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Rapid and Simultaneous Identification of Two *Salmonella enterica* Serotypes, Enteritidis and Typhimurium from Chicken and Meat Products by Multiplex PCR

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Abstract: In order to overcome the large volume of work required to detect and identify food samples that may contain *Salmonella enterica* serotypes Enteritidis and Typhimurium, multiplex polymerase chain reaction (m-PCR) was used as a diagnostic tool to identify *Salmonella enterica*, *Salmonella enterica* ser. Enteritidis and Typhimurium in naturally contaminated meat and poultry products. Three sets of known *S. enterica* and serotypes *S. enteritidis* and *S. typhimurium* specific primers were applied to simultaneous identification of such pathogens in the most frequently used fresh and frozen meat (beef and lamb) and poultry (chicken) products (whole, cut, ground and processed) collected from eight locations within Irbid city (Jordan). Out of 93 samples, Only 21 (22.5%) samples contained serotype *S. typhimurium* alone. Nineteen samples (20%) showed the 312 bp specific band for serotype *S.* Enteritidis specific band. A total of 28 (30%) samples showed only for *S. enterica* genus and 25 (26.8%) samples showed both serotypes *S. enteritidis* and *S. typhimurium*. In general, 46 (49.5%) samples contained serotype *S.* Typhimurium, while 44 (47.3%) samples contained serotype *S. enteritidis*. Multiplex PCR amplification was shown to be an effective and rapid method for the simultaneous identification of such pathogens in food samples.

Key words: *Salmonella*, food products, identification, m-PCR

INTRODUCTION

Members of the genus *Salmonella* are gram-negative and facultative anaerobic, rod-shaped bacteria[1]. Salmonellosis is a major economic problem for the food industry and a public health hazard in many countries[2-5] and also associated with medium to severe morbidity and even mortality in farm-animals and thus represents a major economic and productivity loss in the food and animal industries[6]. *Salmonella*-infected samples must be quickly identified so that they can be isolated and spread of contamination can be controlled[7,8].

A more rapid method for detection of *S. enterica* from food or environmental samples could facilitate bio-security procedures and minimize outbreaks in these environments. Prompt identification of *S. enterica* from such samples is a challenge to laboratory diagnosticians due to the lengthy time required to culture and identify this bacterium by conventional culture methods. Because the average time between acquisition of a sample and definitive identification of a *Salmonella* isolate is between five and eight days[9], it is often difficult to preclude the spread of contamination when conventional culture methods are used.

Several gastroenteritis outbreaks have been reported in Jordan in the pervious years, many of which were owing to *S. enterica* as a causative agent as a result of consumption of contaminated food-products[10-12]. Traditional microbiological methods were mostly used for the detection and identification of *S. enterica* from food samples in Jordan[13].

To minimize the occurrence of salmonellosis cases and to ensure safe products for consumers, a sensitive and rapid detection procedure for even a small number of *S. enterica* is required. Due to the need for detecting and identifying *S. enterica* in such a rapid and reliable manner, the goal of this study was to use multiplex PCR (m-PCR) procedure for the direct identification and identification of *S. enterica*, serotypes *S. enteritidis* and Typhimurium from the pre-enrichment step of naturally contaminated meat and poultry products from the north part of Jordan (Irbid City).

MATERIALS AND METHODS

Chemicals: PCR reagents were purchased by Promega (Madison, WI, USA), PCR primers were synthesized by Bioline Ltd. (UK). Whereas bacteriological media and broths were purchased from Oxiod (Hampshire, England), except where was stated. The rest of the materials, chemicals and reagents used in this study were purchased from Sigma Chemical Co. (St. Louis, MO. USA).

