Comparison of techniques used in isolation and identification of *salmonella spp.* and related genera from raw meat and abattoir environment of Basrah

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ABSTRACT

The present study aimed to detect the presence of *Salmonella spp.* and related genera in raw meat and different parts of abattoir. It aimed also to compare the efficiency of techniques used in identification of *Salmonella spp*. and *Citrobacter freundii* . A total of (298) samples were collected from Basrah abattoir between 16th September 2014 and 21st March 2015. These samples composed of (80) samples from buffalo, (51) samples from cow, (93) samples from sheep, (51) swabs and (23) samples of sewage. Different techniques were used in this study to evaluate the presence of *Salmonella spp.* and phenotypically similar genera, which contaminate the raw meat. These techniques included traditional bacteriological assays, commercial identification kit (API 20 E), serotyping and molecular techniques (multiplex PCR).

Results of these techniques reflected the absence of *Salmonella spp.* from tested samples. However, 35 samples were positive to *Proteus mirabilis*, as identified by API 20 E system. There were differences between API 20 E and serotyping in results, (44) isolates were identified as *Citrobacter freundii* by using API 20 E, whereas, results of serotyping indicated the presence of (31) isolates as *Citrobacter braakii* and (13) *Enterobacter spp.*

The isolates were subjected to mPCR [*invA* gene (243 bp), *spvC* gene (570 bp) and *viaB* gene (516 bp)].

Results of mPCR confirmed only (13) of those isolates as *Citrobacter freundii*, while the other (31) isolates were not confirmed as *Salmonella spp.* or *Citrobacter freundii*. In addition, the results of this study showed that the mPCR is an accurate method for confirmation of *Salmonellaspp.* and *Citrobacter freundii*.

Keywords: *Citrobacter freundii*, *Proteus mirabilis*, *invA*, *spvC* and viaB genes

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الملخص باللغة العربية

هدفت هذه الدراسة إلى الكشف عن وجود السالمونيلا والأجناس ذات الصلة في اللحوم النيئة وأجزاء مختلفة من مسلخ البصرة، كما هدفت إلى إجراء مقارنة بين كفاءة التقنيات المستخدمة في عزل وتشخيص السالمونيلا و *Citrobacter freundii*. تم جمع (298) عينة من مسلخ البصرة خلال الفترة من 16 أيلول / سبتمبر 2014 إلى 21 آذار/ مارس 2015. تكونت العينات من (80) عينة من الجاموس، (51) عينة من الأبقار، (93) عينة من الأغنام، (51) مسحات و (23) عينة من مياه الصرف الصحي. تم استخدام تقنيات مختلفة في هذه الدراسة لتقييم وجود السالمونيلا والأجناس المماثلة لها ظاهريا التي تلوث اللحوم النيئة، شملت هذه التقنيات الاختبارات البكتريولوجية التقليدية ، واختبار (API 20 E) التجاري، التصنيف المصلي وتقنية البلمرة المتعددة.

أشارت نتائج استخدام هذه التقنيات إلى عدم وجود السالمونيلا في العينات التي تم فحصها، ومع ذلك، كانت 35 عينة إيجابية لجنس *Proteus mirabilis*، كما حددها نظام API 20 E . في حين كانت هناك اختلافات بين نتائج API 20 E والفحص المصلي ، حيث تم تحديد (44) عزلة باعتبارها *Citrobacter freundii* باستخدام API 20 E، وأشارت نتائج الاختبار المصلي إلى وجود (31) عزلة *Citrobacter braakii* و (13) *Enterobacter spp.* . خضعت هذه العزلات لتفاعل البلمرة المتعدد للكشف عن جين *invA*، جين *spvC* و جين *viaB* ، وأكدت نتائج تفاعل البلمرة المتعدد أن (13) عزلة فقط من هذه العزلات كانت *Citrobacter freundii* ، بينما لم يتأكد وجود السالمونيلا أو *Citrobacter freundii* في (31) عزلة، كما أظهرت نتائج هذه الدراسة أن تفاعل البلمرة المتعدد يعتبر وسيلة دقيقة لتأكيد عزل وتشخيص السالمونيلا و *Citrobacter freundii*.

INTRODUCTION

Meat and their products are considered as excellent sources of high quality animal protein, vitamins especially B complex, and certain minerals, especially iron (1). They are considered as an ideal culture medium for growth of many organisms because of the high moisture, high percentages of nitrogenous compounds of various degrees of complexity, plentiful supply of minerals, accessory growth factors and some fermentable carbohydrates (glycogen) of a favorable pH for most of the enteric microorganisms (2).

