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Isolation and identification of salmonella spp from beef and chicken meat by polymerase chain reaction test as well as studying the purity and specific genetic virulence gene type of this bacteria

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Abstract
A Study was carried out to identify 10 isolation of salmonella spp previously detected from beef and chicken meats by using pcr and real time pcr as well as 5 isolation were used for sequencening and specific genetics virulence gene type of salmonella spp . our results revealed that 10 isolation (100%) were identified on salmonella Spp by conventional Pcr . our finding also found out 5 isolation which were subjected to the detection of inva by using Rt -Pcr were reported salmonella enterica concerning sequencing and sequence analysis of salmonella 2 beef isolation (out of 2 examined) were reported as S. Agona, which 3 chicken isolates (out of 3 examined) were reported S. Infantis.

Keywords: salmonellosis, InvA. Kerbela

Introduction

Foodborne illness remains a global public health concern. In Canada, foodborne pathogens cause an estimated 4 million cases of gastrointestinal illness annually from known and unspecified agents and serotypes of Campylobacter, Escherichia coli and Salmonella are commonly implicated. (Smith *et al*., 2019). The presence of food pathogens such as non-typhoidal Salmonella (NTS) in meat is also a concern, since this organism is a major cause of food-borne gastroenteritis worldwide (Nhung *et al*., 2010). Meat production is central to livelihoods in many countries, with meat from livestock and poultry being a key protein source in subsistence communities (OECD/FAO, 2016). In many low-resource settings, industrialisation, urbanisation, and the shift from planned to market economies are leading to rapid changes in the way that food is produced, distributed, sold, and consumed (Carron *et al*., 2018; Grace, 2017) [4]. Such market-driven changes within agricultural production towards wider distribution networks, centralised processing, larger-scale and more intensive systems, have been linked to the emergence of zoonotic diseases (Jones *et al*., 2013) [14]. A number of molecular methods for the detection of Salmonella enterica in clinical and food samples have been developed including immunoassays, nucleic acid hybridization, and polymerase chain reaction techniques (X. Li *et al*., 2000). In vitro amplification of DNA by the polymerase chain reaction (PCR) has become potential of a powerful alternative in microbiological diagnostics due to its promptness and accuracy, PCR technique has been widely applied for the detection of food- borne pathogens such as Salmonella enterica, Staphylococcus aureus, Bacillus cereus and other pathogens because of its high specificity and sensitivity, fast response, and low cost. However, single PCR can only detect a single pathogen at a time (Tao *et al*., 2020). Another molecular approach is the Real-time fluorescence quantitative PCR, which has a detection flux of less than six due to the limitation of fluorescence detection channels. These methods are still difficult to meet the needs of non-directional screening of food-borne pathogens. Multiplex PCR, a method which can detect multiple targets in a single reactor tube and another type of PCR technique has been also used (Tao *et al*., 2020). However, due to lack of international validation and standard protocols, as well as, variable quality of reagents and equipment, the methodology has difficulties to move from expert to the end user laboratories. For example, many PCR-based methods published for the detection of Salmonella differ in specificity, detection limit and sample treatment (Malorny *et al*., 2003). Recently, many DNA-based techniques have been developed for the differentiation of pathogenic bacteria including Salmonella enterica. Enterobacterial repetitive intergenic consensus sequence (ERIC) polymerase chain reaction (PCR), PCR-ribotyping and pulsed-field gel electrophoresis (PFGE) techniques have been extensively used for detection of genetic lineages among Salmonella serovars (Kumar *et al*., 2009), PCR has been the most reliable and precise method to rapid, sensitive, and specific detection of Salmonella from poultry samples within a relatively short time. Several PCR assays have been conducted by targeting various Salmonella genes, such as invA, 16S rRNA, agfA, viaB, hilA, sirA, ttr, virulence-associated plasmids (Halatsi *et al*., 2006), and evaluate the nucleotide diversity of the ISRs in rRNA operons (rrnH) for the assignment of Salmonella serotypes (Kipper *et al*., 2019). Another advantage of the molecular based techniques, they are not dependent on the utilization of a substrate or the expression of antigens, thereby circumventing the phenotypic variations in biochemical patterns and lack of detectable antigens (Mirhosseini *et al*., 2009. [20]

The Aims of the study

1. Illustration the diversity Salmonella genotypes that colonizing in local and imported beef, and chickens meat as well as their products by sequencing of a Salmonella spp,
2. Conducting a molecular study by using PCR for rapid isolation and identification of the bacteria

3. Study the purity (sequencing) and specific genetic virulence gene type of this bacteria.

Materials and Methods

1-DNA amplification materials

A: DNA polymerase and molecular weight marker

Table 1: DNA Extraction materials

DNA amplification materials and Content		
1. DNA Extraction Kit (G-spin ™ Genomic DNA) / Intron, Korea		
G-buffer	Pre buffer	Washing buffer A
Washing buffer B	Binding buffer	Elution buffer
Lysozyme powder	Ribonuclease A powder	Proteinase K powder
2. GoTaq® G2 Green Master Mix, 2X / Promega, USA		
Taq DNA polymerase	$MgCl_2$	dNTPs
Reaction buffer	DNA Polymerase,	(blue and yellow) loading dyes
3. SiZer-100 DNA Marker Solution / Intron, Korea		
100 to 1,500 bp		

B: Conventional PCR primers

Table 2: Primers used for the detection of *Salmonella* isolates, Integrated DNA Technologies, USA.

