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Antimicrobial resistance in food-borne pathogens at the human-animal interface: Results from a large surveillance study in India

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ABSTRACT

Background: The burden of foodborne diseases and antimicrobial resistance carried by key foodborne pathogens in India is unknown due to a lack of an integrated surveillance system at the human-animal interface.
Methods: We present data from the WHO-AGISAR (Advisory Group on Integrated Surveillance of Antimicrobial Resistance), India project. Concurrent human and animal sampling was done across a large area across north India. Community-acquired diarrhea cases ($n = 1968$) of all age groups were included. Cross-sectional sampling of stool/ intestinal contents ($n = 487$) and meat samples ($n = 419$) from food-producing animals was done at farms, retail shops, and slaughterhouses. Pathogens were cultured and identified, and antimicrobial susceptibility was performed.
Results: Over 80% of diarrhoeal samples were obtained from moderate to severe diarrhea patients, which yielded EAEC (5%), ETEC (4.84%), EPEC (4.32%), and *Campylobacter* spp. (2%). A high carriage of EPEC (32.11%) and *Campylobacter* spp. (24.72%) was noted in food animals, but the prevalence of ETEC (2%) and EAEC (1%) was low. Atypical EPEC (aEPEC, 84.52%, $p \leq 0.0001$) were predominant and caused milder diarrhea. All EPEC from animal/poultry were aEPEC. Overall, a very high level of resistance was observed, and the MDR rate ranged from 29.2% in *Campylobacter* spp., 53.6% in EPEC, and 59.8% in ETEC. Resistance to piperacillin-tazobactam, cefepime, ceftriaxone, and co-trimoxazole was significantly higher in human strains. In contrast, resistance to ciprofloxacin, aminoglycosides, and tetracycline was higher in animal strains, reflecting the corresponding usage in human and animal sectors. ESBL production was commoner in animal isolates than in humans, indicating high use of third-generation cephalosporins in the animal sector. *C. hyointestinalis* is an emerging zoonotic pathogen, first time reported from India.
Conclusion: In one of the most extensive studies from India, a high burden of key foodborne pathogens with MDR and ESBL phenotypes was found in livestock, poultry, and retail meat.

Abbreviations: ETEC, Enterotoxigenic *Escherichia coli*; EPEC, Enteropathogenic *Escherichia coli*; EAEC, Enteroaggregative *Escherichia coli*; *E. coli*, *Escherichia coli*; CDC, Centers for Disease Control and Prevention; USA, United States of America; WHO, World Health Organization; PGIMER, Post Graduate Institute of Medical Education and Research; AMR, Antimicrobial Resistance; MDR, Multi-Drug Resistance; DPHLs, District Public Health Laboratories; ARB, Antibiotic Resistance Bacteria; AGISAR, Advisory Group on Integrated Surveillance of Antimicrobial Resistance; DEC, Diarrhoeagenic *Escherichia coli*; DNA, Deoxyribonucleic Acid; AST, Antibiotic Susceptibility Testing; CLSI, Clinical and Laboratory Standards Institute; ESBLs, Extended Spectrum Beta Lactamases; MIC, Minimum Inhibitory Concentration; EUCAST, European Committee on Antimicrobial Susceptibility Testing; PCR, Polymerase Chain Reaction; LMICs., Low and middle-income countries; MHA, Mueller Hinton Agar.

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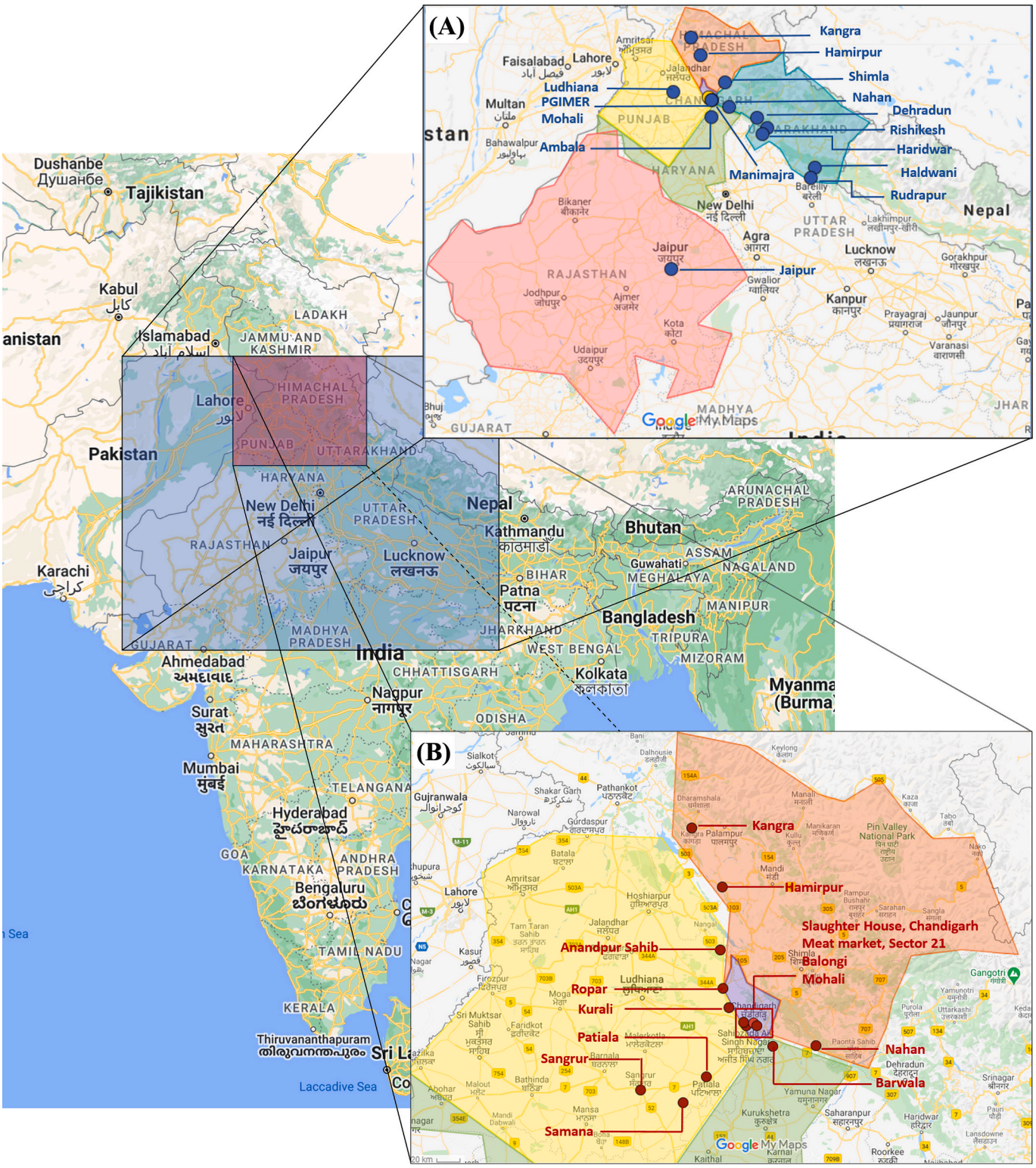

Fig. 1. Human and animal samples collection sites in this study (produced from Google Maps). (A) showing the collection sites for human samples. Different colors represent different states. Red is for Rajasthan, yellow is for Punjab, orange is for Himachal Pradesh, green is for Haryana, and sky blue is for Uttarakhand. (B) showing the animal collection sites. Yellow is for Punjab, Red is for Rajasthan, Purple is for Chandigarh, and Red is for Himachal Pradesh. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

