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Microbiological and molecular investigations of bacterial contaminants of fresh red meat in Al Muthanna Governorate

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Abstract

Red meat is one of the essential foods and sources of protein, fats, and salts for humans worldwide. Meat is a suitable medium for bacterial growth that cause significant economic loss due to rapid spoilage, deadly toxins production, and food poisoning. This study intends to evaluate the microbial contamination in fresh red meat in butcher shops and abattoirs in Samawah city/ Al Muthanna governorate/Iraq and assess their resistance to antimicrobial agents, and determine the presence of tetA resistance genes. Moreover, to determine their resistance or sensitivity to antimicrobial agents and to investigate oxytetracycline resistance gene tet (A) in vitro antibiotic resistance *E. Coli*. Sixteen and five meat samples were collected randomly from butcher shops and abattoir. Samples were cultured on selective media. The isolated bacteria were identified by routine biochemical tests and rapid API tools. At the same time, the disk diffusion method (Kirby-Bauer) was used to determine antimicrobial sensitivity. Additionally, antibiotic-resistant *E.coli* were tested by multiplex PCR to determine the prevalence of tetracycline resistance genes tet (A). Twenty-one microorganisms were isolated from meat samples, and the isolation rate was 100%. The percentages of isolated bacteria were 57.14%, 14.285%, 4.76% , 4.76% , 4.76% , 4.76%, 4.76% and 4.76% for *E. coli*, *Salmonella typhimurium*, *Salmonella typhi*, *Salmonella enteritidis*, other *Salmonella spp*., *Citrobacter freundii*, *Shigella flexneri, and Porteous Vulgaris, respectively*. Multiple drug resistance was observed for the tested antibiotic Tetracycline (TE - 10 µg), Amoxicillin/ Clavulanic (AMC-30 µg), Levofloxacin (LEV-5 µg), Gentamicin (CN-10 µg), ceftazidime (CAZ- 30 µg), and Sulfamethhazol /Trimethoprim (SXT-25 µg). Multiplex PCR revealed the prevalence of tetracycline resistance genes tet (A) in the isolated *E. Coli* (12/12, 100 %). In conclusion, this study approved the presence of various bacterial contaminants in fresh meat sold in butcher shops and the abattoir in Al Muthanna governorate/ Iraq. *Escherichia coli* was isolated at a high percentage, followed by *Salmonella typhimurium*. Most of these isolates revealed multiple drug resistance, and 100 % of isolated *E. Coli* carried tetA resistance genes. Health precautions must be taken during the slaughtering of animals, meat transportation, and displaying meat in butcher shops.

