Extended-spectrum $\beta$-lactamase/AmpC-producing uropathogenic Escherichia coli from HIV patients: do they have a low virulence score?

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Extended-spectrum $\beta$-lactamase (ESBL) production and quinolone resistance are often associated in enterobacteria. Prior exposure to 3G cephalosporins/quinolones accelerates the risk of resistance to both these groups of antibiotics. Hence, information on the antimicrobial resistance pattern of uropathogenic Escherichia coli (UPEC) isolates is important to better formulate the guidelines for the empirical therapy of urinary tract infection in the context of HIV/AIDS. The aim of this study was to determine the incidence of ESBL/AmpC and fluoroquinolone (FQ) resistance among urinary E. coli isolates and to establish the association of extraintestinal virulence and phylogenetic distribution with antibiotic resistance and host immunocompromise. Accordingly, 118 urinary Escherichia coli isolates from HIV (n=76) and non-HIV antenatal patients (n=42) from Chennai, South India, were analysed for the presence of five virulence-associated genes (VAGs): pap, sfa/foc, afa/dra, iutA and kpsMII. Compared with the susceptible HIV isolates, the majority of the ESBL/AmpC/FQ-resistant isolates harboured iutA (66.7 %) and pap (40 %). The FQ-resistant HIV isolates were significantly enriched for iutA (67.8 %) and kpsMII (47.5 %) and qualified as UPEC (54.2 %), while a majority of the FQ-susceptible isolates from the non-HIV patients were found to harbour pap (48.4 %), sfa/foc (41.9 %) and kpsMII (48.4 %) and were classified as UPEC (40.5 %). We conclude that antibiotic-resistant (ESBL/AmpC/FQ-resistant) phylogroup D isolates with limited virulence are competent enough to establish infections in HIV patients, while among non-HIV patients, an array of virulence factors is essential for E. coli to overcome host defences irrespective of antibiotic resistance.

INTRODUCTION

The liberal use of quinolones and $\beta$-lactams has triggered bacterial resistance worldwide. Urinary Escherichia coli are increasingly reported to exhibit resistance to the conventional front-line antibiotic ciprofloxacin (Luthje & Brauner, 2010). The recent EAU (European Association of Urology) guidelines (2011; see http://www.uroweb.org/guidelines/online-guidelines/) recommend the use of Group 3a/b cephalosporins (cefotaxime, ceftazidime, cefoperazone) for the treatment of complicated urinary tract infections and urosepsis (Grabe et al., 2011). However, emergence of $\beta$-lactam resistance mediated by extended-spectrum $\beta$-lactamases (ESBLs) and group 1 AmpC cephalosporinases (especially plasmid-mediated AmpC $\beta$-lactamases – PMACBLs) is a major global problem (Winokur et al., 2001; Lampri et al., 2012). ESBL/AmpC-producing organisms are of paramount concern as they limit therapeutic options, cause treatment failures and increase the cost/duration of hospitalization (Odeh et al., 2002). Emerging resistance in E. coli is frequently due to the irrational use of antibiotics and is associated with high mortality rates. Hence, rapid detection of such strains is a prerequisite to limit their dissemination and for the selection of appropriate antimicrobial therapy, which is frequently non-$\beta$-lactam (Pitout & Laupland, 2008; Lebitasy et al., 2011). Nonetheless, these organisms are increasingly reported to exhibit multiple antibiotic resistance, often being resistant to aminoglycosides, fluoroquinolones (FQs) and trimethoprim-sulfamethoxazole, and are a growing challenge to patient care (Nathisuwan et al., 2001; Paterson & Bonomo, 2005). Recent exposure to third-generation (3G) cephalosporins/FQs is
reported to be a factor associated with infections by ESBL-producing organisms (OR = 3.05 and OR = 9.78 respectively) (Muvunyi et al., 2011). Also, FQ resistance is documented to serve as a prospective marker of broader resistance, including ESBL positivity (van der Starre et al., 2011).

