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Detection of *Salmonella Spp. in* Meat and Meat Products by Culture, Biochemical and Molecular Characterization in Duhok City

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MEAT and meat products are high in protein, essential amino acids, vitamins, lipids, minerals, and other nutrients, although they are nutrient-dense foods for humans. Total of 150 samples of local and imported meat and meat products were collected from different location of Duhok province from November 2021 to August 2022, as follow ,25 samples=(16.66%) from each type of meat included beef meat, burger meat, chicken meat , minced meat , sausage meat and sheep meat. The study molecular methods for detection of the target pathogen. Traditional and biochemical. The percentage of *Salmonella spp* isolated. as 29.3% (44/150) positive samples by using the Traditional culture methods, 23.33% (35/150) positive samples by biochemical method, and 55% (33/60) positive samples by PCR method for molecular confirmation and serological method for serotyping. Accuracy techniques from(*Salmonella,* culture items (counts) and Accuracy % from *Salmonella* True value (100%) and Accuracy 94.47%, sensitivity100%, specificity (92.8%).

Salmonella spp. invAgene was found using the polymerase chain reaction (PCR). These *Sal monella* isolates appeared to possess the invA gene according to the PCR technique since D NA amplification produced one unique band (size 389 bp) when electrophoresed on an agar ose gel. Only 10 isolates were sent to the Gene BankNCBI for registration of the nucleotid e sequences for the 660bp of the 16sRNA gene based on the sequencing method. This was done to diagnose the isolates at a species level, The obtained nucleotide sequences for each isolate searched for their identity and molecular identification of the bacteria implementing the BLAST algorithm of the GenBank database against 16S rDNA sequences of type strains (/ BLAST) at the National Center for Biotechnology Information (NCBI).

Keywords: meat, Salmonella, morphology and PCR, invA gene, DNA sequencing 16sRNA

Introduction

Meat and meat products are highly nutritious for humans due to their abundance of protein, essential amino acids, vitamins, fats, minerals, and other beneficial components [1]. However, these products can also provide an ideal environment for the growth of various organisms due to their high moisture content, nitrogenous compounds, mineral supply, limited fermentable carbohydrates like glycogen, and optimal pH that supports microbial growth [2]. Salmonella is a type of gram-negative rod-shaped bacterium that belongs to the Enterobacteriaceae family. It can survive in low oxygen environments and is considered a mesophile with slow growth rates below 15°C. Salmonella is a facultative intracellular pathogen that can cause a range of diseases from gastroenteritis to typhoid fever. It is consistently identified as one of the leading sources of foodborne illnesses worldwide [3]. Salmonella poses a significant threat to both humans and animals and results in substantial economic losses globally due to various diseases, including mild diarrhea and severe systemic infections like typhoid fever [3]. Consists of

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two species: Salmonella enterica and Salmonella bongori. Salmonellae can ferment glucose, dulcitol, mannitol, and maltose but cannot ferment lactose or sucrose. Salmonella enterica is particularly prevalent among enteropathogenic bacterial species worldwide and encompasses over 2500 serovars [4]. The infection caused by Salmonella remains a major public health concern globally and contributes to the economic burden in both developed and underdeveloped countries through the costs associated with disease surveillance, prevention, and treatment.

Traditional culture methods have long been considered the most reliable way to isolate and identify foodborne bacterial pathogens [5]. These methods involve several steps, including enrichment, plating, and confirmation through various tests. While these methods are sensitive and cost-effective, they are also time-consuming and labor-intensive, taking several days results. Additionally, environmental factors can affect the accuracy of these tests, and they may not detect certain types of bacteria [6]. Alternative methods such as immunoassays and nucleic acid probe analyses have been developed [7], but still have issues with sensitivity and specificity [8]. PCR is a rapid method that offers excellent sensitivity and specificity for detecting pathogenic bacteria in food. However, it can be limited by various factors such as the presence of certain substances in the food matrix [6]. Furthermore, if a pathogen is detected using PCR, traditional confirmation procedures must still be followed [5]. Removing inhibitory chemicals from samples is an important step in preparing them for PCR-based detection of foodborne pathogens. Even though these inhibitors prevent PCR from being used to analyze food samples directly, PCR-based techniques for enrichment broths have been more effective [7]. This study was carried out to evaluate a rapid (12 hour) method for detection of *Salmonella* in food samples and compare it with the conventional method.