1. Introduction

Foodborne illnesses are a serious public health concern worldwide. Centre for Disease Control and Prevention (CDC) states that annually, 1 in 6 people in the USA gets sick, 128,000 are hospitalized, and 3000 die from foodborne diseases [1]. Globally, 1 in 10 people fall sick from consuming contaminated food [2]. Most of these deaths (30%) occur in children under the age of five years [3]. *Campylobacter* spp., *Salmonella* spp., *E. coli, Yersinia* spp., *Vibrio* spp., *Listeria* spp., and *Shigella* spp. are the major bacterial pathogens responsible for acute gastroenteritis,

diarrhea, and vomiting. *Salmonella* spp. is the leading cause of death, followed by *Listeria* spp. and *Campylobacter* spp., while diarrhoeagenic *E. coli* caused most hospitalizations, followed by *Salmonella* spp. and *Campylobacter* spp. [3]. Most of these pathogens are zoonotic agents and spread from animal and animal products to humans [3]. The burden of illnesses associated with bacterial foodborne pathogens is compounded by the increase in infections caused by pathogens resistant to multiple antimicrobials [4]. Antimicrobial use in humans and animals is strongly associated with the development of resistance to known foodborne pathogens [5]. Antibiotics are widely used to prevent infections in India's livestock sector as growth promoters. Worldwide antimicrobial consumption is expected to rise by a staggering 67% between 2010 and 2030 and nearly double in India [6]. Animal husbandry is an integral component of Indian agriculture, supporting the livelihoods of more than two-thirds of the rural population [7]. There is a high demand for livestock as a food source due to the rise in population and change in traditional food habits of young urban Indians [8]. This has resulted in intensive farming methods in limited space, which become a 'Breeding ground' for pathogenic bacteria. The widespread use of antimicrobials in livestock contributes through natural selection to the emergence of antimicrobial-resistant bacteria (ARB). It has significant public health implications: ARB of animal origin can be transmitted to humans through the environment [9] and food products [10]. Residues of these antibiotics remain active, and these residues in foods may alter human intestinal microbiota and cause resistance gene transfer.

Integrated surveillance of AMR in food-producing animals, foods, and humans globally with standardized approaches and timely data sharing is key to identifying potential routes and sources of transmission. The high-income countries have specific organizations that monitor the use of antimicrobials and trends in resistance development in humans and agriculture [11]. However, monitoring/surveillance systems are only available in some low and middle-income countries (LMICs), particularly South East Asia [12]. This is concerning since, in LMICs, the use of antimicrobials is not regulated as in the United States (US) or other high-income countries. There are many hindrances in estimating the burden of AMR. Incongruent data is available from public and private sectors; data are often not appropriately collected and are fragmented. These problems are intensified in LMICs due to problems of inadequate surveillance and poor laboratory infrastructure. One Health is a collaborative effort of multiple health science professions to attain optimal health for people, domestic animals, wildlife, plants, and our environment [13].

The geographical area around Chandigarh (Haryana, Punjab, and Himachal Pradesh) is one of India's significant cattle and poultry rearing areas. Foodborne gastroenteritis is very common, is clubbed with acute diarrhea, and is not notifiable. In a previous study conducted by us, in collaboration with WHO, we found that every year 1400 to 31,000 cases of suspected food- and-water-borne infections were being reported at the district public health labs (DPHLs) across Punjab, Haryana, and Uttarakhand (Unpublished data PGIMER). Most of these infections were not investigated for etiology and antimicrobial resistance. The present study presents integrated surveillance data of AMR in foodborne pathogens generated through the WHO-AGISAR (Advisory Group on Integrated Surveillance of Antimicrobial Resistance), India project. We aimed to concurrently study the prevalence and antibiotic resistance patterns of four major bacterial pathogens, i.e., *Campylobacter* spp., EAEC, ETEC, and EPEC, in humans and animals across a large geographic area in North India.

2. Material and methods

2.1. Study site and ethics statement

The study was conducted at the Enteric Laboratory, Department of Medical Microbiology, PGIMER, Chandigarh, India. The Institutional Ethics Committee approved this study, reference number NK/4458/PhD vide letter no INT/IEC/2018/ 000849 dated 26.5.2018 and no./PGI/IEC/2018/001510 dated 24.09.2018. All human samples included in this study were collected after obtaining informed consent from the patient or their guardian.

2.2. Sample collections

2.2.1. Human samples

We conducted a sustained surveillance from March 2015 to February 2018 for human diarrhoeal disease at the Post Graduate Institute of Medical Education and Research (PGIMER, Chandigarh) and network laboratories in the states of Punjab, Haryana, Rajasthan, Uttarakhand, and Himachal Pradesh. A total of 14 labs across North India participated in the study. (Fig. 1, Supplementary Table 1). Cases referred to PGIMER were also included ($n = 56$). PGIMER is one of the largest tertiary care hospitals in North India and serves patients from across Punjab, Jammu Kashmir, Himachal Pradesh, and Haryana. Diarrhea was the passage of three or more liquid or semi-liquid stools. Diarrhoeal cases originating in the community were included, and all hospital-acquired diarrhea cases (diarrhea occurring in cases after 48 h of presentation to the health care facility) were excluded. Stool samples from patients with community-acquired diarrhea were collected in a sterile container and transported to PGIMER, Chandigarh, in Cary-Blair, transporting the media in a cold chain for further processing. The samples were processed immediately upon receipt. Before sample collection, informed consent from patients or their guardians was taken along with demographic information and clinical details. Vesikari severity score was calculated based on the number of diarrhoeal and vomiting episodes, duration of illness, temperature, and dehydration status. Elements of the ranking include the length of diarrhea (in days; score, 0 to 3 points), the highest number of stools per day during the episode (score, 1 to 3 points), the occurrence of vomiting (score, 0 to 1 point), the maximum number of cases per day during the episode (value, 0 to 3 points), the existence of fever (score, 0 to 1 point), the presence of fatigue (score, 0 to 1 point) and treaties [14]. Based on the score, the cases were categorized as mild (score < 7), moderate (score $>$ 7–10), and severe ($>$10). *Campylobacter* isolation could be done only for 1127 human samples as it took us some time to set up the culture conditions and obtain media.

2.2.2. Animal samples

Concurrently, we conducted cross-sectional sampling of food animal meat products and farm animals (sheep, goats, pigs, and chickens) in the states of Punjab, Haryana, Himachal Pradesh, and Chandigarh from markets and farms in the same areas from where human samples were collected (Fig. 1). The meat shop and farm owners were approached, and those who agreed to provide samples were included in the study (Supplementary Table 2). Samples from goats and pigs were also collected from Slaughter House, Chandigarh, in a repeated cross-sectional manner. We collected samples every Wednesday for 30 weeks, from March 2014 to October 2014. Slaughterhouse under the Municipal Corporation of Chandigarh is a mechanical abattoir that caters to Chandigarh and the neighboring cities of Mohali, Kharar, Panchkula, Manimajra, Zirakpur, Balongi, Meat market sector 21 Chandigarh. Up to 250 goats/sheep and 100 pigs are slaughtered daily. The samples were collected in sterile containers and transported in a cold chain to PGIMER, where they were processed immediately. The total number of samples collected was 906, of which 487 were animal stools/intestinal contents and 352 were meat samples (Supplementary Table 2).

2.3. Microbiological processing

2.3.1. Stool samples

To isolate *E. coli,* a loopful (10 μl) of the sample was directly inoculated onto MacConkey agar (Difco, Maryland, USA) and incubated at 37 °C for 24 h [14]. *Campylobacter* spp. was isolated by directly inoculating on Campy-cefex agar containing 5% sheep blood (Oxoid,

Table 1
Age and gender-wise distribution of patients from different geographical regions.

