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Original Article

Prevalence and Molecular Detection of Virulence Genes among Multidrug-Resistant *Escherichia coli* from Human Clinical Samples and Poultry in Duhok City, Iraq

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Abstract

Background: Extended-spectrum beta-lactamase (ESBL)-producing, carbapenem-resistant Escherichia coli has increased virulence and multidrug resistance (MDR). **Objectives:** This study was designed to ascertain the frequency of some virulence factor genes, antibiotic susceptibility patterns, ESBLs, and MDR, focused on colistin-resistant *E. coli* strains of human and animal origin in Duhok city, Iraq. **Materials and Methods:** Between December 2020 and April 2021, a total of 150 *E. coli* isolates (110 from human clinical specimens and 40 from poultry cloacal swabs) were included in this study. The isolates underwent screening for antibiotic susceptibility, MDR, ESBL, and molecular detection of four virulence genes (*fimA, cnfL, crL,* and *hlyA*) was conducted using the polymerase chain reaction. **Results:** Urine specimens (77.2%) compared to blood, wound, vaginal swab, sputum, and semen from outpatients (71.8%). All strains from humans and poultry showed high resistance to ampicillin (86%–97%), ceftriaxone (74%–47%), tetracycline (72%–85%), ciprofloxacin (48%–97%), and colistin (17%–12%). The lowest levels of resistance were found for carbapenems (4%–4%), and the MDR for the isolates was 63%–93%. Apart from carbapenems, colistin-resistant isolates, especially those from poultry, exhibited significant resistance to other antibiotics, and 57% of these isolates being ESBL producers. Three virulence genes (*fimA, cnfL*, and *crL*) were highly prevalent (92%) in human isolates, with the *crL* gene being predominant (100%). Among poultry isolates, *fimA* was more prevalent (94%) while *crL* was less common (6%). **Conclusion:** The predominance of isolates of colistin-resistant poultry origin and the virulence of isolates of human *E. coli* origin indicate that both strains are currently experiencing an increase in antibiotic resistance.

Keywords: Antibiotics, clinical samples, *Escherichia coli*, poultry, virulence factors

Introduction

Escherichia coli is a bacterium that causes infections in both humans and animals. Genetic variation allows it to flourish in a variety of ecological niches. It is regarded as one of the most well-known bacterial sources of infections, including extraintestinal infections and diarrheal disease caused by food.[1] *E. coli* has a variety of virulence factors that enable it to both cause and survive those infections. Additionally, the virulence factors encourage host colonization and invasion, undermine various host defense mechanisms or disturbance, cause host tissue injury, and/or stimulate noxious host inflammatory reactions.[2] The rate of multidrug resistance (MDR) in Enterobacteriaceae is alarmingly rising as a result of widespread distribution and indiscriminate use of antibiotics in both human and livestock production systems. Additionally, those MDR isolates contain multiple plasmids that have undergone horizontal genetic transfer (between and within species.[3] In recent years, there has been an increase in the transmission of bacterial infections that produce extended-spectrum beta-lactamases (ESBLs) and carbapenem-resistant Enterobacteriaceae in both humans and animals.[4]

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Colistin, a polymyxin antibiotic, has been regarded as a last-resort antibiotic for treating Gram-negative bacterial infections, either alone or in combination with other medications. Colistin has also been applied to livestock and poultry for many years worldwide.[5] The emergence of resistance to colistin has previously been documented.[6,7] It follows that the widespread use of colistin in animals raised for food has contributed to the spread of colistin resistance in human antibiotic therapy.[5] For the treatment of infections brought on by carbapenem-resistant Enterobacteriaceae in humans, colistin is now occasionally used.[8] The extensive use of human antibiotics in agriculture and veterinary medicine, where some nations have actively used colistin in animals raised for food, further jeopardizes this delicate balance between clinical necessity and resistance prevention.[9] No study has ever found characteristics of *E. coli* strains in our setting, as far as we know, in Duhok, Iraq, comparing clinical samples obtained from humans compared to those obtained from poultry. This study was designed to ascertain the frequency of some virulence factor genes, antibiotic susceptibility patterns, ESBLs, and MDR, focused on colistin-resistant *E. coli* strains of human and animal origin in Duhok city, Iraq.

