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Isolation and identification of *Staphylococcus aureus, Listeria monocytogenes, E. coli* O157:H7 and *Salmonella* specious from raw beef and lamb meat in Baghdad by PCR.

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Abstract

The study designed to determine the distribution of a major important food pathogens including *Staphylococcus aureus* , *Listeria monocytogenes*, *E. coli* O157:H7 and *Salmonella* spp from raw beef and lamb meat by using multiplex pcr . A total of 90 raw beef and lamb meat samples were collected from different butcher's shops in Al-Karkh side of Baghdad city and analyzed for the presence of these types of bacteria and their susceptibilities to some antibiotics was investigated ,the results showed that the prevalence of *S. aureus* (5.6%), *L. monocytogenes* (3%), *E. coli* O157:H7 (7.8 %) and *Salmonella* spp (5.6%) from the total samples .The result of the susceptibility test showed that *S. aureus* isolates were susceptible to Amikacin (80%) ,while *L. monocytogenes* isolates were susceptible to the most used antibiotics as following Amikacin, Erythromycin, Oxytetracycline, Nalidixic acid ,Cephalothin, Gentamycin, Ampicillin and Streptomycin (100%). *E. coli* O157:H7 isolates were susceptible to Nalidixic acid and Gentamycin (100%) and *Salmonella* spp isolates were susceptible to Nalidixic acid ,Cephalothin and Gentamycin (80%) .

Keywords: red raw meat, antibiotic susceptibility, food pathogens

عزل و تشخيص جراثيم المكورات العنقودية الذهبية ،اللستيريا ،الاشريشيا القولونية والسالمونيلا من لحوم الابقار والأغنام النيئة في مدينة بغداد بواسطة تفاعل سلسلة البلمرة المتعدد

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الخلاصة

تم تصميم الدراسة لتحديد مدى انتشار بعض الجراثيم الغذائية المهمة ومن ضمنها المكورات العنقودية الذهبية والليستيريا مونوسايتوجينز والاشريشيا القولونية والسالمونيلا من عينات لحوم الابقار والأغنام النيئة بطريقة تفاعل سلسلة البلمرة المتعدد. جمعت 90 عينة من لحوم الابقار والأغنام من محلات القصابة ضمن منطقة الكرخ من بغداد لتحديد وجود البكتيريا ، كما تم اجراء فحص حساسيتها لبعض المضادات الحيوية واظهرت النتيجة تواجد بكتيريا المكورات العنقودية الذهبية بنسبة 5,6% والليستيريا مونوسايتوجينز 3% و والاشريشيا القولونية 7,8% والسالمونيلا 5,6% من مجموع العينات الكلية. نتائج فحص الحساسية سجلت حساسية المكورات العنقودية الذهبية للاميكاسين بنسبة 80% اما الليستيريا مونوسايتوجينز كانت حساسة لمعظم المضادات الحيوية والتي تضمنت (الاميكاسين،الارثرومايسين،الاوكسيتتراسايكلين، حامض الناليدكسيك، السيفالوثين، الجنتمايسين، الامبسلين والستربتومايسين) 100%، الاشريشيا القولونية كانت حساسة لحامض الناليدكسيك والجنتمايسين بنسبة 100% اما السالمونيلا فكانت حساسة لكل من حامض الناليدكسيك ، السيفالوثين والجنتمايسين بنسبة 80% .

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Introduction

Food borne illness considered as a major international health problem that's why, each year millions of peoples become sick from food borne disease even if many cases are not reported .[1] Many risky pathogens are transmitted through contaminated food and water. Protein in many developing countries remains as the main source of energy, this led to increased production and consumption of meats [2].

There are many pathogens recorded as a significant importance for human health and these include *S. aureus* the cause of food poisoning which resulting from the consumption of contaminated food because of staphylococcal enterotoxins. Especially when different food can act as a good medium for *S.aureus* such as raw meat. [3 ,4] *Listeria monocytogenes* is the cause of listeriosis which may lead to death in human , the raw red meat is a direct sources of contamination [5].

E. coli the most significant food-borne pathogens which is a group of *E. coli* which is responsible for a long list of human diseases [6] *Salmonella* spp also reported as a causes of food-borne disease , and show the highest disease at the population scale among bacterial detection from samples [7] Because of the low infective dose of *Salmonella* presence in food the methods for its detection required for the presence of one cell in a defined prove food sample [8].

The present study was carried out to determine the prevalence of *Staphylococcus aureus, Listeria monocytogenes, E.* coli O157:H7and *Salmonella* spp. in raw beef and lamb meat from butcher`s shops in Al-Karkh side of Baghdad city by using a multiplex PCR assay and to determine the susceptibility of the bacterial isolates to some selective antibiotics.

