)	66 (86)	10 (13)	1 (1)	5 (17)	20 (69)	4 (14)	41 (31)	83 (62)	9 (7)
Aztreonam (30 mcg)	6 (46)	0	7 (54)	71 (92)	4 (5)	2 (3)	10 (34.5)	10 (34.5)	9 (31)	85 (64)	28 (21)	20 (15)
Ciprofloxacin (5 mcg)	3 (23)	0	10 (77)	64 (83)	5 (7)	8 (10)	16 (55)	1 (3)	12 (41)	118 (89)	1 (1)	14 (10)
Gatifloxacin (5 mcg)	9 (69)	3 (23)	1 (8)	71 (92)	2 (3)	4 (5)	19 (66)	1 (3)	9 (31)	122 (92)	0	11 (8)
Levofloxacin (5 mcg)	4 (31)	5 (38)	31	72 (94)	1 (1)	4 (5)	16 (55)	4 (14)	9 (31)	121 (91)	1 (1)	11 (8)
Norfloxacin (10 mcg)	3 (23)	1 (8)	9 (69)	67 (87)	1 (1)	9 (12)	14 (48.3)	1 (3.4)	14 (48.3)	110 (83)	3 (2)	20 (15)
Ofloxacin (5 mcg)	5 (39)	2 (15)	6 (46)	69 (90)	0	8 (10)	16 (55.2)	3 (10.3)	10 (34.5)	118 (89)	1 (1)	14 (10)
Chloramphenicol (30 mcg)#	12 (92)	0	1 (8)	63 (82)	7 (9)	7 (9)	4 (50)	2 (25)	2 (25)	20 (77)	3 (11.5)	3 (11.5)

*34 non-*Enterobacteriaceae* family isolates tested, #38 non-*Enterobacteriaceae* family isolates tested. S: Sensitive, I: Intermediate, R: Resistant, ESBL: Extended-spectrum β-lactamase

Molecular detection of extended-spectrum beta-lactamase genes

Among the 252 isolates, 156 (62%) showed positive amplification for ESBL encoding resistant genes, of which 83 (53%) were non-*Enterobacteriaceae* isolates and 73 (47%) were *Enterobacteriaceae* isolates. Among the 83 non-*Enterobacteriaceae* isolates, bla_{TEM}-gene was more prevalent in 94% (78 of 83), followed by bla_{OXA} in 29% (24 of 83) and bla_{SHV} in 4% (3 of 83). Among the 73 *Enterobacteriaceae* isolates, bla_{TEM}-gene was more prevalent in 70% (51 of 73), followed by bla_{OXA}-gene in 42.5% (31 of 73), bla_{SHV}-gene in 36% (26 of 73), and $bla_{CTX-M-1}$ 16% (12 of 73). All the 252 Gram-negative bacterial isolates were subjected to genotypic detection of ESBL encoding resistant genes by mPCR analysis and the results are presented in Table 5 and Figs. 2, 3.

DNA sequencing analysis

For further confirmation and characterization, six amplified products (two amplified with bla_{TEM}-gene-specific primer, two with bla_{SHV}-gene-specific primer, one with bla_{OXA}-gene-specific primer, and one with $bla_{CTX-M-1}$-gene-specific primer) from multidrug-resistant bacterial isolates were subjected to DNA sequencing. A DNA database comparison of partial sequencing of bla_{TEM}, bla_{SHV}, bla_{OXA}-, and $bla_{CTX-M-1}$-genes demonstrated six EBSL enzymes - TEM-193 (JN588556), TEM-195 (JN588557), SHV-142 (JN596240), SHV-143 (JN565741), OXA-162 (JN565742), and CTX-M-15 (KC200081) have been reported in NCBI.

Discussion

ESBL producing Gram-negative bacterial isolates mainly *Enterobacteriaceae* are now an increasing problem worldwide. The emergence and continuous spread of these bacteria seem to be caused mainly by extensive use of broad-spectrum antibacterial agents to treat the infections, and the presence of mobile genes on plasmid encoding ESBLs among the pathogens have been on an increase every year complicating the treatment strategies in patients. ESBL producing organisms are usually reported as hospital-acquired, especially in the Intensive Care Units.[20] Other hospital units that are at risk includes surgical wards, pediatrics and neonatology, rehabilitation units, and oncology wards.[21] Countries with a high rate of prevalence include Turkey (60%), Brazil (45.4%), Western Pacific (24.6%), the Netherlands (22.6%), and Iran (44–74%).[22,23] However, from the current study, the prevalence of ESBL producing ocular pathogens of the *Enterobacteriaceae* and non-*Enterobacteriaceae* family was found to be 47% and 53%, respectively. The previous study reported by Sowmiya *et al*. presented the prevalence of ESBL producing ocular *Enterobacteriaceae* as 77% (57 of 73) at the molecular level. Few surveillance studies that have documented the resistance pattern of ocular pathogens include, Ocular TRUST and The Surveillance Network with the exclusion of ESBL.[24,25] Proper identification of genes involved in ESBL-mediated resistance is a necessity for the surveillance and epidemiological analysis and on this basis the present study was envisaged to determine the prevalence of ESBL encoding bla_{TEM}, bla_{SHV}, bla_{OXA}-, and bla_{CTX-M}- (variant 1, 2, 9, 8/25) resistant genes among ocular Gram-negative bacterial isolates. In the present study, the rate of isolation of ocular *Enterobacteriaceae* and non-*Enterobacteriaceae* is similar to the rates in our earlier study reported by Jayahar Bharathi *et al*., which showed 40% and 60%, respectively.[7]

