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DEVELOPMENT OF PCR PRIMER AND 16s rDNA SEQUENCING FOR GRAM NEGATIVE (*Salmonella* spp) BACTERIA ISOLATED FROM POULTRY MEAT

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ABSTRACT

A Study to Isolate, identify and characterize the *Salmonella* species along with other microbes from poultry/chicken products in Tikrit local markets and supermarkets in Iraq was carried out. A broad-range PCR assay for the detection of bacteria belonging to the *Salmonella* spp was developed in this study. *Salmonella sps* have been widely isolated from raw meats, poultry and poultry products, milk and milk products and the environment. *S. Enteritidis* is more prevalent in poultry and poultry products as it's associated with "salmonellosis" in poultry. Worldwide, epidemiological reports indicate that poultry meat is the food most incriminated in food poisoning. 16s r DNA genes encoded a ribosomal RNA. Each species unique sequence of this common gene can be used as a means for identifying bacterial *sps* like *Salmonella*. Several species of Gram negative pathogenic bacteria were isolated from the poultry meat collected from various places of food markets located in Iraq used in this study. Other bacteria identified in this current study were: *E.Coli*, one type of *Pseudomonas Spp,* 6 types of *Salmonella sps* etc., 8 types of bacteria isolated and characterized with PCR technique; the target genes (*spvB*, *16s rDNA* and *hilA*) produced amplicons at 717bp, 572bp and 854bp which were shown to be 100% specific targeted *Salmonella* Sps bacteria in the collected samples. DNA was isolated from each organism and was amplified by PCR followed by purification and DNA sequencing. The resulting sequences were then analysed to allow for identification of the bacteria isolated. The molecular techniques used in this experiment were performed with the intension of illustrating the usefulness of 16s rDNA sequencing for academic institutions laboratory work in microbiology.

Keywords: Poultry meat, Salmonella, PCR, 16rsDNA, bacteria, Isolation and characterization

1.0 INTRODUCTION

Identification of bacteria is an important for basic biological research and applied clinical microbiology. Salmonellosis, caused by infection with bacteria from the genus *Salmonella*, is one of the most common food borne illnesses and is manifested by diarrhea, mild fever, nausea, and abdominal pains, with the symptoms developing in 12–48 hrs after consumption of contaminated food. Salmonella is a standout amongst the most widely recognized pathogens and a noteworthy reason for foodborne ailments in human around the world. Salmonellosis affects 1.3 billion people worldwide each year with an estimated 3 million annual deaths from non-typhoidal salmonelloses (NTS) [1].

Salmonella infection places significant health and economic burden worldwide [2]. There are many different *Salmonella* serotypes found nearly 2541 as per the CDC report in 2010 [3] and their distribution can change over time [4]. According to Ngan et al [5], *Salmonella Typhi* accounts for more than 25 million infections worldwide, resulting in approximately 200,000 deaths annually. Selected place for the current investigations in Iraq, *Salmonella enterica* and *Salmonella Typhimurium* were the most frequently isolated from humans [6-7]. Among the many rapid methodologies being developed for the detection of Salmonella and other food borne pathogens, the polymerase chain reaction (PCR) has been frequently studied over the past decade because, in addition to being rapid and facile, the method can be highly specific and sensitive [8]. Finally, it is important to use tests that have been appropriately validated for use on clinical/agricultural infected samples. Genetic identification systems may improve Salmonella identification. Several polymerase chain reaction (PCR) assays [9] have been developed by targeting various Salmonella genes, such as *inv*A, 16s rRNA, *flic*, *hil*A, *sir*A , *ttr*AB, virulence-associated plasmids etc., [10]. *Exo*U, *Ecf*X, *Exo*S *phz*M *tox*A *las*B, *gyr*B, *las*I/R etc., for *Pseudomonas aeruginosa* [11] and *UAL*754, *tet*M, *bla* TEM, *aad*A etc for *E.coli* [12]. Traditional methods of identifying bacteria, including culturing, can be time consuming and unreliable. Molecular based methods of sequencing provide quick, reproducible identification. One reason that the 16s rDNA gene sequencing is so widely used to identify salmonella bacteria is that this gene is highly conserved.

