MOLECULAR CHARACTERIZATION OF *ESCHERICHIA COLI* AND *STAPHYLOCOCCUS* SPECIES ISOLATED FROM RAW MEAT OF DIFFERENT ANIMALS IN SULAIMANI CITY, IRAQ

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Abstract. Meat-borne bacteria cause a variety of diseases, some resulting in death and emergence multidrug-resistant bacteria has compounded the problem. Here, the prevalence of *Escherichia coli* and *Staphylococcus* spp contamination were investigated in meat samples (n = 70) together with drug resistance phenotype and presence of resistance genes, and in *E. coli* isolates phenotypic groups and carriage of virulent genes. *E. coli* and *Staphylococcus* spp were detected in 57 and 18% of meat samples respectively, mainly in whole chicken meat and liver, obtained from meat retailors in Sulaimani City, Iraq from December 2018 to January 2019. Resistance to tetracycline was predominant in *Staphylococcus* spp (92%) and *E. coli* (73%) isolates, while both were sensitive to cefotaxime and the latter also to aztreonam and imipenem. Multidrug resistance phenotype was detected in both *E. coli* (42%) and *Staphylococci* spp (69%). Only *tetK* was detected in one tetracycline-resistant *E. coli* and *Staphylococci* spp isolate respectively, and among beta-lactam-resistant isolates only one oxacillin-resistant *Staphylococci* spp isolate carried bla_{OXA} while no resistance genes were detected in *E. coli* isolates. The majority of *E. coli* isolates (62.5%) belonged to phenotypic groups G1 and G2, associated with clinically important and other pathogenic (extra-intestinal) strains. One *E. coli* isolate from a beef sample harbored Shiga-encoded *Stx2*, indicative of a pathogenic enterohemorrhagic strain. In conclusion, raw meat on sale in Sulaimani City contained high levels of contamination by multidrug-resistant *E. coli* and *Staphylococci* spp, a finding that should be of concern to the city public health authorities.

Keywords: antibiotic sensitivity test, beta-lactamase, food-borne bacteria, tetracycline resistance gene, virulent gene

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INTRODUCTION

Animal meat, such as beef, lamb, and chicken meat, provides a good source of nutrition and protein for humans and is consumed daily. On the other hand, animal food acts as a reservoir for transmission of pathogenic microorganisms and is a health hazard. Food-borne diseases constitute a global public health problem (Scallan *et al*, 2011), being a leading annual cause (30%) of food poisoning in developed countries and is responsible for about 2 million cases of mortality in developing countries (Kaper *et al*, 2004; WHO, 2015; FOSAPAC, 2017). One of the main causes of food-borne and transmission of zoonotic disease is contamination of raw meat by pathogenic microorganisms (Bhandare *et al*, 2007; Podpecan *et al*, 2007).

Escherichia coli, a commensal Gram-negative bacterium found mostly in gastrointestinal tract of animals, is non-pathogen, several species are versatile pathogens (Kaper *et al*, 2004; Adams and Moss, 2008; Feng *et al*, 2020; Frederick and Huda, 2011; Adzitey *et al*, 2012), responsible for diarrhea and sometimes life-threatening diseases (Zeinhom and Abdel-Latef, 2014). The presence of *E. coli* in meat is an indication of fecal contamination (Zeinhom and Abdel-Latef, 2014). One of the *E. coli* species transmitted through contaminated raw meat or meat products of public health concern is *E. coli* O157:H7 (Arun *et al*, 2007) and is responsible for many cases of food poisoning annually (Shriver-Lake *et al*, 2007). Complications (~7% of cases) such as hemolytic uremic syndrome may develop after intestinal discomfort and may result in death. The majority (85%) of *E. coli* O157:H7 infection cases are due to the consumption of contaminated food (vegetable, fruit and meat) (Friedman *et al*, 1999; Mead *et al*, 1999), being responsible for about 75,000 cases annually (Perna *et al*, 2001) and contributing to annual outbreaks of infection worldwide (Ferens and Hovde, 2011).

Staphylococcus sp, a Gram- and catalase-positive bacterium, is also a cause of zoonotic diseases stemming from meat contamination (Wang and Ruan, 2017). *S. aureus* is present on skin of a number of animals and can contaminate meat during processing of animals such as cattle, chicken, goat, sheep, and turkey (Olufemi *et al*, 2018). Undercooked and raw meat particularly serve as reservoirs for *S. aureus* food poisoning due to its short incubation period (Fijałkowski *et al*, 2016), with symptoms ranging from abdominal cramp, diarrhea and vomiting to toxic shock (Wang and Ruan, 2017).

