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Antimicrobial resistance pattern of *Bacillus cereus* Strains Isolated from fried rice samples

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Abstract: The aim of the present study was to determine the antimicrobial resistance pattern of *Bacillus cereus* isolates from fried rice samples. A total of 35 isolates of *B cereus* from 70 samples of fried rice were tested for their identification by 16s rDNA test and test sensitivity to fourteen commonly used antibiotics. Antibiotic resistance analysis showed the *B. cereus* isolates were highly resistant to streptomycin 88% (31/35), ampicillin 88% (31/35) and tetracyclin 86% (30/35) followed by resistance towards vancomycin 63% (22/35), gentamcin 57 %(20/35), penicillin G 54 %, (19/35), nalidixic acid 51% (18/35), nitrofurantoin 48% (17/35), kanamycin 45% (16/35), erythromycin (42%, 15/35), ciprofloxacin 42% (15/35), ceftriaxone 37%(13/35) chloramphenicol 34% (12/35) and bactriacin 31% (11/35).
Keywords: Bacillus cereus, isolates, antimicrobial resistance ,pattern, fried rice.

Introduction

Bacillus cereus is regarded as a gram positive bacterium that causes severe food poisoning. This bacterium is found to be rod shaped and forms spores. The spores can stay alive in hot and dry conditions, and remain dormant for many years[1]. The bacterium normally exists in soil, however, it can be found in foods, such as dairy products, rice, cereals and cereals derivatives, dried foods, spices, eggs, vegetables and meats[2,3]. Food poisoning concerning the outbreaks from the past comprises boiled and fried rice, vegetables, cooked meats, soups, and raw vegetable sprouts[4].

According to Food and Drug Administration of the United Stated, food poisonings cause by *B. cereus* are presented into two different clinical syndromes as diarrheal and emetic syndrome. The emetic type results in vomiting after 0.5–6 h of ingestion[5] and diarrhoeal type leads to abdominal pain and diarrhoea after 8 to 16 h of consumption. The diarrheal syndrome has found to be associated with a broad category of food consisting meats, milk, vegetables and fish. On the other hand, the emetic syndrome has been usually correlated with rice products, starchy foods such as potato, pasta, noodles, spaghetti, pastry and cheese products[6]. The diarrheal poisoning is caused by heat-labile enterotoxins produced during vegetative growth of *B. cereus* in small intestine[7]. There are three different enterotoxins in *B. cereus* involved in food poisoning outbreaks are known. Two protein complexes, hemolysin BL (HBL)[8] and nonhemolytic enterotoxin (NHE)[9] and the single protein cytotoxic CytK[10]. HBL is a three-component hemolysin that consist of two lytic components and a binding protein B. The two lytic component are encoded by hblC and hblD. Whereas, protein B is encoded by hblA. Similar with NHE, it also has three component but non hemolytic, encoded by nheA, nheB and nheC. The emetic syndrome is caused by a single heat stable peptide toxin called cereulide[11] encoded by ces gene[12].

It is believed the incidence of *B. cereus* in fried rice is potentially occurred due to food handlers are managed by migrant workers or less education of food hygiene knowledge. The aims of this study are to carry out a study on the prevalence of *B. cereus* in fried rice and analyse the genetic diversity among fried rice isolates of *B. cereus*. The genetic diversity among *B. cereus* will show the locality and the movement of *B. cereus* isolates circulating in different locations. This information is of great value for the purpose of surveillance and control.

Materials and Methods

1 Sample collection

In the present study, A total of 70 (n=70) fried rice samples were purchased from restaurants in Selangor, Malaysia from August 2013 to July 2014. All samples were immediately transported to the laboratory and analyzed within 24 hours. Pure culture of *Bacillus cereus* strain (ATTC 11778) was supplied from Thermos Scientific Sdn. Bhd. (MY.).