Poultry meat and its subordinates are among the food items that reason the most worry to general health experts, inferable from the related dangers of bacterial food harming. The modernization

of chicken ranches and globalization of the bird breeding reproducing exchange likewise have assumed a noteworthy job in "Salmonellosis" disease. According to Panisello PJ et al. (2000), the common reason of salmonella infection mostly reported in humans has been though handling of improper cooked poultry meat, together with poultry carcasses [13]. Abdulla et al (2010) [14] states that chicken/poultry based food products example egg, meat, bakery foods, chicken curry etc., are the major sources of salmonella bacteria. Section of Salmonella into egg or egg might be clarified in two conceivable ways. The main way, transmission by on a level plane, where the tainted excrement enters eggshell pores, or defiles eggs by means of splits on eggs shells. The second way is vertical transmission inside the contaminated chick, where the eggshell layers, yolk and different parts in egg are tainted before oviposion [15].

In this present investigation salmonella bacteria species along with other bacterial isolated from poultry/chicken products collected from selected places in Tikrit city markets in Iraq during July 2018 to August 2018. Antibiotic susceptibility, PCR for virulence genes studies done to assess the relativity among the various parameters.

2.0 MATERIAL AND METHOD

2.1 Samples collection: In the current examination, the isolation and identification gram negative bacteria species like salmonella, Escherichia coli and Pseudomonas from Poultry/Chicken food products collected from the Fast food restaurants, local chicken markets, food restaurants located in the city of Tikrit, Iraq during July to December 2018. A total of 48 samples (10 chicken curry samples/12 Egg Products/ 26 samples of raw chicken met) collected from 12 different locations in the city. A reference sample for *Salmonella* Sps (ATCC 3214) obtained from the department of Microbiology, Ba'quba teaching hospital, Diyala Province is a governorate in eastern Iraq was used as positive control and double distilled water (free from any living organism) used as negative control in the entire study.

***2.2 Bacterial Identification*:** Unadulterated disengages of bacterial pathogens were initially portrayed by colony morphology, hemolytic response on agar medium, gram-stain and catalase test. 48 food samples based on poultry/chicken products were immunized on agar and MacConkey plates, hatched at 37^{o}C for 48 hours for most extreme recuperation of the detaches (Figure 1). Suspected bacterial provinces were recognized by standard bacteriological techniques given by Cheesbrough M. (1999) [16]. These were then subcultured into Deoxychocolate

Citrate Agar. The separates were then subcultured in Kliger Iron Agar (KIA) and Simmon Iron Medium (SIM). These means were trailed by biochemical and stereotyping distinguishing proof. The *Salmonella* positive specimens were then sub-cultured in supplement broth and put away in the fridge at 6°C for anti-microbial susceptibility testing. MacConkey's broth (Adwic), Brilliant green bile 2 % (Oxoid) and Eosine methylene blue agar (Biolife) used to identify the Isolation of *E.coli* isolate and *Aeromonas* base medium (Ryan's formulation) (Oxoid) used to identify Isolation of *Pseudomonas aeruginosa* (Figure 1).

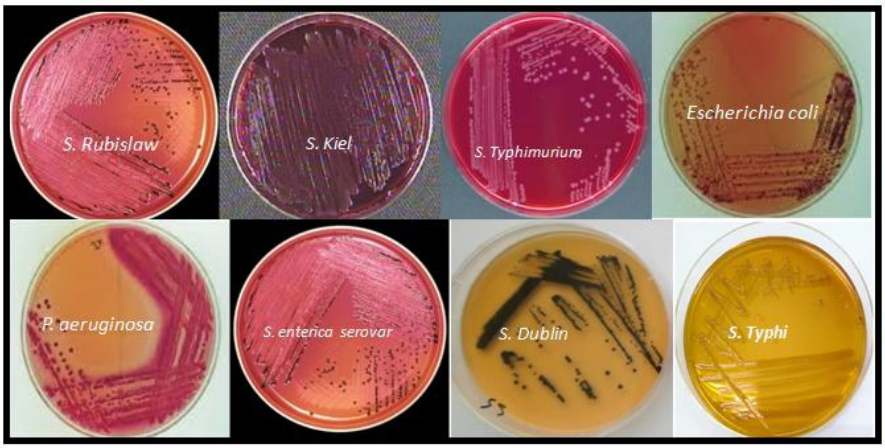

Figure 1 Bacterial colony development of *Salmonella Spp, E Coli* and *Pseudomonas aeruginosa* on agar (characteristics red colonies with black centre)

