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Genotypic and Phenotypic Profiles of *Escherichia coli* Isolates Belonging to Clinical Sequence Type 131 (ST131), Clinical Non-ST131, and Fecal Non-ST131 Lineages from India

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In view of the epidemiological success of CTX-M-15-producing lineages of *Escherichia coli* and particularly of sequence type 131 (ST131), it is of significant interest to explore its prevalence in countries such as India and to determine if antibiotic resistance, virulence, metabolic potential, and/or the genetic architecture of the ST131 isolates differ from those of non-ST131 isolates. A collection of 126 *E. coli* isolates comprising 43 ST131 *E. coli*, 40 non-ST131 *E. coli*, and 43 fecal *E. coli* isolates collected from a tertiary care hospital in India was analyzed. These isolates were subjected to enterobacterial repetitive intergenic consensus (ERIC)-based fingerprinting, O typing, phylogenetic grouping, antibiotic sensitivity testing, and virulence and antimicrobial resistance gene (VAG) detection. Representative isolates from this collection were also analyzed by multilocus sequence typing (MLST), conjugation, metabolic profiling, biofilm production assay, and zebra fish lethality assay. All of the 43 ST131 *E. coli* isolates were exclusively associated with phylogenetic group B2 (100%), while most of the clinical non-ST131 and stool non-ST131 *E. coli* isolates were affiliated with the B2 (38%) and A (58%) phylogenetic groups, respectively. Significantly greater proportions of ST131 isolates (58%) than non-ST131 isolates (clinical and stool *E. coli* isolates, 5% each) were technically identified to be extraintestinal pathogenic *E. coli* (ExPEC). The clinical ST131, clinical non-ST131, and stool non-ST131 *E. coli* isolates exhibited high rates of multidrug resistance (95%, 91%, and 91%, respectively), extended-spectrum-β-lactamase (ESBL) production (86%, 83%, and 91%, respectively), and metallo-β-lactamase (MBL) production (28%, 33%, and 0%, respectively). CTX-M-15 was strongly linked with ESBL production in ST131 isolates (93%), whereas CTX-M-15 plus TEM were present in clinical and stool non-ST131 *E. coli* isolates. Using MLST, we confirmed the presence of two NDM-1-positive ST131 *E. coli* isolates. The aggregate bioscores (metabolite utilization) for ST131, clinical non-ST131, and stool non-ST131 *E. coli* isolates were 53%, 52%, and 49%, respectively. The ST131 isolates were moderate biofilm producers and were more highly virulent in zebra fish than non-ST131 isolates. According to ERIC-based fingerprinting, the ST131 strains were more genetically similar, and this was subsequently followed by the genetic similarity of clinical non-ST131 and stool non-ST131 *E. coli* strains. In conclusion, our data provide novel insights into aspects of the fitness advantage of *E. coli* lineage ST131 and suggest that a number of factors are likely involved in the worldwide dissemination of and infections due to ST131 *E. coli* isolates.

Escherichia coli is the most frequent causal agent of bacterial infections globally, and about 80% of urinary tract infections (UTIs) are caused by extraintestinal pathogenic *E. coli* (ExPEC) isolates (1). These ExPEC isolates are becoming increasingly resistant to frontline antibiotics, like ciprofloxacin and trimethoprim, resistance to which is frequently reported in Europe, America, and much of Asia (2). Moreover, the increasing prevalence of extended-spectrum β-lactamases (ESBLs) in ExPEC, mainly the non-TEM/SHV ESBLs, such as the CTX-M enzymes, has become a serious clinical problem globally and particularly during the last decade (3). Also, ever since the first report of CTX-M-producing *Enterobacteriaceae* (4), several surveys have reported on the presence of ESBLs in Indian clinical isolates (5–7). This has led to an understanding that an endemic incidence of CTX-M ESBL is imminent in many parts of India (8). This scenario could have motivated clinicians to resort to the widespread use of last-line antibiotics, such as carbapenems, to treat various life-threatening infections. Consequently, this could have selected bacteria with novel genes/enzymes that could degrade even the carbapenem group of antibiotics, with the best example being the NDM-1 gene (9, 10). Following the first description of NDM-1 in *Klebsiella pneumoniae* in 2008, a variety of bacterial species positive for NDM-1 carbapenemases was reported globally (5). The emergence of antimicrobial-resistant superbugs is thus a global problem. However, the circumstances predominating in developing countries in particular pose serious concerns (11) despite the fact that there is no credible scientific evidence to indicate that the NDM-1 gene had its origins in India.

The increase in the prevalence of multidrug-resistant (MDR) bacteria in recent years has posed a significant risk to public health (12). Moreover, the dissemination of clonal organisms carrying a heavy antibiotic resistance background has aggravated the problem (13). The strains of sequence type 131 (ST131) form a pandemic clone that is rapidly and boundlessly disseminating in different countries across continents. These clonal pathogens are highly homogeneous in their virulence and antimicrobial resistance properties (14). Pulsed-field gel electrophoresis analysis re-

Received 12 May 2014 Returned for modification 28 June 2014
Accepted 16 September 2014
Published ahead of print 22 September 2014
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doi:10.1128/AAC.03320-14

vealed them to be 85% similar (15). Phylogenetic analysis based on whole-genome sequence data confirmed that the ST131 clones are genetically monomorphic in nature (16, 17). *E. coli* ST131 has emerged globally to become an important pathogen causing urinary tract and bloodstream infections within communities and hospitals (18). This clonal group is also responsible for the recent worldwide spread of CTX-M-15 ESBL types, which are known to frequently harbor fluoroquinolone resistance and for which limited treatment options exist (19). Clinical findings also suggest that the strains belonging to this group are highly virulent and have a fitness advantage (20). If this is the case, then the association of newer mechanisms of antibiotic resistance, such as NDM-1 with ST131 *E. coli* (21), could pose grave complications in the form of pandemics of life-threatening treatment failures.

The dissemination of resistance among bacteria is mainly thought to be due to mobile genes present on self-transmissible plasmids (22). However, a large cache of antibiotic-resistant bacterial clones goes undetected, as these clones often take refuge as normal microbiota in the gut of humans and animals. Moreover, the commensal bacteria are at risk of exposure to orally ingested antibiotics. In this way, they possibly acquire resistance genes and facilitate the spread of such genes to other bacteria of the gut and extant environment (23, 24).