ESBL types, particularly TEM, SHV and CTX-M enzymes, are reported to exhibit a high degree of diversity, of which ESBL positivity (van der Starre et al., 2011) serve as a prospective marker of broader resistance. Furthermore, distinction between chromosomal AmpC enzymes and PMACBLs in E. coli is critical for surveillance and infection control, and the phenotypic tests are often not reliable. Hence, while testing E. coli, the follow-up of screen-positive AmpC isolates with AmpC multiplex PCR is recommended to determine the presence of family-specific PMACBL genes (Pérez-Pérez & Hanson, 2002).

Antibiotic-resistant E. coli strains have been reported to be significantly associated with reduced virulence, non-B2 phylogeny and host immunocompromisation (Johnson et al., 1998). Also, Johnson et al. (2003) have reported that among human clinical E. coli isolates, FQ and/or AmpC resistance was associated with a significant shift towards the non-B2 phylogenetic lineage as well as a reduced virulence profile. However, there is a paucity of data on the antibiotic-resistance profile, phylogeny and prevalence of specific virulence-associated genes (VAGs) among uropathogenic E. coli (UPEC) from HIV patients.

Given the dramatic increase in the incidence of ESBL- and AmpC-producing E. coli that often exhibit co-resistance against FQs, and the experimental evidence which has shown that strains of non-B2 phylogenetic groups with decreased or no virulence determinants can infect compromised hosts, the present study was designed to analyse the association of extraintestinal virulence, phylogeny and ESBL/AmpC/FQ resistance of E. coli with the host immune status.

METHODS

Bacterial isolates. A total of 118 non- replicate community E. coli isolates recovered from HIV (n = 76) patients (with recent exposure to FQs/3G cephalosporins, with CD4 counts <350 cells mm\(^{-3}\) and not on antiretroviral therapy) and non-HIV antenatal patients (n = 42) with UTI were included in the study.

Phylogenetic analysis and virulence genotyping. The phylogenetic status (A, B1, B2 and D) of the E. coli isolates was assessed by a triplex PCR (Clermont et al., 2000). All the isolates were screened by PCR for five VAGs: pap [PapA and PapC], sfa/foc [S and FIC fimbriae], afa/dra [Dr-binding afimbrial adhesin] (Le Bouguenec et al., 1992), iutA [aerobactin receptor] (Johnson & Stell, 2000) and kpsMII [group 2 capsule synthesis] (Johnson & O’Bryan, 2004). The ExPEC (UPEC) status of the E. coli isolates was predicted based on the operational definition of ExPEC: ‘presence of $\geq 2$ of the above-mentioned VAGs’ (Johnson et al., 2003).

ESBL and AmpC detection. E. coli isolates were tested for ESBL production using ESBL combination identification discs. Three sets of combination discs were employed: cefazidime/ceftazidime-clavulanic acid; cefotaxime/cefotaxime-clavulanic acid; and cefepime/cefepime-clavulanic acid. PCR-based detection of ESBL gene families, bla\(_{CTX-M}\), bla\(_{TEM}\) and bla\(_{SHV}\), was carried out using previously described primers (Chang et al., 2001; Pagani et al., 2003; Yagi et al., 2000). Phenotypic detection of AmpC β-lactamase production was performed by an inhibitor (boronic acid)-based method (Coudron, 2005) and further confirmed by AmpC disc test (Black et al., 2005). E. coli ATCC 25922 was used as the negative control. Isolates with an AmpC phenotype were further evaluated for the presence of transferable (plasmid-mediated) ampC genes, using multiplex PCR as described by Pérez-Pérez & Hanson (2002). Isolates that were resistant to 3G cephalosporin(s), cefoxitin and ciprofloxacin (i.e. ESBL+ AmpC+ FQR) were designated multiple antibiotic-resistant isolates.

DNA sequencing. DNA sequencing was performed using an Applied Biosystems 3130 Genetic Analyzer with ABI PRISM BigDye Terminators V3.1 (at GenOmb Biotechnologies, Pune, India) to confirm the identity of the amplified PCR products and to establish positive controls. The nucleotide sequences were submitted to the GenBank database (see title page for accession numbers).