1.1 Isolation of *Bacillus cereus*

Samples have been analyzed, using the standard procedure for the detection of *B. cereus*[13] with modifications which have been explained in details by[14]. Generally a total of 25 g of each sample has been put in a stomacher bag and it has been added with 225 ml of Tryptic Soy Broth (TSB; BactoTM,), and it has been homogenized in a stomacher (Interscience, France) for 60 s followed by incubation at 30°C for 12 h. *B.cereus* count has been determined on the basis of ISO 7932l2004 by the surface plating method with mannitol egg yolk polymyxin (MYP) agar (Oxide CM0929). In addition, the dilutions of the stomached fluid have been prepared. Tryptic Soy Broth (TSB; BactoTM). 0.1 ml portions of each dilutions of the fluid have then been transmitted into three tubes and incubated at 30°C for 18 to 24 h. Then a loopful of culture from each tube has been streaked onto Mannitol Egg Yolk Polymyxin Agar Base (MYP; Difco) and added with sterile Polymyxin B Selective Supplement (Difco) and sterile Egg-Yolk Tellurite Emulsion 20% (V/V) (Merck) which is a particular media for the isolation of the Rough and bright pink colonies with a zone of egg yolk precipitation, and use chromogenic medium (chromagarTM *B. cereus* base)add supplement(France) have then been transmitted to nutrient agar slants, *B. cereus.* Identification is consequently confirmed by 16 s r DNA partical sequence[15].

2 Identification by 16 s rDNA Partical Sequence

2. 1 DNA Extraction using DNA Purification Kit

The DNA extraction using DNA Purification Kit (Promega, MY) was conducted as escribed in the manufaturer's manual. The cells were grown in 1 ml of Nutrient broth (NB) for 20 hours at 30°C. One ml of culture was centrifuged for 2 minutes at 13,000 rpm. The supernatant was discarded. The cells was suspended in 480 µl 50 mM Ethylene-diamine-tetraacetic acid (EDTA). A total of 120 µl lysozyme was added and incubated for 60 minutes at 37°C. The cells were centrifuged for 2 minutes at 13,000 rpm and the supernatant was removed. A total of 600 µl Nuclei Lysis Solution was added to the tube and incubated for 5 minutes at 80°C, then cold down to room temperature. Three µl of RNase Solution was then added and incubated for 60 minutes at 37°C, then cold down to room temperature. A total of 200 µl of protein precipitation solution was added, vortex and incubated in ice for 5 minutes, then centrifuged for 3 minutes at 13,000 rpm. The supernatant was transferred to a clean tube containing 600 µl of isopropanol, mixed and centrifuged for 13,000 rpm at 2 minutes. The supernatant was discarded and 600 µl of 70% (v/v) ethanol was added, mixed and centrifuged for 2 minutes at 13,000 rpm. The centrifuge tube was then air-dried for 10 minutes. A total of 100 µl of Rehydration Solution was added and placed into water bath for 1 hour at 65°C. The concentration and purity of the extracted DNA were determined by absorbance at 260 nm and 280 nm using Maestro Nano Spectrophotometer (Maestro Gen, USA). The genomic DNA were then stored at 4^{o}C until use.

2.3 PCR Amplification

The 16S r DNA of strains were PCR amplified using universal primers and PCR conditions described by[15] as shown in (Table 1) which produced 711 bp amplicon. The PCR reaction was performed in a 25 µl volume containing of 12.5 µl of DreamTaq^{TM} PCR MasterMix (Fermentas, MY), 1µl forward and reverse 1µl

of oligonucleotide primer 16S rDNA (1st Base, MY), 7.5 µl of sterile nuclease free water (NFW) and the 3 µl of (50-100 ng) DNA template. The amplicon was purified using a PCR purification kit (Promega, MY). Amplification was performed in an Eppendorf Mastercycler (Eppendorf, Germany) with a temperature program consisting of the initial denaturation at 94 °C for 3 min followed by 25 cycles of denaturation at 94 °C for 1 min, annealing for 1 min at 50 °C and polymerization at 72 °C for 1 min. Final elongation was at 72 °C for 10 min. Negative controls (NFW) were included in each PCR amplification, in order to verify the PCR efficiency and to detect contamination[15].

