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Increasing secondary bacterial infections with *Enterobacteriaceae* harboring $bla_{CTX-M-15}$ and bla_{CMY-6} in patients with bronchogenic carcinoma: an emerging point of concern

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ABSTRACT

Objective: To look for secondary bacterial infections in bronchogenic carcinoma (BC_A) with resistant organisms harboring *bla* genes considering the paucity of relevant studies. **Methods:** A total of 137 confirmed cases of BC_A and 34 healthy volunteers were studied for the occurrence and prevalence of bla_{CTX-M} and and bla_{AmpC} harboring-enterobacteriaceae. A subset of these patients (n=69) was previously reported for the secondary infection with the *Aspergillus* species. Bronchoalveolar lavages (BAL) were subjected for bacterial and fungal cultures and the bacterial isolates were screened by multiplex PCRs for the presence of bla_{CTX-M} and bla_{AmpC}. The isolates were also screened for the association of insertion sequence *(IS26)* by PCR and characterized by RAPD for any clonal relatedness. **Results:** A total of 143 bacterial isolates were obtained from 137 BAL specimens of BC_A patients. The *Enterobacteriaceae*-isolates were multidrug-resistant showing concomitant resistance to fluoroquinolones and aminoglycosides. Both bla_{CTX-M} and bla_{AmpC} of CIT family were detected in 77.4% and 27.4% isolates, respectively. Sequencing revealed the presence of $bla_{CTX-M-15}$ and bla_{CMY-6}. Twenty one percent of the isolates were simultaneously harboring bla_{ampC} and $bla_{CTX-M-15}$. *IS26* PCR and RAPD typing revealed the presence of diverse bacterial population but no predominant clone was identified. The present study also suggests strong association of aspergillosis with lung cancer and further strengthens the potential use of non-validated serological tests suggested earlier. **Conclusions:** We emphasize that all patients of bronchogenic carcinoma should also be screened for secondary bacterial infections, along with secondary fungal infections, so as to introduce early and specific antimicrobial therapy and to prevent unwanted deaths.

1. Introduction

The patients of bronchogenic carcinoma (BC_A) are debilitated and immunocompromised, and hence, are more prone to secondary pulmonary infections, both bacterial and fungal, due to cancerous involvement of the pulmonary tissue itself, and in particular, due to long term steroid and anticancer therapy. The early diagnosis of these secondary infections in such patients could be rewarding. Recently, we described frequent association between BC_A and secondary aspergillosis especially with non-small cell lung cancer[1]. While performing bacterial cultures of bronchoalveolar lavages (BALs) of those patients we also noticed high occurrence of bacterial resistance to third and fourth generation cephalosporins especially in *Escherichia coli (E. coli)* and *Klebsiella pneumoniae (K. pneumoniae)* isolates.

In recent years, CTX-M extended spectrum β-lactamases (ESBLs) have been increasingly reported in various clinical specimens[2-7]. However, we could not get any published study specifically looking for CTX-M and AmpC producing *Enterobacteria* in patients of BC_A. It must again be emphasized here that early detection of such resistant bacteria could help improved outcome of the patients.

Keeping in view the above facts, especially the paucity of literature, globally, we found worth looking frequency and occurrence of CTX-M and AmpC genogroups/genotypes,

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their genetic environment; and to compare any relatedness or diversity in CTX-M carrying isolates obtained from BC_A patients with those of isolates obtained from other clinical sources, including environmental isolates obtained from T.B. and Chest Diseases inpatients' wards.

2. Material and methods

2.1. Study population

The present study was conducted on patients of BC_A attending the outpatient department or admitted in the wards of TB & Chest Diseases of J N Medical College & Hospital, AMU, Aligarh, India, From June 2003 to February 2007. During this period, bronchoscopy was performed in 213 patients, of which 159 were suspected cases of bronchogenic carcinoma. A total of 95 confirmed patients of BC_A (including 69 patients whose data on secondary fungal infections have previously been reported[1]), were included. A collection from 42 patients of BC_A of the year 2001-2002 was also included. Therefore, the study population for this study comprised of a total of 137 patients. Remaining 64 suspected patients of BC_A were excluded because of some other co-existing underlying immunosuppressive disorders or because the patients were taking antibacterial/ antifungal therapy. Thirty-four, age and sex matched volunteers, not having history suggestive of any lung disease in the recent past were included as healthy controls. Informed written consent was taken prior to bronchoscopy in all study and control groups and the investigations were performed after the approval by Institutional Ethical Committee.

