Available online at sjuoz.uoz.edu.krd

Science Journal of University of Zakho
Vol. 12, No. 2, pp. 149 – 152 April-June, 2024

p-ISSN: 2663-628X
e-ISSN: 2663-6298

journals.uoz.edu.krd

IDENTIFICATION OF SALMONELLA TYPHIMURIUM AND SALMONELLA ENTERITIDIS IN THE IMPORTED FROZEN POULTRY

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Received: 4 Feb., 2024 / Accepted: 26 Mar., 2024 / Published: 24 Apr., 2024. https://doi.org/10.25271/sjuoz.2024.12.2.1260

ABSTRACT:

Salmonellosis, a zoonotic illness caused by Salmonella spp., is mostly transmitted to humans through the consumption of raw animal products, particularly poultry meat. Salmonella enterica serovar Typhimurium and Salmonella enterica serovar Enteritidis are the predominant strains responsible for human infection. This study aimed to assess the prevalence of S. Enteritidis and S. Typhimurium infection in frozen imported poultry using a triplex PCR technique. A total of 110 samples were chosen using the cluster sampling technique from the Ibrahim Khalil border throughout the summer and autumn of 2023. Following the cultivation of the meat samples in enrichment and selective media, suspicious colonies were obtained. Subsequently, DNA extraction was conducted, and the Salmonella isolates were identified using triplex PCR. Three (3) sets of primer pairs were utilised to target the invA gene for the Salmonella genus, the prot6 gene for the S. enteritidis serovar, and the fliC gene for the S. typhimurium serovar. The prevalence of Salmonella infection in frozen poultry meat was 12% (13 out of 110 samples). Among the contaminated samples, 38% (5 out of 13) were recognised as S. enteritidis and 30% (4 out of 13) were recognised as S. typhimurium. The findings of this finding suggest that frozen poultry meat pose a significant danger for zoonotic infections caused by S. typhimurium. and S. enteritidis.

KEYWORDS: Salmonella Enteritidis, Salmonella typhimurium, Zoonotic diseases, Poultry.

1. INTRODUCTION

1.1 Instructions

Salmonella is a Gram-negative, facultatively anaerobic bacterium that is a member of the family Enterobacteriaceae that is found in the environment (Steve Yan *et al.*, 2004). Within environment, Salmonella species can be found in a broad variety of locations; nevertheless, the intestinal tracts of animals are the primary habitat for these bacteria (Ellermeier & Slauch, 2006). The ingestion of infected items, such as eggs, milk, and chicken meat, can lead to the transmission of the *Salmonella* bacteria among individuals (Gillespie *et al.*, 2003).

It is estimated that twenty percent (20%) of all poultry products around the world are infected with Salmonella. Salmonella able remain for an extended period of time in the habitats of animals and humans, as well as in facilities, due to the production of biofilms (Vestby et al., 2009). S. enteritidis and S. typhimurium serovars have been discovered in the majority of the salmonellosis outbreaks that have been attributed to the eating of poultry products (Vose *et al.*, 2011).

Polymerase Chain Reaction (PCR) is a powerful technique used to investigate outbreaks of food-borne illnesses and identify specific pathogens (Riyaz-Ul-Hassan *et al.*, 2004; Tayeb *et al.*, 2020). PCR offers rapid outcomes and a notable level of specificity. Combining routine PCR testing with standard culture methods should enhance the accuracy of determining the existence of this disease in grill carcasses (Carrasco *et al.*, 2012). The primary cause of *Salmonella* contamination in carcasses is the presence of *Salmonella* in the digestive system, skin, and feathers of chickens. This contamination can occur along to the processing line.

This paper was carried out on the frozen poultry meat at the central laboratory of the Ibrahim Khalil border, Zakho, Duhok region, to assess the level of contamination of poultry carcasses with Salmonella spp., specifically S. typhimurium and S. enteritidis. The contamination was evaluated using a combination of culture and triplex PCR methods, which involved the detection of prot6E and fliC genes.