Table 1: Characteristics of PCR primers used for 16S r DNA.

Traget gene	Primer codes	primer sequences (5'-3')	Product size (bp)	References
16S rDNA	*16S rDNA F* *16S rDNA R*	AGAAGTTTGATCCTGGCTCAG AAGGAGGTGATCCAGCCGCA	711 bp	Devereux et al. (2004)

2.4 Gel Electeriophoresis

The extracted DNA from the bacteria were separated by electrophoresis technique on 1.5 % (w/v) agarose gel in 1X TAE buffer (40 mMTris-acetate, 1 mM EDTA, pH 8.0) (1st Base, Malaysia) at 100 V for 40 minutes following PCR amplification. The gel was pre-stained with Maestrosafe™ Nucleic Acid (V-Bio Science, Malaysia) while GeneRuler™ 1 kb DNA ladder and 100 bp (Fermentas, MY) were used as DNA size marker. Finally, all gels were viewed and captured by UV trans-illuminator Gel Documentation System (Syngene, UK).

3 Antibiotic Susceptibility Test

A colony of *B. cereus* isolate were inoculated into 10 ml Nutrient broth and grown the culture for 20 h at 30 °C. A 0.1 ml were then spread onto Mueller-Hinton agar (Sigma, Switzerland). Antimicrobial sensitivity discs (Sensi Discs, BBL, Becton Dickinson) were placed on each plate. Two discs were used for each of the 14 antibiotics. The discs contained; Ampicillin (30µg) ,Bacitracin (10U) Ceftriaxone (30 µg) Ciprofloxacin (5µg) Chloramphenicol (30 µg), Erythromycin (15 µg), Gentamcin (10 µg), Kanamycin (30 µg), Nitrofurantoin (300 µg) Nalidixic acid (30µg), Penicillin (10U), Streptomycin (10 µg), Tetracyclin(30 µg) and Vancomycin (30 µg), and plates were incubated at 30º C for 24 h after the addition of the respective antibiotics and the diameter of the zone of resistance around each disc was measured with reference to interpretive standards methods[16,17]

Results and Discussion

All *B. cereus* isolates specifically known as BC1 to BC35, were further identified by 16S rDNA with similarity of 80%-100% when it blast in the NCBI data base (Figure 1-2) shown the amplicons of *B. cereus* isolates using 16s rDNA universal primers which produced 711 bp in size.