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Introduction

Meat has been an essential human food since ancient times. It is a significant source of high nutritional valuable animal protein, containing the essential amino acids necessary for the sustainability of life. Furthermore, it also contains major and minor minerals and trace elements such as selenium and zinc. Meats are a source of energy-rich food containing long-chain fatty acids and vitamins, especially the Vitamins B group (Robinson, 2001). The safety of food is a complex issue. Meats and meat products are generally considered high-risk food because their composition encourage rapid growth of large number of spoilage and pathogenic microorganisms (Bersisa *et al*., 2019). Meat and its products tend to be contaminated from the preliminary stage of production to consumption. Meat Contamination is a crucial origin of food-borne sicknesses, and death is caused by causative agents that penetrate the body via ingestion (WHO, 2007). Food-borne diseases occur from ingesting bacteria, toxins, and cells produced by microorganisms present in food (Okonko *et al.,* 2010). All parts of the slaughtered animals contain several microorganisms, and previous publications have confirmed the presence of many bacteria in meat. Streptococcus.; Staphylococci, Clostridium, and Salmonella spp. were isolated from the lymph nodes of red meat of various animals (Frazier & Wsethoff, 2003). The natural origins of meat contamination are the environment, the workers, and the slaughtered animals themselves, and to a minor degree, contamination from the air via aerosols and from carcass dressing water (Birhanu *et al*., 2017; Bell and Hathaway,1996). Furthermore, the animal coat, stomachs, intestines, and faces are the primary source of the contaminated bacteria (Norrung *et al*., 2009). The environment of abattoirs provides an appropriate opportunity for microorganisms to grow on the surfaces of carcasses. It can be contaminated during bleeding, skinning, washing with water, and cutting, packing, and shipping. Contamination also occurs from any other environmental source present in the abattoirs; thus, the growth of microorganisms increases on meat surfaces (David *et al*., 2015). Bacteria are the most significant food-borne risk from fresh meat. It can cause human diseases (pathogenic bacteria), such as Staphylococcus aureus and Escherichia coli O157: H7 (Khairi,2020). However, Salmonellae species, Staphylococcus aureus, Listeria monocytogenes, and Campylobacter species require only a few bacteria to cause food poisoning in humans. Contaminated utensils, water, and unhygienic practices such as poor handling and transportation of meat promoted bacterial growth in abattoirs and meat-selling tables. The common contaminated bacteria are Staphylococcus aureus and Shigella (Fasanmi *et al*., 2010; Ali *et al*., 2010). Microbiological testing is required to estimate the operation processing of slaughtering and selling meat for the population to lower and control meat contamination (MIG, 2006). Bacterial counts can be estimated as a good indicator of the quality of meat hygiene. The abattoir's inadequate infrastructural facilities also contribute to the high bacterial load in meat. Therefore, by assessing the bacterial counts, the threat that occurred to human health can be confirmed (Birhanu *et al*., 2017).

Food-borne diseases commonly happen in developing countries due to poor food handling and sanitation practices. Other factors are insufficient food protection regulations, ineffective regulatory systems, lack of financial aid to support secure equipment, and lack of education for food handlers (WHO, 2004). Without appropriate hygienic control, the abattoirs and butcher's atmosphere can be an important source of bacterial contamination (Gill *et al*., 1998).

All Iraqi towns have a high request and utilize animal products such as fresh meat. In different areas of Baghdad (Aldura, Almahmodia, Algehad, Alsader city, Alkhademia, Albyiaa), researchers studied the contamination of local and imported red meat (from Saudi, Indian, and Australia). They found high bacterial contamination in the imported meat, mainly of Coliform, Staphylococcus aureus, and Salmonella. However, a high count of non-diagnosed bacteria was recorded and called "different species "of bacteria (Aseel *et al*., 2010). Thus, the counts of all these bacterial isolates reached the maximum level of the acceptable range specified by the "Central Organization for Standardization and Quality Control." Nevertheless, the local meat's average bacterial count was less than the normal range of limits. Furthermore, Salmonella bacteria showed negative results in all samples. (Samir *et al*., 2013). In another previous study, the researcher isolated various types of bacteria from beef and sheep meat samples collected from different Baghdad regions. Various bacterial species were isolated in different percentages from raw sheep meat % 23.8, 29.4 %, 14.7 %,% 4.9, %3.5, %14.7, %14.7 and %2.1 for *Streptococcus group D*, *Staphylococcus aureus*, *E.coli*, *Salmonella sp.*, *pseudomonas aeruginosa*, *Proteus sp.,* and *Listeria sp* respectively. In contrast, the percentages of isolation from raw beef meat were %5.55, %8.14, %5.18, %3.7, 1.85 %, %1.85, % 1.48, 1.48 % for *Staphylococcus aureus*, *Streptococcus group D*, *E.coli, Proteus sp*, *Salmonella sp.*, *pseudomonas aeruginosa*, *Bacillus cereus*, and *Klebsiella sp.* respectively. The demand and consumption of meat and animal products are increased in many Iraqi governorates, including Al Muthanna governorate. Nonetheless, reviewing the literature on the hygienic situations and management practices of meat in abattoirs and butcher shops revealed scarce publication. Additionally, no similar information is available regarding the assessment of food safety practices, food-borne diseases, and microbial load of meat in the abattoir and butcher shops of the study area. Consequently, this study was designed to isolate and determine the bacterial load, identify pathogenic bacteria in meat from abattoir and butcher shops in Al Muthanna governorate/Iraq, assess their resistance to antimicrobial agents, and determine the presence of tetA resistance genes.

