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SHORT COMMUNICATION

Enterotoxigenic *Bacillus cereus* from cooked chicken meat: A potential public health hazard

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ABSTRACT

Aims: This study was conducted to isolate *Bacillus cereus* from raw and cooked chicken meat from selected retail shops and wet markets in Kota Bharu and to determine the antimicrobial resistance patterns of *B. cereus.*
Methodology and results: A total of sixty samples (30 from raw and 30 from cooked chicken meat) were tested for presence of *B. cereus*. Isolation and identification of *B. cereus* was done by using routine bacterial culture and Polymerase Chain Reaction (PCR). *Bacillus cereus* was detected in 16.67% (10/60) of the samples tested. All isolates were negative for the enterotoxigenic gene, *nhe* genes, however, six of the isolates were found to be positive for *hbla* genes. *B. cereus* isolates showed 100% resistance towards beta lactam antibiotics.
Conclusion, significance and impact study: Although only 60 samples are analysed in the current study, the fact that toxigenic strains of *B. cereus* were isolated in cooked chicken meat intended for human consumption implies the potential public health risk it might pose. Further study with increased sample size, screening other toxigenic strains of *B. cereus* and molecular typing is recommended to have a more detailed understanding of the occurrence of the bacteria in chicken meat in Kota Bharu. It is necessary to educate the public on the risks of food contamination by bacteria that may cause food borne illnesses. Some precautions such as routine checking of the freshness of food before consumption, hygienic preparation and proper cooking of food can be implemented to reduce the risks of food borne illnesses related *B. cereus* and other potentially dangerous bacteria.

Keywords: Chicken meat, food poisoning, *Bacillus cereus*, enterotoxigenic genes, PCR

INTRODUCTION

Microbial food safety and food-borne infections are important public health concerns worldwide. Ingestion of contaminated food such as chicken meats has been resulting in a number of food-borne illnesses. Pathogens that play role in food-borne diseases are mostly zoonotic in origin (Busani *et al*., 2006). Food-borne infections and intoxication have been assumed to be strongly associated with health hazard in the recent years. According to some studies, the primary cause of human food poisoning is contaminated poultry meat (Mulder, 1999). The abilities to form thermoduric endospore, to grow and survive at refrigeration temperature and toxin production are the major factors that make *B. cereus* a potential threat to food processing (Okanlawon *et al.*, 2009). According to the Food and Drug Administration of United State (FDA) reports, *B. cereus* group are associated with two different clinical syndromes of food poisoning which are diarrheal and emetic (vomiting) syndromes (Tallent *et al.*, 2012). In Malaysia, the first outbreak of food poisoning due to *B. cereus* was reported by Rampal in 1984 (Rampal, 1984). The outbreak was reported to affect 114 students staying in the hostel of a religious secondary school in Klang. Even though the outbreak of food poisoning involving *B. cereus* is rare, still there are reports that indicate the importance of the bacteria in causing food borne illnesses (Eglezos *et al*., 2010; Tallent *et al.*, 2012). Although there are very few reports as to the contamination of food by *B. cereus* in Malaysia, no study was reported on the contamination of raw or cooked chicken meat in Kelantan. Therefore, this study was conducted to isolate *B. cereus* from raw and cooked chicken meat from selected retail shops and wet markets in Kota Bharu and to determine the antimicrobial resistance patterns of *B. cereus* towards selected antibiotics including beta-lactams.

MATERIALS AND METHODS

Sample collection

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Thirty (n=30) cooked chicken meat samples were collected from twenty restaurants, eight from festival food and two from fast food restaurants. Likewise, 30 (n=30) raw chicken meat samples were collected from different twenty roadside stalls, seven wet markets and three supermarkets. Each sample was placed into ziplock-bags and 10 mL normal saline water was added into each sample collection bag. Then the samples were homogenised using stomacher and 1 mL of suspension was transferred into 10 mL buffered peptone water and incubated at 37 °C for 12 h.