Material and Methods

Collection of samples and Sample Preparation

One hundred and fifty food samples, including beef, burger, chicken, minced meat, sausage, and sheep meat, collected from (super markets restaurant and abattoir). The samples were labeled, recorded, and analyzed promptly. If there was a delay, the samples would be refrigerated at 0-4°C for no more than 24 hours after collection. The pre-enrichment process using a modified method [9] involved mixing 25 grams of each sample with 225 mL of Buffered Peptone Water (BPW) medium and dividing it into two portions. The first portion underwent pre-enrichment culture for 6 hours, while the second portion was incubated at 37°C for 24 hours. DNA extraction was performed on the first portion using the boiling method, while the second portion was used to confirm the presence of Salmonella through standard cultural methods and subsequent biochemical test. The isolation and identification of *Salmonella* were conducted according to International Organization for Standardization (ISO) 6579 (9).

Isolation and Identification of Salmonella spp.:

Twenty-five gm of each sample were placed in separate sterile plastic bags along with 225 mL of non-selective enrichment in Buffered Peptone Water (BPW). These bags were then incubated at 37°C for approximately24 hours. Afterwards, 1 mL of the pre-enriched solution was transferred to 10 mL of Rapaport Vassiliadis (RV) broth and left overnight. A loop full of this broth was streaked onto the surfaces of Salmonella-Shigella agar (S.S) (Oxoid, UK) Xylose lysin deoxycholate agar (XLD) (Oxoid, UK) , and salmonella chromogenic agar plates(Oxoid,UK) . These plates were then incubated at 37°C for 24 hours. Colonies resembling Salmonella were observed on these plates. On xylose lysine deoxycholate (XLD) agar streaks, pink colonies with or without black centers were seen. On salmonella chromogenic agar streaks, magenta-colored colonies with a metallic sheen occasionally appeared. On Salmonella-Shigella (S.S) agar streaks, black colonies were observed. These results were confirmed through standard biochemical tests [10].

Biochemical test for *Salmonella ssp*

An isolated or purified suspicious colony was biochemically confirmed. Biochemical confirmation was carried out using specific tests according to WHO [14], such as catalase +ve, citrate, capsule -ve, Gram staining -ve ,Gas-ve, H_2S +ve , indol -ve ,oxidase -ve, Triple sugar iron TSI +ve ,urea's –ve Gas.

Salmonella SSP. serotyping

Salmonella was serotyped to identify serovars in meat and meat products. *Salmonella* serotypes were identified by serological technique, Serological confirmation tests typically use polyvalent antisera for flagellar (H) and somatic (O) antigens. Isolates with a typical biochemical profile, which agglutinate with both H and O antisera are identified as Salmonella spp.

Molecular method.

DNA extraction:

Isolates were subculture onto Salmonella-Shigella agar (S.S), Deoxycholate citrate agar (XLD), and salmonella chromogenic agar agar media To obtain pure bacterial colonies for DNA extraction. DNA extraction was performed using a modified boiling method described in reference [11]. Pure colonies

with similar morphology were selected and added to tubes containing 500 ml of deionized sterile distilled water. The mixture was vortexed and heated at 95°C for 10 minutes, then cooled on ice. After centrifugation, the supernatant containing DNA was collected and used for PCR amplification. The purity and concentration of extracted DNA were assessed using a Nanodrop Spectrophotometer (DNA purity between 1.9-2.0). The extracted DNA was stored at -20°C for further molecular identification purposes.

Primers:

The primers are carefully chosen to target the desired DNA sequence. [12,13].

TABLE 1.Oligonucleotide Primers used in the current study, their name, sequence, size and reference.

No.	Genes	Primer Sequence (5′ -′3)	PCR product size (bp)	Reference
1	*invA*(F)	GCTGCGCGCGAACGGCGAAG	389	Ferretti, *et al*.(2000)
	invA(R)	TCCCGGCAGAGTTCCCATT		
2	*16sRNA*(F)	GGAACTGAGACACGGTCCAG	660	Rohwer *et al*.(2001)
	16sRNA(R)	CCAGGTAAGGTTCTTCGCGT		

The *invA* Gene Detection:

Salmonella spp. was detected using specific *invA* gene primers in PCR. The PCR amplification mixture (20µl) for *invA* gene detection consisted of 10 µl of Qiagen-Germany master mix (1x) containing Taq DNA polymerase and dNTPs with yellow and blue dyes, 2.5µl of template DNA, 1µl each of forward and reverse primers 10 pmol/ µl, and 5.5 µl of nuclease-free water. The mixture was transferred to PCR tubes and the reaction was initiated in a thermocycler (Stuart.SH130, Korea). The reaction included an initial denaturation at 95°C for 5 minutes, followed by 35 cycles with denaturation at 95°C for 90 sec, annealing at 62°C for 60 sec, extension at 72°C for 90 sec, and final extension at 72°C for 7min.