Our previous study from India reported important data on clinical isolates of *E. coli* (7). However, a thorough comparative work describing ESBL-producing ST131 *E. coli* and other non-ST131 ESBL *E. coli* isolates has not been reported so far. Therefore, the objectives of this study were to assess the occurrence, diversity, and distribution of antimicrobial-resistant genotypes and ESBL phenotypes in order to deduce a molecular epidemiological scenario involving commensal bacteria, clinical ST131 types, and non-ST131 isolates of *E. coli*. We also determined their phylogeny and genetic diversity using PCR-based approaches. The virulence attributes of the three categories of isolates were determined by genotyping as well as phenotypic methods. This comprehensive profiling of the Indian isolates of *E. coli* forms an essential molecular epidemiological perspective on the predominance and diversity/clonality of such isolates that will aid planning for mitigation with better control strategies.

MATERIALS AND METHODS

Sample collection and screening for ST131 strains. A total of 350 *E. coli* isolates were collected from symptomatic UTI patients from a tertiary care hospital (D. Y. Patil Medical College and Hospital, Pimpri, Pune, India) in India between January 2009 and March 2011. Fifty *E. coli* isolates were also cultured from the feces of healthy individuals who had reported for routine health check-ups at the hospital. All isolates were tested for purity and were identified by routine laboratory methods. The O types of all *E. coli* isolates were initially determined with an allele-specific PCR targeting the *rfbO25* subgroup gene locus (25). ST131 strains were further confirmed by PCR-based detection of the *pabB* allele as described earlier (25). After this screening procedure, only 83 out of the 350 isolates and 43 fecal *E. coli* isolates were finally selected for further studies. Therefore, a total of 126 *E. coli* isolates were subjected to the thorough investigation described below. All microbiological study protocols for these *E. coli* isolates were approved by the Institutional Biosafety Committee of the University of Hyderabad.

Antibiotic susceptibility testing. Antibiotic susceptibility profiles were obtained for all *E. coli* isolates by using the standard disc diffusion method on Mueller-Hinton agar. The Clinical and Laboratory Standards Institute (CLSI) recommendations for antimicrobial susceptibility testing were followed (7). The antibiotic panel used was as follows: gentamicin (120 μg), ciprofloxacin (5 μg), nalidixic acid (30 μg), chloramphenicol (30 μg), sulfamethoxazole-trimethoprim (25 μg), and tetracycline (30 μg). All strains were tested for the ESBL production phenotype using the CLSI method, as described previously (26). Resistance to carbapenems was detected by the Etest (HiMedia, India) method, with MICs being determined after a 24-h incubation at 37°C. Susceptibility was defined according to the breakpoints of the CLSI criteria. Additionally, the modified Hodge test was performed to detect carbapenemase production (27).

Antimicrobial resistance gene testing. The presence of genes encoding antimicrobial resistance was determined by PCR amplification of the respective genes. PCR amplification of the $bla_{\text{CTX-M}}$, bla_{TEM}, and bla_{SHV} genes was carried out on all *E. coli* isolates using the PCR conditions and primers previously described (28). The presence of other antibiotic resistance genes, such as those conferring resistance to tetracycline [namely, *tet*(*A*), *tet*(*B*), and *tet*(*C*)], sulfonamides (*sul1*, *sul2*, *sul3*), streptomycin (*strA*, *strB*), aminoglycosides (*aadA1*-like, *aac4*), and trimethoprim (*dhfr*, *dfr*), and some other plasmid-mediated quinolone resistance determinants, such as *aac* (6′)-*Ib*, was determined by PCR (29–34). Four well-known carbapenemase resistance genes, $bla_{\text{OXA-48}}$, bla_{KPC}, bla_{VIM}, and $bla_{\text{NDM-1}}$, were also amplified as described previously (29, 35). All the *E. coli* isolates were screened by PCR for the presence of the integron-associated *intI1* gene encoding the class 1 integrase with the help of primers described elsewhere (36).

Conjugation and plasmid analysis. Conjugative transfer of ESBL genes was tested by broth mating experiments using plasmid-free and sodium azide-resistant *E. coli* strains as the recipients (32). Putative transconjugants were selected on eosin-methylene blue agar plates containing sodium azide (100 μg/ml) and cefotaxime (4 μg/ml), and these were checked by PCRs for the presence of the relevant *bla* genes. Plasmids were classified into two major replicon types according to their incompatibility groups by using a PCR-based replicon-typing scheme (37).

Phylogenetic grouping and virulence gene identification. All the isolates were tested for the determination of their phylogenetic groups using PCR according to the method described by Clermont et al. (38). All the *E. coli* isolates were screened for 6 major ExPEC-associated virulence genes by PCR, as described previously (28, 39). The virulence score for each isolate was determined on the basis of the number of virulence genes detected.

Zebra fish lethality assay and biofilm formation. Zebra fish were anesthetized by immersion in water containing 168 μg/ml of tricaine (Sigma) and then intraperitoneally injected with 10 μl of phosphate-buffered saline (PBS) containing *E. coli* (1×10^6 CFU/ml) using a 26.5-gauge syringe (Becton Dickinson, USA). After injection, the fish were returned to water tanks and closely monitored for mortality every 3 h for up to 1 week, as described earlier (40). Biofilm formation by *E. coli* isolates from the three groups was assayed by use of a 96-well polystyrene microtiter plate in the static biofilm model, as described in our previous study (7).

Metabolic profiling. The three categories of *E. coli* isolates were tested against 35 different substrates to check their metabolizing abilities by use of a KB009 Hi carbohydrate kit (HiMedia, Mumbai, India). Fifty microliters of inoculum was added into the wells of the strip. Upon incubation, the organisms underwent metabolic changes, which were indicated by a spontaneous color change in the medium due to a change of pH.

