ORIGINAL ARTICLE

Gut resistome with special reference to beta-lactamase-producers from human, poultry, and cattle from North-Indian region: a step towards "One Health" approach

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ABSTRACT

Background: There is lack of systematic studies simultaneously comparing antibiotics resistance, and related genes (ARGs) from gut of animals and humans, and subsequently comparison with clinical isolates.

Methods: 137 Gram-negative bacteria from gut of poultry, cattle, and healthy human volunteers and a subset of 74 GNB were studied for frequency/patterns for antibiotics resistance, and prevalent ARGs (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{ampC}$ and *bla*$_{NDM-1}$) by PCR. Comparative analyses for resistance rates and patterns, and existing genes were done. Representative PCR amplicons were sequenced and analysed for precise *bla* type and RAPD typing of the human faecal and clinical isolates was done to see any clonal relatedness/diversity.

Results: Varying frequency of resistance was noticed in gut isolates from poultry, cattle and healthy human volunteers and the patterns were different. Resistance rates were much higher in clinical isolates than the gut flora, including from healthy human volunteers. No resistance was seen with colistin in neither clinical nor gut isolates from poultry, cattle, and healthy human volunteers. Resistance to minocycline and tigecycline was noticed in 28.78% and 30.30% clinal isolates, respectively. From 139 faecal isolates, a total of 7 CTX-M (5.03%; 7/139), 10 TEM (7.19%; 10/139), and 4 SHV (2.87%; 4/139) were detected. CTX-M was more prevalent in the human gut isolates (13.89%; 5/36) as compared to poultry (1.69%; 1/59) and cattle (2.27%; 1/44), whereas TEM was found to be more prevalent in poultry isolates (13.56%; 8/59). On the other hand, AmpC was present in significant proportion (58.27%; 81/139) of the gut isolates from all the three test groups and was almost equally distributed with the highest occurrence in cattle. Among clinical isolates, maximum occurrence of *bla* genes was of *bla*$_{CTX-M}$ (58%), followed by *bla*$_{ampC}$ (40%), *bla*$_{SHV}$ (26%), and *bla*$_{TEM}$ (12%). Sequencing of representative isolates showed presence of CTX-M-15, TEM-1 and SHV-38. There was no clonal relatedness between human faecal isolates and clinical isolates.

Conclusions: Multi-drug-resistance of varying frequency was noticed in faecal isolates and patterns were different between faecal and clinical isolates. There appears to be frequent and wider dissemination of class C beta-lactamase (AmpC) at animal and human interface, however wider dissemination of class A ESBL (CTX-M, TEM, SHV) has not yet established. Though no resistance to colistin in animal- and human-faecal and clinical isolates is a sign of relief, appearance of resistance to reserved drug such as tigecycline is alarming.

Keywords: gut resistome; antibiotics resistance genes; poultry; cattle, healthy human volunteer; clinical bacterial isolates; comparative study; India.

N Z J Med Lab Sci 2022; 76(1): 18-25

INTRODUCTION

The pressing issue of antimicrobial resistance is continuing worldwide (1), which is worrisome. This is not only due to the known reasons such as irrational use of antibiotics in human health sector, but is also due to improper use of antimicrobials for food-animals and veterinary medicine.(2) Perceiving the seriousness of this continuously expanding issue of antimicrobial resistance, WHO initiated the slogan "combat antimicrobial resistance" (3). Following which, the journal *Lancet* acted proactively and published a series on this concerning issue of antimicrobial resistance, in which, one of the authors of this manuscript also contributed on exploring evidence base for policy interventions (both national and regional) to combat resistance.(4) In that article, it was advocated to adopt a "One Health" approach enabling development of sensitive policies on concerned sectors and it was emphasized on addressing the concerns of specific regions and countries.

The faecal flora represents a large potential reservoir for source of antimicrobial resistance, as well as the site where resistance genes can be transferred from commensal flora to virulent microorganisms (5,6). "Gut resistome" is the name given to this reservoir of antibiotic-resistance genes (7,8). Thus, the level of resistance of commensal bacteria can be considered as a good indicator of selection pressure by antibiotic use, and it can also give the magnitude of the problem to be expected in pathogenic bacteria.

During the last decade a few studies from India reflected the region (along with other neighbouring countries) as a hub for high antibiotics resistance, including bacteria harbouring NDM-metallo-beta-lactamases, which were shown to be present both in clinical and environmental strains (9,10). However, realizing the scarcity of systematic studies exploring gut resistome from India, including in the animal sector, and the lack of comparison between human and animal gut resistome therein, we performed this preliminary study with the aim of exploring antibiotics resistance patterns and commonly existing *bla* genes (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{AmpC}$ and *bla*$_{NDM-1}$) in the gut flora of animals (cattle and poultry) and healthy human volunteers. We also compared the antibiotics resistance rates and patterns, and *bla* genes, between the isolated human and animal bacterial isolates. Moreover, we compared the patterns of these resistance profiles with those in a subset of clinical isolates to see if these strains from animal gut have already made the species jump to humans.

METHODS

Place of study and sample collection

The study was conducted in the Department of Microbiology, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh during October 2017 to October 2019, after obtaining institutional ethical approval. The samples were collected during

during the initial six months of the study period. A total of 139 Gram-negative bacterial isolates, which were obtained from 60 faecal samples (20 each from poultry, cattle, and healthy human volunteers) were studied.

The faecal samples from animals were collected in a sterile, leak proof container from local farms in the Aligarh city and the stool samples from healthy human volunteers were also from the same city. A subset of 74 random Gram-negative bacterial clinical isolates (66 Enterobacterales and 8 *Pseudomonas* spp.) from the same geographic region were selected for comparing their antibiotics resistance profiles and the prevalent beta-lactam resistance genes with those of isolates obtained from the gut of animals and healthy human volunteers. These clinical isolates were selected from the clinical specimens sent to the hospital laboratory for routine culture and sensitivity. The clinical isolates had been stored for further molecular studies later. Only 50 of the 74 clinical isolates thus stored were used for the detailed molecular study for antibiotics resistance genes. The faecal samples were inoculated on blood agar and MacConkey agar and the plates were incubated overnight at 37ºC in aerobic conditions and were identified using standard biochemical techniques (11).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed on all faecal isolates by the Kirby-Bauer method on Mueller Hinton Agar, using commercial antibiotics discs from Himedia (India) and results were interpreted as per Clinical and Laboratory Standards Institute (CLSI) breakpoints (12). The antibiotics used are shown in Figure 1A-1C. For clinical isolates, the antibiotics were as per Gram-negative antibiotics panel used in our diagnostic laboratory (Figure 1D).