Statistical methods. Comparisons of proportions involving two groups were tested using the chi-squared test or Fisher’s exact test (2-tailed) as appropriate. Comparisons involving virulence scores were assessed using the Mann–Whitney U test. The threshold for statistical significance was $P<0.05$.

This study was reviewed and approved by the institutional human ethical committee, Dr ALM PGBMS, University of Madras, India.

RESULTS

Phylogenetic background of E. coli isolates

The highly virulent phylogroup B2 was the most represented among strains isolated from non-HIV antenatal patients compared to those from HIV patients (42.9 % vs 4 %) ($P<0.0001$, OR = 18.25, CI = 4.942, 67.3937), while the less virulent phylogroup D was the most represented among the isolates from HIV patients (50 % vs 14.3 %; $P=0.00027$, OR = 6, CI = 2.2648, 15.8951) (data not shown).

ExPEC (UPEC) status

The prevalence of the five ExPEC-defining VAGs among the HIV isolates ranged from 1.3 % (sfa/foc) to 56.6 % (iutA), while 34 (44.7 %) isolates were designated UPEC (ExPEC VAG score 2, 18/34, 52.9 %; score 3, 16/34; 47.1 %). Of the 42 E. coli isolates from non-HIV patients that were screened, 21 (50 %) isolates harboured two or more of the five VAGs, and the prevalence of VAGs ranged from 2.4 % (afa/dra) to 42.9 % (iutA) (Table 1). The majority (9/21; 42.9 %) of the UPEC isolates from non-HIV patients had a VAG score of 4, followed by score 2 (8/21; 38.1 %) and score 3 (4/21; 19 %). Comparison of the
mean virulence scores of UPEC isolated from the HIV and the non-HIV patients showed a statistically significant difference (2.471 vs 3.048, \( P=0.03 \)). The majority (16/21, 76.2\%) of the UPEC isolates from the non-HIV patients belonged to the virulent phylotype B2, while most (32/34, 94.1\%) of those from HIV patients were of non-B2 phylogenetic background [especially group D (79.4\%), \( P<0.0001, \text{OR}=51.2, \text{CI}=8.931, 293.5228 \)].

**Antibiotic resistance**

A significant difference was observed in the incidence of FQ resistance (77.6\% vs 26.2\%, \( P<0.0001 \)) among the HIV and non-HIV isolates (Table 1). Also, the incidence of ESBL and AmpC co-producers differed significantly between the isolates from HIV and non-HIV patient populations (71.1\% vs 35.7\%, \( P=0.000233 \)), while ESBL producers were more common among non-HIV isolates compared to the HIV isolates (16.7\% vs 3.9\%, \( P=0.04407 \)) (Table 1). Significantly more of the ESBL/AmpC producers from the HIV patients were found to co-exhibit FQ resistance compared to the non-HIV isolates (83.3\% vs 53.3\%, \( P=0.0219 \)). Also, multiple antibiotic resistance, ESBL\(^+\) AmpC\(^+\) FQ\(^8\), was significantly higher among the HIV isolates compared to the non-HIV isolates (59.2\% vs 19.0\%, \( P=0.000036 \)) (Table 1).

**bla genotyping**

Among the total isolates (n=118) that were screened for bla genes, 39 (33.1\%) and 47 (39.8\%) carried bla\(_{TEM}\) and bla\(_{CTX-M}\) respectively, while none harboured bla\(_{SHV}\). All the bla\(_{CTX-M}\)-harbouring isolates were found to be of ESBL phenotype. A majority of the E. coli isolates from HIV patients were found to harbour a bla gene [55 (72.4\%) vs 18 (48.9\%), \( P=0.0027, \text{OR}=3.4921 \)]. When stratified based on host group, a significant difference was observed in the incidence of bla\(_{CTX-M}\) among the isolates from HIV and non-HIV patients [40 (52.6\%) vs 7 (16.7\%), \( P=0.000156, \text{OR}=5.5556 \)]. However, the difference in prevalence of bla\(_{TEM}\) was not statistically significant [28 (36.8\%) vs 11 (26.2\%), \( P=0.3077 \)]. Interestingly, all the 13 strains that possessed multiple bla genes were isolated from HIV patients (\( P=0.00387, \text{OR}=5.6411 \)).