Materials and Methods

Study design, subjects, and specimen collection

In Duhok city, Iraq, three governorate hospitals and one central health lab were involved in this cross-sectional study. The patients were received as inpatients (hospitalized) or attended different clinic departments (out-patient) at the Azadi Teaching Hospital, Emergency Hospital, Heevi Pediatric Hospital, and Central Health Lab in Duhok, Iraq. Before the study commenced, all participants provided written informed consent, and the confidentiality of the information gathered was ensured.

The Ethics Committee for Duhok University and Duhok General Health Directorate approved the study protocol. The *E. coli* isolates used in this investigation were collected between December 2020 and April 2021.

As part of standard hospital laboratory procedures, *E. coli* strains were isolated and were further identified and verified in the laboratory. Out of 1600 clinical specimens, 110 non-duplicate, non-consecutive clinical isolates of *E. coli* were found in 110 cases of human infections. On another hand, this study included 40 *E. coli* isolates obtained from poultry cloacal swabs. Of those 110 human cases, 82 were female and 28 were male, with ages ranging from 1 day to 89 years and a mean age of 32.40 years. The clinical isolates were obtained from a urine sample ($n = 85$), wound swab ($n = 15$), vaginal swab ($n = 7$), sputum ($n = 2$), blood ($n = 2$), and semen fluid ($n = 2$). The Duhok abattoir in Duhok city, Iraq, provided the veterinary samples (50 poultry cloacal samples). Patients who had not received antibiotics 3 days before enrollment were included; those who had received antibiotics within 3 days, pregnant women, and patients who refused to provide a sample or participate in the study were excluded.

Sample processing and identification

By using a sterile loop, all samples (human and poultry) were streaked on blood agar, MacConkey agar, and chrome agar medium (Oxoid, UK). The samples were then incubated at 37°C for 24–48 h. Isolates were only included after a pure culture with $> 10^5$ cfu/mL was obtained from urine samples that were cultured using a standard sterile calibrated loop. For blood culture, 5 mL of blood from adult patients was drawn into sterile syringes, suspended in 45 mL of brain heart infusion broth (Oxoid), and then incubated at 37°C for 5–7 days. They were then streaked on blood and MacConkey agar by a sterile loop and incubated overnight at 37°C.[10] Colony morphology and traditional biochemical tests (Gram staining, oxidase, IMVIC, and Triple Sugar Iron Agar) were used to identify the isolates (Alpha Chemika, India), and microbial species-level identification was achieved using the VITEK-2 Compact system (Biomerioux. France) and through uidA gene amplification using the primers listed in Table 1 and a Master Cycler® (Applied Biosystems, Singapore) personal polymerase chain reaction (PCR).

Antibiotic susceptibility test

An agar disk diffusion method was used to determine the bacteria's susceptibility to a panel of 16 antibiotics of different classes such as beta-lactam antibiotics (ampicillin, amoxillin– clavulanic acid, piperacillin–tazobactam, ceftriaxone, ceftazidime, cefepime, cefoxitin, cefotaxime, meropenem, and imipenem), aminoglycoside (gentamicin), fluoroquinolone (ciprofloxacin), trimethoprim/sulfamethoxazole, chloramphenicol, tetracycline, and colistin. Pure cultures of recognized bacteria were developed in 0.85% saline to 0.5 McFarland turbidity norms and distributed on Muller–Hinton Agar media using sterile swabs. The Clinical and Laboratory Standards Institute[11] provided guidelines for the interpretation of the findings. The majority of the data that were collected were qualitative (resistant, intermediate, or susceptible). MDR isolates are resistant to three or more antimicrobials from various classes.[12]

Phenotypic detection of extended-spectrum β-lactamase production

According to Drieux *et al.*[13] all isolates underwent a double disc synergy test with four antibiotics: amoxicillin/clavulanic acid (AMC, 20/10 mcg), cefotaxime (30 g), ceftazidime (30 g), and aztreonam (ATM, 30 g) to determine whether they produced ESBLs.

Preparation of DNA templates for PCR testing

Sixty *E. coli* isolates (42 from humans and 18 from poultry) were chosen for chromosomal extraction using the heat shock method and used as templates for PCR assays. As reported by Yang *et al*.,[14] five pure and fresh colonies were suspended in 200 µL of dry weight, and the bacterial cells were lysed by boiling at 100°C for 20 min (in a water bath). The other cellular components were separated by centrifuging them at 9000 rpm for 10 min after they had been placed on ice for 40 min. The DNA template was then created using the supernatant.

