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Non-haemolytic enterotoxigenic *Bacillus cereus* strains from raw and pasteurized milk and milking utensils in Kelantan, Malaysia

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Abstract

The toxigenic strains of *Bacillus cereus (B. cereus)* produce toxins such as haemolytic and non-haemolytic toxins. Fresh milk, pasteurized milk and swab samples were collected from milking utensils in a diary farm. Routine microbiological examination for *B. cereus*, antimicrobial sensitivity tests and PCR detection of the *Bacillus* group specific genes and genes encoding for haemolytic enterotoxin and non-hemolytic eneterotoxin genes were conducted. *Bacillus cereus* was isolated from raw bulk mlik, pasteurized milk and milking utensils swabs collected. Detection of *B. cereus* was higher in milking utensils compared to raw and pasteurized milk. Bacillus cereus was also detected in corn-flavoured pasteurized milk and milking utensils of dairy colonies. There were non-hemolytic enterotoxin gene positive isolates and most of them were susceptible to Gentamicin, Chloramphenicol and Ciprofloxacin compare to other beta lactam antibiotics. As control and prevention stratgies, increase in public awareness through public education, proper hygiene practices in farms and dairy processing plants, regular surveillance and quality control including intensive screening for milk and milk products need to be in place. However, further in-depth study based on larger and diversified sample and detection of other toxigenic genes are recommended.

Introduction

Bacillus cereus is a Gram-positive, aerobic-to-facultative, spore-forming rod widely distributed in the environmentally and is closely related to other *Bacillus* species both phenotypically and genetically (Bottone, 2010). *Bacillus cereus* is commonly implicated in food-borne illnesses which are often reported to be mild and self-limiting. However, In addition to food poisoning, *B. cereus* causes a number of systemic and local infections in both immunologically compromised and immunocompetent individuals (Bottone, 2010). The *B. cereus* group are associated with two different clinical syndromes of food poisoning which are diarrheal and emetic (vomiting) syndromes (Sandra *et al*., 2012).

The presence of *B. cereus* in raw and pasteurized milk is major concern to dairy industry because contamination by this microorganism produces proteinases and lipases that may deteriorate the quality of milk and milk products (Miller *et al*., 2008). Recent researches from various countries have suggested that Gram-positive spore-forming microorganisms such as *B. cereus* are the predominant microorganisms isolated from pasteurized milk and raw milk. Spoilage of pasteurized milk occurs due to heat-resistance spores produced by *Bacillus* species in the raw milk. In addition, inadequately cleaned and sanitized vessels, churns, pipe lines, and equipment due to clean-in-place (CIP) and packaging machines were also implied as a source of *B.cereus* contamination of pasteurized (Christiansoon, 2001; Merin *et al*., 2002; Soleimaninanadegani, 2013). In dairy farms, contamination of raw milk by *B.cereus* is most commonly associated with insufficient hygiene control systems (Soleimaninanadegani, 2013).

Numerous studies have been conducted to determine the incidence of *B. cereus* in various foods and drinks, but recent study on the detection of toxigenic strains of *B. cereus* in milk in Malaysia in general and Kelantan state in particular is poorly reported or unknown. Therefore, the aim of this study was to detect presence of *B. cereus* in raw bulk milk, pasteurized milk and milking utensils in a dairy colonies and Dairy Industry Development Centre in Kelantan. In addition, this study was conducted to detect toxigenic strains of *B.cereus* using PCR and determine antibiotic resistance patterns of *B. cereus* isolates from milk samples and milking utensils.

Materials and Methods

Sample collection

Milk samples were collected from milk churns of dairy farmers in Pasir Puteh, Ketereh and Tanah Merah Kelantan. A total of 60 samples comprised of

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18 raw bulk milk, 16 pasteurised milk samples and 26 swab samples from milking utensils were collected. The raw milk in the churns was pipetted using 10 ml disposable pipette, placed into 20 ml sterile universal bottles and labelled appropriately. The swab samples from milking utensils and pasteurized churns in Dairy Industry Development Centre also collected and placed into transport media containing Amies and were collected and stored into nutrient broth. Refrigerated pasteurized milk in bottles were bought and placed in ice box container before transporting to bacteriology laboratory at Faculty of Veterinary Medicine, Universiti Malaysia Kelantan.

