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Original Article

Detection of *Salmonella* spp. by Traditional and PCR Assays in Raw Milk, Maysan, Iraq

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Abstract

Salmonella spp are characterized as rod- shaped, motile, gram- negative bacteria which has the ability to infect animals and human. *Salmonella spp* occasionally causes sickness while in most cases not lead to severe symptoms. Analyzing milk for *Salmonella* spp. is not routine but traditional culture methods are used to evaluate the health condition of the dairy products. However, the antibody-based and nucleic-acid- based methods are practical for identifying *Salmonella* spp. Therefore, this research was designed to evaluate the use of traditional culture methods and PCR in detection of the presence of *Salmonella* spp. in raw milk samples in, Maysan Iraq. A total number of 130 raw milk samples collected from Maysan Iraq. All the samples were analyzed for the presence of *Salmonella* spp. using traditional culture method and polymerase chain reaction (PCR). The culture method used in this experiment were done by using pre-enrichment, enrichment, selective plating and biochemical tests. The results of this traditional technique were compared with the results obtained from PCR method. The PCR was performed using a 284bp sequence of the *invA* gene. The results showed that 8 (7.07%) of samples were identified as *salmonella* positive using traditional culture technique but 14 (12.3%) samples were detected as *salmonella* positive by PCR method. The results of the current research revealed that the traditional culture based methods are generally time costuming and labor intensive but the development of new rapid methods including DNA based methods such as PCR are more sensitive and have dramatically decreased the time necessary for the detection of bacteria.

Keywords: PCR**,** biochemical tests, raw milk**,** *Salmonella* spp.

1. Introduction

Salmonella spp. are characterized as rod- shaped, motile, gram- negative bacteria which has the ability to infect animals and human. *Salmonella* spp. occasionally causes sickness while in most cases not lead to severe symptoms (1, 2). It has been well documented that the most common reported causes of foodborne sickness are caused by *Salmonella* spp. (3). More than half of the Salmonella infections occurred due to the utilization of non-pasteurized and contaminated animal products such

as dairy and poultry products (4). The source of bacteria in raw milk include, feces, teat colonization or mastitis infection bedding (4), cross contamination from the milking staff and auto-milker (5).

Consumption of non-pasteurized milk or dairy products is the common amount the rural and urban populations due to some cultural reasons or for perceived health benefits of natural and unprocessed food (4, 6). About the consumption of the raw and non-pasteurized dairy products consequently caused foodborne disease, there have been limited epidemiologic published data. However, it has been accepted that some factors like farm management procedure, season, number of animals on the farm, geographical area, and hygiene significantly affect the prevalence rate of *Salmonella* spp. (7, 8).

Analyzing milk for *Salmonella* spp. is not routine but traditional culture methods are used to evaluate the health condition of the dairy products. However, the antibody-based and nucleic-acid- based methods are practical for identifying *Salmonella* spp. (5). Conventional culture methods for the identification of salmonella spp. are generally laboratory-based, time-consuming, and labor-intensive (9, 10). However PCR targeting specific genetic markers represents a major advance in term of speed, specificity and sensitivity to improve food safety (9, 10).

Therefore, this research was designed to evaluate the use of traditional culture methods and PCR in detection of the presence of *Salmonella* spp. in raw milk samples in, Maysan, Iraq.

2. Materials and Methods

2.1. Sample Collection

In the first step the city of Maysan was divided into 10 region, then using cluster sampling method 130 raw milk samples were collected. All the collected samples were transported to the laboratory at 4°C.

2.2. Traditional Culture Method

The milk samples were inoculated in to selective enrichment broths. To do this a milk sample of 25 ml was added directly to 225 ml of Selenite Cystine broth and Tetrathionate Broth then incubated at 43°C and 37°C, respectively, for 18-24 h. Selective enrichment cultures were subcultured on to Brilliant Green Agar and Xylose Lysine Decarboxylase Agar. Following a period of 18-24h at 37°C, up to three identified colonies were subcultured on Nutrient Agar at 37°C for 18-24h. Following the incubation completion confirmation was performed using biochemical tests, including TSI, urease, and LI agar (11).