PCR amplified products had been resolved by 1.2% agarose gel electrophoresis and visualized using a UV-transilluminator, and photographed.

Sequencing and analysis of 16SrRNA.

After PCR conformation of 16SrRNA for isolates, 50 µl of the PCR products have been sequenced by Korea company Bioneer for DNA sequencing, implementing an ABI3730 XL automatic DNA analyzer and the primer pair 16SF and16SR.from meat and meat products on the 16sR NA gene, and the PCR product was purified from agarose gel by employing (DNA Gel E xtraction), was carried out for the confirmative identi fication of salmonella ssp. Samples of the 16s RNA s equencing were transferred to the Korea company Bi oneer for DNA sequencing [14]. The genomic sequences were assembled and submitted to GenBank-NCBI. The housekeeping gene of *Salmonella* spp., -16s rRNA, was obtained from NCBI GenBank with forward primer ,resulting in a product size of 660 bp. For PCR preparation, a master mix of 25 µl was used along with DNA template (5 µl), forward and reverse primers (1.5 µl each at a concentration of 10 Pmole), and DNase-RNase free water to reach a volume of 50 µl. The mixture was mixed by vortexing and the reaction was carried out in a PCR thermocycler apparatus with denaturation at 94°C for 5 minutes, followed by 35 cycles as follows: Denaturation was performed at 94°C for 30 s, annealing at 57°C for 4 5 sec, extension at 72°C for 90 sec, and final extensio n at 72°C for 7min. All final products were tested using an electrophoresis apparatus.

Statistical analyses

The prevalence of positive outcomes of microorganisms among different types of meats was determined by dividing the number of positive cases confirmed by culture, chemical, and PCR assays by the total number of tested cases and multiplied by 100. The comparisons of culture, chemical and PCR outcomes of microorganisms by sources of meats were examined in chi-squared test. The significant level of difference was determined in a $p<0.05$. The statistical calculations were performed using the JMP Pro 14.3.0. Statistical software (https://www.jmp.com/en_us/home.html)

Results

Isolation of some food borne pathogens bacteria from meat and meat products

The samples of the current study were collected from fresh and frozen meat and meat products from some locations of Duhok city and classified into 6 categories (beef meat, , burger meat, chicken meat, minced meat , sausage meat and sheep meat) as presented in table2

TABLE 2. Sources of meats included in the study

Sources of meat samples	Number	Percentage
Meats		
Beef meat	25	16.66
Burger meat	25	16.66
Chicken meat	25	16.66
Minced meat	25	16.66
Sausage meat	25	16.66
Sheep meat	25	16.66
Total	150	

Prevalence of *Salmonella SSP.* isolates according to diagnostic tests

The study comprised between traditional, biochemical and PCR test in (Table 3). The percentage of *Salmonella spp.* isolation was 29.3% (44/150) positive samples by using the Traditional culture methods of meat samples on enrichment and selective media. 23.33% (35/150) positive samples by biochemical method and 55% (33/60) positive samples by PCR method

TABLE 3. Prevalence of positive *Salmonella* among sources of meats by different assays

Microbes	Assays no (%) culture and biochemical n=150 PCR =60		
	Culture	Chemical	PCR
Salmonella			
Negative	106(70.6)	115(76.66)	27 (45.00)
Positive	44 (29.3)	35 (23.33)	33 (55.00)

Traditional culture method of *Salmonella ssp.*

The percentage 0f *Salmonella spp* , According to the traditional culture methods were as follow, 36.00% (9/25) positive samples from beef meat , 24.00% (6/25) samples were positive from burger meat , 36.00% (9/25) positive samples from chicken meat 32.00%(8/25) samples from minced meat ,20.00%(5/25) samples from sausage meat and 28.00% (7/25) samples from sheep meat. The rate Hight of the *salmonella* in chicken meat and beef meat. Lower ratio in the sausage meat and significantly (p-value < 0.3190).That showed in (Table 4).

