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ISOLATION, CONVENTIONAL AND MOLECULAR IDENTIFICATION OF *BACILLUS CEREUS* AND ITS IDENTIFICATION IN BEEF SAMPLES FROM QUETTA



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Abstract

The current study was conducted to examine the presence of *Bacillus cereus* in beef samples. Study was designed to isolate *B. cereus* from 50 beef samples, among them 29 (58 %) of the samples were positive. A huge number of bacteria in n=29 out of n=50 samples were observed in beef samples. Isolates were analyzed through different biochemical tests like catalase, citrate utilization, glucose/galactose fermentation, oxidase, motility and Gram staining. A number of isolates were found positive when checked by biochemical tests as well as through PCR (Multiplex polymerase chain reaction). Major degree of genetic similarity is found in the groups of *Bacillus cereus* bacteria. Thus, to efficiently identify and differentiate the *Bacillus cereus* group, a multiplex polymerase chain reaction method was performed for immediate detection using the *groEL* and *gyrB* gene as diagnostic markers. For *B. cereus* group *groEL* gene of 400 bp and 475 bp amplicon size for *gyrB* gene of the *B. cereus* was yielded. In result, the multiplex PCR is proved efficient, simple and rapid for the immediate identification of *B. cereus* group bacteria in beef samples. It shows that Multiplex PCR is proved simple useful and efficient method for the detection of *B. cereus* in beef samples of Quetta.

Keywords: *Bacillus cereus*, conventional identification, biochemical tests, *groEL* and *gyrB* genes, Multiplex PCR

INTRODUCTION

Contaminated food is a serious health problem and it may result in serious diseases when consumed without proper treatment. Several types of pathogens are involved in deterioration of food items. Consumption of food contaminated with *bacillus cereus* will result in syndromes including Diarrheal and Emetic syndromes, as it is an opportunistic pathogen which infects soft tissues in human (1).

It is a spore forming bacteria when consumed produces toxins which results in vomiting and diarrhea and symptoms last up to 24 hours. It is found in the soil and can survive at

normal temperatures used for cooking. It is flagellated, Gram-positive and rod-shaped belongs to the genus of *Bacillus* having multiple species i.e. *B. toyonensis*, *B. thuringiensis*, *B. mycoides*, *B. cytotoxicus*, *B. sensu stricto*, and, *B. anthracis*, *B. pseudomycoides*, *B. weihenstephanensis*. Out of these *B. cereus*, *B. thuringiensis* and *B. anthracis* are 99% similar when checked through genomic sequencing 16S rRNA sequencing (2).

B. cereus group is wide spread and is saprophytic present in the soil sprouting, budding and sporulating (3). Several strains of *Bacillus cereus* and other *Bacillus* species are responsible for food poisoning (3) and many other infections. Once *B. cereus* senses that it is in danger it forms spores, which can resist the environmental stress and are tough as a compared to the vegetative cell (4). *B. cereus* causes two sorts of symptoms i.e. vomiting and diarrhea. Vomiting is caused by the emetic toxins which are produced outside the body while growing in the food while diarrhea is caused by the diarrheal toxins which are produced within the small intestine (5). *B. cereus* is present in numerous food products including meat; however, few food species are free from this organism. Enterotoxin produced by *B. cereus* is also responsible to cause diarrhea because half of *B. cereus* strains produce diarrheal enterotoxin.

There are two strains of *B. cereus* which include psychotropic and mesophilic, the psychotropic strains well grow at refrigerating temperatures and grow poorly at 37°C, while mesophilic strain grows well at 37°C but grows weakly at 10°C (5). The *B. cereus* can grow in anaerobic conditions but shows optimal growth in aerobic conditions. Mesophilic strains show greater acid resistance. The spores of *Bacillus cereus* show more resistance to heat in fatty and oily foods, while the spores show more resistance to dry heat than in moist heat while radiation does not affect spores but kills vegetative cells. To prevent the spores germination benzoate, sorbates and ethylenediaminetetraacetic acid can be used for prevention of vegetative cell growth. While *B. cereus* causes emetic syndrome, which is caused by cyclic peptide toxins known as Cereulide having a short incubation (1-5 hours) and recovery (6-24 hours), symptoms include nausea stomach cramps and vomiting which is produced in food during growth (6). While *B. cereus* cause Diarrheal syndrome inside the host intestine by producing enterotoxins. Its incubation period can be up to 8-16 hours while recovery time can be 12-14 hours' symptoms include watery diarrhea with abdominal pain (7). *B. cereus* is rarely life threatening. Although it is mostly food born but *B. cereus* can be found associated with non-food illnesses rarely. It has been found causing infections in post-traumatic and post-surgical wounds and may be in immunocompromised individuals (8).

