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Molecular genotyping of *Salmonella* spp. isolated from cheese samples of local stores in Al-Diwaniyah city, Iraq

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Abstract

Background: Food safety is an important subject that the global cheese industry increases awareness of. This urges these economic sectors to elevate the level of research to minimize cheese contamination with pathogenic bacteria, such as *Salmonella*.

Aim: Based on these merits, this study was conducted to genotype *Salmonella* spp. isolated from cheese samples of local stores in Al-Diwaniyah City, Iraq.

Methods: The study used 41 samples of local fresh unsalted white cheese in a selective-growth-based isolation of *Salmonella*. These isolates were confirmed utilizing a slide-agglutination (SA) test and VITEK® 2 system (V2S). Then, the isolates were subjected to conventional PCR and sequencing techniques that both targeted the *16S rRNA* gene. For subtyping, the *Salmonella* isolates were subjected to a random amplified polymorphic DNA (RAPD)-PCR method.

Results: The results of both SA and V2S revealed the presence of 14 (34.2%) isolates of *Salmonella* spp. in the cheese samples. The PCR confirmed 6 (42.9%) of these isolates, which further were defined with close nucleotide similarity (98.03%) and (97.88%) to different world isolates, such as *Salmonella enterica* subsp. *Arizonae* and *Salmonella enterica* subsp. *enterica serovar Typhi*, respectively. The RAPD-PCR findings showed different fragments for all the tested isolates.

Conclusion: The present study indicates that the samples of the local fresh unsalted white cheese contain different *Salmonella* genotypes, which could be originated from different contamination sources.

Keywords: Cheese, Foodborne pathogens, RAPD-PCR, *Salmonella*, VITEK® 2.

Introduction

Foodborne diseases (FBDs) are a major cause of illness and death across all demographics and a barrier to progress in both the economic and social sectors worldwide. The World Health Organization (WHO) reported in 2010 that foodborne illness was responsible for 600 million cases. In most cases, foodborne illnesses are brought on by either pathogens, including bacterial, viral, parasitic, fungal, mycotoxin, and prion-based infections, or by environmental elements, such as contamination throughout the manufacturing, preparation, transportation, and storing stages. Bacterial pathogens are the leading cause of FBDs globally, with *Salmonella*, *Vibrio parahaemolyticus*, *Listeria monocytogenes*, *Staphylococcus aureus*, and a few others being the most common and significant (Kadariya *et al.*, 2014; Torgerson *et al.*, 2015; Martinović *et al.*, 2016; Bintsis 2017; Jordan and McAuliffe 2018; Faour-Klingbeil and Todd 2020; Gallo *et al.*, 2020; Dutta *et al.*, 2021).

Salmonella, a key bacterium linked to FBDs, is often found in raw poultry, raw milk, raw meat, raw eggs, and other products. *Salmonella* is responsible for anything from 200 million to over a billion infections every year, resulting in 93 million incidents of gastrointestinal tract infections and diseases and 155,000 fatalities. These food items could become contaminated during production via cross-contamination. *Salmonella* thrived in meat, poultry, and dairy products owing to the abundance of nutrients and water in these foods (Whiley and Ross 2015; Hung *et al.*, 2017; Kore *et al.*, 2017; Yeh *et al.*, 2017; Milczarek *et al.*, 2019).

In addition, food items, including fruits and vegetables contaminated by livestock fecal microorganisms, may serve as a cultivation environment for *Salmonella*. *Salmonella* causes flu-like symptoms in humans, including nausea, vomiting, stomach discomfort, and diarrhea. There are now over 2,500 known serotypes of *Salmonella*, with some countries having over 200 of them, such as in China. Between 1996 and 2014, the

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three most prevalent serotypes documented by the CDC in the United States were *S. typhimurium*, *S. enteritidis*, and *S. newport* (Crump *et al.*, 2015; Chlebicz and Śliżewska, 2018; Powell *et al.*, 2018; Sun *et al.*, 2021; Wessels *et al.*, 2021).

The purpose of FBD tracking is to keep an eye on food contamination and dangerous components so that we can lessen the number of sick people caused by eating contaminated food. Twenty distinct kinds of FBD surveillance systems exist now, covering event-based, indicator-based, and integrated monitoring of the whole food supply chain. *Salmonella* has been shown to live for a long time in low-moisture foods. Bacterial pathogens may spread during cheese production, ripening, and storage as a result of direct or indirect contamination circumstances that take place in commercial, household, and retail settings. Although raw milk is generally thought to be the most common cause of cheese contamination, cross-contamination during manufacturing has been linked to the likelihood of bacteria building biofilms and surviving on food surfaces (André *et al.*, 2008; Kousta *et al.*, 2010; Podolak *et al.*, 2010; Tiwari *et al.*, 2014; Ford *et al.*, 2015; Schön *et al.*, 2016; Jordan *et al.*, 2018; Chen *et al.*, 2022).