Region	Age group (years)	Male (%)	Female (%)	Total (%)
Chandigarh	0–2	96 (14.81)	49 (7.56)	145 (22.37)
	>2–5	36 (5.55)	17 (2.62)	53 (8.17)
	>5–15	47 (7.25)	31 (4.78)	78 (12.03)
	>15–40	140 (21.6)	110 (16.9)	250 (38.58)
	>40	60 (9.25)	62 (9.56)	122 (18.82)
		379 (58.48)	269 (41.5)	648
	Details not available			0*
$n = 648$	Sub-total			**648**
Punjab	0–2	101 (26.16)	39 (10.1)	140 (36.26)
	>2–5	31 (8.03)	10 (2.5)	41 (10.6)
	>5–15	33 (8.54)	25 (6.47)	58 (15.02)
	>15–40	66 (17.09)	31 (8.03)	97 (25.12)
	>40	32 (8.29)	18 (4.6)	50 (12.9)
		263 (68.13)	123 (31.8)	386
	Details not available			0*
$n = 386$	Sub-total			**386**
Haryana	0–2	77 (22.64)	49 (14.4)	126 (37.05)
	>2–5	21 (6.17)	12 (3.52)	33 (9.7)
	>5–15	17 (5)	10 (2.94)	27 (7.9)
	>15–40	42 (12.35)	45 (13.2)	87 (25.58)
	>40	36 (10.58)	31 (9.11)	67 (19.7)
		193 (56.76)	147 (43.2)	340
	Details not available			0*
$n = 340$	Sub-total			**340**
Rajasthan	0–2	10 (11.49)	3 (3.44)	13 (14.9)
	>2–5	2 (2.29)	2 (2.2)	4 (4.59)
	>5–15	4 (4.59)	7 (8.04)	11 (12.6)
	>15–40	18 (20.68)	21 (24.13)	39 (44.8)
	>40	13 (14.94)	7 (8.04)	20 (22.9)
		47 (54.02)	40 (45.9)	87
	Details not available			0*
$n = 87$	Sub-total			**87**
Himachal Pradesh	0–2	55 (21.4)	34 (13.2)	89 (34.6)
	>2–5	14 (5.4)	8 (3.1)	22 (8.5)
	>5–15	9 (3.5)	9 (3.5)	18 (7)
	>15–40	37 (14.39)	32 (12.45)	69 (26.8)
	>40	34 (13.2)	25 (9.7)	59 (22.9)
		149 (57.9)	108 (42.02)	**257**
	Details not available			5*
$n = 262$	Sub-total			**262**

Area	Age group	Male	Female	Total
Uttarakhand	0–2	12 (6.62)	12 (6.6)	24 (13.2)
	>2–5	11 (6.07)	8 (4.4)	19 (10.5)
	>5–15	31 (17.1)	12 (6.62)	43 (23.75)
	>15–40	25 (13.8)	26 (14.36)	51 (28.17)
	>40	25 (13.8)	19 (10.49)	44 (24.3)
		104 (57.4)	77 (42.5)	**181**
	Details not available			8*
$n = 189$	Sub-total			**189**
Others*	0–2	10 (17.8)	0	10 (17.8)
	>2–5	1 (1.8)	3 (5.4)	4 (7.2)
	>5–15	2 (3.6)	2 (3.6)	4 (7.2)
	>15–40	20(35.7)	9 (16.0)	29 (51.7)
	>40	7 (12.5)	2 (3.6)	9(16.1)
	Total	40 (71.4)	16 (28.6)	**56**
	Details not available			0*

Hampshire, England, U·K) and incubated under microaerophilic conditions at 42 °C for 48 h. The samples were also selectively enriched in Bolton broth (Oxoid, Hampshire, England, U·K) supplemented with 5% sheep blood for 48 h under microaerophilic conditions and then subcultured on Campy-cefex agar. Microaerophilic conditions were created using a Campy-Gen gas pack (ThermoFisher Scientific, Victoria, Australia) or an automated gassing system (Don Whitley Scientific, Bingley, England, U.K) [15].

2.3.2. Meat samples

For meat samples, 25 g of meat were inoculated into 225 ml buffered peptone water (BPW) (Difco, Maryland, USA) and incubated at 37 °C for 24 h. A loopful from BPW was inoculated on MacConkey agar (Difco, Maryland, USA) and incubated at 37 °C for 24 h for *E. coli* isolation. Meat sample 25 g added to 225 ml Bolton broth and incubated for 48 h at 42 °C under microaerophilic conditions. A loopful from Bolton broth was inoculated on Campy-cefex agar for 48 h at 42 °C under microaerophilic conditions for *Campylobacter* spp. isolation.

After incubation, presumptive colonies of non-lactose fermenting colonies of greyish brown colonies of *Campylobacter* spp. and round, smooth pink colonies (all morphological variants) of *E. coli* were confirmed by matrix-assisted laser desorption and ionization-time of flight mass spectrometry (MALDI-TOF-MS) bacterial identification system (Bruker Daltonics, Bremen, Germany). Species were assigned for

scores of ≥2.0 and genera for ≥1.7 but <2.0. If scores were lower than 1.7, no identification was assigned as it was unreliable. Confirmed isolates were preserved for further use by suspending them in fresh, sterilized Trypticase soy broth (TSB) (Difco, Maryland, USA) containing 15% glycerol at −70 °C. For *Campylobacter* spp. isolates, TSB with 20% glycerol was used.

2.3.3. *E. coli* pathotype identification by PCR

DNA from *E. coli* colonies was extracted by boiling method [16]. A multiplex PCR was put in two parts to identify diarrhoeagenic *E. coli* (DEC) pathotypes. These included the following targets: ETEC enterotoxins (heat-labile [LT] and heat-stable [ST]), EPEC (*eae*) protein bundle forming protein (*bfp*), Shiga toxins (*stx1*, *stx2*), VTcom for EHEC and EAEC (*pCVD432*) [14,17].

The following PCR reaction conditions for Multiplex I & II were used: Hot start 95 °C for 2 mins, Denaturation 95 °C for 15 s, Annealing 52 °C for 8 s, Extension 72 °C for 10 s, Final extension 72 °C for 2 mins. All PCRs were performed in the thermocycler (Applied Biosystems Veriti 96 Well Thermal Cycler, Life Technologies, ThermoFisher Scientific, California, USA). Amplified samples were viewed in a 1.5% agarose gel (Sigma Aldrich, Missouri, USA) run in 0.5× Tris–borate–EDTA (TBE) and stained with ethidium bromide. *E. coli* positive for ETEC, EAEC, and EPEC genes were stored for further experiments.

2.4. Antimicrobial susceptibility testing (AST)

2.4.1. Disc diffusion method

AST was tested by the Kirby-Bauer disk diffusion method [18] on Mueller Hinton II agar (Difco, Maryland, USA) for ETEC and EPEC for following antibiotics and interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2014). ETEC, and EPEC: ampicillin (10 μg), cefoxitin (μg), gentamicin (10 μg), amikacin (30 μg), piperacillin-tazobactum (100/10 μg), cefepime (30 μg), ceftriaxone (30 μg), ciprofloxacin (μg), levofloxacin (μg), imipenem (10 μg), cotrimoxazole (μg), ertapenem (10 μg). For EAEC, the following antibiotics were tested: Ampicillin (10 μg), Ciprofloxacin (μg), Amikacin (30 μg), Imipenem (10 μg), Levofloxacin (μg), Gentamicin (10 μg), Cefixime, Piperacillin-Tazobactam, Ertapenem (10 μg), Cotrimoxazole (μg), Cefoxitin and Ceftriaxone (30 μg).

The strains were also checked for ESBL production by combination disc method according to CLSI guidelines where ceftazidime (10 μg), ceftazidime-clavulanic acid (10/30 μg), and cefotaxime cefotaxime-clavulanic acid (10/30 μg) were placed to size the zone size difference [19]. MDR isolates were defined as non-susceptibility to at least one antimicrobial agent in three or more classes of antibiotics [20].