Detection of *bla* genes, sequencing, and RAPD typing

The detection of class A ESBLs (bla_{CTX-M}, bla_{TEM} and bla_{SHV}), class C beta-lactamases (bla_{ampC}) and metallo-beta-lactamase (bla_{NDM-1}) was done as per our previously published protocols (13,14). A total of 11 representative amplicons from CTX-M, TEM, SHV, and AmpC were purified and sequenced commercially by Eurofins Genomics India Pvt Ltd. (Bangalore, India). RAPD typing of human isolates (faecal isolates from healthy volunteers and clinical isolates) was done as described previously (15).

Statistical analysis

Statistical analysis was performed by applying chi-square test, using SPSS 22.0 software (SPSS, Inc., Chicago, Ill). A *p*-value of <0.05 was taken as indicative of statistical significance and a *p*-value of <0.01 was considered highly significant.

RESULTS AND DISCUSSION

Bacteria isolated from faecal samples

Sixty faecal samples yielded the growth of a total of 139 GNB numbering 59, 44 & 36 isolates from poultry, cattle and healthy human volunteers, respectively. Gram-positive bacteria were not included in this study. The most common bacteria isolated from these three test groups were *E. coli* (52.54% in poultry, 75.00% in cattle, 58.33% in human volunteers), however, the proportion of other bacterial species differed with each sample source. Poultry samples yielded higher percentage of *Proteus* and *Pseudomonas* species (25.42% & 13.56%) in comparison to cattle and healthy human volunteers' samples. *Klebsiella* species were highest in healthy human volunteers' samples (33.33%) as compared to the other two sample sources (Table 1). Collectively, Enterobacterales were the dominant group isolated from faeces of poultry, cattle, and healthy human volunteers (n=51, n=42 and n=36, respectively).

Antibiotics-resistance in gut bacteria and its comparison

In *E. coli* from poultry-gut (which was also the most common organism isolated from the cattle and human faeces), maximum resistance among beta-lactam antibiotics was seen with piperacillin (61.29%) followed by cephalosporins such as cefepime and ceftazidime. Alarmingly, resistance to carbapenems, such as imipenem and meropenem, was significant (19.36% and 25.81%, respectively). Concomitant resistance to other clinically relevant groups such as fluoroquinolones (ciprofloxacin) and aminoglycosides (amikacin) was also observed. The alarmingly high levels of resistance to doxycycline (93.55%) may be due to tetracycline being the commonly used drug in poultry production. However, there are also reports of tetracycline resistance in poultry even without the administration of this antibiotic, with some geographic variations (19,20).

Since Enterobacterales were predominant in all the three cohorts of gut flora (poultry, cattle, and healthy human volunteers), we chose to compare the resistance profile of Enterobacterales, leaving aside the comparison with *Pseudomonas* spp. as they were less in number and because the antipseudomonal antibiotics panel is different from Enterobacteriaceae panel. On comparing resistance rates in Enterobacterales from faecal samples of poultry (n=51) and cattle (n=42), resistance to cephalosporins, cephamycin (cefoxitin) and monobactam (aztreonam) was generally higher in cattle isolates. Carbapenem resistance was more in poultry isolates, with imipenem- and meropenem-resistance of 35.29% and 37.25% in poultry, and 21.43% and 26.19% in cattle isolates. The difference was not statistically significant. The resistance to doxycycline was statistically highly significant with resistance at 90.20% in poultry vs. 21.43% in cattle isolates (Figure 1A). While earlier studies reported either very low or no resistance to carbapenem group of antibiotics, we found resistance to imipenem and meropenem in significant proportion of poultry isolates, which is alarming (21,22).

Similar type of resistance pattern was noticed in cattle isolates with beta-lactam antibiotics (penicillins and cephalosporins) and aminoglycosides; however, the percentage of resistance to these beta-lactam antibiotics was higher in cattle as compared to poultry isolates (Figure 1A). This could be due to increasing use of injectable antibiotics in cattle, whereas oral antibiotics use is more common in poultry feed. Again, resistance to carbapenems (imipenem and meropenem) was comparatively low in cattle isolates as compared to those of poultry, and resistance to ciprofloxacin and doxycycline was also lower in cattle than in poultry isolates (Figure 1A). This could possibly be due to lesser use of these drugs in cattle than poultry. No resistance to colistin and gentamicin was seen in either poultry or cattle isolates. Reports on high frequency of antimicrobial resistance in cattle are fragmentary; however, on the other hand some global studies depicted very low resistance in *E. coli* from healthy cattle faeces (23,24).

Table 1. Bacterial species isolated from faecal samples.