MATERIAL AND METHODS

SAMPLE COLLECTION

Present study was conducted from March 2019 to March 2020. A total of 50 samples were collected from different areas of District Quetta randomly. After collection, all the samples were immediately transferred to the laboratory with proper aseptic method for further processing. The inoculated samples were incubated overnight at 37°C as previously described by Tiwari *et al.*, 2015 (9).



ISOLATION AND ENUMERATION OF *BACILLUS CEREUS*

For *B. cereus* isolation all the samples were prepared as per procedure of Rhodehamel and Harmon (1998) with proper methodology. Twenty-five grams of beef samples were taken in a plastic bag containing 225 ml peptone water (PW) to prepare 1:10 serial dilutions, homogenized and 0.1 ml dilution of each sample was spread slightly onto the surface of the plates. The plates were incubated for 24-48 hours at 35 °C. The precipitate zone surrounded around the MYP colonies with eosin pink color indicates the lecithinase which is known to be *B. cereus*. For more identification a typical colony which is supposed to be *B. cereus* was shifted to the slants of nutrient agar and incubated for 24-48 hours at 35 °C. For more identification all the probable colonies together of *B. cereus* were exposed to the biochemical as well as morphological tests for species identifications (10).

IDENTIFICATION OF *BACILLUS CEREUS* THROUGH BIOCHEMICAL TESTS

Identification of the specie was confirmed by the characters of growth on selective media as well as by conventional biochemical assessments, including Gram staining, oxidase, indole, and catalase, MR (methyl red, motility, VP (voges proskauer), glucose, galactose, fermentation tests and citrate utilization test (11).

MOLECULAR IDENTIFICATION

Extraction of DNA

A loop full colony of the *B. cereus* was taken and transferred for culture to 5 ml (BHI) Brain Heart Infusion incubated for 18-24 hours at 35 °C. For DNA extraction the cultured broth was processed through boiling, chilling and centrifugation. 1 ml of broth culture before harvesting was taken into a tube and centrifuged at 12,000 rpm for 3 min after the supernatant was discarded. One ml of distilled water was added to the pellet and vortex. The vortexed samples were then boiled at 97 °C for 12 min and frozen quickly at -20 °C for 15 minutes. The tubes were centrifuged at 12,000 rpm for 3 min and the supernatant was used as a DNA template (12).

PCR for DNA Template

For the conformation of *Bacillus cereus* PCR (polymerase chain reaction) methods are used daily. By using (groEL and gyrB genes) all the biochemical tests positive samples were identified by Multiplex polymerase chain reaction (13).

Reaction Mixture for each sample

PCR mixture was prepared as (10 ul master mix, 5ul DNA, 1.5ul MgCl, 1ul each primer, 0.5 ul dNTPs, 2.5 ul 10X PCR buffer, 0.2 ul Taq polymerase. A total reaction of 20ul was made by adding PCR grade H₂O (12). The amplification was accomplished with a thermal cycler (Eppendorf, Germany). The initial step was denaturation with 94°C for 5min followed by 30 cycles of 94°C for 30 seconds, annealing 63 °C for 30sec, extension 72°C for 30 sec, and final extension was 72°C for 5min (13).

Multiplex PCR for B. cereus

For the performance of Multiplex PCR two types of primers (groE & gyrB gene) were used (Table 4). The reaction mixture for each sample was as following (10 ul master mix, 5ul DNA, 1.5ul MgCl, 1ul each primer, 0.5 ul dNTPs, 2.5 ul 10X PCR buffer, 0.2 ul Taq polymerase. A total reaction of 20ul was made by adding PCR grade H₂O (14).

Conditions for amplification

The initial step was denaturation with 94°C for 5min followed by 30 cycles of 94°C for 30 seconds, annealing at 63°C for 30 seconds, extension 72°C for 30 seconds and final extension was 72°C for 5 minutes (13, 14).

Gel electrophoresis

For the preparation of gel electrophoresis 1X TAE buffer, 1% agarose gel and 3µl ethidium bromide stain was used. Electrophoresis process was performed with 1X TAE buffer at 100 volts for 45 minutes. For the conformation and length of amplified product (50 bp) DNA ladder was used (13).