Primer		Sequence 5'-3'	Amplicon size	References
ITR 1– 2 NF	F	GTGAAATTATCGCCACGTTCGGGCAA	ranged 284bp	(Sunar *et al*. 2014)
	R	TCATCGCACCGTCAAAGGAACC		

Methods

Study design and Specimens collection

A Cross-sectional study was performed to collect a total of 160 samples, for searching about *Salmonella enterica spp*. in beef and chickens . These samples were gathered from different locations in Kerbala province and cultured in appropriate media according to internationally known protocols for bacterial cultivation and identification (MacFaddin 2000). Then it followed by the initial bacterial isolation process on the special and distinctive culture media of salmonella such as SS agar and the followed propagation (NHS 2017) [24]

The nucleic acid materials of the germ were then collected, in order to target a specific genomic gene of the *Salmonella enterica* genome (invA gene) to be amplified. The whole DNA was extracted from isolated bacteria, then the PCR products were subjected to gel electrophoresis, DNA bands were illuminated using a gel documentation system (Salm-surv and June 2010). The sample isolated amplicons were sent to MACROGEN® for sequencing using the Sanger sequencer. The resulted sequences were cleaned and quality trimmed using quality threshold of more than 20. High quality sequences (reverse or forward) of nucleotides were identified and compared with archives available in the National Center for Biotechnology Information (NCBI) and classified through the use of bioinformatics algorithms and programs specified for this type of analysis, these steps of isolation and identification of *Salmonella enterica* . (HPA, 2008) [12, 13].

Results

Molecular identification of *Salmonella*

1. **Polymerase chain reaction results of *Salmonella* isolates**

All the 10 isolates were subjected to the conventional PCR by using specific primers with amplicon size ranged from 284bp. The electrophoresis results are demonstrated in Figure (4-4).

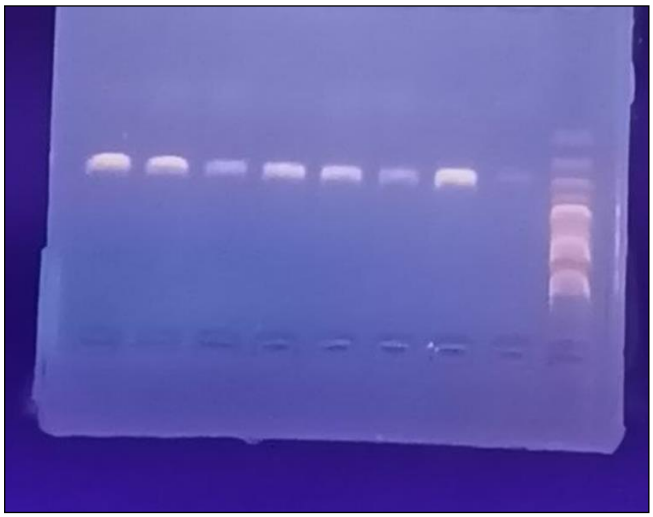

Fig 2: Gel electrophoresis results of *Salmonella* isolated from beef and Chicken.

2. **Real Time PCR results of *Salmonella* isolates.**

The next step was the real time identification method for the final diagnosis of five PCR diagnosed Salmonella isolates, as a final step before the sequencing; all five isolates was subjected to the detection of invA by using RT-PCR, the figure (4-5) demonstrate the cycle threshold of the invA amplification result.

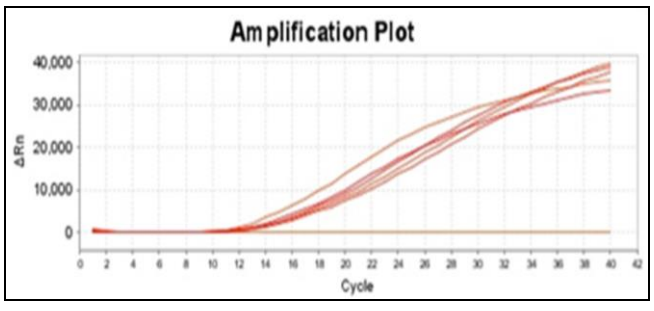

Fig 3: cycle threshold of RT-PCR technique.

3. Sequencing and sequence analysis of *Salmonella* isolates

Among the 54 positive isolates of the *Salmonella* genus five isolates selected for sequencing, only two serotypes were identified by using DNA based sequence search. All sequences of the isolates were submitted to the BLASTn search tool of National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov). The two strains *S.* Infantis (3) isolates and *S.* Agona (2) isolates were identified. The significance was measured at P value level ($p<0.01$ and $p<0.05$).

4. Characterization of *Salmonella* serotypes.

Two strains were detected among the 5 isolates of the *Salmonella* genus that isolated from red meat and chicken. The BLASTn search hits to NCBI records were as mentioned in Table (3).