Bacterial species isolated	% Poultry (n)	% Cattle (n)	% Healthy human volunteers (n)
E. coli	52.54 (31)	75 (33)	58.33 (21)
Klebsiella species	6.78 (4)	9.1 (4)	33.33 (12)
Proteus species	25.42 (15)	2.27 (1)	5.55 (2)
Citrobacter species	1.69 (1)	9.09 (4)	2.78 (1)
Pseudomonas species	13.56 (8)	4.54 (2)	0 (0)
Total isolates	**59**	**44**	**36**

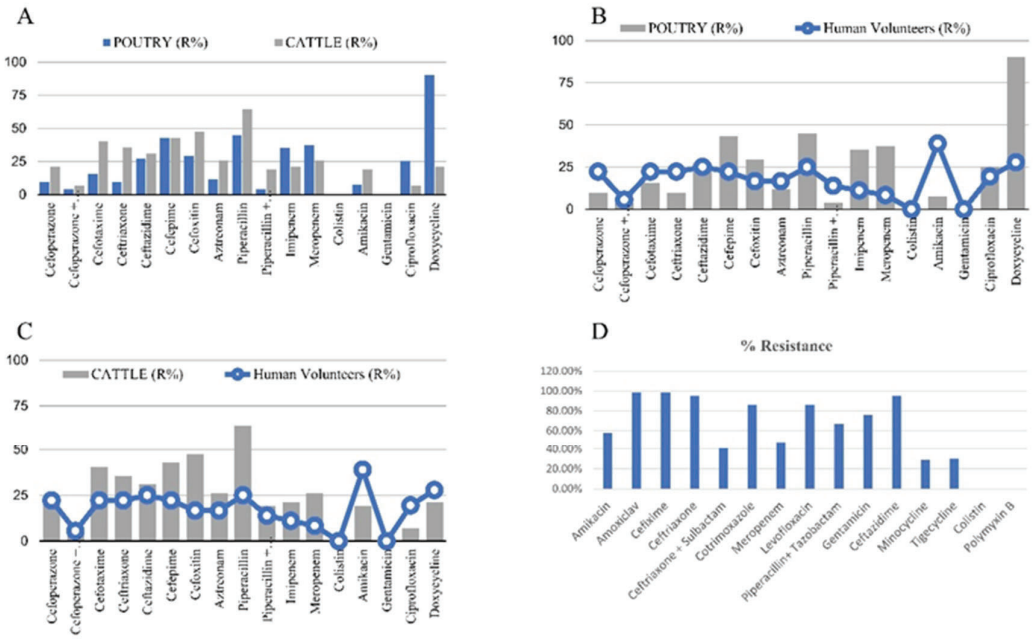

Figure 1. **(A):** Resistance rates and patterns in Enterobacterales from poultry (n=51) and cattle (n=42). **(B):** Comparison of antibiotic resistance rates and pattern in bacterial gut isolates from healthy human volunteers (n=36) and poultry (n=51). **(C):** Comparison of antibiotic resistance rates and pattern in bacterial gut isolates from healthy human volunteers (n=36) and cattle (n=42). **(D):** Antibiotic susceptibility rates in Enterobacterales (n=66) isolated from clinical specimens.
Footnote to Figure 1: None of the gut isolates was resistant to colistin and gentamicin. Ceftriaxone-sulbactam was tested in clinical isolates while cefoperazone-sulbactam was tested in healthy human volunteers and animal isolates. Levofloxacin was tested in clinical while ciprofloxacin was tested in healthy human volunteers isolates and animal isolates.

In our study, Enterobacterales from healthy human volunteers' faecal isolates showed the maximum resistance to amikacin (38.89 %); however, on the contrary there was no resistance to gentamicin. Doxycycline resistance was seen in 27.78% isolates. Among penicillin and cephalosporin groups, resistance rates were in the range from ~22% to ~25%. Among carbapenems, resistance to imipenem and meropenem was 11.11% and 8.33%, respectively (Figure 1B). This must be emphasised here that geographic variation in human gut flora has been reported in numerous studies (25,26).

On comparing the resistance profile of gut isolates from healthy human volunteers with poultry, it was noticed that resistance to cephalosporins and aminoglycosides (amikacin) was generally higher in healthy human volunteers; however, resistance to carbapenems (imipenem and meropenem) was higher in poultry isolates. Similarly, resistance to doxycycline was higher in poultry isolates compared to healthy human volunteers' gut isolates. This is likely due to lesser consumption of tetracycline group of antibiotics in humans. Resistance to fluoroquinolones (ciprofloxacin) was similar in healthy human volunteers and poultry. One possible reason could be high use of fluoroquinolones in poultry with flumequine and enrofloxacin accounting for 14% of all antibiotic use in poultry (27). However, it is also to be noted that over the counter availability of fluoroquinolones and the high self-medication rates reported for this group of antibiotics for enteric infections in humans may be the possible reason for difference not being statistically significant. While comparing human gut isolates with cattle, in contrast to poultry, the resistance to penicillins, cephalosporins and carbapenems was higher in cattle isolates which possibly indicates higher use of these antibiotics in the cattle industry. Resistance to amikacin, ciprofloxacin and doxycycline was higher in healthy human volunteers indicating its comparatively higher usage in humans as compared to use in cattle. Again, no resistance was noticed for gentamicin and colistin in human gut isolates (Figures 1B and 1C).

Antimicrobial susceptibility rates in clinical Enterobacterales

Figure 1D shows antibiotic resistance rates in Enterobacterales (n=66) in clinical samples. Maximum resistance was seen with cefixime and amoxiclav (98.48% each), followed by ceftriaxone and ceftazidime (95.45% each). Ceftriaxone in combination with sulbactam showed resistance in 40.91% isolates. Meropenem from carbapenem group showed resistance in 48.48% isolates. From fluoroquinolones, levofloxacin showed resistance in 86.36% of clinical isolates. Cotrimoxazole also showed a high level of resistance at 86.36%. Among aminoglycosides, gentamicin and amikacin showed resistance in 75.75 %, and 57.57%, respectively. Minocycline and tigecycline showed resistance in 28.78% and 30.30% of cases, respectively. There was no resistance observed with colistin and polymyxin B in clinical isolates.

Resistance in clinical isolates vs. isolates from human faecal samples

The resistance rates were much higher in clinical isolates than the gut flora from healthy human volunteers. Cephalosporin resistance in clinical isolates was much higher with resistance to ceftriaxone and ceftazidime in 95.45% (each) vs. 22.22% and 25.00%, respectively, in healthy human volunteers' isolates. Carbapenem resistance was also higher in clinical isolates, with meropenem resistance in clinical isolates of 48.48% vs. 8.33% in healthy human volunteers' isolates. Among aminoglycosides, amikacin was resistant in 57.57% of clinical isolates, and 38.89% of human gut isolates, while gentamicin was resistant in 75% of clinical isolates, but none of the healthy human isolates was resistant to gentamicin. In fluoroquinolone group of antibiotics, clinical isolates showed 89.36% resistance to levofloxacin, while healthy human isolates showed only 19.44% resistance to ciprofloxacin. However, no resistance was seen with colistin in neither clinical nor healthy human isolates (Figure 2A).

