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Occurrence of extended-spectrum β-lactamase, AmpC, and carbapenemase-producing genes in gram-negative bacterial isolates from human immunodeficiency virus infected patients

Marimuthu Ragavan Rameshkumar[a], Narasingam Arunagirinathan[a,b,*], Balasubramanian Senthamilselvan[a], Chinnambedu Ravichandran Swathirajan[c], Sunil Suhas Solomon[d,e], Ramachandran Vignesh[c,f], Pachamuthu Balakrishnan[c], Reem M. Aljowaie[g], Khalid S. Almaary[g], Tse-Wei Chen[h]

[a] Department of Microbiology and Biotechnology, Presidency College (Autonomous), Affiliated to University of Madras, Chennai, Tamil Nadu, India
[b] Central Research Laboratory, Meenakshi Academy of Higher Education and Research (Deemed to be University), Chennai, India
[c] Infectious Diseases Laboratory, Y.R. Gaitonde Centre for AIDS Research and Education, Voluntary Health Services Hospital Campus, Chennai, India
[d] HIV Clinic, Y.R. Gaitonde Centre for AIDS Research and Education, Voluntary Health Services Hospital Campus, Chennai, India
[e] Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, USA
[f] Preclinical Department, Faculty of Medicine, Universiti Kuala Lumpur Royal College of Medicine Perak (RCMP UniKL), Ipoh, Malaysia
[g] Department of Botany and Microbiology, College of Science, King Saud University, P.O. 2455, Riyadh, Saudi Arabia
[h] Department of Materials, Imperial College London, London, SW7 2AZ, United Kingdom

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ABSTRACT

Background: Progressive decline of immune response in HIV patients makes them susceptible to frequent bacterial infections. High usage of antibiotics influences the emergence of multidrug-resistant bacteria and worsens the clinical outcomes. In this study, the occurrence of drug-resistant genes in Gram-negative bacterial isolates from HIV patients in South India was analyzed.
Methods: A total of 173 Gram-negative bacterial (GNB) isolates from HIV patients were screened for antibiotic susceptibility profile using the Kirby-Bauer diskdiffusion method. Positivity of drug-resistant genes was analyzed using polymerase chain reaction method.
Results: In this study, 72.8% of bacterial isolates were obtained from urine specimens, and *Escherichia coli* (47.4%) was the predominantly isolated bacterium. Overall, 87.3% and 83.2% of GNB were resistant to 3rd generation cephalosporin antibiotics such as cefotaxime and ceftazidime, respectively, 56.6% were resistant to cephamycin (cefoxitin) and 43% to carbapenem (imipenem) antibiotics. Extended-spectrum β-lactamases (ESBL) production was noted among 79.5% of GNB isolates, followed by AmpC (57.1%) and Metallo β-lactamases (37.3%). Molecular analysis revealed that ESBL genes such as *bla*TEM (94.1%), *bla*CTX-M (89.2%), and *bla*SHV (24.2%) were detected at higher levels among GNB isolates. Carbapenemase-producing genes such as *bla*OXA-48 (20%), *bla*OXA-23 (2.6%), and both *bla*OXA-23 and *bla*OXA-51 like genes (2.6%) and AmpC producing genes such as *bla*CIT (26.7%), *bla*DHA (3.6%), and *bla*ACC (1.8%) were detected at low-level.
Conclusions: This study concludes that ESBL producing genes are detected at high level among gram-negative bacterial isolates from HIV patients in South India.

Introduction

The progressive decline of immune responses makes people living with Human Immunodeficiency Virus (PLHIV) highly susceptible to various bacterial infections. Among PLHIV in developing countries, bacterial infections caused by the Enterobacteriaceae represent the second most common cause of death after tubercu-

* Corresponding author at: Department of Microbiology and Biotechnology, Presidency College (Autonomous), Chennai, 600 005, Tamil Nadu, India.
E-mail address: n_arunagiri@yahoo.co.in (N. Arunagirinathan).