Material and Methods

A total of 90 random samples of raw beef and lamb meat were collected from different butcher's shops in Al-Karkh side of Baghdad city between March and November 2015. All collected samples were transported to the laboratory in iced boxes within 2 hours. 25 g each sample were homogenized by using a stomacher with 225 mL of enrichment broths for *S. aureus*.[9] Enrichment broth for *L. monocytogenes,* trypticase soy broth (TSB) containing 0.5 mg/ml novobiocin as an enrichment broth for *E. coli* O157:H7 and Rappaport-Vassiliadis enrichment broth for *Salmonella* . Enrichment broths were incubated for 24 hours at 37ºC, while *Salmonella* broth was incubated at 42ºC. At the end of the incubation period loopfull from each of the selective enrichment broths was streaked on prepared plated medium BBL™ CHROMagar™*S.aureus* medium(Becton Dickinson, Franklin Lakes, NJ). The growth of mauve-colored colonies at 24 is considered positive for *S.aureus* , Columbia Blood Agar Base which consist of polymyxinB sulfate, acriflavine-HCl, lithium chloride and ceftazidime PALCAM agar and the typical colonies of *L. monocytogenes* were small round mignonette colonies surrounded by brownish-black hydrolysis halo [10].

After incubation, *E. coli* O157:H7 colonies have black or gray coloration on Rainbow Agar(RBA) and *Salmonella-Shigella* (SS) agar and incubated at 37°C for 24 h. The plates were examined for the presence of typical colonies of *Salmonella* which were transparent colonies with black centers then biochemical tests were used for complete characterization .

Extraction of DNA and Multiplex Polymerase Chain Reaction

The extraction of DNA was done at the laboratories of the Iraqi Biotechnology Co. in Baghdad using boiling method. [11] After incubation a 1 mL of the enrichment broths were centrifuged for 3 minutes. Then the formed bacterial pellets were suspended in 1 mL of sterile saline solution (0.85% NaCl). after the centrifugation the supernatants were replaced with 50 µL of sterile distilled water and incubated for 10 minutes at 100ºC for DNA extraction , then the clear supernatants centrifuged for 5 minutes at 14000 rpm, they were stored at -20ºC till using. The extracted DNA were mixed and used for the multiplex PCR reactions. The following oligonucleotide primers are used in this study showed in Table 2 and the multiplex PCR procedure is done as reported by Karami et al . [12] In the multiplex PCR with mixed DNA samples, the thermal cycler of the reaction steps are initial denaturation at 94ºC for 3 minutes and denaturation at 94ºC for 45 seconds then primer annealing at 54ºC for 45 seconds and extension at 72ºC for 60 seconds. The final cycle included a 5-minute additional extension at 72ºC. 2% agarose gels using to observe the PCR products .

Table 1- types of Oligonucleotide primers that used in the study

Bacterial name	Sequence used (5′→ 3′)	Target Gene	(bp)	Reference
S.aureus	ForwardCTGGCATATGTATGGCAATTG Reverse AATGCACTTGCTTCAGGACC	nuc	397	[13]
L monocytogenes	Forward:CTGGCACAAAATTACTTACAACGA Reverse AACTACTGGAGCTGCTTGTTTTTC	iap	454	[14]
E. coli O157:H7	Forward:GATAGACTTTTCGACCCAACAAAG Reverse:TTGCTCAATAATCAGACGAAGATG	stx	208	[14]
Salmonella spp.	Forward:GAATCCTCAGTTTTTCAACGTTTC Reverse TAGCCGTAACAACCAATACAAATG	invA	678	[14]

Antibiotic susceptibility

Antibiotic susceptibilitywas monitored with the disk diffusion assay (Kirby–Bauer) recommended by the National Committee for Clinical Laboratory Standards on Muller Hinton agar (Oxoid, Milan,Italy), the zone of inhibition was interpreted according to NCCLS guidelines [15].

These antibiotic discs were used Amikacin (30µg), Erythromycin (15 μg), Vancomycin (30 μg), Oxacillin (1 μg), Oxytetracycline (30 μg), Nalidixic acid (30 μg), Cephalothin (30 μg), Gentamycin (10 μg), Ampicillin (10μg) and Streptomycin (10 μg) supplied by HiMedia Laboratories Pvt. Ltd., Mumbai (India), were placed onto Mueller-Hinton agar plates. The plates were incubated at 37◦C for 24 h. The zone diameter was measured and results were interpreted based on CLSI [16].

The reference strains *E. coli* ATCC 25922 and *S. aureus* ATCC 6538 were used as a control .

Statistical Analysis

The Chi-square test used for statistical analysis. A P value <0.05 was used for statistical significance to compare rate of isolation of the various pathogens in beef and lambs. [17].