RESULTS AND DISCUSSION

BIOCHEMICAL AND CONVENTIONAL IDENTIFICATION

The current study was conducted to examine the presence of *Bacillus cereus* in beef samples. 50 samples were collected for the isolation of *B. cereus*, among them n=29 (58 %) samples were *B. cereus* positive (Table I). Isolates were analyzed through different biochemical tests like catalase, citrate utilization, glucose/galactose fermentation, oxidase, motility and Gram staining. *B. Cereus* Mannitol Egg Yolk (MYP) Agar was used with supplements for the selective and differential enumeration of *Bacillus cereus* from beef samples (Fig. 1.) because the MYP agar has been considered the standard media for plating *B. cereus* and it has been established to be extremely efficient for detecting *B. cereus* even for ratios as testing as one cell of *B. cereus* to 10⁶ cells of other organisms (16).

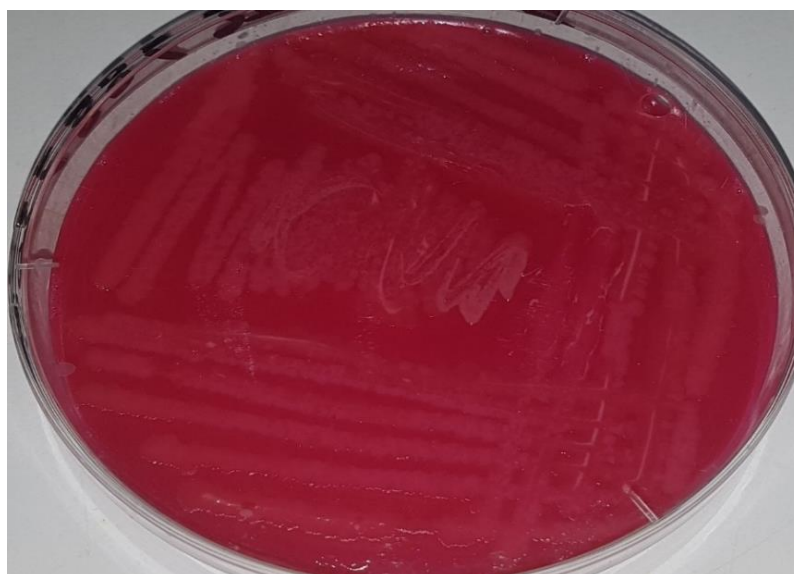


Fig. 1. *Bacillus cereus* on Mannitol Egg Yolk (MYP) agar

Table I. Biochemical tests for *B. cereus* (Different Biochemical tests results)

Biochemical tests	Positive	Negative
Catalase	Positive +ve	
Motility	Positive +ve	
Oxidase		Negative -ve
Citrate	Positive +ve	
Galactose		Negative -ve
MR		Negative -ve
Glucose	Positive +ve	
Oxidase		Negative -ve
VP	Positive +ve	
Indole		Negative -v

SENSITIVITY OF PRIMER PAIRS AND SPECIFICITY OF MULTIPLEX PCR

In the present study, two primer sets were selected to simultaneously detect 2 different species of the *B. cereus* group using the Multiplex polymerase chain reaction. The 2 species-specific primers were synthesized using the *gyrB* gene, plus one specific primer for the *B. cereus* group was constructed from the *groEL* gene. To verify and evaluate the specificity of the primers used, a single PCR was performed with DNA templates prepared from the 50 different bacterial type strains, including positive control strains. The primers for BCGSH-1F and -1R which produced a 400 bp amplicon size from the *B. cereus* group and the primers for BCJH-F and 1R which yielded a 475 bp amplicon size from *B. cereus* (Fig. 2). To avoid nonspecific amplification, the multiplex PCR conditions were optimized with respect to the primer concentrations (15). Following careful comparison of various reactions, the primer concentrations for *B. cereus* and the *B. cereus* group were set up. Thus, multiplex PCR was performed with templates from *B. cereus*, only amplicons of the predicted size were formed *B. cereus* group bacteria. Nonetheless, despite this high homology, the primers used in this study were still able to distinguish *B. cereus* group bacteria with specificity. The *groEL* gene has already been used in a PCR to detect the *B. cereus* group (15). However, the current study constructed a new *B. cereus* group primer set based on the *groEL* gene to enable *B. cereus* group bacteria with different sizes of PCR product to be distinguished from other *Bacillus* species in a multiplex PCR.