**AmpC genotyping**

Of the 57 AmpC-positive E. coli isolates from HIV patients that were screened for PMACBls, seven harboured ampC genes that belonged to two different families, CIT (n=3) and DHA (n=3), while one harboured both CIT and DHA.

**Virulence genotyping of the resistant strains**

When stratified based on host status, compared with the 17 FQ-susceptible HIV isolates, the majority of the FQ-resistant HIV isolates harboured iutA [40/59 (67.8\%), \( P=0.0003, \text{OR}=9.8246, \text{CI}=2.5183, 38.3287 \), \( P<0.002065, \text{OR}=8.8899, \text{CI}=1.8645, 42.3772 \)] and hence a higher proportion of the FQ-resistant isolates from HIV patients qualified as UPEC (\( P=0.0027, \text{OR}=2.7692, \text{CI}=1.0763, 7.1249 \)) and pap [18 (40\%), \( P=0.0114, \text{OR}=4.5, \text{CI}=1.3451, 15.0551 \)]. pap, sfa/foc and kpsMII were more common among the FQ-susceptible isolates (n=31) from non-HIV patients compared to the FQ-resistant isolates (n=11) and hence a majority of the UPEC isolates were found to be FQ-susceptible (\( P=0.0324, \text{OR}=7.125, \text{CI}=1.3093, 38.7724 \)). Also, among the non-HIV isolates, a significant difference was observed in the incidence of sfa/foc [4/23 (17.4\%)] and

![Table 1. Incidence of VAGs/resistance phenotypes among E. coli isolates (n=118) from HIV and non-HIV patients](http://jmm.sgmjournals.org)

<table>
<thead>
<tr>
<th>VAG/characteristic</th>
<th>Prevalence of VAG/characteristic [n (%)]</th>
<th>P-value</th>
<th>Odds ratio</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All isolates (n=118)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>HIV isolates (n=76)</td>
<td></td>
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<td></td>
<td>Non-HIV isolates (n=42)</td>
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<tr>
<td>iutA</td>
<td>61 (51.7)</td>
<td>43 (56.6)</td>
<td>18 (42.9)</td>
<td>0.18 1.7374</td>
</tr>
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<td>pap</td>
<td>39 (33.1)</td>
<td>22 (28.9)</td>
<td>17 (40.5)</td>
<td>0.2246 1.6691</td>
</tr>
<tr>
<td>sfa/foc</td>
<td>16 (13.6)</td>
<td>1 (1.3)</td>
<td>15 (35.7)</td>
<td>&lt;0.0001 41.667</td>
</tr>
<tr>
<td>afia/dra</td>
<td>7 (5.9)</td>
<td>6 (7.9)</td>
<td>1 (2.4)</td>
<td>0.4189 3.5143</td>
</tr>
<tr>
<td>kpsMII</td>
<td>48 (40.7)</td>
<td>31 (40.8)</td>
<td>17 (40.5)</td>
<td>1 1.0131</td>
</tr>
<tr>
<td>ExPEC</td>
<td>55 (46.6)</td>
<td>34 (44.7)</td>
<td>21 (50)</td>
<td>0.7002 1.2353</td>
</tr>
<tr>
<td>FQ(^8)</td>
<td>70 (59.3)</td>
<td>59 (77.6)</td>
<td>11 (26.2)</td>
<td>&lt;0.0001 9.7807</td>
</tr>
<tr>
<td>ESBL</td>
<td>10 (8.5)</td>
<td>3 (3.9)</td>
<td>7 (16.7)</td>
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<tr>
<td>AmpC</td>
<td>4 (3.4)</td>
<td>3 (3.9)</td>
<td>1 (2.4)</td>
<td>1</td>
</tr>
<tr>
<td>ESBL(^+) AmpC(^+) FQ(^8)</td>
<td>69 (58.5)</td>
<td>54 (71.1)</td>
<td>15 (35.7)</td>
<td>0.000233 4.4182</td>
</tr>
</tbody>
</table>