Results and discussion

The results showed that the prevalence of *S. aureus* (5.6%), *L. monocytogenes* (3%), *E. coli* O157:H7 (7.8 %) and *Salmonella* spp (5.6%) from the total samples there were no significant differences between two groups at $P < 0.05$ as shown in Table-2.

Table 2- The incidence of *S. aureus* , *L. monocytogenes*, *E. coli* O157:H7 and *Salmonella* spp. in beef and lamb meat

Types of Meat	Samples No.	*S. aureus*	*L. monocytogenes*	*E. coli* O157:H7	*Salmonella* spp
Beef	45	3(6.7%)	2(4.4%)	3(6.7%)	2(4.4%)
Lamb	45	2(4.4%)	1(2.2%)	4(8.9%)	3(6.7%)
total	90	5 (5.6%)	3(3%)	7 (7.8%)	5 (5.6%)

Multiplex Polymerase Chain Reaction was used for the determination of pathogens in two kinds of meat, Table 2 showed that *S. aureus* isolates detected in 6.7% of beef and 4.4% of lamb raw meat , in other study in Egypt *S. aureus* was isolated at a rate of 2% beef raw meat . In China, raw beef and lamb meat found as major food types contaminated by *S. aureus* reaches to 38% [18]. Other study in Tehran showed the prevalence of *S. aureus* 3.7 % in beef and 1.6% in lamb [19]. The level of contamination can substantially increase by poor slaughter procedures, contaminated tools and equipment, personnel and clothing play an important role in contamination [20].

L. monocytogenes isolates detected in 4.4% of the beef and 2.2% of the lamb samples while in other Iranian study about 2.6% of the beef and 6% of the lamb samples detected the presence of this pathogen [21]. In Thailand the prevalence of *L. monocytogenes* in raw meats marketed was 15.4% from the total samples and 3% from total beef samples.[22]. Meat in different butcher`s shops have brought from different sources beside the absence of good hygienic meat processing and handling, the pH and water activity play significant role in the growth of *L. monocytogenes* [23,24]. And this might be one of the reasons behind the different prevalence of *L. monocytogenes* found in the studies.

The study showed that 6.7 % of the beef and 8.9 % of the lamb samples contaminated with *E. coli* O157:H7 which found in the carcass also the work surfaces and material used for processing of meat products but we should say that with good hygienic practices in handling the level of carcass contamination will significantly decrease the carriage rate [25]. In Denmark, 3.2% of the beef

carcasses were contaminated with *E. coli* O157:H7 and another study in the United States *E. coli* O157:H7 was detected in 1.6 of the lamb slaughtered carcasses. In South Yorkshire they isolate *E. coli* O157:H7 from 1.4% of beef and 0.7% of lamb carcasses [26, 27]. And in Rome they found that the prevalence of *E. coli* O157:H7 in slaughtered lambs was 0.2% [28]. This difference in the prevalence rates could be because of the sizes and varieties of samples, the used methods, and the sampling times. the presence of *E. coli* O157:H7 in meats threats the public health and it may cause severe disease in children, pregnant women, the immune-compromised and the elderly.

In the present study the prevalence of *Salmonella* spp was 4.4% of the beef and 6.7% of the lamb samples. And this disagree with another study in United Kingdom , as *Salmonella* was detected in 1.7% of lamb, 1.1% of beef [29]. While other studies in USA found that *Salmonella* contamination in beef products was 1.0–1.9% [30]. The presence of *Salmonella* spp. in meat commonly indicates contamination that may be directly introduced by the hands of workers and contaminated equipment.

The result of the Susceptibility test showed that *S. aureus* isolates were susceptible to Amikacin (80%) and resistant to the most other antibiotics as shown in Table 3. and these results agree with other study which found that *S. aureus* resistance of 90% to oxacillin [31], but other study reported high sensitivity of *S. aureus* to vancomycin and high resistance to ampicillin [32]. The use of antibiotics as growth promoters in animal husbandry especially of those commonly used for both human and animal care should be avoided.

L. monocytogenes isolates were susceptible to the following antibiotics Amikacin, Erythromycin, Oxytetracycline, Nalidixic acid ,Cephalothin, Gentamycin, Ampicillin and Streptomycin (100%) as shown in Table-4. And this agree with other study that found all *L. monocytogenes* strains were susceptible to 90% from the tested antibiotics [33].

While disagree with another studies that found *L. monocytogenes* strains have a natural resistance phenotypes to the cephalosporins and nalidixic acid, the resistance for this antibiotic could be due to the illegal using in animal's farms [34,35].

Table 3- Antibiotic sensitivity and resistance of *S. aureus* isolates .