***2.3 PCR development, Molecular characterization and Stereotyping*:** The Isolated bacterial culture were platted on Hekton Enteric Agar and cultured 48hours in a 37°C in incubator, these obtained bacterial cultures used to DNA extraction. The Prep man Isolation kit was used for isolation of bacterial DNA method developed by Anonymous (2000) [17].

The PCR mix consist of 10µL of the bacterial DNA in a clean 1000□□L micro centrifuge tube 25µL each (250 picomoles) *spv*B primer 5'-*ACC GCA CCC GCC TCA TTA GGC CCA CTA GTA CCA CCT-3' and 16s* rDNA primer 5'-CCT CCA GGA CCT CCC AAG TTA CCA CCC GGT CCA CCG-3' and *hil*A primer 5'-CGA GGT CCC CCA CCC CAC CGA CCA GTA CCT CCA CCC-*3'* 25µL HotStar Taq master PCR mix (Shimadzu) and 10µL of distilled water was added to the reaction mixture was place in a PCR machine, which was programmed according with manual. The PCR product was run in a 2.5% agarose electrophoresis get and the results were given in figure 2A, 2B & 2C. Samples showing an appropriate size band were saved for purification and DNA sequencing. PCR products were purified using the QIAquick PCR purification Kit (Qiagen Valencia, CA) and eluted with water. DNA sequences were analysed by

submission to the ribosomal database project website [18] for identification.

***2.4 Antimicrobial susceptibility test*:** Culture media was prepared by reconstituting commercial powder in distilled water and sterilized at 121ºC for 15 minutes in an autoclave per the manufacturer's instructions [19]. The isolated microbial species were cultured on Hicrome agar Media. The isolates were tested by disc diffusion method for drug susceptibility according to National Committee for Clinical Laboratory Standards guidelines [20]. This was then impregnated with antimicrobial sensitivity discs using sterile forceps and then gently pressed down onto the agar. The antibiotic disc sensitivity was done using Ampicillin (25µg); Azithromycin (20µg); Clindamycin (10µg); Ciprofloxacin (10µg) Erythromycin (20µg) ; Ofloxacin (10µg); Gentamicin (15µg); Tetracycline (20µg) and Ceftriaxone (15µg) (HiMedia, India). The isolates were grown on autoclaved Mueller Hinton broths (HiMedia, India) for 18 hrs at 37°C. About 100 µl of the inoculums was spread on Mueller Hinton agar using sterile disposable Lshaped spreader and antibiotic discs were placed onto the plate using sterile forcep. The plates were incubated at 37 °C for 24 hrs and observed for zone of inhibition. The results (Table 2) were categorized as sensitive, moderately sensitive and resistant based on diameter of zone of growth inhibition corresponding to different isolates.

3.0 RESULTS AND DISCUSSION

The poultry meat, egg products and curry samples of any food from its source are usually free from pathogenic bacteria, but during processing in different stages contamination occur leading to introduction of dangerous salmonella and other pathogen isolates enter into the poultry food products. The source of these pathogens may be endogenous from the gastrointestinal tract or from surrounding environment in farm and/ or slaughterhouse. Poultry are the most common food vehicle of human infection with enters pathogens throughout the world.

In the Current study total 8 microbes were identified from 48 samples of Chicken/poultry products. Based on PCR and biochemical studies identified microbes named as *S. Kiel, S. Rubislaw, S. Typhimurium, S. Typhi , S. Dublin, S. enterica serovar, Escherichia coli* and *Pseudomonas aeruginosa.*

- The prevalence of *S. Kiel* identified as /10(60.00%) in Chicken curry samples, in Egg Samples 4/12(33.33%) and in Raw chicken Meat samples 11/26 (42.31%) respectively (Table1). Gharieb RM et al (2015) [21] isolated *salomonella* spp from poultry meat and

diarrhoeic patients in Egypt. All the isolates were serologically identified into *Salmonella typhimurium*, S. Derby, S. Kiel, S. Rubislaw.