2.4.2. Estimation of Minimum inhibitory concentration (MIC)

MIC values were estimated by *E*-test method for ETEC & EPEC for the following antibiotics: ciprofloxacin (0.0625–16 μg/ml), azithromycin (1–256 μg/ml), gentamicin (0.5–128 μg/ml), ertapenem (0.125–32 μg/ml), colistin (0.125–16 μg/ml), tetracycline (0.25–64 μg/ml), and ceftriaxone (0.25–64 μg/ml) (*bioMérieux*, Lyon, France).

The susceptibility of *Campylobacter* spp. was tested by estimating the MIC values for ciprofloxacin (0.002–32 μg/ml), azithromycin (0.016–256 μg/ml), tetracycline (0.016–256 μg/ml) and gentamicin (0.016–256 μg/ml) antibiotics by *E*-strip method (*bioMérieux*, Lyon, France).

The organisms from a fresh culture plate were emulsified in normal saline, and their turbidity was adjusted to 0.5 McFarland (Himedia, Mumbai, India) and plated on Mueller Hinton agar (MH agar, Difco, Maryland, USA) plate for EAEC, ETEC, and EPEC. MH agar was supplemented with 5% sheep blood using a sterile swab (Himedia, Mumbai, India). Antibiotic containing *E*-strip was placed at the center of the plate with alcohol-sterilized forceps and incubated under microaerophilic conditions at 42 °C. After 48 h of incubation, the results were recorded and interpreted using CLSI (M45) for EAEC, EPEC, and ETEC. For *Campylobacter,* both CLSI and EUCAST guidelines were used.

Table 2
Distribution of severity scores from community acquired diarrhea in different regions.

Name of center	Total sample	Severe (%)	Moderate (%)	Mild (%)
Chandigarh	642	282 (43.9%)	186 (29.0%)	174 (27.1%)
Haryana	337	175(51.9%)	105 (31.1%)	57 (16.9%)
Himachal Pradesh	190	120 (63.1%)	62(32.6%)	8(4.2%)
Panjab	378	195(51.5%)	118 (31.2%)	65 (17.2%)
Rajasthan	53	29 (54.7%)	9 (16.9%)	15 (28.3%)
Uttarakhand	176	103(58.5%)	52 (29,5%)	21(11.9%)
Total	1776*	904(50.9%)	532 (30.0%)	340 (19.1%)

Table 3
Table showing region wise isolation of *Campylobacter* spp. and DEC pathotypes from human stool samples.

State	n	*Campylobacter*	DEC Pathotypes		
			EPEC	ETEC	EAEC
Chandigarh	648	9	35	38	36
Haryana	340	5	18	19	23
Himachal Pradesh	262	2	8	10	9
Punjab	386	3	13	20	3
Rajasthan	87	0	4	2	8
Uttarakhand	189	4	6	5	19
Total	1968	23/1127* (2%)	84 (4.32%)	94 (4.84%)	98 (4.97%)

3. Results

3.1. Human samples

In total, 1968 diarrheal stool specimens were collected from children and adults suffering from acute watery diarrhea from March 2015 to February 2018 from various laboratories across north India (Fig. 1, Table 1). Overall, males predominated over females. Thirty-six percent of cases were from children under five years of age, and 50% of cases were from adolescents and adults. A region-wide severity score was available from 1776 specimens, showing that 50.9% of samples were obtained from patients with severe diarrhea and 30% from moderate diarrhea (Table 2). The severity scores were consistent across the different regions. This reflects that mainly moderate and severe diarrhea cases are present in healthcare facilities.

3.2. Isolation of foodborne pathogens

From 1968 human specimens, 309 (15.6%) tested positive by PCR as DEC pathotypes: ETEC in 94 (4.78%), EAEC in 98 (5%), EPEC in 84 (4.23%), and STEC in 28 (1.42%). A total of 23/1127 (2.0%) samples were positive for *Campylobacter* spp., and 25/1968 (1.2%) were positive for *Salmonella* spp. Five additional samples tested positive for multiple DEC pathotypes and so were likely to represent mixed infections comprised of multiple pathotypes (Table 3).

3.3. Animal samples

Out of a total of 906 samples collected, 637 (70.3%) were positive for foodborne pathogens, out of which 224 (24.7%) were positive for *Campylobacter* spp., 104 (11.47%) were positive for *Salmonella* spp., 291 (32.1%) for EPEC and 18 (2%) for ETEC, 12 (1%) for EAEC (Fig. 2).

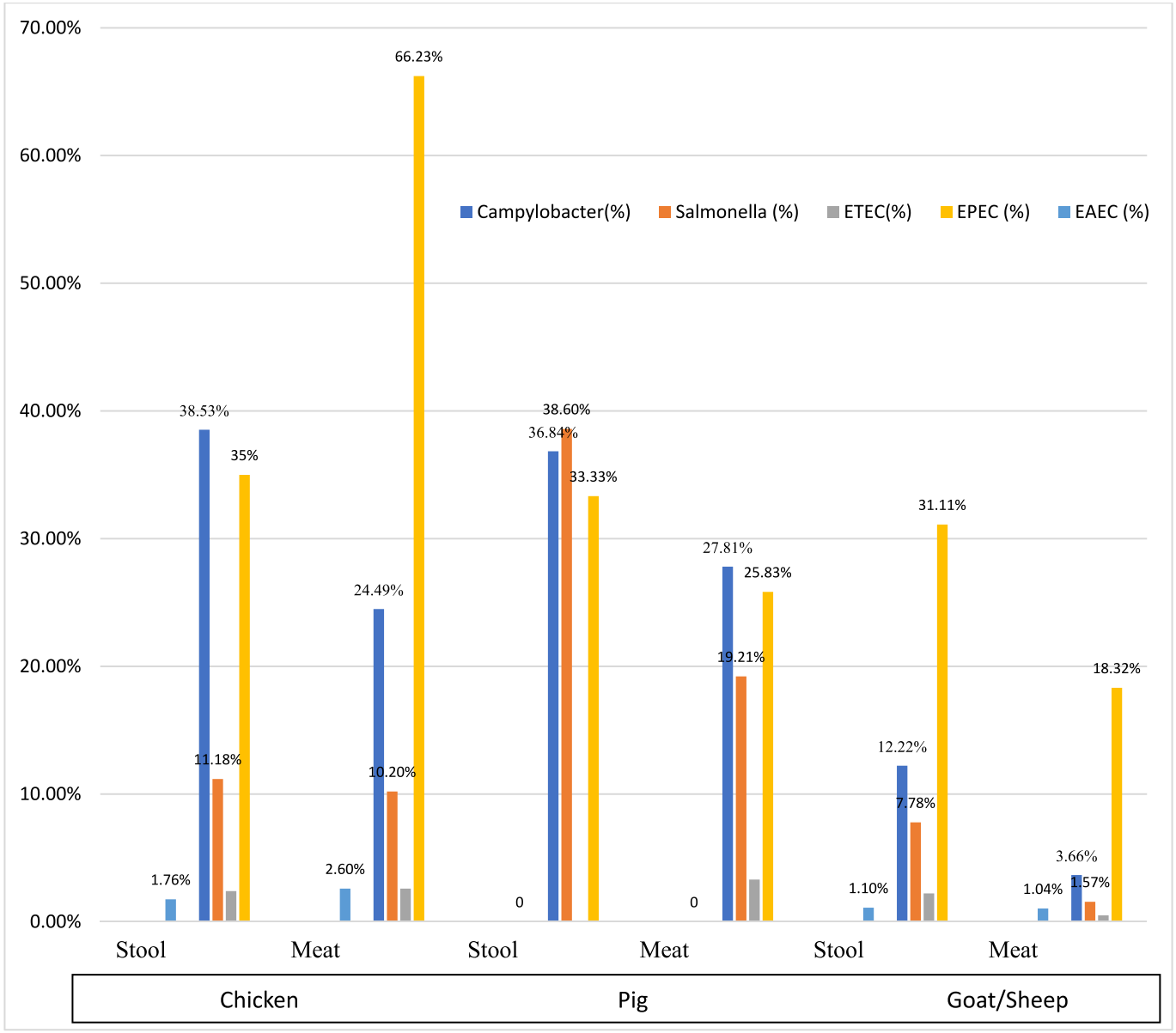

Fig. 2. Isolation of *Campylobacter* spp. and DEC pathotypes in Animal Samples.

