

Detection of Yersinia enterocolitica in the Shami goat's milk in the North Sinai Governorate

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Considerable difficulties are associated with the isolation of *Yersinia enterocolitica* from food particularly milk and milk products. Most methods are time consuming require enrichment steps and are unable to differentiate pathogenic isolates from non pathogenic ones. The purpose of this study was to evaluate the detection of *Yersinia enterocolitica* in milk by both polymerase chain reaction (PCR) and conventional culturing methods. Fifty milk samples were collected from Shami goats in North Sinai governorate.

Two primers (DG26 and DG63) were used in PCR and the size of the PCR-product was 440bp. The results obtained by PCR technique were in good agreement with that obtained by conventional culturing method. Five samples (10%) were positive by PCR while 4 samples were positive by conventional culturing method. Interestingly, PCR results are obtained within few hours. Moreover, it solved the problem of interpretation of classical biochemical and serological typing in one step without necessity of using additional examinations. This makes diagnosis in food control laboratories much faster and more efficient.

Yersinia species are one of the important etiologic microorganisms of food poisoning. *Yersinia* has a particular importance for the study of consumers, because of its capability for growing in raw milk and remains viable for long periods of time at refrigeration temperatures. Furthermore, low temperature conditions may enhance the growth rate of this potential pathogen relative to the growth of spoilage microorganisms (Ursing *et al.*, 1980).

Although *Y. enterocolitica* is an ubiquitous microorganism, the majority of isolates recovered from foods are non-pathogenic, and thus, it is important to determine the pathogenic significance of isolates (Kapperud, 1991 and De Boer *et al.*, 1986). This can be done with several phenotypic tests, but these are time-consuming and not always reliable (Kwaga *et al.*, 1992). PCR assay has been used to verify the pathogenic strains of *Y. enterocolitica* isolates rapidly and

with high specificity (Kapperud *et al.*, 1990; Wren and Tabaqchali, 1990 and Bhaduri *et al.*, 1997). This method is based on specific segments of the virulence plasmid that have known virulence functions such as *yadA* gene. The virulence plasmid is essential for *Y. enterocolitica* to survive and multiply in lymphoid tissues (Cornelis *et al.*, 1998). The *yadA* gene codes the major outer membrane protein YadA (Lachica and Zink, 1984 and Skurnik and Wolf-Watz, 1989), which forms a fibrillar matrix on the surface of *Y. enterocolitica* (Kapperud *et al.*, 1987) and is only expressed at 37°C (Portnoy and Martinez 1985). *YadA* plays a protective role in *Y. enterocolitica*, with several different functions (Table 1). The PCR procedure is a rapid *in vitro* enzymatic amplification of the copy numbers of target DNA sequence (Mullis *et al.*, 1994). The development of these rapid and sensitive PCR techniques increases the sensitivity

Table (1): Role of YadA protein in the virulence of *Yersinia enterocolitica*.

Function of YadA protein	
Serum resistance	Heesemann <i>et al.</i> , 1983
Surface hydrophobicity	Lachica and Zink, 1984
Autoagglutination	Skurnik <i>et al.</i> 1984
Adhesion to epithelial cells	Heesemann and Gruter 1987
Expression of fibrils on the surface	Kapperud <i>et al.</i> , 1987
Haemagglutination	Kapperud <i>et al.</i> , 1987
Binding to intestinal brush border membranes	Paerregaard <i>et al.</i> , 1991
Resistance to killing by polymorphonuclear leukocytes	Ruckdeschel <i>et al.</i> , 1996

Table (2): Primers used in the PCR technique.

Primers	Sequence (5'-3')	Polarities	Product length	Gene bank accession number
DG62	TAG TGT TGC CAG AAC AAC TT	Sense	440	AF056092
DG63	CAT TGT ACA TGA CAT CCG AG	Anti-sense		AF056092

for detection of a DNA sequence that may be present in trace amounts in foods (Bej *et al.*, 1994, Millar *et al.*, 1996, and Trkov *et al.*, 1999). In this study, the PCR procedure using *yadA* gene was a trial for the direct detection of *Yersinia enterocolitica* in Shami Goats' milk in comparison with the conventional methods.

Material and Methods

Bacterial strain. *Yersinia enterocolitica* ATCC 23715 was grown at 25°C in tryptic soya broth containing 0.6% yeast extract (TSBY).

Primer design. The DNA primers used in the present study were synthesized in Bio-System and are listed in table (2). The *yadA* gene was chosen, as it is virulence determinant in *Yersinia enterocolitica* and has been tested in food (beef and pork) for the specific detection of *Yersinia enterocolitica* (Kapperud *et al.*, 1993).

Collection of raw milk samples. Goat milk samples were collected from different locations representing the extensive system in North Sinai governorate (El-Arish, El-Sheikh Zoid, Rafah, Al-Qosima and Nekhle). Fifty milk samples were collected under complete aseptic condition where 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 250ml sterile bottles and transmitted to the laboratory for bacteriological examination and DNA extraction.