- *S. Rubislaw* found in 4 Chicken curry samples with the rate of 40.00%, 3 Egg samples with the rate of 25.00% and 13 samples out of 26 Chicken meat samples with the existence rate of 50.00%.
- *S. Typhimurium* 9 (90.00%), 10 (83.33%), 20 (76.92%), *S. Typhi* , 8 (80.00%), 4 (33.33%), 11 (42.31%), *S. Dublin* in 2 (20.00%), 5 (41.67%), 6 (23.08%) and *S. enterica serovar* in 6 (60.00%), 7(58.33%), 9 (34.62%) respectively in chicken curry samples (n=10), egg samples (n=12)and Raw chicken meat samples (n=26).

Table 1: Frequency of isolated Salmonella *spp* along with other microbes in three type's poultry products

Bacteria Name	Chicken curry samples (n=10)		Egg Samples (n=12)		Raw chicken Meat samples (n=26)	
	No of Samples	% Found	No of Samples	% Found	No of Samples	% Found
S. Kiel	6	60.00%	4	33.33%	11	42.31%
S. Rubislaw	4	40.00%	3	25.00%	13	50.00%
S. Typhimurium	9	90.00%	10	83.33%	20	76.92%
S. Typhi	8	80.00%	4	33.33%	11	42.31%
S. Dublin	2	20.00%	5	41.67%	6	23.08%
S. enterica serovar	6	60.00%	7	58.33%	9	34.62%
Escherichia coli	7	70.00%	2	16.67%	4	15.38%
Pseudomonas aeruginosa	2	20.00%	2	16.67%	4	15.38%

- On the other hand, the occurrence of *E.coli* was the considerable amount in all three types of chicken products. 70.00% (Chicken curry samples), 16.67% (Egg Samples) and in Raw chicken Meat samples it as 15.38%. The existence of ***E.coli*** in poultry products (raw meat, egg products, chicken curry) due to the effect of freezing environment and preservative conditions, in accordance to James et al., (1992) [22] who found that existence concentration of *E.coli* count in poultry based on cooling conditions.
- Finally, *Pseudomonas aeruginosa* found in 2/10 (20.00%), 2/12 (16.67%) and 4/26 (15.38%) in chicken curry samples, egg samples and Raw chicken meat samples respectively
- The presence of multiple drugs resistant *Salmonella Spp* in poultry product indicated high prevalence of *Salmonella* in raw chicken meat and makes the food chain unsafe from farm to the Table 1, which considered as a major global threat to public health. It requires continuous surveillance of situation including the antimicrobial resistance pattern, because it is a serious public health problem in the world, with an increasing concern for the emergence and spread of antimicrobial-resistant strains [23].
- From our study, some conclusions with potential implications for the isolation and identification of Salmonella from poultry meat could be drawn. First,

contamination of chicken meat with *Salmonella*, even in low incidence, indicates bad microbiological quality of chicken meat.

3.1 PCR results and gene sequencing

Gel analysis of PCR products showed 9 bands along in 5 bands in PCR amplicon for *spvB* gene fragmentation at 717bp (Figure 2b), out of 9 bands three bands are negative control and five are positive control represents 5 Salmonella serovers. In the figure 2a, PCR amplification for *16s rDNA* gene fragments shows 12 bands including 1 negative and 11 positive control bands obtained at 572bp along with reference band, finally PCR amplification for *hilA* gene fragments observed in figure 2c shows nine bands with one negative and 8 positive control at 854bp along with reference sample M in figure2c. Approximate sized DNA bands were also obtained following PCR form of 12 bacterial isolates of blood, urine and stool samples. Among the three gene targets all 12 isolates responded positively to *spvB* and 16sr DNA genes. 16sr DNA is moderately reflected in the current multi PCR technique. Proportion of bacterial strains isolates that were positive for the spvB, 16s rDNA and hilA genes were given in table 3. The primer 16S rRNA proved to be specific for the PCR detection of all Salmonella isolates with various serogroups. For these reasons, we used the primers 16S rRNA gene and found that all Salmonella spp. isolates identified by conventional tests gave positive bands with PCR. Maciorowski et al. (2000) [24] used PCR to detect 63% salmonella spp in poultry samples by conventional methods. Löfström et al. (2004) [25] developed PCR procedure for routine analysis of viable Salmonella spp. in 14 different feed samples and 8% of the samples were positive by PCR, compared with 3% with the conventional method.