Antibiotic-resistant bacteria are increasing in number and their presence in food is a another cause of public health problem (Johnson *et al*, 2007; Pesavento *et al*, 2007; Rahim *et al*, 2020). Antibiotics form the main

therapeutic intervention in humans and animals, but their use in animal husbandry for prophylaxis and growth promotion have led to rapid spread of drug resistance (Aarestrup, 2005). This is aided by the ability of bacteria to exchange drug resistance genes through horizontal transfer (Stokes and Hall, 1989; Rowe-Magnus *et al*, 2002).

Here, prevalence of contamination by *E. coli* and *Staphylococcus* spp together with their antibiograms and antibiotic resistance genes were determined in animal and poultry meats obtained from meat retailers in Sulaimani City, Iraq. These findings should provide baseline information regarding meat-borne bacteria contamination in raw meat, which should be of use to public health authorities in Sulaimani City.

MATERIAL AND METHODS

Sample collection and processing

Meat samples (beef, n = 10; chicken (liver), n = 10; chicken (whole), n = 20; fish, n = 10; sheep, n = 10; and turkey, n = 10) were collected from meat retailers in Sulaimani City, Iraq from December 2018 to January 2019. Samples were collected under aseptic condition and kept on ice until delivery at the laboratory within one to two hours, where 10 g of each meat sample were homogenized with 90 ml of Neogen® nutrient broth (Neogen Culture Media, Neogen Corporation, Lansing, MI) and incubated at 37°C with agitation for 24 hours.

Isolation and identification of *E. coli* and *Staphylococcus* sp

A loop of broth culture was streaked onto MacConkey agar (Neogen Culture Media, Neogen Corporation, Lansing, MI) and Eosin Methylene Blue (EMB) Agar Levine (Neogen Corporation, Lansing, MI) and incubated at 37°C overnight for isolation and subsequent identification. *E. coli* was identified using an IMViC test (HiMedia Laboratories Private Limited, Maharashtra, India) and *Staphylococcus* spp by growth on mannitol salt agar (HiMedia Laboratories Private Limited, Maharashtra, India), Gram staining, and catalase (Reiner, 2010) and coagulase tests (Bennett *et al*, 1986).

Molecular characterization of *E. coli* and *Staphylococcus* sp

DNA of *E. coli* isolates was extracted by boiling a fresh colony in 100 µl of distilled water for 15 minutes, centrifuged at 11,000 g for 5 minutes and supernatant collected (Dashti *et al*, 2009). DNA of *Staphylococcus* sp was obtained from one colony grown in mannitol salt broth (HiMedia Laboratories Private Limited, Maharashtra, India) for 24 hours at 37°C and five ml aliquot was centrifuged at 3000 g for 5 minutes and pellet used for DNA extraction by a Monarch Genomic DNA

Purification Kit (New England BioLabs, Ipswich, MA). Polymerase chain reaction (PCR) *E. coli* identification was performed using *uidA*-specific primers (Chen and Griffiths, 1998; Heijnen and Medema, 2006) and that of *Staphylococcus* sp using a genus-specific 16S DNA primers (Mason, 2001). PCR was carried out in 20 µl solution containing 10 µl of 2X RedTaq DNA polymerase premix (SBS Genetech, Beijing, China), 0.5 µM each primer and 2 µl of DNA. Thermocycling was performed using Applied Biosystems 2727 thermal cycler (Applied Biosystems, Waltham, MA) as follows: 95°C for 7 minutes; 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds; and a final step of 72°C for 7 minutes. Amplicons, 166 base pairs (bp) and 791 bp for *E. coli* and *Staphylococcus* sp respectively, were separated by 1% DNA agarose gel-electrophoresis, stained with Good View Nucleic Acid Stain (SBS Genetech Co Ltd, Beijing, China) dye and recorded using a SmartDoc 2.0 Imaging System (Accuris Instruments, Edison, NJ).