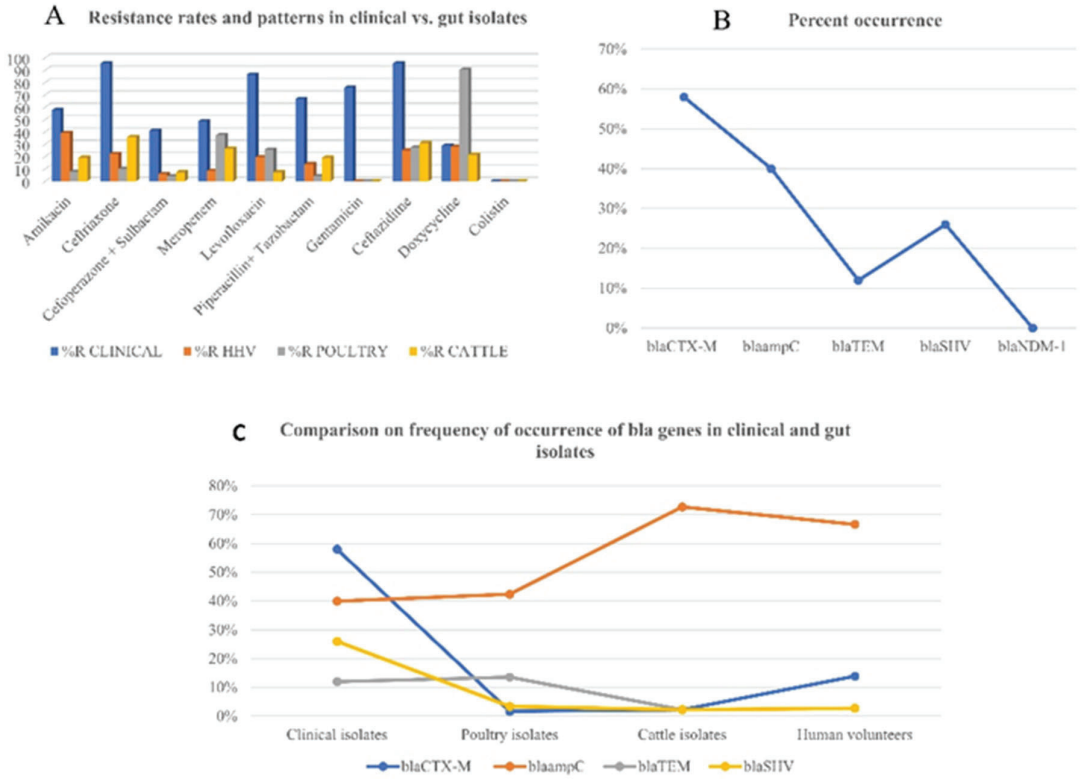

Figure 2. (A): Comparison between resistance rates and patterns in Enterobacterales from clinical isolates (n=66) and gut isolates from healthy human volunteers (n=36), poultry (n=51) and cattle (n=42). **(B):** Percent occurrence of various *bla* genes from clinical isolates tested (n=50**). (C):** Comparison on frequency of occurrence of *bla* genes in clinical and gut isolates.

Resistance in clinical isolates vs animal isolates

Meropenem resistance was found to be highest with clinical isolates (48.48%), followed by 37.25% in poultry isolates, and 26.19% in cattle isolates. Aminoglycoside resistance was much higher in clinical isolates. Amikacin was resistant in 57.57% of clinical isolates, 7.84% of poultry isolates and 19.04% of cattle isolates. Gentamicin resistance was seen with 75% of clinical isolates; however, no resistance was seen with any of the animal isolates. Resistance with fluoroquinolones group of antibiotics was much higher in clinical isolates; 89.36% of clinical isolates were found to be resistant to levofloxacin, while 25.49% of poultry isolates and 7.14% of cattle isolates were found to be resistant to ciprofloxacin. No resistance with colistin was observed with any of the clinical and animal faecal isolates (Figure 2A).

Detection of ARGs from gut isolates from poultry, cattle, and healthy human volunteers

From the tested class A and class C beta-lactamases, a total of 7 CTX-M (5.03%; 7/139), 10 TEM (7.19%; 10/139), 4 SHV (2.87%; 4/139) and 81 AmpC genes (58.27%; 81/139) were detected. CTX-M was more prevalent in the human gut isolates (13.89%; 5/36) as compared to poultry (1.69%; 1/59) and cattle (2.27%; 1/44), whereas TEM was found to be more prevalent in poultry isolates (13.56%; 8/59). On the other hand, it was interesting to note that AmpC was present in significant proportion (58.27%; 81/139) of the gut isolates from all the three test groups and was almost equally distributed with the highest occurrence in cattle (figure 2C).

Of the detected CTX-M genes, most were seen in *E. coli* (n=6, 85.71%; 6/7). Similarly, out of detected TEM genes, most were seen in *E. coli* (70%; 7/10). SHV was found to be equally distributed among *E. coli, Klebsiella* spp.*, Citrobacter* spp.*, and Pseudomonas* spp.*,* one in each (25%; 1/4). The details of the distribution and frequency of occurrence of these *bla* genes (either single or in combination) are shown in table 2 for poultry, cattle, and healthy human volunteers' isolates. Occurrence of *E. coli* harbouring ESBLs/AmpC in food-producing animals has been reported worldwide (28-30). While $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX M-15}}$ is the most common type reported regardless of the geographical origin, bla_{TEM}, bla_{SHV} and bla_{ampC} have been reported with varying but lesser frequency (31-33). In addition, European countries also showed high prevalence of $\text{bla}_{\text{CTX-M-1}}$ which was rarely reported from other regions (34). In our study, among the poultry isolates, bla_{TEM} gene detection was the most prevalent (13.56%) followed by bla_{SHV} (3.39%) and lowest prevalence was for $bla_{\text{CTX-M}}$ gene (1.69%). Whereas, among the cattle isolates, $bla_{\text{CTX-M}}$, bla_{TEM}, and bla_{SHV} was found in 2.27% each.