Genotyping by ERIC-PCR. *E. coli* strains were typed by enterobacterial repetitive intergenic consensus PCR (ERIC-PCR). Crude DNA obtained from the *E. coli* isolates by direct cell lysis was analyzed by ERIC sequence-based PCR with the ERIC1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) primers; PCR was performed as previously described (41). Amplicons were separated on a 1.5% agarose gel containing ethidium bromide (5 μg/ml) at 50 V for 3 h. The banding pattern of 126 *E. coli* isolates was analyzed using BioNumerics software (Applied Maths, Belgium). Bands were scored by the use of Dice similarity indices based on the unweighted-pair group method with arithmetic mean (UPGMA), and dendrograms were generated to draw relationships among the ERIC profiles.

TABLE 1 Contribution of ST131, non-ST131, and stool *E. coli* isolates to overall antimicrobial resistance rates

Resistance trait	Overall prevalence of resistant phenotype in population (%)	Estimated fraction due to:		
		ST131 clinical isolates	Non- ST131 clinical isolates	Stool non-ST131 isolates
Quinolone or fluoroquinolone				
Ciprofloxacin	76	0.40	0.25	0.34
Nalidixic acid	95	0.38	0.23	0.38
Sulfonamide-trimethoprim, co-trimoxazole	68	0.33	0.23	0.43
Aminoglycosides, gentamicin	45	0.47	0.21	0.31
Phenicols, chloramphenicol	28	0.51	0.17	0.31
Tetracyclines, tetracycline	67	0.35	0.27	0.37
ESBL phenotype	87	0.33	0.30	0.35
MBL phenotype[a]	20	0.48	0.52	0.00
Multidrug resistance	92	0.33	0.32	0.33

[a] Fisher's exact test did not identify any significant differences between the three groups of strains for the different traits analyzed, except for the MBL phenotype, in which the *P* value for ST131 and non-ST131 isolates was <0.05 in comparison with stool *E. coli* isolates.

RESULTS

ST131 status and patient demography. A total of 350 *E. coli* isolates were screened by PCR with the help of a pair of primers directed at the *pabB* fragment, a specific signature of the ST131 lineage. Of these, 43 isolates (12.2%) were positive for ST131-specific amplicons. These ST131 strains were additionally tested for the *rfbO25b* locus by PCR, and their ST131 status was thus reconfirmed. None of the 43 stool isolates belonged to the ST131 lineage. Among the 43 ST131 *E. coli* isolates, 26 (60%) were from female patients with a mean age of 38 years and 17 (40%) were from male patients with a mean age of 48 years, whereas among the 40 non-ST131 *E. coli* isolates, 21 (52%) were from female patients and 19 (47%) were from male patients with mean ages of 35 and 45 years, respectively. Male patients (for both ST131 and non-ST131 isolates) were more likely to develop prostatitis than common UTI. Among the different wards/sections of the hospital, a majority of the ST131 isolates originated from surgical wards (30%) and the intensive care unit (33%), while 28% of the isolates were from different medical wards. Only 9% of the ST131 isolates came from the outpatient department. All the clinical non-ST131 isolates originated from nonsurgical medical wards.

Phenotypic and molecular screening for antimicrobial resistance. A total of 126 isolates (comprising three groups of 40 ± 3 randomly selected isolates each) representing ST131, non-ST131, and stool non-ST131 *E. coli* isolates underwent antimicrobial susceptibility testing. Disc diffusion test entailing 6 antibiotics of 5 different non-β-lactam classes showed high levels of resistance among all *E. coli* isolates regardless of their genetic lineage or clinical status (Table 1). The antimicrobial resistance rates for the three groups of strains, namely, ST131, non-ST131, and stool non-ST131 isolates, were as follows: for ciprofloxacin, 90%, 92%, and 76%, respectively; for tetracycline, 63%, 75%, and 86%, respectively; for gentamicin, 60%, 50%, and 42%, respectively; for co-trimoxazole, 63%, 75%, and 86%, respectively; and for chloramphenicol, 40%, 33%, and 25%, respectively. *E. coli* isolates within the three groups showed a nearly equal rate of resistance (95%) toward nalidixic acid. ST131 and non-ST131 isolates, including the fecal isolates, did not differ significantly in the prevalence of resistance to any of the particular antibiotics tested. However, aggregate resistance scores (resistance to all the 6 antibiotics) differed marginally among the three groups and were the highest among the ST131 isolates (35%) and the lowest among the fecal isolates (14%). Among the three groups of strains, the proportions of multidrug-resistant (MDR) isolates were as follows: for ST131 and fecal isolates, 95% each; for non ST-131 isolates, 91%. With regard to ESBL production, 37 (86%) of the 43 ST131 isolates, 39 (91%) of the 43 fecal isolates, and 33 (83%) of the 40 non-ST131 isolates were ESBL producers. The rate of ESBL production among the three groups differed slightly, with higher rates of ESBL production being found in stool isolates, followed by ST131 isolates and then clinical non-ST131 isolates (Table 1).

The prevalences of the major genes for resistance to tetracycline, aminoglycosides, fluoroquinolones, sulfonamides, and trimethoprim in our isolates are reported in Table 2. All 126 isolates were negative for the *sul3*, *aac*, and *tet*(*C*) genes. The correlation between resistance genotypes (presence or absence of resistance genes) and phenotypes (resistance or susceptibility) was high for fluoroquinolones (94% agreement), co-trimoxazole (90% agreement), and aminoglycosides (88% agreement), whereas the agreement between resistance to tetracycline and the presence of *tet*(*A*) and *tet*(*B*) was poor (60%). The agreement between the ESBL genotypes and the ESBL-positive phenotype in the ST131 and stool isolates was 100%, and for non-ST131 isolates it was 84%. The main gene contributing to the ESBL phenotype among the ST131 strains was $bla_{\text{CTX-M-15}}$, whereas for non-ST131 and fecal strains, it was $bla_{\text{CTX-M-15}}$ plus bla_{TEM}. $bla_{\text{CTX-M-15}}$ was found to be strongly associated with ST131 status (93%). The possible combinations of ESBL production–CTX-M-15 production, ESBL production-fluoroquinolone resistance, and CTX-M-15 production–fluoroquinolone resistance gave rise to six subgroups with two subgroups per combination (Table 3). Among these, the dual-positive subgroup (ESBL positive, CTX-M-15 positive) was the most predominant (57%), followed successively by the CTX-M-15-positive–fluoroquinolone-resistant subgroup (55%), the ESBL-positive–CTX-M-15-negative subgroup (33%), the ESBL-negative–fluoroquinolone-resistant subgroup (8%), and the ESBL-negative–fluoroquinolone-susceptible and CTX-M-15-positive–fluoroquinolone-susceptible subgroup (with a low prevalence of 6% each). Within these subgroups, the ST131 strains contributed more or less equal numbers of representatives compared to the numbers contributed by the stool and clinical non-ST131 strains, except for subgroups 1 and 5. The prevalence of metallo-β-lacta-