Materials and Methods

Description of the Study Area

Al Muthanna governorate is located in southwestern Iraq, at a distance of 280 Kilometers from Baghdad, the country's capital city. It borders Saudia-Arabia and shares internal boundaries with Najaf, Qadissya and Thi-Qar, governorates. Al Muthanna governorate shows a desert climate, in summer temperatures over 40°C, while rainfall is scarce and limited to the winter months, with average High Temperatures are 15°C (January) to 42°C (July), while the average Low Temperatures are 7°C (January) to 30°C (July) (Figure. 1) (Al Salihi & Kathem, 2021).

Figure.1: Shows a location of Al Muthanna governorate (Red color) in southwestern of Iraq

This cross-sectional study was done by randomly collecting meat samples from the butcher shops and the governorate's abattoir from November 2021 to January 2022. Twenty-one meat samples, including 16 and 5 from butcher shops in Samawah city and Al Muthanna abattoir, were collected in sterile plastic bottles using necessary disinfection and sterilization measurements. The samples were transferred immediately in an icebox to the central microbiology laboratory/ Al Muthanna teaching veterinary hospital for bacteriological investigation (Figure.2).

Figure.2: Shows meat samples collected from butcher shops and abattoir in sterile plastic bottle

Isolation and Identification of Bacteria.

A small piece (about 25 grams) of each raw meat sample was transferred to a blender, emulsified with 150 mL of 0.1% sterile buffered peptone water, and kept in the incubator for 24 h at 37◦C for the Pre-enrichment period. A loopful of the enriched broth was streaked on various media, including Nutrient agar, Blood agar, MacConkey agar plates (Oxoid), Eosin Methylene Blue agar, and Salmonella-Shigella agar, and incubated at 37◦C for 24 hrs. All media were prepared according to the manufacturer's instructions. The growing colonies were examined macroscopically for colonies description, including shape, color, size, blood hemolysis, and consistency. Gram staining and appropriate biochemical tests were carried out according to the standard microbiological procedure, including VP (Voges Proskauer), Simmon Citrate test, Indole test, TSI, Urease, Catalase, and Oxidase. The reactions of each isolate were recognized by comparing their morphological and biochemical characteristics with standard reference organisms of known taxa, as described in Bergey's Manual for Determinative Bacteriology (Buchanan & Gibbons, 1984). The API 20 E identification strips were used for rapid biochemical tests. The test was conducted by emulsifying 1-3 well-isolated colonies of young culture into 5ml normal saline, afterward adjusted to 0.5 McFarland's standard tube. A 5ml disposable syringe was used to distribute bacterial suspension in API strip tubules. Cupules and tubules were filled for citrate, Voges-Proskauer, and gelatin tests, whereas only the cupules were filled for the other tests. The sterile paraffin oil was covered in the following tests: Arginine Dihydrolase (ADH), Lysine Decarboxylase (LDC), Ornithine Decarboxylase, Hydrogen sulphite production (H2S), and urease activity tests. Later, the inoculated strips were placed in the incubation box containing a little volume of water at the bottom to prevent dehydration and incubated at 37°C for 18 hours. After incubation, and according to the manufacturer's instructions, some specific reagents were added to some tests, and the results were read and recorded.

