Possible Acquisition of ESBL-mediated Antimicrobial Resistance by Farmers from Aquatic Reservoir used for Bathing and Cleaning of Water Buffalos (Bubalus bubalis) with Intestinal Carriage of ESBL Producing Escherichia coli

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Abstract

Little information is available on the risk of human subjects for acquisition of antimicrobial resistance (AMR) from aquatic environment other than those treated with antimicrobials for aquaculture. Carriage of extended-spectrum beta-lactamase (ESBL) and carbapenemase categories of AMR by enteric bacteria in livestock have been frequently reported. Dissemination of these categories of AMR to the environment thus poses a threat for their transmission to farmers engaged in livestock care posing a severe public health hazard. A study on the prevalence of ESBL- and carbapenemase-mediated AMR among Escherichia coli isolated from earth pond environment used for bathing and cleaning of buffalos (Bubalus bubalis) and from human subjects engaged in such activity revealed isolation rate of ESBL positivity to be higher in human subjects engaged in washing and bathing of buffalos (37.5%) compared to those without engagement in such activities (20.7%) with CTX-M type ESBL, a group of class A ESBL, as the predominant molecular type (97.4%). While no carbapenemase positivity could be detected among E. coli isolated from pond environment or buffalos, small percentage of carbapenemase could be detected among the E. coli isolated from human subjects although the risk was not higher than those not associated with bathing and cleaning of buffalos. Bathing and cleaning of buffalos could potentially facilitate transmission of ESBL resistance from livestock to human subjects in pond environment.

Keywords: Ponds, buffalos, ESBL, CTX-M, carbapenemase
INTRODUCTION
Most of the small-scale dairy farmers in the rural belt of Haryana state of northern India earn their livelihood by rearing water buffalos (*Bubalus bubalis*) for production of milk. These buffalos are taken to earth ponds frequently for bathing and cleaning which also helps to increase their production of milk (Fig. 1). The earth ponds get contaminated with enteric pathogens from the fecal matter of buffalos during the process of cleaning and bathing and thus may serve as source for acquisition of these pathogens by human subjects engaged in such activity.

While antibiotics are not commonly used as growth promoters in animals in India since they are not used as food animals in the country, there has been a perceptible increase in antibiotic resistance in the livestock in the country due to their indiscriminate use in unregulated veterinary sector for treatment of various infections.

*Escherichia coli*, being a member of normal enteric flora of both human and animals, is commonly utilized as an indicator organism to determine the fecal carriage rate of antimicrobial resistance (AMR). Misuse of third-generation cephalosporins in both human and livestock created a selection pressure that led to the establishment and spread of Extended-spectrum beta-lactamase (ESBL)-producing *E. coli* in both the sectors. Among the ESBL genes, TEM- and SHV-ESBLs dominated the ESBL landscape in the 1980s and 1990s of the previous centuries, mostly associated with *E. coli* and *Klebsiella pneumoniae* outbreaks in hospitals. However, since 2000s, the CTX-M type of ESBL gene has almost replaced the other variants and it has been identified as the most common among community strains, and evidence of CTX-M-producing isolates being isolated from livestock and domestic pets is increasing, which is concerning because they could serve as a reservoir for resistant organisms' acquisition. Carbapenems are antibiotics used as a last option against drug-resistant bacteria, such as *E. coli* that produces ESBL. Although carbapenem resistance has been observed in isolates obtained from livestock, these antibiotics are not licenced for use in livestock. Thus, different AMR *E. coli* carried by livestock could contaminate the environment and pose a serious AMR threats to public health especially among farmers. A study was taken up to find out prevalence of AMR in ESBL- and carbapenemase producing *E. coli* and their molecular variants in samples collected from pond environment, buffalos and from human subjects exposed to pond environment.

MATERIALS AND METHODS
The protocol of the study was approved by both independent institutional research and ethical committees of SGT University (SGTU/ FMHS/MICRO/341). Information sheet was provided to the owners of the buffalos included in the study and informed consent was obtained for collection of samples from them as well as from the buffalos owned by them.

Selection of ponds, human subjects and buffalos

Ponds
The study was limited to two earth ponds, one each located in two villages in the rural belt of Haryana state, India used by small scale dairy farmers for bathing and cleaning of buffalos. These ponds were shallow (knee deep), surrounded by agricultural fields and were never used for aquaculture. The selected ponds were located away from human inhabitation without any healthcare center within 2 km distance. The pond edges were not known to be used for defecation by human subjects and did not have connection with any sewage effluent from the community.