2.2. Collection and processing of specimens

BALs were collected for direct microscopy, bacterial and fungal culture, and BAL-PCR for fungal detection, and blood samples for serological tests detecting anti-*Aspergillus* antibodies. Bronchoscopy was performed by the expertise in T.B. Chest department. Two consecutive BAL specimens were collected in sterile containers with all aseptic precautions. Each BAL specimen was divided in three parts for direct microscopy, culture and PCR.

2.3. Bacterial culture and identification

BALs were subjected to bacterial culture on Blood agar (BA), Mac Conkey's agar (MCA), and chocolate agar (CA). The BA and MCA plates were incubated aerobically, and CA plates in CO_2 enriched environment, in candled jar, at 37 ℃ overnight, and the organisms were identified as per standard techniques[8]. Of the various bacterial isolates obtained from the culture of BAL specimens, only the isolates of *E. coli* and *K. pneumoniae* were selected for further phenotypic and molecular studies.

2.4. Antibiotics susceptibility testing

Disk susceptibility tests were performed and interpreted according to CLSI (formerly NCCLS) criteria[9] by using Mueller-Hinton agar (HiMedia Lab. Ltd., India). The antibiotics (HiMedia, India) and their concentrations (mg) are shown in Table 1. *In vitro* efficacy of ceftriaxone/ sulbactam (kindly provided by Ranbaxy Lab. Ltd., Gurgaon, India), which is a recently launched combination in India, was also evaluated. Since there are no published breakpoints for ceftriaxone/sulbactam combination, the susceptibility criteria used for this combination was as adopted in our earlier study[10].

Table 1
Antibiotics resistance rates in *E. coli* and *K. pneumoniae* isolates* obtained from BAL against first and second line antibiotics tested.

Antibiotics tested (μ g)	Resistance [*n* (%)]	
	E. coli (*n*=21)	*K. pneumoniae* (*n*=41)
Ampicillin (10)	95.2 (20)	NT
Co-trimoxazole (1.2/23.8)	95.2 (20)	100.0 (41)
Tetracycline (30)	95.2 (20)	95.1 (39)
Ciprofloxacin (1)	90.5 (19)	73.2 (30)
Gentamicin (10)	66.7 (14)	63.4 (26)
Amikacin (30)	47.6 (10)	56.1 (23)
Tobramycin (10)	61.9 (13)	36.6 (15)
Cefotaxime (30)	90.5 (19)	92.7 (38)
Ceftazidime (30)	85.7 (18)	92.7 (38)
Cefpodoxime (10)	95.2 (20)	97.6 (40)
Cefpirome (30)	71.4 (15)	58.5 (24)
Cefepime (30)	71.4 (15)	58.5 (24)
Cefoxitin (30)	38.1 (8)	51.2 (21)
Aztreonam (30)	85.7 (18)	73.2 (30)
Trimethoprim (2.5)	85.7 (18)	85.4 (35)
Piperacillin (100)	100.0 (21)	100.0 (41)
Piperacillin/tazobactam (100/10)	90.5 (19)	97.6 (40)
Ticarcillin (75)	100.0 (21)	100.0 (41)
Ticarcillin/clavulanate (75/10)	100.0 (21)	100.0 (41)
Meropenem (10)	14.3 (3)	0.0 (0)
Ceftriaxone/sulbactam (30/15)	14.3 (3)	0.0 (0)

*The isolates were resistant to multiple antibiotics.
NT = not tested.

2.5. Phenotypic ESBL detection by disk synergy tests

ESBL detection was performed by disk synergy tests using co-amoxiclav[11] and piperacillin-tazobactam discs[10]. Phenotypic detection was also performed by combination disk method using cephalosporin disks alone, and in the presence of clavulanate.