Table 3: NCBI database search results using BLASTn search tool.

Number	Scientific Name	No. of isolates	Percent Identity
1.	*Salmonella* enterica subsp. enterica serovar Infantis strain	1	100%
2.	*Salmonella* enterica subsp. enterica serovar Infantis strain	1	99.05%
3.	*Salmonella* enterica subsp. enterica serovar Infantis strain	1	98.75%
4.	*Salmonella* enterica subsp. enterica serovar Agona	2	100%

Discussion

Polymerase chain reaction result of salmonella isolates

The invA gene was chosen in current study because it involves sequences distinctive to the genus Salmonella, and has been agreed as an appropriate target for the polymerase chain reaction (PCR) with diagnostic capability (Shanmugasamy *et al*,. 2011). This gene encodes for a protein found in the inner membrane of bacteria and is responsible for the invasion of the host's epithelial cells (Wang *et al*. 2009). This gene is also necessary for complete virulence of Salmonella, and is believed to initiate the internalization needed for deeper tissue invasion (Oladapo *et al*. 2013). Furthermore, the use of the invA gene can significantly reduce the false-negative reports faced by most laboratories (Salehi *et al*,. 2005). Accordingly, rapid detection of Salmonella spp. by PCR targeting the invA gene is recommended (Ifeanyi Smith 2015).

In the current study, the optimization trial for PCR showed successful amplification for the specific invA gene at different annealing temperatures. However, the temperature of 55°C was used later in the next experiments as it showed slightly sharp bands (on the agarose gel) than the other temperatures. The findings of the current study were in accordance with (Sunar *et al*. 2014) who found (100%) *Salmonella* isolate carrying this gene among 25 samples.

Real Time PCR results of *Salmonella* isolates.

5 isolates were confirmed as Salmonella based on RT-PCR figure (4-5). The same level of accuracy was observed in the previous reports (Monteiro *et al*. 2016) [21]. In a recent study, the identification of S. enterica by RT-PCR was considered rapid but high expensive compared with conventional methods (Kasturi, 2020). The current data are in accordance with a report presented by (González-Escalona *et al*. 2009) [9], stating that RT-PCR gives accurate detection of S. enterica and saves time with high efficiency, making it an ideal method of diagnosis (Salman *et al*., 2021).

Many animals can be infected with non-typhoidal *Salmonella* with public health concerns since the ingestion, colonization, and shedding events typically cause no harm to the animals and *Salmonella* that is ubiquitous in the environment. *Salmonella* can therefore contaminate poultry meat (from fecal shedding) or during processing from intestinal leakage and meat slicer, resulting in one of the leading causes of *Salmonella* infections in humans (Najmin *et al*. 2018) [22].

Sequencing of salmonella isolates

Despite the serious clinical consequences recently caused by *S.* Agona there is a lack of information about the prevalence of these to *Salmonella* or the antimicrobial resistance profile of these serotypes in Iraq. Generally, according to our knowledge, this is the first report of *S.* Agona serotype isolated from minced meat-derived samples in Iraq.

The results of the current study showed that the prevalence of *S. Agona* was 40% of the isolates, this result was in accordance with (Modarressi and Thong, 2010) in Malaysia that reported 40% of the isolated strain were *S.* Agona from meat samples. In Senegal, (*et al*. 2021) reported (4%) among broiler samples that found a lower prevalence rate of *S.* Agona.

These findings supported that *S.* Infantis was the predominant strain among the samples of the current study, this distribution may be due to inhibition of this serovar the colonization of the other *Salmonella* serovars, and probably its ability to resist antibiotics. Generally, the results of the current study revealed that the prevalence rate of *S.* Infantis isolates was higher than *S.* Agona among the poultry and meat derived samples, in fact, the differences in the rates can be explained by several causes such as antimicrobial resistance are enzymes designated as "extended-spectrum beta-lactamases" (ESBL) and "ampC beta-lactamases" (AmpC). The bacteria require certain "resistance genes" to produce these enzymes, these genetic properties can be passed on from one bacterial generation to the next through cell division during propagation. However, it can also be passed on from one bacterial cell to another on transmissible gene sections such as plasmids (Franco *et al*. 2015) [8].

This study shed light on an important finding, which is the increasing rate of isolated non-typhoidal *Salmonella* with a public health concern. .Sequence similarity searching is a very important bioinformatics task, the Salmonella genome size ranged from 4.6 to 5.1 mega bases (Mbs), and it is organized into several operons (Dhanani *et al*., 2015). For this purpose many softwares have been used for nucleotide editing and sequence alignment such as SnapGene software (www.snapgene.com), and BLAST of NCBI (blast.ncbi.nlm.nih.gov/Blast.cgi) and many more**.** Basic Local Alignment Search Tool (BLAST) is one of the most popular bioinformatics tools, it is a sequence similarity search program that can be used to quickly search a sequence database for matches to a query sequence. Several variants of BLAST have been existed to compare all combinations of nucleotide or protein queries (is called BLASTp) against a nucleotide or protein database (Camacho *et al*., 2009) [3]; (*Hashim et al*,2020) [16, 18].

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