TABLE 2 Molecular determination of antimicrobial resistance in 126 *E. coli* isolates

Antibiotic class	Specific trait	No. (%) of isolates			*P* value[a]	
		ST131 *E. coli* isolates (group 1; $n = 43$)	Non-ST131 *E. coli* isolates (group 2; $n = 40$)	Fecal *E. coli* isolates (group 3; $n = 43$)	Group 1 vs group 2	Group 1 vs group 3
Tetracyclines	*tet(A)*	24 (56)	17 (43)	7 (16)		<0.001
	tet(B)	5 (12)	22 (55)	10 (23)	<0.001	
Aminoglycosides	*strA*	33 (77)	19 (48)	24 (56)	0.006	0.040
	strB	15 (35)	7 (18)	16 (37)	0.073	
Fluoroquinolone	*aac(6′)-lb-cr*	36 (84)	17 (43)	36 (84)	<0.001	
Sulfonamides	*sul1*	20 (47)	17 (43)	10 (23)		0.024
	sul2	5 (12)	12 (30)	22 (51)	0.038	<0.001
Trimethoprim	*dfr*	18 (42)	7 (18)	21 (49)	0.016	
	dhfr	15 (35)	3 (8)	5 (12)	0.002	0.011

[a] *P* values (determined by Fisher's exact test, two-tailed) are shown when *P* is <0.05.

mase (MBL)-positive strains was only 20% (25 out of a total 126 isolates), having a median resistance and resistance range higher than those of MBL-negative isolates (Table 3). Molecular detection revealed that two such MBL producers belonged to the ST131 group and, more importantly, harbored the $bla_{\text{NDM-1}}$ metallo-β-lactamase gene, in addition to the $bla_{\text{CTX-M-15}}$ ESBL gene. This suggests the emergence of metallo-β-lactamase NDM-1-producing ST131 *E. coli* isolates in India. Overall, resistance to antimicrobial agents other than those specified (Table 1) was common and differed insignificantly between the three groups.

Conjugation and plasmid analysis. Plasmid-based replicon typing of the two major incompatibility replicons, FIA and FIB, revealed that the ST131 strains were strongly associated with FIA and FIB at frequencies of 84% and 81%, respectively, and the non-ST131 strains were associated with FIA and FIB at frequencies of 15% and 20% respectively, whereas only 9% and 16% of stool *E. coli* strains were positive for the FIA and FIB replicons, respectively. For identification of the plasmids that were responsible for ESBL production, 10 representative strains from each group were tested by conjugation with the laboratory strain J53, which does not harbor plasmids of its own. We were successful in getting 10 transconjugants out of the total of 30 representative strains tested. These comprised transconjugants derived from 2 non-ST131, 3 ST131, and 5 stool *E. coli* isolates. The transconjugants were subjected to an ESBL production test by the double-disc synergy method, and it was confirmed that all the 10 transconjugants contained an ESBL plasmid. They were also checked for their ability to confer coresistance to other classes of antibiotics, as described in Fig. 1. As observed, the conjugation propensity was higher among stool *E. coli* strains, followed by ST131 and non-ST131 strains. The transconjugants were found to confer resistance to none or a maximum of four antibiotics, but none of these transconjugants were coresistant to all the antibiotics to which their parent clinical strains were resistant.

Phylogenetic grouping and virulence gene identification. All the 126 strains (comprising 43 ST131 strains, 40 non-ST131 strains, and 43 fecal strains) formed four distinct phylogenetic groups (groups A, B1, B2, and D), as shown in Table 4. Among the total population, group B2 was most prevalent phylogenetic group, corresponding to 49% of the isolates, followed in prevalence by the other groups: A (27%), D (16%), and B1 (8%). All the ST131 isolates were from group B2 (accounting for 43 [70%] of

TABLE 3 Antimicrobial resistance scores and range toward 6 non-β-lactam antibiotics among 6 groups of strains defined by the possible combination of ESBL and CTX-M-15 production, ESBL production and fluoroquinolone resistance, and CTX-M-15 production and fluoroquinolone resistance, including those for MBL-positive and MBL-negative strains

Subgroup	Subset definition	All isolates		ST131 isolates		Non-ST131 isolates		Fecal isolates	
		No. of isolates	Median (range) score	No. of isolates	Median (range) score	No. of isolates	Median (range) score	No. of isolates	Median (range) score
1	ESBL positive, CTX-M-15 positive	72	4 (1–6)	35	5 (2–6)	21	4 (2–6)	16	4 (2–5)
2	ESBL positive, CTX-M-15 negative	41	4 (1–6)	15	4 (3–6)	2	3 (3)	24	5 (1–6)
3	ESBL negative, fluoroquinolone resistant	10	4 (3–6)	4	3 (3–4)	5	4 (4–6)	1	0 (5)
4	ESBL negative, fluoroquinolone susceptible	7	1 (0–3)	2	0 (0)	2	2 (1–3)	3	1 (0–3)
5	CTX-M-15 positive, fluoroquinolone susceptible	69	4 (2–6)	38	5 (2–6)	20	4 (2–6)	11	4 (2–5)
6	CTX-M-15 positive, fluoroquinolone susceptible	7	3 (0–4)	3	3 (0–3)	1	0 (3)	3	3 (3–4)
7	MBL positive	25	5 (2–6)	12	5 (2–6)	13	4 (2–6)	0	0
8	MBL negative	58	4 (0–6)	31	4 (0–6)	27	4 (1–6)	0	0

