Detection of *Salmonella typhimurium* in chicken meat imported in the local markets of Diwaniya city By using PCR technique

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Abstract:

The aim of this study to detected contamination with *Salmonella spp*. In imported chicken meat in the local markets of Al- Diwaniyia city . to protect health of consumer and Determintion the most contaminated origin with *salmonella spp*. A toatl of 100 chicken meat samples collected from different origin. The bacteria cultured and isolated in enrichment and selective media . Salmonella isolates were subjected to some biochemical tests show positive productive results H2S .TSI . SIM And its give negative for indole , vo-gs Proskauer and ureas , Biochemical identification was carried out using API 20-E test ..the result showed isolation sample (33\55)60% on bismuth sulphate agar and the results of isolation on chromogenic agar were 87.8 |%(29\33) .according to reading Api20-E system the results of confirmation of isolates 92%(25\26) In this study,(23) *Salmonella* isolates were detected by polymerase chain reaction (PCR) by using *16s rRNA and invA* gene these primers were selected specifically for the detection of *Salmonella* to amplify a 406bp and 558 bp DNA fragments, respectively. Only 7 isolates out of 23 were identified as *S. typhimurium* the results of this study showed the highest percent of s.typhimurim isolates was (50%) (3/6) for India origin and the lowest was Turkish origin

Introduction

Food borne diseases caused typhoid Salmonellosis represent an important public health problem worldwide. It is estimated that approximately 70%–80% of food borne bacterial outbreaks were caused by *Salmonella* spp. in China (1). In the United States Salmonella infections (approximately 32,000 annually) were reported during 1998–2002 (2). , beef and poultry /chicken meat have been recognized as significant sources of human salmonellosis (3). *Salmonella* serotypes, (*S.*) *Typhimurium* is one of the most important agents of food borne Salmonellosis in humans . [4] It was estimated that approximately 75% of human salmonellosis cases were due to contaminated food products, such as beef, pork, poultry and Chicken products are recognized as an important source molecular methods such as polemear chain reaction (PCR), have shown high sensitivity and specificity for detecting target pathogens, including *Salmonella*, in different types of foods, and the time required to obtain results can be as short as 12 h (5) The use of *16s r RNAgene* or *invA gene* specific PCR method in most diagnostic and research laboratories is possible and through the molecular basis of Salmonella identification techniques, this method is the simplest and less expensive (6) the 16S rRNA genes are highly conserved among isolates belonging to the same bacterial species ,(7) *invA* is located on the pathogenicity island 1 of *Salmonella spp*. encoding proteins of a type (T3SS) III secretion system this gene is highly conserved among the *Salmonella spp*. and is associated with the adherence and invasion of mammalian cell.

Material and Methods

1-Collection of samples

Chicken samples were collected from different market in al-diwaniyia city with different origin include different trademark (al-kafeel ,al murad , thighs U.S.A, Turkish Chicken, Chicken JD) the sample transport by ice box about 25 g from meat sample were placed in 225 ml of enrichment medium tetrathionate broth in microbiological laboratory in veterinary collage for 18-24 h at 37°C.. this study took place during the period from December 2011 and carry on June2012.

2- Isolation and identification of *salmonella spp.:-*

The samples were cultivated on to selective medium such as bismuth sulphate agar and chromogenic agar For identification of *salmonella* colonies, incubation at 37c˚ for 18-24 hr samples were subjected to biochemical tests such as (TSI), Sulfide-Indole- (SIM), (MRVP), Urea, and finally confirmed by using Api20-E system, Colonies that showed biochemical characteristics similar to that of *Salmonella* spp. were tested by API20-E system and the confirmation was identified by PCR with *16s rRNA* and *invA* genes primers for the detection of*salmonellaspp*