Isolation and identification of Bacillus cereus

One ml of milk samples either raw or pasteurized were added into 9 ml buffered peptone water in universal bottle and was incubated aerobically at 37°C overnight. Likewise, swab samples were incubated in 5 ml of buffered peptone water and were incubated aerobically at 37°C overnight. The enriched milk and swab samples were directly streaked onto blood agar and the primary culture were incubated at 37°C for 24 hours. Subsequent colony morphology identification, Gram-staining, sub-culturing and biochemical tests were conducted to routinely identify *B. cereus* species. Presumptive *B. cereus* isolates were stock cultured on nutrient agar slant for further identification and characterization.

Antibiotic sensitivity test

The antibiotic sensitivity test was carried out for all *B. cereus* isolates identified through routine microbiology according to Kirk-Bauer method. The antibiotics used were beta lactamase antibiotics and antibiotics that are clinically used in human medicine including Ampicillin 10, Oxytetracyline 30, Streptomycin 10, Bacitracin 10, Erythromycin 15, Nalidixic Acid 30, Ciprofloxacin 5, Cepoxitin 30, Ceftazidime 30, Cefataxime 30, Tetracycline 30, Novobiocin 5, Amoxicillin and Clavulanic Acid 30, Gentamicin 10 and Chloramphenicol 30. The results were interpreted according to Clinical and Laboratory Standards Institute (CLSI, 2012).

Amplification of B. cereus *group specific gene and toxigenic genes (*hbl *and* nhe*)*

Genomic DNA was extracted from all the 19 presumptive *B. cereus* isolates by using Promega DNA extraction kit (USA) following the manufacturer's instructions. For this study, three sets of primers were used namely, *B. cereus* group specific BaIF, 5' TGC AAC TGT ATT AGC ACA AGC T 3' and BaIR, 5' TAC CAC GAA GTT TGT TCA CTA CT 3', hemolytic enterotoxin *(hbl)* gene specific HbIA15' GCT AAT GTA GTT TCA CCT GTA GCA AC 3' and HbIA2, 5' AAT CAT GCC ACT GCG TGG ACA TAT AA 3' (Das *et al*., 2009) and a primer pair for non-hemolytic enterotoxin *(nhe)*, nhe A 5' TAC GCT AAG GAG GGG CA 3' and nhe B, 5' GTT TTT ATT GCT TCA TCG GCT 3' (Hansen and Hendriksen, 2001). Two microliter of DNA template was amplified in 10 µl of readymade 2x master mix (Promega), 10 µm forward and reverse primers and 4 µl of deionized water respectively. PCR amplifications were conducted using MyCycler™ Thermal Cycler (Bio-Rad, USA). The amplification protocols used were with initial denaturation at 95°C for 3 minutes, consisted of 30 cycles of 94°C for 45 seconds (denaturation), 55°C for 45 seconds (annealing), 72°C for 45 seconds (extension) and 72°C for 5 minutes for final extension. While for HblA1 and HblA2 primers, the PCR reaction was performed with an initial denaturation at 95°C for 3 minutes, 94°C for 30 seconds, annealing at 58°C for 45 seconds, extension at 72°C for 60 seconds in 30 cycles and 72°C for 5 minutes for final extension. To amplify the non-haemolytic enterotoxin gene, the protocol was set with initial denaturation at 95°C for 3 minutes, denaturation at 94°C for 45 seconds, annealing at 55°C for 45 seconds and extension at 72°C for 45 seconds and final extension at 72°C for 5 minutes. Gel electrophoresis of the PCR products were done in 1.5% of agarose gel in 1X TBE buffer and the gels were analysed using GelDoc™ EZ Imager gel documentation system (Bio-Rad, USA).