Antibiotic sensitivity test

The antibiotic sensitivity test of the isolated bacteria was determined by the standard disc-diffusion test (Bauer *et al*., 1959; Bauer *et al*., 1966; Kirby–Bauer, 1997; Vandepitte et al*.,* 2003; Clinical Laboratory Standards Institute,2006; Jorgensen and Turnidge, 2007). Various commercially available antibiotic discs and Muller – Hinton agar (MHA) were also used in this test. Bacterial suspensions were prepared and adjusted to 0.5 McFarland Standard. Instantly after standardization, a sterile cotton swab was dipped in bacterial suspension and streaked on the surface of Muller Hinton agar. The following commercial available antibiotic discs: Tetracycline (TE -10 µg), Amoxicillin/ Clavulanic (AMC-30 µg), Levofloxacin (LEV-5 µg), Gentamicin (GN-10 µg), Ceftazidime (CAZ- 30 µg), Trimethoprim/ Sulfamethhazol (SXT-25 µg) were arranged on the surface of inoculated plates. The plates were incubated at 370 C for 16-18 hours. Then the results were evaluated, and the inhibition zones around each disc were measured and compared with standard measurement data (CLSI, 2017, https://clsi.org/media/1795/catalog2017_web.pdf).

Detection of Tetracycline (tet) Resistance Genes

According to the instructions of the company (Qiagen/German), DNA extraction was done using overnight bacterial isolates culture and tet AC F, tetAC R, Tet(A)-F, and Tet(A)-R primes (Table.1). The PCR amplification cocktail Tet(A) was prepared (Table. 2). The PCR Amplification Program for Tet (A) and PCR Amplification Program tetAC were displayed in Table. 3 and 4, respectively.

Table. 1: The designed primers use for confirmation of the tet A resistance gene *in E. coli*

Prime	**Sequence (5'-3')**	**Size of amplified product (bp)**	**References**
*tet*AC F	5'CGCYTATATYGCCGAYATCAC-3'	417bp	(Balasubramaniam, *et al.,* 2003)
tetAC R	5'CCRAAWKCGGCWAGCGA-3		
Tet(A)-F	5'- GTGAAACCCAACATACCCC-3'	888bp	(Maynard *et al*., 2003)
Tet(A)-R	5'-GAAGGCAAGCAGGATGTAG-3		

Table. 2: The volum of PCR Amplification cocktail Tet(A)

	Reagents	Quantity
1.	Green Master Mix. Tube	10 µl
2.	F Primer	1 µl
3.	R Primer	1 µl
4.	DNA template	6 µl
5.	Nuclease-free water	32 µl

Table. 3: PCR Amplification Program Tet(A)

Stages		Steps	Temperature (C°)	Time	No. of cycles
First		Initial Denaturation	94	5 min	1
Second	I	Denaturation	94	40 s	40
	II	Annealing	57	1	
	III	Extension	72	1 min	
Third		Final Extension	72	10 min	1

Table. 4: PCR Amplification Program tetAC

Stages		Steps	Temperature (˚ C)	Time	No. of cycles
First		Initial Denaturation	94	5 min	1
Second	I	Denaturation	94	45 s	30
	II	Annealing	55	1 min	
	III	Extension	72	45s	
Third		Final Extension	72	10 min	1

Results

This study showed that all samples were contaminated with microorganisms, and the isolation percentage was 100%. The routine biochemical test and API 20E investigated the following bacteria: *E. coli, Salmonella typhi, Salmonella typhimurium, Salmonella enteritidis, other Salmonella sp*., *Citobacter freundii, Shigella flexner* and *Porteous vulgaris,* moreover, the percentages of isolation were 57.14%, 4.76%, 14.285, 4.76%, 4.76%, 4.76%, 4.76%, and 4.76% respectively (Figure.3).

