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Insights into biochemical characterization, antimicrobial resistance, and molecular characterization of *Bacillus cereus* from market chevon

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Abstract
In the present study, 165 Chevon samples were gathered from multiple meat shops located in the vicinity of Anand city. Among these 165 specimens, 48 tested positive for *Bacillus cereus* using selective media Polymixin egg-yolk mannitol bromothymol blue agar (PEMBA), that displayed typical bluish growth surrounded by lecithinase activity. All isolates underwent catalase test and VP test and were found positive, and a majority of them also tested positive for nitrate and exhibited motility. Tests for sugar fermentation depicted variability. All isolates underwent testing against a panel of six antibiotics. Antibiotic susceptibility testing revealed Meropenem, Imipenem, and Clindamycin as the most effective. PCR confirmed *B. cereus* identity using the *gro*EL gene. Virulence gene analysis showed the presence of *gyr*B, *bce*T, *hbl*A, and *cyt*K, indicating potential toxin production and cytotoxicity.

Keywords: *Bacillus cereus*, Chevon, foodborne illness, antimicrobial resistance, genes

1. Introduction
In India, meat production totals approximately 8.11 million tonnes, with chicken, buffalo, goat, sheep, pork, and beef contributing about 50.06%, 19.05%, 13.35%, 8.36%, 4.98%, and 4.02% respectively (BAHS, 2019) [2]. Poultry, particularly chicken, is the most popularly consumed meat in the country. However, sheep and goat are the primary sources of red meat. Gastroenteritis, mainly foodborne, is a significant cause of death and illness in developing countries, including India (Desai and Varadraj, 2009) [4].

Foodborne illnesses represent a substantial amount of worldwide health issues, involving various diseases from contaminated food items resulting from viruses, chemicals, microbes, or parasites. These diseases, particularly diarrheal illnesses, can result in severe health issues and, in some cases, death. Commonly isolated bacterial pathogens found in food items are following species of *Bacillus cereus* group- *B. cereus, B. weihenstephanensis, B. pseudomycoides, B. mycoides, B. thuringiensis*, and *B. anthracis*. These pathogens with others, such as Clostridium botulinum, Escherichia coli O157:H7, Campylobacter Jejuni, Listeria monocytogenes, and various Salmonella and Shigella species, pose substantial risks to food safety and public health (WHO, 2007; Ehling-Schulz *et al*., 2005; Lindback and Granum, 2006) [24, 5, 12].

Since last twenty-five years, *Bacillus cereus* posed as the second most concerning among Bacillus species affecting both animals and humans. This bacterium has been found to contaminate different kinds of foods, including boiled rice, milk, meat, desserts, sauces, and fish. *B. cereus* is recognized for causing two major types of symptoms when affected: 1. Emetic, 2. Diarrheal (more common). Both symptoms are manifested by two different enterotoxins. The incubation period for the diarrheal illness is typically 8-16 hours and is associated with wide range of food items, while for the emetic illness is 1-5 hours and is commonly linked to cereal products like rice.

Bacillus species are ubiquitous in the environment and can be very easily found in dust, soil, water, and air. There are approximately 50 described species of Bacillus, some of which have industrial applications, likely in environmental applications, or enzyme production, eg. insecticides.

Whereas, few species tend to be pathogenic to humans as well as animals. The most significant pathogenic species are part of the *B. cereus* group, which comprises *B. cereus*, *B. thuringiensis*, *B. anthracis*, *B. mycoides* and currently identified species are *B. weihenstephanensis* and *B. pseudomycoides*.

2. Materials and Methods

Samples were processed to isolate *B. cereus*. (Tallent *et al*., 2001) [18] as per the standard Bacteriological Analytical Manual (BAM), U.S. Food and Drug Administration (USFDA) procedure.

2.1 Sample collection

Initially, aseptic collection of a total of 165 chevon samples was done from various meat shops in Anand, Gujarat. After this, the samples were placed in an icebox and sent to the laboratory for detailed assessment. Each chevon sample was transferred into 10 ml of Brain Heart Infusion broth (BHI) and incubated for a period of 18-24 hours at 37 °C. The incubation period promotes the growth of *Bacillus cereus*, which would enable deeper analysis of the samples.