*Significant (\( P<0.05 \)).

http://jmm.sgmjournals.org
Phylotyping of the resistant strains

ESBL-positive E. coli isolated from HIV patients predominantly (94.9%) belonged to non-B2 from non-HIV patients belonged to the phylogroups B2 corresponded to phylogroup D (47.4%) whereas those phylogroup B2 (88.9%) the resistant ones were associated with non-B2 phylogroups (81.8%) (data not shown).

**DISCUSSION**

Exposure to antibiotics has been considered as a significant factor that strongly influences the emergence and spread of antibiotic resistance (Cantón & Morosini, 2011; Andersson & Hughes, 2010). Currently, FQs and β-lactams are the broad-spectrum antimicrobials widely used in clinical medicine. However, ESBLs have been reported among 51–90% of *Enterobacteriaceae* in Asia, especially in India (Mathai et al., 2002; Hawkey, 2008). Over the years, the percentage prevalence of ESBL-producing urinary isolates of *E. coli* has increased in India, ranging from 26.6% to 76.9% (Khurana et al., 2002; Tankhiwale et al., 2004; Babypadmi & Appalaraju, 2004; Singhal et al., 2005; Kumar et al., 2006; Tanjau et al., 2008; Agrawal et al., 2008; Shiju et al., 2010). In line with these reports, we found that the overall percentage positivity of ESBL-producing *E. coli* was 66.9%. Among the *E. coli* isolates that harboured *blaTEM* 65.9% exhibited an ESBL phenotype, while all the *blaCTX-M* + isolates exhibited an ESBL phenotype. SHV- derived ESBL enzymes were not detected in the present study, which is in agreement with other Indian studies (Baby Padmini et al., 2008; Alfaresi & Elkoush, 2010). Conversely, Sharma et al. (2010) reported that 12 (48%) of the 25 ESBL-producing *E. coli* isolates they studied possessed a *blaSHV* gene. Also, a Turkish study reported TEM- (65.1%) and SHV- (28.6%) mediated resistance to be more common among the cefazidime-resistant ESBL-producing *E. coli* (Hoşoğlu et al., 2007).

The first Indian study on the occurrence of AmpC β-lactamases revealed that 20.7% of the strains harboured AmpC enzymes (Manchanda & Singh, 2003). In our study, 61.9% of the *E. coli* isolates expressed an AmpC phenotype. Conversely, other Indian studies have reported a lower incidence of AmpC production among *E. coli*, ranging from 6.97% to 47.3% (Singhal et al., 2005; Patel et al., 2010; Hemalatha et al., 2007). Among the AmpC- positive isolates, 4 (5.5%) were pure AmpC producers, while co-production of ESBL along with AmpC was detected in 69 (94.5%) isolates. Our results agree with those of another Indian study in which the majority (86.1%) of the AmpC-positive isolates co-produced ESBLs while only 9.2% were pure AmpC producers (Hemalatha et al., 2007). The majority of our AmpC screen positive isolates showed negative results for PMACBLs, which indicates that they were either AmpC hyperproducers or harboured other mobile *ampC* genes that were not detected by our multiplex PCR.

Previous studies have documented an undoubted link between the increased use of cephalosporins and the emergence of ESBL-producing *Enterobacteriaceae* (Meyer et al., 2010). As anticipated, the majority of our *blaTEM* (68.3%) and *blaCTX-M* (83.3%) positive isolates were from HIV patients. The co-carriage of ESBL genes (*blaTEM* and *blaCTX-M*) among *E. coli* from HIV patients further increases the risk of treatment failure. This is in agreement with a recent study in Central Taiwan which reported the co-existence of two or more ESBL genes in about 40% of the *E. coli* isolates (Lin et al., 2010). We found that the majority of our study isolates from HIV patients who were on cephalosporin treatment against lower respiratory tract infection exhibited an ESBL+ AmpC+ phenotype, suggesting that repeated antibiotic exposure plays a key role in the emergence of drug resistance – a common phenomenon in these patients.