3.2 Antibacterial Resistance

Resistance has many consequences. Resistance also compels the use of more toxic or more expensive alternative drugs. The evaluation of antibacterial resistance typically reveals some organisms that are naturally resistance and that appear to be susceptible, thus defining the spectrum of activity for the specified agent. In the current investigation few common antibiotics were used to test the antibacterial resistance against the isolate of 6 *salmonella* spp in three types of samples and average susceptibility values (%) are given ion table 2. It was identified that out of nine antibiotics tested in this study Clindamycin (10µg), Azithromycin (20µg) and Ampicillin (25µg) shows between 70-80% susceptibility against all *salmonella* isolates and next antibiotic in order is Ceftriaxone (15µg) active against major isolates.

Table 2: The Percentage of antibiotic susceptibility pattern of *Salmonella* spp, *E.coli* and *Pseudomonas Spp* isolates from Poultry products

	% of Antibiotic susceptibility pattern in Chicken meat Samples								
Bacteria Isolate	Ampicillin (25µg)	Azithromycin (20µg)	Clindamycin (10µg)	Ciprofloxacin (10µg)	Erythromycin (20µg)	Ofloxacin (10µg)	Gentamicin (15µg)	Tetracycline (20µg)	Ceftriaxone (15µg)
S. Kiel	43.19	44.05	44.93	30.10	20.17	20.57	20.98	21.40	21.83
S. Rubislaw	77.62	79.17	80.76	54.11	36.25	36.98	37.72	38.47	39.24
S. Typhimurium	62.46	63.71	64.99	43.54	29.17	29.76	30.35	30.96	31.58
S. Typhi	63.65	64.92	66.22	44.37	29.73	30.32	30.93	31.55	32.18
S. Dublin	61.44	62.67	63.93	42.83	28.70	29.27	29.86	30.45	31.06
S. enterica serovar	62.46	63.71	64.99	43.54	29.17	29.76	30.35	30.96	31.58
Escherichia coli	62.24	63.49	64.75	43.39	29.07	29.65	30.24	30.85	31.46
Pseudomonas aeruginosa	43.19	44.05	44.93	30.10	20.17	20.57	20.98	21.40	21.83
Max	77.62	79.17	80.76	54.11	36.25	36.98	37.72	38.47	39.24
Min	43.19	44.05	44.93	30.10	20.17	20.57	20.98	21.40	21.83
Std Dev.	11.38	11.61	11.84	7.94	5.31	5.42	5.53	5.64	5.75
	% of Antibiotic susceptibility pattern Egg Samples								
Bacteria Isolate	Ampicillin (25µg)	Azithromycin (20µg)	Clindamycin (10µg)	Ciprofloxacin (10µg)	Erythromycin (20µg)	Ofloxacin (10µg)	Gentamicin (15µg)	Tetracycline (20µg)	Ceftriaxone (15µg)
S. Kiel	47.08	48.02	48.98	32.82	21.99	22.43	22.87	23.33	23.80
S. Rubislaw	84.61	86.30	88.02	58.98	39.51	40.30	41.11	41.93	42.77
S. Typhimurium	74.64	76.13	77.66	52.03	34.86	35.56	36.27	36.99	37.73
S. Typhi	69.38	70.77	72.18	48.36	32.40	33.05	33.71	34.39	35.07
S. Dublin	66.97	68.31	69.68	46.68	31.28	31.90	32.54	33.19	33.86
S. enterica serovar	42.10	42.94	43.80	29.35	19.66	20.06	20.46	20.87	21.28
Escherichia coli	43.10	43.96	44.84	30.04	20.13	20.53	20.94	21.36	21.79
Pseudomonas aeruginosa	47.08	48.02	48.98	32.82	21.99	22.43	22.87	23.33	23.80
Max	84.61	86.30	88.02	58.98	39.51	40.30	41.11	41.93	42.77
Min	42.10	42.94	43.80	29.35	19.66	20.06	20.46	20.87	21.28
Std Dev.	16.45	16.77	17.11	11.46	7.68	7.83	7.99	8.15	8.31
	% of Antibioticsusceptibility pattern in Chicken Curry Samples								
Bacteria Isolate	Ampicillin (25µg)	Azithromycin (20µg)	Clindamycin (10µg)	Ciprofloxacin (10µg)	Erythromycin (20µg)	Ofloxacin (10µg)	Gentamicin (15µg)	Tetracycline (20µg)	Ceftriaxone (15µg)
S. Kiel	ND	ND	ND	ND	ND	ND	ND	ND	ND
S. Rubislaw	46.24	47.16	48.11	32.23	21.60	22.03	22.47	22.92	23.38
S. Typhimurium	51.27	52.30	53.34	35.74	23.94	24.42	24.91	25.41	25.92
S. Typhi	ND	ND	ND	ND	ND	ND	ND	ND	ND
S. Dublin	27.57	28.12	28.68	19.22	12.88	13.13	13.40	13.66	13.94
S. enterica serovar	30.64	31.25	31.88	21.36	14.31	14.60	14.89	15.19	15.49
Escherichia coli	31.64	32.27	32.92	22.06	14.78	15.07	15.37	15.68	16.00
Pseudomonas aeruginosa	40.84	41.66	42.49	28.47	19.07	19.46	19.84	20.24	20.65
Max	51.27	52.30	53.34	35.74	23.94	24.42	24.91	25.41	25.92
Min	27.57	28.12	28.68	19.22	12.88	13.13	13.40	13.66	13.94
Std Dev.	9.54	9.74	9.93	6.65	4.46	4.55	4.64	4.73	4.82