Colonisers of gut, particularly Enterobacterales carrying ESBL genes, are being disseminated worldwide both to animals and humans (35). Before 2008, ESBL-producing *E. coli* colonising rates were reported to be less than 10%, rising sharply to 60% after 2008, specially in some lower middle-income countries (36). India and China have been reported as some of the largest reservoir of ESBL genes (37). Colonization rates of ESBL producing *E. coli* in healthy human was reported 14% globally and relatively higher (~22%) from South-East Asia (38). In livestock, the prevalence of various ESBL/AmpC types have been reported ranging from 0.6% and 44.7% (studies mostly from European countries); however, from Asia it was 1.7% to 11.8% (34). Geographic variations for ESBL-producing *E. coli* in food producing animals (poultry and cattle) have been reported from India ranging from 6% in Odisha to 87% in Punjab (21,39). In the present study, 12.95% of faecal isolates (healthy human volunteers + poultry + cattle) were tested positive by genotypic method (i.e., shown presence of any of the three ESBL genes, i.e $bla_{\text{CTX-M}}$, bla_{TEM} or bla_{SHV}). Highest prevalence was seen in poultry (18.64%, 11/59), followed by healthy human volunteers (13.89%, 5/36), and the least in cattle (4.54%, 2/44).

Simultaneous occurrence of class A and class C beta -lactamases

Simultaneous occurrence of class A and class C beta-lactamases was seen in a total of 12 (8.63%; 12/139) of faecal isolates {seven (11.86%; 7/59) poultry isolates, four (11.11%; 4/36) healthy human volunteers' isolates, and one (2.27%; 1/44) cattle isolate}. No isolate showed simultaneous occurrence of all three class A genes together (viz. CTX-M, TEM and SHV) or all four types of genes (viz. AmpC, CTX-M, TEM and SHV). Faecal isolates resistant to imipenem or meropenem or both were tested for $bla_{\text{NDM-1}}$ gene but were found to be negative.

Detection of ARGs in clinical isolates

Out of 74 clinical isolates, 50 were further selected for detailed molecular characterisation. These isolates were *E. coli* (n=25*), Klebsiella* spp. (n=9), *Citrobacter* spp. (n=7), *Pseudomonas* spp. (n=6) and *Proteus* spp. (n=3). Among the *bla* genes detected in clinical isolates, maximum occurrence was for $bla_{\text{CTX-M}}$ in 58% (29/50) isolates followed by bla_{ampC} in 40% (20/50) isolates. The frequency of occurrence of *bla* genes is shown in Figure 2B**.**

Out of total 29 CTX-M genes detected from clinical isolates, maximum occurrence {16 (55.17%)} was in *E. coli* followed by in *Klebsiella* species {eight (27.59%)}. Out of 20 AmpC genes detected in clinical isolates, maximum (70%) was seen with *E. coli* (n=14), while only three (15%), 2 (10%), and 1 (5%) was found in *Klebsiella* species, *Citrobacter* species, and *Proteus* species, respectively. Simultaneous occurrence of class A and class C beta lactamases was seen in 16 (32%) of clinical isolates (Table 2).

Molecular detection of $bla_{\text{NDM-1}}$ in clinical isolates

Isolates resistant to imipenem or meropenem or both were tested for $bla_{\text{NDM-1}}$ gene but were found to be negative. This may be due to the presence of metallo-beta-lactamases other than NDM-1, or some other mechanism(s) of resistance.

Comparison of frequency of occurrence of *bla* genes in clinical- and faecal-isolates from poultry, cattle, and healthy human volunteers

In this study bla_{ampC} was the predominant gene in gut (faecal) isolates; its occurrence was noticed in 72.72% isolates from cattle gut and in 66.67% in healthy human volunteers. Other *bla* genes were in significantly lower number except for $bla_{\text{CTX-M}}$ in human gut (13.89%) and bla_{TEM} in poultry gut (13.56%). Clinical isolates had class A and class C beta-lactamase genes in comparative higher percentage to that of gut isolates; CTX-M being the most common in 58% of isolates followed by AmpC in 40% ofisolates (Figure 2C).

RAPD analysis of human faecal- and clinical-isolates

Based on RAPD typing, out of total 36 human faecal isolates, only four could be typed by RAPD, and 32 were found to be un-typable. Out of 50 clinical isolates, 19 could be typed by RAPD, and 31 were found to be un-typable. Among these, 11 isolates were grouped in two clusters based on similar banding pattern, and the remaining isolates showed unique banding pattern signifying genetic unrelatedness.

Sequencing of representative *bla* genes

Sequencing of representative CTX-M-positive amplicons demonstrated presence of CTX-M-15 type, TEM as TEM-1 type, and SHV as SHV-38 type. The AmpC sequence analysis (based on the BLAST search) demonstrated ampC-like sequence. CTX-M amplicons from all three different sample sources, i.e., humans, cattle, and poultry, showed presence of $bla_{\text{CTX-M-15}}$ type based on the sequencing results. It is evident that $bla_{\text{CTX-M-15}}$ is identified from humans, both in hospital and community, its presence in poultry and cattle probably indicate a common past source of contamination with introduction of ESBL-producing Enterobacterales carriers (or crossover of plasmids carrying $bla_{\text{CTX-M}}$ genes) and their diffusion due to close contact in livestock. However, these $bla_{\text{CTX-M}}$ carrying Enterobacterales are mainly established in clinical settings and not yet established widely in guts, especially in animal guts.