Human subjects
All the households of farmers from the two villages owning the buffalos taken regularly to the selected ponds were identified (subgroup I or Sgr I). The residential premises of these households were shared by the human inhabitants as well as by the buffalos reared by them. The following two sub-categories of human subjects from the selected households were enrolled for the study viz. (i) those involved in regular bathing and washing of the buffalos for past one year (all males, subgroup 1a or Sgr 1a) and (ii) age matched males in the same households involved in other activities related to care of buffalos viz. milking, feeding etc. but not involved in bathing and cleaning of buffalos (subgroup 1b or Sgr 1b) for the same duration. The rationale for limiting the history regarding the duration of association with buffalos as preceding one year in the present study was based on the maximum reported duration of colonization by drug resistant *E. coli* (less than
6 months) and the purpose of the study being assessment of the prevalence of recently acquired resistant strains by the human subjects at the time of sampling.\textsuperscript{11,12} Those subjects sharing dual responsibilities catered by Sgr Ia and Sgr Ib were not included. Age matched male subjects from an additional randomly selected 60 households, 30 each from the same two villages inhabited by Sgr Ia and Sgr Ib subjects were selected (subgroup II or Sgr II) where the households did not have any livestock in their premises and no member in the household had history of direct exposure to livestock (i.e. they were engaged in different occupations for living e.g. shopkeepers, vegetable vendors, school teachers, etc).

**Buffalos**
All the buffalos in the selected households inhabited by Sgr I subjects that were taken to the earth ponds regularly by their owners for bathing and cleaning were included in the study for sampling.

**Collection of specimens**

**Identification of sites for sampling of pond and pond environment**
This was carried out weekly for 12 months, 4 months (16 weeks) during each season e.g. summer (March-June), monsoon (July-October) and winter (November-February) in the year 2017-2018. Each pond was radially marked in four perpendicular intersecting radial directions. In each direction, three points were identified for sampling viz. pond surface water at 20 feet and at 2 feet distance from the edge of the pond and soil at 6 inches away from the soil-water interface in the same radial direction.

**Sampling**

**Pond water**
One hundred ml of surface water sample was collected from each of the selected point in the pond stepwise by submerging pre-sterilized stoppered water bottle up to the depth of approximately 20 cm below the surface of water, opening the stopper, holding the bottle horizontally allowing water to flow freely in the bottle and then closing the mouth of the container by stopper while holding the bottle at the same level.\textsuperscript{13} Each week a total of 8 surface water samples were collected from each pond, one each at 2 ft and 20 ft from the edge in 4 radial directions. Thus, a total of 128 surface water samples (8 samples X 16 weeks) were collected from each pond per season totaling to 256 samples from the two ponds.

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**Fig. 1.** Human subject engaged in washing and cleaning of buffalos in earth pond.
Pond bed sludge

Sediment sludge sample was collected from the pond bed at each of the same points from where surface water samples were collected using wide mouth screw capped pre-sterilized glass containers sequentially by opening the lid of the container at the bottom of the pond, dragging the container with mouth open along the pond bed for short distance and recapping the container at the same level before bringing up on the surface. A total of 128 pond bed sludge samples were collected in each season from each pond totaling to 256 samples from the two ponds.

Soil

Soil samples were collected in an area 6 inches away from water soil interface in each of the 4 radial directions selected for sampling of pond water and pond bottom sludge. Sampling was carried out at the selected point covering an approximate area of 10 cm by 10 cm square with five longitudinal, five latitudinal and two diagonal strokes of sterile swabs pre-moistened with nutrient broth, placed in polypropylene tube containing 1.5 ml of nutrient broth and was immediately transported to laboratory in ice pack within 30 minutes.

Buffalos

This was carried out as a cross sectional study among all the buffalos taken to the two ponds for bathing and cleaning. Fresh fecal samples from all the buffalos were sampled within one minute of deposition outside the pond e.g. in the residential premises of their owners. Sterile swabs moistened with nutrient broth were immersed in the fecal material taking care to sample the area not touching the soil, placed in polypropylene tube containing nutrient broth and was immediately transported to laboratory in ice pack within 30 minutes.