Table 3: Proportion of bacterial strains isolates that were positive for the *spvB*, 16s rDNA and *hilA* genes

Name of the Identified Bacteria	% of salmonella Sps (+) ve, *E.coli* and *P. aeruginosa* for the targeted genes		
	spvB	*16s rDNA*	*hilA*
S. Kiel,	64.46	63.89	24.37
S. Rubislaw	70.78	66.61	70.28
S. Typhimurium	59.36	51.09	78.73
S. Typhi	70.78	48.82	86.30
S. Dublin	69.52	71.35	80.57
S. enterica serovar	60.10	52.28	70.05
Escherichia coli	10.42	6.98	5.68
Pseudomonas aeruginosa	11.70	12.88	18.64

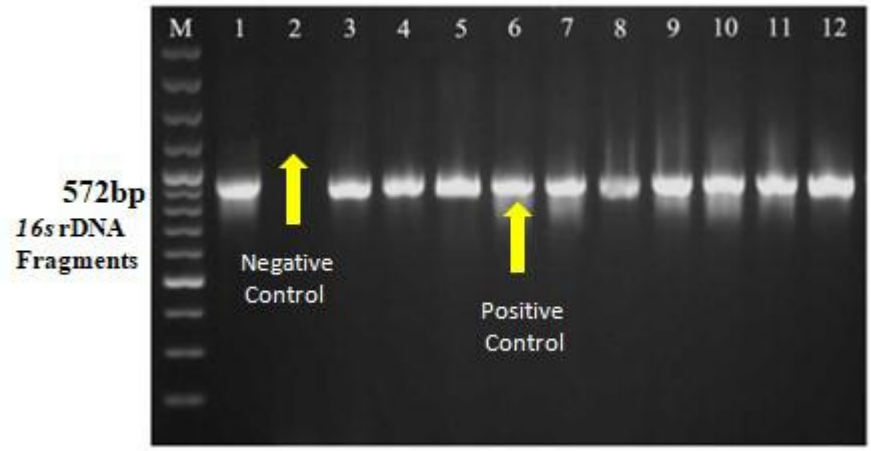

Figure 2a: Electrophoresis image for amplification of 16s rRNA gene (572bp) of *Salmonella species*; shape of the line-Rod like, lane 1,3 to 12 positive control and lane 2 for negative control results