2.3. PCR Amplification

The DNA extraction was performed using a commercial kit (Diatom DNA Prep 100, Galart-Diagnosticum, Russia). The DNA samples were obtained from the enriched culture and extracted according to the manufactures instruction. Then the purified DNA samples were considered as a template for PCR tests. It has been previously mentioned that S139 and S141 primers are highly specific for the identification of the invA gene expressed in *Salmonella* spp. The primers sequence is tabulated in table 1. A 25µl amplification mixture composed of 2.5 µl 10x PCR buffer (500mM KCl, 200mM Tris HCl), 1.25µl dNTPs (10mM), 1.5µl $MgCl_2$ (50mM) was used to perform PCR reaction using these primers. Also, the volume of 0.5 µl of each primer, combined with 0.5µl of Taq DNA polymerase (Fermentase) and 2µl of extracted DNA from enriched cultured samples were mixed with an amplification mixture for performing the PCR assay. A gradient thermocycler (Bio Rad, icycler, USA) was used for performing the DNA template amplification. The PCR cycling program was as follows: an initial incubation occurred at 95 ºC for 5 min. The denaturation step was done at 35 cycles at 94°C for 60 sec, followed by an annealing procedure which takes place at 56º C for 30 sec, followed by elongation at 72°C for 30 sec, and final extension happened at 72ºC for a period of 10 min. For the final evaluation of the PCR products, the amplicons were electrophoresed in 1.2% agarose gel. A 100bp DNA ladder was used as a size reference. A gel documentation apparatus was used for gel documentation, and the staining procedure with ethidium bromide was performed prior to the gel documentation. In this study negative and positive controls were deionized distilled water (DDW), and *S. typhymurium* (ATCC: 25923), respectively (11).

Table 1. Sequence of oligonuclotid used as primers in the PCR assay

Primer	Sequence (5́-3́)	Target gene	Amplicon fragment (bp)	Reference: No
S139-F S141-R	GTG AAA TTA TCG CCA CGT TCG GGC AA TCA TCG CAC CGT CAA AGG AAC C	Inv A	284	(15)

3. Results and Discussion

Previously published research revealed that several illnesses are transmissible via the consumption of raw dairy products. It has been well established that these products are considered major vehicles for the transmission of pathogens (12, 13). Due to the high prevalence of Salmonellosis and pervasiveness of *Salmonella* spp. isolation and characterization of these food-transmitted pathogens has of great importance for the prevention and control of the disease prevalence (14). There have been introduced several different technology for detection of *Salmonella* spp. including traditional and novel culture technique (14), immunomagnetic separation, EIA and ELISA-based assay incorporating fluorescent and molecular techniques such as DNA hybridization and PCR-based assay. Almost all rapid test protocol need a selective enrichment stage (15). In this study we compared traditional culture method with PCR- based assay, to compare number of 130 raw milk samples from 10 different regions of the city of maysan were analyzed. The results of the current research showed that out of 130 raw collected milk samples from 10 dairy markets from different region of maysan by using traditional culture technique 9 (7.06%) samples were characterized as *Salmonella* positive, while with using PCR method 16 (12.4%) samples were identified as *Salmonella* – positive (Figure 1).