.**3-Specific Primers Sequence Used for PCR Amplification:**

The primers used for the detection specific sequence of *16s rRNA* gene ribosomal genes of *Salmonella* spp **[8].** And *invA* gene encoding proteins of a type (T3SS) III secretion system **[9]**These primers are specific for designed in this study by using NCBI Gene Bank and Primer: online and provided by (Bioneer company, Korea) as following Table(1):

Table(1):Specific primers used for the detection specific sequence of *16s rRNA gene* and *invA gene*

Sequence	Orientation	Position	Size of PCR product(bp)
CGG.,ACG,GGT,GAG,TAA,TGT ,CT	Forward	*16s rRNA*	406
GTT,AGC,CGG,TGC,TTC,TTC,. TG	Reverse		
ATG,CCC,GGT,AAA,CAG.ATG, ATG,AG	Forward	*invA*	558
CTC,GCC,TTT,GTC,GGT,TTT,A G	Reverse		

4- Polymereas chain reaction PCR

4.1 Genomic DNA extraction

Salmonella spp. isolates were cultured on brain heart broth for 18-24 h at 37°C; the extraction of DNA was performed according to Genomic DNA kit provided by geneaid company (USA).

4.2 DNA Amplification:

The amplified DNA products from *Salmonella spp* .specific-PCR were analyzed

with electrophoresis on 1% agarose gels stained with ethidium bromide and visualized by UV illumination. depending on DNA marker (2000 bp DNA ladder).

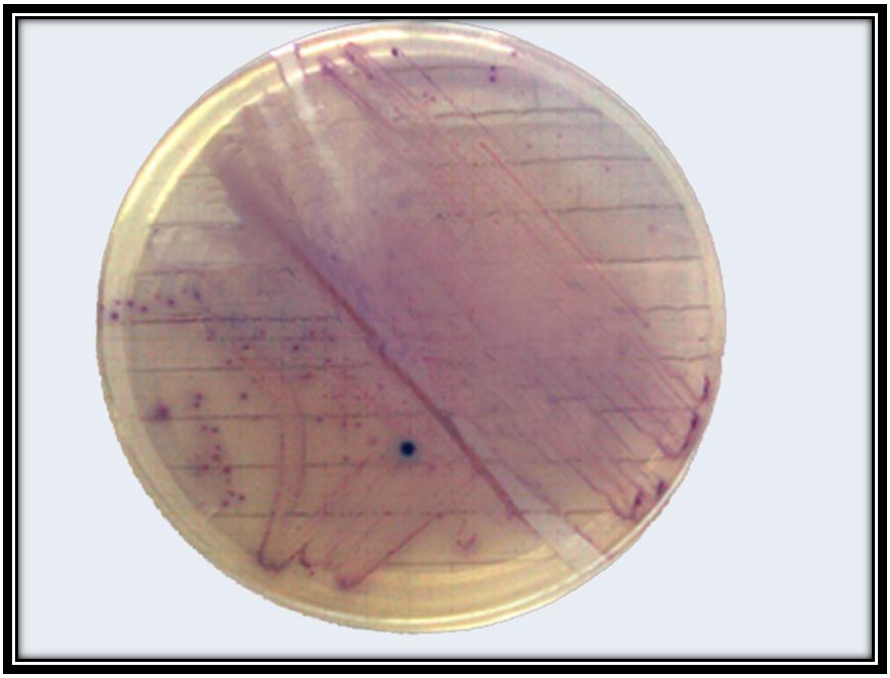
4.3 Preparation master mix for Detection of *16s rRNA* and *invA* genes