Antibiogram profiling

Antibiogram profiling for all isolates was carried out using a Kirby Bauer disc diffusion test according to protocols of the Clinical and Laboratory Standards Institute (CLSI, 2012). In brief, a fresh colony was suspended in one ml aliquot of normal saline and cultured on Mauler Hilton agar (Neogen Corporation, Lansing, MI). Each disc (Bioanalyse, Ankara, Turkey) contained 10 µg of amikacin, 25 µg of amoxicillin, 10 µg of ampicillin, 30 µg each of amoxicillin + clavulanic acid, 30 µg of azithromycin, 10 µg of aztreonam, 30 µg of cefotaxime, 10 µg of ciprofloxacin, 10 µg of gentamycin, 10 µg of imipenem, 30 µg of nalidixic acid, 30 µg of novobiocin, 1 µg of oxacillin, 10 IU of penicillin, 5 µg of rifampin, 10 µg of tetracycline, 25 µg of co-trimoxazole (trimethoprim/sulfamethoxazole 1:19), or 30 µg of vancomycin.

PCR detection of *β*-lactamase and tetracycline resistance genes

Multiplex PCR was used to amplify two tetracycline resistance genes (*tetK* and *tetM*) (Strommenger *et al*, 2003; Nouri Gharajalar and Onsori, 2019) and five common β-lactamase resistance genes (bla_{CMY}, bla_{CTX}, bla_{SHV}, $bla_{TEM\text{-}1}$, and bla_{OXA}) using two separate reactions, one for amplification of and bla_{CMY} and $bla_{TEM\text{-}1}$ (group G1) and the other for bla_{CTX}, bla_{OXA} and bla_{SHV} (group G2) (Ahmed *et al*, 2007; Ahmed *et al*, 2009). PCR reaction was performed in 20 µl solution containing 10 µl of 2X RedTag DNA polymerase premix (SBS Genetech Co Ltd, Beijing, China), 0.2 µM each primer and 2 µl of DNA. Thermocycling was carried out as described above using the following conditions: 95°C for 7 minutes; 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds; with a final step of 72°C for 5 minutes.

Phylogenetic grouping of *E. coli* strains

A multiplex PCR was employed to amplify *chuA* and *yjaA* and uniplex PCR for DNA fragment TSPE4.C2A of the *E. coli* strains (Clermont *et al*, 2000). Amplicons were separated by 2% agarose gel-electrophoresis and analyzed as described above. Phylogenetic grouping of *E. coli* strains was conducted as previously described (Clermont *et al*, 2000).

Determination of *E. coli* virulent genes, *eaeA, Stx1* and *Stx2*

Virulent *E. coli* strains, enteroaggregative *E. coli* (EAEC) and enterohemorrhagic *E. coli* (EHEC), were identified by amplifying *eaeA* (encoding intimin) and *Stx1* and *Stx2* (encoding Shiga toxins) as previously described (Guion *et al*, 2008). Amplicons (248, 150 and 255 bp of *eaeA*, *Stx1* and *Stx2* and, respectively) were separated by 2% agarose gel-electrophoresis and analyzed as described above.

RESULTS

Prevalence of bacterial contamination in meat samples

A total of 70 meat samples were examined for the presence of two common bacterial genera, namely, *E. coli* and coagulase-negative *Staphylococcus* spp. *E. coli* was detected in 40 samples (57%) and *Staphylococcus* spp in 13 samples (18%), mostly in whole chicken meat and liver (50.9 %).

Antibiogram profiles

Antibiotic sensitivity testing for all *E. coli* (*n* = 40) and *Staphylococcus* spp (*n* = 13) isolates was carried out using the Kirby Bauer disc diffusion test according to CLSI (2012). Most of the *E. coli* strains were resistant to tetracycline (29 isolates), followed by amoxicillin, (20 isolates), while all isolates were sensitive to aztreonam, cefotaxime and imipenem (Fig 1). The majority of *Staphylococcus* spp isolates were resistant to tetracycline (12 isolates) followed by azithromycin and novobiocin (8 isolates each), while and no isolate was resistant to cefotaxime (Fig 2). Multidrug resistance bacteria were found in both types of bacteria, but prevalence was higher among *Staphylococcus* spp, (9 isolates, 69%) than *E. coli* (17 isolates, 42%).

Phylogenetic grouping of *E. coli* isolates

When *E. coli* isolates were grouped according to the criteria of Clermont *et al* (2000), the highest number of isolates (*n* = 13) belonged to group B1 (presence of DNA fragment TSPE4.C2), followed by 12 isolates to group B2 (presence of *chuA* + *yjaA* or *chuA* + *yjaA* + DNA fragment TSPE4.C2), 6 isolates to group A (presence of *yjaA*), 4 isolates to group D (presence of *chuA* or *chuA* + DNA fragment TSPE4.C2), and 5 isolates to unclassified group (presence of *yjaA* + DNA fragment TSPE4.C2) (Fig 3).