Contamination of raw meat is one of the main sources of foodborne illnesses (3,4) Unfortunately, The presence of microbial contaminants in meat products cannot be detected visually (5), which raise both the risks associated with foodborne pathogens and the incidence of human diseases (6). Microbial contamination of raw meat starts during slaughter, when the carcass become contaminated with microorganisms residing on external surfaces, the gastrointestinal tract and lymph nodes of the animal, and in the plant environment (7). The incidence of carcasses contamination depends on various factors including stress during transportation, time spent in lairages and hygienic level during slaughter (8). In fact, tissue from healthy animal are sterile however, it has been pointed that during slaughter, dressing and cutting, microorganisms came chiefly from the exterior of the animal and its intestinal tract but that more added from knives, cloths, air, carts and equipment in general. External contamination of meat is a constant possibility from the moment of bleeding until consumption (9).

The gram-negative bacteria account for approximately 69% of the cases of bacterial food borne diseases (10). *Salmonella* is one of the microorganisms most frequently associated with food-born outbreaks of illness (11). Furthermore, it remains a leading cause of food poisoning in the developed world, resulting in multiple cases of absenteeism, illness, hospitalization and death each year (12). *Salmonellae* can be frequently found in sewage, sea, and river water and can contaminate a variety of foods (13).

Citrobacter freundii is an environmental bacteria, which can be opportunistic and that also can be found in many animals (14,15). Moreover, *Citrobacter* species are occasional inhabitants of human, animal intestines, soil, water, sewage, and food (16).These organisms are isolated from variety of clinical specimens like urine, pus, blood, and cerebrospinal fluid (17).

Environmental pathogenic contaminant *Proteus mirabilis* are capable of growth in low nutrient conditions (18). Thus, this bacteria is able to grow in water distribution systems (19), manure, and soil, where it plays an important role in decomposing organic matter of animal origin (20). *Proteus* was recovered from hides and work surfaces within the abattoir, from carcasses, butchered meat as well as from environmental samples in meat processing plants (21,22).

Multiplex PCR (mPCR) approaches have been largely applied to detect different species of several microbial niches, to differentiate closely related species and to recognize single species (23).

Aims of the study

1. Isolation and identification of *Salmonella spp*. and related genera from raw meat and environment of the abattoir by conventional microbiological, serological and molecular techniques.
2. Comparison of the efficiency of techniques used in identification of *Salmonella spp*. and *Citrobacter freundii*.

MATERIALS AND METHODS

Samples collection

A total of 298 samples were collected between 16th September 2014 to 21st March 2015. The samples were collected from slaughtered animals, workers and different places of Basra abattoir. Table (1) indicates the sources, types and number of samples.

Table (1): sources, types and number of the collected samples

Source of sample	Type of sample	No. of samples
Muscular tissue	Buffalo	50
Liver tissue		10
Bile		20
Muscular tissue	Cows	27
Liver tissue		14
Bile		10
Muscular tissue	Sheeps	57
Liver tissue		24
Bile		12
Sewage		23
Wall	Swabs	17
Ground		17
hands of workers		17
Total		**298**

Isolation of bacteria

Laboratory analyses of samples were achieved according to (24) by pre-enrichment in buffered peptone water and then enrichment in tetrathionate broth. After enrichment of samples with tetrathionate broth for 18 hrs., a loop-full of culture was streaked into plates of xylose lysine deoxycholate (XLD) agar at 37°C for 18 hrs. and checked for growth of colonies of *Salmonella spp*., *Proteus spp.* and *Citrobacter spp*.

Conventional microbiological tests

The Presumptive isolates were cultured on MacConkey agar to differentiate between lactose and non-lactose fermenting. Suspected colonies were stained by Gram staining as described by (25). The presumptive isolates were submitted to conventional biochemical tests, urease test was done according to (26), triple sugar iron (TSI) agar slant reaction according to (27), citrate utilization test using Simmon's citrate agar according to (28) and oxidase test was accomplished like described by (29).

Confirmatory identification test for isolates

1. Analytical Profile Index (API 20E) test: The identities of all presumptive isolates were confirmed using analytical profile index API 20E test. The test was performed according to manufacturer's protocol (BioMerieux Inc., France). The results were read with reagents and the indices based on phenotypic profiles of isolates were used to determine the identity of isolates by referring to the analytical profile index**.**

2. Serological methods: Serological identification of isolates was done at the Department of microbiology, Central Public Health Laboratory in Baghdad.

3. Molecular characterization of isolates:

3.1 Extraction of genomic DNA: Genomic DNA was extracted from all isolates using a DNA extraction kit (Genaid, Korea) according to manufacturer's protocol. The DNA extracted was stored at 4°C until PCR analysis.