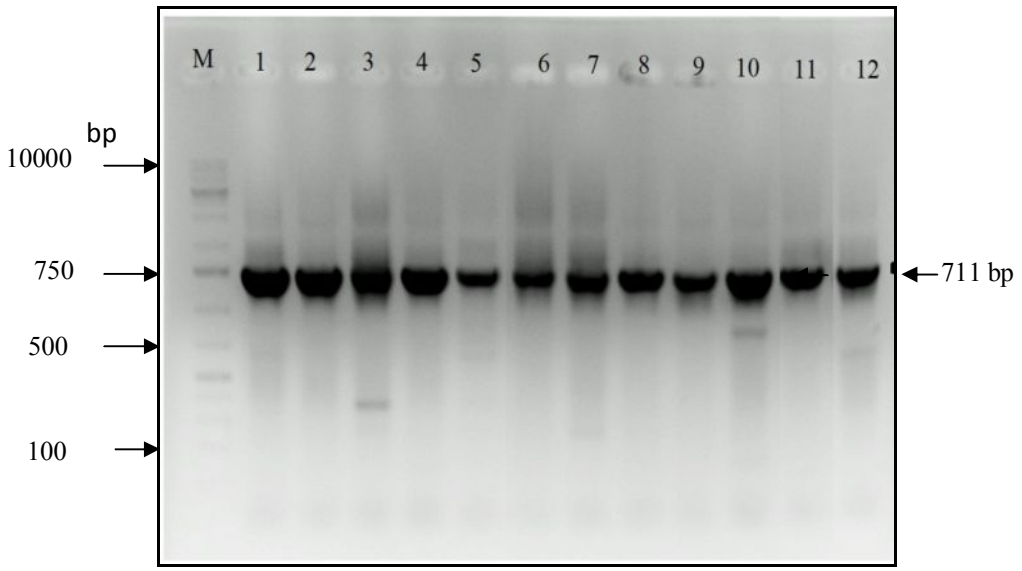

Figure1: Amplicons of *Bacillus cereus* isolates using 16s rDNA universal primers on 1.5 % (w/v) agarose gel. Lane M: 1 kb DNA ladder; Lane 1-11: *B. cereus* isolates (BC1-BC11); Lane 12: Positive control.

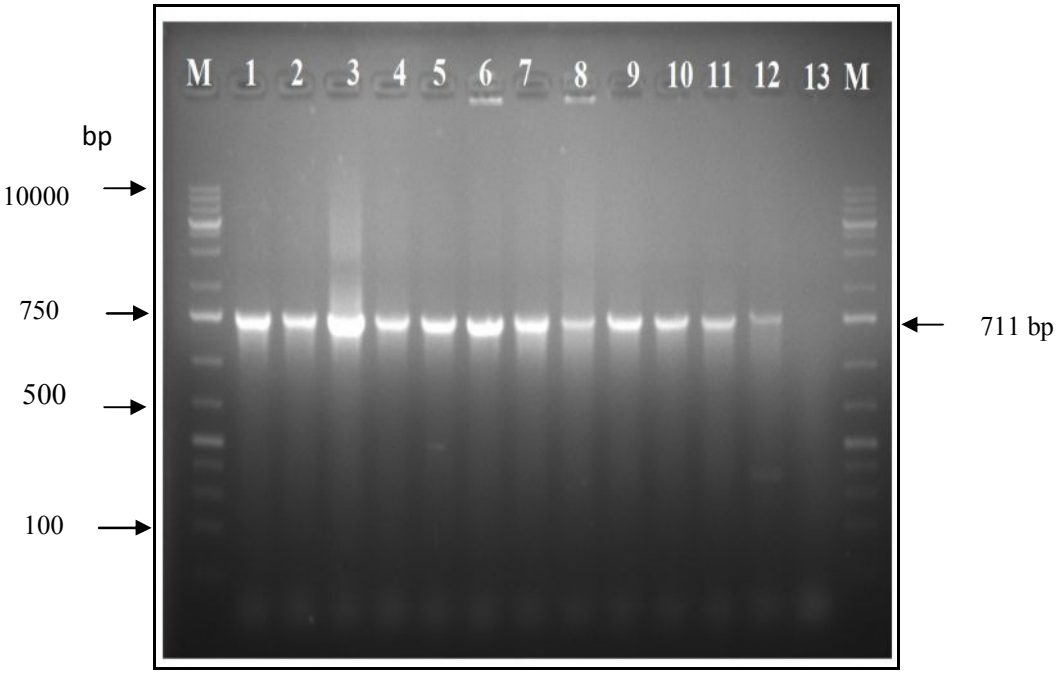

Figure 2: Amplicons of *Bacillus cereus* isolates using 16s rDNA universal primers on 1.5 % (w/v) agarose gel. Lane M: 1 kb DNA Lane 1- 11: *B. cereus* isolates (BC12-BC22); Lane 12: Positive control.

All data was transformed as follows before analysis. Antibiotic resistance values were coded as 0 while susceptibility was coded as 1. As shown in (figure 1) and (Table 2-3) , it was observed that 88 % (31/35) of the isolates were resistant to both Streptomycin (S) and Ampicillin (AMP). This was further explained by the result of chi square analysis which showed that there was no statistical difference ($p > 0.005$) in the resistance of Streptomycin (S) and Ampicillin (AMP) among the isolates. Thirty (30) isolates were resistant to Tetracycline (TE). Similarly, 63% of the isolates were resistant to Vancomycin (VA), 57 % resistant to Gentamicin (CN) and Penicillin (P) while 54 % isolates were resistant to Nalidixic Acid (NA) and Nitrofurantoin=F respectively. One way analysis of variance (ANOVA) and t test of the data showed that there was no significant difference ($p > 0.005$) in the resistance of the isolates to antibiotics tested.