2. MATERIALS AND METHODS

2.1 Mode of sampling

The sampling was performed at the Central laboratory of Ibrahim Khalil border Duhok region, For the purpose of this investigation, a total of one hundred and ten (110) frozen poultry samples were collected using cluster sampling techniques. This process was carried out between summer and autumn of 2023.

2.2 Bacterial isolation and identification

The protocol was conducted according to the ISO 6579-2020 manuals and a previously published protocol (Sharif & Tayeb, 2021). Briefly, 25 g of frozen meat sample were added to 225 mL Buffer peptone water (BPW) (CONDALAB, SPAIN) and then were incubated at 37℃ Celsius degrees for 18 h. Then, one mL of each re-enriched sample was put into 9.9 mL of Rappaport-Vassiliadis soya peptone broth (RVS broth), and the mixture was then incubated at 41.5 ℃ for 24 hr, and 1 mL was transport to a10 mL of Muller-Kauffmann-Tetrathionate Novobiocin (MKTTn, Merck, Germany), then incubated at 37 ℃ for 24 h. Following the incubation period, a loopful of each culture was streaked onto Xylose Lysine Deoxycholate agar (XLD agar) (Condalab-Spain) Salmonella Chromogenic Medium which was then preserved at 37 ℃ for 24 h. The urease and triple sugar iron (TSI), lysine decarboxylase, were utilised in order to validate the presence of presumed Salmonella colonies through biochemical analysis (Jamshidi *et al.*, 2010).

2.3 Extraction of DNA

After the results of the biochemical tests indicated that the bacterial colonies were really *Salmonella* species, they were grown on nutrient agar for an overnight incubation. For DNA extraction from the suspected colonies, a previous published protocol was followed (Ameen *et al.*, 2016). Briefly, a small amount of Salmonella from agar plates was mixed with 100 µL

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of sterile distil water in a 1.5 mL Eppendorf tube and vortexed to create a bacterial suspension. Following this, the bacterial suspension underwent boiling at 95-100ºC for 10 a min and subsequent centrifugation at 10,000x g for 10 min. The resulting supernatant served as the DNA template for qPCR.

2.4 Triplex PCR method

The PCR process was conducted in a final volume of 25 µL, which included 2.5 µL of 10x PCR buffer comprising 500 mM of KCL and 200 mM of Tris–HCL, 1.25 µL of deoxynucleotide triphosphate (10 mM), 1.5 µl of $MgCl_2$ (2 mM), 0.5 µL of Taq DNA polymerase (Fermentas), 2 µL of extracted DNA, and 0.5 µL of each primer. A Rotor-Gene Q7 (Qiagen kit) was used to perform the amplification reaction and cycling conditions. The procedure consisted of an initial incubation at 95 °C for five minutes, then following by thirty-five cycles of denaturation at 94 °C for sixty seconds, annealing at 56 °C for thirty seconds, extension at 72 °C for thirty seconds, and a final extension period for ten minutes at 72 °C (Jamshidi *et al.*, 2009).
Primers of S141 and S139, which were target for the invA gene, were utilised for the testing of the genus *Salmonella*. Primers of prot6e-6 and prot6e-5, which were specific for the prot6E gene, were utilised for the identification of *S. enteritidis*. Additionally, Tym and Fli15primers, which were targetted for the fliC gene, were utilised for the identification of *S. typhimurium* (Table 1) (Afshari,*et al.*2018)

These two strains of S. typhimurium (ATCC: 14028) and S. enteritidis (ATCC-13076) served as positive controls obtained from New standard company, while sterile distilled water served as the negative control.

Table 1: displays the oligonucleotide sequence utilised as primers in the triplex-PCR (Qiagen triplex Kit)

