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ORIGINAL ARTICLE

Multiplex polymerase chain reaction detection of Shiga toxin genes and antibiotic sensitivity of *Escherichia coli* O157:H7 isolated from beef meat in Quetta, Pakistan

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Abstract

The objective of this study was to conduct a qualitative analysis of raw beef meat sold in the city of Quetta, Pakistan for presence and drug sensitivity of the potentially pathogenic *Escherichia coli* strain O157:H7. The study used 200 raw beef meat samples collected from retail butcher shops. Conventional and rapid biochemical tests, latex agglutination and multiplex polymerase chain reaction (PCR) using primers designed for the rfb_{O157} and $flic_{H7}$ genes were used to detect *E. coli* O157:H7. All O157:H7 isolates were also tested for Shiga toxin genes stx_1 and stx_2. The prevalence of *E. coli* O157:H7 in collected beef meat samples was 10%. Detection through PCR was found more sensitive than detection of O and H antigens. The quantity of *E. coli* O157:H7 isolates positive for Shiga toxins was 50% (20% for stx_1, 45% for stx_2 and 10% for both stx_1 and stx_2). Season wise variation showed highest *E. coli* O157:H7 prevalence during summer months. A further concern is that *E. coli* O157:H7 isolates were resistant to a range of common antibiotics. The results indicate an urgent need for applying proper food hygiene practices in the Quetta region to reduce incidence of foodborne diseases and they also emphasize the global problem of antimicrobial resistance.

Practical applications

E. coli O157:H7 is as a potentially threatening foodborne pathogen. A significant prevalence of *E. coli* O157:H7 detected in raw beef meat from retail outlets in the city of Quetta indicates an urgent need for applying proper food hygiene practices in the Quetta region to reduce the incidence of foodborne diseases. Furthermore, resistance of the *E. coli* O157:H7 isolates to a range of commonly used antibiotics emphasizes the global problem of antimicrobial resistance. The multiplex PCR method used here is a reliable, sensitive, and relatively rapid technique for

detecting *E. coli* O157:H7 in food and environmental samples and important for ongoing surveillance to minimize contamination of raw meat products and associated cross contamination by *E. coli* O157:H7.

1 | INTRODUCTION

Foodborne infections have large effects on public health and on the economy worldwide (Havelaar et al., 2015; Hoffmann et al., 2017; Kirk et al., 2015; Yang, Lin, Aljuffali, & Fang, 2017). Indeed, microbial contamination of meat due to improper handling and processing poses great health risks around the globe (Ahmed & Sarangi, 2013; Antwi-Agyei & Maalekuu, 2014; Bakhtiary et al., 2016; Hassan Ali, Farooqui, Khan, Khan, & Kazmi, 2010; Jayathilakan, Sultana, Radhakrishna, & Bawa, 2012; Kim & Yim, 2017). Among foodborne pathogens, *Escherichia coli* O157:H7 is broadly categorized as a threatening pathogen (Armstrong, Hollingsworth, & Morris Jr., 1996; Bidet et al., 2005; Kiranmayi, Krishnaiah, & Mallika, 2010).

E. coli is a Gram-negative bacterium from the family Enterobacteriaceae that lives in the intestines of humans and other animals and does not usually cause any health risks. Some strains, however, have modified themselves to become equipped with toxigenic or pathogenic factors. By virtue of their pathogenic factors, such strains can cause several lethal diseases. On the basis of somatic 'H' (flagellar) antigens, 'O' (lipopolysaccharide) antigens and 'K' (capsular) antigens, *E. coli* are classified as different serotypes (Fratamico et al., 2016; González Garcia, 2002; Orskov & Orskov, 1992; Yang et al., 2016). Infections caused by pathogenic *E. coli* strains are wide-ranging depending on their serotypes and include: hemolytic uremic syndrome (HUS), urinary tract infection, neonatal meningitis, respiratory illnesses, and intestinal diseases. Pathogenic or diarrheagenic *E. coli* strains are divided into different pathotypes (STEC, EPEC, ETEC, EIEC, EAEC, and DAEC) depending on the nature of the disease caused by the specific strain: enteropathogenic (EPEC), Shiga toxin-producing/enterohemorrhagic (STEC/EHEC), Shigella/enteroinvasive (EIEC), enteroaggregative (EAEC), enterotoxigenic (ETEC), and diffusely adherent (DAEC) (Ibrahim, 2015).