Antimicrobial resistance is continuously emerging as a silent pandemic. Antimicrobial resistance in humans has been studied extensively, though antimicrobial resistance in animals is now gaining wider interest. Antibiotics are extensively used as prophylactic, therapeutic and growth promoting agents in animal production. While promoting better health and productivity, it has also played a significant role in evolution of antibiotic-resistant strains (16).

Poultry and livestock are considered most important reservoirs for pathogenic Enterobacterales due to use of antimicrobials in animal farming that in turn has led to the emergence, selection, and dissemination of antimicrobial-resistant microorganisms. India, China, the United States, Brazil, and Germany account for 3% of global consumption of antibiotics used in animal production, with India on top of the list (17). A two-thirds increase of worldwide consumption of antibiotics in animals is estimated for 2030, again with India projected to have its use of antibiotics in animal feed increase by 82% by 2030 (17). Penicillins, tetracyclines, and quinolones are the most widely used antibiotics globally in animal feeds (17). Human gut flora of healthy humans also remains a large potential reservoir of antimicrobial resistance, providing an environment where resistance genes can be transferred from the commensal flora to virulent microorganisms (18).

The potential pathways for transmission of antimicrobial resistance from cattle farm environment to humans have been identified but they are complex in nature (40). Some of the theoretical pathways for antimicrobial resistance transmission from cattle and their environment to humans are through the food chain, faecal-oral route, and clinical contact between animals and their handlers (41). However, the actual contribution of each pathway is undetermined. Evidence of this transmission is still equivocal; however, poultry and swine seem to be more likely source compared to cattle (42), probably because of the routine use of antimicrobials in these production systems. Human to human transmission is also a possibility as ESBL-producing Enterobacteriaceae were also isolated from humans and human sewage (43). Based on our molecular results in faecal isolates from all three test groups, it seems $bla_{\text{CTX-M}}$ has not widely established/jumped to other bacterial species, except for *E. coli* and to a lesser extent for *Klebsiella*. On the other hand, species jump in clinical isolates for these *bla* genes (CTX-M, TEM, SHV, AmpC) has already established as evident by noticing presence of these ARGs in different bacterial species. It can be speculated, though primitive, that healthcare settings are a more enriched environment for ARGs transfer as opposed to animal and human gut; however more studies on large sample size are required to establish this fact.

We previously reported the presence of bla_{SHV} (18.52%), followed by $bla_{\text{CTX-M}}$ and bla_{TEM} (11.11% each) from the environmental samples (water, sewage and drain) in the same region (14). AmpC detection in our present study was quite high (72.72% in cattle, 66.67% in healthy human volunteers, and 42.37% from poultry), even more than CTX-M which indicates frequent and wider dissemination on bla_{ampC} at all the three transactional interfaces (animal, human and environment) in our geographic region. This hypothesis is backed by the findings of our earlier study on environmental samples (water, drain, sewage) where we found presence of $blaa_{\text{mpC}}$ in 48.5% samples (14). Also, simultaneous occurrence of class A ($bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}) and class C beta-lactamases (AmpC) in various combinations in our study is further evidence for the increasing antimicrobial resistance worldwide.

The findings of this study are interesting as they reflect that the resistance markers (and thus the selection pressure) are diverse at the clinical and gut interface (both in animals and humans). It also suggests that the selection pressure for resistance genes in clinical and animal interface are quite different and the resistance markers have not yet widely disseminated, especially for class A ESBLs. Similarly, the frequency of these resistance markers in clinical isolates and human faecal samples was quite different thus suggesting difference in selection pressure in clinics and in the community.

Table 2. Distribution and frequency (either single or in combination) of *bla* genes in gut (healthy human volunteers, poultry and cattle) and clinical isolates.

Sample source	No. of isolates	No. of isolates found positive for genes	*bla* genes singly or in various combinations									
			CTX-M only	TEM only	SHV only	AmpC only	CTX-M+AmpC	CTX-M+TEM+AmpC	CTX-M+SHV+AmpC	TEM+AmpC	CTX-M+TEM+SHV	CTX-M+SHV
Human volunteers	36	24	-	-	-	19	3	1	1	-	-	-
Poultry	59	29	-	2	2	18	1	-	-	6	-	-
Cattle	44	33	-	-	1	31	-	1	-	-		
Total faecal	**139**	**86**	**-**	**2**	**3**	**68**	**4**	**2**	**1**	**6**	**-**	
Clinical	50	36	6	-	3	4	11	2	3	-	4	3
Total clinical	**50**	**36**	**6**	**-**	**3**	**4**	**11**	**2**	**3**	**-**	**4**	**3**

One of the major limitations of our study was not attempting detection of carbapenemases other than NDM-1 despite the high occurrence of resistance to imipenem and meropenem. This non-attempt was due to financial constraints. Similarly, the broth microdilution method or PCR for detecting mcr-genes was not performed, which can be seen as another limitation.

In conclusion, this study strengthens the argument for a rational use of antibiotics in humans and animals. It was also interesting to note that comparatively the frequency of bla_{ampC} was higher in normal human faecal samples compared to human clinical isolates which suggests that some other selection pressure (apart from the use of antibiotics) might be playing a role in the community which has yet to be explored. Moreover, increasing resistance to minocycline and tigecycline is alarming. This study further underscores the emphasis on effective policy implementations within the "One Health" framework (4). With increased globalisation of the food trade, particularly meat and dairy products, that now includes a sizeable trade in live animals, antimicrobial resistance has become a pandemic, albeit silent.

ACKNOWLEDGEMENTS

The secretarial support by Mr. Tomy Kaitharath in editing the manuscript is highly appreciated. The authors also wish to thank Prof. Fazal K. Dar (FRCPath) for reviewing the draft manuscript.