2.6. Phenotypic detection of AmpC production

Since there are no published CLSI criteria for phenotypic screening or confirmatory test for AmpC β-lactamases, a modified three-dimensional test (MTDT), as described previously[12], was used for the detection of AmpC β-lactamase. The isolates selected for the MTDT were those which were screen positive i.e. those with cefoxitin zone diameter of < 18 mm and cefoxitin susceptible isolates (based on cefoxitin disk susceptibility) but were found to carry bla_{ampC} alleles.

2.7. Multiplex PCRs for detection of bla_{CTX-M} and bla_{ampC}

Multiplex PCRs for the detection of bla_{CTX-M} and bla_{ampC} were carried out using the primer sets and conditions as described by Woodford *et al*[13] and Perez-Perez & Hanson[14], respectively. After running the amplicons on 2% agarose gel, the precise size of the band was determined using the "Quantity One" software provided with the Gel documentation system (BioRad U.S.A.).

2.8. Nucleotide sequencing

Representative isolates were randomly selected for DNA sequencing of the bla_{CTX-M} open reading frame (ORF) and bla_{AmpC} in order to find out the specific types. Whole ORF of bla_{CTX-M} was amplified using primers IS*Ecp1* U1 (5′-AAA AAT GAT TGA AAG GTG GT-3′) and P2D (5′-CAG CGC TTT TGC CGT CTA AG-3′). For AmpC- sequencing, the same primer set (CIT Group) as used for multiplex PCR were used for sequencing. The PCR protocol used was that adopted in our previous studies[7].

2.9. Association of IS26 with blaCTX-M-15

All CTX-M-15-carrying isolates were screened using the PCR protocol described by Woodford *et al*[15]. A separate collection of bacteria from different specimens, collected for a parallel ongoing study, and detected to carry bla_{CTX-M}, was also analyzed for the *IS26* elements and compared with the isolates of this study in order to determine any similarity or dissimilarity in *IS26* amplicon insertions in the two collections.

2.10. RAPD-PCR typing

All isolates found to carry bla_{CTX-M} and bla_{ampC} were typed using the RAPD-PCR[16] to determine whether any predominant clone existed in this isolate collection. A separate collection of bacteria from different specimens, collected for a parallel ongoing study, and detected to carry bla_{CTX-M} and bla_{ampC}, were also typed by RAPD and compared with the isolates of this study to determine any common circulating clone or diversity in clinical isolates obtained from different samples.

3. Results

3.1. Patients' demographic information and clinical presentations

Of 137 patients, majority (56.9%) were in the age group of 45-<60 years, followed by 60-<75 years (35.8%). The median age of the study population was 58 years and male to female ratio was 11.5:1. A total of 55 (40.1%) and 36(26.3%) patients underwent chemotherapy or took steroids 6 months prior to the study, respectively.

The most common presentation was with cough, which was noticed in 70% patients followed by expectoration and chest pain noticed in 52.6% and 50.4% patients, respectively. The detailed presenting features of the patients are shown in Figure 1.

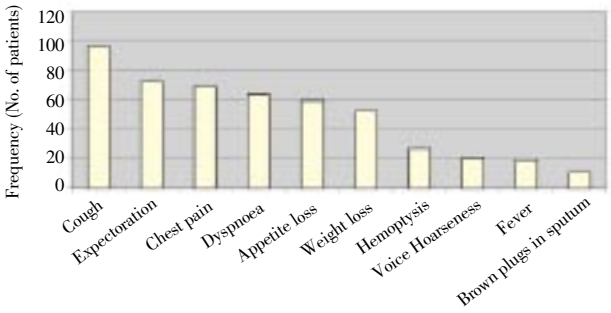

Figure 1. Presenting features of the patients with bronchogenic carcinoma included in the present study.
* Most of the patients presented with multiple presenting features.

3.2. Bronchoscopic features and bronchogenic carcinoma types

The detailed bronchoscopic features of the patients, as revealed during bronchoscopy, are shown in Table 2. The most common finding noticed in the bronchoscopy was intraluminal growth observed in 79.6% cases. Most of the patients presented with mixed bronchoscopic findings. The bronchogenic carcinoma types, based on histopathological examinations, are shown in Figure 2.