Human subjects

Each of the study participants was explained regarding collection of feces in sterile leak proof containers provided by the laboratory. Self-collected early morning fecal samples were transported to the Microbiology laboratory of SGTH within one hour of collection in insulated carriers with ice packs provided to the participants.

Bacteriological screening of ESBL producing E. coli and identification of the isolates

Ten-fold dilutions of water and sediment sludge samples were prepared in physiological saline. Soil swabs and fecal swabs of buffalos collected in polypropylene tube containing nutrient broth were vortexed for 2 mins. For human sample, approximately 0.5 g of faecal sample was suspended in 1.5 ml of nutrient broth and vortexed for 2 mins. One hundred microliter of each sample viz. water, sediment sludge, soil, buffalo feces and human feces was inoculated on two MacConkey agar plates, one supplemented with 2μg of cefotaxime per ml (Mac-CTX) and the other supplemented with 2μg of ceftazidime per ml (Mac-CAZ). The inoculated plates were incubated aerobically at 37°C for 24-48 h. One lactose fermenting colony representing each distinct colonial morphotype suggestive of E. coli was regrown in the same selective plate and further identified using Vitek 2 system (BioMerieux, France). One isolate of each morphotype from each sample was selected for ESBL characterization.

Phenotypic confirmatory test for detection of ESBL production

Double disc synergy test (DDST)

E. coli isolates were subjected to DDST, a phenotypic confirmatory test for ESBL detection. Two pairs of antibiotic discs, ceftazidime (30 µg) and ceftazidime plus clavulanic acid (30 µg plus 10 µg) discs as first pair and cefotaxime (30 µg) and cefotaxime plus clavulanic acid (30 µg plus 10 µg) discs as second pair were placed on a plate of Mueller Hinton agar inoculated with the suspension (turbidity of 0.5 MacFarland standard) of isolates. K. pneumoniae ATCC 700603 and E. coli ATCC 25922 were used as control strains.

Molecular identification of blaTEM, blaSHV and blaCTX-M genes

Polymerase chain reaction (PCR) for detection of blaTEM, blaSHV and blaCTX-M was carried out for strains showing ESBL positivity in DDST using the pre-published sequences viz. TEM primers (TEMF ATGAGTATTCAACATTTCCGTG, TEMR TTACCAATGCTTAATCAGTGAG) amplifying 840-bp fragment, SHV primers (SHVSF ATTTGTCGCTTCTTTACTCGC, SHVSRTTTATGGCGTTACCTTTGACC) amplifying
1051-bp fragment and CTX-M primers (CTX-MF TTTGCGATGTGCACTTACCA, CTX-MR CGATATCGTTGGTGCCGATA) amplifying 544-bp fragment. Following antibiotic susceptibility testing

**Antibiotic susceptibility testing**

All the ESBL positive isolates were subjected to antibiotic susceptibility testing (AST) by Kirby-Bauer disc diffusion method on Mueller-Hinton agar and the results were interpreted based on zone of inhibition validated as per CLSI guidelines. Following antibiotics were tested as per disc concentration and strength indicated: ampicillin (10 µg), amoxicillin-clavulanic acid (20/10 µg), piperacillin/tazobactam (100/10 µg), amikacin (30 µg), gentamicin (10 µg), cefotaxime (30 µg), ceftriaxone (30 µg), cefepime (30 µg), aztreonam (30 µg), ciprofloxacin (5 µg), co-trimoxazole (25 µg), ertapenem (10 µg), meropenem (10 µg).

Table 1. Positivity and molecular types of ESBL among *E. coli* isolated from pond environment