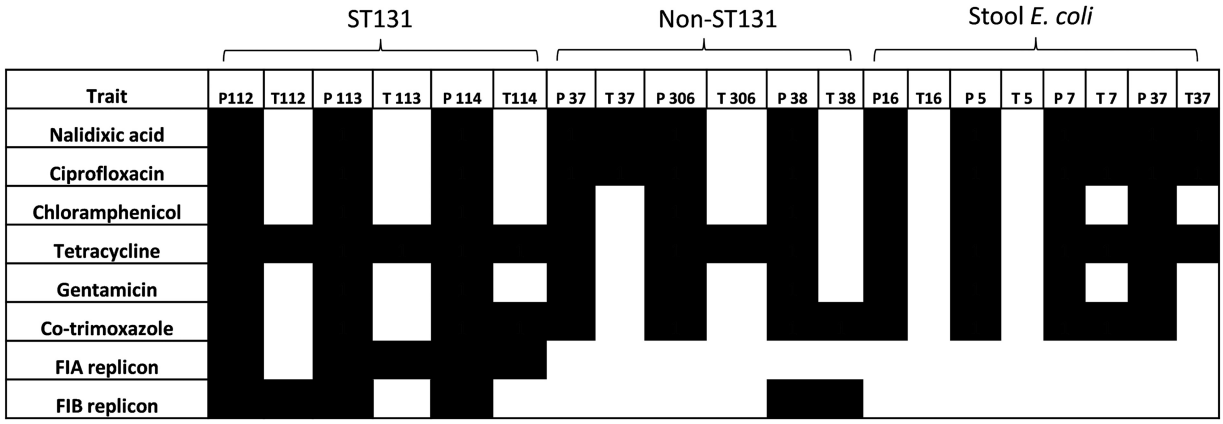

FIG 1 Agar diffusion test and screening of major replicon genes between clinical strains (designated P) and their respective transconjugants (designated T). Black squares, resistance to the indicated antimicrobial or the presence of the indicated replicon; white squares, susceptibility to the indicated antimicrobial or the absence of the indicated replicon.

the 62 isolates of the B2 type), whereas the non-ST131 and fecal isolates exhibited diverse phylogenetic groups, with B2 and A being predominant in the two groups, respectively. Although the non-ST131 isolates were likely to be from group B2 (38%), the phylogenetic group distribution was not significantly associated with CTX-M-15-positive and/or CTX-M-15-negative isolates. In contrast, the stool isolates were predominantly from group A, followed by groups D, B2, and B1. Overall, the distribution of isolates into phylogroups varied significantly among the three groups of *E. coli* isolates (except for group D, with which both non-ST131 and fecal isolates were affiliated in equal proportions).

The genotypes for 6 ExPEC-associated virulence markers were determined for all the 126 isolates comprising the three groups defined by ST131 type and clinical status. All the 6 virulence genes that we explored were detected once in at least 1 isolate. Among the three groups, the median virulence score and the virulence score range values increased progressively from a high of 2 (range, 0 to 5) for ST131 isolates to a low of 1 (range, 0 to 1) for stool isolates, with the intermediate score, and a score of 1 (range, 0 to 3) for non-ST131 isolates. The ST131 isolates harbored a slightly higher and discrete virulence profile than the non-ST131 and stool isolates (Table 4). In the ST131 group, an aggregate of 81 isolates was positive for the 6 different virulence markers, whereas 25 and 29 isolates within non-ST131 and fecal groups, respectively, were positive for the 6 different virulence markers. Particularly, *sfa* and *foc* (encoding S fimbria and FIC fimbriae, respectively) and *papA* (encoding type 1 fimbrial adhesin) were predominant in ST131 strains. Coinciding with the greater prevalence of a few ExPEC-associated virulence markers among the ST131 isolates, a more significant proportion of ST131 isolates (58%) than non-ST131 and fecal isolates (5% each) technically fell in the ExPEC category. Moreover, among the ST131 isolates, the presence of CTX-M-15 was significantly associated with ExPEC status (98%).

TABLE 4 Phylogenetic and virulence characteristics of 126 *E. coli* isolates

Specific trait	No. (%) of isolates with trait			
	Total (n = 126)	ST131 isolates (n = 43)	Non-ST131 isolates (n = 40)	Fecal isolates (n = 43)
Phylogenetic group				
Group A	34 (27)	0 (0)	9 (22.5)	25 (58)
Group B1	10 (8)	0 (0)	8 (20)	2 (5)
Group B2	62 (49)	43 (100)	15 (37.5)	4 (9)
Group D	20 (16)	0 (0)	8 (20)	12 (28)
Virulence genes				
afa	16 (13)	7 (16)	1 (3)	8 (19)
sfa-foc	4 (3)	4 (9)	0 (0)	0 (0)
papA	45 (36)	29 (67)	12 (30)	4 (9)
aer	55 (44)	32 (74)	9 (23)	14 (33)
hlyD	11 (9)	6 (14)	2 (5)	3 (7)
cnf	4 (3)	3 (7)	1 (3)	0 (0)

Biofilm formation. We explored the biofilm-forming ability of the three categories of strains and found that the ST131 strains were indeed superior in their ability to form a biofilm, but their numbers were not significantly higher. This was followed by non-ST131 strains. The stool strains were weaker biofilm formers than the ST131 and non-ST131 strains (Fig. 2). This indicates that biofilm formation could possibly contribute to the resilience and predominance of ST131 strains, which offers them an advantage to

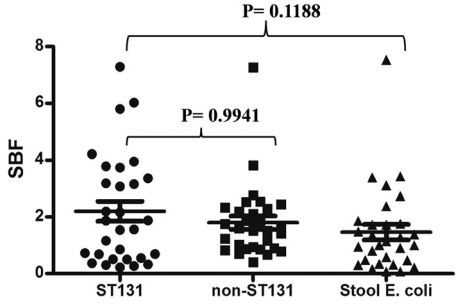

FIG 2 Comparison of the specific biofilm formation (SBF) capacities of ST131, non-ST131, and stool *E. coli* isolates. The mean value for each population is indicated by a dark line. The Mann-Whitney test shows that the differences between the three groups are statistically insignificant.