2.2 Isolation and identification of the species

Polymyxin pyruvate egg-yolk mannitol-bromothymol blue agar (PEMBA) was used as the selective medium to isolate *Bacillus cereus*. A loopful of the inoculum from the enrichments was smeared on PEMBA, and it was again incubated for 24 hours at 37 °C which resulted into typical blue colonies encircled by an egg yolk precipitate of the same color. For storage, the putative isolates were streaked on nutrient agar slants, and then additional biochemical testings were conducted to determine their identities.

2.3 Biochemical characterization of *B. cereus* isolates

The following Biochemical tests were conducted: 1. nitrate reduction test, 2. catalase test, 3. motility test, 4. Voges-Proskauer (VP) test and Numerous sugar fermentation tests such as: Fructose, Inositol, Mannitol, Dextrose, Sucrose, Dulcitol and Salicin were carried out on all suppositional *B. cereus* colonies. The Presumptive *Bacillus cereus* isolates were confirmed and identified using biochemical tests proposed by Guven *et al*. (2006) [7], Harmon (1979) [9], Schiemann (1978) [14] and Tewari and Singh (2015) [18].

2.4 *B. cereus* Antimicrobial resistance to different antibiotics

The antibiotic susceptibility tests were performed as per the procedure described by Bauer *et al*. (1966) [3] to rule out the pattern of antibiotic resistance of all *Bacillus cereus* isolates. *In vitro* antibiotic sensitivity testing was performed using the paper disc diffusion method with discs supplied by HiMedia Laboratories Pvt. Ltd., Mumbai, India. The isolates were tested against six antibiotics:

1. Meropenem (10 µg).
2. Clindamycin (2 µg).
3. Imipenem (10 µg).
4. Levofloxacin (5 µg).
5. Erythromycin (15 µg).
6. Ciprofloxacin (5 µg).

Tryptone Soya Broth (TSB) (HiMedia) was used to culture *Bacillus cereus* isolates for 12- 18 hours. The grown cultures were then swabbed on the Muller-Hinton agar plates (HiMedia Pvt. Ltd.) using sterile cotton swabs and then left to dry for 30 minutes for pre-diffusion. Thereafter, different antibiotic discs were placed on the agar surface approximately 2 cm apart using ethanol- dipped and flamed forceps. To ensure complete contact with the medium, discs were gently pressed with the forceps and then the plates were incubated for 18-24 hours at 37 °C. After incubation, inhibition zones were formed around each antibiotic disc, and the diameter of each zone was measured and compared with an informative chart provided by the manufacturer. The zones were then graded as per the criteria into a) sensitive, b) intermediate, or c) resistant.

2.5 Determination of genes by Polymerase chain reaction (PCR)

All *Bacillus cereus* isolates underwent a screening process to detect group-specific genes. PCR protocols designed for different genes (groEL, gyrB, hblA, bceT, cytK) were used, with separate protocols for each gene. The PCR protocols were standardized following methods by Park *et al*. (2007) [12] and Desai and Varadraj (2009) [4]. A standard *B. cereus* strain (MTCC-25061) was used for protocol standardization.

3. Results and Discussion

3.1 Isolation on selective media

48 samples out of 165 studied samples showed positive cultural characteristics on the selective medium Polymyxin egg-yolk mannitol bromothymol blue agar (PEMBA). The positive isolates exhibited typical bluish growth surrounded by lecithinase activity on PEMBA. The study pointed out that 29% of the isolates depicted distinctive features of *B. cereus* in chevon samples, which is consistent with findings of Floristean *et al*. (2008) [6] and Guven *et al*. (2006) [7], who reported positivity rates of 22.5% and 22.4% respectively. Tewari and Singh (2015) [18] found a prevalence of 27.3%, while Tewari *et al*. (2015) [18] recorded a higher prevalence of 30.9% in meat samples.

3.2 Biochemical characterization of *Bacillus cereus* isolates

Bacillus cereus isolates in this study revealed characteristics such as, they were gram- positive with spores centrally situated and largely sporulated rods with unswollen sporangia. Confirmation and identification of the presumptive isolates were done based on various biochemical tests such as nitrate reduction, catalase, Voges-Proskauer, and sugar fermentation tests. The tests which yielded positive isolates were considered to be *Bacillus cereus*.