<table>
<thead>
<tr>
<th>Season</th>
<th>ESBL</th>
<th>Location for collection of pond sample</th>
<th>Edge soil (LS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2 ft from collection of pond sample</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sub-surface water (L1a)</td>
<td>Sediment sludge (L1b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n = 128</td>
<td>n = 128</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No (%)</td>
<td>No (%)</td>
</tr>
<tr>
<td>Summer</td>
<td>ESBL</td>
<td>41 (32%)</td>
<td>30 (23.4)</td>
</tr>
<tr>
<td></td>
<td>CTX-M alone*</td>
<td>35 (85.4)</td>
<td>26 (86.7)</td>
</tr>
<tr>
<td></td>
<td>CTX-M with other ESBL genes*</td>
<td>3 (7.3)</td>
<td>2 (6.7)</td>
</tr>
<tr>
<td></td>
<td>Other ESBL genes alone*</td>
<td>3 (7.3)</td>
<td>1 (3.3)</td>
</tr>
<tr>
<td>Monsoon</td>
<td>ESBL</td>
<td>56 (43.8%)</td>
<td>46 (35.9)</td>
</tr>
<tr>
<td></td>
<td>CTX-M alone*</td>
<td>48 (85.7)</td>
<td>39 (84.8)</td>
</tr>
<tr>
<td></td>
<td>CTX-M with other ESBL genes*</td>
<td>5 (8.9)</td>
<td>5 (10.9)</td>
</tr>
<tr>
<td></td>
<td>Other ESBL genes alone*</td>
<td>3 (5.4)</td>
<td>2 (4.3)</td>
</tr>
<tr>
<td>Winter</td>
<td>ESBL</td>
<td>30 (23.4%)</td>
<td>20 (15.6)</td>
</tr>
<tr>
<td></td>
<td>CTX-M alone*</td>
<td>24 (80)</td>
<td>18 (90)</td>
</tr>
<tr>
<td></td>
<td>CTX-M with other ESBL genes*</td>
<td>4 (13.3)</td>
<td>2 (10)</td>
</tr>
<tr>
<td></td>
<td>Other ESBL genes alone*</td>
<td>2 (6.7)</td>
<td>0</td>
</tr>
</tbody>
</table>

*Calculated as no (%) of the total ESBL positive isolates

**Statistical comparisons**

@ L1a vs L2a: Summer *P = 0.001;* Monsoon *P = 0.004;* Winter *P = 0.001

<table>
<thead>
<tr>
<th>Comparison of seasons</th>
<th>L1a</th>
<th>L1b</th>
<th>L2a</th>
<th>L2b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monsoon vs Summer</td>
<td>0.05</td>
<td>0.03</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>Monsoon vs Winter</td>
<td>0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.003</td>
</tr>
<tr>
<td>Summer vs Winter</td>
<td>NS (0.1)</td>
<td>NS (0.1)</td>
<td>NS (0.1)</td>
<td>NS (0.6)</td>
</tr>
</tbody>
</table>

Note: (i) None of the samples in any location of pond environment yielded *E. coli* isolate with carbapenem resistance
(ii) There was no statistical difference in rate of ESBL positivity among *E. coli* isolated from sub-surface water and sediment sludge at both the locations in pond (L1 and L2) regardless of season.
and imipenem (10µg) and tigecycline (15µg). In addition, a third-generation cephalosporin viz. ceftiofur known to be used only in veterinary sector, was also included, (EFT disc, 30µg, Oxoid Ltd., United Kingdom) included and the results were interpreted as per CLSI guidelines for drugs used for veterinary use.21

**Statistical analysis**

Prevalence of ESBL, CTX-M and other ESBL types and carbapenem resistance were expressed in terms of percentage resistant and were compared by chi-square test as categorical variables. P-value less than 0.05 was considered to be statistically significant.

**RESULTS**

During each of the three seasons, a total of 256 pond surface water samples, comprising of 128 collected from each pond and 256 pond bed sludge samples collected from same locations were analyzed in the present study. Fecal specimens from 104, 82 and 196 human subjects belonging to categories Sgr 1a, Sgr 1b and Sgr II respectively and a total of 286 buffalos sharing residential premises with Sgr 1a and Sgr 1b human subjects were subjected to similar analysis.

Prevalence of ESBL producing E. coli was detected to be maximum during rainy season followed by summer and winter in all locations of pond environment i.e. surface water, pond bed sludge and soil. Out of the three sites in each radial direction chosen for sampling of pond environment, samples collected at 2 ft distance from water edge showed higher prevalence of ESBL producing E. coli in surface water as well as in pond sludge compared to other two sites regardless of the season. However, there was no statistical difference in the prevalence of ESBL producing E. coli between samples collected from surface water and that from sludge at the same point of collection from pond. Analysis of molecular types of the ESBL positive isolates revealed CTX-M variety alone to be the predominant type regardless of the point of collection from pond environment with very few isolates found to harbor CTX-M with other ESBL genes and other ESBL genes alone (Table 1).