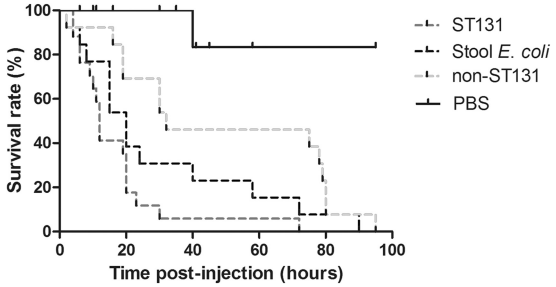

FIG 3 Survival rates of adult zebra fish infected with *E. coli* isolates belonging to the three *E. coli* groups tested. Each experimental group contained 12 zebra fish, and each experiment was performed in duplicate. Using Kaplan-Meier analysis, we observed differences in survival rates. Although the virulence of ST131 isolates differed significantly ($P = 0.0032$) from that of the clinical non-ST131 *E. coli* isolates, it did not differ significantly ($P = 0.1820$) from that of stool non-ST131 *E. coli* isolates.

infect and cause chronic infections; a previous study has also shown that ST131 strains from global sources harbor virulence factors that potentially associate with *in vitro* biofilm formation (42).

Zebra fish lethality assay. The zebra fish infection model is considered a valuable tool to resolve the diverse virulence phenotypes of closely related ExPEC strains. The survival rate of the infected fish was determined to evaluate the virulence potential of ST131 strains and compare it with that of non-ST131 and stool *E. coli* isolates. In this study, three bacterial strains per category were tested; each bacterial strain was injected into four replicates of fishes. The control fish were injected only with PBS. The control fish were all healthy and survived even after 5 days postinjection. Bacterium-infected zebra fish exhibited apparent signs of discomfort, including an inflamed peritoneal area, improper swimming, and reduced gill movement. The ST131 strains were significantly more virulent than the non-ST131 and stool strains. Almost 90% of the zebra fish injected with ST131 strains died within 24 h postinjection. The lethality of the stool *E. coli* isolates was moderate, followed by the level of lethality of the non-ST131 clinical isolates. These results indicate that the ST131 strains were highly lethal for zebra fish (Fig. 3). This observation is consistent with the presence of several classical virulence factors (such as *hlyA*, *cnf*, and *aer*) in our ST131 isolates. Virulence in stool *E. coli* isolates could partly be accounted for by the presence of the *hlyA* and *aer* genes; in addition, the possibility that other unknown toxins or virulence factors are produced cannot be ruled out.

Metabolic profiling. To find an alternative explanation for the success of the prevalent ST131 *E. coli* isolates, we compared carbohydrate utilization among the three categories of *E. coli* isolates. Thirty-five API test reagents (HiMedia, India) were used to perform metabolic profiling (carbohydrate utilization) on 6 ST131, 6 clinical non-ST131, and 6 stool non-ST131 *E. coli* isolates. The aggregate utilization percentages for ST131, non-ST131, and stool non-ST131 *E. coli* isolates were 53%, 52%, and 49%, respectively. Although not statistically significant, the bioscore for the ST131 isolates was slightly higher than that for the non-ST131 *E. coli* isolates; similarly, none of the biochemical traits was significantly associated with ST131 clones. However, they exhibited a fair trend in their ability to utilize only two substrates: salicin and sorbose.

Genotyping. To illustrate the relationship of ST131 with other *E. coli* groups and to establish evidence for ST131's prominence,

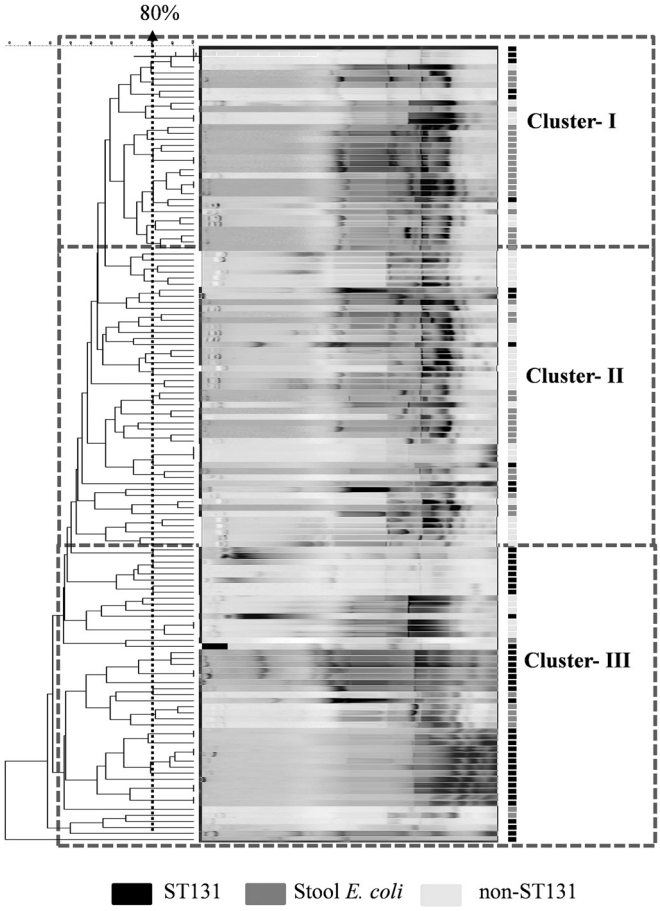

FIG 4 ERIC-PCR-based dendrogram of 126 *E. coli* isolates comprising 43 ST131, 40 non-ST131, and 43 stool *E. coli* isolates produced by use of the UPGMA algorithm based on Dice similarity coefficients. The ST131 strains were genetically homogeneous (cluster III). The homogeneity of the ST131 strains was followed by that of non-ST131 (cluster II) and stool *E. coli* (cluster I) isolates.