E. coli serotype O157:H7 was discovered in 1982 (Riley et al., 1983), it has produced outbreaks worldwide and is ranked among the most studied foodborne pathogens (Adamu, Shamsul, Desa, & Khairani-Bejo, 2014; Bell et al., 1994; Ibrahim, 2015; Kieckens et al., 2015; Torso et al., 2015). Natural reservoirs of this pathogen include cattle, sheep, and goats, and the modes of transmission of the infections are animal to person, waterborne, foodborne, and person to person (Ateba & Mbewe, 2011; Ferens & Hovde, 2011). *E. coli* O157:H7 is within the class EHEC that produces Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2), which are regulated by the genes stx_1/stx_2 along with other virulence factors (intimin and enterohemolysin) (Parsons, Zelyas, Berenger, & Chui, 2016). Symptoms of *E. coli* O157:H7 infections include bloody diarrhea (hemorrhagic colitis), non bloody diarrhea, HUS, and thrombotic thrombocytopenic purpura (Griffin et al., 1988), which can be life-threatening if not treated successfully. Conventional methods to detect *E. coli* O157:H7 are laborious, time consuming, and suffer from poor sensitivity (Deisingh & Thompson, 2004). To ensure food safety, rapid and reliable diagnostic tools are essential for detecting *E. coli* O157:H7 in different food products. Multiplex real-time polymerase chain reaction (PCR) has emerged as the most attractive alternative to culture-based and immunological-based methods for detecting *E. coli* O157:H7 (Jinneman, Yoshitomi, & Weagant, 2003; Li, Liu, & Wang, 2017; Tabashsum, Nazneen, Ahsan, Bari, & Yasmin, 2016; Van Giau, Nguyen, Nguyen, Le, & Nguyen, 2016; Zhou et al., 2017).

The main objective of this study was to estimate the prevalence of Shiga toxin producing *E. coli* O157:H7 in raw beef sold in Quetta, Pakistan by using biochemical tests, latex agglutination, and PCR methods. Another objective was to test the sensitivity of *E. coli* O157:H7 isolates to commonly available antibiotics.

2 | MATERIALS AND METHODS

2.1 | Sample collection

This study used 200 raw beef samples (100 samples each of diced and ground/minced) that were collected randomly during January 2016–August 2016 from 100 different retail butcher shops in Quetta, Pakistan. These shops were a minimum distance of 100–300 m from each other. Samples were stored in sterilized plastic containers in an ice box and brought to the Center for Advanced Studies in Vaccinology and Biotechnology (CASVAB) Bacteriology laboratory, University of Balochistan for immediate processing.

2.2 | Culture conditions and biochemical identification

Approximately 25 g of sample was transferred aseptically to modified trypticase soy broth (mTSB) (225 mL) and this was incubated at 37 °C for 24 hr. Later, non-sorbital fermenting (NSF) colonies were selected from MacConkey agar with sorbitol, cefixime, and tellurite (CT-SMAC) plates and streaked onto eosin methylene blue (EMB) agar plates, which were further incubated at 37 °C for 24 hr. On EMB, typical *E. coli* with a metallic shine were further characterized by biochemical tests (indole, methyl red, vogesproskauer, citrate, and lysine decarboxylase tests) as reported previously (Lee & Choi, 2006; You et al., 2006). For further confirmation that the isolated colonies were *E. coli*, the Remel RapID ONE System (Thermo Fisher Scientific, U.K.) was also used, which identifies medically important Enterobacteriaceae and other oxidase-negative Gram-negative bacilli based on enzyme technology.