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losis. Continuous usage of prophylactic antibiotic therapy to treat frequent bacterial infections leads to emergence of drug-resistant bacterial pathogens [1]. Bacterial pathogens with multidrug-resistance (MDR) profile have become a severe public health threat that limits the availability of effective antibiotics [2]. MDR bacterial infections are associated with increased morbidity and mortality, prolonged hospital stays, direct and indirect costs, prolonged usage of antibiotics, and greater opportunities to spread in the community [3]. Increasing resistance to 3rd generation cephalosporins (3GCs) due to the production of extended-spectrum β-lactamases (ESBLs) enhances the usage of carbapenem antibiotics as a drug of choice for the treatment. Nowadays, resistance against carbapenem antibiotics also developed due to persistent exposure and the emergence of carbapenemase (e.g. Metallo β-lactamases) producing bacteria [4]. Cephalosporins and carbapenem-resistant bacteria spread rapidly, causing a severe outbreak and also threatens human life. In the global priority pathogens list (global PPL), World Health Organization (WHO) categorized the carbapenem-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* and carbapenem-resistant and ESBLs producing Enterobacteriaceae as critical priority bacteria [5]. Communicable and Infectious Disease Steering Committee (CIDSC) also classified critical priority drug-resistant bacteria as priority pathogens for antimicrobial surveillance [6]. In HIV patients, the reports of antibiotic resistance in Gram-negative bacteria (GNB) causing infections are very limited among the Indian population [7]. In this study, molecular profile of ESBL, carbapenemase, and AmpC beta-lactamase-producing genes was analyzed among GNB isolated from HIV patients in South India.

Materials and methods

Bacterial isolates and antibiotic susceptibility test

Bacterial strains were isolated from clinical specimens collected from HIV-infected patients attending YR Gaitonde Centre for AIDS Research and Education (YRG CARE), Voluntary Health Services Hospital, India, using standard culture and biochemical tests [8]. Antibiotic susceptibility of bacterial isolates was screened by Kirby–Bauer disc [9] diffusion method according to CLSI guidelines [10]. In this method, lawn culture was made using culture suspension (adjusted to 0.5 McFarland's standards) of bacterial test isolates on the surface of Mueller–Hinton agar (MHA) plates. Standard antibiotics such as ampicillin (10 μg), piperacillin (100 μg), piperacillin-tazobactam (100/10 μg), amikacin (30 μg), gentamicin (10 μg), aztreonam (30 μg), erythromycin (15 μg), cefoxitin (30 μg), cefpodoxime (30 μg), cefoperazone (75 μg), ceftriaxone (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), imipenem (10 μg), ertapenem (10 μg), ciprofloxacin (5 μg), co-trimoxazole (25 μg), doxycycline (30 μg), tetracycline (30 μg), chloramphenicol (30 μg) and trimethoprim (5 μg) (HiMedia, India) were used to screen the sensitive, intermediate and resistant patterns of bacterial isolates. *E. coli* ATCC 25922 was used as quality control strain for the antimicrobial susceptibility test.

Phenotypic detection of β-lactamase producers

ESBL, Metallo β-lactamases (MBL) and AmpC productions in GNB were screened using the combination disc method (CDM). ESBL producers were detected using cefotaxime (30 μg) and cefotaxime-clavulanic acid (30 μg/10 μg) discs along with ceftazidime (30 μg) and ceftazidime-clavulanic acid (30 μg/10 μg) (HiMedia, India) discs. An increased zone of inhibition of ≥5 mm was interpreted as positive for ESBL production [10]. MBL production was detected using imipenem (10 μg) discs, with and without anhydrous EDTA (750 μg), and an increased zone of inhibition of >4 mm around the imipenem-EDTA disc compared to the imipenem alone was considered as positive for MBL [11]. Cefoxitin-resistant bacteria were also checked for the production of AmpC β-lactamases using cefoxitin (30 μg) and cefoxitin/cloxacillin (30/200 μg) discs [12] and an increased zone of inhibition of >4 mm around the cefoxitin/cloxacillin disc compared to the cefoxitin alone was considered as positive for AmpC.