3.4. Isolation of EPEC, EAEC, and ETEC

EPEC infections were seen in all age groups, but children below five years (63.1%, $p = 0.0007$) were most affected. Overall, EPEC infections were more associated with males (61.9%, $p = 0.0021$) than females, representing 38.1% of cases (Table 4).

Typical EPEC were defined by the presence of both the eae and bfp genes, whereas strains that were positive for eae but negative for the *bfp* gene were classified as aEPEC. Among our isolates, aEPEC (84.52%, $p \leq 0.0001$) was predominant compared to typical EPEC present in 15.48% (13/84) (Table 5). The tEPEC was more prevalent in children below five (84.61%, $p = 0.0005$) than in 15.38% of cases above five years. No such significant age-related association was noted among aEPEC cases (Table 5). On the Vesikari severity scale, tEPEC isolates caused more severe infections ($p = \mathbf{0.0292}$), whereas aEPEC isolates were associated with mild diarrhea episodes ($p = \mathbf{0.0278}$).

From 906 animal samples, 291 (32.11%) EPEC were isolated; the prevalence of EPEC was higher in chicken samples (40.77%, $p \leq 0.0001$) followed by pigs (27.88%) and then goat (22.42%). The stool samples constituted 34.1% EPEC, while 29.83% came from meat samples ($p = 0.1702$). None of the animal isolates carried the *bfp* gene, so all the isolates were aEPEC.

3.5. Isolation of EAEC

From 1968 human stool samples, 98 (4.9%) EAEC were isolated. These isolates were obtained from 6 out of 7 geographical regions, and their prevalence varied from 0.7% to 10.5%. Maximum prevalence was noted in the Uttarakhand region, followed by Rajasthan and Haryana (Table 1). EAEC infections were seen in all age groups, but children below five years old (65%) were most affected. Overall, EAEC infections were more associated with males (65%) than females, representing 35% of cases (Table 4). Also, there was a statistically significant association of EAEC infection with age group 0–2 ($p = 0.04$) age group.

The presence of the aggR gene defined typical EAEC, whereas strains that were positive for the aggR gene were classified as aEAEC. Among our isolates, tEAEC (72%) was predominant compared to atypical EAEC prevalence was 27.5%. The tEAEC was more prevalent in children below five years of age than in cases above five years. On the Vesikari severity scale, tEAEC (44%) isolates caused more severe infections, whereas

Table 4
Age and gender-wise distribution of ETEC, EPEC and ETEC cases.

	Age groups	Total (%)	Male (%)	Female (%)	*p*-value
ETEC	≤2 years	22 (23.2)	17 (17.9)	5 (5.4)	0.2014
	>2–5 years	20 (21.4)	12 (12.5)	8 (8.9)	0.7161
	>5–15 years	5 (5.4)	3 (3.6)	2 (1.8)	0.8677
	>15–40 years	24 (25)	10 (10.7)	14 (14.3)	0.7091
	>40	24 (25)	13 (14.3)	11 (10.7)	0.7091
	Total	*N* = 94	55 (58.5)	39 (41.4)	**0.0194**
EPEC	0–2*	37 (44.05%	22 (59.46%)	15 (40.54%)	0.1060
	>2–5*	16 (19.05%)	9 (56.25%)	7 (43.75%)	0.4864
	>5–15 20	10 (11.9%)	6 (60%)	4 (40%)	0.3833
	>15–40	12 (14.29%)	8 (66.67%)	4 (33.33%)	0.1098
	>40	9 (10.71%)	7* (77.78%)	2 (22.22%)	**0.0220**
	Total	84	52* (61.9%)	32 (38.1%)	**0.0021**
EAEC	0–2	24 (24.4%)	18 (75%)	6 (25%)	0.04*
	>2-5	40 (40.8%)	26 (65%)	14 (35%)	0.1
	>5–15	14 (14.2%)	8 (57%)	6 (42%)	0.7
	>15–40	9 (9.1%	6 (66%)	3 (33%)	0.6
	>40	11 (11.2%)	6 (54%)	5 (45%)	1.0
	Total	98	64 (65%)	34 (35%)	0.01*

* p-value < 0.05 was considered significant.

aEAEC isolates were associated with mild (46%) diarrhoeal episodes. Also, there were statistically significant associations of tEAEC and aEAEC infection with age group 0–2 ($p = 0.03$) and 2–5 years (0.005) of the age group. We found a very low prevalence of EAEC 1.32% in animal samples originating from all species sampled goat, sheep, and chicken.

3.6. Isolation of ETEC

Forty-four percent of cases were from children under five years of age, and 51.06% were from persons above 15 (Table 4). Males represented 58.5% of cases, significantly higher ($p = 0.0194$) than females, representing 41.4%. Severity scores varied from 5 to 14 on the Vesikari scale, with a median score of 11 and an average severity score of 11.06. Most patients presented with severe diarrhea (55.31%, $p = 0.0001$), while 23.21% and 21.42% presented with moderate and mild diarrhea respectively (Table 6).

ETEC was defined by the presence of one or more toxin genes. Heat-stable toxins were found to be most common, with STp and STh present in 34% (32/94) and 32% (30/94) isolates, respectively, while the heat-labile toxin gene was present in 31% (29/94). A small number of strains carried a combination of heat labile and heat stable (3%) genes, like LT with STh was present in 2% (2/94) and Lt with STp in 1% (1/94). Among the animal samples, only 18 (2%) isolates of ETEC were obtained. Isolates were obtained from all animal species i.e., chicken (55.56%), goats/sheep (27.78%), and pigs (16.67%). By multiplex PCR, STp was the predominant toxin gene present in 44% (8/18) isolates, followed by STh 27.8% (5/18) and LT 22% (4/18) genes. A single isolate was found to carry a combination of LT + STh genes.

3.7. Isolation of Campylobacter spp

A total of 1127 human samples were processed by microbiological culture methods to isolate *Campylobacter* spp., where 23 (2.04%) samples tested positive (Fig. 3). Culture-positive samples mainly belonged to *C. coli* (13/23, 56.52%) and *C. jejuni* (10/23, 43.48%) species. From 906 animal samples, 224 (24.72%) *Campylobacter* spp. isolates were obtained. The majority of the animal isolates were obtained from poultry (34.29%; 143/417, $p \leq 0.0001$), followed by pigs (30.29%; 63/208) and goats (6.4%; 18/281). Among the animal samples, *C. coli* (133/224, 59.4%) was the major *Campylobacter* spp. followed by *C. jejuni* (86/224, 38.4%) and *C. hyointestinalis* (5/224, 2.2%). There was variation in species distribution among the animal categories. Members of all three species were identified in goats, with *C. jejuni* (66.7%) being the predominant species, followed by *C. coli* (27.8%) and *C. hyointestinalis* (5.6%). Poultry samples carried both *C. coli* (48.3%) and *C. jejuni* (51.7%) species almost equally. *C. jejuni* was not detected in pig samples, and carriage of *C. coli* (93.7%) was significantly higher than *C. hyointestinalis* (6.3%). Isolations were obtained from stool (163/487, 33.47%) and meat (61/419, 14.56%).(Table 7).