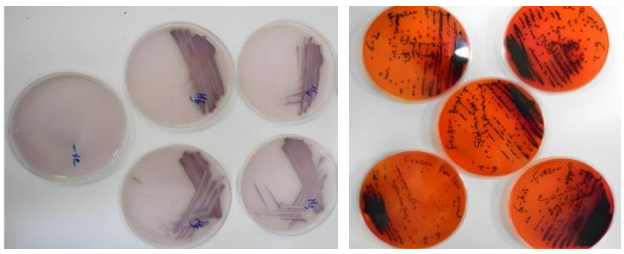
Primers	Sequences (5′–3′)	Target gene
S139-F	GTG AAA TTATCG CCA CGT TCG GGC AA	*InvA*
S141-R	TCA TCG CAC CGT CAA AGG AAC C	
Fli15-F	CGG TGT TGC CCA GGT TGGTAAT	*fliC*
Tym-R	ACT CTT GCT GGC GGT GCG ACTT	
Prot6e-5-F	ATA TGG TCG TTG CTGCTT CC	*Prot6e*
Prot6e-6-R	CATTGT CCA CCG TCA CTTTG	

F: forward primer; R: reverse primer. (Afshari,*et al.*2018)

3. RESULTS

3.1 Morphological characteristics of Salmonella

Based on the morphology of Salmonella colonies observed on both selective and non-selected media, a total of 14 (12%) meat samples were tested as positive for the presence of Salmonella. The distinctive characteristics of the colonies, such as colour, size, and shape, allowed for accurate differentiation and confirmation. The morphological characterization of the black and mauve colonies as shown in Figure 1.



A B

Figure 1: Typical Salmonella Spp on chromogenic agar, showing mauve color (A), and on XLD agar red, black colonies (B).

3.2 Biochemical confirmation

The suspected samples were confirmed as positive by traditional culturing methods were confirmed by biochemical tests. The samples which were initially suspected of harbouring specific microorganisms were subsequently verified as positive through conventional culturing techniques, a confirmation that was further substantiated by biochemical assays
Lysine ,urase test ,TSI as shown in Figure 2.

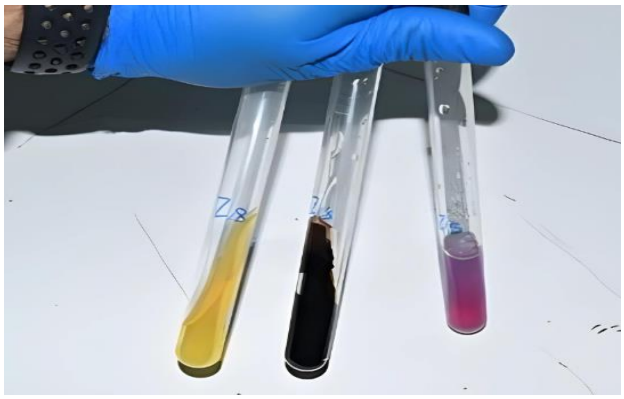


Figure 2: Biochemical test performance. Left side tube: urea is negative; in the middle: H_2S gas production; right side tube: Lysin decarboxylase shows positive result.

3.3 Confirmation by PCR

We used real time PCR (Triplex kit) in this finding. Further confirmation was done by using PCR techniques (fragment of the invA gene). Using a triplex PCR assay, five of the fourteen isolates were determined to be related to S. enteritidis (fragment of the prot6e gene) (Figure 3), and four of the isolates were related to S. typhimurium (fragment of the fliC gene) (Figure 4). It was determined that the remaining isolates did not belong to the species S. enteritidis or S. typhimurium.

Figure 3: Shows the genus of Salmonella plus S. enteritidis and S. typhimurium detected by qPCR. The yellow channel is for S. typhimurium.

Table 2: Show the prevalence of *salmonella* in chicken meat

Number of sample	Total *Salmonella spp detected*	Positive *S. enteritidis*	Positive *S. typhimurium*	*Salmonella spp*
110	13	(5 out of 13)	(4 out of 13)	(4 out of 13)

4. DUSCUSSION

Determining the prevalence of microorganisms in food is the initial stage in implementing techniques to prevent food poisoning. The study yielded a 12 % isolation rate of the *Salmonella* genus using both the culture technique and triplex PCR. Jamshidi *et al.* utilized the same procedure to isolate Salmonella from 11.66% of the samples. The contamination rate in other research demonstrated a prevalence of 33% (124 out of 376 samples) in meat chicken and the beef meat samples from market shops in Tehran (Dallal *et al.*, 2010). Similarly, in England, 25% (60 out of 241 samples) of complete raw chicken samples were found to be contaminated (Jørgensen et al., 2002). In the USA, the contamination rate was observed to be 3% in poultry carcasses (Zhao *et al.*, 2001). In Duhok region , Ahmed and his team in 2023 found around 68%of *Salmonella spp* in poultry carcasses.