For the detection of *Salmonella* spp. and *S. typhimurium* by PCR. The PCR amplification mixture (20µl) which was used for the detection each gene includes 5 µl of (PCR PreMix Lyophilized**)**,which provided by Bioneer (Korea .)include: bacterially derived Taq DNA polymerase; dNTPs which include: 400 µM of each dATP, dGTP, dCTP, dTTP; 3mM of Mgcl2; Yellow and blue dyes as loading dye), 5 µl of template DNA, 1.5 µl of each forwarded and reversed primers and 7. µl pcr water to complete the amplification mixture to 20 µl. The PCR tubes containing an amplification mixture were transferred to thermocycler and started the program for amplication of the *16s rRNA and invA genes* . 30 cycles of PCR, with 1initial denteration 1 cycle 95C˚ for I min then 5 min at 95C˚ (denaturation), 30 s at 55 C˚ (annealing),and 45s at 72 C˚ (extension). And 1 cycle for 7 min at 72c˚ (final extension

Results

5.1 Culture methods :-

The total percentage of isolation on tetrathionate broth , bismuth sulphate agar, chromogenic agar was 55% (55/100), 60 % (33/55) , 87.8% (33/29) , the highest percent of isolation was from (India origin) . The colonies of *salmonella spp.*on chromogenic agar were Variable in size convex and mauve in color. Figure(1) . the Results of isolation *salmonella spp*. using cultural methods. present in table (2).



Figeur (1) Colonies of *salmonella spp*. on chromogenic salmonella agar (The arrow shows variable size and mauve in color.

Table (2)Results of *salmonella spp*. Isolation by using culture methods from chicken meat sample.

Culture media / Sample origin	Tetrathionate broth			Bismuth sulphate agar			Chromogenic agar		
Origin	No. of tested sample	No.of positive	%	No.of tested sample	No.of positive	%	No.of tested sample	No.of positive	%
Jordan chicken JD	20	12	60	12	7	58.3	7	6	85.7
Turkish casken oglo	20	9	45	9	5	55.5	5	5	100
Brazil al-kafeel	20	10	50	10	6	60	6	5	83.3
India al-murad	20	11	55	11	7	63.6	7	7	100
U.S.A. thighs	20	13	65	13	8	61.5	8	6	75
Total	100	55	55	55	33	60	33	29	87.8

5.2 Confirmatory isolation of *salmonella spp*.and *S.typhimurium* by using Api20-E and PCR technique:-

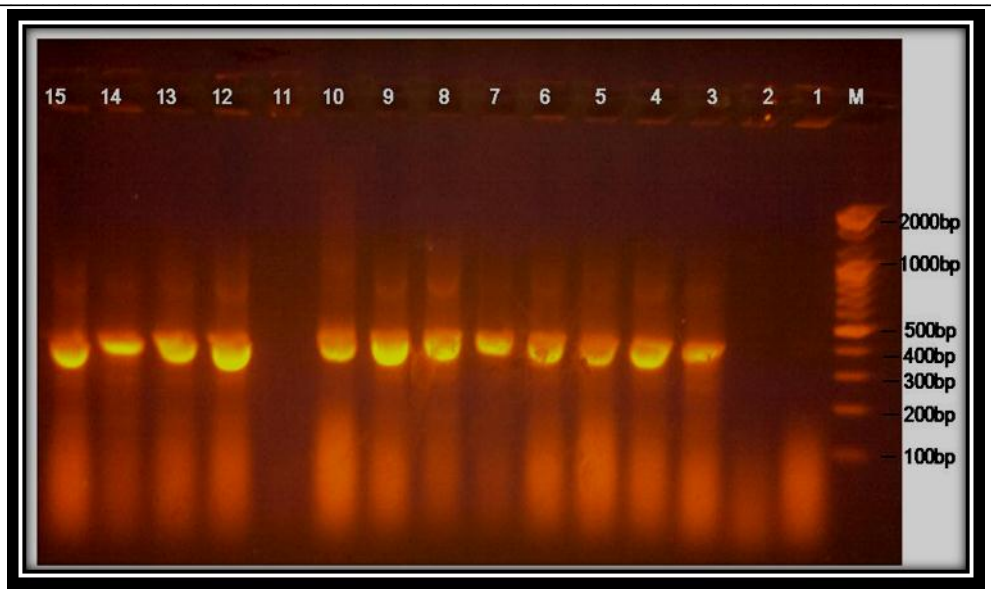
Salmonella isolates were subjected to some biochemical tests show positive productive results H2S .TSI . SIM And its give negative for indole , vo-gs Proskauer and ureas , The total percentages of these tests 89.6% (29 \ 26). And according to the reading of API 20-E system show that 25 isolated positive to API20-Esystem from 26 with percentage 96.1%**.**the results in table (3).