3.8. Antibiotic resistance

3.8.1. EPEC, ETEC, and EAEC

In human isolates, high-level resistance was noted against ampicillin, cotrimoxazole, ciprofloxacin, ceftriaxone, cefepime and levofloxacin. Moderate to low level resistance was seen for piperacillin-tazobactam, gentamicin, ertapenem and amikacin. Similar to human isolates, in animal isolates, the highest resistance was observed against ciprofloxacin, followed by ampicillin, tetracycline, and third-generation cephalosporins. ESBL production ranged from 28.5 to 54% in humans and 51% to 72% in animals (Table 8). MIC value estimation also showed high resistance to ciprofloxacin, tetracycline, and ceftriaxone, consistent with disk diffusion results.

3.9. Antimicrobial susceptibility of Campylobacter

The *E*-test estimated the minimum inhibitory concentrations for

Table 6
Distribution of EPEC, EAEC and ETEC cases on Vesikari severity score for diarrhea.

DEC types	Severity	aEPEC (*n* = 71)	tEPEC (*n* = 13)	p-value
EPEC	Mild	28 (39.44%)*	1 (7.7%)	**0.0278**
	Moderate	17 (23.94%)	3 (23.08%)	0.9470
	Severe	26 (36.62%)	9 (69.24%)*	**0.0292**
	Severity	**aEAEC (n = 13)**	**tEAEC (n = 71)**	**p-value**
EAEC	Mild	4 (31%)	25 (35.2%)	**1.0**
	Moderate	6 (46%)	15 (21%)	0.2
	Severe	3 (23%)	31 (44%)	**0.5**
	Severity	**No of Isolates (%)**		
ETEC	Severe	52 (55%)		
	Moderate	22 (23.21%)		
	Mild	20 (21.42%)		
	Total	94		

* p-value < 0.05 was considered significant.

Table 5
Age and gender-wise distribution of tEPEC and aEPEC cases, tEAEC and aEAEC cases.

Age groups	N = EPEC	aEPEC (%)	tEPEC (%)	p-value	N = EAEC	tEAEC	aEAEC	P-value
0–2	37	31 (83.78%)*	6 (16.22%)	**<0.0001**	24 (24.4%)	19 (79%)	**aEAEC (%)**	**p-value**
>2–5	16	11 (67.75%)*	5 (31.25%)	**0.0421**	40 (40.8%)	31 (77%)	5 (21%)	**0.03***
>5–15	10	10 (100%)*	0	**<0.0001**	14 (14.2%)	9 (64%)	9 (23%)	**0.005***
>15–40	12	10 (83.33%)*	2 (16.67%)	**0.0014**	9 (9.1%)	4 (44%)	5 (35%)	**0.5**
>40	9	9 (100%)*	0	**<0.0001**	11 (11.2%)	8 (72%)	5 (55%)	**1.0**
Total	84	71 (84.52%)*	13 (15.48%)	**<0.0001**	98	71 (72%)	3 (27%)	**0.2**

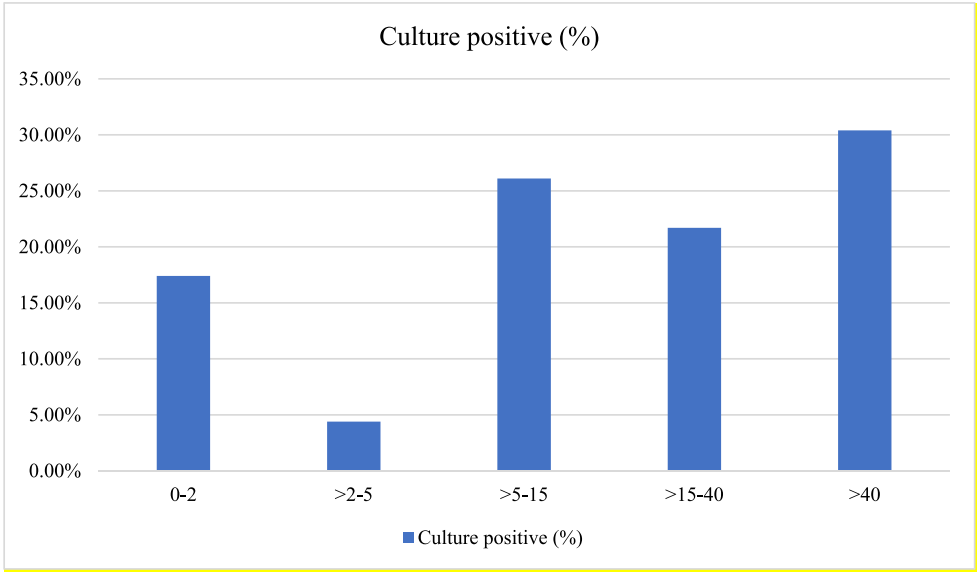

Fig. 3. Culture positive of *Campylobacter* spp. in different age groups.

Table 7
Distribution of *Campylobacter* spp. in food animals.

Source	Sample (n)	*Campylobacter* (%)	
Goat/sheep (281)	Stool (90)	11 (22.22%)	18 (8.04%)
	Meat (191)	7 (3.66%)	
Pig (208)	Stool (57)	21 (36.84%)	63 (28.13%)
	Meat (151)	42 (27.81%)	
Chicken (417)	Stool (340)	131 (38.53%)	143 (63.84%)
	Meat (77)	12 (24.49%)	
Total	906	224 (24.72%s)	

ciprofloxacin, tetracycline, azithromycin, and gentamicin. We used EUCAST guidelines to interpret MIC values for *Campylobacter* spp. and CLSI guidelines for fastidious organisms (M45). Since *Campylobacter* spp. breakpoints were unavailable in either guideline; antibiotic gentamicin was interpreted using breakpoints for *Enterobacteriaceae* from CLSI. No significant difference in resistance was observed when interpreted using two different guidelines. A single strain was found to be susceptible to all antibiotics tested. Resistance against at least one antibiotic was noted in 98.46% (64 /65), and 29.23% (19/65) were MDR isolates.

Out of the four antibiotics tested, maximum resistance against ciprofloxacin (96.92%, $p \leq 0.0001$) followed by tetracycline (50.77%) was noted. Resistances to azithromycin and gentamicin were noted in 23.07% and 13.85% isolates, respectively. Both *C. hyointestinalis* isolates were susceptible to gentamicin and tetracycline. *C. jejuni* species was more resistant as the proportion of resistance to 3 out of four antibiotics (azithromycin [$p = 0.0216$], gentamicin [$p = 0.0022$], tetracycline [$p = 0.0001$]) and several MDR strains were significantly higher in *C. jejuni* as compared to *C. coli*. Resistance to ciprofloxacin was equally high in both species (Fig. 4).

4. Discussion

This is one of the most extensive surveillance studies on integrated surveillance from India, where concurrent human and animal sampling was done in Chandigarh, Haryana, Himachal Pradesh, Punjab, Rajasthan, and Uttarakhand regions. Our sampling strategy was designed to include human and animal samples concurrently in the same spatio-temporal frame. We modeled our study on integrated surveillance as delineated in the WHO AGISAR project. Human samples included stool samples from acute gastroenteritis cases across all age groups. Fourteen laboratories participated in the study in Punjab, Haryana, Rajasthan, Uttarakhand, and Himachal Pradesh, north India. The Vesikari score measured clinical severity. Parallelly, a cross-sectional study design was used to collect stool/intestinal contents of food animals along with meat from retail shops and slaughterhouses. Samples from the slaughterhouse were collected repeatedly, cross-sectionally once a week, as each time would represent a different farm. For poultry, we sampled both big (10000-15,000) and moderate-sized (2000−3000) commercial poultry farms. Pig rearing is a budding industry in north India, and very little information is available in India regarding them being reservoirs of multidrug-resistant foodborne pathogens.

