DETECTION OF BACILLUS CEREUS ISOLATES FROM GOAT'S MILK WITH CONVENTIONAL AND PCR METHODS

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SUMMARY

Bacillus cereus is a gram-positive spore-forming rod that is ubiquitous in the environment. B. cereus is a common contaminant in dairy products. Food poisonings from the consumption of B. cereus-contaminated milk products have been reported. So, the aim of this study was establishing an easy and accurate method for detection and identification of this pathogen. Therefore, eighty raw milk samples were collected from different locations (El-Arish vally, El-Shekh Zoid, Rafah, Al-Qosima and Nekhle) where Shami goats are raised under grassing conditions representing the extensive system in North Sinai governorate. Physiological features were studied including lecithinase activity and an assay based on the PCR has been developed to facilitate the detection and identification of B. cereus in milk. Two pairs for the PCR have been designed within the sequence for cereolysin A (cerA), a cytolytic determinant that encodes lecithin hydrolyzing of B. cereus. The organism was isolated from 33 raw milk samples (41%) from which 28 samples (84%) produced lecithinase enzyme by conventional culturing method, while 5 samples (15%) did not show lecithinase activity. With PCR assay, all isolated strains of B. cereus gave amplification of the cerA gene with selected primers (Pf-Pr). Results demonstrate a high specificity of the primers selected for isolates of B. cereus.

INTRODUCTION

Bacillus cereus is a gram-positive spore-forming rod that is ubiquitous in the environment. B. cereus is a common contaminant in dairy products **(Lund and Granum, 1996)**. Food poisonings from the consumption of B. cereus-contaminated milk products have been reported. Two types of illness have been attributed to the consumption of foods contaminated with B. cereus. The first and better known is characterized by abdominal pain and diarrhea; it has an incubation period of 4-16 h and symptoms that last for 12-24 h. The second, which is characterized by an acute attack of nausea and vomiting, occurs within 1-5 h after consumption of contaminated food. B. cereus strains can grow at temperatures between 4 and 37°C **(Van Netten et al., 1990 and Vaisanen et al., 1991)**, and psychrotrophic B. cereus strains can produce enterotoxin

(Christiansson et al., 1989, Granum et al., 1993 and Griffiths, 1990) both aerobically and anaerobically. Furthermore, the spores survive heat treatment, especially in foods that contain a lot of fat as milk and its by-products **(Kramer and Gilbert, 1989)**.

Present detection methods for B. cereus rely on standard plate counting. Hydrolysis of lecithin (egg yolk reaction) is a major criterion for detection and identification of B. cereus on plating media, since most strains of the B. cereus group (e.g., B. cereus, B. mycoides, and B. thuringiensis) possess lecithinase activity. Detection of low numbers of B. cereus is especially difficult if the milk is heavily contaminated with other microorganisms. Therefore, PCR amplification using specific primers would facilitate direct detection of B. cereus in milk. At present, no sequence data which could serve to design specific primers for B. cereus are available, and on the basis of 16S sequencing data, it has been suggested that members of the B. cereus group represent a single species **(Ash et al., 1991)**.

In the present study, primers have therefore designed for phospholipase C of B. cereus; these primers were expected to amplify DNA of isolates of the B. cereus group. Such a DNA test would provide additional information about the potential risks involved with milk and its by-products and could replace the plating and other conventional methods for detecting the presence of enterotoxic B. cereus.

Materials and Methods

2.1 The bacterial strain:

Bacillus cereus strain was obtained from American Type Culture Collection (ATCC), catalogue No., 14579, Parklawn Drive, Rockville, USA. The strain, grown in tryptic sorya broth (Oxoid LTD, Basingstoke, Hampshire, UK) after addition of glucose in concentration of 0.1%. Incubation was at 37°C for 24 hours and then washed three times by means of centrifugation (8,000Xg for 10 min) in saline solution.

2.2 Collection of raw milk samples :

Goat milk samples collected from different locations representing the extensive system in North Sinai governorate **(El-Arish, El-Sheikh Zoid, Rafah, Al-Qosima and Nekhle)**. Eighty 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.

2.3 Cultivation and biochemical typing:

A total of thirty three Bacillus cereus strains were isolated from raw goat's milk Biochemical characteristics of examined samples was determined by biochemical tests in accordance with **Microbiology-General guidance for the enumeration of B. Cereus (1993)**. Samples were spread onto the surface of Mannitol-egg yolk-polymyxin (MYP) agar plates which supplemented with polymexin B. and incubated for 24 h at 30°C and observed for colonies surrounded by precipitate zone, which indicates that lecithinase was produced. Haemolytic activity was determined on blood agar (tryptic soya agar supplemented with 5% of defibrinated sheep blood).