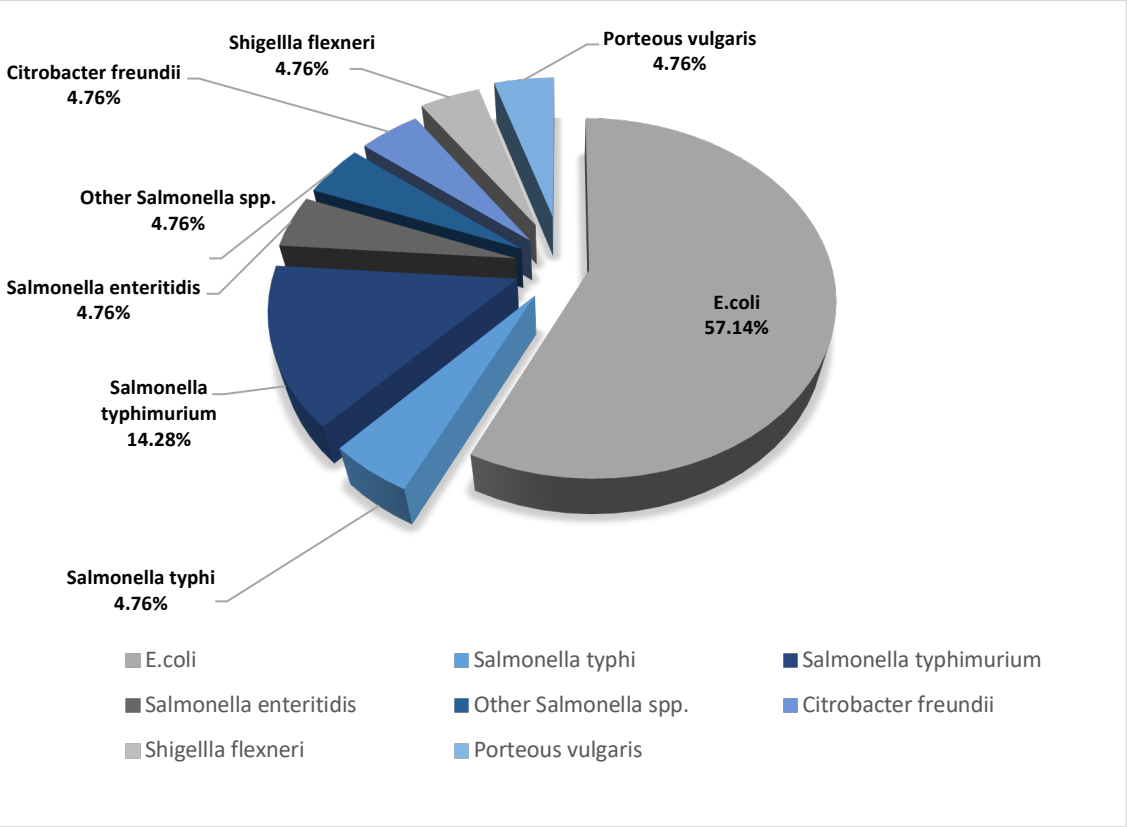

Figure.3: Shows the isolated bacteria from meat samples and its percentages

This study also showed high percentages of *Escherichia coli*, followed by *Salmonella typhimurium*, *salmonella typhi*, *Salmonella enteritidis.*, *other types of salmonella*, *Porteous vulgaris* and *Citobacter freundii.* The antibiotic sensitivity test showed multiple drug resistance for the examined bacterial isolates. The number and percentage of resistant and sensitive bacteria were18 (85.71%) and 3 (24.29%), respectively (Figure. 4 &5). Moreover, 12 out of 12 isolated *E. coli* were revealed Tetracycline (TE -10 µg) resistant in vitro. These isolates were tested to identify the prevalence of tetracycline resistance genes tet (A). 12 out 12 (100 %) of *E. Coli* isolates carried the tetA gene (Figure.6). PCR amplified the tet (A) gene of strains with two sets of primers targeting the tetracycline efflux gene (tetA) (Figure. 6).

