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DETECTION OF *SALMONELLA TYPHIMURIUM* IN THE MILK OF NORTH SINAI SHAMI GOATS BY PCR AMPLIFICATION OF THE *INVA* GENE SEQUENCE

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SUMMARY

Salmonella Species are widely distributed in the environment, and cause salmonellosis in humans and animals. Salmonella typhimurium often transmit to humans through milk and dairy products. A collection of 50 milk samples from North Sinai Damscus (Shami) goats were examined for detection of Salmonella typhimurium by both convenential cultural and PCR methods. PCR was performed by amplification of nucleotide sequence within the InvA gene of Salmonella typhimurium. It was found that 5 samples (10%) were positive by standard culturing method while 7 samples (14%) were positive by using PCR technique from which 2 samples were positive by PCR assay but negative with culturing. The remaining samples were negative by both PCR and bacteriological culture. PCR technique was shown to be an effective, rapid, reliable and sensitive method for detection of Salmonella typhimurium than the conventional cultural method.

Keywords: Shami goats, Salmonella typhimurium, milk, PCR, InvA

INTRODUCTION

Salmonella is the most prevalent food poisoning microorganism. Members of the genous *Salmonella* are gram negative and facultative anaerobic, rod shape bacteria (Le Minor, 1992). *Salmonella* species are widely distributed in the environment, and cause salmonellosis in humans and animals. Milk as a food of animal origin, is frequently implicated in human infection because the high prevalence of *Salmonella* strains in domestic animals (International Association of Microbiological Society, 1978). The established culture based methods used to detect *Salmonellae* in milk are laborious; need intensive culture technique, time consuming and often not specific enough. The standard methods used today for analyzing *Salmonellae* involve pre-enrichment in buffered peptone water (BPW), selective enrichment in Rappaport Vassiliadis soy broth, plating on selective agar and subsequent identification by biochemical tests and then confirmed serologically (Charlotta *et al*., 2004).

The complete test requires three to four days to obtain a negative result and up to seven days to get a confirmed positive result (Anomymous, 1995). This is not parallel with the control of infection which depends mainly on the accuracy of rapid methods and precise diagnostic tests for monitoring the infection. For this purpose several rapid methods have been developed. These methods include enzyme-linked immunosorbant assays using polyvalent, polycolonal or monocolonal antibodies (Krysinki, 1977 and Mottingly, 1984) against somatic or flagellar antigens and DNA

hybridization assays using polynucleotide or oligonucleotide probe. Morever, there are still problems with their sensitivity and specificity. The PCR technique in particular has been found to be highly specific molecular diagnostic tool (Rahn *et al*., 1992 and Hoorfar *et al*., 2000) in which a few copies of target DNA can be amplified to a level detectable by gel electrophoresis (Rassmussen *et al*., 1994).

The pathogenicity of *Salmonella* species is dependant upon the ability of these organisms to invade and gain access to cells that are normally non phagocytic. The gene which responsible for this character of pathogenicity is a chromosomal encoded locus, *InvA*, has been determined (Galan and Crutiss, 1991), in addition, InvA gene is present and functional in most, if not all, virulent *salmonella* strains. So, The study was designed to develop and apply a simple, rapid and sensitive polymerase chain reaction (PCR) based protocol for routine detection of viable *Salmonella* in contaminated milk samples depending on the presence of *InvA* gene.

MATERIALS AND METHODS

Bacterial strain

Salmonella typhimurium strain was obtained from American Type Culture Ciollection (ATCC), catalogue No., 25566, Parklawn Drive, Rockville, USA. The strain, grown in tryptone soy broth (Oxoid LTD, Basingstoke, Hampshire, UK) at 37^{o}C for 24h, was washed three times by means of centrifugation (8,000 Xg for 8 min) in a 0.8% NaCl solution.

Collection of raw milk samples

Fifty milk samples were collected; udder halves were cleaned and disinfected prior to sampling. The first three squirts of milk were discarded from each teat and samples were collected into 250 ml sterile bottles and transmitted to the laboratory for biological examination and DNA extraction.