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Table 1: Bacterial strains used in this study

Bacteria
***Salmonella* serotypes**[a,b]
Salmonella enterica serotype Enteritidis
Salmonella enterica serotype Hadar
Salmonella enterica serotype Heidelberg
Salmonella enterica serotype Infantis
Salmonella enterica serotype Paratyphi B
Salmonella enterica serotype Paratyphi C
Salmonella enterica serotype Typhi
Salmonella enterica serotype Typhimurium
Salmonella *enterica* serotype Brandenburg
Salmonella enterica serotype Virchow
Non-*Salmonella* strains[c]
Escherichia coli
Klebsiella sp.
Bacillus cereus
Proteus sp.
Pseudomonas aeruginosa
Serratia marcescens
Staphylococcus aureus

[a] All the above *Salmonella* serotypes were gift from Pasteur Institute, Paris, France
[b] Nomenclature of *Salmonella* serotypes is according to Brenner *et al.*[5]
[c] All the non-*Salmonella* strains were clinical isolates

Table 2: Primers used for m-PCR amplification

Primer	Sequence (5` → 3`)	Target sequence	Product size (bp)	Reference
ST11	AGCCAACCATTGCTAAATTGGCGCA	Random fragment	429	Aabo *et al.*[17]
ST15	GGTAGAAATTCCCAGCGGGTACTG			
Sef167	AGGTTCAGGCAGCGGTTACT	*sefA* gene	312	Soumet *et al.*[8]
Sef478	GGGACATTTAGCGTTTCTTG			
Fli15	CGGGTGTGCCCAGGTTGGTAAT	*fliC* gene	620	Soumet *et al.*[7]
Typ04	ACTGGTAAAGATGGCT			

Bacterial strains and food samples: The reference bacterial strains used in this study and their sources are listed in Table 1. Different food samples containing *S. enterica*, previously identified and confirmed by m-PCR and bacteriological methods[14], were used for the simultaneous identification of *S. enterica* serotypes of *S. enteritidis* and *S. typhimurium.*

Extraction of genomic DNA: DNA was extracted according to the direct lysis method[15].

***Salmonella enterica* serotypes enteritidis and typhimurium specific primers:** Primers ST11, ST15, Sef167, Sef478, Fli15 and Typ04 were used for the identification of *S. enterica*, serotypes *S. enteritidis* and *S. typhimurium*, respectively. Primer sequences are shown in Table 2.

Specificity of the m-PCR: To test the specificity of the m-PCR, a panel of *Salmonella enterica* reference serotypes was used as positive controls to determine the specificity and the best PCR conditions for the detection. Whereas non-*Salmonella enterica* reference strains were also used as negative control.

Detection of *Salmonella enterica* serotypes enteritidis and typhimurium by m-PCR: PCR amplification was carried in 50 μl reaction mixture, which contained: 0.3 μ mol l^{-1} each of the ST11, ST15, Sef167, Sef478, Fli15 and Typ04 primers, 1X PCR buffer ($MgCl_2$ free), 2 m mol l^{-1} $MgCl_2$, 100 μ mol l^{-1} each of the deoxynucleotide Triphosphate (dNTPs); 0.5 U Taq DNA Polymerase, 5 μl (0.5 μg μl^{-1}) template DNA and Nuclease-Free water was used to bring the reaction volume to 50 μl, using 0.5 ml thin walled, nucleases free PCR tubes (Advanced Biotechnology, UK) by Perkin Elmer thermocycler model 480 (Perkin Elmer, USA) programmed for: 30 cycles of 30 s at 95°C, 45 s at 60°C, 1 min at 72°C with a final extension for 10 min at 72°C. Each PCR reaction was overlaid with 100 μl of mineral oil. Samples were kept at 4°C until processed.

The amplified products were then electrophoresed[16] at 95 V for 3 h in 2% agarose gel using 1X TBE buffer and visualized under UV light after staining with a solution of ethidium bromide (0.5 μg ml^{-1}). Gels were photographed with Nikon F3 camera (Nikon, Japan) using Agfa pan films (ISO 25).