PCR amplification

PCR reactions were performed in a final reaction volume of 50 µL containing 8 µL of DNA, 4 µL (2 µL forward + 2 µL reverse) of 30 pmol of each of the primers, 25 µL of Master Mix (200 mM of dNTP and 1.25 U Taq DNA polymerase in 1X PCR buffer containing 1.5 mM $MgCl_2$), and 13 µL DDW. The PCR thermal cycler device (BioRad-USA) was employed for amplification of bacterial DNA. Those sixty selected human and poultry *E. coli* isolates were screened for four virulence factors genes: *fim*A (fimbria), *hly*A (hemolysin), *cn*f1 (cytotoxin protein), and *cr*L (siderophore). PCR primers (Macrogen, South Korea) and cycling conditions are listed in Table 1. The amplification was carried out in a thermal cycler machine, and conditions consisted of an initial denaturation at 94°C for 10 min, followed by 35 cycles of denaturation at 94°C for 2 min, annealing at a specific temperature for 30 s [Table 1], and extension at 72°C for 1 min. Then, 10-mL aliquots of the PCR product underwent gel electrophoresis on 1.5% agarose, followed by staining with DNA SafeStain (Hydrgreen, Czech Republic). The size of the amplicons was estimated by comparing them on gel electrophoresis with a 100-bp DNA marker. Amplified DNA fragments of specific sizes were detected by ultraviolet (UV)-induced fluorescence.[15-20]

The final reaction volume for PCR reactions was 50 µL, which contained 8 µL of DNA, 4 µL (two forward and two reverse) of 30 pmol of each primer, 25 µL of the master mix (200 mM of dNTP and 1.25 U Taq DNA polymerase in 1× PCR buffer containing 1.5 mM $MgCl_2$) and 13 µL DDW. The PCR thermal cycler (BioRad, USA) was used to amplify the bacterial DNA. The four virulence factor genes *fim*A (fimbria), *hly*A (hemolysin), *cn*f1 (cytotoxin protein), and *cr*L (siderophore) were screened for in those sixty chosen human and poultry *E. coli* isolates. Table 1 lists the PCR primers (Macrogen) and cycling conditions. The conditions for the amplification, which was done in a thermal cycler machine, were as follows: initial denaturation at 94°C for 10 min, followed by 35 cycles of denaturation at 94°C for 2 min, annealing at a specific temperature for 30 s, and extension at 72°C for 1 min. Then, 10-mL aliquots of the PCR product underwent gel electrophoresis on 1.5% agarose, followed by staining with DNA SafeStain. By comparing the amplicons' sizes on gel electrophoresis to a 100-bp DNA marker, their sizes were estimated. DNA fragments that have been amplified and are of a certain molecular weight were detected by UV-induced fluorescence.

Statistical analysis

Data analysis was performed on the study sample, and the statistics were described using means, standard deviations, frequencies, and percentages. Data were analyzed using statistical package for the social sciences v23 (SPSS Inc., Chicago, IL, USA).

Results

A total of 150 isolates, including 40 from poultry and 110 from humans, were identified as belonging to the *E. coli* species after PCR amplification targeted *uid*A gene (147 bp), and conventional biochemical tests were conducted. Urine samples from outpatients (71.8%), women (74.2%), and those of the age range of 21–30 years (20%) at Azadi Teaching Hospital (28.1%) were the most frequently implicated in clinical *E. coli* isolates from humans. Blood, sputum, and semen fluid yielded lowest rates (each at 1.8%). Out of 50 poultry cloacal swabs taken, 40 (80%) contained the *E. coli* isolates [Table 2].