Bacteriological examination

The obtained milk samples were pre-enriched by placing of 25 ml from each milk sample in a sterile flask with 225ml of 1% buffered peptone water (BPW) and incubated at 37^{o}C for 24 hours (Poemla and Silliker, 1976). The samples were enriched by inoculation of 0.1 ml from each pre-enriched sample in 10ml of Rappabort Vassiliadis (Merck) broth and 1ml to tetrathionate broth (Merck) and incubated at 42^{o}C and 37 ^{o}C for 24 hours respectively. One loopful of both cultures were streaked onto XLD (Oxoid) and SS agar (Oxoid) media. The plates were incubated at 37^{o}C for 24h. Presumptively positive colonies, red colonies with black center from XLD agar and creamy ones from SS agar were picked up and inoculated into Triple sugar iron agar (Oxoid), Motility indole urea agar (Oxoid) and Simmon's citrate agar (Oxoid). All plates and tubes were incubated at 37 ^{o}C for 24h. At least two colonies were selected and streaked into two separate slope agar tunes for biochemical identification which was done according to Edwards and Ewing, 1972 and Cruickshank *et al*., 1975. Confirmation was done serologically by using *Salmonella* polyvalent O and H agglutination sera obtained from Staten Serum Institute (SSI), Copenhagen, Denmark.

***Serological examination*:** according to Kauffman, 1974

Isolates that the preliminary identified biochemically as *salmonella* were subjected to serological identification according to Kauffman white scheme, suspected *salmonella* isolates were cultured on nutrient agar slopes for 24 hours at 37^{o}C. Then, subjected to the following examinations:

Slide agglutination technique:

A loopful was suspended in a drop of physiological saline solution on a slide, so as to make a homogenous suspension. A drop of *salmonella* anti-sera was added to the suspension with a standard loop and mixed thoroughly to bring the organisms in close contact with anti-sera. Positive agglutination occurred within a minute and could be easily seen with the naked eye. A delayed or partial agglutination was considered as negative or false.

A- ***Determination of "O" somatic antigen***

Polyvalent "O" anti-sera were first tried to assure that the suspected isolates are *salmonella*. Positive cultures were then tested with each of the "O" grouping sera followed by the respective mono-specific "O" anti-sera factor in order to determine the complete antigenic formula.

B- ***Determination of "H" flagella antigen***

Polyvalent "H" anti-sera for both phase 1 and phase 2 were applied to determine the antigenic formula of the isolates.

DNA extraction from milk

Each milk sample (10ml) was pretreated with one ml of 25% sodium citrate. The DNA was extracted using a modification of the method described previously by Drake *et al*., 1996. Briefly, petroleum ether (2ml), 100% ethanol (2ml) and saturated ammonium hydroxide (4ml) were added, mixed, and the solution centrifuged at 8000g for 10 min at room temperature. The supernatant was discarded and the pellet resuspended in 500-900µl STET buffer (80% sucrose, 0.5% Triton-X-100, 50mmol EDTA, 50 mmol Tris-HCl at pH 8.0) and transferred to a 2ml microcentrifuged tube. The tube was vortexed occasionally for 10 min. An equal volume of phenol, chloroform and isoamyl alcohol was added, mixed, and the tube centrifuged at 17000g for 10 min at room temperature. The supernatant fluid was transferred to a new tube and an equal volume of chloroform was added, mixed, and centrifuged as above. The clear aqueous phase was pipetted to a new tube and the DNA precipitated with 0.1 volume 3 molar sodium acetate, pH 5.4, 1 volume cold isopropanol and 30µg glycogen. The sample was centrifuged at 12000g for 30min at room temperature to pellet the DNA. The air dried pellet was resuspended in 15 µl nuclease-free water and quantified at 260 nm using UV spectrophotometer.