Isolation and identification of *B. cereus*

The samples were taken from enrichment media and streaked on Horse Blood Agar. The streaked blood agar plates were incubated at 37 °C for 24 h. After incubation, the colonial morphologies of the growths were analysed. All the typical colonies of *B. cereus* with irregular, rough and haemolytic colony were subjected to Gram staining. All the colonies that show Gram positive rod were sub-cultured on Horse Blood Agar and were incubated at 37 °C for 24 h and biochemical tests including oxidase, catalase, motility and MRVP tests were conducted for further identification.

Detection of *B. cereus* and enteroxin gene (*hbla*) by Polymerase Chain Reaction

DNA extraction was conducted by using commercial DNA extraction kit (Promega®, USA), following the procedures recommended by the manufacture. All suspected *Bacillus cereus* colonies were screened for *B. cereus* group and two enterotoxigenic genes. The primer pair Ba1F/Ba1R is specific for *B. cereus* group. While the primer pairs Hb1A1/Hb1A2 and nheA344S/nheA843A were used to screen the presence of *hbla* and *nhe* genes respectively. Five microlitres of template was amplified in 25 µL of 2× *Taq* Master Mix (Vivantis) consisting of 5 µL *Taq* DNA Polymerase , 2× Vibuffer A, 0.4 mM dNTPs and 3.0 mM $MgCl_2$ and 5 µL of respective primers (Das *et al*., 2009). Oligonucleotide primers were provided from First Base. PCR reaction using Ba1F and Ba1R primers consisted of 94 °C for 45 sec (denaturation), 55 °C for 45 sec (annealing) and 72 °C for 45 sec (extension). While PCR condition for the primer Hb1A1/ Hb1A2 was 94 °C for 30 sec (denaturation), 58 °C for 45 sec (annealing) and 72 °C for 1 min (extension). On the other hand, a cycling condition for nheA344S/nheA843A was 94 °C for 15 sec (denaturation), 55 °C for 45 sec (annealing) and 72 °C for 2 min (extension). In each PCR, total number of cycles was adjusted to 30. In all of the PCR reactions, an initial denaturation was performed at 95 °C for 3 min and after completion of 30 cycles, final extension was carried out at 72 °C for 5 min. The PCR products were analyzed in 1.5% agarose gel (Agarose Vivantis) prepared in 1× TBE buffer added with 1.0 µL of Midori green. Electrophoresis was carried out at 85 V and 400 A for 45 min in a gel electrophoresis system. Finally, the gel was analysed by using Gel Doc^{TM} EZ Imager BIO-RAD.

Antibiotic sensitivity test

Antimicrobial drugs used in this study are beta-lactam antibiotics (Penicillin G, Oxacillin, Clavamox, Ampicillin, Cefotixin, Cefotoxime and Ceftazidine) and others (Tetracycline, Nalidixic acid, Ciprofloxacin, Chlorampenicol, Gentamycin and Novobiocin). Mueller-Hinton Agar (MHA) was used for antimicrobial sensitivity test. A swab of single colony from nutrient agar plate was transferred into the eight milimetres of 0.9% normal saline. Then, the turbidity of samples was compared with 0.5% of McFarland standard and suspension was uniformly streaked on MHA. The selected antibiotics, Penicillin G, Oxacillin, Clavamox, Ampicillin, Cefotixin, Cefotaxime and Ceftazidine, Tetracycline, Nalidixic acid, Ciprofloxacin, Chlorampenicol, Gentamycin and Novobiocin discs were placed on the agar plate using sterile forceps. All the MHA plates were incubated at 37 °C for 24 h. Diameters of the inhibition zone were measured and interpreted according to criteria set by Clinical Laboratory Standards Institute (CLSI, 2011).

RESULTS

Isolation and identification of *B. cereus*

Out of the 60 samples tested, 18.3% (11/60) samples comprised of 9.1% (1/11) and 90.9% (10/11) isolates respectively from raw and cooked chicken meat samples were positive for *B. cereus* based on routine phenotypic isolation and identification. The samples showed the typical colony of *B. cereus* which is irregular, rough and haemolytic colony and Gram positive rod.