RESULTS

Specificity of the m-PCR: All *Salmonella enterica* serotypes amplified with the three sets of primers under the pre-mentioned conditions gave the 429 bp band specific to *S. enterica.* The serotypes *S. enteritidis* and

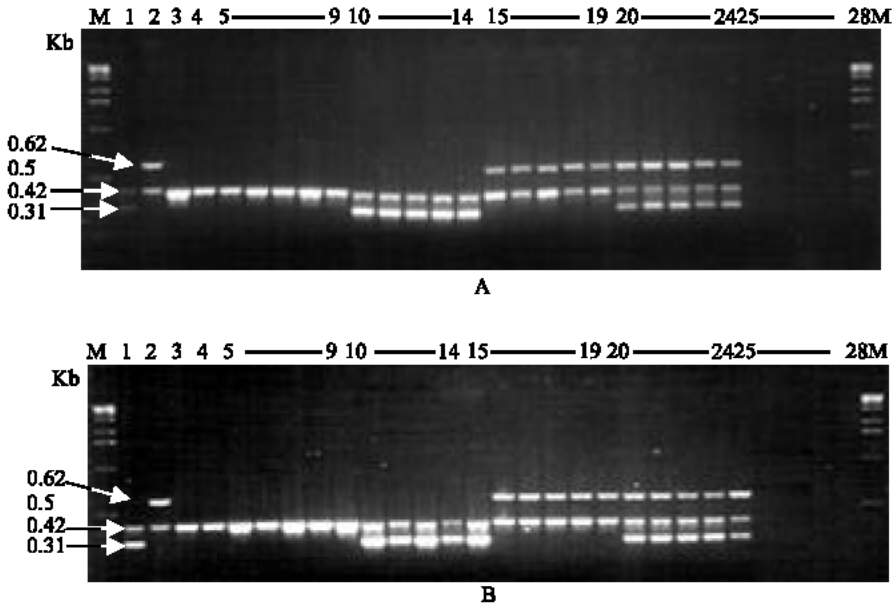

Fig. 1: Amplification of DNA from different *Salmonella* and Non-*Salmonella* strains

(A) Lane M: 1 Kb DNA ladder molecular weight marker; Lane 15: Sample 16; Lane 1: *Salmonella enterica* ser. Enteritidis; Lane 2: *Salmonella enterica* ser. Typhimurium; Lane 3: *Salmonella enterica* ser. Hadar; Lane 4: *Salmonella enterica* ser. Infantis; Lane 5: Sample 20; Lane 6: Sample 30; Lane 7: Sample 43; Lane 8: Sample 48; Lane 9: Sample 52; Lane 10: Sample 42; Lane 11: Sample 46; Lane 12: Sample 55; Lane 13: Sample 68; Lane 14: Sample 48; Lane 16: Sample 45; Lane 17: Sample 49; Lane 18: Sample 57; Lane 19: Sample 59; Lane 20: Sample 38; Lane 21: Sample 41; Lane 22: Sample 51; Lane 23: Sample 63; Lane 24: Sample 74; Lane 25: Sample 15; Lane 26: *Staphylococcus aureus*; Lane 27: *Escherichia coli*; Lane 28: PCR negative control (dd H_2O).

(B) Lne M: 1 Kb DNA ladder molecular weight marker; Lane 1: *Salmonella enterica* ser. Enteritidis; Lane 2: *Salmonella enterica* ser. Typhimurium; Lane 3: *Salmonella enterica* ser. Paratyphi B; Lane 4: *Salmonella enterica* ser. Virchow; Lane 5: Sample 76; Lane 6: Sample 80; Lane 7: Sample 88; Lane 8: Sample 243; Lane 9: Sample 248; Lane 10: Sample 101; Lane 11: Sample 106; Lane 12: Sample 115; Lane 13: Sample 133; Lane 14: Sample 251; Lane 15: Sample 70; Lane 16: Sample 75; Lane 17: Sample 82; Lane 18: Sample 100; Lane 19: Sample 130; Lane 20: Sample 85; Lane 21: Sample 86; Lane 22: Sample 93; Lane 23: Sample 105; Lane 24: Sample 200; Lane 25: Sample 252; Lane 26: *Bacillus cereus*; Lane 27: *Klebsiella* spp.; Lane 28: PCR negative control (dd H_2O)