Prevalence of ESBL producing E. coli along with its CTX-M variety was more in the Sgr 1a human subjects compared to those belonging to Sgr 1b and Sgr II (Table 2). There was high prevalence of ESBL producing E. coli among the isolates from buffalos (89 out of 286 i.e. 31.1% of buffalos) that belonged predominantly to CTX-M type alone (72 out of 89 i.e. 80.9%) (data shown in footnote of Table 2). Carbapenem resistance could be detected in human subjects belonging to various subgroups with comparable prevalence without being detected in any sample from pond environment.

### Table 2. Prevalence of AMR in human subjects with or without companion animals (water buffalos)

<table>
<thead>
<tr>
<th>Type of resistance</th>
<th>Samples from human subjects</th>
<th>Statistical comparisons</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With companion animals</td>
<td>Without companion animals</td>
</tr>
<tr>
<td></td>
<td>Sgr Ia (n=104)</td>
<td>Sgr Ib (n=82)</td>
</tr>
<tr>
<td></td>
<td>ESBL</td>
<td>39 (37.5)</td>
</tr>
<tr>
<td></td>
<td>CTX-M alone*</td>
<td>31 (79.5)</td>
</tr>
<tr>
<td></td>
<td>CTX-M with other ESBL genes*</td>
<td>7 (17.9)</td>
</tr>
<tr>
<td></td>
<td>Other ESBL genes alone*</td>
<td>1 (2.6)</td>
</tr>
<tr>
<td></td>
<td>Carbapenemase</td>
<td>3 (7.7)</td>
</tr>
<tr>
<td></td>
<td><strong>ESBL</strong></td>
<td><strong>39 (37.5)</strong></td>
</tr>
<tr>
<td></td>
<td><strong>CTX-M alone</strong></td>
<td><strong>31 (79.5)</strong></td>
</tr>
<tr>
<td></td>
<td><strong>CTX-M with other ESBL genes</strong></td>
<td><strong>7 (17.9)</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Other ESBL genes alone</strong></td>
<td><strong>1 (2.6)</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Carbapenemase</strong></td>
<td><strong>3 (7.7)</strong></td>
</tr>
</tbody>
</table>

*Calculated as no. (%) of the total ESBL positive isolates

Note: Prevalence of ESBL-EC in samples from companion animals (buffalos) was found to be 31.1% (89 out of 286), of which 80.9% (72 out of 89), 12.4% (11 out of 89) and 6.7% (6 out of 89) were found to harbour CTX-M alone, CTX-M with other ESBL genes and other ESBL genes alone respectively. None of the isolates from pond environment or companion animal showed evidence of carbapenemase production.
Table 3. Co-resistance pattern of the ESBL producing *E. coli* isolated from human subjects, companion animals (buffalos) and pond environment

<table>
<thead>
<tr>
<th></th>
<th>Human subjects</th>
<th>Companion animals</th>
<th>Pond environment</th>
<th>Statistical comparison between various categories of human subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sgr Ia</td>
<td>Sgr Ib</td>
<td>Sgr II</td>
<td>n=89</td>
</tr>
<tr>
<td></td>
<td>n=39</td>
<td>n=17</td>
<td>n=44</td>
<td>No (%)</td>
</tr>
<tr>
<td>AMC (20/10 μg)</td>
<td>14(35.9)</td>
<td>6 (35.3)</td>
<td>18 (40.9)</td>
<td>21 (23.6)</td>
</tr>
<tr>
<td>GEN (10 μg)</td>
<td>7 (20.5)</td>
<td>1 (5.9)</td>
<td>3 (6.8)</td>
<td>21 (23.6)</td>
</tr>
<tr>
<td>CTR (30 μg)</td>
<td>39(100)</td>
<td>17 (100)</td>
<td>42 (95.5)</td>
<td>84 (94.4)</td>
</tr>
<tr>
<td>EFT (30 μg)</td>
<td>13(33.3)</td>
<td>3 (17.6)</td>
<td>0</td>
<td>50 (56.2)</td>
</tr>
<tr>
<td>CPM (30 μg)</td>
<td>11(28.2)</td>
<td>4 (23.5)</td>
<td>12 (27.3)</td>
<td>16 (18)</td>
</tr>
<tr>
<td>AT (30 μg)</td>
<td>39 (100)</td>
<td>17 (100)</td>
<td>44 (100)</td>
<td>89 (100)</td>
</tr>
<tr>
<td>CIP (5 μg)</td>
<td>10 (25.6)</td>
<td>3 (17.6)</td>
<td>8 (18.2)</td>
<td>17 (19.1)</td>
</tr>
<tr>
<td>COT (25 μg)</td>
<td>12 (30.8)</td>
<td>2 (11.8)</td>
<td>2 (4.5)</td>
<td>21 (23.6)</td>
</tr>
<tr>
<td>ETP (10 μg)</td>
<td>3 (7.7)</td>
<td>2 (11.8)</td>
<td>4 (9.1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>TE (10 μg)</td>
<td>17 (43.6)</td>
<td>4 (23.5)</td>
<td>7 (15.9)</td>
<td>41 (46.1)</td>
</tr>
</tbody>
</table>