TABLE 4. Outcomes of *Salmonella* among sources of meats (culture)

	Meats no (%)						
	Beef meat	Burger meat	Chicken meat	Minced meat	Sausage meat	Sheep meat	P
Salmonella							
Negative	16 (64.%)	19 (77%)	16 (64. %)	17 (68%)	20(80%)	18(72. %)	0.3190
Positive	9 (36%.)	6 (24. %)	9 (36. %)	8 (32%)	5 (20%)	7 (28%)	

Biochemical identification of *Salmonella ssp.*

The percentage of *Salmonella* and the results of biochemical testes were as follow (28.00%)7/25 Positive samples of beef meat (20%), 5/25 positive samples from burger meat (20%) ,5/25 positive chicken meat samples (27.50%) 7/25 positive minced meat samples. 5/25 positive assuage meat sample (20.00%) and 6 /25positive sheep meat samples (24.00%).

Beef and minced meat has the largest concentrati ons of salmonella germs, followed by sheep meat, w hile hamburger meat, chicken meat and sausage meat had the lowest concentration (Table 5). All utilized in biochemical tests, including urea's, catalase, oxidase, gas, Gram staining, H2S, and indol. (p-value < 0.6437).

TABLE 5. Outcomes of *Salmonella* among sources of meats (chemical assay)

	Meats						P
	Beef meat	**Burger meat**	**Chicken meat**	**Minced meat**	**Sausage meat**	**Sheep meat**	
Salmonella							
Negative	18 (72.00%)	20 (80%)	20 (80.%)	18 (72.5%)	20 (80%)	19 (76%)	0.6437
Positive	7 (28.00%)	5 (20%)	5 (20%)	7 (27.5%)	5(20%)	6 (24%)	

Detection of Salmonella using PCR-technique.

A total of 60 samples of *Salmonella* samples and the percentage have been investigated in this study, As shown as in (Table 6) , 33/60 samples were positive of all kind samples. 4/10 positive sample from beef meat (40%) ,5/10positive samples from burger meat (50%) , 6/60 positive samples from chicken meat (50%) ,6/10 positive samples from minced meat (60%) ,6/10 positive samples from sausage meat (60%) ,6/10 positive samples from sheep meat (60%) of *Salmonella* ,the hight rate in chicken ,minced ,sheep and sausage meats then burger meat and less in beef meat, the significantly (p-value < 0.9228) .

TABLE 6. Outcomes of *Salmonella* among sources of meats (PCR assay)

	Meats (n=60 for each meat type)						P
	Beef meat	**Burger meat**	**Chicken meat**	**Minced meat**	**Sausage meat**	**Sheep meat**	
Salmonella							
Negative	6 (60%)	5 (50%)	4 (40%)	4 (40%)	4 (40%)	4 (40%)	0.9228
Positive	4 (40%)	5 (50%)	6 (60%)	6 (60%)	6 (60%)	6 (60%)	

Sensitivity and specificity of culture over biochemical

Data describing the sensitivity and specificity of culture over biochemical assay are presented in (Table 7) assays. Accuracy techniques from(*Salmonella,* culture items (counts) and Accuracy % from *Salmonella* True positive (35),true negative(115),false positive (42)%, false negative(0%),positive predictive value (80.8%) negative predictive value (100%) and Accuracy 94.47%, sensitivity100%, specificity(92.8%).

TABLE 7. Sensitivity and specificity of assays.

Accuracy techniques (culture over chemical)	**Accuracy items (counts)**				**Accuracy (%)**				
	TP	**TN**	**FP**	**FN**	**PPV**	**NPV**	**Accuracy**	**Sensitivity**	**Specificity**
Salmonella	35	115	42	0	80.8	100	94.47	100	92.8

TP: True positive; **TN**: true negative; **FP**: False positive; **FN**: false negative; **PPV**: Positive predictive value; **NPV**: Negative predictive value

Distribution of *Salmonella* serotype among isolates that are confirmed by conventional PCR. included 10 positive sample sent to Baghdad public health center and the results were *Salmonella enteritidis.*

TABLE 8. Distribution of *Salmonella* serotype among *Salmonella* isolates that are Confirmed by Conventional PCR.