S. Typhimurium gave either the specific band for *S. enteritidis* or *S. typhimurium* only. In order to test the general applicability of the primer pairs, the same PCR amplification conditions were performed on different Non-*Salmonella* strains and no nonspecific products were amplified (Table 3).

The primers used in this study showed specificity and ease of application for identifying *S. enterica* and serotypes *S. enteritidis* and *S. typhimurium*, since; the amplified DNA was specifically from *S. enterica*, *S. enterica* serotype *S. enteritidis* and *S. enterica* serotype *S. typhimurium* and not from the other tested organisms.

Simultaneous direct detection of *Salmonella enterica*, *Salmonella enterica* serotypes Enteritidis and Typhimurium in pre-enriched food samples by m-PCR: Out of 93 samples, Only 21 (22.5%) samples contained *S. typhimurium* alone and showed two bands; the general band (429 bp) for *S. enterica* and the other is the specific band (620 bp) for Typhimurium serotype (Fig. 1a and b, lanes 15 to 19), which is similar to that obtained when using DNA from cerateype *S. typhimurium* reference strain (Lane 2, Fig. 1a and b). On the other hand, only 19 samples (20%) showed only the serotype *S. enteritidis* specific band (312 bp), in addition to the *S. enterica* general band (429 bp) (Lane 10 to 14, Fig. 1a and b) similar to that appeared when DNA from serotype *S. enteritidis* reference strain was used (Lane 1, Fig. 1a and b). The m-PCR was applied to DNA extracted from the pre-enriched food samples showed that out of 93 samples, a total of 28 (30%) samples showed only the specific band (429 bp) for *S. enterica* (Lane 5 to 9, Fig. 1a and b) similar in size to the band obtained when using DNA from *S.* Hadar, *S.* Infantis, *S. paratyphi* B and *S.* Virchow

Table 3: Specificity and detection of multiplex PCR

	PCR result[a]		
Bacterium	ST11/ ST15 429 bp	Sef167 /Sef478 312 bp	Fli15/ Typ04 620 bp
S. enteritidis	+	+	-
S. hadar	+	-	-
S. heidelberg	+	-	-
S. infantis	+	-	-
S. paratyphi B	+	-	-
S. paratyphi C	+	-	-
S. typhi	+	-	-
S. typhimurium	+	-	+
S. brandenburg	+	-	-
S. virchow	+	-	-
Escherichia coli	-	-	-
Klebsiella sp.	-	-	-
Bacillus cereus	-	-	-
Proteus sp.	-	-	-
Pseudomonas aeruginosa	-	-	-
Serratia marcescens	-	-	-
Staphylococcus aureus	-	-	-

[a] +: PCR positive. -: PCR negative

reference strains (Fig. 1a and b, lanes 3 and 4, respectively). Performing m-PCR reactions using the three primer pairs on the isolated DNA revealed 25 (26.8%) samples containing both serotypes *S. enteritidis* and *S. typhimurium* (Lane 20 to 24, Fig. 1a and b). All in all, 46 (49.5%) samples contained *S. typhimurium*, while 44 (47.3%) samples contained serotype *S. enteritidis*.

No bands where observed in the negative samples (Lane 25, Fig. 1a and b) or when DNA from related and non-related bacteria were used as a template in the m-PCR (Lanes 26 and 27, Fig. 1a and b).