3.2 Multiplex PCR (mPCR) assay: The mPCR primer pairs used in this study were to detect only *Salmonella spp.* and *Citrobacter freundii*. mPCR was performed by using three primer pairs according to (30); these primers were designed to identify 14 clinically important bacterial organisms, including *Citrobacter freundii, S. enteric* serovars *Typhi* and *Paratyphi* C, *Dublin*, and other non-typhoidal *Salmonella* that harbor a virulence plasmid (table 2).
PCR reactions were performed standard 25µl volumes containing 3µl of DNA template, 12.5 µl of Green master mix, 5µl of Nuclease free water and 0.75µl of each oligonucleotide primers. PCR amplification was performed with a DNA thermal cycler, using the following conditions: 95^{o}C for 5 min., followed by 35 cycles of 95^{o}C for 35 sec., 55^{o}C for 30 sec., 72^{o}C for 35 sec. and a final extension at 72^{o}C for 10 min.

Table (2): Oligonucleotide primers for mPCR amplification

Primer	Sequence (5´→ 3´)	L (bp)	S	P (bp)
F	ACAGTGCTCGTTTACGACCTGAAT	24	*invA* gene	243
R	AGACGACTGGTACTGATCGATAAT	24		
F	ACTCCTTGCACAACCAAATGCGGA	24	*spvC* gene	570
R	TGTCTTCTGCATTTCGCCACCATCA	25		
F	TGTCGAGCAGATGGATGAGCAT	22	*viaB*gene	516
R	ACGGCTGAAGGTTACGGACCGA	22		

RESULTS AND DISCUSSION

The collected samples were analyzed for the presence of *Salmonella spp*. and related genera. The growing colonies on XLD agar were small, discrete, round, smooth, convex and transparent with black centre. The suspected colonies were analyzed by using preliminary tests (lactose and non-lactose fermenting on MacConkey agar, Gram staining, urease test, Simmons citrate agar test, triple sugar iron agar test and oxidase test) (table 3).
According to the confirmatory identification test (API 20E test), the proportion of isolates that satisfied these tests were revealed that, 14.7% (44/298) of isolates were identified as *Citrobacter freundii* (figure 1) and only 11.7% (35/298) of isolates were identified as *Proteus mirabilis* (figure 2), whereas no one of the isolates were identified as *Salmonella spp*. (table 4).

Table (3): Comparison of the isolates according to the conventional biochemical results

Biochemical tests	Results	No. of results of lactose fermenting	No. of results of lactose non-fermenting
TSI test	Positive	44	35
	negative	0	0
Urease test	Positive	0	35
	negative	44	0
Simmon'scitrate	Positive	34	32
	Negative	10	3
Oxidase test	Positive	0	0
	negative	44	35

Figure (1): *Citrobacter freundii*

Figure (2): *Proteus mirabilis*

Table (4): Distribution of *Citrobacter freundii* and *Proteus mirabilis* identified by using API 20 E

Source of sample	Type of sample	No. of sample	Total identified *C. freundii* by API 20E	Total identified *P.mirabilis* by API 20E
Muscular tissue	Buffalo	50	12	9
Liver tissue		10	2	1
Bile		20	2	1
Muscular tissue	Cow	27	15	4
Liver tissue		14	2	2
Bile		10	0	0
Muscular tissue	Sheep	57	0	8
Liver tissue		24	0	1
Bile		12	0	0
	Sewage	23	7	5
Wall	Swabs	17	1	1
Ground		17	3	2
hands of workers		17	0	1
Total		**298**	**44**	**35**

This isolation rate of *Citrobacter freundii* in agreement with (31), who isolated *C. freundii* from fresh meat in ratio 13.9%, however the isolation rate of *Proteus mirabilis* was similar to (32), who reported that the isolation rate of *Proteus mirabilis* was12%. Concerning the isolation rate of *Salmonella spp.* from fresh meat, the results were in agreement with (31), who reported negative results of *Salmonella spp.* isolation. In this study, the identification rate of conventional biochemical tests was similar to that of API 20 E. (table 5).

A total of 44 isolates were identified as *Citrobacter freundii*, were subject to serotyping. However, a large proportion 70.4% of these isolates were identified as *Citrobacter braakii* and a small proportion of these isolates 29.5% were identified as *Enterobacter spp*., while nil of *Salmonella spp.* This result in agreement with (33), who reported negative results for the *Salmonellaspp.* from fresh beef by serological methods (table 6).