Table (3) Results of Biochemical test and API20-E system .

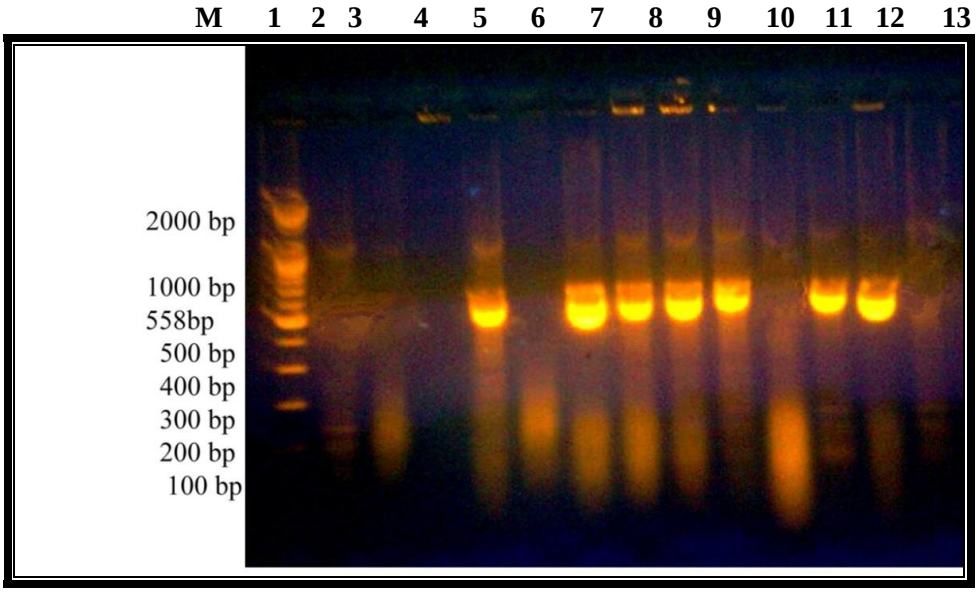
Test / Sample origin		Biochemical test			API20-E system		
Origin	tradmark	No. of tested sample	No. of positive	(%)	No .of tested sample	No. of positive	(%)
Jordan	chicken JD	6	5	83.8	5	5	100
Turkish casken oglo		5	4	80	4	4	100
Brazil	al-kafeel	6	6	83.3	6	5	80
India	al-murad	6	5	83.8	5	6	100
U.S.A.	thighs	6	6	100	6	6	100
Total		29	26	89.6	26	25	96.1

5.3 Molecular confirmatory detection using Single plex PCR:-

The confirmed diagnosis of *Salmonella* spp. were performed by using single plex PCR to detect *16s rRNA* gene the total percentage was 92 %(23/25) for chicken meat and the higher percent for isolation salmonella spp. by *16s rRNA* gene was from al-kafeel and U.S.A thighs 100% while the lower percent was from Turkish origin 75% **.** the total percentage for detect *invA* gene for *S.typyimuirim* serotype was 30.4 %(7/23). And the highest percent of isolation of *S.typyimuirim* was 50% from India origin while the lower was 0 % from Turkish origin.(Figure 2) and (Figure 3).



(Figer:- 2)) DNA amplification of a 406 bp of salmonella spp.detecting *16s r RNA* gene using singleplex PCR lane 1 controll, lane 2,11 negative results ,lane 3,4,5,6,7,8,9,10,12,13,14,15 positive results as *salmonella spp*. Lane M 2000bp marker (ladder).