We targeted *Campylobacter* spp. and diarrhoeagenic *E. coli* as indicator foodborne pathogens as *Campylobacter* spp. DEC causes maximum hospitalizations. Among the different pathotypes of *E. coli,* Enteroaggregative *E. coli* (EAEC), Enteropathogenic *E. coli* (EPEC), and Enterotoxigenic *E. coli* (ETEC) cause acute diarrhea in children, especially in children below five years of age [21]. In India, the frequency of DEC pathotypes among diarrhea cases is as follows- ETEC (4.2–4.5%), EPEC (1.3–3.4%), and EAEC (6.8–7.3%) [22]. Enteroinvasive *E. coli* (EIEC) and Enterohaemorrhagic *E. coli* (EHEC) are uncommon.

The prevalence of foodborne pathogens causing diarrhea in our geographic region was 4.84% for ETEC, 4.32% for EPEC, and 2% for Campylobacter. The tEPEC were associated with more severe infections ($p = 0.0292$), whereas aEPEC isolates were associated with mild diarrhea ($p = 0.0278$). ETEC infections were most common in children under five years of age (44%) but were also seen in adolescents and adults. Most patients presented with severe diarrhea (55.31%, $p = 0.0001$), while 23.21% and 21.42% presented with moderate and mild diarrhea respectively. Over 80% of samples were obtained from patients with moderate to severe diarrhea, indicating that most moderate to severe diarrhea presents to health-care facilities even at the primary health-care level.

Very few studies are available for animal reservoirs of DEC in India. The reason is a requirement of molecular methods to detect DEC, which are only sometimes available. DEC is most frequently reported from cows, although other animals like chickens, deer, sheep, and pigs have also been known to carry it [18,23]. Various foods have been implicated, meat and meat products being the most common. Meat gets contaminated during slaughter when contents from the intestines of an infected animal or their feces come in contact with the carcass. The prevalence of ETEC was 3.03% in poultry from Mumbai [24], while from Kashmir, an

Table 8
Antibiotic resistance data of EPEC, ETEC, and EAEC in human and animal isolates.

Organism	Antibiotics	AK	AM	CAZ	CFM	CIP	COT	CTR	CTX	ESBL	ETP	FEP	FOX	GM	IMP	LVX	PIP-TAZ	TET
EPEC isolates	Human (n = 84)	6 (−7.14%)	65 (−77.39%)	31 (−36.9%)	NA	42 (−50%)	48 (−54.15%)	38 (−45.24%)	14 (−16.67%)	24 (28.57%)	1 (−1.19%)	23 (−27.38%)	NA	8 (−9.52%)	NA	27 (32.14%)	14 (−16.67%)	28 (−33.33%)
	Animal(n = 291)	19 (−6.53%)	239 (−82.13%)	79 (−27.15%)	NA	245 (−84.2%)	96 (−32.99)	79 (−27.15%)	147 (−50.52%)	169 (−58.08%)	11 (−3.78%)	19 (−6.53%)	NA	37 (−12.71%)	NA	81 (−27.84%)	7 (−2.4%)	141 (−48.45%)
	Total (n = 375)	25 (−6.67%)	304 (81.07%)	110 (29.33%)	NA	287 (76.53%)	144 (38.4%)	117 (31.2%)	161 (42.93%)	193 (−51.47%)	12 (−3.2%)	42 (11.2%)	NA	45 (12%)	NA	108 (28.8%)	21 (5.6%)	169 (−45.07%)
	P value	0.8437	0.3293	0.0842	NA	**<0.001**	**0.0004**	**0.0016**	**<0.0001**	**<0.0001**	0.2354	**<0.0001**	NA	0.4286	NA	0.4439	**<0.0001**	**0.0143**
ETEC isolates	Human (n = 94)	3 (3.19%)	79 (84.04%)	NA	NA	74 (78.72%)	37 (39.36%)	63 (67.02%)	NA	49 (52.13%)	3 (3.19%)	32 (34.04%)	36 (38.3%)	15 (15.96%)	0	46 (48.94%)	18 (19.15)	NA
	Animal (n = 18)	5 (27.78%)	14 (77.78%)	NA	NA	18 (100%)	5 (27.78%)	9 (50%)	NA	13 (72.22%)	4 (22.22%)	0	9 (50%)	4 (22.22%)	0	5 (27.78%)	2 (11.11%)	NA
	Total (n = 112)	8 (7.14%)	93 (83.04%)	NA	NA	92 (82.14%)	42 (37.5%)	72 (64.29%)	NA	62 (55.36%)	7 (6.25%)	32 (28.57%)	45 (40.18%)	19 (16.96%)	0	51 (45.54%)	20 (17.86%)	NA
	P value	**0.0002**	0.5187	NA	NA	**0.0316**	0.3547	0.1693	NA	0.1179	**0.0023**	**0.0035**	0.3558	0.5155	–	0.0995	0.4166	NA
EAEC isolates	Human (n = 98)	5	83 (84.6%)	NA	NA	65 (66.3%)	69 (70.4%)	62(63.2%)	NA	53 (54%)	2(2.04%)	NA	30 (30.6%)	18 (18.3%)	9(9.1%)	51 (52%)	9 (9.1%)	NA
	Animal (n = 12)	0	12 (100%)	NA	4 (32%)	9 (75%)	9 (75%)	9 (75%)	NA	7 (58.33%)	0	NA	4 (32%)	2 (16%)	1 (8%)	9 (75%)	2 (16%)	NA
	Total (n = 110)		95 (86.36%)	NA	–	74 (67.27%)	78 (70.90%)	71 (64.54%)	NA	60 (58.18%)	–	–	34 (30.90%)	20 (18.18%)	10 (9.09%)	60 (54.54%)	11 (10%)	NA
	P-value	1.0	0.8	–	–	0.8	1.0	0.8		1.0	–	–	1.0	1.0	1.0	0.47	0.6	–

p-value <0.05 was considered significant.
AK-amikacin, **AM**-ampicillin, **CAZ**-ceftazidime, **CFM**-Cefixime, **CIP**-ciprofloxacin, **COT**-co-trimoxazole, **CTR** ceftriaxone, **CTX** cefotaxime, **ESBL**-Extended-spectrum beta-lactamases, **ETP**-ertapenem, **FEP**-cefepime, **FOX**-Cefoxitin, **GM**-gentamicin, **IMP**-imipenem, **LVX**-levofloxacin, **PIP-TAZ**-piperacillin-tazobactum, **TET**- tetracycline.

8% prevalence of ETEC was reported from diarrhoeagenic calves [25]. A high carriage of EPEC (32.11%) and *Campylobacter* spp. (24.72%) was noted in food animals, but the prevalence of ETEC (2%) and EAEC (1%) was low. The meat was an important source of EPEC, contributing to 43% of animal isolates, indicating cross-contamination of meat samples with animal feces. None of the animal isolates carried the *bfp* gene, so all the isolates were aEPEC.

There is hardly any data on antibiotic resistance of DEC from humans and animals. In a study by Rasheed et al. from Hyderabad, 14.7% resistance to one or more antibiotics in *E. coli* isolates from various foodstuffs collected from local markets, with the majority of resistance to ampicillin and amoxicillin followed by tetracycline was found [26]. Overall, in our study, a very high level of resistance was observed, and on comparing resistances between human and animal isolates, resistance to piperacillin-tazobactam, cefepime, ceftriaxone, and co-trimoxazole was significantly higher in human strains. In contrast, animal resistance to ciprofloxacin, aminoglycosides, and tetracycline was higher, reflecting the corresponding usage in human and animal sectors. The MDR rate ranged from 29.2% in *Campylobacter* spp., 53.6% in EPEC to 59.8% in ETEC. ESBL production was commoner in animal isolates than in humans, indicating high use of third-generation cephalosporins in the animal sector. Carbapenems were the most susceptible class of antibiotics in all pathogens, where only 3.2% EPEC and 6.25% ETEC were resistant to ertapenem. The highest resistance was observed to fluoroquinolones in all pathogens, with 67% in EPEC, 60% in ETEC, and 96.9% in *Campylobacter* spp. resistant to ciprofloxacin.