TABLE 1 Oligonucleotide primers for PCR amplification of the genes rfb_{O157}, $flic_{H7}$, stx_1, and stx_2

Target gene	Primer sequence 5′–3′	Product size	Thermal cycle program	Reference
rfb_{O157}	F-CGGACATCCATGTGATATGG R-TTGCCTATGTACAGCTAATCC	259 bp	Initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 30 sec, elongation at 72 °C for 1 min and extension at 72 °C for 10 min.	Gannon et al. (1997)
$flic_{H7}$	F-GCGCTGTCGAGTTCTATCGAG R-CAACGGTGACTTTATCGCCATTCC	625 bp		
stx_1	F-ACACTGGATGATCTCAGT GG R-CTGAATCCCCCTCCATTATG	614 bp	Initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, elongation at 72 °C for 2 min and extension at 72 °C for 10 min.	Manna et al. (2006)
stx_2	F-CCATGACAACGGACAGCAGTT R-CCTGTCAACTGAGCACTTTG	779 bp		

2.3 | Serotyping

Serotyping was performed by latex agglutination using a Remel-Wellcolex *E. coli* O157:H7 latex agglutination kit (Thermo Fisher Scientific) to detect both O157 (somatic antigen) and H7 (flagellar antigen) (Ewing, 1986). Isolates positive for the O157 antigen were further cultivated on blood agar overnight to detect the H7 antigen. Blue color agglutination indicates a positive result for the H antigen and red color agglutination indicates the O antigen.

2.4 | Oligonucleotide primers

The primers (Macrogen, South Korea) used for PCR amplification of the rfb_{O157} and $flic_{H7}$ genes (Gannon et al., 1997) and the Shiga toxin stx_1/stx_2 genes (Manna, Brahmane, Manna, Batabyal, & Das, 2006) are shown in Table 1.

2.5 | DNA extraction and PCR conditions

The boiling method was used to extract DNA from each isolate (Radu et al., 2000). An Applied-Biosystems (U.K., 2720) PCR thermocycler was used and programmed using standard conditions (Chen & Griffiths, 1998). Following polymerization, the sample was loaded onto an agarose gel, which was run at 100 V for 35 min, and DNA bands were visualized using a Wealtec Dolphin-view system. A positive control of *E. coli* O157:H7 (already characterized and PCR confirmed) was obtained from the CASVAB seed bank and sterile distilled water was used as the negative control.

2.6 | Antibiotic sensitivity testing

Resistance or antibiotic sensitivity of the isolated strains was tested on Mueller–Hinton agar (MHA, oxoid) by the standard single disk method as described previously (Bauer, Kirby, Sherris, & Turck, 1966) and recently (Kassim, Omuse, Premji, & Revathi, 2016). Antibiograms of the isolates were critically examined.

Briefly, the *E. coli* O157:H7 inoculum was prepared in normal saline, its turbidity was matched according to 0.5 McFarland standards (McFarland, 1907; Swenson & Thornsberry, 1984) and was spread evenly on MHA plates. Using sterilized forceps, antibiotic disks were placed on the agar surface and the plates were then incubated at 37 °C for 24 hr. After incubation the plates were observed for appearance of a zone of inhibition. The zone of inhibition was measured according to the diameter of the clear zone (no bacterial growth) around the antibiotic disks. The sensitivity and resistivity of *E. coli* O157:H7 against each antibiotic was evaluated from the zone of inhibition and categorized into three different groups: resistant (R), intermediate (I), and sensitive (S). The standard break points for sensitivity and resistance were adopted from Clinical and Laboratory Standards Institute (CLSI) standards (CLSI. M100-S22, 2012 [http://www.facm.ucl.ac.be/intranet/CLSI/CLSI-M100S22-susceptibility%20testing-2012-original.pdf]; CLSI. M100-S23., 2013 [http://www.facm.ucl.ac.be/intranet/CLSI/CLSI-M100S23-susceptibility-testing-2013-no-protection.pdf]).