The findings of this study are parallel to those reported by Tewari and Singh (2015) [18] in which all isolates were found to be gram-positive bacilli, positive for catalase test and Voges- Proskauer test and exhibited egg yolk reaction. All isolates showed typical growth on Polymyxin egg-yolk mannitol bromothymol blue agar (PEMBA), were positive for motility, but showed slight variability in nitrate reduction, hemolysis, and Voges-Proskauer tests.

All sugar fermentation tests on 48 samples revealed the highest utilization rate (90%) for Fructose, followed by Salicin (70%), Dextrose (55%), Sucrose (45%), and Inositol (5%) by *Bacillus cereus*. Utilization of Mannitol or Dulcitol was done by none of the isolates. This finding aligns with previous studies by Guven *et al*. (2006) [7], Floristean *et al*. (2008) [6], and Tewari and Singh (2015) [18], in which none isolates produced acid by mannitol fermentation.

45% of isolates were acid producers from sugar which is consistent with Schiemann (1978) [14] who reported 46% of isolates utilizing sucrose. This contrasts with the study by

Shinagawa (1990) [15], where 90.1% of isolates utilized sucrose. However, 30% of isolates fermented salicin in Schiemann's study, contrasting with the present study where 70% of isolates utilized salicin. The higher utilization of salicin is parallel to the study of Hafeez *et al*. (2012) [8] who also observed a higher proportion of *Bacillus cereus* isolates utilizing salicin.

3.3 Pattern of Antimicrobial resistance in isolates

The resistance to antibiotics observed in *B. cereus* showed the highest resistance rates for Levofloxacin (10.42%) and Ciprofloxacin (10.42%), followed by Erythromycin (2.00%). Conversely, the highest sensitivity was observed for Imipenem (77.08%), Clindamycin (72.91%), and Meropenem (66.66%).

Floristean *et al*. (2008) [6] and Aklilu *et al*. (2016) [1] reported that *Bacillus cereus* isolates exhibited complete resistance (100%) to beta-lactam antibiotics, specifically Ampicillin and Penicillin G, which is in line with the present findings of resistance to Imipenem and Meropenem. Kamat *et al*. (1989) also reported complete resistance to ampicillin. Tewari *et al*. (2012) [19] arrived at an 82.8% resistance rate, while Rather *et al*. (2012) [13] reported a 91.75% resistance rate towards ampicillin. However, Tahmasebi *et al*. (2014) [16] observed a lower resistance rate of 33.33%, contrasting with the present study's findings. Found that *Bacillus cereus* isolates exhibited a 100% sensitivity rate to Ciprofloxacin, which contradicts the present study's findings.

3.4 Standard polymerase chain reaction and molecular detection of genes

The PCR amplification assay was conducted following the manufacturer's recommendations with slight modifications, targeting the groEL gene sequence to confirm the *Bacillus cereus* group. The aim was to amplify a DNA fragment of 400 base pairs (bp). All 48 samples previously identified as *Bacillus cereus* isolates yielded specific-sized fragments of 400 bp, confirming their membership in the *Bacillus cereus* group. Furthermore, it was found that 22 isolates (45.83%) carried the gyrB genes. The study also examined the existence of the hblA, bceT, and cytK genes among the isolates. The bceT gene was prominently present, found in 21 isolates, accounting for 43.75% of the sample pool. In contrast, the hblA gene was less prevalent, identified in 9 isolates (18.75% of the samples). The cytK gene showed the lowest occurrence, detected in only 5 isolates, comprising 10.41% of the total samples.

4. Conclusion

In summary, a total of 165 chevon samples were processed, with 48 (29%) samples testing positive for *B. cereus*. This highlights the importance of illiberal monitoring and inspection to ensure practices that are effectively hygienic. *Bacillus cereus* is a bacterium commonly found in soil and food products, including chevon. To prevent *B. cereus* contamination and subsequent foodborne illness, it is crucial to handle and cook chevon properly. This includes storing meat at the correct temperature, cooking it thoroughly to kill any bacteria, and avoiding leaving cooked meat at room temperature for extended periods. Proper food hygiene practices can significantly reduce the risk of *B. cereus* contamination and protect against foodborne illness.

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