PCR design and amplification conditions

Primers were selected from the published sequence of *InvA* gene of *Salmonella* as described previously (Rhan *et al*., 1992), were synthesized in Bio-Synthesis, for InvA1 with sequence 5' ACA GTG CTC GTT TAC GAC CTG AAT 3', and for InvA2 with sequence 5' AGA CGA CTG GTA CTG ATC GAT AAT3'. InvA1 and InvA2 were used for amplify the amplified fragment of 284 base-pair level. From

pure culture or genomic DNAs of *Salmonella typhimurium*, amplification of the *InvA* gene was achieved on the thermocycler (model 9600, Perkin-Elmer) as follows: 20 cycles of PCR, with 1 cycle consisting of 1 min at 94°C (denaturation), 30 seconds at 58°C (annealing), and 1 min at 72°C (primer extension). An additional cycle of 1 min at 94°C, 30seconds at 58°C, and 5 min at 72°C was also included. A 25 µl PCR mixture contained the following: 50 mM Tris-HCl (pH 8.3); 200uM (each) dATP, dCTP, dGTP and dTTP; 0.075 µM (each) primer; 0.65µ of AmpliTaq; and 2.5 mu $MgCl_2$. Genomic DNA at a concentration of 4 ng/ul, a single colony, or cells from serially diluted sample of *Salmonella* were used in the PCR mixture. *Salmonella* choleraesuis subsp. choleraesuis (smith) Weldin serotype typhimurium deposited as *Salmonella typhimurium* (ATCC 25566) was used as a positive control. A template control (sterile water) was included to monitor contamination of the PCR reagents in each PCR assay.

RESULTS

Total 50 milk samples were collected from Shami goats in North Sinai governorate and examined by the PCR- based detection system for *Salmonella typhimurium*. The target PCR product at 275 bp (fig:1) was amplified in a total of 7 samples (14%) while *Salmonella typhimurium* was isolated by culturing in 5 samples only (10%) as illustrated in table (1). Comparing results of PCR and that of conventional cultural assays (table 2), it was found that 5 samples in both PCR and culture were positive, 2 samples were positive by PCR assay but negative with culturing. The remaining 43 samples were negative by both PCR and bacteriological culture.

Table 1. Frequency of isolation of *S. typhimurium* from milk samples by conventional cultural technique and PCR

No. of samples	Conventional		PCR	
	No.	%	No.	%
50	5	10	7	14

Table 2. Comparative analysis between the PCR and conventional cultural technique for *S. typhimurium*

Culture	PCR	No.	%
+ Ve	+ Ve	5	10
	- Ve	0	0
- Ve	+ Ve	2	٤
	- Ve	43	86
No. of examined samples		٥٠	100

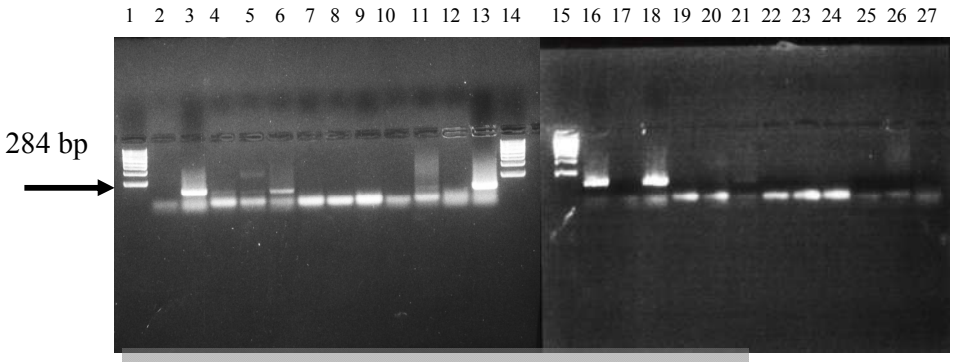

Figure 1. PCR amplification of DNAs from milk samples. Lanes:1, 14 &15 1 kb DNA ladder; 4, 5, 6,11, 13, 16 & 18 are positive samples for *S.typhimurium* and Lanes 7, 8 ,9 ,10, 12, 17 & 20-28 are negative samples while Lane 3 showing positive control and Lane 2 negative control (no DNA).

DISCUSSION

Milk and milk by products captured a great importance because they are one of the cheapest source of animal protein and are of high nutritive value, palatability and digestibility. This importance increased in desert and new reclaimed areas such as North Sinai governorate where the Bedouins depend mainly on the goat's milk and its by-products. Unfortunately, Milk as a highly nutritive substrate for growth of many microorganisms especially in low hygienic areas, may contain multiple bacteria species of major public health significance. *Salmonella* species are the most important microorganism that threat the human health where *Salmonella* species were identified in 84.5% of the outbreaks for which a causative agent was reported (Schmidt *et al*., 1995).