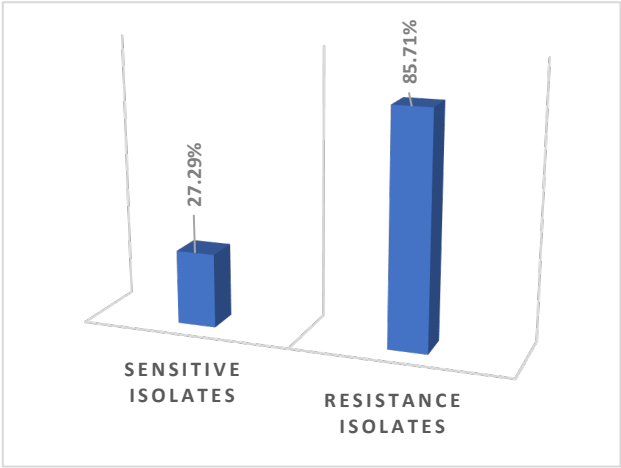

Figure.4: Shows the percentages of sensitive and resistance isolates in the antibiotic sensitivity test

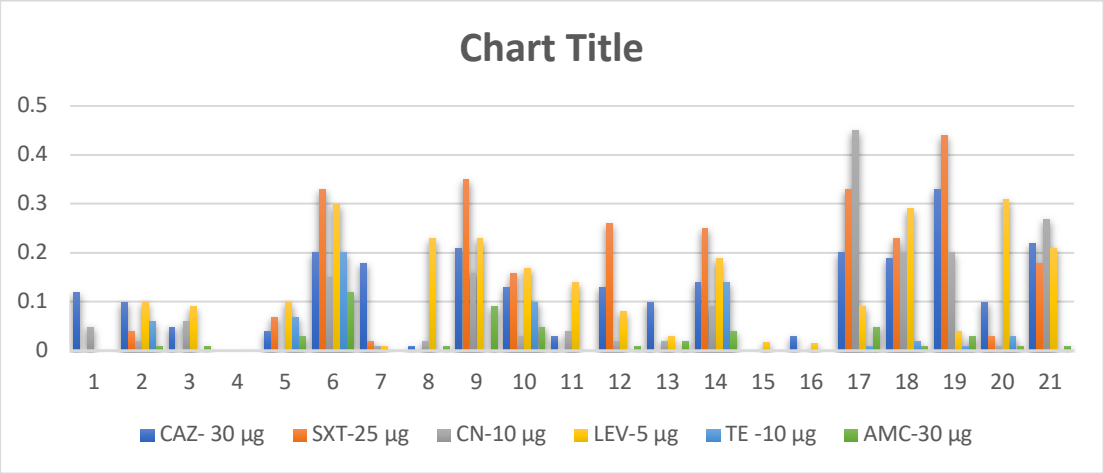

Figure.5 Shows the results of inhibition zone antibiotic sensitivity test for the isolated bacteria (1 to 21 bacterial isolates)