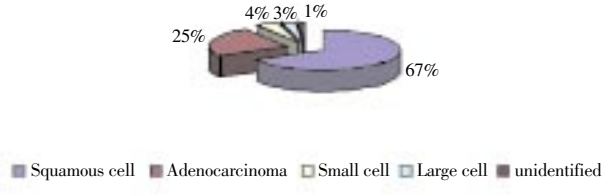

Figure 2. Bronchogenic carcinoma cell-types as identified by histopathological examination.

3.3. Bacterial isolates

Out of 137 BAL specimens of BCA patients, 104-yielded bacterial growth on aerobic incubation, and a total of 143 bacterial isolates (many samples showed mixed growth) were obtained. The details of the isolated bacteria are shown in Table 3. *K. pneumoniae* was the most frequent isolated organism noticed in 29.9% samples followed by *Pseudomonas aeruginosa* which was noticed in 26.3% BAL samples. *E. coli* was isolated from 15.3% samples. None of the BAL specimens from healthy volunteers provided growth of *Enterobacteriaceae* (data not shown). The bacterial population consisting of *E. coli* (n=21) and *K. pneumoniae* (n=41) was subjected for further testing.

3.4. Antibiotic susceptibility

The detailed results of the antibiotic susceptibility

Table 2
Bronchoscopic features according to the cell-type of bronchogenic carcinoma (n=137).

Carcinoma-type	No. of cases	Vocal cord palsy	Widened carina	Intraluminal growth	Compressed bronchi	Congestion	Secretions
Squamous cell	91	26	26	86	24	41	56
Adenocarcinoma	34	5	–	14	19	6	8
Small cell	6	–	–	5	–	–	–
Large cell	4	–	–	4	–	–	–
Unidentified	2	–	–	–	–	2	2
Total	137	31	26	109	43	49	66

* Many patients presented with multiple bronchoscopic findings.

are presented in Table 1. The isolates of *E. coli* showed maximum susceptibility to ceftriaxone-sulbactam, a combination recently launched in India, and to meropenem in 85.7%, each, followed by cefoxitin (53.2%) and amikacin (46.8%). Concomitant high resistance to other antibiotics, including tetracycline (95.2%), co-trimoxazole (95.2%), ciprofloxacin (90.5%), aztreonam (85.7%), trimethoprim (85.7%), gentamicin (66.7%), was also noticed.

All the *K. pneumoniae* isolates were susceptible to ceftriaxone-sulbactam and imipenem. Similar to *E. coli* isolates, *K. pneumoniae* also showed concomitant high resistance to other antibiotics tested (Table 1).

Table 3
Bacterial species isolated from 137 BAL fluids of BCA patients.

Bacteria isolated	No. of isolates*	Percentage
K. pneumoniae	41	29.9
Pseudomonas aeruginosa	36	26.3
E. coli	21	15.3
β *-hemolytic streptococci*	15	10.9
Citrobacter spp.	9	6.6
Acinetobacter spp.	8	5.8
Staphylococcus aureus	6	4.4
Staphylococcus epidermidis	4	2.9
Diplococcus pneumoniae	3	2.2
Total	143	–

* Many specimens showed mixed bacterial growth.

3.5. Phenotypic ESBL and AmpC detection

Comparative results of ESBL detection by these three tests are shown in Figure 3.

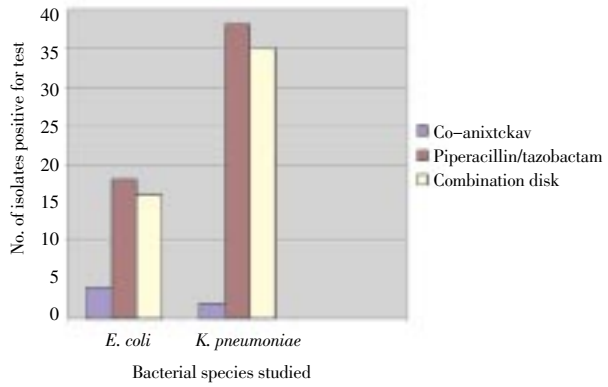

Figure 3. Comparative analysis of the ESBL detection by three phenotypic tests used in the present study.