Several obstacles are encountered when analuzying *Salmonellae* in milk samples, namely law concentration and uneven distribution in the milk (Charlotto *et al*., 2004). Polymerase chain reaction (PCR) is a molecular biology technique which has taken up as increasingly significant space in the field of laboratory diagnostics, allowing the detection of many pathogens such as *Listeria monocytogenes*, *Campylobacter sp*., *Yersinea enterocolitica*, *Vibrio cholera*, *Shigella flexneri*, *Escherichia coli* and *Salmonella* in different kinds of food such as meat and milk (Luciana *et al*., 2001). Moreover, PCR method able to detect *Salmonella* even in poorly contaminated products (Luciana *et al*., 2004).

One of the critical points of the PCR technique used in the choice of the sequence to be amplified, which must be common to most of the serovars and do not present homology with other organisms. In our study, when using primers which are derived from gene InvA and code for proteins related to cell invasion, a specific amplification of DNA of *Salmonella* was observed at 284 bp as shown in figure (1). The figure shows lacking of non specific bands during amplification plus the lack of false positive results makes this method unique and indicating high specificity of this PCR assays. This appeared to be similar to results obtained by Wang *et al*., 1997 and Rhan

et al., 1992 who studding 630 isolates of *Salmonella* species including *Salmonella typhimurium*, amplified all samples of *salmonella*, except *S. Litchfield* and *S. senftenberg*. In the outhers' opinion, the apparent absence of gene InvA in these two serovars suggests that these organisms are not invasive, or that they have alternative pathways to penetrate cells with an as yet unknown pathogenic potential.

However, in the present study two primers which specifically amplify an 284 bp fragment in strains of the genus *salmonella* were selected and synthesized. The primers were selected completely internal to the InavA gene. By selecting primers completely internal to the InavA gene, all non *Salmonella* strains responded negatively to the amplicon of InvA gene.

All samples were cultured according to conventional technique by using procedure outlined in Material and Method, and the data cleary indicate the accordance between the results of the biochemical methods and PCR as shown in table (2). Comparing results of PCR and that of conventional culture assays (table 1&2). PCR was shown to be more sensitive in *Salmonella typhimurium* detection than culture method as the latter method yield 5 samples positive only. The difference in the positive result between the two methods may attributed to the sensitivity of the PCR assay as it could be detected very law number of *Salmonella typhimurium* up to 1 cell / 25 ml of milk and this in agreement with the results obtained by Moffezzoli *et al*., 1995.

The results confirm that the InvA gene is unique to detect *Salmonella* strains and PCR assay serve as an effective, rapid reliable and sensitive technique for the detection of *Salmonella* strains.

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الكشف عن ميكروب السالمونيلا تيفيميوريم في حليب الماعز الشامي بشمال سيناء باستخدام تفاعل البلمرة المتسلسل بتكبير تتابع جين *InvA*

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ينتشر ميكروب السالمونيلا على نحو واسع في البيئة مسبباً مرض السالمنيللوزس للإنسان والحيوان على حد سواء. وينتقل ميكروب السالمونيلا إلى الانسان بطرق كثيرة ومن أهمها الحليب ومنتجاته. فقد تم فحص عدد خمسون عينة من لبن الماعز الشامي المتواجد في محافظة شمال سيناء للكشف عن مدى تواجد ميكروب السالمونيلا وذلك باستخدام الطريقة التقليدية للزرع والعزل والتقنيات الحديثة باستخدام تفاعل البلمرة المتسلسل لتكبير تتابع جين *InvA* وتم عمل المقارنة بين الطريقتين. فأوضحت النتائج أن ٥ عينات بنسبة ١٠% من المجموع الكلي للعينات كانت إيجابية بالطرق المثالية للعزل والتصنيف بينما أعطت ٧ عينات نتيجة إيجابية بإستخدام تفاعل البلمرة المتسلسل والتي منها أعطت عدد ٢ عينة نتيجة سالبة بإستخدام طريقة العزل والتصنيف ولم تعطي باقي العينات أي إيجابية بإستخدام الطريقتين. وقد إتضح أن طريقة تفاعل البلمرة المتسلسل هي الأكثر دقة وسرعة وفعالية في الكشف عن ميكروب السالمونيلا.