Food safety is an important subject that the global cheese industry increases awareness of. This urges these economic sectors to elevate the level of research to minimize cheese contamination with pathogenic bacteria, such as *Salmonella*. Based on these merits, this study was conducted to genotype *Salmonella* spp. isolated from cheese samples of local stores in Al-Diwaniyah City, Iraq.

Materials and Methods

Bacterial isolation and identification

A total of 41 samples of local Fresh unsalted white cheese were collected randomly from local stores in Al-Diwaniyah City, Iraq, during the period from October 2022 to February 2023. The samples were directly cool-transferred to the Laboratory of Public Health, College of Veterinary Medicine, University of Al-Qadisiyah, Al-Diwaniyah City, and analyzed immediately without storage. In brief, cheese samples at 25 g/each were 225 ml-buffered peptone-suspended and incubated at 37˚C for 24 hours for pre-enrichment cultivation. Later, 1 ml of the pre-enrichment broth was utilized for selective enrichment cultivation in 10 ml tetrathionate broth and incubated at 42˚C for 24 hours. After that, 1 loop-full of the tetrathionate broth was placed onto *Salmonella shigella* agar and Brilliant Green Agar and incubated at 37˚C for 24 hours. Following that, the suspected *Salmonella* colonies were transferred to tubes that contained Kligler and Urea agar base and incubated at 37˚C for 24 hours. SA and V2S tests were performed.

Molecular identification of Salmonella isolates

DNA extraction

The DNA of the bacterial isolates was performed using the Wizard Genomic DNA Purification Kit (Promega, USA). The protocol of the kit was employed to complete the process. In short, 1 ml of the BHI broth (2 minutes–13,000 rpm centrifugation) pellet was used as a starting material. The resulting DNA was measured for its quality and quantity using a NanoDrop. The DNA was −20˚C-stored until further analyses.

Conventional PCR

The PCR was conducted using "TACGGYTACCTTGTT-ACGACTT" and "AGAGTTTGATCMTGGCTCAG" primers. At 25 µl of the total volume of the PCR reaction, the following components were inserted as: 12.5 µl (1×) master mix, 1 µl (1 µM) of each primer, 3 µl DNA, 7.5 µl water for molecular use. The following reads for a thermocycler were used; initial denaturation (denaturation, annealing, and extension) and final extension at 95˚C, (95˚C, 60˚C, and 72˚C), and 72˚C, respectively, for 5 minutes (30, 30, and 60 seconds), and 7 minutes, respectively, under 1, 30, and 1 cycle, respectively. The PCR-1%-agarose gel electrophoresis was done as described by Green and Sambrook (2019). The run was under 80–90 V for 60 minutes. The gel was visualized using a UV-light imager.

RAPD-PCR

The PCR was conducted using the OPB primer (Shekhawat *et al.*, 2019) "CGT CTG GGA C." At 50 µl of the total volume of the PCR reaction, the following components were inserted; 25 µl (10×) (EconoTaq® PULS GREEN 2X Master Mix (Lucigen), 0.5 µl primer, 1 µl DNA, and 23.5 µl water for molecular use. The following reads for a thermocycler were used; initial denaturation (denaturation, annealing, and extension) and final extension at 94˚C (94˚C, 35˚C, and 72˚C), and 72˚C, respectively, for 5 minutess (60, 60, and 120 seconds) and 7 minutes, respectively, under 1, 45, and 1 cycle, respectively. The PCR solution at 10 µl/each was mixed with 2 µl (6×) loading dye (Thermo Fisher Scientific, USA). The gel was visualized using

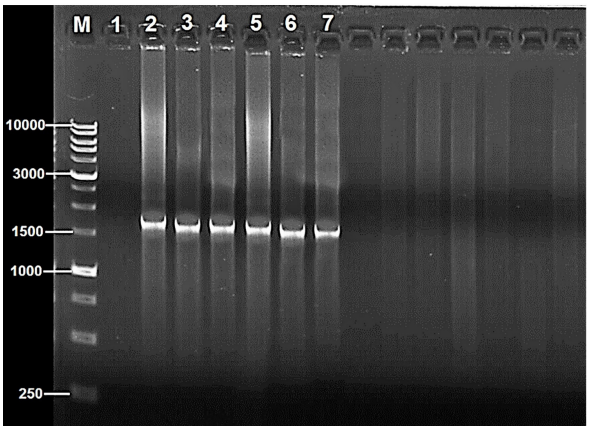

Fig. 1. PCR amplification based on *16S rRNA* gene of 14 *Salmonella* bacterial isolates from local fresh white cheese on 1% agarose gel stained with ethidium bromide, M: 1K-ladder; 1: negative control, and 2–7: positive identification.