None of the isolates, out of 62, could be detected as ESBL-producers by applying amoxicillin-clavulanate at 30 mm, center to center (Figure 4 A-F). By applying amoxicillin-clavulanate at a distance of 20 mm, we could only detect 9.7% (6/62) isolates as ESBL-producers, of which 4 isolates were of *E. coli* and 2 were *K. pneumoniae*. Most of the isolates in the present study either did not or showed very small zones of inhibitions to cephalosporins.

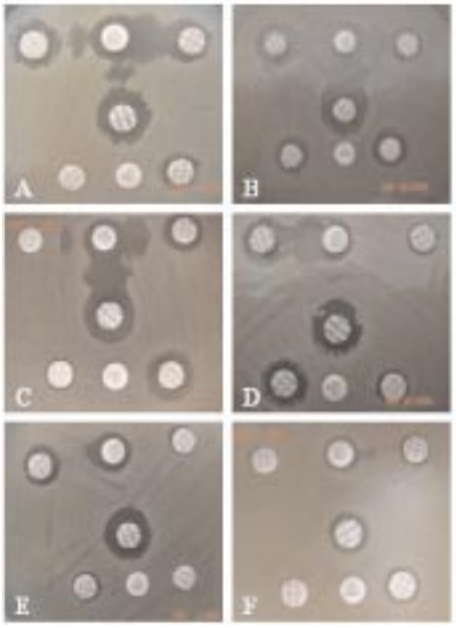

Figure 4. Photograph of $bla_{CTX-M-15}$ carrying isolates showing synergy between cephalosporins and piperacillin-tazobactam discs at 30 mm. Non-detection of ESBL by co-amoxyclav even at 20 mm (A to D). Isolates not giving good synergy but still detected as ESBL-producers by ceftriaxone and cefotaxime and not by ceftazidime by using piperacillin-tazobactam disk (E & F).

In the modified test, where piperacillin-tazobactam was used in place of co-amoxyclav disk, we could detect ESBLs in total of 90.3% (56/62) isolates, and of which, it could be detected in 85.7% (18/21) and 92.7% (38/41) isolates of *E. coli* and *K. pneumoniae*, respectively. Excellent synergy between piperacillin-tazobactam and cephalosporins, in all the isolates, was seen when the discs were placed 20 mm apart. When the discs were placed at 30 mm, still ESBL could be detected in all the isolates, most of the isolates giving excellent synergistic patterns as shown in Figure 4 A-D, however few of the isolates gave patterns as shown in Figure 4 E & F. Comparative sensitivity of ceftriaxone, cefotaxime

and ceftazidime in ESBL detection by using piperacillin-tazobactam discs was found to be 90.3% (56/62), 88.7% (55/62), and 67.7% (42/62), respectively.

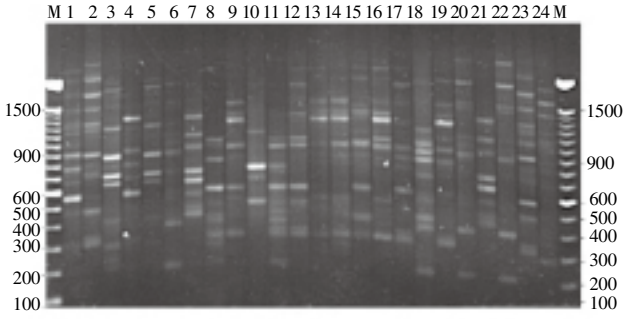

Figure 5. RAPD profile demonstrating diversity of the representative isolates carrying bla_{CTX-M} and bla_{ampC} obtained from patients of bronchogenic carcinoma as well as from other clinical and environmental specimens.
Lanes M show the molecular weight markers.

Using the CDT method, ESBL production was detected in total of 82.3% (51/62) isolates. Of these, ESBLs could be detected in 76.2% (16/21) of *E. coli* and 85.4% (35/41) of *K. pneumoniae* isolates.