Source of sample	*Salmonella enteritidis* serotype and percentage
Chicken meat(local and imported)	10=100%
Total	10

Polymerase chain reaction results of *Salmonella,* isolates

Detection of *salmonella ssp.*

PCR assay was carried out for the DNA from 60 meat samples to detect the presence of S*almonella ssp* by *invA* gene in six type of meat samples. The size of PCR products produced by specific primers for *Salmonella ssp* were 389 bp . The electrophoresis results are demonstrated in (Figure 1).

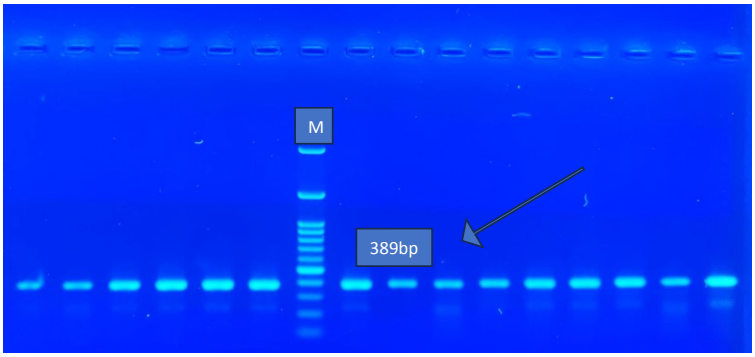

Fig. 1. Represents an example of the PCR amplification products of invA gene for tested samples, electrophoresed in 1.2% agarose gel at 5-8 v/cm. Lane M100-bp DNA ladder : Lane 1: Control positive. Lanes 2, 3, 3, 4and 11 positive invA gene of Salmonella strains (389 bp).

Identification of bacteria using 16s RNA amplification.

The PCR results of tested samples using 16SrRNA shown in (Fig. 2) The identity of sequenced samples with NCBI database reference was 99%. Numbers Amplification of the 16s RNA gene illustrated. amplified fragment through the PCR was 660 bp amp licon.

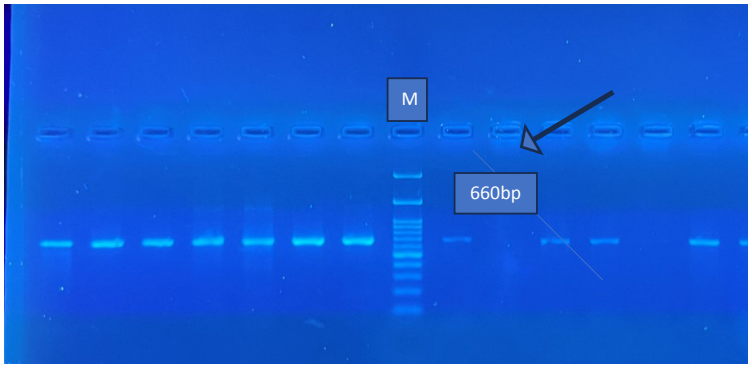

Fig.2. Represents an example of the PCR amplification products of16s RNA gene for tested samples, electrophoresed in 1.2% agarose gel at 5-8 v/cm. Lane M100-bp DNA ladder represents the molecular weight marker (660 bp). Numbers from 1 to 8,10,11,13,14, positive samples and 9,12 negative samples… represent the tested sample.

Salmonella16s RNA genus isolates were sequenced and analyzed.

The nucleotide sequences of 16S rRNA for both forward and reverse strands had determined for all 10 positive isolates. The obtained nucleotide sequences for each isolate searched for their identity and molecular identification of the bacteria implementing the BLAST algorithm of the GenBank database against 16S rDNA sequences of type strains (/

BLAST) at the National Center for Biotechnology Information (NCBI).

The increase in cases of Salmonella-related food poisoning has led to recurring outbreaks, some of which have resulted in fatalities. Most illnesses are caused by consuming animal-based foods contaminated with Salmonella, such as beef, chicken, and other types of meat products [15]. Detecting Salmonella in food is crucial for public health, as its presence can lead to health problems. In this study, 150 food samples suspected of being contaminated with Salmonella were tested using three different methods: culture, biochemical analysis, and PCR. The results from the culture method showed that 44 samples (29.3%) were positive for Salmonella. The commonly accepted gold standard for this purpose is culture techniques, which can be used to detect *Salmonella* and other bacterial pathogens in food products (16). These processes often take longer than PCR-based techniques (17). Compared to PCR-based approaches, and are less sensitive (18). The contamination rate of all types of meat by Salmonella was 35 samples (23.33%) according to the biochemical test, while the PCR method detected 33 positive samples (55%). Cultural techniques rely on providing nutrients and identifying specific metabolic products produced by Salmonella species [19]. However, these techniques involve multiple sub-culturing stages and subsequent biochemical and serological confirmation tests, which can take up to 7 days to obtain a confirmed positive result.