The results presented in Table 3 showing the antibiotic susceptibility patterns of *E. coli* isolates from human

Table 1: Primer sets and PCR cycling conditions used for *E. coli* confirmation and amplification of virulence factor genes

Primer	Sequence 5′ → 3′	Amplicon size	Annealing temperature (°C)	References
fimA	F: GCACCGCGATTGACAGC R: CGAAGGTTGCGCCATCCAG	132	56	Nojoomi and Ghasemian[15]
hlyA	F: GGT GCA GAA AAA GTT GTAG R: TCT CGC CTG ATA GTG TTT GGT	1551	59	Karch *et al.*[16]
cnf1	F: GGGGGAAGTACAGAAGAATTA R: TTGCCGTCCACTCTCACCAGT	1112	60	Raisch *et al.*[17]
Crl	F: TTTCGATTGTCTGGCTGTATG R: CTTCAGATTCAGCGTCGTC	250	59	Maurer *et al.*[18]
uidA	F: AAAACGGCAAGAAAAAGCAG R: ACGCGTGGTTACAGTCTTGCG	147	58	Ramirez-Martinez *et al.*[19]

Table 2: Frequency of *E. coli* isolates in human clinical specimens and poultry samples

General characteristics (human = 110 isolate)	Statistics	
	No. of positive isolates	Percentage
Age (range: 1 month–85 years)	Mean = 32.40	SD = 20.98
Age category (years)		
1–10	21	19.09
11–20	12	10.91
21–30	22	20.00
31–40	19	17.27
41–50	13	11.82
51–60	14	12.73
61–70	5	4.55
71–80	2	1.82
81–90	2	1.82
Gender		
Female	82	74.2
Male	28	25.8
Clinical specimens		
Blood	2	1.82
Semen	2	1.82
Sputum	2	1.82
Urine	85	77.27
Vaginal swab	7	6.34
Wound	12	10.91
Hospitals		
Azadi	31	28.18
Emergency	30	27.27
Heevi	24	21.82
Central lab	25	22.73
Patients		
Inpatient	31	28.18
Outpatient	79	71.82
E. coli prevalence (n = 1600)	110	6.88
Poultry cloacal swab (50)	40	80

clinical specimens and poultry samples. All human and poultry strains generally exhibited significant resistance to penicillin, cephalosporins, aminoglycosides, and fluoroquinolones. The lowest resistance was found for imipenem and meropenem (4%–2%), respectively, indicating that carbapenems were the most effective antibiotics.

According to our findings, 69 (63%) and 37 (93%) of the *E. coli* isolates from clinical and poultry samples, were MDR, respectively. The 69 MDR clinical isolates were found in the following specimens: blood (2), sputum (2), wound swabs (5), HVS (4), urine (45), and wound swabs (5). Clinical *E. coli* isolates that were colistin-resistant showed high resistance to beta-lactams (apart from carbapenem), relatively moderate resistance to ciprofloxacin and trimethoprim/sulfamethoxazole, and low resistance to gentamicin. On the other hand, only cephalosporins and carbapenems were effective antibiotic colistin-resistant poultry *E. coli* isolates [Table 4].

The highest ESBL producers (57%) were found among human clinical isolates that were also resistant to colistin, which were statistically significant [Table 5]. A total of 60 *E. coil* isolates (42 humans; 18 poultry) were chosen for the molecular detection of four virulence genes (*fim*A, *cnf*L, *cr*L, and *hly*A), high rates (80%–90%) of human and poultry isolates were carried by double genes (*fim*A and *cr*L), with the *cr*L gene being predominant (100%). Frequency of those genes was less detected in poultry isolates, where *fim*A was prevalent (94%) and *cr*L was only 6%. Neither clinical isolates nor isolates from poultry contained the *hly*A gene [Table 6].

Discussion

Detection of *E. coli* virulence traits and antimicrobial resistance from strains of various origins are crucial for

Table 3: Antibiotic susceptibility patterns of *E. coli* isolates from human clinical specimens and poultry samples