Confirmation of B. cerens was done as lecithinase-positive colonies from MYP agar plates were selected and transfered to nutrient agar slants. Slants were incubated for 24 h at 30°C. Gram-stained smears were prepared from slants and examined microscopically. B. cereus appeared as large Gram-positive bacilli in short-to-long chains; spores were ellipsoidal, central to sub-terminal, and did not swell the sporanglum. Loopful of culture from each slant was transferred to tube containing 0.5ml of sterile phosphate-buffered dilution water and the culture was suspended in diluent with Vortex mixer. Suspended cultures were used to inoculate the following confirmatory media: **Phenol red glucose broth, Nitrate broth, Modified VP medium and Lysozyme broth**. Isolates which 1) produce large Grain-positive rods with spores that did not swell the sporanglum; 2) produce lecithinase and did not ferment mannitol on MYP agar; 3) grow and produce acid from glucose anaerobically; 4) reduce nitrate to nitrite 5) produce acetylmethylcarbinol (VP-positive); 6) decompose L-tyrosine; and 7) grow in the presence of 0.001% lysozyme were typed as B. cereus.

2.4 DNA extraction :

Total genomic DNA was isolated from the bacterial strains by phenol-chloroform extraction. Bacteria were cultured in brain heart infusion broth (30°C, 18 to 48 h). To harvest the cells, 3 ml of the broth culture was centrifuged (14,000 3 g, 10 min). After washing with 0.85% sterile NaCl, the bacteria were suspended in 400 ml of sucrose solution (6.7% [wt/vol]). 25 ml of lysozyme (10 mg/ml) was added, and the mixture was incubated for 30 min at 37°C. Afterwards, 50 ml of 20% (wt/vol) sodium dodecyl sulfate (SDS) was added, and the mixture was incubated for 30 min and digested with 5 ml of proteinase K solution (20 mg/ml) at 37°C for 30 min. Genomic DNA was extracted twice with 1 volume of phenol-chloroform-isoamyl alcohol. After adding 0.1 volume of 3 M sodium acetate, DNA was precipitated in ethanol, air dried, and dissolved in 80 ml of sterile water. The minigel method **(Sambrook et al., 1989)** was used to quantify the extracted DNA.

2.5 PCR amplification.

PCRs were carried out in 50-ml volumes which consisted of 33 µl of ultrapure sterile water, 0.5 µl of 10% dimethyl sulfoxide, 5 µl of a 10X reaction buffer (100 mM Tris-HCl, 15 mM MgCl2, 500 mM KCl), 5 µl of each deoxynucleoside triphosphate (0.1 mM), 0.75 µl of each primer (0.1 µg/µl); The primers sequences showed in table (1), and 5 ml of phenol-chloroform-extracted DNA (4 ng/ml). These mixtures were held at 70°C for 3 to 5 minutes, 1 U of Taq DNA polymerase (Boehringer Mannheim) was added. Thirty-five amplification cycles were performed in an automated DNA thermal cycler (model 480; Perkin-Elmer Cetus, Norwalk, Conn.) with the following parameters; denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and elongation at 72°C for 1 min. During the last cycle, the elongation temperature was held for a total of 4 min. Five microliters of the PCR amplifications was run on submarine 1.3% (wt/vol) agarose gels in Tris-acetate buffer (1.5 h at 40 V), stained with ethidium bromide, and photographed on a UV transilluminator.

a) Position of primer within the nucleotide sequence of cereolysin A gene (cerA)

RESULT AND DISCUSSION

The development of B. cereus diagnostic assays is important because this organism produce proteolytic enzymes, many of which are toxic for animals and human. Several selective plating methods have been described for detecting B. cereus **(Mossel et al., 1967, Kim and Goepfert, 1971, Holbrook and Andersson, 1980 and Meira and Rabinovitch, 1998)**.

The selection is based on, for instance, the ability of B. cereus to grow in the presence of polymexin B and its lecithinase activity. These methods often require, up to 4 days to be performed, including confirmatory testing. This is too much time-consuming when inspecting products with short shelf-live as milk. Another disadvantage of using selective media is that the growth of other microorganisms is not totally inhibited by any of the media diagnosed to detect B. cereus. Therefore, we wanted to develop a rapid and reliable method for deteeting B. eereus based on the sequence encoding for phospholipase C (cer A).

Phospholipase C encoded by the gene cerA is responsible for lecithin degradation, a major criterion for detection and identification of B. cereus. Searching the GenBank database **(Benson et al., 1993)** for complementary sequences showed 100% homology with the primers of gene encoded for phospholipase of B. cereus **(Johansen et al., 1988a; Johansen et al., 1988b; Yamada et al. 1988 and Kuzmin et al., 1993)**. No highly homologous sequences were found for other bacteria, indicating an excellent specificity of the primers. In this study, eighty samples were

collected. B. cereus strains were isolated from 33 samples (41%) from which 28 samples (84%) produced lecithinase enzyme by culturing method on Mannitol-egg yolk-polymyxin (MYP) agar plates while 5 samples (15%) did not show lecithinase enzyme production and considered as negative result. When the same samples re-examined by PCR assay using primer set Pf-Pr (their sequence showed in table1) that amplifies a 1.4 kb portion of cerolysin AB gene as shown in fig. 1, all thirty three samples from which B. cereus were isolated showed positive result (100%), it means that all of them had lecithinase activity. Positive result by PCR assay established with an egg-yolk negative B. cereus isolates may indicate that this set of primer could also be specific for atypical strains of B. cereus **(Schraft and Griffiths, 1995)**.