DISCUSSION

The m-PCR used in this study take advantage of one *S. enterica* specific primer pair ST11/ST15, which was shown by Aabo *et al.*[17] to be specific to *S. enterica* and targeted a random fragment of 429 bp specific to *S. enterica*. The second primer pair is Sef167/Sef478, which was developed by Soumet *et al.*[8] and targeted a unique 312 bp fragment in the *sefA* gene of *S. enterica* serotype *S. enteritidis*. The third primer pair, Fli15/Typ04, generated from the *fliC* gene[7] sequence and was shown to be specific for serotype *S. typhimurium* and gives a band of 620 bp. The three primer sets were used simultaneously in this study to assure the identification of *S. enterica* and to identify the serotypes *S. enteritidis* and *S. typhimurium* in the same PCR even in the presence of any other related and non-related bacteria. *S. enteritidis* and *S. typhimurium* serotypes were chosen because they are the most prevalence serotypes in Jordan[21].

The results in this study showed that m-PCR was fast and specific in identifying *S. enterica* and *S. enterica* serotypes *S. enteritidis* and/or *S. typhimurium* in food samples (Fig. 2a and b). Mahon *et al.*[9], Soumet *et al.*[7] and Vantarakis *et al.*[18] showed similar results to that obtained here.

Taking in our consideration that a contaminated food sample may contain several bacterial species[19], multiplex PCR proved to be a specific method with a superior ability to detect *S. enterica* and the serotypes *S. enteritidis* and/or *S. typhimurium* in the presence of other bacteria simultaneously. The results here indicated the efficiency of m-PCR as suitable and as a method of choice for the identification of both *S. enteritidis* and/or *S. typhimurium* serotypes in food.

The identification of *S.* Enteritidis and/or *S. typhimurium* in food was applied on DNA isolated from the pre-enrichment step; because *Salmonella* is not uniformly distributed throughout contaminated samples[20]. Consequently, it is essential to increase the processed quantity of the sample to enhance the probability of detecting such bacterium and to avoid sampling errors.

The m-PCR procedure described here is able also to identify dead *S. enterica*, *S. enterica* serotype *S. enteritidis* and/or *S. enterica* serotype *S. typhimurium* that are present in a contaminated food sample (data not shown). This finding recommends also the use of conventional microbiological methods in such investigations, where PCR could be used to gain a quick and a reliable idea about the status of the sample and microbiological techniques could be used to further confirm the presence of certain microorganism and/or isolating it.

The m-PCR procedure described here may also be extended for the routine detection and/or identification of *S. enterica*, *S. enterica* serotype *S.* Enteritidis and/or *S. enterica* serotype *S. typhimurium* from environmental and clinical samples.

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REFERENCES

1. Le Minor, M., 1992. The genus *Salmonella*. In The Prokaryotes (Eds. A. Balows, H.G. Truper, M. Daworkin, W. Harder, K.H. Schleifer), 2nd Ed. New York, Springer-Verlag.