To avoid bias due to confusing in the results obtained by different methods, the PCR was used to eliminate that confusion and because *Salmonella* was genetically closer to *Citrobacter* (34) and species of *Citrobacter* share about 50 to 55% of their nucleotide sequences with *Salmonella* (35), because of these reasons the mPCR primer pairs used in this study were to detect only *Salmonella spp.* and *Citrobacter freundii*. All the 44 isolates were subjected to mPCR analysis for characters of *Salmonellaspp*., this was achieved through amplification of the (*invA*) gene fragments specific for *Salmonella* isolates and (*spvC*) gene for seven serovars of *Salmonella spp*. have been reported to harbor the virulence plasmid: *S.Typhimurium*, *S.Enteritidis*, *S.Abortusovis*, *S.Choleraesuis*, *S. Dublin*, *S.Gallinarum-Pullorum*, and *S.Sendai*. While primer pairs of (*viaB*) gene using to identified *Citrobacter freundii* ,*S. Typhi* , *S. Paratyphi* C. and *S. Dublin* (30).

The mPCR results of this study revealed that, despite the fact that none of the isolates possessed the (*invA* and *spvC*) gene, a small proportion 13(4.3%) were positive for the (*viaB*) gene fragments, and identified as *Citrobacter freundii*. This result in agreement with (30) who reported *viaB* gene is specific for *Citrobacter freundii* (figure 3).

Results of comparison of the different methods clarified that there was similarity in the results between API 20E and conventional techniques. While there are dissimilarity between traditional serotyping and multiplex PCR, the differences are statistically highly significant (table 7). This result was in agreement with (36) who reported that, the deficiencies of conventional serotyping have led to the development of alternative molecular strategies to replace or complement conventional serotyping. Also (37-41) employed PCR-based approaches such as mPCR to determine different O and H antigens as a means to replace serologic identification of these antigens. While the results of this study disagreement with (42), who reported that the correlation between traditional serotyping and multiplex PCR was 100%.

Table (5): Comparison of *Citrobacterfreundii* and *Proteus mirabilis*identified by using conventional biochemical tests and API 20 E

No. of examined samples	Identified bacteria	Positive isolates identified by conventional		Isolates identified by API 20E System	
		No.	%	No.	%
298	*Citrobacter freundii*	44	14.7	44	14.7
298	*Proteus mirabilis*	35	11.7	35	11.7

Note: Percentages resulted from dividing the number of cell by total number of raw

Table (6): Serotyping results of isolates with their percentages

Serotype	No.	Percentage
Citrobacter braakii	31	70.4%
Enterobacter spp.	13	29.5%

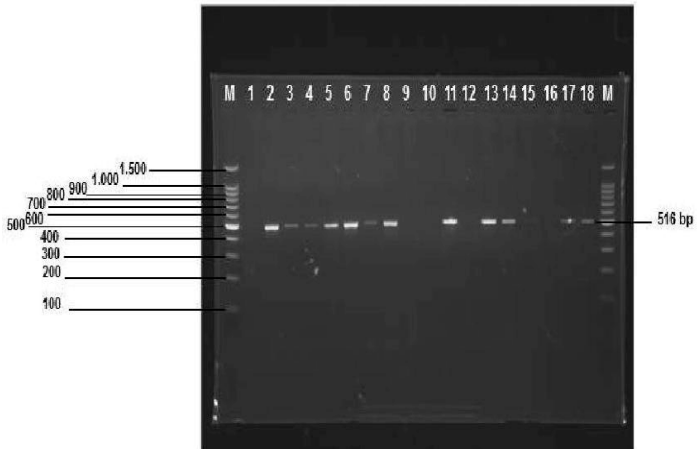

Figure (3): mPCR amplification mixture was run on 1.2% agarose gel stained with ethidium bromide. Lanes: M, Marker. 2-8, 11,13,14,17 and 18 positive for *viaB* gene as *C.freundii*

Table (7): Overall results of *Citrobacter freundii* obtained by different methods of isolation and identification

Type of test	Total no. of isolates	Positive tested samples	Percentage (%)
Positive cultural result on XLD	298	79	26.5
Conventional biochemical test	79	44	55.6
API 20 E	44	44	100
Serological	44	0	0
PCR	44	13	29.5

Chi-Sq = 129.654; Df = 4; P-Value = 0.000

Also the results of this study disagreed with many previous studies (43-46), who used the techniques of conventional and serology as only diagnostic

tools. While this study was in agreement with (47,48), who reported that, the serology method has a number of deficiencies, including the inability to serotype between 5 and 8% of isolates and incorrect typing due to the loss of surface antigens. In addition, cell surface antigens are sometimes horizontally transferred; a phenomenon that can cause classification of genetically unrelated strains within the same serovar, also this method does not reveal the genetic relationships of strainswithin the same or different serotypes (49). However, (50) and (51) demonstrated that serotyping approach can present a risk of false positives for *Salmonella*.

The present study concluded that, the isolated strains were identified as *Citrobacter freundii* and *Proteus mirabilis*, however, nil *Salmonella* species were identified in the raw meat and abattoir environment. In addition, this study concluded that, the mPCR is an accurate method for confirmation of *Salmonella spp.* and *Citrobacter freundii*.

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