The percentages of infected samples with Salmonella spp collected from local markets in our study were as follows: 36% from beef meat, 24% from burger meat, 36% from chicken meat, 32% from minced meat, 20% from sausage meat, and 28% from sheep meat. These contamination rates were similar to a previous study [20]. However, there were fewer salmonella isolates found in minced meat compared to another study [21]. These results were consistent with the outcomes of the biochemical tests conducted on the sources of meats. Out of the 150 samples tested, we recovered a total of 44 positive samples (28% beef, 20% burger, 20% chicken, 27% minced, 20% sausages, and 24% sheep). The prevalence of Salmonella was higher in beef flesh compared to what was found in another study [22]. We also used molecular screening techniques to detect nucleic acids. Following a nonselective enrichment and PCR method suggested by [23], we were able to detect Salmonella spp within a maximum of 12 hours. By utilizing genomic DNA acquired through boiling technique, we increased the number of samples identified by PCR to 60%. Specifically, we found that 40% of beef meat samples were positive for Salmonella, while it was 50% for burger meat, and 60% for chicken meat, minced meat, sausage meat, and sheep meat.

Discussion

When culture was considered the standard, PCR had a sensitivity of 100% and a specificity of 92.8%, with a positive predictive value of 80.8% and a negative predictive value of 100%. This demonstrated the accuracy of the 12-hour pre-enrichment-PCR method. Comparing this method to traditional culture, it could be a fast and effective tool for detecting Salmonella in food samples. Other studies have also found PCR to be more sensitive than culture and biochemical methods for detecting Salmonella in food, particularly in meat products [24, 25]. In this study, we used a PCR technique without an internal amplification control (IAC) according to Ferretti et al 2001, but it would be beneficial to include an IAC to prevent false negative results and manage inhibitory substances that may affect amplification effectiveness [26, 27]. Overall, our results showed that enriching the samples for 6 hours followed by PCR was a practical strategy for identifying *Salmonella* within 12 hours of receiving the meat samples.

Conclusion

The results indicate that the meat and meat products are considered as a reservoir of many food pathogens at the super markets, restaurant and abattoir 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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الكشف عن السالمونيلا في اللحوم ومنتجاتها حسب الطريقة التقليدية والخصائص البيوكيميائية والجزيئية في مدينة دهوك

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[3] رئيس جامعة زاخو- جامعة زاخو- العراق.

تشير النتيجة الحالية الموضحة أن مجموعة 150 عينة من اللحوم ومنتجات اللحوم المحلية والمستوردة و تم جمعهم من مواقع مختلفة في محافظة دهوك مما بين الفترة من نوفمبر 2021 إلى أغسطس 2022 كما يلي: 25 عينة = (16.66%) من كل نوع من اللحوم المتضمنة لحم البقر، لحم برجر، لحم دجاج، لحم مفروم، لحم سجق، لحم غنم وتضمنت الدراسة كانت نسبة السالمؤتيلا المعزولة بطريقة التقليدية والكيميائية الحيوية وطريقة PCR.كانت النسبة الايجابية 29.3% (150/44).اما النتيجة الايجابية بطريقة الكيموحيوية كانت 23.33% (150/35)والعينات الايجابية ل PCR كانت33% (60/33) .وكذلك تاكد العينات بطريقة المصلية. تقنيات الدقة٪ للسالمونيلا القيمة الحقيقية (100%) والدقة 94.47% والحساسية 100% والنوعية (92.8%). لفحص السالمونيلا يستخدم جين invA لتفاعل البوليميراز المتسلسل .(PCR) ذات حجم bp389 هلام الاغاروز .
. تم إرسال 10 عزلات فقط إلى بنك الجينات NCBI لتسجيل النيوكليوتيدات استخدمت جين 16Srna ذات حجم bp660.وقد تم تشخيص العزلاتعن طريقgene back والحصول على رقم الانضمام (BankIt2717092 Salmonella

الكلمات الدالة: اللحوم، السالمونيلا، التشكل وتفاعل البلمرة المتسلسل، جين invA، تسلسل الحمض النووي sRNA16