Molecular detection of drug-resistant genes

Bacterial DNA extracted by boiling lysis method was used as a template for detecting drug resistance genes in the bacterial isolates using polymerase chain reaction (PCR). Genes responsible for ESBLs production such as *bla*TEM, *bla*SHV, *bla*CTX-M, *bla*CTX-M group 1, *bla*CTX-M group 2, *bla*CTX-M group 5, *bla*CTX-M group 9, *bla*CTX-M group 25, *bla*OXA, *bla*VEB, and *bla*GES were screened using conventional PCR [13–17]. MBL producing genes such as *bla*IMP, *bla*VIM, *bla*GIM, *bla*SPM, *bla*SIM, and other carbapenemase-producing genes such as *bla*OXA-48, *bla*OXA 23 like, *bla*OXA 24 like, *bla*OXA 53 like, *bla*OXA 58 like, and *Klebsiella pneumoniae* Carbapenemases (KPC) and AmpC producing genes such as *bla*MOX, *bla*CIT, *bla*DHA, *bla*ACC, *bla*EBC, and *bla*FOX were screened using multiplex PCR [18–23]. Class 1 and class 2 integrons were also detected using PCR with specific primers. In addition, sulfamethoxazole resistance genes such as *sul1* and *sul2* were detected among integron-positive GNB isolates [24–27].

Amplified PCR products were separated using 2% agarose gel electrophoresis. Ethidium bromide (EtBr) solution was prepared by dissolving 5 mg of EtBr powder in 10 mL of distilled water, and 0.5X TBE (Tris-borate-EDTA) buffer was used for electrophoresis. For separation of amplified genes, 10 μL of PCR product was mixed with 2 μL of gel loading dye, and PCR products have been resolved at 100 V for 20 min. The size of DNA fragments in the gel was determined using 5 μL of 100 bp DNA ladder (GeneDireX, India), and the agarose gels were visualized using the BioRad gel documentation system. The Chi-square test was used for the determination of the significance of the association. The p-value ≤ 0.05 was considered as significant.

Results

In this study, a total of 173 Gram-negative bacterial strains were isolated from 183 HIV patients and among them, 38.3% were males and 61.7% were females. The CD4+ T lymphocyte cell counts of the HIV patients included in this study were ranged from 05 to 929 cells/mm^3 (median cell count was 296 cells/mm^3). *Escherichia coli* (47.4%) was the predominantly isolated bacterium, followed by *Klebsiella pneumoniae* (17.3%). Specimen-wise distribution showed that a high percentage (72.8%) of bacterial isolates were obtained from the urine specimens followed by pus specimens (15%) (Table 1).

Antibiotic susceptibility profile of the bacterial isolates

Antibiotic susceptibility profile of the GNB isolates revealed that 87.3% and 83.2% of the bacterial isolates were resistant to 3GC such as cefotaxime and ceftazidime, respectively, and 43% were resistant to carbapenem antibiotic, namely imipenem. The resistance rate of bacterial isolates to cefoxitin was 56.6%. Also, 96% of bacterial isolates were resistant to cefoperazone, followed by 94.8% to ampicillin, 92.5% to cefpodoxime, 92% to aztreonam, 87% to trimethoprim, 83.2% to piperacillin, and 77.4% to piperacillin-tazobactam. A high level of sensitivity was noted against ertapenem (77.5%), followed by amikacin (74%) and chloramphenicol (71.1%).

Table 1
Sample-wise distribution of bacterial isolates along with number of organisms producing β-lactamases from HIV patients.

Samples	Organisms						
	E. coli	*K. pneumoniae*	*K. oxytoca*	*P. vulgaris*	*P. mirabilis*	*P. aeruginosa*	*A. baumannii*
Urine	72	18	17	1	10	8	0
Pus	6	6	1	2	2	7	2
Sputum	1	5	3	0	1	6	0
Blood	2	0	0	0	0	0	0
Skin Scrapping	0	1	0	0	0	0	0
Vaginal Swab	1	0	0	0	0	0	0
Fine Needle Aspiration Cytology	0	0	0	0	1	0	0
Total	82	30	21	3	14	21	2
%	47.4	17.3	12.1	1.7	8.1	12.1	1.2
p Value	0.002*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
β-lactamases							
ESBL (120)	68	20	7	2	9	14	0
MBL (28)	12	6	5	0	1	3	1
AmpC (56)	17	15	12	2	6	4	0

NA = Not Applicable.
E. coli = Escherichia coli; *K. pneumoniae = Klebsiella pneumoniae*; *K.oxytoca = Klebsiella oxytoca*; *P. aeruginosa = Pseudomonas aeruginosa*; *P. vulgaris = Proteus vulgaris*; *P. mirabilis = Proteus mirabilis*; *A. baumannii = Acinetobacter baumannii*.
* Statistically significant.