Table 1. Comparison between the nucleotide sequences of current study *Salmonella* isolates with GenBank sequences (BLAST software).

Isolate code	Names of GenBank isolates	Accession No.	Source	Similarity %
CD1	*Salmonella enterica* subsp. *enterica serovar Enteritidis*	OQ254756	Cheese	97.50%
CD2	*Salmonella enterica* subsp. *enterica serovar Typhimurium*	OQ254757	Cheese	96.78%
CD3	*Salmonella enterica* subsp. *enterica serovar Typhi*	OQ254758	Cheese	97.88%
CD4	*Salmonella enterica* subsp. *enterica serovar Typhimurium*	OQ254759	Cheese	97.34%
CD5	*Salmonella enterica* subsp. *salamae*	OQ254760	Cheese	96.71%
CD6	*Salmonella enterica* subsp. *enterica*	OQ254761	Cheese	97.59%
CD7	*Salmonella enterica* subsp. *arizonae*	OQ254762	Cheese	98.03%

a UV-light imager, and the images were analyzed by recruiting ImageJ software (version 1.8.0_112).

DNA sequencing

The DNA from the PCR was sent to sequencing using the Sanger ABI3730XL system (Macrogen Inc., Korea). The phylogenic study and tree were computed using NCBI websites and MEGA X software.

Results

The results of both SA and V2S revealed the presence of 14 (34.2%) isolates of *Salmonella* spp. in the cheese samples. The PCR confirmed 6 (42.9%) of these isolates by amplifying the *16S rRNA* gene piece at 1,500 bp (Fig. 1).

The sequencing defined nucleotide similarity of (98.03%) and (97.88%) with different world isolates, such as *Salmonella enterica* subsp. *Arizonae* and *Salmonella enterica* subsp. *enterica serovar Typhi*, respectively. Table 1 and Figure 2 show details about the close identity of global isolates of *Salmonella* spp. The RAPD-PCR findings showed different fragments for all the tested isolates.

The RAPD-PCR findings showed different fragments for all the tested isolates. Some isolates showed universal and distinct bands (Fig. 3).

Discussion

The present study revealed that the local Iraqi white cheese has different *Salmonella* isolates with different subtypes. Sanitize industrial machinery, microorganism-free technological water, and sanitary conditions during packing and storage at the distributor are all necessary for a safe and effective manufacturing process. Cheese is a fantastic medium for bacterial development because of its contents of protein, lactose, and water content. Thus, the short shelf life

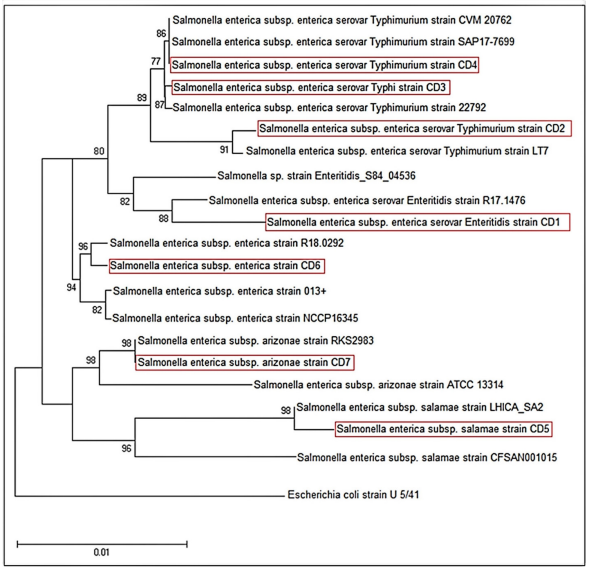

Fig. 2. Phylogenetic tree based on *16S rRNA* gene of 7 *Salmonella* bacterial isolates from local fresh white cheese. Neighbor-joining method.