A different approach to detect *B. cereus* is amplifying a specific, unique sequence of the organism's DNA. This sort of DNA test would give additional information about the probable hazards concerned with a food product and possibly will, if successful, substitute the immunological and plating methods for detecting the existence of enterotoxigenic *B. cereus*. To test for the presence of different bacterial strains in food, two approaches have been suggested: animal tests or immunological tests. There are commercial kits available for detecting enterotoxins produced by *B. cereus* bacteria through immunological reactions (17, 18).

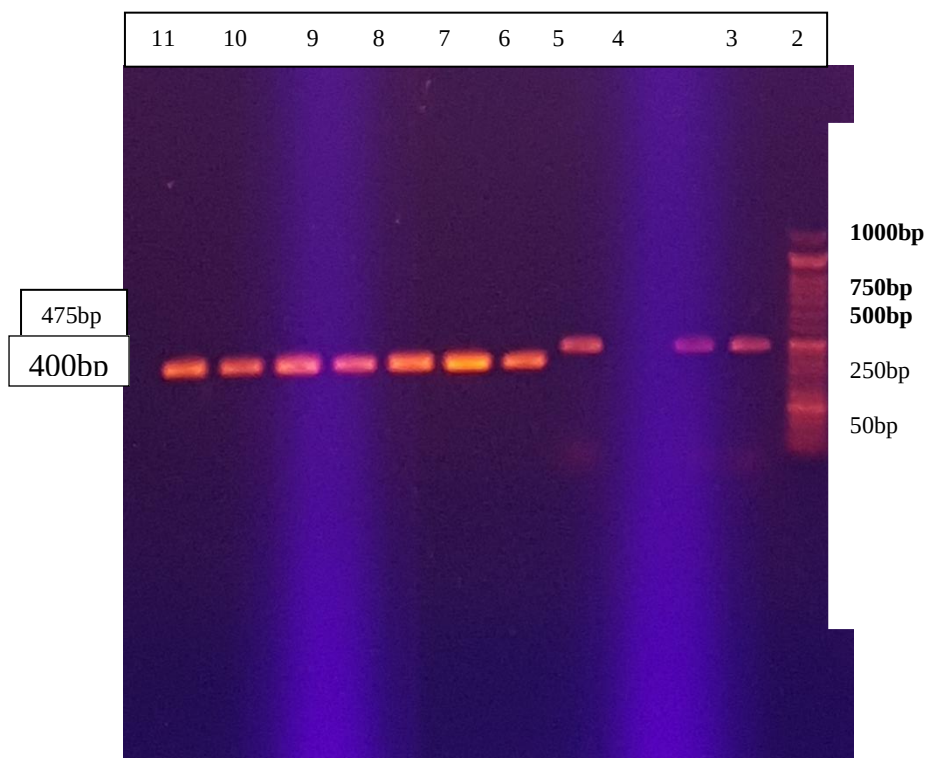


Fig. 2. Genomics DNA product of Multiplex PCR for the *B. cereus* group; Lane 1: DNA ladders (50bp). Lane 2, 3, 4: Positive control *gyrB* gene, Lane 5, 6, 7, 8, 9, 10, 11: *groEL* gene of *Bacillus cereus*

Table II. Primer sequences of *groEL*, and *gyrB* genes of *Bacillus cereus*.

Genes	Direction	Sequences (5'-3')	Amplicon Size
<i>groEL</i>	Forward	GTG CGA ACC CAA TGG GTC TTC	400bp
<i>gyrB</i>	Reverse	CCT TGT TGT ACC ACT TGC TC	475bp
	Forward	TCA TGA AGA GCC TGT GTA CG	
	Reverse	CGA CGT GTC AAT TCA CGC GC	

Furthermore, a rapid and reliable method for detecting *B. cereus* for the enterotoxin is very important to ensure that food products are safe. Since contamination levels may be low, the detection method should be sensitive enough to be able to detect low numbers of *B. cereus* organisms as quickly as possible. The PCR method for testing the desired sequence is a sensitive and fast method for testing food products and determining their enterotoxic potential.

CONCLUSION

In conclusion, the projected multiplex PCR is a trustworthy, simple, quick and effective technique for the immediate identification of *B. cereus* group bacteria in all food products especially beef meat samples.

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