In India, the incidence of ESBL mediated resistance was observed among 60–68% of clinical pathogens that were isolated from major hospitals,[26] but there are very few reports for ocular pathogens from the *Enterobacteriaceae* family which shows the incidence to be ranging from 7% to 77%.[7,12] In our

Table 5: Distribution of extended-spectrum β-lactamases encoding resistance genes among Gram-negative bacteria isolates recovered from ocular infections

Name of the bacterial isolates	Total number of isolates tested	Number of positive genes (%)	Positive by mPCR for ESBL-encoding resistance genes (*n*=156)								
			bla_{TEM} alone	bla_{TEM} + bla_{OXA}	bla_{SHV} alone	bla_{TEM} + bla_{SHV}	bla_{OXA} alone	bla_{TEM} + bla_{OXA} + $bla_{CTX-M-1}$	bla_{TEM} + bla_{SHV} + bla_{OXA}	bla_{OXA} + $bla_{CTX-M-1}$	Other ESBL genes*
Ocular non-*Enterobacteriaceae*	162	83 (53)	58 (70)	18 (22)	-	1 (1)	4 (5)	-	1 (1)	-	1 (1)
Pseudomonas aeruginosa	112	53	34	16	-	1	2	-	-	-	-
Alcaligenes denitrificans	20	9	8	-	-	-	1	-	-	-	-
Pseudomonas alcaligenes	12	7	5	1	-	-	1	-	-	-	-
Alcaligenes faecalis	9	6	5	1	-	-	-	-	-	-	-
Aeromonas hydrophila	5	5	3	-	-	-	-	-	1	-	1
Acinetobacter lwoffii	4	3	3	-	-	-	-	-	-	-	-
Ocular *Enterobacteriaceae*	90	73 (47)	18 (24.7)	17 (23.3)	15 (20.5)	6 (8.2)	2 (2.7)	6 (8.2)	3 (4.1)	2 (2.7)	4 (5.5)
Enterobacter agglomerans	25	17	4	7	3	1	-	1	1	-	-
Klebsiella pneumonia	25	20	5	2	11	1	-	-	1	-	-
Escherichia coli	13	13	2	-	-	1	2	5	-	2	1
Serratia marcescens	12	12	3	7	-	1	-	-	1	-	-
Enterobacter aerogenes	4	4	1	-	1	1	-	-	-	-	1
Enterobacter cloacae	2	1	-	-	-	-	-	-	-	-	1
Klebsiella oxytoca	2	2	1	-	-	-	-	-	-	-	1
Morganella morganii	2	1	-	1	-	-	-	-	-	-	-
Proteus mirabilis	2	2	1	-	-	1	-	-	-	-	-
Citrobacter diversus	1	0	-	-	-	-	-	-	-	-	-
Citrobacter freundii	1	1	1	-	-	-	-	-	-	-	-
Citrobacter koseri	1	0	-	-	-	-	-	-	-	-	-
Total in numbers (%)	252	156 (62)	76 (49)	35 (22)	15 (10)	7 (4.5)	6 (4)	6 (4)	4 (2.5)	2 (1)	5 (3)

*Other ESBL genes: Ocular non-*Enterobacteriaceae* include bla_{SHV}+bla_{OXA} (*n*=1) and ocular *Enterobacteriaceae* include $bla_{CTX-M-1}$ alone (*n*=1), bla_{TEM} + $bla_{CTX-M-1}$ (*n*=1), bla_{SHV} + $bla_{CTX-M-1}$ (*n*=1), and bla_{SHV} + bla_{OXA} + $bla_{CTX-M-1}$ (*n*=1). ESBL: Extended-spectrum β-lactamase, mPCR: Multiplex polymerase chain reaction