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REFERENCES

1. Shahid M. Environmental dissemination of NDM-1: time to act sensibly. *Lancet Infect Dis* 2011; 11(5): 334-335.
2. Argudin MA, Deplano A, Meghraoui A, et al. Bacteria from Animals as a Pool of Antimicrobial Resistance Genes. *Antibiotics (Basel)* 2017; 6(2): 12.
3. Hoffman SJ, Caleo GM, Daulaire N, et al. Strategies for achieving global collective action on antimicrobial resistance. Bull World *Health Organ* 2015; 93(12): 867-876.
4. Dar OA, Hasan R, Schlundt J, et al. Exploring the evidence base for national and regional policy interventions to combat resistance. *Lancet* 2016; 387 (10015): 285-295.
5. Frost JA, Rowe B, Vandepitte J. Acquisition of trimethoprim resistance in epidemic strain of Shigella dysenteriae type 1 from Zaire. *Lancet* 1982; 1(8278): 963.
6. Shanahan PM, Thomson CJ, Amyes SG. Beta-lactam resistance in normal faecal flora from South Africa. *Epidemiol Infect* 1995; 115(2): 243-253.
7. Casals-Pascual C, Vergara A, Vila J. Intestinal microbiota and antibiotic resistance: Perspectives and solutions. *Human Microbiome J* 2018; 9: 11-15.
8. Kim Y, Leung MHY, Kwok W, et al. Antibiotic resistance gene sharing networks and the effect of dietary nutritional content on the canine and feline gut resistome. *Anim Microbiome* 2020; 2(1): 4.
9. Kumarasamy KK, Toleman MA, Walsh TR, et al. Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. *Lancet Infect Dis* 2010; 10(9): 597-602.
10. Walsh TR, Weeks J, Livermore DM, Toleman MA. Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study. *Lancet Infect Dis* 2011; 11(5): 355-362.
11. Collee JG, Mackie TJ, McCartney JE. Mackie & McCartney practical medical microbiology. New York: Churchill Livingstone; 1996.
12. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial disk susceptibility tests. 27 ed. Wayne, PA: CLSI document M100. Clinical Laboratory Standards Institute; 2017.
13. Shahid M. Citrobacter spp. simultaneously harboring blaCTX-M, blaTEM, blaSHV, blaampC, and insertion sequences IS26 and orf513: an evolutionary phenomenon of recent concern for antibiotic resistance. *J Clin Microbiol* 2010; 48(5): 1833-1838.