ESBL, MBL & AmpC

Of the 151 cefotaxime resistant GNB isolates, 79.5% were positive for ESBL production by CDM. A high level of ESBL positivity was noted among *E. coli* (56.7%), followed by *K. pneumoniae* (16.6%). MBL production was observed among 37.3% of the 75 carbapenem (imipenem)-resistant isolates, and a high level of MBL production was noted among *E. coli* (42.8%). It was found that 57.1% of the 98 cefoxitin-resistant isolates showed positive for AmpC production, and among them, 53.4% were also positive to ESBL production (Table 1).

Molecular analysis of drug-resistant genes

Molecular analysis of ESBL producing isolates showed that 94.1%, 89.2%, and 24.2% were positive for ESBL genes *bla*TEM, *bla*CTX-M, and *bla*CTX-M, respectively. The *bla*CTX-M genes were further sub-typed and 91.5% ($p < 0.001$) of *bla*CTX-M genes were belonging to *bla*CTX-M group 1 (415 bp), 7.3% ($p < 0.001$) to *bla*CTX-M group 25 (327 bp) and 1.2% ($p < 0.001$) to *bla*CTX-M group 9 (205 bp). It was also found that 58.3% of the ESBL producing isolates showed positive for *bla*OXA gene (885 bp) (Fig. 1). *bla*TEM, *bla*CTX-M, and *bla*OXA genes were sequenced and identified as *bla*TEM-116, *bla*CTX-M-15, and *bla*OXA-1, respectively.

Among 75 carbapenem-resistant isolates, 20% ($p < 0.001$) were found to be positive for *bla*OXA-48, 2.6% for *bla*OXA 23 like, and another 2.6% for both *bla*OXA 23 like and *bla*OXA 51 like genes (Table 1). Furthermore, 18.3% of the cefoxitin-resistant isolates exhibited positive for AmpC producing genes, and among them, 26.7% were tested positive for *bla*CIT, followed by 3.6% for *bla*DHA, and 1.8% for *bla*ACC (Figs. 1 and 2). It was noted that 17% of the isolates positive for ESBL, MBL and AmpC production. Among the multiple β-lactamases producing isolates from HIV patients, *K. pneumoniae* (50%) was the predominant isolates followed by *E. coli* (25%), *K. oxytoca* (12.5%) and *P. mirabilis* (12.5%). These isolates showed 100% positive to both *bla*TEM and *sul1*, followed by 87.5% to *bla*OXA, 75% to *sul2* and 56.2% to both *bla*CTX-M and *Int1*. The percentage of drug-resistant genes detected among multiple β-lactamase producing isolates from HIV patients was given in Fig. 2.

Analysis of integrons among multidrug-resistant GNB revealed that more isolates had *IntI1* ($p < 0.001$) as compared to *IntI2* ($p < 0.001$). Among class 1 integron harboring isolates, 33.3% each showed positive for *bla*TEM, *bla*CTX-M, *bla*SHV and *bla*OXA; *bla*TEM, *bla*CTX-M and *bla*OXA, 12.2% each for *bla*TEM and *bla*CTX-M; *bla*CTX-M, *bla*SHV and *bla*OXA and 3% each for *bla*TEM and *bla*OXA; *bla*TEM and *bla*SHV; *bla*TEM, *bla*CTX-M and *bla*SHV. Among class 2 integron harboring isolates, 16.7% showed positive for *bla*CTX-M and *bla*OXA, 11.1% each for *bla*TEM and *bla*CTX-M; *bla*CTX-M, *bla*OXA and *bla*CITM; *bla*TEM, *bla*CTX-M, and *bla*OXA; *bla*TEM, *bla*CTX-M, *bla*SHV and *bla*OXA, 5.5% each for *bla*TEM, *bla*OXA and *bla*TEM; *bla*TEM and *bla*DHA; *bla*TEM, *bla*CTX-M, and *bla*DHA, 3.8% for *bla*TEM and *bla*OXA, 5.4% for *bla*TEM, 5.2% for *bla*CTX-M, 4.8% for *bla*OXA, 3.2% for *bla*SHV. Class 1 and class 2 integrons harboring bacterial isolates were further tested for sulfamethoxazole resistance genes and observed that 57.6% of the class 1 integron harboring isolates showed positive for *sul2* gene and 42.4% of them for both *sul1* and *sul2* genes. Of class 2 integron harboring isolates, 61.1% showed positive for *sul1* and *sul2* and 38.9% for *sul2* (Fig. 1 & Supplementary Table 1).