Antibiotics	Human no. (%)			Poultry no. (%)		
	Resistant	Intermediate	Sensitive	Resistant	Intermediate	Sensitive
Ampicillin (Am)	95 (86)	0 (0.0)	15 (13)	39 (97)	1 (2)	0 (0)
Amoxillin + clavulanic acid (AMC)	61 (55)	16 (14)	33 (300)	26 (65)	13 (32)	1 (2)
Piperacillin–tazobactam (TZP)	25 (23)	10 (9)	75 (68)	2 (5)	0 (0)	38 (95)
Ceftriaxone (CRO)	82 (74)	0 (0.0)	28 (25)	19 (47)	1 (2)	20 (50)
Ceftazidime (CAZ)	84 (76)	0 (0.0)	26 (24)	12 (30)	2 (5)	26 (65)
Cefepime (FEB)	83 (75)	0 (0.0)	27 (25)	11 (27)	4 (10)	25 (62)
Cefoxitin(CFM)	53 (48)	5 (4)	52 (47)	6 (15)	5 (12)	29 (72)
Cefotaxime (CTX)	73 (66)	1 (0.9)	36 (33)	10 (25)	3 (7)	27 (67)
Meropenem (MRP)	4 (3)	0 (0.0)	106 (96)	1 (2)	0 (0)	39 (97)
Imipenem (IMI)	5 (4)	1 (0.9)	104 (94)	1 (2)	0 (0)	39 (97)
Gentamicin (CN)	37 (33)	0 (0.0)	73 (66)	22 (55)	0 (0)	18 (45)
Tetracycline (TE)	79 (72)	1 (0.9)	30 (27)	34 (85)	1 (2)	5 (12)
Trimethoprim/sulfamethoxazole (SXT)	59 (54)	1 (0.9)	50 (45)	25 (62)	0 (0)	15 (37)
Chloramphenicol (C)	33 (30)	2 (2)	75 (68)	30 (75)	0 (0)	10 (25)
Ciprofloxacin (CIP)	53 (48)	2 (2)	55 (50	39 (97)	0 (0)	1 (2)
Colistin (CS)	19 (17)	7 (6)	84 (76)	5 (12)	7 (17)	28 (70)

Table 4: Resistance rates of colistin-resistant *E. coli* isolates to other antibiotics

Isolates	Antibiotic resistance rates													
	AM	*AMC*	*CRO*	*CAZ*	*FEP*	*CFM*	*CTX*	*MEM*	*IPM*	*CN*	*TE*	*SXT*	*C*	*TZP*	*CIP*
Human	95	68	79	79	84	58	73	0.5	0.5	26	79	58	42	42	58
Poultry	100	40	40	40	10	0	40	0	0	80	100	80	80	0	100

understanding epidemiological data of causative agents, allowing doctors to offer alternative treatment options, improve patient outcomes, and optimize excellent infection control methods.[21] In our study, most clinical *E. coli* isolates were obtained from urine (77.2%) compared to blood, sputum, and semen fluid (each at 1. 8%), and females had a higher incidence of *E. coli* in urine samples (59%) than males (40.95%). Urine was the most frequent clinical specimen, and females composed the majority of the study participants. This result was in accordance with those of several authors worldwide.[5,22,23] The majority of the samples used in our study were from adults in their middle years. Nevertheless, Spencer *et al*.[5] found that male patients with an average age of 68.5 years (range, 53–93 years) had a high rate of UTI. In this study, out of 50 poultry cloacal swabs collected, 40 (80%) contained *E. coli* isolates.

The percentage of human and poultry isolates with MDR in this study was 63%–93%, respectively, for most of the tested antimicrobials. This is consistent with the observation in other studies alike.[24,25] According to the study's findings, ampicillin, amoxicillin–clavulanic acid, ceftazidime, trimethoprim–sulfamethoxazole, cefoxitin, ceftriaxone, cefepime, and cefotaxime were most ineffective against isolates of human and animal origin in percentages of 86%–97%, 55%–65%, 76%–30%, 54%–62%, 48%–15%, 74%–47%, 75%–27%, and 66%–25%, respectively, and has led to challenges in clinical practice. This research is in line with those on human isolates, supporting the findings of Yılmaz *et al.*[26] and Salman *et al.*[27] and disagreeing with those of Maleki *et al*.[28] in Iran, stating that ceftazidime and cefotaxime had 26.1% and 30% resistance rates, respectively. Our findings that MDR was very high in poultry strains were supported by studies that found that poultry *E. coli* isolates had higher levels of antibiotic resistance than beef isolates.[29,30] The resistance might be due to the ESBL enzyme, which is essential for clavulanic acid resistance, the efflux pump mechanism, as well as frequent use of these antibiotics in empirical therapy, especially in domestic/companion animals and agricultural fields.[26-28,31]