14. Shahid M. Environmental dissemination of NDM-1: time to act sensibly. *Lancet Infect Dis* 2011; 11(5): 334-335.
15. Argudin MA, Deplano A, Meghraoui A, et al. Bacteria from Animals as a Pool of Antimicrobial Resistance Genes. *Antibiotics (Basel)* 2017; 6(2): 12.
16. Hoffman SJ, Caleo GM, Daulaire N, et al. Strategies for achieving global collective action on antimicrobial resistance. Bull World *Health Organ* 2015; 93(12): 867-876.
17. Dar OA, Hasan R, Schlundt J, et al. Exploring the evidence base for national and regional policy interventions to combat resistance. *Lancet* 2016; 387 (10015): 285-295.
18. Frost JA, Rowe B, Vandepitte J. Acquisition of trimethoprim resistance in epidemic strain of Shigella dysenteriae type 1 from Zaire. *Lancet* 1982; 1(8278): 963.
19. Shanahan PM, Thomson CJ, Amyes SG. Beta-lactam resistance in normal faecal flora from South Africa. *Epidemiol Infect* 1995; 115(2): 243-253.
20. Casals-Pascual C, Vergara A, Vila J. Intestinal microbiota and antibiotic resistance: Perspectives and solutions. *Human Microbiome J* 2018; 9: 11-15.
21. Kim Y, Leung MHY, Kwok W, et al. Antibiotic resistance gene sharing networks and the effect of dietary nutritional content on the canine and feline gut resistome. *Anim Microbiome* 2020; 2(1): 4.
22. Kumarasamy KK, Toleman MA, Walsh TR, et al. Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. *Lancet Infect Dis* 2010; 10(9): 597-602.
23. Walsh TR, Weeks J, Livermore DM, Toleman MA. Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study. *Lancet Infect Dis* 2011; 11(5): 355-362.
24. Collee JG, Mackie TJ, McCartney JE. Mackie & McCartney practical medical microbiology. New York: Churchill Livingstone; 1996.
25. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial disk susceptibility tests. 27 ed. Wayne, PA: CLSI document M100. Clinical Laboratory Standards Institute; 2017.
26. Shahid M. Citrobacter spp. simultaneously harboring blaCTX-M, blaTEM, blaSHV, blaampC, and insertion sequences IS26 and orf513: an evolutionary phenomenon of recent concern for antibiotic resistance. *J Clin Microbiol* 2010; 48(5): 1833-1838.
27. Shahid M, Khan HM, Sobia F, et al. Molecular epidemiology of various antibiotics resistance genes, including blaNDM-1, in Indian environmental and clinical bacterial isolates: A comparative study: *N Z J Med Lab Sci* 2014; 68(1): 9–14.
28. Ensor VM, Shahid M, Evans JT, Hawkey PM. Occurrence, prevalence and genetic environment of CTX-M beta-lactamases in Enterobacteriaceae from Indian hospitals. *J Antimicrob Chemother* 2006; 58(6): 1260-1263.
29. Sharma C, Rokana N, Chandra M, et al. Antimicrobial Resistance: Its Surveillance, Impact, and Alternative Management Strategies in Dairy Animals. *Front Vet Sci* 2018; 4: 237.
30. Van Boeckel TP, Brower C, Gilbert M, et al. Global trends in antimicrobial use in food animals. *Proc Natl Acad Sci U S A* 2015; 112(18): 5649-5654.
31. van Schaik W. The human gut resistome. *Philos Trans R Soc Lond B Biol Sci* 2015; 370(1670): 20140087.
32. Agyare C, Boamah VE, Zumbi CN, Osei FB. Antibiotic Use in Poultry Production and Its Effects on Bacterial Resistance. In: Kumar Y (Editor). Antimicrobial Resistance - A Global Threat: Intech Open 2018. p. 1-20.
33. van den Bogaard AE, Stobberingh EE. Epidemiology of resistance to antibiotics. Links between animals and humans. *Int J Antimicrob Agents* 2000; 14(4): 327-335.
34. Brower CH, Mandal S, Hayer S, et al. The Prevalence of Extended-Spectrum Beta-Lactamase-Producing Multidrug-Resistant Escherichia Coli in Poultry Chickens and Variation According to Farming Practices in Punjab, India. *Environ Health Perspect* 2017; 125(7): 077015.
35. Islam M, Sultana S, Das KK, et al. Isolation of plasmid mediated multidrug resistant Escherichia coli from poultry. *Int J Sustain Crop Prod* 2008; 3(5): 46-50.
36. Aasmäe B, Häkkinen L, Kaart T, Kalmus P. Antimicrobial resistance of Escherichia coli and Enterococcus spp. isolated from Estonian cattle and swine from 2010 to 2015. *Acta Vet Scand* 2019; 61(1): 5.
37. Olowe OA, Adewumi O, Odewale G, et al. Phenotypic and Molecular Characterisation of Extended-Spectrum Beta-Lactamase Producing Escherichia coli Obtained from Animal Fecal Samples in Ado Ekiti, Nigeria. *J Environ Public Health* 2015; 2015: 497980.
38. Monira S, Shabnam SA, Ali SI, et al. Multi-drug resistant pathogenic bacteria in the gut of young children in Bangladesh. *Gut Pathog* 2017; 9: 19.
39. Rutgersson C, Fick J, Marathe N,et al. Fluoroquinolones and qnr genes in sediment, water, soil, and human fecal flora in an environment polluted by manufacturing discharges. *Environ Sci Technol* 2014; 48(14): 7825-7832.
40. van den Bogaard AE, Breeuwsma AJ, Julicher CH, Mostert A, Nieuwenhuijs JH, Vaarkamp H, *et al*. [Guidelines for veterinary use of antibiotics: recommendations of a work group. Beleidsgroep Veterinaire Apotheek, Koninklijke Nederlandse Maatschappij voor Diergeneeskunde]. *Tijdschr Diergeneeskd*. 1994; 119(6): 160-183. Article in Dutch.
41. Carattoli A. Animal reservoirs for extended spectrum beta-lactamase producers. *Clin Microbiol Infect* 2008; 14 Suppl 1: 117-123.
42. Ewers C, Grobbel M, Bethe A, et al. Extended-spectrum beta-lactamases-producing gram-negative bacteria in companion animals: action is clearly warranted! *Berl Munch Tierarztl Wochenschr* 2011; 124(3-4): 94-101.
43. Smet A, Martel A, Persoons D, et al. Broad-spectrum beta -lactamases among Enterobacteriaceae of animal origin: molecular aspects, mobility and impact on public health. *FEMS Microbiol Rev* 2010; 34(3): 295-316.
44. Dandachi I, Sokhn ES, Dahdouh EA, et al. Prevalence and Characterization of Multi-Drug-Resistant Gram-Negative Bacilli Isolated from Lebanese Poultry: A Nationwide Study. *Front Microbiol* 2018; 9: 550.
45. Madec JY, Lazizzera C, Chatre P, et a*l*. Prevalence of fecal carriage of acquired expanded-spectrum cephalosporin resistance in Enterobacteriaceae strains from cattle in France. *J Clin Microbiol* 2008; 46(4): 1566-1567.
46. Schmid A, Hörmansdorfer S, Messelhäusser U, et al. Prevalence of extended-spectrum beta-lactamase-producing Escherichia coli on Bavarian dairy and beef cattle farms. *Appl Environ Microbiol* 2013; 79(9): 3027-3032.
47. Ewers C, Bethe A, Semmler T, et al. Extended-spectrum beta-lactamase-producing and AmpC-producing Escherichia coli from livestock and companion animals, and their putative impact on public health: a global perspective. *Clin Microbiol Infect* 2012; 18(7): 646-655.
48. Hasan B, Drobni P, Drobni M, et aol. Dissemination of NDM-1. *Lancet Infect Dis* 2012; 12(2): 99-100; author reply 101-102.
49. Woerther PL, Angebault C, Jacquier H, et al. Massive increase, spread, and exchange of extended spectrum beta-lactamase-encoding genes among intestinal Enterobacteriaceae in hospitalized children with severe acute malnutrition in Niger. *Clin Infect Dis* 2011; 53(7): 677 -685.
50. Hawkey PM. Prevalence and clonality of extended-spectrum beta-lactamases in Asia. *Clin Microbiol Infect* 2008; 14 Suppl 1: 159-165.

51. Karanika S, Karantanos T, Arvanitis M, et al. Fecal Colonization With Extended-spectrum Beta-lactamase-Producing Enterobacteriaceae and Risk Factors Among Healthy Individuals: A Systematic Review and Metaanalysis. *Clin Infect Dis* 2016; 63(3): 310-318.
52. Kar D, Bandyopadhyay S, Bhattacharyya D, et al. Molecular and phylogenetic characterization of multidrug resistant extended spectrum beta-lactamase producing Escherichia coli isolated from poultry and cattle in Odisha, India. *Infect Genet Evol* 2015; 29: 82-90.
53. Collis RM, Burgess SA, Biggs PJ, et al. Extended-Spectrum Beta-Lactamase-Producing Enterobacteriaceae in Dairy Farm Environments: A New Zealand Perspective. *Foodborne Pathog Dis* 2019; 16(1): 5-22.
54. Boerlin P, Reid-Smith RJ. Antimicrobial resistance: its emergence and transmission. *Anim Health Res Rev* 2008; 9(2): 115-126.
51. Lazarus B, Paterson DL, Mollinger JL, Rogers BA. Do human extraintestinal Escherichia coli infections resistant to expanded-spectrum cephalosporins originate from food-producing animals? A systematic review. *Clin Infect Dis* 2015; 60(3): 439-452.
52. Mesa RJ, Blanc V, Blanch AR, et al. Extended-spectrum beta-lactamase-producing Enterobacteriaceae in different environments (humans, food, animal farms and sewage). *J Antimicrob Chemother* 2006; 58(1): 211-215.