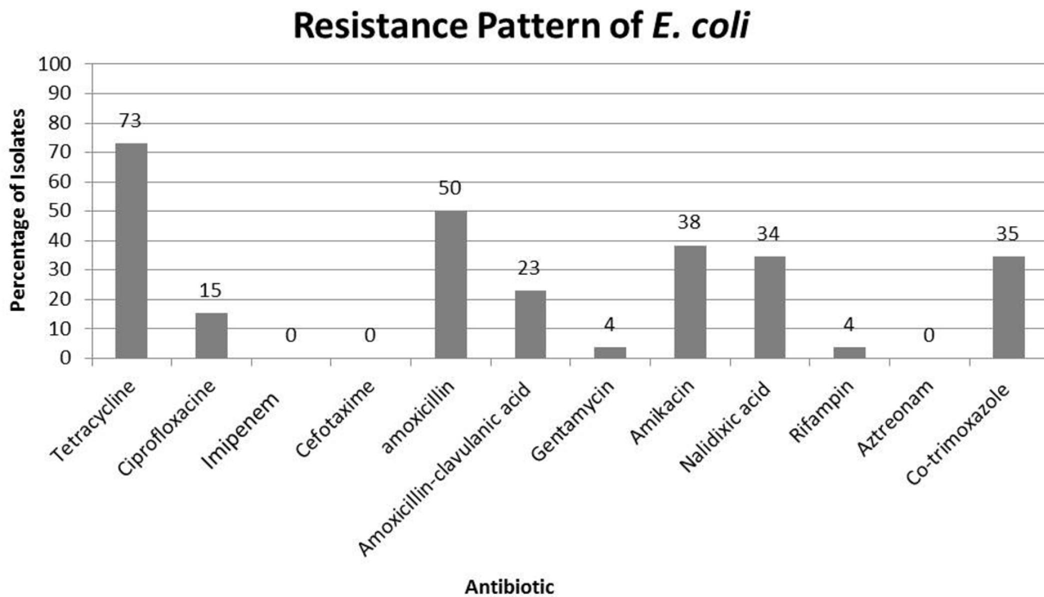

Fig 1 - Antibiogram profile of *Escherichia coli* isolates (n = 40) from 70 meat samples obtained from meat retailers in Sulaimani City, Iraq (December 2018 - January 2019)

Antibiotic sensitivity tests of all *E. coli* isolates were carried out using the Kirby Bauer disc diffusion test according to CLSI (2012).

Prevalence of resistance genes in *E. coli* and *Staphylococcus* spp. isolates

Resistance genes to tetracycline (*tetK* and *tetM*) and beta-lactams (bla_{CMY}, bla_{CTX}, bla_{OXA}, bla_{SHV}, and bla_{TEM-1}) were chosen for analysis in *E. coli* and *Staphylococcus* spp isolates using PCR. Of the *E. coli* isolates, only one isolate (2.5%) was positive for *tetK* and none for the other resistance test genes. Similarly, of the *Staphylococcus* spp isolates, one isolate (7%) was positive for *tetK* and one for bla_{OXA}, while none for the remaining test genes. It is worth noting that 29 (72%) and 12 (92%) *E. coli* and *Staphylococcus* spp isolates respectively were phenotypically resistant to tetracycline, and 29 (72%) and 9 (69%) respectively were phenotypically resistant to beta-lactams.

Prevalence of *E. coli* isolates carrying virulent genes

Using multiplex PCR, only one *E. coli* isolate (2.5%, from beef) carried the virulent *Stx2* and all 40 *E. coli* isolates lacked virulent *eaeA* and *Stx1*.

DISCUSSION

Contamination of meat by intestinal *E. coli* is common during slaughtering of animals and subsequent meat processing (Zweifel *et al*, 2008; Thanigaivel and Anandhan, 2015). As *Staphylococcus* spp usually reside in skin, meat contamination by these microorganisms is likely to be related to the animal skin flora (Olufemi *et al*, 2018). Less likely sources of bacterial contamination are hands of abattoir workers, utensils and animal carcass storage places (Bhandare *et al*, 2007; Adzitey, 2011; Adzitey and Huda, 2012). Our study revealed over 50 and 15% raw meat from local shops in Sulaimani City, Iraq were contaminated by *E. coli* and *Staphylococci* spp respectively, in keeping with previous studies from Switzerland and Chennai, India (Zweifel *et al*, 2008; Thanigaivel and Anandhan, 2015). Bacterial contamination of raw meat is related to poor hygienic measures in processing of meat and lack of good abattoir management practice, and poses a threat to human public health (Holds *et al*, 2008; Zweifel *et al*, 2008; Salih *et al*, 2019).