Bacteriological examination. Five ml of milk samples were transferred aseptically to 45 mls of sterile phosphate buffered saline (PBS), mixed well and incubated to 22-25°C for 24 hours, then 0.5 ml of the sterile PBS that treated with alkali by transferring it into 4.5 ml of 0.5% KOH - 0.5 % NaCl. After mixing for 3-4 seconds, loopfuls of this broth (Alulisio *et al.*, 1980) were streaked onto MacConky (oxid) with 5 g/L of tween 80 (Lee, 1977), and on cefsuladin Irgasan Novobiocin (CIN) agar (Biolife) and then were incubated at 30 °C for 2 days (Schiemann and Wauters, 1992). Five to six colonies showing

Yersinia characteristics (dark red "bull's eye" surrounded by a transparent border) were picked up and streaked onto nutrient agar slant which were incubated at 25°C for 24 hours. Colonies were tested for catalase, Gram staining, motility at 25°C and 37°C, growth on triple sugar iron agar (TSI), urea broth and lysine decarboxylase. Pure cultures that were urea positive, producing acid in triple sugar iron agar, and negative for gas production, H₂S, lysine decarboxylase and motility at 25°C were considered *Yersinia enterocolitica*.

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 was centrifuged at 8000g for 10 min at room temperature. The supernatant was discarded and the pellet was resuspended in 500-900µl STET buffer (80% sucrose, 0.5% Triton-X-100, 50 mmol EDTA, 50 mmol Tris- HCl at pH 8.0) and transferred to a 2ml micro-centrifuged 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 mentioned above. The clear aqueous phase was transferred to a new tube and the DNA was precipitated with 0.1 volume 3 molar sodium acetate, pH 5.4, one volume cold isopropanol and 30 µg glycogen. The sample was centrifuged at 12000 g. for 30 min. at room temperature to pellet the DNA. The air-dried pellet was resuspended in 15 µl nuclease-free water and the DNA concentration was measured at 260 nm using UV spectrophotometer.

PCR design and amplification conditions.

Table (3): Comparison between PCR reaction and traditional methods for *Yersinia enterocolitica* detection in raw milk samples

Total samples	PCR				Traditional culture			
	+ Ve	%	- Ve	%	+ Ve	%	- Ve	%
50	5	10	45	90	4	8	46	92

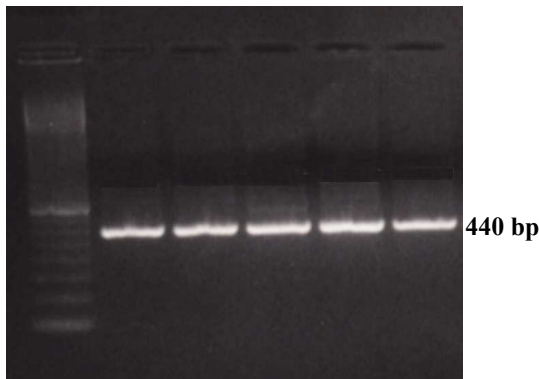


Fig. (1): Detection of *Yersinia enterocolitica* in raw milk samples by PCR from milk samples using DG62 and DG63 primers. Lane1 shows DNA Ladder. Lane2 control positive sample for *Yersinia enterocolitica*; lanes 3, 4, 5 & 6 are Positive samples.

DG62 and DG63 primers were used for amplification of fragment length of 440bp. The amplification of the extracted DNA from both milk samples and that of the reference strain of *Yersinia enterocolitica* was achieved on thermocycler Perkin-Elmer as follows. Each 50ul of PCR reaction mixture contained 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM MgCl₂, 200uM dNTPS, 2.5 unit of Taq DNA polymerase, 100Pmol of each primer and varying amounts of template DNA.

Samples were denatured at 94°C for 5 min and subjected to amplification cycles in the thermocycler. Each PCR cycle consisted of a 45 seconds denaturation step (94°C), a 45 seconds annealing step (55°C), and a 45 seconds extension step (72°C). Finally, products were extended for 7 minutes at 72°C. At the completion of amplification cycles, PCR products were analyzed by 1.5% agarose gel electrophoresis and stained with ethidium bromide.

Result and Discussion

Milk captured a great importance in the Bedouin life because it is one of the cheapest

sources of protein and considered one of animal protein sources in their diet. On the other hand, milk may be microbiologically contaminated by multiple bacterial species of major public health significance especially in low hygienic areas; one of them is *Yersinia enterocolitica*.

Since the primers DG62 and DG63 were designed for selective detection of the sequences within the *yadA* gene of *Yersinia enterocolitica* after checking with the GenBank Blast program, it was expected to be specific and not to amplify other food poisoning bacteria. The total DNA was extracted from *Yersinia enterocolitica* and was subjected to the PCR with DG62/DG63 primers. As expected, DG62/DG63 specifically amplified 440 bp fragment, as shown in fig.1.

As shown in Table 3, the result obtained from PCR method to large extent was in agreement with the traditional method of culture. The little difference between the results may be attributed to the presence of too few numbers of this pathogen cells to grow or a pathogen was dead and failed to grow on the culture media. Moreover, for direct detection by PCR, the whole procedure work took only five hours to identify *Yersinia enterocolitica* in milk. In contrast, the conventional method usually requires enrichment in liquid media, isolation of pure cultures on selective differential plating method and identification of isolates by biochemical and serological tests that take 1–2 weeks to be completed (Swaminathan *et al.*, 1982). Therefore, PCR-based method provides more convenient and faster way than the conventional method to detect *Yersinia enterocolitica* in milk.

The finding of a pathogen like *Yersinia enterocolitica* in milk supports the well known need for greater care regarding health hazard critical control point associated with production and handling of milk. There is also an urgent need to develop methods that will decrease the incidence of contamination, especially in ready to consume product as milk. Furthermore, adequate

heating must be ensured, and all personnel involved in milking, distribution, storage, handling should be aware of the potential risks of cross contamination.

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