(Figer 3) DNA amplification of a 558 bp of salmonella spp .detecting *invA* gene using singleplex PCR lane 1 conterol results ,lane,4, 6,7,8,9,10,12, positive results as *S. typhimuirim* spp. Lane 2,3 5,13negative result , lane M 2000bp marker (ladder).

Table (4):- Results of detecting *salmonella spp*. By Single plex PCR *16s rRNA* gene and *in vA* gene

Sample origin		Single plex PCR Detect *16s r RNA* gene			Single plex PCR detect *inVA* gene		
Origin	trademark	No. tested sample	No. of positive	%	No. tested sample	No. of positive	%
Jordan	chickenJD	5	4	80	4	1	25
Turkish	casken oglo t	4	3	75	3	0	0
Brazil	al-kafeel	4	4	100	4	1	25
India	al-murad	6	6	100	6	3	50
U.S.A.	thighs	6	6	100	6	2	33.3
Total		25	23	92	23	7	30.4

Disscuse

Chicken meat, is one of the most important sources and ,a good compromise for the growth and transfer of *Salmonella spp*. and causing cases Food poisoning and that its presence in the fresh chicken meat, chilled and not well cooked Pose a threat to public health and a source of contamination of food through the stages food during preparation stages and food preparation.(10) . To determine the level of contamination with *salmonella spp.* in imported poultry meat in the markets of the city of Al- Diwaniya, this study included several methods to isolated and detected a hundred samples of chicken meat from different origins the results of isolation on Tetrathionat broth as enrichment media were 55 % (55/100) this results came compatible with (11) which his result (58.6%) from chicken meat when used Tetrathionat broth as pre enrichment media 42 ˚C , and higher than those obtained by (12) (48%.)and, (13) (31.4%) . several bacteriological selective media have been used to isolating *Salmonella spp.* them bismuth sulphate agar. Where results of isolation on this media 60 %(33/55) and this results higher than (14) when use Bismuth sulphate agar to isolated salmonella from a ported chicken in market of Baghdad city which his results was 24.76% . chromogenic agar

considers One of the latest techniques that used in recent decade to rapid isolation of pathogenic agent in water and food is. These media are very specific and their component act as substrate for specific enzyme and depending on enzyme exhibit special color. (15*)* . *salmonella* spp. Was isolated (29/33) samples when inoculation on this agar with percent (87.8 %) which was significantly higher what has been reached in the study (16). the cause of this difference in the percent of isolated salmonella between studies due to the difference in the number of samples examined and health standards in the massacres . the results of isolation in chromogeninc agar were refer to the accuracy and specificity of this media for bacterial isolation of *Salmonella spp.* in compare with other diagnosis methods chromogenic media have more advantage and can be an appropriate alternative for conventional and routine procedure. Chromogenic media eliminate the need of subculture in addition to shortest period of time pathogenic agent can be identified.

Biochemical test and Api-20 E system :-

The API 20-E diagnostic, which detects 20 biochemical reactions, is a traditional method for the identification of *Salmonella enterica* and other *Enterobacteriaceae* (**17)** . the present study shows that the total percentage of isolation *salmonella spp*. According to the reading of API 20-E system the confirmation of 25 isolates were done from 27 with percentage 92.5 % and this percentage was very closer to (18) that was his result 99%) when evaluated API 20-E as indicator for *salmonella enterica*. And this results show that API 20 E system is a universal method supported in most laboratories global diagnostic the results don't show any difference or variation in the characteristics of bacteria (bacteriological .biochemical characteristic) and this gives us more confidence for all subsequent steps related to this research .

Molecular confirmatory detection of *salmonella spp*.