A 100% resistance to ciprofloxacin in animal isolates of ETEC was noted. The use of broad-spectrum antimicrobials in food animals and humans has led to the emergence of multi-resistant *E. coli*. The development of resistance in *E. coli* is problematic due to their tendency to spread antimicrobial resistance genes. Resistance genes have been traced from *E. coli* in animals to *E. coli* in humans [27]. A recent example is the emergence of plasmid-mediated resistance to colistin that was identified on a pig farm and later reported from 20% percent farm animals and 15% raw meat in China. Colistin is a last resort antibiotic used only in necessary situations where no other antibiotic options are available. However, physicians are forced to prescribe more colistin due to global AMR calamity [28].

Despite using state-of-the-art culture techniques and high-quality media, *Campylobacter* spp. isolation for human samples was far lower than animal isolation. Since *Campylobacter* is a microaerophilic organism and transforms to a viable but nonculturable (VBNC) state when exposed to oxygen stress, we suspected that 2.04% positivity in human samples was not an accurate estimation and, therefore, molecular /antigen-based detection may be more sensitive. The majority of the animal isolates were obtained from poultry (34.29%), followed by pigs (30.29%) and goats (6.4%). Among the animal samples, *C. coli* (59.4%) was the major *Campylobacter* spp. followed by *C. jejuni* (38.4%) and *C. hyointestinalis* (2.2%). There was variation in species distribution; in goats, *C. jejuni* (66.7%) was the predominant species, followed by *C. coli* (27.8%) and *C. hyointestinalis* (5.6%). Poultry samples carried both *C. coli* (48.3%) and *C. jejuni* (51.7%) equally. *C. jejuni* was not detected in pig samples, and carriage of *C. coli* (93.7%) was significantly higher than *C. hyointestinalis* (6.3%). Meat samples contributed to 61 (27.2%) of animal isolates. *Campylobacter hyointestinalis* is an emerging zoonotic pathogen isolated from India for the first time. This pathogen has been associated with extra-intestinal invasive infections in world literature. *Campylobacters* were highly resistant to ciprofloxacin (96.92%, $p \leq 0.0001$) and tetracycline (50.77%). Resistances to azithromycin and gentamicin were noted in 23.07% and 13.85% isolates, respectively. *C. jejuni* species were more resistant, and MDR strains were significantly higher in *C. jejuni* than in *C. coli*. A recent study of 18 years on antibiotic resistance in Campylobacter showed a decreasing trend in AMR. However, the rates are still high despite decades of reduced animal antibiotic usage [20,29,30].

The shedding of microorganisms into the environment may signify a

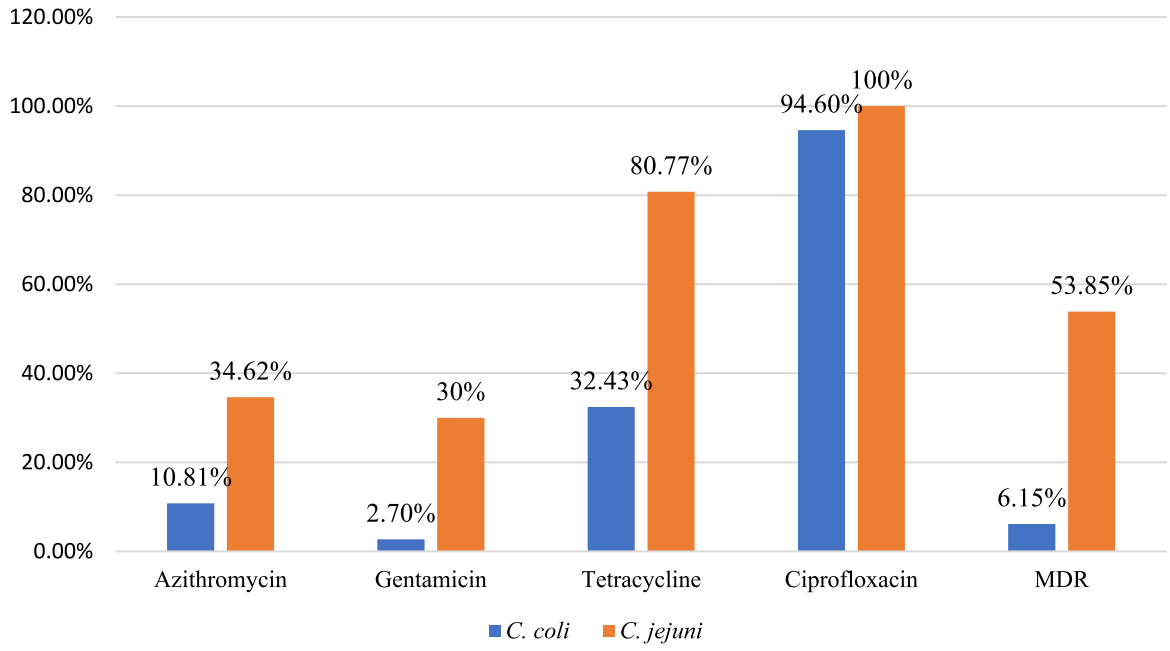

Fig. 4. Comparison of AMR between *C. coli* and *C. jejuni* species.

link between food and water contamination and human infections. Hence, the concept of one health comes into the picture. There are many lacunae in Indian settings regarding the use of antimicrobials in livestock, such as lack of implementation of guidelines on antibiotic use in feed, extensive use of antibiotics of human disease treatment as growth promoters in animals, limited knowledge of antibiotic resistance patterns or resistance gene pool in food animals as well as limited knowledge of the rate of transmission of antibiotic resistance from animals to humans. Hence, there is a need for veterinary surveillance of antimicrobial resistance and antimicrobial use, raising awareness among professionals and farmers, and strengthening the national drug regulatory authorities in the animal health sector.

The power of whole genome sequencing is increasingly employed to address the public health challenge of AMR, supporting surveillance, outbreak investigation, and improving diagnostics and therapeutics. Further mathematical analysis and modeling can be used to address the transmission pathways and control the spread of infections [31–33].

5. Conclusion

Our study is one of the most extensive surveillance study based on the integrated surveillance of AMR in foodborne pathogens, as delineated in the WHO-AGISAR protocol. This study fills important gaps in our knowledge of the burden of key foodborne pathogens and AMR. A high burden of foodborne pathogens was found in animal samples. Also, high resistance levels were observed towards fluoroquinolones, tetracyclines, third-generation cephalosporins, and aminoglycosides. Interestingly, ESBL production was commoner in animal isolates than in human isolates. Despite using state-of-the-art culture techniques and high-quality media, isolation of *Campylobacter* spp. for human samples was far lower than animal isolation. Whole genome sequencing can elucidate the characterization of AMR genes and transmission pathways at the human-animal interface.

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CRediT authorship contribution statement

Jaspreet Mahindroo: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Chandradeo Narayan:** Investigation, Methodology, Data curation, Formal analysis. **Vinay Modgil:** Data curation, Investigation, Methodology, Formal analysis. **Harpreet Kaur:** Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Varun Shahi:** Data curation, Formal analysis, Investigation, Methodology. **Bhawna Sharma:** Formal analysis, Methodology, Visualization. **Ruby Jain:** Resources. **Siddhartha Thakur:** Conceptualization, Funding acquisition, Resources. **Balvinder Mohan:** Supervision. **Neelam Taneja:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

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