FQs are increasingly relied on for the empirical therapy of UTI; hence the increased FQ resistance observed among our HIV isolates poses a substantial threat, escalating the risk of treatment failure. Also, a significant association exists between quinolone resistance and ESBL production among enterobacteria, and wide use of quinolones and β-lactams often results in cross-resistance (Frank et al., 2011). Pitout & Laupland (2008) highlighted that CTX-M-producing *E. coli* strains isolated from hospital and community sites often exhibit co-resistance to trimethoprim-sulfamethoxazole and ciprofloxacin. Dual FQ and β-lactam resistance is quite problematic, especially among immunocompromised patients. In agreement with other Indian studies, the majority of our ESBL/AmpC-producing *E. coli* exhibited a high level of resistance towards quinolone (93.1%) and FQ (75%), which would have therapeutic implications (Baby Padmini et al., 2008). Several reports have documented a strong correlation between *qnrA* prevalence and ESBL positivity in *E. coli* isolates, especially the CTX-M type (Jacoby et al., 2003; Mammeri et al., 2005; Lavigne et al., 2006), which could frequently be due to the presence of these resistance determinants on mobile genetic elements (Jacoby & Sutton, 1991; Partridge et al., 2001; Preston et al., 2003; Villa et al., 2000; Wang et al., 2003). In our study, of the 34 isolates that possessed *blaCTX-M* 30 (88.2%) were FQ
resistant, and of the 25 isolates that possessed \( \text{bla}_{\text{TEM}} \), 12 (48%) were FQ resistant. Among the 13 isolates that harbour both \( \text{bla}_{\text{TEM}} \) and \( \text{bla}_{\text{CTX-M}} \) ESBL genes, 12 (92.3%) were FQ resistant. Also, a Turkish study by Oktem et al. (2008), reported that \( \text{qnrA} \)-positive isolates predominantly harbour ESBLs of the SHV and CTX-M types, and both the \( \text{bla}_{\text{CTX-M}} \) and \( \text{qnrA} \) genes were located on the same transferable plasmid.

There have been only few studies which addressed the incidence of UTI caused by ESBL/AmpC-producing \( E.\ coli \) among HIV patients, and the present study provides further insights into the degree of multiple drug resistance (ESBL\(^+\) AmpC\(^+\) FQ\(^R\)) exhibited by the UPEC. Our findings reveal that antibiotic-resistant (ESBL\(^+\) AmpC\(^+\) and/or FQ\(^R\)) phylogroup D isolates with limited virulence (mean virulence score 2.471) are competent enough to establish infections in immunocompromised (HIV) patients. In contrast, in immunocompetent hosts (non-HIV patients) who have not had recent/repeated exposure to FQ/cephalosporins, an array of virulence factors (mean virulence score 3.048) is essential to overcome host defences irrespective of antibiotic resistance.

The virulence potential and multiple drug resistance observed among the \( E.\ coli \) of non-B2 phylogroups further strengthens the claim that horizontal gene transfer might play a major role in the emergence of drug resistance among these originally avirulent/less virulent strains which can no longer be called ‘innocent bystanders’. Also, prior exposure to 3G cephalosporins/FQs probably serves as a driving force for the selection of multidrug-resistant isolates primarily of endogenous origin to establish UTI among HIV patients. Multidrug resistance exhibited by these UPEC signifies the urgent need to modify the current guidelines on the empirical therapy of UTI, especially among immunocompromised patients. Also, combined FQ resistance with ESBL/AmpC production exhibited by these \( E.\ coli \) indicates the need for prompt diagnosis and effective treatment of UTI in order to avoid haematogenous spread of these pathogens, which could eventually result in bacteremia of urinary tract origin.

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