The majority of *Salmonella enterica* infections in Iran occur in poultry, with serovar *S. enteritidis* being detected in

51.4% (35/68) of the samples (Zahraei-Salehi *et al*., 2005). Sixty percent of salmonellosis cases in Europeans and the majority of cases globally are caused by *Salmonella enterica, serovar Enteritidis* (Thorns, 2000). The majority of cases of Salmonellosis in the US are caused by *S. typhimurium* (Afshari et al., 2018).

The disparity between the findings of this study and those of previous research may be attributed to variables such as adherence to rigorous manufacturing standards and the conducting of Hazard Analysis and Critical Control Points (HACCP) during the slaughter process. Additionally, the specific stage of the process chosen for sampling, the time of year when the slaughter occurred, and the methodology employed for sampling and culturing could also contribute to the observed differences (Yin *et al.*, 2016).

A previous study revealed that the lowest rate of Salmonella spp. contamination (6%) in slaughtered birds occurred after stunning, whereas the greatest contamination rate (52%) was observed prior chilling (Yin *et al.*, 2016).

The main serovars used to manage risk factors of salmonellosis are *S. enteritidis* and *S. typhimurium*. These serovars can be transmitted from parent hens to their offspring at the hatchery (Heyndrickx *et al*., 2002). The findings indicated that *S. enteritidis* was present in 38% (5/13) of the samples, while *S. typhimurium* was detected in 30% (4/13) of the samples. The other isolates (35%) likely belonged to serovars that were not assessed in this study. According to a previous report, the prevalence of *S. enteritidis* contamination in poultry carcasses was found to be 1.8% (Ulloa *et al.*, 2010).

Previous research revealed that 8.2% of the 1154 chicken meat samples and 12.8% of the chicken piece samples were contaminated (Alexandre *et al.*, 2000). The most common serotype found was *S. enteritidis*. In another study performed in Mashhad-Iran, documented that the prevalence of *S. typhimurium* contamination in poultry carcasses was 8.2% and 6.10% (Jamshidi *et al.*, 2009). The serological type *S. enteritidis* was shown to be the most prevalent in a study done by Mikołajczyk and Radkowski (Zhao *et al.*, 2001). De Freitas and colleagues (de Freitas et al., 2010) detected the presence of *S. enteritidis* in 1.37% of poultry samples in Brazil using multiplex polymerase chain reaction (triplex PCR).

Currently, molecular approaches are highly accurate and sensitive in detecting and identifying *Salmonella* spp, particularly when dealing with a large number of samples (Malorny *et al.*, 2007). Additionally, it has been noted that employing two procedures can substantially enhance the identification of isolates, as PCR is unable to differentiate between deceased and viable cells. Therefore, it is recommended to utilize PCR in conjunction with microbiological assays. In this investigation, both the classical methodology (enrichment broth, selective media, and biochemical tests) and the molecular technology were employed for detection.

Another study found that *Salmonella* was detected in 32 samples (16%) using standard culture methods and in 38 samples (19%) using the PCR approach. When the data from both methods were merged, the identification of the pathogen climbed to 45 out of the 198 samples (23%) (Whyte *et al*., 2002). On the other hand, a further finding was done on the isolation of Salmonella species in poultry products, revealing that 4.4% were contaminated based on traditional culturing methods and 1.8% when assessed using qPCR (Ameen *et al*., 2016).

CONCLUSION

Ultimately, the findings of this study suggest that imported frozen chicken possesses the potential to transmit Salmonella spp. infections to individuals. Consequently, it is imperative for the poultry sector to prioritize the implementation of stringent control measures aimed at minimizing the transmission of infections during production operations.

ACKNOWLEDGEMENTS

The author would like to express their gratitude for the support provided by the Central Laboratory of Ibrahim Khalil Border in facilitating and contributing to this work.

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