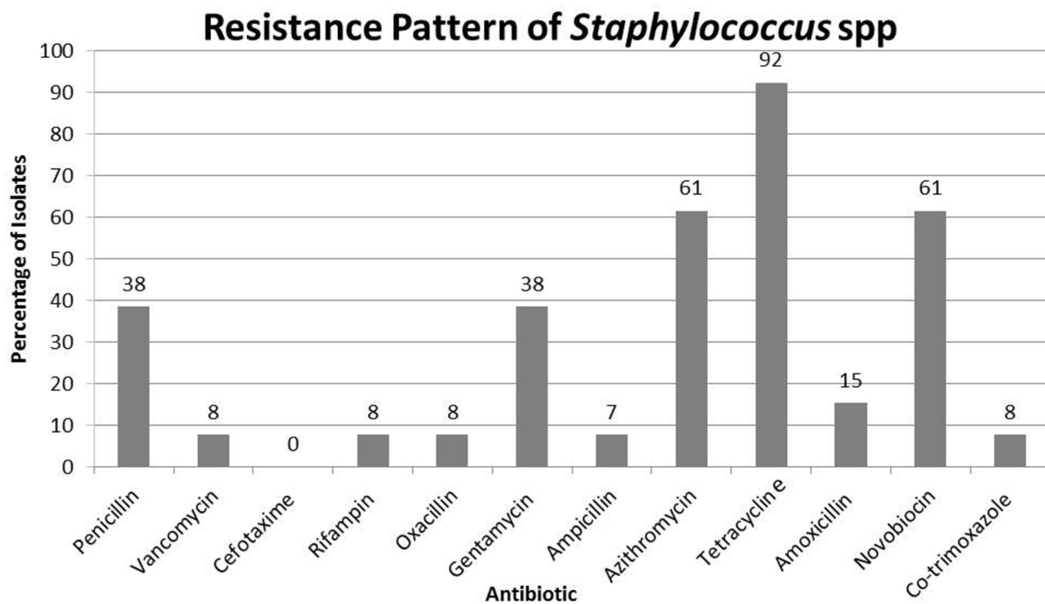

Fig 2 - Antibiogram profile of *Staphylococcus* spp. isolates (n = 13) from 70 meat samples obtained from meat retailers in Sulaimani City, Iraq (December 2018 - January 2019)

Antibiotic sensitivity tests of all *Staphylococcus* spp isolates were carried out using the Kirby Bauer disc diffusion test according to CLSI (2012).