3 | RESULTS

Two hundred raw beef samples (100 each of diced and ground/minced) were collected randomly from 100 different retail butcher shops in Quetta, Pakistan to check for the prevalence of *E. coli*. Importantly, only one diced and one minced beef sample was collected from each shop and these shops were a minimum distance of 100–300 m from each other, as they were scattered throughout the city of Quetta. Multiplex PCR results (Figure 1) showed that the prevalence of *E. coli* O157:H7 was 8% (n = 8/100) in diced samples and 12% (n = 12/100) in ground/minced samples. The overall prevalence of *E. coli* O157:H7 in both diced/minced beef meat samples was therefore 10% (n = 20/200). Conventional culture methods using primary enrichment on mTSB and plating showed 22 (11%) NSF colonies on CT-SMAC. When tested through the RapID One biochemical system, latex agglutination and PCR, two of the isolates were confirmed not to be O157 (Table 2). Two out of 20 isolates gave negative results for the H7 antigen by latex agglutination, but when tested using PCR, the H7 gene was detected. When multiplex PCR was used to test for the presence of Shiga toxin genes in the 20 O157:H7 isolates (Figure 2), 11/20 (55%) were positive for stx_1 or stx_2, 4/20 (20%) were positive for stx_1, 9/20 (45%) were positive for stx_2, and 2/20 (10%) were positive for both stx_1 and stx_2 Shiga toxins (Table 2). Month wise detection of *E. coli* O157:H7 in the beef samples showed highest prevalence during April–August, with the highest prevalence in June (Table 3).

Antibiotic sensitivity was tested for 21 antibiotics of different classes (Table 4) and results showed that all of the isolates were sensitive to aminoglycosides, fluoroquinolones, chloramphenicol, and imipenem. All

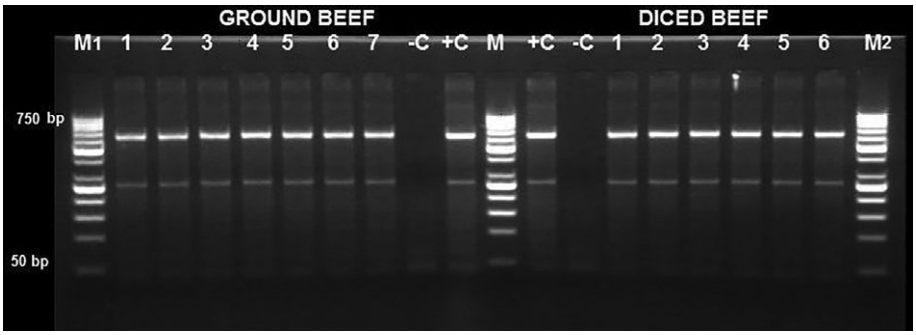

FIGURE 1 Agarose gel (2%) electrophoresis showing amplification fragments of the rbf_{O157} (259 bp) and $flic_{H7}$ (625 bp) genes by multiplex PCR. Ground beef: Lanes 1–7 show positive amplification of *E. coli* O157:H7. Diced beef: Lanes 1–6 show positive amplification of *E. coli* O157:H7. M1/M2 = 50 bp DNA markers, +C = positive control, −C = negative control

TABLE 2 Detection of Shiga toxin producing *E. coli* O157:H7 in beef meat samples using culture, biochemical, serological, and PCR methods

Isolates	NSF on CT-SMAC	Biochemical confirmation by RapID one system	Latex agglutination		Multiplex PCR		Multiplex PCR Shiga toxins	
			O	H	rfb_{O157}	$flic_{H7}$	stx_1	stx_1
1	+	+	+	+	+	+	−	+
2	+	+	+	+	+	+	−	+
3	+	+	+	−	+	+	−	−
4	+	+	+	+	+	+	−	−
5	+	+	+	+	+	+	+	−
6	+	+	+	+	+	+	+	+
7	+	−	−	−	−	−	−	−
8	+	+	+	+	+	+	−	+
9	+	+	+	+	+	+	−	−
10	+	+	+	−	+	+	−	+
11	+	+	+	+	+	+	−	−
12	+	+	+	+	+	+	+	−
13	+	−	−	−	−	−	−	−
14	+	+	+	−	+	+	−	+
15	+	+	+	+	+	+	−	+
16	+	+	+	+	+	+	−	−
17	+	+	+	+	+	+	−	−
18	+	+	+	+	+	+	−	−
19	+	+	+	+	+	+	−	+
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21	+	+	+	+	+	+	−	−
22	+	+	+	+	+	+	−	−