current analysis, the rate of ESBL production among ocular pathogens from *Enterobacteriaceae* family was 14% (13 of 90) and non-*Enterobacteriaceae* family 18% (29 of 162). ESBLs have been reported most frequently in *E. coli* and *K. pneumoniae* from nosocomial and community-acquired infections.[27] Similarly, the present study shows the prevalence of ESBL-production was 54% in ocular *Enterobacteriaceae* family isolates. *E. coli* was isolated from patients with conjunctivitis and dacryocystitis case indicated the existence of community-associated strains. Isolation of ESBL producing non-*Enterobacteriaceae* family isolate *P. aeruginosa* (55%) in the case of postoperative endophthalmitis also indicated that the strains were associated with hospital-acquired predominantly during surgery notwithstanding all standard aseptic preventive measures, includes the use of povidone-iodine, proper instilling of preoperative antibiotics, dedicated sterilized instruments, and intraocular lenses. Similarly, our current study reports well correlated with our earlier study reported by Jayahar Bharathi *et al*.[7]

ESBL producing ocular *Enterobacteriaceae* family isolates showed increased susceptibility to chloramphenicol (92%) and amikacin (85%). A higher degree of resistance was seen to cefazolin (85%), cefotaxime, ceftazidime, and ciprofloxacin (each 77%). In a study conducted at our hospital by Bharathi *et al*., in 2010,[3] ocular *Enterobacteriaceae* family isolates from community-acquired ocular infections showed the highest percentage of susceptibility to amikacin 92% and gatifloxacin 93%. The switch in the resistance rates of cefotaxime and ceftazidime from 40% and 45% to 77% demonstrate the widening spread of drug resistance among ocular pathogens. ESBL positive ocular non-*Enterobacteriaceae* family isolates showed increased susceptibility to gatifloxacin (66%), followed by ofloxacin and gentamicin (each 55%) and a higher degree of resistance to cefazolin, cefotaxime, and ceftazidime 87.5%, 80%, and 52%, respectively. This also confirms the development of resistance rates of cefotaxime and ceftazidime from 35% and 18% to 80% and 52%, respectively.

The prevalence of ESBLs types greatly vary with geographical regions, and in most cases,[11,19] bla_{TEM}-gene has been reported with a higher frequency, while in some places,[28,29] bla_{SHV}-gene are more prevalent than other types. The present study reports the most prevalent gene detected to be bla_{TEM}-gene (83%) followed by bla_{OXA}-gene (35%) and bla_{SHV}-gene (18.5%) alone or together. Our present study shows the greater difference when compared with previous findings. It was higher than the previous study conducted by Sowmiya *et al*. showed that bla_{TEM}-gene (77%) was more prevalent followed by bla_{SHV}-gene (35%) and bla_{OXA}-gene (33%). In addition to TEM, SHV, and OXA-types, another class of ESBL, CTX-M type-β-lactamases has been reported to be more active against cefotaxime and ceftriaxone than ceftazidime, but a possibility of point mutation can always increase the activity against ceftazidime.[30] The prevalence of bla_{CTX-M}-gene also stands reported indicating 73% in Indian hospitals among clinical isolates, especially among

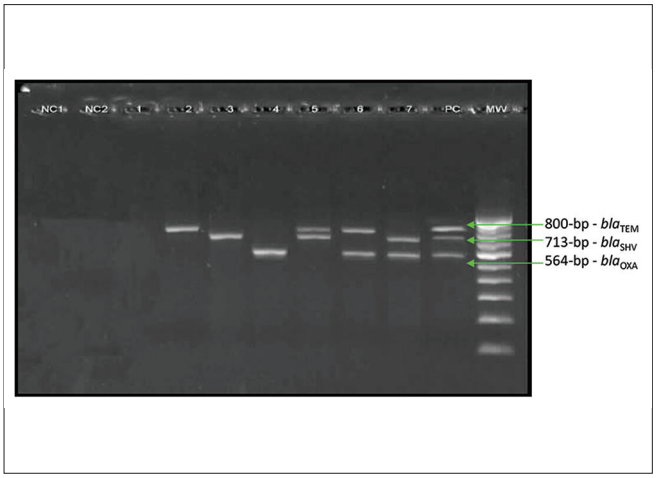

Figure 2: Agarose gel electrophoresis of multiplex polymerase chain reaction products for bla_{TEM}, bla_{SHV}, and bla_{OXA}-genes. Lane NC1 and NC2: Negative controls, Lane 1: *E. coli* ATCC 25922 (negative control for extended-spectrum beta-lactamase genes), Lane 2: *P. aeruginosa* 13134 strain positive for bla_{TEM}-gene (800 bp) alone, Lane 3: *E. agglomerans* 4245 strain positive for bla_{SHV}-gene (713 bp) alone, Lane 4: *P. aeruginosa* 13111 strain positive for bla_{OXA}-gene (564 bp) alone, Lane 5: *P. aeruginosa* 4211 strain positive for both bla_{TEM} and bla_{SHV}-genes (800 bp and 713 bp), Lane 6: *P. aeruginosa* 12839 strain positive for both bla_{TEM} and bla_{OXA}-genes (800 bp, 564 bp), Lane 7: *A. hydrophila* 4019 strain positive for both bla_{SHV} and bla_{OXA}-genes (713 bp, 564 bp), Lane PC: Positive control, Lane MW – Marker 100 bp DNA ladder. *E. coli*: *Escherichia coli*, *P. aeruginosa*: *Pseudomonas aeruginosa*, *A. hydrophila: Aeromonas hydrophila*, *E. agglomerans*: *Enterobacter agglomerans*