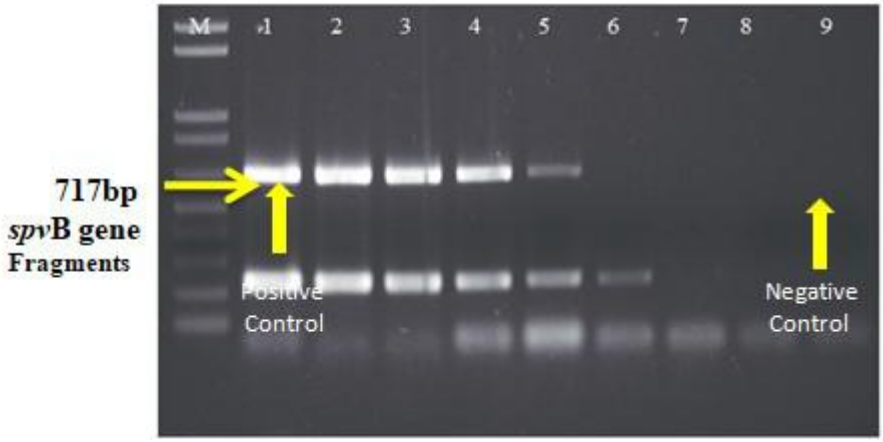

Figure 2b: Electrophoresis image for amplification of *spv*B gene (717bp) of *Salmonella species*; shape of the line-Rod like, lane 1 to 5 positive control and lane 6, 7,8 and 9 for negative control results

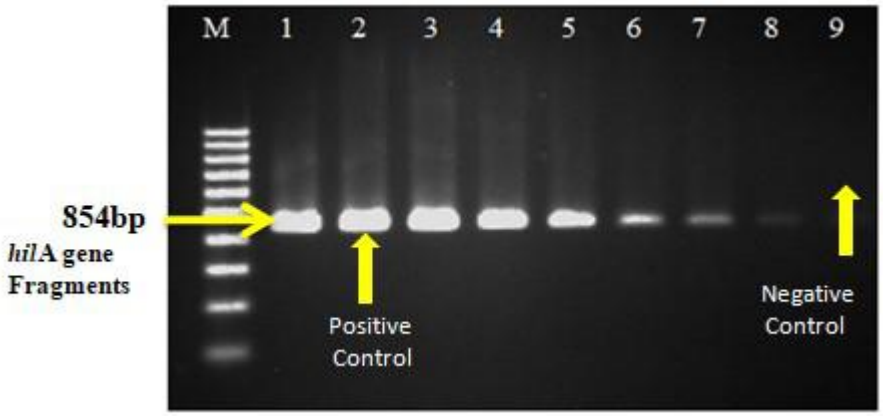

Figure 2c: Electrophoresis image for amplification of *hil*A gene (854bp) of *Salmonella species*; shape of the line-Rod like, lane 1 to 8 positive control and lane 9 for negative control results

4.0 CONCLUSION

To conclude, this short segment of the 16s rDNA mitochondrial gene could be a very good candidate for a rapid, accurate, low-cost and easy-to-apply and interpret method to identify animal species by PCR amplification that can be easily incorporated in integrated conservation and forensic programmes. The ability of the designed pair of primers to identify animal species through non-invasive approaches by examining poultry products could also be very helpful in various studies. A prevalence of Salmonella was observed from chicken meat and curry samples expected based on environment, knife, wooden table and hands from slaughtering places in local chicken shops in Tikrit city, Iraq. This study reported the isolation of Salmonella spp. from egg products, chicken curry samples for the first time in Tikrit city and emphasized that poultry products were the most important vehicles for chicken salmonellosis.

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