By using Single plex PCR Technique :

Traditional methods for detection of *Salmonella* in food have included culturing the food item on selective media followed by characterization of suspect colonies with additional biochemical tests and immunoassays. In general, this process requires multiple days for successful identification of the pathogen. To overcome the protracted nature of traditional detection methods, and to enhance the sensitivity and specificity of detection, a number of molecular diagnostic methods have been developed, including methods that utilize Polymerase Chain Reaction (PCR). The use of *16s r RNAgene* or *invA gene* specific PCR method in most diagnostic and research laboratories is possible and through the molecular basis of Salmonella identification techniques, this method is the simplest and less expensive (19). the results *salmonella spp*. detection by using *16s r RNA* gene in present study from chicken meat samples were 92% (23/25) Table (16) than the percent of isolation serotype *S.typhimurium* from these sample were 30.4 %(7/23) , the results agreed with previous study (20) , (21) obtained the ratio of contamination of salmonella in chicken meat 36%(9/25) , 38% respectively when using *invA* gene .. The ability of *Salmonella* specific primers to detect *Salmonella* species rapidly and accurately in the present study is primarily due to the primer sequences that are selected from the gene *inv*A of *S. typhimurium* as reported by (22) The amplified PCR products which were carried out using the universal bacterial *16srRNA* and *invA* primers and visualized by UV illumination showed the expected bands of about 406 bp Figure (6) 558pb Figure (7) The results demonstrated a correct genus identification of examined *Salmonella* isolates. The final results of

present study were 23% (23 /100) it closer to (14) which his result 30% while his result to isolated *S.typhimurim* were 7.7%(2/26) , also similar to previous studies obtained by (23) (20%), While lower than (24) that his result 60% of 192 chicken samples . This variation of results between studies may be associated with different factors such as, season of the study, number of samples and the methods applied. The predominant serotypes differ indifferent countries, hygienic conditions in storage and cross contamination during transport .

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عزل وتشخيص جرثومة السالمونيلا تايفيميوريم من لحوم الدواجن المستوردة في اسواق مدينة الديوانيه بأستخدام تقنية الPCR

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الخلاصة

الهدف من هذه الدراسة هو الكشف عن التلوث بجرثومة *salmonella spp*. في عينات لحوم الدواجن المستوردة في السوق المحلية لمدينة الديوانية وذلك لحماية صحة المستهلك .وقد تم جمع 100 عينة من لحوم الدجاج ومن مناشئ مختلفة . وقد استخد مت عدة اوساط اغنائية وانتقائية لعزل الجرثومة وأظهرت هذه الدراسة عزل 55 عزلة على مرق tetrarathionate و(33 \ 55) 60٪ على وسط bismuth sulphate وكانت نتائج العزل على وسط chromogenic 87،8٪ (29 \ 33) . .كما استخدمت عدة اختبارات كيميوحيويه لعزلات السالمونيلا حيث اعطت هذه العزلات نتائج ايجابية الانتاج . H2S .TSI .SIM, MRD واختبار الحركة ونتائج سالبة للاندول والفوغس بروسكاور واليوريز ووفقا لقراءة لنظام Api20-E كانت نتائج العزلات 96.1 ٪ (26 \ 25) و في هذه الدراسة قد تم تأكيد عزل 23 عزله من السالمونيلا من اصل 25 بنسبة 92% عن طريق تقنية تفاعل البلمرة المتسلسل (PCR) باستخدام البادئ النيوكليوتيدي *16s rRNA* لنوع السالمونيلا والبادئ النيوكليوتيدي و*invA* تم اختيار هذه البادئات خصيصا للكشف عن السالمونيلا لتضخيم الحمض النووي bp406 و bp 558، على التوالي. وقد تم تحديد 7 عزلات من أصل 23بنسبة 30.4% للنمط المصلي *S.typhimurium*. نتائج هذه الدراسة أظهرت أن أعلى نسبة من عزلات السالمونيلا تايفيموريم كانت 50% للمنشأ الهندي (3\6) واقل منشأ تلوثا المنشأ التركي.