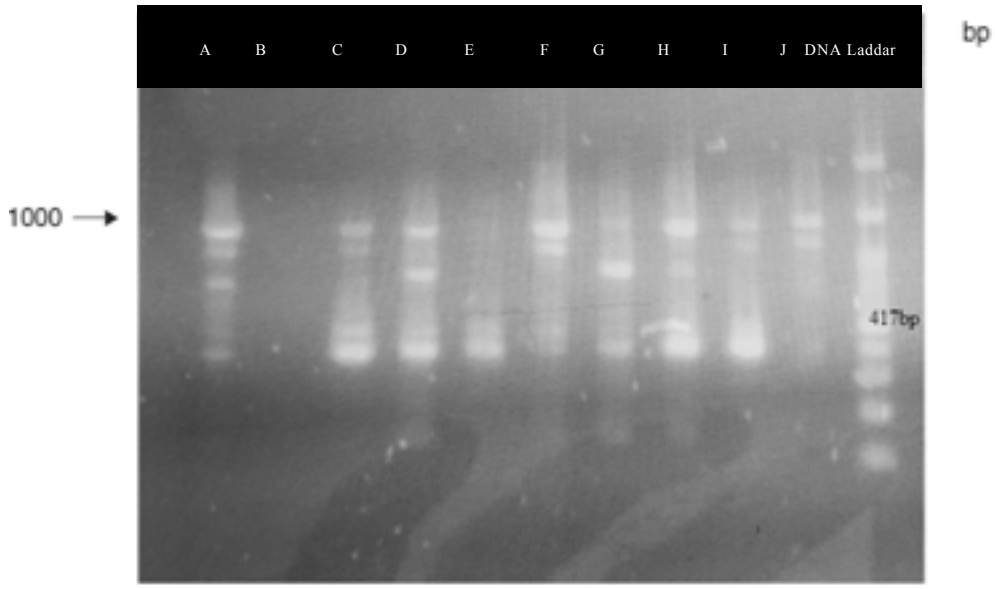

Figure.6: *E. coli* isolates genomic DNA profiles obtained with multiplex PCR. Shows the results of tetracycline-resistant *E. coli* isolates obtained from meat samples. Multiple bands obtained from isolates.

Discussion

The abattoir and butcher shops contribute to the appearance and distribution of food-borne diseases if no public health measurements are taken (Roberts *et al.,* 2009). The results of the current study revealed bacterial contamination with eight bacterial species from fresh red meat in the Al Muthanna governorate. The high percentage of isolation was 57.14% for *Escherichia coli,* which is compatible with the previous study (Al Kayita, 2006; Ukut *et al.,* 2010) that approved isolation of E. coli O157; H7 at high percentages reached 80% and 65% for local and imported bovine minced meat respectively. The researcher explained that minced meat is more exposed to contamination than non-minced fresh meat. This study's results are also compatible with a previous study (Aseel *et al.,*2010), who isolated *E.coli* at a percentage of 5.81%, though the isolation percentage was lower than the current study. The results of this study are also compatible with a previous study in Palestinian (Adwan & Adwan, 2004), who isolated E. coli at 14.7% from bovine meat in north Palestinian. However, according to the Australian ACT Health, the isolation percentage of *E. coli* was 16.6 %. At the same time, the isolation percentages of E.coli were 19 % (White *et al*., 2001), who mentioned the importance of quick evisceration of the internal organs such intestine for the reduction of bacterial contamination. Nonetheless, other researchers referred to the elevation of bovine meat *E.coli* contamination in the rain season (Sheridan., 1998), while the contamination rate of *E.coli* in sheep meat was 14.7% (Rigobelo *et al.,* 2008). In the current study, *Salmonella typhimurium*, *Salmonella typhi*, *Salmonella enteritidis.*, *other types of Salmonella*, *Porteous vulgaris, Shigella flexneri,* and *Citrobacter freundii* were also isolated from meat samples, *and their* percentages *of isolation* were, 14.285, 4.76%, 4.76%, 4.76%, 4.76%, 4.76%, and