Polymerase Chain Reaction

Eleven phenotypically positive samples were tested on PCR for further confirmation of *B. cereus* by using Ba1F/Ba1R primer. Ten out of 11 (16.67%) of the suspected isolates were found to be positive for *B. cereus.* Figure 1 represents the results of PCR products for detection of *B. cereus* by using *B. cereus* group specific primer Ba1F/Ba1R and ten isolates yielded 533 bp amplified product that represents the *B. cereus* specific gene. Out of the 10 samples of confirmed to be *B. cereus*, 6 were positive for *hbl*a, the enterotoxigenic gene. Figure 2 shows the results of PCR products for detection of *hbla genes.* The presence of *hbl*a gene was confirmed by detecting a PCR product with a size of 834 bp. However, out of 10 samples of *B. cereus*, all samples were negative for *nhe* genes.

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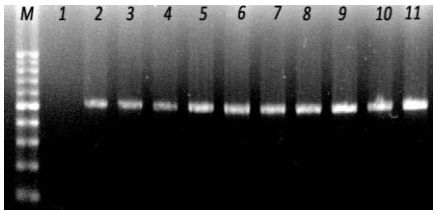

Figure 1: PCR amplification of *Bacillus cereus* specific group gene. Lane M, 100 bp DNA Ladder; Lane 1, negative control; Lane 2, positive control; Lanes 3-11, representative test samples.

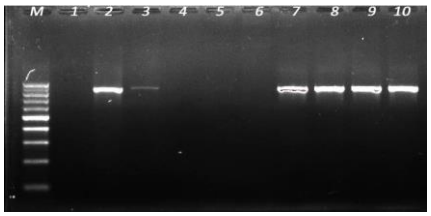

Figure 2: PCR amplification of enterotoxigenic gene (*hbla) of B. cereus*. Lane M, 100 bp DNA Ladder; Lane 1, negative control; Lane 2, positive control; Lanes 3, 7, 8, 9 and 10, representative test samples.

Antibiotic sensitivity test

Mostly, the *B. cereus* isolates were resistant towards beta-lactam antibiotics including Penicillin G (100%), Oxacillin (100%), Amox/Clavalunic Acid (100%), Ampicillin (100%), Cefotaxime (100%), Ceftazidime and Cefoxitin (90%). All *B. cereus* isolates also show resistance towards Novobiocin antibiotic. However all the isolates were susceptible to Gentamycin (Figure 3).