Note: All the isolates were sensitive to piperacillin/tazobactam, amikacin and tigecycline and resistant to ampicillin, and cefotaxime.
environment or from animal source i.e. from buffalos.

Prevalence of the co-resistance pattern among the ESBL resistant *E. coli* isolated from the three subgroups of human subjects showed identical pattern for all the antibiotics tested except that for gentamicin, ciprofloxacin and ceftiofur that were found to be more prevalent among the Sgr Ia i.e. the subgroup of human subjects engaged in bathing the buffalos in the pond compared to Sgr Ib i.e., the human subjects in the same residential premises involved in other types of care of buffalos as well as to Sgr II human subjects that were not associated with direct contact with any livestock. Such difference was more marked for Ceftiofur compared to other two antibiotics i.e. gentamicin and ciprofloxacin (Table 3).

**DISCUSSION**

Numerous studies on ESBL-producing *Enterobacteriaceae* isolated from aquatic environments have been focused on reservoirs fed by antimicrobials in aquaculture. There is an isolated report on ESBL-producing *Enterobacteriaceae* in water reservoir serving as source of drinking water in rural area. To the best of our knowledge there is hardly any study on assessment of risk for acquisition of ESBL producing *Enterobacteriaceae* from earth pond used for bathing and cleaning of livestock by humans.

In the present study, prevalence of AMR in pond environment was found to be maximum during the rainy season compared to other seasons regardless of the type of sample i.e. pond edge soil or sediment sludge or surface water and location of sampling in the pond i.e. 2ft or 20 ft away from pond edge. In a study by Hoa, et al. in Vietnam higher prevalence of Tetracycline and Ampicillin resistant bacteria were recorded in the rainy season than other seasons. Heavy rains during the monsoon season leads to considerable flow of run-off water from soil and anthropogenic sources into aquatic environments carrying bacteria and accompanying resistant genes. The load of different varieties of AMR was found to be maximum in both surface water and sediment samples at the point closer to the edge of the pond (at 2 ft from edge) compared to the similar samples collected further away from the edge (20 ft away from the edge) and least from the dry soil on the edge. While the ponds selected in the present study were away from any health care setting (> 2 km distance), we could not rule out the possible contribution of runoff water from surrounding agricultural soils that are frequently irrigated with canal water receiving untreated community sewage. Moreover, local birds e.g. common egrets, pigeons visiting the pond may contribute to ESBL producing *E. coli* as reported in elsewhere. These factors may explain highest prevalence of ESBL producing *E. coli* detected in surface water and sludge samples drawn from the area closer to the edge of the pond compared to the location further away from the edge and the pond edge soil samples that is exposed to sunlight and dry atmosphere providing the least favorable environment for persistence or growth of bacteria. In the present study *E. coli* with ESBL positivity were isolated from the pond bottom sludge samples with same frequency as that of surface samples at the same site. This appears to be a paradoxical observation considering that pond sediment is known to be rich in organic matters and coliforms are facultative anaerobes. Probable explanation for such observation could be shallowness of pond insignificant enough to result in difference at the two levels and vigorous mixing of water due to movement of buffalos and humans.

ESBL producing isolates, especially those producing CTX-M type enzymes, are able to colonize into almost any kind of setting and are now being increasingly acquired by the human community. As a consequence, the CTX-Ms have emerged to be the dominant type of ESBL over the years replacing the TEM and SHV types in both human, veterinary sector as well as in environment which is also reflected in the present study showing the CTX-M variety being the predominant molecular variety among the ESBL isolates from all sources.