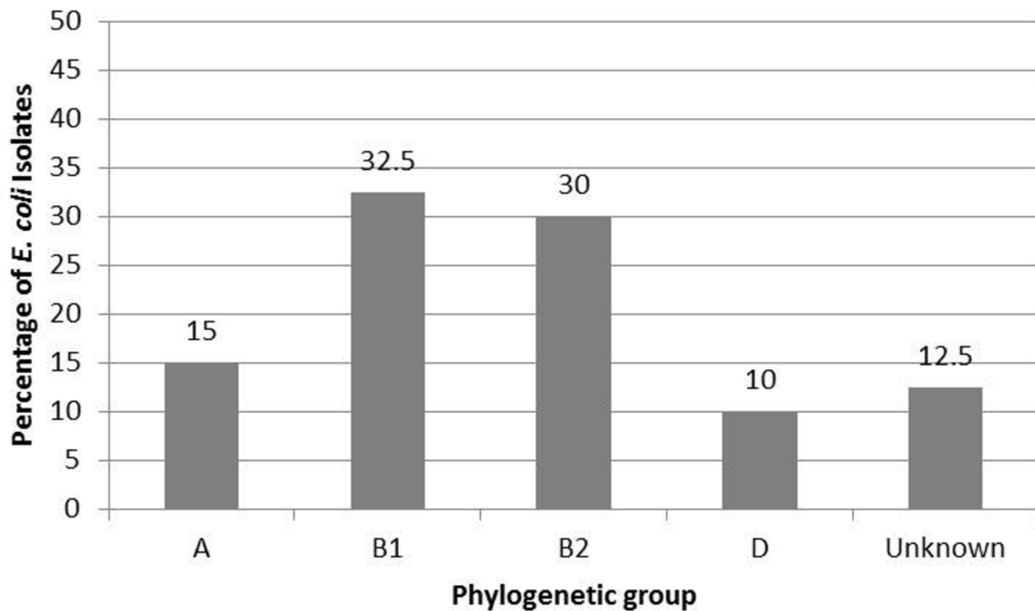

Fig 3 - Phylogenetic grouping of *Escherichia coli* isolates (n = 40) from 70 meat samples obtained from meat retailers in Sulaimani City, Iraq (December 2018 - January 2019)

E. coli isolates were classified into five different groups, namely, A, B1, B2, D, and unknown based on PCR detection of *chuA*, *yjaA* and DNA fragment TSPE4.C2A (Clermont *et al*, 2000).

Antibiotics are commonly used in animal husbandry as prophylactics and to stimulate growth, thereby increasing the probability of developing drug-resistant pathogenic bacteria. Prevalence of tetracycline resistance in *E. coli* and *Staphylococci* spp from the meat samples was very high as was resistance to beta-lactams, particularly in *E. coli* isolates. These findings may reflect the use of these antibiotics in the meat industry of Sulaimani City. Multidrug-resistant bacteria have previously been reported in various types of raw meat from Southern Taiwan, Norway, and Bangladesh (Yan *et al*, 2004; Sunde, 2005; Nahar *et al*, 2014). Among the tetracycline-resistant *E. coli* (n = 29) and *Staphylococci* spp (n = 12) isolates, only one isolate from each bacterial species was found to harbor *tetK* (and none carried *tetM*). These isolates were completely resistance to tetracycline and this may be due to the effect of their *TetK* gene. Similarly, among *E. coli* (n = 29) and *Staphylococci* spp (n = 9)

beta-lactam-resistant isolates, only one *Staphylococci* spp isolate, carried bla_{OXA}, corresponding to the only isolate resistant to oxacillin and it is resistant to all beta-lactam antibiotics used in this study including penicillin, ampicillin, and amoxicillin.

Phylogenetic grouping of *E. coli* allows classification of strains according to their pathogenicity, with clinically important and other pathogenic (extra-intestinal) strains belonging to groups B1 and D (Bingen *et al*, 1998). In the current study some 60% of *E. coli* isolates were of groups B1 and B2, indicating the majority raw meat purchased from meat retailers in Sulaimani City may pose a health problem to consumers. However, 12.5% of the *E. coli* isolates did not belong to any of the four classification groups defined by Bingen *et al* (1998) and their pathogenicity needs investigation.

Further analysis of the virulence genes, *eaeA*, *Stx1* and *Stx2*, were carried out on the *E. coli* isolates. EHEC serovar O157:H7 contains intimin and causes serious complications in human diseases of the gastrointestinal tract (Shriver-Lake *et al*, 2007; Mead *et al*, 1999), and Shiga toxins present in Shiga-toxin producing *E. coli* (STEC) strains lead to serious illnesses such as hemolytic uremic syndrome, due mainly to Shiga toxin encoded by *Stx2* (Moussa *et al*, 2010). Of the 40 *E. coli* isolates analyzed, only one isolate (from minced beef) carried *Stx2* which belongs to phylogenetic group B1 and no isolate harbored *eaeA* and *Stx1*. Although the prevalence of *Stx2* in STEC isolates from raw meat was low (2.5%), this should be brought to the attention of the relevant public health authority sectors as STEC strains producing *Stx2*-encoded Shiga toxin have been reported to cause outbreaks in several countries (Hussein, 2007).

In summary, our recent analysis of *E. coli* and *Staphylococcus* spp in raw meat available from local shops in Sulaimani City reveals a high prevalence of these two bacteria genera. Of concern is that the majority of isolates were phenotypically resistant to tetracycline and beta-lactams, and over half of *E. coli* isolates belonged to phylogenetic groups associated with pathogenicity. Attempts to identify antibiotic resistance genes met with limited success. Nevertheless, the overall data collected indicate that consumption of raw or improperly cooked meat obtained from retailers of meat in Sulaimani City posed a potential health problem to consumers and should be of urgent concern to public health authorities.

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CONFLICTS OF INTEREST DISCLOSURE

The authors declare no conflicts of interest.

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