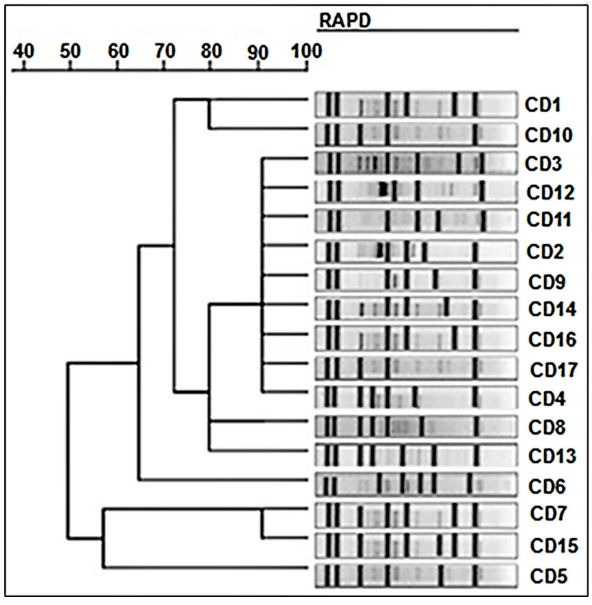

Fig. 3. RAPD-PCR-based dendrogram of subtyping for *Salmonella* bacterial isolates from local fresh white cheese. Average linkage unweighted group pair method with arithmetic averages (UPGMA).

of local cheeses may be due in part to bacteria in milk or arise from secondary contamination. An efficient cleaning process, as well as hygienic conditions for packing and storing the product, are essential for achieving the highest possible hygiene requirements (Schön *et al.*, 2016).

Inadequate sanitary practices during milking and on farms lead to the prevalence of coliform bacteria in raw milk. Furthermore, coliform bacteria may be used as a gauge of the sanitation of dairy processing facilities. The presence of coliform bacteria was identified in 45% of the examined samples in research by Lobacz and Zulewska (2021) that assessed the microbiological quality of tvarogs from 15 producers. All of the samples from the raw milk cheese tested positive for the presence of fecal bacteria, with an average starting count of 6 log cfu/g (Lobacz and Zulewska, 2021).

Given that *S. enteritidis* may survive in the products for many months, *Salmonella* infection in sheep poses a genuine concern to public health. Sheep milk is often not pasteurized before cheese manufacture. Raw milk cheese was shown to be the origin of infection after a microbiological and environmental examination, as well as molecular typing of *S. enteritidis* isolates (Doosti *et al.*, 2017; Napoleoni *et al.*, 2021).

Our sequencing results indicated close identity with global isolates of *Salmonella* spp. Many facts can play a role in introducing new base pairs to local bacterial species. One of the important factors is the travel and import of animals from endemic areas and countries. This process can bring in a novel bacterial species, and with the presence of different genetic tools, such as horizontal gene transfer, a new bacterial sequence can be produced. Then, the newly produced bacterial isolates can show close nucleotide similarity with global sequences, which can hit 98% similarity (Wang *et al.*, 2017; Bokhary *et al.*, 2021; Sridhar *et al.*, 2021).

All of the *Salmonella* spp. isolates examined were able to be fingerprinted using RAPD-PCR. *Salmonella* isolates clustered into seven distinct RAPD-PCR types in a dendrogram constructed using the RAPD-PCR technique. Dendrogram fingerprint analysis revealed four distinct fingerprint types among all *Salmonella* isolates, clustering them into seven distinct clonal groupings. The *Salmonella* isolates showed average genetic similarity. Some isolates revealed similar bands, which might indicate a conserved sequence between isolates. The current study results agree with those of Shekhawat *et al*. (2019), who identified different subtypes of four *Salmonella* isolates from different foods. Shekhawat *et al*. (2019) found that *Salmonella gallinarum* isolates from chickens had the most genetic diversity (Shekhawat *et al.*, 2019).

It is important to notice that detecting pathogenic bacteria in cheese samples brings high attention to public health concerns. This is true since these bacteria may carry antibiotic-resistance genes in their genomes that can be transmitted between the same bacterial species or to other bacterial species via genetic carriers, such as plasmids. Chahouri *et al.* (2022) studied the presence of fecal bacteria and other bacterial organisms and their antibiotic resistance pattern in marine and river ecosystems. The authors detected the presence of *Salmonella* spp. in their samples and were susceptible to all antibiotics tested except amoxicillin+Ac clavulanic, ampicillin, and chloramphenicol (Chahouri *et al*., 2022). However, this indicates that the current study isolates may carry the same pattern or with a broader range of antibiotic resistance, which makes cheese as one of the main sources of this health risk.

The present study indicates that the samples of the local fresh unsalted white cheese contain different *Salmonella* genotypes, which could have originated from different contamination sources.

Acknowledgments

None.

Conflict of interest

All authors of the current article confirm that this work has no conflict of interest.

Authors contributions

All authors participated in the sample, Lab work, data analysis, and writing, revising, and approving the manuscript.

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Data availability

Data of the current study are available upon request.

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