Antibiotics	***S. aureus* n=5**		
	S	**I**	**R**
Amikacin	4(80%)	0	1(20%)
Erythromycin	2(40%)	0	3(60%)
Oxacillin	0	1(20%)	4(80%)
Oxytetracycline	1(20%)	0	4(80%)
Nalidixic acid	0	1(20%)	4(80%)
Cephalothin	2(40%)	0	3(60%)
Gentamycin	1(20%)	0	4(80%)
Ampicillin	1(20%)	0	4(80%)
Streptomycin	1(20%)	0	4(80%)

Table 4- Antibiotic sensitivity and resistance of *L. monocytogenes* isolates.

Antiboitics	***L. monocytogenes* n=3**		
	S	**I**	**R**
Amikacin	3 (100%)	0	0
Erythromycin	3(100%)	0	0
Vancomycin	2(67%)	0	1(33%)
Oxacillin	2(67%)	0	1(33%)
Oxytetracycline	3(100%)	0	0
Nalidixic acid	3(100%)	0	0
Cephalothin	2(67%)	0	1(33%)
Gentamycin	3(100%)	0	0
Ampicillin	3(100%)	0	0
Streptomycin	3(100%)	0	0

E. coli O157:H7 isolates were susceptible to Nalidixic acid and Gentamycin (100%), and these finding were disagree with other result of *E. coli* serotype O157:H7 they were 70% resistant to

Nalidixic acid and all the isolates were sensitive to Gentamicinhe, high level of resistance to these antimicrobials was probably an indication of their extensive usage in the veterinary sector for therapeutic and prophylactic purpose both for *E. coli* and other infections. [36].

Our findings on some of the effective antibiotics agree with the other reports [37,38]. The susceptibility might have contributed to the effectiveness of these antimicrobials mostly against gram negative bacteria like those of the family of *Enterobacteriaceae* to which *E. coli* O157:H7 belongs.
On the other hand *Salmonella* spp isolates were susceptible to Nalidixic acid ,Cephalothin, Gentamycin (80%) and resistant to Oxytetracycline (100%).As shown in Table 5. And this agree with other study in morocco as they found that 71% (75/105) of *Salmonella* spp isolates were susceptible to ciprofloxacin, ceftazidim, cefotaxime, cefamandol, gentamycin and mecillinam while the most common resistance observed was to tetracycline and ampicillin ,Another study in Iran ,all isolates, from meat samples were resistance to Ampicillin, Amoxicillin, Nitrofuranthoin, Tetracycline, and were susceptible to Gentamycin and Ceftriaxone [39,40].

Table 5- Antibiotic sensitivity and resistance of *E. coli* O157:H7 and *Salmonella* spp isolates.

Antiboitics	***E. coli* O157:H7 n=7**			***Salmonella* spp n=5**		
	S	**I**	**R**	**S**	**I**	**R**
Amikacin	2(29%)	0	5(71%)	1(20%)	0	4(80%)
Vancomycin	1(14%)	1(14%)	5(71%)	1(20%)	0	4(80%)
Oxacillin	0	0	7(100%)	1(20%)	0	4(80%)
Oxytetracycline	1(14%)	0	6(86%)	0	0	5(100%)
Nalidixic acid	7(100%)	0	0	4(80%)	0	1(20%)
Cephalothin	5(71%)	0	2(29%)	4(80%)	0	1(20%)
Gentamycin	7(100%)	0	0	4(80%)	1(20%)	0
Ampicillin	1(14%)	2(29%)	4(57%)	2(40%)	0	3(60%)
Streptomycin	2(29%)	1(14%)	4(57%)	1(20%)	0	4(80%)

This study shows that multiplex PCR is a very useful tool for the detection of food-borne pathogens, specifically S.*aureus L. monocytogenes*, *E. coli* O157:H7 and *Salmonella* spp. The method represents a rapid and cost-effective way for detection of these pathogens in beef and lamb meat.
When comparing our results to other studies, the discrepancies could be partly due to differences in sampling techniques and the detection methods. Besides, slaughter hygiene, cross contamination of the products at different stages throughout the food chain should be considered.

The results of the present study can provide a significant contribution to both regulatory agencies and the meat industries.

Conclusion

The results indicate that the beef and lamb raw meat is considered as a reservoirs of many food pathogens at the butcher's shops and this maybe because the absence of sanitary hygiene and due to the potential hazard of these pathogenic bacteria, it is necessary to put more emphasis on meat hygiene, so, the surveillance of potential contaminant bacteria in different kinds of meat is crucial to safeguard the public health. and the isolated bacteria were highly susceptible to a number of antibiotics which could use as a treatment of infections caused by these pathogens .

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