Present study showed that the activities like bathing and cleaning of the livestock in earth ponds could serve as important risk factors for transmission of AMR from the livestock to human since prevalence of various categories of AMR studied were significantly higher in the subjects engaged in bathing and cleaning of livestock (sgr Ia) compared to the age and sex matched members...
in same families without such engagement (sgr lb) as well as to the group of villagers without having direct exposure to livestock (sgr II). Considering that the ponds selected in the present study in Northern India were not used for any activity like aquaculture or had any access to effluents from health care establishment, the apparent source of AMR demonstrated in E. coli isolated in pond water and pond bed sludge could be the fecal contamination from buffalos that showed high prevalence of intestinal carriage of similar organisms in the present study. In contrast to developing countries, livestock are not used as food animals in India and thus application of antibiotics as growth promoters does not seem to account for such high prevalence of AMR in them. However, in contrast to the use of antibiotics in human, there are no well-defined regulatory guidelines on veterinary use of antibiotics in India resulting in their indiscriminate use. Thus, unregulated use of antibiotics for various ailments in livestock could account for high AMR in them encountered in the present study. A survey published in 2005 by the OIE (World Organization for Animal Health) revealed that lack of regulatory guidelines controlling the use of antimicrobial agents in livestock is not unique for India. Even in developed countries like United States, many antibiotics are readily available to the livestock farmers without any prescription. Transmission of resistant bacteria from animals to humans through oral route have been documented in numerous reports among subjects in close association with livestock. Our observation adds another scenario for transmission of antibiotic resistant bacteria through oral route hitherto unreported. Most of the data on carbapenem resistance in rural community from this part of the country, although limited, are hospital based showing prevalence rate as 7.8 to 22.1%. Observation in our laboratory indicated a steady rise in the prevalence of carbapenemase producing E. coli and K. pneumoniae in clinical specimens among patients from the local rural community attending hospitals over the period between 2015 and 2018. However, there is hardly any report on estimation of carriage rate of carbapenem resistance in rural community other than those seeking health care that may be an important determinant of the burden in the population.

Comparable prevalence of carbapenem resistance in various subgroups of human subjects i.e. those involved in the activity of bathing and cleaning of buffalos, those involved in other activities related to other types of care of buffalos and those without any direct exposure to the buffalos suggest lack of association with livestock as the primary cause of such resistance. In an earlier study on surveillance of carbapenem resistance in a subpopulation of hospital attending patients (mostly farmers with livestock) from the same rural community in which the ponds in the present study are located the prevalence of carbapenem resistant among E. coli was found to be (7.8%) that could be epidemiologically linked with history of prior exposure to hospital environment rather than association with livestock. Carbapenems are not permitted for animal use globally. To the best of knowledge, there has not been any report from India on carbapenem resistance among animals that supports lack of any isolation of carbapenem resistant E. coli in samples from buffalos in the present study.

Fluoroquinolones, third-generation cephalosporins and tetracyclines are the most commonly used antibiotics in veterinary sector in many developing countries, resulting in injudicious use of these antibiotics. Moreover, use of penicillins, tetracyclines, macrolides and aminoglycosides are pronounced in veterinary medicine, and these have been used for more than 50 years in livestock for treatment of ailments like mastitis, pneumonia and metritis. An interesting observation of the present study was the finding of resistance to ceftiofur exclusively among human subjects having companion livestock, in significantly higher proportion among those engaged in bathing and cleaning of buffalos compared to those without such engagement. Ceftriaxon, a third-generation cephalosporin is known to be used exclusively for veterinary ailments and is not considered for human use due to its toxicity. However, ceftiofur has been reported to be capable of transferring resistance to other members of third generation Cephalosporins of human therapeutic importance.

**CONCLUSION**

Higher carriage rate of antibiotic resistance detected in the human population...
engaged in the bathing and cleaning of buffalos, especially for the antibiotics used in veterinary therapy suggests the activities associated with bathing and cleaning of buffalos as definite risk factors towards acquisition of such resistance.

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CONFLICT OF INTEREST
The authors declare that there is no conflict of interest.

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AUTHORS' CONTRIBUTION
LSD contributed in specimen collection, conducting laboratory work, data analysis and manuscript writing. DC contributed in study conception and designing the protocol, supervised the overall work and manuscript writing. Both the authors read and approved the final manuscript for publication.

DATA AVAILABILITY
The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT
This study was approved by the Institutional Ethics Committee, SGT University under the protocol no. SGTU/FMHS/MICRO/341.

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