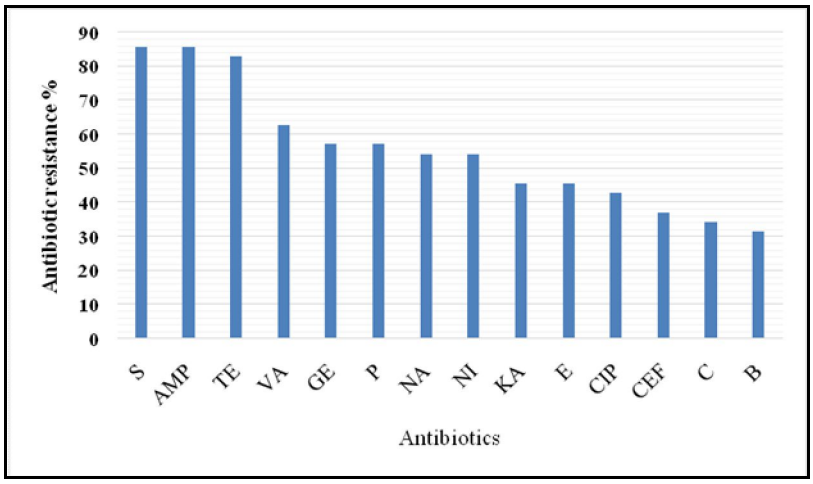

Figure 1: Antibiotic resistance of isolates. Penicillin=P, Vancomycin=VA Tetracycline=TE, Streptomycin=S, Nalidixic Acid=NA, Ampicillin=AMP, Nitrofurantoin=F, Erythromycin=E, Kanamycin=K, Ciprofloxacin=CIP, Chloramphenicol=C, Bacitracin=B, Gentamicin=CN, Ceftriaxone=CEF.

Conducted[18] a study considering 300 isolates of *B. cereus;* those are taken from nine different food of Nigeria for examining their sensitivity towards 10 types of commonly used antibiotics. It explored that all *B. cereus* isolates are susceptible to ciprofloxacin (5 mg), chloramphenicol (30 mg), ofloxacin (10 mg). The study also found that tested foods have less than 10% resistance to gentamicin (10mg) and nalidixic acid (10mg).Overall resistance to penicillin G (82%), cefotaxime (56.7%), ceftriaxone (53.3%) and ampicillin (44%) were most frequent, whereas isolates were least resistance to tetracycline (6.7%) nalidixic acid (3%) and gentamicin (1%). Whereas our study is line with[18] in some aspects of such as ampicillin , gentamcin, penicillin G, ceftriaxone, ciprofloxacin , and chloramphenicol. However, there is some contrasting findings in terms of nalidixic acid , and tetracycline. conducted[19] a study considering a total of sixty samples (30 from raw and 30 from cooked chicken meat), where they found 16.67% of *B. cereus* in the total sample. The study also found that tested foods have resistance to Penicillin G (100%), Oxacillin (100%),Amox/Clavalunic Acid (100%), Ampicillin (100%), Cefotaxime (100%), Ceftazidime and Cefoxitin (90%). All *B. cereus* isolates also had resistance towards Novobiocin antibiotic. However all the isolates were susceptible to Gentamycin. focused[20] on 34 type of ice-cream to detect *B. cereus* isolated and they found the presence of 8 forms of commonly used antibiotics and resistant to ampicillin (29.5%), penicillin (29.5%),trimethoprim-sulphamethoxazole (12%), tetracycline 3% and , erythromycin, 3%. However, the study found no in terms of vancomycine, gentamicin and ciprofloxacin. conducted[21] a study on 50 smaple of rabbit meat where they found 36 % of *B. cereus* , while antibiotic resistance of *B. cereus* isolates was found to be 100% penicillin, 94.4% ampicillin, 27.7% streptomycin, 22.2% gentamicin and erythromycin, and no resistance was detected to chloramphenicol and vancomycin.