Results

Based on routine bacterial culture and biochemical test results, 31.67% (19/60) were persumptively identified as *B.cereus*. However, only 47.37% (9/19) of the isolates were confirmed to be B.cereus by using PCR amplification of the isolates (table 1). Out of the 9 confirmed *B.cereus* isolates, three were from pasteurized milk samples of different flavours, i.e one from orange flavoured and the other two from corn flavoured milk. Antibiotic sensitivity test results shown that all the 9 isolates were susceptible to Gentamicin 10, Chloramphenicol 30 and Ciprofloxacin 5. However, four of the 9 isolates were resistant to the beta-lactam antibiotics namely, Ampicilin 10, Cefoxitin 30, Ceftazidime 30 and Cefataxime 30 (Figure 1).

Out of the nine *B. cereus* isolates six were found to be positive for the non-hemolytic enterotoxigenic gene *(nheA)*, however, none of the isolates were positive for the haemolytic eneterotoxigenic gene

Table 1. Isolation and identification of *B.cereus* from bulk milk, pasteurised milk and milking utensils using PCR.

Samples	Quantity	Positive for *B. cereus* group specific gene (*Bcl*)	Detection rate (%)
Raw bulk milk	18	1	5.56
Pasteurized milk	16	3	18.75
Milking utensils	26	5	19.23
Total	60	9	15

Table 2. Comparison between toxigenic strains detection of *B. cereus* in raw bulk milk, pasteurized milk and milking utensils.

Samples	Positive *B. cereus*	Positive *nhe*A enterotoxin (%)	Positive *hbl*a enterotoxin (%)
Raw bulk milk	1	1 (100)	0(0)
Pasteurized milk	3	3(100)	0(0)
Milking utensils	5	2(50)	0(0)
Total	9	6(66.67)	0(0)

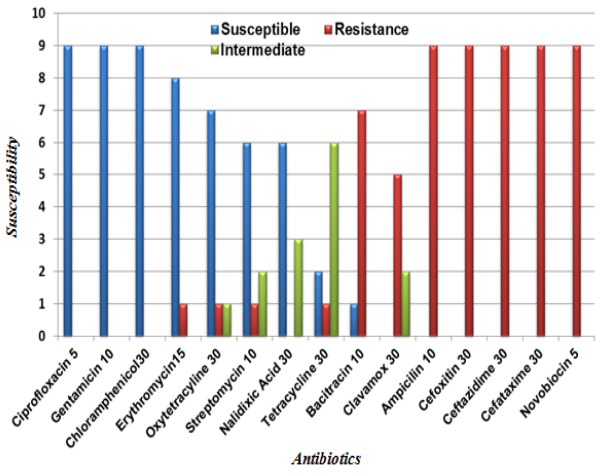

Figure 1. Antibiotic susceptibility patterns of *B.cereus* isolates from from bulk milk, pasteurised milk and milking utensils

(hblA) (Table 2).

Discussions

In this study, *B. cereus* was detected in 15% (9/60) of the samples including raw bulk milk, pateurised milk and milking utensils. Moreover, 6 out of the 9 isolates were positive for non-hemolytic enterotoxigenic (*nhe*A) strains as confirmed by PCR. However, no hemolytic enterotoxigenic strains were detected. In a study conducted in China, *B. cereus* was found in 71.4% and 33.3% in spring and in autumn samples of full-fat milk (Zhou *et al*., 2008). Another study conducted in Poland, Bartoszewicz *et al.* (2008) reported that *B. cereus* group spores were detected in 19 of 20 milk samples.