A total of 19.4% (12/62) isolates were detected as AmpC-producers by CDT, and of which 3 were *E. coli* and 9 were *K. pneumoniae*. Among the 29 cefoxitin-resistant isolates studied by modified three-dimensional test, 12 (3 *E. coli* and 9 *K. pneumoniae*) were detected as AmpC-producers, and 3 isolates (All *K. pneumoniae*) were inferred as AmpC-intermediate. Simultaneous production of ESBLs and AmpC enzymes could be inferred in 14.5% (9/62) isolates by the phenotypic method described above.

3.6. Detection and characterization of bla_{CTX-M} and bla_{AmpC} alleles based on multiplex-PCR and sequencing

bla_{CTX-M} could be detected in 77.4% (48/62) isolates using the multiplex PCR. Noting the detection of bla_{CTX-M} alleles in *E. coli* and *K. pneumoniae*, we found their presence in 81% (17/21) and 75.6% (31/41) isolates, respectively. Based on the PCR-amplicon size, all belonged to CTX-M phylogenetic Group 1. Of the 62 isolates, bla_{ampC} of CIT-family were detected in 27.4% (17/62) isolates. Of these bla_{ampC}-carrying isolates, 23.8% (5/21) were *E. coli* and 29.3% (12/41) were *K. pneumoniae*. The PCR assay detected bla_{ampC} in 15/29 (51.7%) of cefoxitin-resistant isolates. Two isolates found to carry bla_{ampC} were categorized as being susceptible to cefoxitin using the zone breakpoint of <18 mm to infer resistance.

3.7. Co-carriage of bla_{ampC} and $bla_{CTX-M-15}$

Of the 62 isolates studied, 13 (21%) were simultaneously harboring bla_{ampC} and $bla_{CTX-M-15}$. The frequency of simultaneous occurrence in the respective species was noticed in 19% (4/21) and 22% (9/41) of *E. coli* and *K. pneumoniae* respectively.

3.8. Sequencing of representative isolates

Further confirmation by sequencing in representative isolates confirmed the presence of $bla_{CTX-M-15}$ (GenBank Accession numbers EF 371799 and EF 371800) and bla_{CMY-6} (GenBank Accession Nos. EF 103125 and EF 103126).

3.9. Detection of IS26 element

IS26 was detected in 20 of 48 (41.7%) isolates carrying $bla_{CTX-M-15}$ (9 *E.coli*, 11 *K. pneumoniae*). Amplicons of ~650 bp, ~750 bp, ~850 bp, ~950 bp, ~1 100 bp, and >1 400 bp were obtained, the most common being ~850 bp (Table 5). Random analysis of the bacterial isolates, obtained from other clinical samples, for a parallel ongoing study also showed this diverse picture of varying *IS26* fragments and even multiple banding patterns.

Table 4
Distribution and patterns of IS26 insertion in CTX-M-15-producing isolates.

Fragment size	Detected in		Total
	E. coli	*K. pneumoniae*	
~650 bp	1	2	3
~750 bp	1	1	2
~850 bp	5	4	9
~950 bp	1	1	2
~1 100 bp	1	1	2
>1 400 bp	0	2	2
Total	9	11	20

* Most of the patients presented with multiple presenting features

3.10. RAPD analysis

RAPD-typing revealed the presence of diverse bacterial population and no predominant clone was identified in our Indian bacterial collection (Figure 5).

3.11. Brief mycological data (detailed data not presented)

BAL culture of 42 (30.7%) patients showed growth of *Aspergillus* species and 19 of these 42 patients revealed fungal elements in direct microscopic examination of BAL. Of these 42 culture-positive cases, 18 were categorized as cases of definite-IPA and 24 of probable-IPA as described in our earlier study[1]. Of the *Aspergillus* spp. isolated, *Aspergillus fumigatus* was the predominant species isolated from 26 (61.9%) cases followed by *Aspergillus flavus* in 38.1% (16/42) patients. On analysis of the isolation of *Aspergillus* spp. in various forms of IPA, it was noticed that *Aspergillus fumigatus* was isolated from 12 and 14 cases of definite- and probable-IPA, respectively, whereas, *Aspergillus flavus* was isolated from 6 and 10 cases of definite- and probable-IPA, respectively. No *Aspergilli* was isolated from cases of possible and non-IPA, and none among the healthy controls showed growth of *Aspergilli* in the culture. On serological testing, the anti-*Aspergillus* antibodies could be detected in 48 (35%) patients, each, by DID and DBA. However, the