Table 2: Antibiotics resistant of *Bacillus cereus* with and without plasmid DNA.

Antibiotic tested	Total no.(%) of isolates[a] resistant
Ampicillin	31 (88%)
Streptomycin	31 (88%)
Tetracyclin	30 (86%)
Vancomycin	22 (63%)
Gentamcin	20 (57 %)
Penicillin G	19 (54 %)
Nalidixic acid	18 (51%)
Nitrofurantoin	17(48%)
Kanamycin	16 (45%)
Erythromycin	15 (42%)
Ciprofloxacin	15 (42%)
Ceftriaxone	13 (37%)
Chloramphenicol	12 (34%)
Bactriacin	11 (31%)

a The total number of isolates tested were 35

Table 3: Antimicrobial resistance of *Bacillus cereus* isolated from fried rice samples.

Number of *B.cereus* strains	Antibiotic resistance patterns[a]	Types of antibiotic Resistance patterns
BC 1	AmpBCipFKPT	U1
BC 2	AmpCipFPSVa	U2
BC 3	AmpFEPS	U3
BC 4	AmpCipPS	U4
BC 5	AmpGPSVa	U5
BC 6	AmpCip	U6
BC 7	AmpCefPSVa	U7
BC 8	BKPSTVa	U8
BC 9	AmpPSVa	U9
BC 10	AmpGNAPSVa	U10
BC 11	AmpCipGNaPSTVa	U11
BC 12	AmpCipFNa	U12
BC 13	CipCef F	U13
BC 14	AmpCef Cip FGPST	U14
BC 15	AmpCCipEFPSVa	U15
BC 16	AmpCipEFKP	U16
BC 17	AmpCip EGKS	U17
BC 18	AmpCCipEGKNaS	U18
BC 19	AmpC CefCipEGKNaST	U19
BC 20	CefCipGKST	U20
BC 21	CefGKNaPSTVa	U21
BC 22	AmpCefFGKNaPSTVa	U22
BC 23	AmpBCCefFGKSTVa	U23
BC 24	AmpBC FGKNaPT	U24
BC 25	AmpBCCefFGKNaSTVa	U25
BC 26	AmpB CefCip FKSTVa	U26
BC 27	B CEFKNaPVa	U27
BC 28	AmpBCefEFGKNaPSTVa	U28
BC 29	AmpBC CefE KFNaVa	U29
BC 30	AmpCCefEFGK SVa	U30
BC 31	AmpENaSTVa	U31
BC 32	AmpBCCip EGNa STVa	U32
BC 33	AmpBCE, NaS Va	U33
BC 34	AmpC EG Na S Va	U34
BC 35	AmpBC EGNaVa	U35

Bactracin(B), ciprofloxacin(Cip), streptomycin(S),chloramphenicol(C), erythromycin(E), Nitrofurantoin (F), tetracycline(T), gentamycin(G), kanamycin(K), ceftriaxone(Cor), nalidixicacid(Na), penicillin G (P), ampicillin (Amp), vancomycin(Va). Descriptive statistical analysis of obtained data was analysed using IBM SPSS 22.

Conclusion

Based on the findings which result from this thorough analysis of 35 selected *B. cereus* strains on 16S r DNA sequences, strain categories were screened on potential dangerous features which could lead to intoxication if conditions are favorable, The Antibiotics resistance of the isolates was examined against 14 commercial antibiotics. The results revealed that there was no statistical difference ($p > 0.005$) in the resistance of Streptomycin (S) and Ampicillin (AMP) among the (31) isolates. Thirty (30) isolates were resistant to Tetracycline (TE). Correspondingly, 63% of the isolates were resistant to Vancomycin (VA), 57 % resistant to Gentamicin (CN) and Penicillin (P) while 54 % isolates were resistant to Nalidixic Acid (NA) and Nitrofurantoin.

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