Detection of *B. cereus* in raw bulk milk, pasteurized milk and milking utensils can be due to several factors. Contamination of raw milk by *B. cereus* may increase with use of contaminated utensils such as improper cleaning and disinfectantion of milking machine, churns and use of dirty clothes to clean the equipments. Types of rearing system of dairy cows such as semi- intensive may also contribute to contamination as there is tendency for teat contamination during grazing. It has also been suggested that soil is the primary source of *B. cereus* contamination in raw milk and there were significant correlations between dirtiness of the teats and access alley and contamination of raw milk with spores (Christiansson *et al*., 1999). According to investigation in the Netherlands, *B. cereus* spores were detected in 23% of bulk tank milk samples collected from farms at which the cows were pastured whereas *B. cereus* was found in only 4% of bulk tank milk samples from farms at which the cows were housed throughout the year (Slaghuis *et al.*, 1997).

Poor hygiene in terms of cleaning and disinfection

of milking utensils, improper utensils storage and source of water used in process of cleaning may lead to contamination of *B. cereus* in milking utensils (Peng *et al*., 2001). Almost all of the farm stores the milking utensils exposed to environment, high moisture area and on wooden shelf which increase the chance of adherence of spores and vegetative cell to metal surface and later cross contaminate the raw and pasteurized milk. *Bacillus cereus* can naturally be found in the environment including water. Therefore source of water is also important to be considered as one critical point where contamination can take place (Peng *et al.*, 2001). The fact that *B. cereus* are spore forming bacteria added to the spores' ability to survive pasteurization process makes it difficult to get rid off contamination risks by *B. cereus* even by pasteurization. According to Schoeni and Wong (2005), instead of destroying spores, the heat treatment can trigger spores to germinate, and temperatures between 65 and 75°C have been reported as optimal for heat activation of *B. cereus* spores. Furthermore, recontamination of the pasteurization machine by *B.cereus* spores as a result of improper hygiene practices were also reported to contribute to the presence of the bacteria in the pasteurized milk (Lin *et al.*, 1998).

Contamination of milk by *B. cereus* is important both from economic and public health points of view as it may result in degradation of milk and milk products and may result in foodborne illness. As it grows in milk, *B. cereus* may produce lipases and proteinases that degrade milk components. The term "bitty cream" is used to describe quality defects caused by the activity of lipases, produced by *B. cereus*, leading to aggregation of fat globules and development of rancid and fruity off-flavours (Meer *et al*., 1991). *Bacillus cereus* also produces proteinases that in turn cause quality defects called "Sweet curdling". It was also reported that the action of proteinases may lead to "sweet curdling" gelation of the milk in the absence of a reduction in pH and development of bitter off-flavours (Meer *et al*., 1991). The detection of toxigenic strains of *B. cereus* in fresh and pasteurised milk samples in this study also underscores the importance of routine screening of dairy products to safeguard the public health.

Attempts to control *B. cereus* may be reasonably challenging as contamination of milk may occur at any point in the production and processing lines. This is due to the fact that contamination of raw milk, milking utensils, processing facilities the processed milk products by spores is possible at any stage. Presence of *B. cereus* in sufficient infective dose in milk and milk products may lead to illnesses and may pose public health threat. Food borne illness caused by *B. cereus* may result in vomiting and diarrhoea which may often be related to other more potent and common bacterial pathogens such as *Salmonella* spp. and *E. coli*. Due to huge margin of potential sources based on findings, appropriate intervention strategies are recommended. Recommendations such as control and prevention to increase public awareness through public education, proper hygiene practices in farms and dairy processing plants, regular surveillance and quality control including intensive screening for milk and milk products have to be in place. This research had certain limitation so further study is recommended by increasing samples in term of size and diversity for better understanding of the occurrence of *B. cereus* in milk and milk products. Prevalence and association between risk factors that may contribute to presence of *B. cereus* in milk can be studied further to have more significant result. Enumeration can be done to know the infective dose of *B. cereus* in the isolates which can lead to clinical signs. Furthermore, detection of other toxigenic strains that cause emetic syndromes in addition to diarrheal toxins and molecular typing of the isolates can be done to trace the spread and origin of the bacteria.

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