Discussion

HIV infected persons are having frequent episodes of bacterial infections due to compromised immune system. Furthermore, the development of drug resistance in bacterial pathogens could cause serious challenges in treating infections in the immunocompromised patients [28,29]. In this study, *E. coli* caused a high rate of bacterial infections in HIV patients followed by *K. pneumoniae*, *K. oxytoca*, *P. aeruginosa*, *P. mirabilis*, *P. vulgaris*, and *Acinetobacter baumannii*. The bacterial isolates from this study exhibited a high level of resistance to cephalosporins, imipenem, ciprofloxacin, and trimethoprim-sulfamethoxazole (TMP-SMX) antibiotics. It was also found that high level of infections were caused by MDR bacterial pathogens. The selection of standard antibiotic regimens in treating bacterial infections is challenging for MDR bacteria as they are resistant to the most recommended antibiotics [30]. In their study, Iweriebor et al. [31] reported that bacterial isolates were highly susceptible to second-generation cephalosporins and imipenem. In one study, it was reported that bacterial infections by ESBL producing *E. coli*, *Klebsiella* spp., and *Proteus mirabilis* led to adverse clinical outcomes when treated with cefuroxime, cefotaxime, ceftriaxone, ceftazidime, or cefepime antibiotics [32]. In another study, it was reported that ESBLs and AmpC producing bacteria serve as an emerging source of increased morbidity and mortality in immunocompromised patients due to treatment failure [33]. In this study, ESBL production was found at higher level among GNB (79.5%), when compared to the study of Osazuwa et al. [34] who observed that only 17.3% of GNB were ESBL producers.

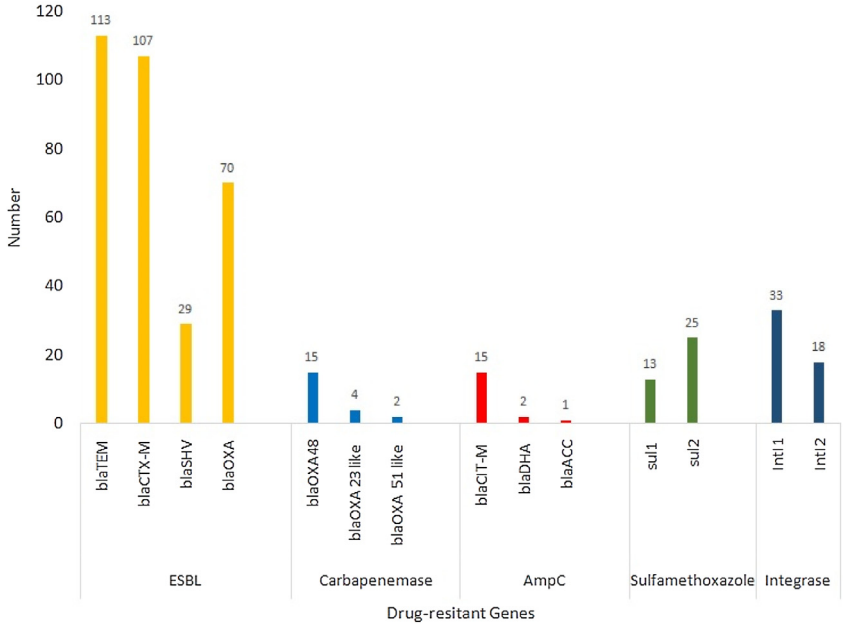

Fig. 1. Number of drug-resistant genes detected in Gram-negative bacterial isolates from HIV patients.