For Enterobacteriaceae, specially for *Salmonella* growth there have been at least 8 plate agar media commercially produced. Most of selective agar media for isolation of *Salmonella* spp. rely on one or both of two identifiers including lactose fermentation and hydrogen sulfide production, in combination with various concentrations of inhibitors (14). Bennett, Greenwood (11) reported that 5% of serotypes of *Salmonella* isolated from clinical samples did not produce hydrogen sulfide. The hydrogen sulfide production measurements combined with acid production tests from lactose are inadequate for the identification of Salmonella spp. from commensal bacteria. Also, the majority of the media do not easily yield colorless *Salmonella* colonies (non-lactose fermenting colonies). In a previously published study it is revealed that milk samples may carry several microorganisms that may compete with *Salmonella* in the enrichment medium. Therefore, the coexistence of *Salmonella* spp. with other residence bacterial in milk that some of them have very similar biochemical properties to those of *Salmonella* spp. may increase the false-positive tested samples or keeping the total number of *Salmonella* lower than the detection limit on culture plate (5).

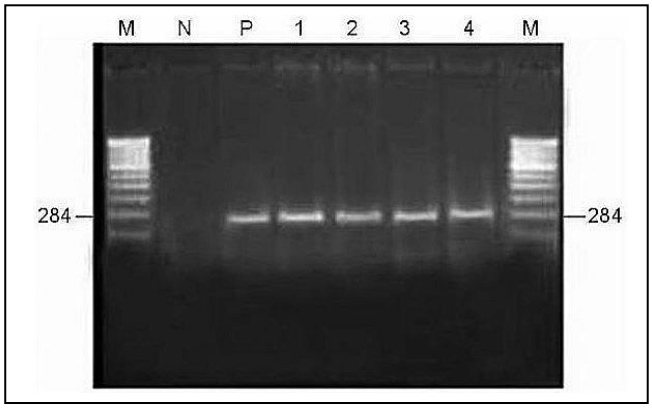

Figure 1. Agaros gel electrophoresis of PCR product of InvA gene of *Salmonella* spp.:Lane M ,100 bp Marker; Lane N: Negative control; Lane P:Positive control (S.Typhimurium: ATCC: 25923); Lane 1to4 *Salmonella* spp. isolated from raw

Timing plays a pivotal role in quality control in the processing of a food product, therefore any attempts to carry out routine monitoring must take into consideration. The development of new rapid techniques including DNA-based techniques in

identifying bacterial species has dramatically reduced the necessary time for the characterization of bacteria (5). Previously in a study for the validation and standardization of PCR for the detection of five major foodborne pathogens including *Salmonella* conducted by Chen the results showed that the most selective primer set was found to be 139-147, which targets the *invA* gene (15). These set of primers could identify all types of *Salmonellae* and showed no cross reactions with other organisms (1, 2).

The results of the previous published studies have shown a wide range of *Salmonella* contamination in raw milk (1, 3, 6). The results of a study conducted by Steele, McNAB (16) showed only 0.17% Salmonella positive samples from of 350 bulk tank. Kessel (5) found *Salmonella* spp. in 12% in Maryland, USA from 200 bulk tank milk samples. The results of a study conducted by Ekici, Bozkurt (17) revealed that from 36 dairy farms in Van, Turkey *Salmonella* spp. did not isolated in any of the samples. The findings of a study conducted by Jayarao and Henning (8) showed that out of 131 bulk tank milk sample 6.1% of the samples were *Salmonella* positive. In all the above mentioned studies enrichment culture techniques was used followed by isolation on selective agars.

Therefore we can conclude that raw milk contamination with *Salmonella* should not be considered a rare case. The results obtained from bacteriological quality control of raw milk in the current research showed relatively high contamination rate of *Salmonella*. The growth potential for Salmonella in improperly stored raw milk and in other dairy products, represents a serious public health risk, particularly for susceptible populations.

Authors' Contribution

Study concept and design: A. F. A. and M. S. A.
Acquisition of data: N. M. H.
Analysis and interpretation of data: H. D. S.
Drafting of the manuscript: D. A. H.
Critical revision of the manuscript for important intellectual content: S. H. A., S. H. and D. M. A.
Statistical analysis: H. A. H.
Administrative, technical, and material support: S. A. H. and D. M. A.

Ethics

All the procedures were approved by the ethics committee of the Al-Manara College for Medical Sciences, Maysan, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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