In conclusion, The finding of a pathogen like B. cereus in milk support the well known need for greater care regarding health hazard critical control 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.

Acknowledgment :

This study was supported by USAID Middle east Regional Cooperation (MERC) program under project entitled "Multinational Approaches to Enhance Goat Production in Middle East. Great thanks are to prof. D. El-Shaer, H.M. the main investigator for his continuous encouragement, guiding, reading and revision the manuscript.

Table (1): Sequence of the used primers.

Primer	Orientation	Sequence	Position [a]
Pf	Forward	5' GAG TTA GAG AAC GGT ATT TAT GCT GC 3'	250 - 275
Pr	Reverse	5' CTA CTG CCG CTC CAT GAA TCC 3'	638 - 658

TABLE (2): Results of lecithinase activity and PCR assay for *B. cereus* strains.

Total No. of examined samples	No. of *B.cereus* strains				
	Total No. of isolated *B. cereus*	Tested for lecithinase activity		Tested byPCR assay	
		Positive	Negative	Positive	Negative
80	33	28	5	33	0

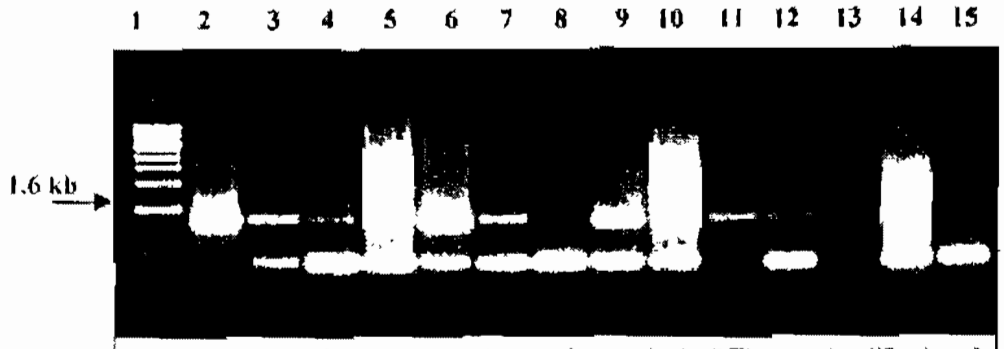

Fig (1): Detection of *Bacillus cereus* in raw milk samples by PCR assay. Amplification of DNAs from milk samples using Pf and Pr primers. Lane1 shows DNA Ladder 1-kb (Bethesda Research Laboratories, Inc.). Lane2 control positive sample for *Bacillus cereus*; lanes 3, 4, 6, 7, 9, 11and 12 are Positive saoples while lanes 5, 8, 10, 13 and 15 shows negative samples

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الملخص العربى

"الكشف عن وجود ميكروب الباسيلاس سيرس في لبن الماعز الشامى بإستخدم الطرق التقليدية وتفاعل البلمرة المتسلسل"

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يعتبر ميكروب الباسيلاس سيرس من الميكروبات الشائعة في تلوث المنتجات الغذائية وعلى وجه الخصوص اللبن ومنتجاته. وهو ميكروب عصوي موجب لصبغة الجرام قادر على التجرثم في الظروف الغير ملائمة. فكان الهدف من الدراسة هو إيجاد وسيلة سهلة ودقيقة لعزل وتصنيف هذا الميكروب لمعرفة مدى تواجده في لبن الماعز الشامي. لهذا الغرض تم أخذ ٨٠ عينة لبن من أماكن متفرقة من محافظة شمال سيناء (وادى العريش، الشيخ زويد، رفح، القسيمة ونخل) ممثلة النظام المكثف في التربية. تم عزل وتصنيف الميكروب بطرق الزرع التقليدية وكذلك بطريقة تفاعل البلمرة المتسلسل باستخدام البادىء (Pf-Pr) وهو تتابع لجين (cerA) المسئول عن إنتاج إنزيم الليسيسينيز. تم عزل الميكروب فى عدد ٣٣ عينة (٤١٪) وجد منهم ٢٨ عينة (٨٥٪) إيجابية لوجود إنزيم الليسيسينيز بينما أعطت ٥ عينات (١٥٪) نتائج سلبية. وباستخدام تفاعل البلمرة المتسلسل أعطت جميع العينات المعزولة والتى صنفت على أنها باسيلاس سيرس نتائج إيجابية لوجود إنزيم الليسيسينيز، برهنت النتائج على حساسية البادىء المستخدم ويمكن الاعتماد عليه فى عزل وتصنيف ميكروب الباسيلاس سيرس بدرجة عالية الدقة والكفاءة عن الطرق التقليدية.