antibodies could be detected in 54 (39.4%) patients by ELISA. None among the healthy volunteers showed anti-*Aspergillus* antibodies in any of the serological test. *Aspergillus*-DNA could be detected in 51.8% (71/137) samples by PCR. The *Aspergillus*-DNA was detected in all the patients categorized as cases of definite-IPA and probable-IPA, however, it could be detected in 69.7% (23/33) and 9.7% (6/62) cases of possible- and non-IPA, respectively. Based on the present study, a strong association between a positive PCR test and all forms of aspergillosis ($P<0.05$) was noticed, with the strongest association correlation found with definite- and probable-IPA ($P< 0.001$).

When the data was compared statistically with entire dataset, the sentitivity of PCR and serological tests was noted 100% while specificity ranged between 65%-83%. This clearly denotes high diagnostic values of these tests in such cases. However, in cases of possible- IPA, the sensitivity, specificity, PPV, and NPV of PCR were 69.7%, 65.2%, 32.4%, and 90%, respectively. These values for ELISA, in possible-IPA, were were 24.2%, 66.7%, 14.8%, and 78.6%, respectively. This strongly suggest that PCR has greater diagnostic role in possible-IPA as compared to those of serological tests. The diagnostic role of PCR was also higher than serological tests in cases with non-IPA. However, ELISA do play a diagnostic role in non-IPA, to an extent, as opposed to DID and DBA.

4. Discussion

The frequency of secondary bacterial lung infection is high in lung malignancies due to the immunosuppressive state. The studies looking for the bacterial profile in BAL-fluids from patients of bronchogenic carcinoma, and especially the extended-spectrum β-lactamases (ESBLs) producing isolates, are fragmentary.

The first CTX-M class of extended spectrum β-lactamase (ESBL) was described in Germany[17], and since then more than 90 variants have been reported (http://www.lahey.org/studies/inc_webt.asp). In India, the very first report of the presence of CTX-M-producing *Enterobacteriaceae* came from New Delhi on six isolates[18]. Since then several Indian surveys have reported the presence of ESBLs in clinical isolates based on phenotypic tests. We were unable to find any Indian report in our search of the literature using the Entrez PubMed database provided by the National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov/entrez) that specifically looked for $bla_{\text{CTX-M}}$ and then determined the precise genotype present, except one of our recently published report[7]. Moreover, we could not get any report, globally, looking for the secondary bacterial infection harboring *bla* genes in bronchogenic carcinoma patients.

It is interesting to note that CTX-M-15 is the only existing type, reported to date, in India however, diversity of CTX-M types within a single country have been reported [19, 20]. Therefore, we found worth looking occurrence of CTX-M genogroups, specific CTX-M types, and their genetic environment in CTX-M carrying- *E. coli* and *K. pneumoniae* isolates obtained from bronchogenic carcinoma patients; and to compare any relatedness or diversity with those of isolates obtained from other clinical sources. In this study, 48 of 62 (77.4%) isolates produced CTX-M-15 and no other CTX-M-type ESBL was found. This finding was surprising: other surveys have reported a diversity of CTX-M types within a single institution or country[19-21]. We found a high level of co-resistance in isolates producing CTX-M-15 ESBL, which has been reported for many CTX-M producing isolates[22, 23]. In our survey, a total of 64.5% isolates were resistant to gentamicin, 85.5% to trimethoprim and 79% to ciprofloxacin. Resistance to cefoxitin was noted in 38.1% *E. coli* and 51.2% *K. pneumoniae* and was thought to be due to the presence of plasmidic bla_{ampC}. This transferable resistance determinant is clinically significant as it confers resistance in the host to beta-lactam-beta-lactamase inhibitor combinations as well as to narrow spectrum penicillins and expanded spectrum cephalosporins but spares fourth-generation cephalosporins such as cefepime and cefpirome. A total of 14.3% (3/21) isolates of *E. coli* were resistant to meropenem, whereas all *K. pneumoniae* isolates were susceptible to meropenem.