2. Rumeu, M.T., M.A. Suarez, S. Morales and R. Rotger, 1997. Enterotoxin and cytotoxin production by *Salmonella enteridis* strains isolated from gastroenteritis outbreaks. J. Applied Microbiol., 82: 19-31.
3. Wan, J., K. King, H. Carven, C. Mcauley, S.E. Tan and M.J. Coventry, 2000. Probelia™ PCR system for rapid detection of *Salmonella* in milk powder and ricotta cheese. Letters of Applied Microbiol., 30: 267-271.
4. Zdragas, A., P. Tsakos and P. Mavrogeni, 2000. Evaluation of two assays, MSRV and RV for the isolation of *Salmonella* spp. from wastewater samples and broiler chickens. Letters of Applied Microbiol., 31: 328-331.
5. Brenner, F.W., R.G. Villar, F.J. Angulo, R. Tauxe and B. Waminathan, 2000. *Salmonella* nomenclature. J. Clinical Microbiol., 38: 2465-2467.
6. Bean, N.H. and P.M. Griffin, 1990. Foodborne disease outbreaks in the United States, 1973–1987: pathogens, vehicles and trends. J. Food Protection, 53: 804–816.
7. Soumet, C., G. Ermel, N. Rose, V. Rose, P. Drouin, G. Salvat and P. Colin, 1999a. Evaluation of a multiplex PCR assay for simultaneous identification of *Salmonella* sp., *Salmonella enteritidis* and *Salmonella typhimurium* from environmental swabs of poultry houses. Letters in Applied Microbiol., 28: 113-117.
8. Soumet, C., G. Ermel, N. Rose, V. Rose, P. Drouin, G. Salvat and P. Colin, 1999b. Identification by a multiplex PCR-based assay of *Salmonella typhimurium* and *Salmonella enteritidis* strains from environmental swabs of poultry houses. Letters in Applied Microbiol., 29: 1-6.
9. Mahon, J., C.K. Murphy, P.W. Jones and P.A. Barrow, 1994. Comparison of multiplex PCR and standard bacteriological methods of detecting *Salmonella* on chicken skin. Letters in Applied Microbiol., 19: 169-172.
10. Khuri-bulos, N.A., M. Abu Khalaf, A. Shehabi and K. Shami, 1994. Foodhandler-associated *Salmonella* outbreak in a university hospital despite routine surveillance cultures of kitchen employees. Infection Control and Hospital Epidemiol., 15: 311-314.
11. Youssef, M., A. Shurman, M. Bougnoux, M. Rawashdeh, S. Bretagne and N. Strockbine, 2000. Bacterial, viral and parasitic enteric pathogens associated with acute diarrhea in hospitalized children from northern Jordan. FEMS Immunology and Medical Microbiology, 28: 257-263.
12. Shehabi, A.A., 1995. Extra-intestinal infections with multiply drug-resistant *Salmonella typhimurium* in hospitalized patients in Jordan. European J. Clinical Microbiol. and Infectious Dis., 14: 448-451.
13. Sharif, L., T. Abdul Aziz and M.F. Sedik, 1996. Prevalence of *Salmonella* in broiler caeca and carcasses in Northern Jordan. Assoc. Vet. Med. J., 35: 76-81.
14. Malkawi, H. and R. Gharaibeh, 2003. Multiplex PCR for the direct detection of *Salmonella* from chicken, lamb and beef food products. J. Basic Microbiol., 43: 328-336.
15. Dickinson, J.H., R.G. Kroll and K.A. Grant, 1995. The direct application of the polymerase chain reaction to DNA extracted from foods. Letters in Applied Microbiol., 20: 212-216.
16. Sambrook, J., E.F. Fritsch and T. Maniatis, 1987. Molecular Cloning: A Laboratory Manual, 2nd Ed. NY, USA, Cold Spring Harbor, pp: 150-478.
17. Aabo, S., L. Rossen, O.F. Rasmussen, P.D. Sorensen and J.E. Olsen, 1993. *Salmonella* identification by the polymerase chain reaction. Molecular and Cellular Probes, 7: 171-178.
18. Vantarakis, A., G. Komninou, D. Venieri and M. Papapetropoulou, 2000. Development of a multiplex PCR detection of *Salmonella* spp. and *Shigella* spp. in mussels. Letters in Applied Microbiol., 31: 105-109.
19. Wang, R.F., W.W. Cao and C.E. Cerniglia, 1997. A universal protocol for the detection of 13 species of foodborne pathogens in foods. J. Applied Microbiol., 83: 727-736.
20. Quinn, C., J. Ward, M. Griffin, D. Yearsley and J. Egan, 1995. A comparison of conventional culture and three rapid methods for the detection of *Salmonella* in poultry feed and environmental samples. Letters in Applied Microbiol., 20: 89-91.
21. Shehabi, A.A. and J.M. Haddadin, 1982. *Salmonella* serotypes isolated from patients at two main medical centers in Amman, 1976-1980. Jordan Med. J., 16: 49-54.