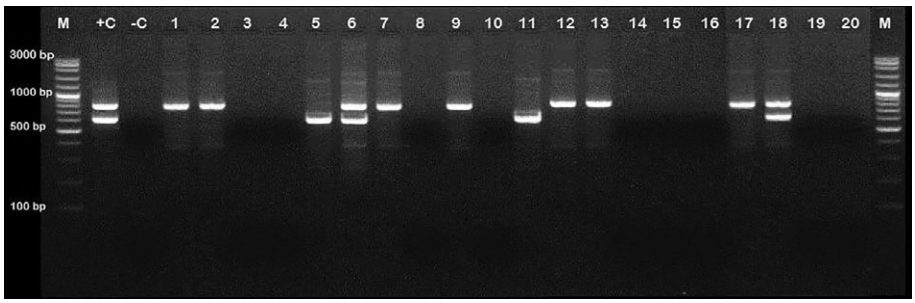

FIGURE 2 Agarose gel (2%) electrophoresis showing amplification fragments of the stx_1 (614 bp) and stx_2 (779 bp) genes by multiplex PCR. Lanes 1, 2, 7, 9, 12, 13, and 17 are positive for stx_2. Lanes 5 and 11 are positive for stx_1. Lanes 6 and 18 are positive for both stx_1 and stx_2. M1/M2 = 100 bp DNA markers, +C = positive control, −C = negative control

TABLE 3 Seasonal (month wise) prevalence of *E. coli* O157:H7 in beef meat samples

	Month								
Sample type	January	February	March	April	May	June	July	August	Total positive
Diced beef	0/10	0/12	0/13	2/13	1/13	2/13	2/13	1/13	8/100
Ground beef	0/10	1/12	1/13	1/13	2/13	4/13	2/13	1/13	12/100
Total	0/20	1/24	1/26	3/26	3/26	6/26	4/26	2/26	20/200

of the isolates were resistant to novobiocin, cephradine, bacitracin, clindamycin, metronidazole, sulfamethoxazole + trimethoprim, penicillins, and tetracyclines. Clarithromycin and nalidixic acid showed intermediate zone of inhibition (Table 4).

4 | DISCUSSION

In this study, two of the isolates did not exhibit H7 reaction in the latex agglutination test, which is in agreement with previous studies (Kim et al., 2005). These results are also in agreement with March and Ratnam (1986), who evaluated latex agglutination as an efficient and rapid test for the O157:H7 serotype. Interestingly, PCR assay for H7 gene in these isolates showed positive results. A possible explanation is that the O157:H7 strain did not express characteristically or the antigenic protein got saturated by host antibodies. Recent advances in diagnostic techniques have made several protocols available for isolation and characterization of *E. coli* O157:H7 from food, fecal, and environmental samples (Deisingh & Thompson, 2004; Tamerat, Muktar, & Shiferaw, 2016). Further, to classify and elaborate the function of the main virulence genes, such as stx_1 and stx_2, and genes encoding accessory STEC virulence factors, such as *eaeA*, *hlyA*, and $flic_{H7}$, different PCR assays have been described (Al-Ajmi et al., 2006; Gannon, King, Kim, & Thomas, 1992; Holland, Louie, Simor, & Louie, 2000; Kumar, Grover, & Kumar Batish, 2013; Pan, Chen, & Su, 2002; Sharma, 2006; Sharma & Dean-Nystrom, 2003).

Our findings are similar to those from other studies on beef meat samples reported from several countries around the world, in most of which the stx_2 gene was more prevalent than *stx1* in *E. coli* O157 isolates. For example, studies conducted on beef cattle meat in Argentina revealed a high presence of stx_2 in the *E. coli* O157 serotype (Blanco et al., 2004). Our findings to some extent are similar to previous studies by Kalin and Öngör (2014) who reported 16.6% stx_1 and 50.0% stx_2 in minced beef samples in Turkey. Sallam, Mohammed, Ahdy, and Tamura (2013) revealed the presence of 46.7% stx_1 and 86.7% stx_2 genes in *E. coli* O157 isolates from retail beef samples in Egypt. However, our findings are dissimilar to some studies that reported a higher prevalence of stx_1 than stx_2 (Kiranmayi et al., 2011). The prevalence of *E. coli* O157:H7 in beef meat samples from our study is comparable with the results of Heuvelink, Zwartkruis-Nahuis, Beumer, and de-Boer (1999), who reported a prevalence rate of 10.4% in beef from the Netherlands. The occurrence of *E. coli* O157:H7 in beef samples was reported as more than 8% in South Africa (Hajian, Rahimi, & Mommtaz, 2011), 6.8% in