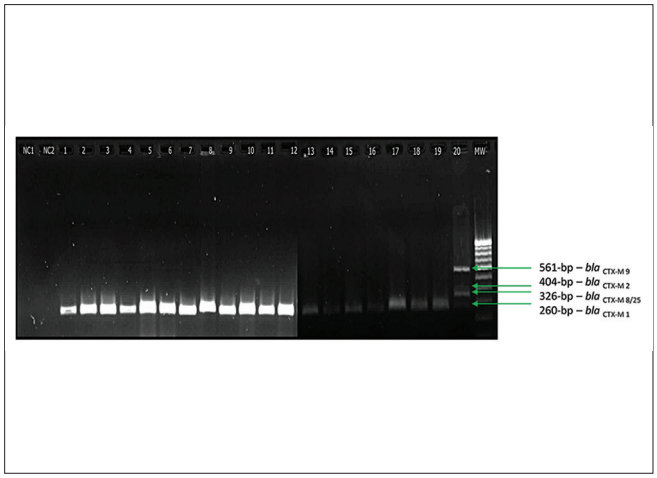

Figure 3: Agarose gel electrophoresis of multiplex polymerase chain reaction products for $bla_{CTX-M-1}$, $bla_{CTX-M-2}$, $bla_{CTX-M-8/25}$, and $bla_{CTX-M-9}$-genes. Lane NC1 and NC2: Negative controls, Lane 1-19: Amplification of $bla_{CTX-M-1}$, $bla_{CTX-M-2}$, $bla_{CTX-M-8/25}$, and $bla_{CTX-M-9}$ primers sets with template DNA of study subjects. Lane PC: positive control, Lane MW - Marker 100 bp DNA ladder

E. coli and *K. pneumoniae*.[31] However, from the current study shows a prevalence of $bla_{CTX-M-1}$ gene (8%) majorly among *E. coli* (67%); more specifically CTX-M-15 type. It was lower than the previous study reported by Sowmiya *et al*. showed that bla_{CTX-M}-gene (56%). CTX-M enzymes like CTX-M-9 have been reported from Spain, CTX-M-2 from most South American countries, Japan, and Israel, whereas CTX-M-15 exist worldwide.[32]

Our current study demonstrates that the high prevalence of common beta-lactamase genes, bla_{TEM} (16%) were detected more from patients with corneal ulcer and contact lens-related conjunctivitis cases, followed by bla_{OXA}-genes (25%) detected more from contact lens-related conjunctivitis cases. Current findings undoubtedly could be ascribed to microbial contamination of contact lens storage, especially by Gram-negative bacteria that lead to the development of contact lens-related corneal and conjunctival infections.[33] The contaminating source of contact lens during storage appear to be mainly of external origin, especially water contaminated with Gram-negative bacteria leading to a widespread incidence of EBSL producing strains. Topical broad-spectrum aminoglycoside and fluoroquinolones were used to treat these infections. In nosocomial settings, the prevalence of bla_{TEM} gene (9%) were detected more among *P. aeruginosa* (17%) isolated from vitreous specimens collected from postoperative endophthalmitis cases referred from outside hospital. The major source of the ESBL producing *P. aeruginosa* strains is contaminated irrigating fluids, contaminated instruments, and poor operating room environments. Topical or intraocular piperacillin/tazobactam has been used for the treatment of such cases.[34,35]

Conclusion

The present study shows the low prevalence of ESBL encoding resistant genes among ocular pathogens isolated in our hospital setup. The prevalence of ESBL producing isolates is of crucial importance among ocular *Enterobacteriaceae* and *P. aeruginosa*. Molecular detection and characterization of ESBL producing ocular pathogens confirm the presence of common ESBL genotypes majority from community-acquired infection and nosocomial settings. It is a real challenge to an ophthalmologist and one should limit the therapeutic problem. Our data suggest that the prevalence of ESBL producing Gram-negative ocular pathogens in nosocomial and community settings in developing countries is probably under-reported. Our single center study report could not reflect a broader regional context among ocular isolates and study data suggest more study has to be conducted in different parts of South India.

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Conflicts of interest

There are no conflicts of interest.

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