4.76% descendingly. These results are compatible with previous studies (Yang *et al.,* 2010; Xiaojuan *et al.,* 2019). Nonetheless, the current study's isolation percentages were equal to those reported in China, 14.1% (Li *et al*.,2014). Another study reported high contamination percentages, 33.3% from the current study. Additionally, these results disagree with other studies from other Iraqi governorates (Ali, 1986), who reported 8 % isolation percentage in meat from Al Dora abattoir / Baghdad. While other researchers reported contamination of sheep meat with *Salmonella* in Mosul (Hadad & Jemel, 1985). Therefore, the high percentage of *Salmonella typhimurium* is a serious human food pathogen. In the current study, *Porteous vulgaris* and *Citrobacter freundii* were also isolated from meat samples at 4.76% and 4.76%, respectively, which are lower than the results reported in Egypt at 13.3 % (Gwida *et al.,* 2014). However, these isolation percentages are not acceptable because these microorganisms are pathogenic and can cause diarrhea and enteritis. Additionally, these results are compatible with results reported previously in Iran (Ebrahim *et al*., 2017), who found multiple drug resistance microorganisms in hotdogs and burgers and considered causes of gastritis and food-borne diseases. In the current study, the results of the antibiotic sensitivity test revealed a multidrug-resistant including TE -10 µg: Tetracycline, AMC-30 µg: Amoxicillin/ Clavulanic, LEV-5 µg: Levofloxacin CN-10 µg: Gentamicin, CAZ-30 µg: ceftazidime: SXT-25 µgTrimethoprim/ Sulfamethhazol. Only three bacterial isolates (24.29 %) revealed sensitivity to some antibiotics, while 18 (85.71%) isolates were resistant to the antibiotic in vitro. These results are compatible with previous studies that approved the isolation of resistance microorganisms for at least one antibiotic from meat in abattoirs and butcher shops at percentages of 83.0% and 65.6% respectively (Xiaojuan *et al.,* 2019).

Moreover, 12 out of 12 isolated *E. coli* were revealed Tetracycline (TE -10 µg) resistant in vitro. In the present study, 12 out of 12 (100%) isolated *E. coli* revealed the presence of the tetA gene, which is compatible with the higher percentages of tetracycline-resistant isolates reported in the previous study (Koo & Woo, 2011). This study found that 98.3% of meat-borne *E. coli* contained at least one of the tetA to tetD genes and was able to transfer tetracycline resistance to a tetracycline-susceptible recipient strain of *E. coli*. These results approved that tetA gene can be spread more quickly in the environment than tetB. Moreover, direct or indirect contact methods can spread antimicrobial resistance to humans and animals via consumed food/feed and through the environment. Hence, the epidemiology and mechanisms of antimicrobial resistance's emergence and spread need to be evaluated (Entedhar *et al*., 2019). The reasons for the high isolation percentages of the current study might be related to several factors. These include the process of slaughtering, transferring, and preserving the carcass until selling, the health condition of the workers that play an essential role in contamination of meat by human pathogenic microorganisms like Salmonella sp., Mycobacteria sp. and Staphylococcus aureus. The worker's clothes, dirty hands, and instruments used in skinning and cutting meat also play an important in spreading microorganisms and contamination of meat. The other important factor in the high percentage of *E.coli* in meat samples was the non-appropriate transportation method for the carcasses. The carcasses are transported by the same vehicle that transports the live animals to the abattoir, which often contains the urine and feces of these animals that contain a population of pathogenic and non-pathogenic microorganisms. The absence of a cold and clean transportation method for the carcasses and the use open non-refrigerator, a small vehicle called Sitota, exposed the meat to high weather temperature and dust and

promoted the growth of carcass surface bacteria found in low concentration after slaughtering. These results are compatible with previously reported studies ((AL-Sheddy *et al.,* 1995; Hinkins *et al.,* 1995; Hassan, 1992; Sheet *et al.,* 2009; Al-aboudi *et al*., 1987).

In conclusion, this study approved the contamination of fresh meat by various types of pathogenic bacteria that can threaten the lives of people consuming these meats. The study also approved the absence of hygienic procedures during slaughtering the animals in abattoirs accompanied by poor transportation of meat after slaughter by open-air vehicles that are not refrigerated and exposing them to bacteria, dust, and vehicle exhaust. Unhygienic meat storage, hanging carcasses for long hours in butcher shops, and indiscriminate slaughter outside the abattoirs was also approved. The authors recommend another future study focusing on hygienic measurement in the abattoir to establish strict abattoir hygienic regulations.

Ethical statement

This study was approved by research and animal ethical committee / Department of Biology /College of Education for Pure Sciences / Al Muthanna University/ Iraq.

Conflict of interest

No conflict of interest in publishing this article is declare by the authors.

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Authors contributions

All authors perceived , research, write and review this manuscript equally.

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