Ciprofloxacin resistance was 48% in human isolates and extremely high 97% in those of poultry origin. Studies on human isolates correspond with those of Tajbakhsh *et al*.[32] and Ramírez-Castillo *et al*.[33] who found ciprofloxacin resistance ratios of 56% and 47.3%, respectively. High ciprofloxacin resistance is brought on by bacterial biofilm formation, overuse of quinolones in veterinary medicine and UTI treatment, as well as food-borne transmission from animal sources to human.[34,35] According to our data, imipenem and piperacillin–tazobactam resistance rates in humans and poultry were 40–5% and 4–2%, respectively. Closer to the findings of Al-No'aemy[31] who recorded low resistance (4.7%) to imipenem, while in contrast to Zaman *et al*.[36] stated that the piperacillin–tazobactam resistance rate was 8%. This demonstrates that these antibiotics were successful in treating *E. coli* once, but due to self-medication

Table 5: Association of ESBL production with colistin and carbapenem susceptibility patterns of human clinical *E. coli* isolates

Antibiotics	ESBL	Antibiotic susceptibility No. (%)			p-value
		Resistant	Intermediate	Sensitive	
Colistin	Negative	8 (42)	4 (57)	53 (63)	0.2422
	Positive	11 (58)	3 (43)	31 (37)	
Meropenem	Negative	3 (75)	0 (0.0)	62 (58)	0.6433
	Positive	1 (25)	0 (0.0)	44 (42)	
Imipenem	Negative	4 (80)	1 (100)	60 (57)	0.4316
	Positive	1 (20)	0 (0.00)	44 (43)	

Pearson chi-squared tests were performed for statistical analyses

Table 6: Frequency of virulence factor genes among *E. coli* isolates from human clinical specimens and poultry samples

Virulence factors	Human (n = 42)		Poultry (n = 18)		*P* value
	Number	Percentage	Number	Percentage	
*fim*A					
Negative	9	21.43	1	5.56	0.0102
Positive	33	78.57	17	94.4	
*hly*A					
Negative	42	100	18	100	NA
Positive	0	0	0	0	
*cnf*I					
Negative	33	78.57	17	94.4	0.1305
Positive	9	21.43	1	5.56	
*Cr*L					
Negative	0	0	0	0	NA
Positive	42	100	18	100	

Fishers' exact test was performed for statistical analyses

and excessive antibiotic use, both of which are quite common in this region, antibiotic resistance is growing.

ESBLs are one of the most important virulence factors that help in destroying the β-lactam ring in certain antibiotics and, thus, increase antibiotic resistance and virulence of *E. coli*. Indeed, this study found that 63% of human isolates produced ESBLs, with a frequency of 57% and 20% among colistin-resistant and carbapenem-resistant isolates, respectively. A similar study conducted in Egypt reported that 20% of ESBL-*E. coli* producers had carbapenem resistance.[37] This could be attributed to the widespread use of carbapenems as an empirical treatment for ESβLsinfections.[22] With the growing emergence of the carbapenem-resistant Enterobacteriaceae, some old antibiotics like colistin were used for treatment of ESBL infections with marked increase in usage. Unexpectedly, our data observed high ESBL producers (58%) among colistin-resistant human isolates. This finding is alarming because it raises the possibility of treatment failure and calls for the adoption of new antibiotic prescription guidelines, especially in the veterinary field where antibiotics are still used as growth promoters at sub-inhibitory concentrations.

In this study, all human and poultry isolates tested positive for at least one of the four examined virulence genes, which are as follows: single gene (7%–5%), double gene (80%–90%), and triple gene (13%–5%). The frequency of the expressed genes was *fim*A (human = 78%; poultry = 94%), *cr*L (human = 100%; poultry = 0%); and *cnf*1 (human = 22%; poultry = 6%). *cr*L in humans and *fim*A in poultry are the two most predominant genes, with a prevalence of 100% and 94%, respectively.

Conclusion

The study concluded that poultry *E. coli* strains are characterized by harboring more MDR and less virulence factors when compared with those isolates from human origin that harbored more virulence genes and colistin resistance determinants that pose a threat and limit therapeutic options. All isolates harbored single, double, and triple gene collections of virulence genes. For this reason, more detailed studies are needed to determine the correlation between virulence traits, antibiotic resistance, and certain *E. coli* clones that cause various infections in humans and veterinary sources. Antibiotic overuse, particularly at sub-inhibitory concentrations, in livestock systems needs to be reevaluated.

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Conflict of interest

There are no conflicts of interest.

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