ERIC-PCR-based fingerprinting was employed to assess the genomic diversity among the clinical ST131, clinical non-ST131, and stool non-ST131 *E. coli* isolates. The isolates in an ERIC-PCR-based dendrogram of 126 *E. coli* isolates could be broadly classified into three clusters, as defined by the ability of the same category of strains to cluster together, although the three categories did not form distinct clusters with respect to ST131 and/or clinical status. Nevertheless, the ST131 strains were more clonal and more genetically similar, followed by the non-ST131 strains and then the fecal strains (Fig. 4). Consistent with this, the ST131 isolates were more likely to exhibit similar ERIC-PCR-based fingerprinting profiles (30%) than the non-ST131 (18%) and stool *E. coli* (9%) isolates. The two NDM-1-positive isolates formed identical ERIC-PCR-based fingerprinting profiles, suggesting vertical transmission. However, several dissimilar isolates belonging to all the three categories harboring $bla_{\text{CTX-M-15}}$ were scattered throughout the dendrogram, suggesting possible horizontal gene transfer (regardless of ST131 and clinical status). These observations confirm our previous results (7) that the ST131 strains are evolving clonally, whereas the non-ST131 fecal isolates are genetically diverse, with non-ST131 clinical strains being intermediate in their genetic heterogeneity.

DISCUSSION

Recent studies have shown a significant increase in the number of CTX-M-producing *E. coli* isolates possibly due to the dominant mechanisms of clonal expansion of *E. coli* ST131 (15, 16, 43). The association of ST131 with several ExPEC-associated virulence genes and broad-spectrum drug resistance, together with their propensities for rapid expansion and geographic dispersal, has led to the worldwide predominance of ST131 among extraintestinal pathogenic *E. coli* infections. However, the exact underlying mechanism conferring this behavior has not been identified yet. Having said this, it is very pertinent at this juncture to understand the epidemiology of the clone ST131. Past studies have analyzed selected factors for their association with ST131 and non-ST131 isolates (42, 44, 45). However, comprehensive analyses to effectively measure the association of several key factors with clinical ST131 isolates, non-ST131 isolates, and *E. coli* isolates from healthy adult subjects, particularly from developing countries like India, remain scarce. In this study of MDR *E. coli* isolates, obtained from clinical samples and stool specimens from India, we sought to define the associations of some of the key factors related to resistance profiles and transmissibility, virulence genotypes, and pathogenicity and also assessed the genetic affinities and metabolic capabilities of the three populations of *E. coli*. Such a study is also important since ST131 *E. coli* is presumed to be still evolving in terms of the acquisition of newer resistance and virulence genes, and the global expansion of such clones poses a significant public health threat. The current findings extend those from our previous study (7) and offer valuable insights into the basis of the epidemiology of CTX-M-15 dissemination in general and the success of ST131 *E. coli* in particular.

Our analysis of 126 systematically selected isolates comprised 43 (group B2) ST131 clinical *E. coli* isolates and 40 non-ST131 clinical isolates involved in nosocomial and community-acquired infections, along with 43 isolates from healthy individuals, including both ESBL producers and non-ESBL producers. We found that although *E. coli* clonal group ST131 was present in the wider uropathogenic *E. coli* (UPEC) population, it was particularly discerned among MDR ESBL producers. However, their prevalence was moderate; this is consistent with the idea that *E. coli* is a highly diverse species and the clinical isolates are usually represented by several small clonal groups. Remarkably, none of the stool *E. coli* isolates belonged to the ST131 clonal group. Moreover, we identified very few cases of ST131 *E. coli* infection in individuals in outpatient departments. We found a predominance (93%) of $bla_{\text{CTX-M-15}}$ among ESBL-producing ST131 *E. coli* isolates. Notably, over approximately 50% of non-ST131 and fecal isolates also harbored $bla_{\text{CTX-M-15}}$, providing evidence of the widespread distribution of $bla_{\text{CTX-M-15}}$ and a strong association of ST131 with $bla_{\text{CTX-M-15}}$, but not vice versa. The estimated overall comparable contributions of ST131, non-ST131, and stool *E. coli* isolates to non-β-lactam antimicrobial resistance phenotypes suggest not only that it is ST131 that is responsible for the recent increase in antimicrobial resistance prevalence but also that the possibility that other factors contribute to the spread and maintenance of high resistance rates cannot be ruled out. Between the three *E. coli* groups, no single group demonstrated any unique resistance gene profile which was reflective of the observed phenotypes. Despite the similarity of our study to that of Kumarasamy et al. (5), our results were quite different: only two of the total clinical samples analyzed by us were positive for NDM-1. Also, none of the ESBL-producing *E. coli* strains of fecal origin harbored NDM-1. Given this, the prevalence rates appear to be contrasting, although both the studies were performed on clinical samples from India. Plasmid-based replicon typing did not identify plasmid replicons common to the ST131, non-ST131, and stool *E. coli* isolates. Nevertheless, the ST131 isolates shared common plasmids, particularly plasmids positive for FIA-FIB replicons. Earlier studies also showed that ST131 strains are enriched with FIA-FIB replicons (14). Moreover, the transmissibility of these plasmids, together with their propensities to acquire new genes, implies an alarming potential to spread and diversify. The fluoroquinolones and extended-spectrum cephalosporins as well as aminoglycosides and β-lactam antibiotics are generally prescribed as empirical therapy for the treatment of hospital-acquired infections and urinary tract infections in particular. Having said this, the clinical and the public health implications of the observed high prevalence of multidrug resistance and resistance to extended-spectrum cephalosporins and, in particular, the high rates of fluoroquinolone resistance among the clinical as well as the stool *E. coli* isolates are alarming, as it might narrow or deplete the treatment options.

Our results and the observation of a high prevalence of phylogroup B2 isolates among the ST131 isolates compared to its prevalence among the non-ST131 and stool *E. coli* isolates suggest the clonal expansion of ST131 from a common phylogenetic ancestor; this observation reinforces the explanations for ST131's remarkable epidemiological success, as phylogroup B2 is thought to be epidemiologically associated with extraintestinal virulence by means other than through the defined extraintestinal virulence traits.

Furthermore, the molecularly inferred virulence profiles as well as the pathogenicity of the ST131 isolates substantially exceeded those of the non-ST131 and stool *E. coli* isolates in zebra fish, and the ST131 isolates were slightly stronger biofilm formers. This profound virulence advantage observed for ST131 strains may compensate for their modest disadvantage for the development of resistance compared to the possibility of resistance development in the other non-ST131 and stool *E. coli* isolates.