The association of insertion sequences with antibiotic resistance genes has been reported previously[24, 25]. IS*Ecp1* is always found upstream of $bla_{\text{CTX-M-15}}$ and is strongly implicated in the mobilization of this antibiotic resistance gene[26]. *IS26* has also been reported in association with $bla_{\text{CTX-M}}$ including $bla_{\text{CTX-M-15}}$ and more specifically, has been found to insert within IS*Ecp1*, although interestingly, the insertion of *IS26* differs from strain to strain[27]. In our study of highly diverse *E. coli* and *K. pneumoniae* producing CTX-M-15, IS*26* was detected in 41.7% isolates, indicating high frequency of association of this element with $bla_{\text{CTX-M-15}}$. It was interesting to note that PCR products of variable sizes were detected, and an amplicon of 850 bp was most frequently observed. Such diversity of varying *IS26* amplicons was also noticed in other collections of bacterial isolates from different clinical samples. Numerous reports from India have indicated a high prevalence of ESBLs in Gram-negative bacteria, which must be presumed to have taken a considerable time to reach a high prevalence. Our present consecutive study also suggests that CTX-M-15 is the most common, or maybe the only, CTX-M type in India.

Acquisition of ampC-type genes by plasmids in *K. pneumoniae* and *E. coli* has been known since 1989, but only recently they are being reported with increasing frequency[28]. Despite of increasing recognition of this resistance mechanism worldwide, there is paucity of data from India, and most studies only present phenotypic data[29]. In our search of the scientific literature from the Indian subcontinent, we found only two publications that have characterized bla_{ampC} at the molecular level, and these report the presence of $bla_{\text{BIL-1}}$-like and $bla_{\text{CMY-4}}$[30, 31]. We report here the presence of bla_{ampC} of the CIT family in Indian *Enterobacteriaceae* in significant numbers (27.4%). Sequencing of representative isolates showed the presence of $bla_{\text{CMY-6}}$, which is in addition those previously published reports.

We noticed slightly higher frequency of occurrence of bla_{ampC} in *K. pneumoniae* (29.3%, 12/41) as opposed to *E. coli* in (23.8%, 5/21) and this finding is in contrast to earlier reports[32]. We presume that plasmid host range and success amounts for these differences.

Simultaneous occurrence of non-CTX-M ESBLs and AmpC β-lactamases has been reported in the literature[33, 34]. However the data is still fragmentary on the simultaneous occurrence of AmpC and CTX-M enzymes, and recently reports start coming on such combination resistance mechanisms[35, 36]. Analyzing our bla_{ampC} carrying isolates for co-carriage of bla_{CTX-M}, we noticed simultaneous occurrence in 21% isolates. Spreading of ESBL-producing strains with ampC genes is a concern and poses a serious therapeutic problem, as carbapenems remain the only active β-lactam against these organisms[37]. This information is crucial for controlling the spread of resistance and for decision of empirical selection of antimicrobial agents. RAPD-typing of the isolates demonstrated great diversity and no predominant type, indicating either horizontal transfer and/or mobilization of these genes on plasmids.

To the best of our knowledge, this is the first report characterizing the occurrence and prevalence of CTX-M- and AmpC-producing enterobacteria from patients of bronchogenic carcinoma. We report here a 3GC resistance rate of 96.8% of all *E. coli* and *K. pneumoniae* obtained from BAL of BC_A patients and in these, a CTX-M carriage rate of 77.4%. bla_{ampC} of CIT-family were detected in 27.4% of isolates. Our report supports the urgent need for regular screening and national surveillance characterizing the CTX-M types and to implement strict antibiotics policy to limit the irrational use of antibiotics, including cephalosporins, so as to minimize antibiotic selective pressure. We also emphasize that all patients of bronchogenic carcinoma should also be screened for secondary bacterial infections, along with secondary fungal infections, with these resistant bugs so as to introduce specific antibacterial therapy and to prevent unwanted death of the bronchial carcinoma patients because of these secondary infections. In consequence to our earlier report, present study also suggests strong association of aspergillosis with lung cancer and further strengthens the potential use of non-validated serological tests suggested earlier.

Conflict of Interest Statement

We declare that we have no conflict of interest.

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