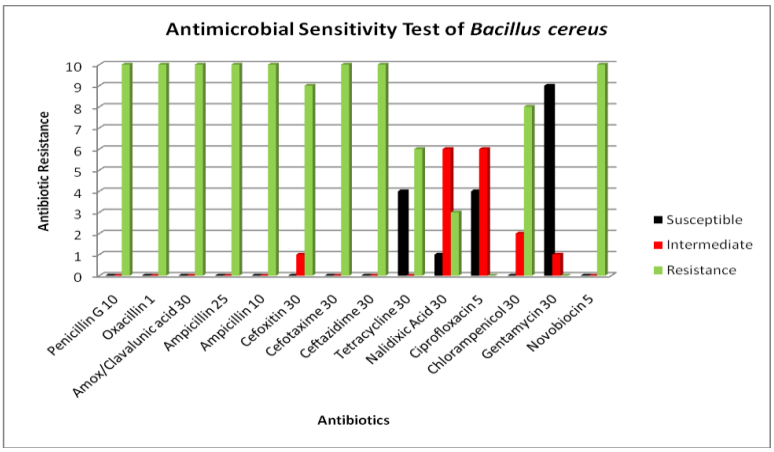

Figure 3: Antimicrobial resistance patterns of *B. cereus.*

DISCUSSION

Bacillus cereus is a spore forming, Gram-positive bacterium commonly found in the environment as well as in a variety of contaminated foods. Some species of *B. cereus* produce toxins that cause vomiting or diarrhea. In this study, *B. cereus* was detected in 16.67% (10/60) of the samples tested by using PCR. One of the positive samples was from raw chicken meat while the other nine were from cooked chicken meat. This may show that cooked chicken meat has high incidence of contamination with *B. cereus.* In Australia, the cooked foods that contain chicken components were reported to be associated with 8 of 18 outbreaks of food poisoning which were commonly related to *B. cereus* infection (Eglezos *et al*., 2010). In a previous research in Malaysia, five local varieties of raw rice samples were negative for *B. thuringiensis* but all (100%) were positive for *B. cereus* (Sandra *et al*., 2012). Furthermore, according to Floristean *et al.* (2007) the highest incidence of contamination with *B. cereus* was found in processed poultry products compared to raw poultry meat. The high detection rate in cooked chicken meat in this study might be due to the possible contamination from food additives or ingredients added to chicken meat during cooking or due to cross contamination by the food handlers, cooking utensils or the environment. Besides that, the spores of *B. cereus* are able to survive in harsh environments including normal cooking temperatures. Earlier report by Floristean *et al.* (2007) has also attributed the contamination of cooked chicken meat to the use of food additives or ingredients. The presence of *B. cereus* in raw chicken meat is maybe due to contamination during processing or handling of the meat. In this study, only one of the raw chicken meat samples collected from supermarket was positive for *B. cereus*. The presence of

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the bacteria in this sample can be due to contamination at any of the processing stages including slaughtering and processing at meat the processing plants, during delivery or transportation, storage and supply. Inadequate temperature during heating or chilling the raw chicken meat also facilitates bacterial growth (Floristean *et al.*, 2007).

Bacillus cereus can cause emetic and diarrheal food poisoning syndromes. *Bacillus cereus* produces one emetic toxin (ETE) and three different enterotoxins including hemolysin BL (HBL), nonhemolytic enterotoxin (NHE) and enterotoxin T (ENT). In this study, the *B. cereus* isolates were screened for two enterotoxin encoding genes namely, hemolysin BL (*hbla*) and non-hemolytic enterotoxin (*nhe*). Six of the isolates were found to be positive for *hbla* genes; however, all the isolates were negative for *nhe* genes. All the six hbla positive *B. cereus isolates were* from cooked chicken meat. The *hbla* genes are known to encode enterotoxins that can cause diarrheal food poisoning syndrome (Wong, 1988). The detection of these genes in the isolates from cooked chicken meat implies the public health risk that might be posed to consumers, especially for the young, elderly and other consumers with weaker immunity.

As to the antimicrobial sensitivity test, the *B. cereus* isolates showed 100% resistance towards beta lactam antibiotics which are Penicillin G, Oxacillin, Amox/Clavalunic Acid, Ampicillin, Cefoxitin, Cefotaxime and Ceftazidime. Reports by others research also show that *B. cereus* is often resistant towards beta-lactam antibiotic. According to the study by Whong *et al.* (2007) the majority of *B. cereus* isolates were resistant to penicillin (80.0%), cefotaxime (56.7%), cefriaxone (53.3%) and ampicilin 44.0%. In addition, the antibiogram pattern from this current study shows that *B. cereus* is susceptible to Gentamycin. Other reports also shown that *B. cereus* isolates were highly susceptible to Gentamycin (99.0%) (Whong *et al.*, 2007). As beta lactam antibiotics are often used for the treatment of food poisoning caused by *B. cereus*, the occurrence of resistance strains towards these antibiotics can be a subject of concern as it may compromise the effectiveness of treatments and hampers the control and prevention efforts. However, this study was conducted on smaller scale and the fact that smaller number of samples analysed investigated in this study compared to other reports may indicate some of its limitations. However, the detection rate, in particular, detection of toxigenic strains of *B. cereus* and the multi-resistance nature of the bacteria revealed in this study casts the light on the importance of such studies which can be conducted more comprehensively and on a larger scale. Therefore, it is recommended to increase sample size, use *B. cereus* selective media and screen for other toxigenic strains to have a more detailed understanding of the occurrence of the bacteria in chicken meat and its virulence factors. Further study can also be done by employing molecular typing methods to better understand the epidemiology and spread of the *B. cereus.* It is also necessary to educate the public on the risks of food contamination by bacteria that may cause food borne illnesses. Some precautions such as routine checking of the freshness of food before consumption, hygienic preparation and proper cooking of food can be implemented to reduce the risks of food borne illnesses related *B. cereus* and other potentially dangerous bacteria.

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