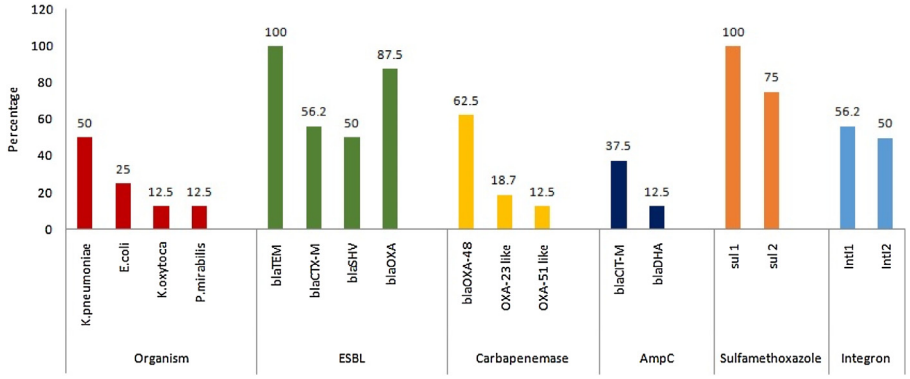

Fig. 2. Percentage of various drug-resistant genes in multiple β-lactamases producing bacterial isolates from HIV patients.
K. pneumoniae – *Klebsiella pneumoniae*; *E. coli* – *Escherichia coli*; *K. oxytoca* – *Klebsiella oxytoca*; *P. mirabilis* – *Proteus mirabilis*; ESBL – extended spectrum β-lactamases; AmpC – AmpC β-lactamases.

Padmavathy et al. [33] reported that among uropathogenic *E. coli*, 72.7% were ESBL producers, and only 4.5% were AmpC producers. Among ESBL producers from this study, *bla*TEM was detected at higher level than *bla*CTX-M and *bla*SHV. In another study Padmavathy et al. [35] documents that ESBL producers showed more positivity to *bla*CTX-M (59.5%) than *bla*TEM (31.7%). The findings of this study revealed that 3G cephalosporins resistant isolates harbored multiple resistant genes that are responsible for ESBL production.

The production of Metallo-β lactamases mainly caused carbapenem resistance by the gram negative bacteria. Among carbapenem-resistant isolates in this study, the *bla*OXA-48 gene was detected in more isolates, and *bla*OXA23 like and *bla*OXA 51 like genes were detected only in few isolates. In their study, Amudhan et al. [36] detected *bla*OXA-51 like gene (93.39%) at high level among carbapenem-resistant isolates, and they also detected other genes such as *bla*OXA-23, *bla*VIM, and *bla*IMP that are responsible for MBL production. Garg et al. [37] in their study reported that the imipenem/meropenem resistant isolates also showed co-positive for ESBL and AmpC production. In this present study, production of ESBL, MBL, and AmpC β-lactamases was observed in 17% of the MDR isolates. The multiple β-lactamases production is the main factor contributing to MDR bacterial profile. In our previous study, we reported that ESBL, MBL, and AmpC β-lactamases producing MDR isolates were highly sensitive to ertapenem [38]. In this study, AmpC producing genes such as *bla*CIT, *bla*DHA, and *bla*ACC were detected at lower levels and also found that all AmpC gene-positive isolates showed co-positive to any one of the ESBL genes studied. AmpC gene positivity observed in this study was higher than that of Arora and Bal (2005), who reported that only 6.7% of cefoxitin-resistant isolates were positive to AmpC genes [39]. Padmavathy et al. [40], in their study also reported that AmpC genes were found only in *E. coli* isolates from HIV patients. In the current study, AmpC genes were detected in *E. coli* and *K. pneumoniae*. HIV patients are frequently exposed to antibiotics particularly trimethoprim-sulfamethoxazole, cephalosporins, and carbapenems for treating recurrent bacterial infections in the intestinal, respiratory, and urinary tracts and bloodstream, which might lead to the emergence of multidrug-resistant bacteria [41].

Results of this study showed that *IntI1* was detected at higher level as compared to *IntI2* in GNB isolates. While *IntI1* positive isolates harbored only ESBL genes, *IntI2* positive isolates harbored both ESBL and AmpC genes. Apart from β-lactamases genes, the high positivity of sulfamthoxazole drug-resistant genes was found in

IntI1 and *IntI2* harboring bacterial isolates. Dissemination of sulfamethoxazole resistant genes among *IntI1* and *IntI2* harboring isolates certainly complicates the treatment of bacterial infections in clinical settings [7]. This study concludes that bacterial isolates from HIV patients have a multidrug-resistant profile and showed a higher level of resistance to 3rd generation cephalosporin antibiotics than carbapenem and cephamycin antibiotics. Molecular analysis revealed that ESBL genes were detected at high level in the gram-negative bacterial isolates from HIV patients in South India. Findings of this study alarm that HIV patients are at high risk of infections by ESBL producing multidrug-resistant bacteria.

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Competing interests

None declared.

Ethical approval

This study was approved by Institutional Review Board of Y. R. Gaitonde Centre for AIDS Research and Education (YRG: 209A), Voluntary Health Services Hospital Campus, Chennai, India.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jiph.2021.11.008.

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