TABLE 4 Antibiotic sensitivity of *E. coli* O157:H7 isolates from beef meat samples

No.	Antibiotic	Class	Result
1	Novobiocin (NV-30 μg)	Aminocoumarin	Resistant
2	Amikacin (AK-30 μg)	Aminoglycoside	Sensitive
3	Gentamicin (CN-10 μg)		Sensitive
4	Kanamycin (K-5 μg)		Sensitive
5	Tobramycin (TOB-10 μg)		Sensitive
6	Chloramphenicol (C-10 μg)	Amphenicol	Sensitive
7	Imipenem (IPM-10 μg)	Carbapenem (β-lactam)	Sensitive
8	Cephradine (CE-30 μg)	Cephalosporin (β-lactam)	Resistant
9	Bacitracin (B-10 μg)	Cyclic peptide	Resistant
10	Enrofloxacin (ENR-5 μg)	Fluoroquinolone	Sensitive
11	Ofloxacin (OFX-5 μg)		Sensitive
12	Clindamycin (DA-2 μg)	Lincosamide	Resistant
13	Clarithromycin (CLR-2 μg)	Macrolide	Intermediate zone of inhibition
14	Metronidazole (MTZ-5 μg)	Nitroimidazole	Resistant
15	Amoxycillin + clavulanic acid (AMC-3 μg)	Penicillin (β-lactam)	Resistant
16	Ampicillin (AMP-2 μg)		Resistant
17	Methicillin (MET-5 μg)		Resistant
18	Nalidixic acid (NA-30 μg)	Quinolone	Intermediate zone of inhibition
19	Sulfamethoxazole + trimethoprim (SXT-25 μg)	Sulfonamide	Resistant
20	Oxytetracycline (OT-30 μg)	Tetracycline	Resistant
21	Tetracycline (TE-10 μg)		Resistant

China, 1.5–8% in Iran (Abdul-Raouf, Ammar, & Beuchat, 1996; Maktabi, Zarei, & Mohammadpour, 2016), 9% in raw beef in Saudi Arabia, 9% in Egypt, 8.2% in Thailand, and 3.7% in the U.S. (Hessain et al., 2015; Rahimi, Kazemeini, & Salajegheh, 2012).

Summer and fall are the seasons with highest prevalence of *E. coli* O157:H7 in meat samples, which are in agreement with the findings from previous studies on beef and sheep (Byrne et al., 2003; Elder et al., 2000; Rahimi et al., 2012). Moreover, our results showed presence of the H7 antigen in all of the O157 isolates tested, which is in accordance with previous studies reporting presence of the H7 antigen in isolates of human and bovine origin (Rahimi et al., 2012).

Our antibiotic sensitivity results are in agreement with previous studies reporting 100% resistance of *E. coli* O157:H7 isolates to trimethoprim, ampicillin, and amoxicillin/clavulanic acid followed by 80% resistance to tetracycline and 40% resistance to nalidixic acid (Fard, Bokaeian, & Qureishi, 2008). Further, our findings agree with this study that revealed sensitivity of *E. coli* O157:H7 to ciprofloxacin, imipenem, gentamycin, and amikacin (Fard et al., 2008). A further important observation is that the *E. coli* O157:H7 isolates were resistant to greater than three different classes of antimicrobials, providing evidence of multidrug resistance.

5 | CONCLUSIONS

Despite frequent contamination of raw meat products by potentially virulent *E. coli* O157:H7, to date, no clinical data about the impact on human health are available in Pakistan. Our study detected *E. coli* O157:H7 in raw beef meat from retail butcher shops in Quetta, Pakistan. Therefore, it is recommended that appropriate hygiene conditions, food safety measures, and improved surveillance are used in Quetta for minimizing contamination of such raw meat products and associated cross contamination by *E. coli* O157:H7. For this purpose, multiplex PCR is a reliable, sensitive, and relatively rapid technique for detecting *E. coli* O157:H7 in food and environmental samples. Of further concern is that the O157:H7 isolates were resistant to a range of commonly used antibiotics, thus emphasizing the global problem of antimicrobial resistance.

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CONFLICT OF INTEREST

The authors declare that they have no potential conflicts of interest.

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