The outcome of the zebra fish lethality assay added to our understanding that *E. coli* ST131 isolates possess an enhanced virulence potential and, thereby, an enhanced ability to cause invasive disease (20, 46, 47), although this may not consistently corroborate the findings of different studies performed in different settings and with different isolates (48–52). Nevertheless, the assay provided a much needed *in vivo* snapshot to establish the virulence attributes of our ST131 strains in adult zebra fish, even though the number of strains in each group was limited. However, scant numbers of strains have previously been used in some of the landmark studies conducted in this area (15, 53). Recently, two reports (15, 48) have shown that *E. coli* ST131 strains do not possess a higher virulence potential in causing invasive infections than other UPEC or other B2 group *E. coli* isolates. This disagreement with respect to virulence could possibly be due to several factors, such as differences in experimental conditions, the ST131 virulence reporter itself, and differences in study populations. Thus, these findings suggest that the ST131 isolates are not (universally) more virulent than the *E. coli* isolates of other sequence types. It is also possible that ST131 strains would act as efficient opportunistic pathogens in humans, given the fact that ST131 is currently the dominant extraintestinal human pathogen. How-

ever, the possibility that other host and environmental factors play a role cannot be ruled out. Furthermore, this discrepancy deserves an investigation by whole-genome comparative analysis of ST131 strains from different geographic origins, along with an experimental assessment of their role in pathogenesis. Overall, the ST131 strains were significantly more likely to qualify as ExPEC strains, and because the ST131 isolates were significantly more likely to be from group B2 than the non-ST131 and stool *E. coli* isolates, they may have enhanced fitness over other *E. coli* isolates. In addition to this, the ST131 strains were also found to be significantly more likely to be associated with the CTX-M-15 gene while also being resistant to a wide range of β-lactam and non-β-lactam antibiotics. This supplemental property seems to provide a competitive advantage to ST131 *E. coli* isolates over other *E. coli* isolates.

Studies have reported variations in the virulence-associated gene profile (VAGF) among ST131 strains, which causes one to question the role of VAGF in the current success of ST131 (49, 50), and therefore, these studies have proposed other factors, such as an enhanced metabolic potential, that contribute to the fitness of this dominant clone. Also, it is well established that bacterial metabolic potential can enhance fitness, leading to increased pathogenesis. For example, a previous study has shown that carbohydrate metabolism in enterobacteria may increase bacterial virulence (51). In this connection, we tested and compared sugar source utilization and found that the ST131 strains were only moderately superior to the other *E. coli* strains tested in metabolizing the 35 sugars tested. Our data are not in total agreement with those of Gibreel et al. (52) or Alqasim et al. (49) because our study did not show either a significantly enhanced or a significantly reduced metabolic capacity of ST131 strains in comparison with the metabolic capacity of other non-ST131 *E. coli* strains. This discrepancy could be due to differences in the types of tests employed, differences in the study populations, and/or comparisons of biased samples. Given this, it will be necessary to profile a collection of strains that is as diverse as possible and as many strains from different geographic regions of the world as possible, and in particular, Indian isolates should be analyzed in a comparative genomics study and by use of phenotypic screens employing other well-known and completely sequenced isolates of the ST131 lineage.

Genotyping by ERIC-PCR showed that the ST131 isolates of this study formed a more or less distinct cluster representing the majority of ST131 strains when these strains were compared to the other *E. coli* groups. This strongly suggests clonal expansion from a common ancestor. Even though it has been years since the emergence and dissemination of ST131 *E. coli* strains, they still maintain their clonal nature. This clonal nature, on the one hand, holds the danger of potentially causing disease outbreaks but, on the other hand, may facilitate effective control strategies involving vaccine development and transmission prevention.

In summary, we utilized comprehensive genotyping and phenotyping approaches to compare clinical ST131 *E. coli* isolates with clinical non-ST131 *E. coli* and stool non-ST131 *E. coli* isolates. Although our isolates displayed diversity in virulence, resistance, metabolic activity, and clonality, neither their affiliation with ST131 nor clinical status was significantly linked to the observed differences. Furthermore, our comprehensive data suggest that none of the key traits examined as described above *per se* contributed exclusively to the epidemiological success of ST131 *E. coli* strains. Rather, the cumulative effect of some of the factors, such as their being of phylogenetic group B2, their sustained carriage of CTX-M-15 plasmids, and their slightly enhanced virulence, antimicrobial resistance, and metabolic activity, along with the high degree of clonal structure and some undefined evolutionary mechanisms, could provide a multilayered fitness advantage to ST131 strains. Hence, the biological basis for the success of ST131 could be a combination of some of the key traits explained above, but none of them could be the sole or exclusive characteristic responsible for the remarkable success of ST131 strains. This is also suggested by the fact that analysis of ST131 *E. coli* strains from different origins and comparison of these strains with well-characterized strains showed inconsistent results with respect to some of the key traits (42, 50). Further investigation based on the functional molecular infection epidemiology of ST131 *E. coli* isolates from different geographic origins and their comparison with other well-characterized strains from *E. coli* phylogroup B2 are needed to exactly determine the basis for the success of ST131 *E. coli*. Regardless of this, the broad multidrug resistance profile observed among all the three categories of *E. coli* strains and especially the continued acquisition of new antibiotic resistance determinants (NDM-1) by ST131 may limit therapeutic options in the future and warrant attention from the public health and clinical communities.

ACKNOWLEDGMENTS

We acknowledge help from the Indo-German International Research Training Group, Internationales Graduiertenkolleg (GRK1673), on functional molecular infection epidemiology, an initiative of the German Research Foundation (DFG) and the University of Hyderabad (India), for which N.A. is a speaker. We thankfully acknowledge the support received from a University of Malaya High Impact Research Grant (reference no. UM.C/625/1HIR/MOHE/02 [A000002-5000 1]) in molecular genetics, for which N.A. is a lead collaborator. N.A. is an adjunct professor at the Academy of Scientific and Innovative Research (ACSIR), India.

We thank Lothar H. Wieler and Christa Ewers for stimulating discussions.

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