Prevalence of Bacillus cereus in milk and some milk products in Egypt

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ABSTRACT

Bacillus cereus is a spore-forming bacterium that causes food spoilage and poisoning. This study aimed to evaluate the prevalence of entero-toxigenic Bacillus cereus in 180 dairy samples across three cities (Tanta, El-Santa, and El-Mahalla El-Kubra) in El-Gharbia Governorate, Egypt, including raw milk, milk powder and Ras cheese (60 of each). Samples were collected from December 2022 to April 2023 and analyzed using traditional isolation, biochemical identification with VITEK 2, and PCR targeting the nheA gene as a virulence factor for Bacillus cereus. Traditional isolation on selective media indicated a prevalence of 1.66%, 24.99% and 4.99% in raw milk, milk powder and Ras cheese, respectively. However, VITEK 2 only confirmed that Bacillus cereus in milk powder isolates was 24.99%. PCR detection of the nheA gene revealed that Bacillus cereus in the examined samples completely matched with traditional isolation. Overall, 10.5% of samples were positive for enterotoxicogenic Bacillus cereus by PCR. Compared to PCR, traditional isolation showed 100% sensitivity and specificity, while VITEK 2 had 79% sensitivity and 100% specificity. Although biochemical methods like VITEK 2 enabled rapid automated identification, PCR detection of virulence genes provided superior sensitivity for identifying enterotoxicogenic Bacillus cereus of clinical significance from dairy samples. Traditional culture techniques remain essential for isolating target bacteria prior to PCR. This study provided insights into the prevalence of enterotoxicogenic Bacillus cereus contamination in some Egyptian dairy products.

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1. INTRODUCTION

Bacillus cereus is a spore-forming bacterium that is a common cause of foodborne illness worldwide (Sadek et al., 2018). Raw milk appears a significant source of contamination, with Bacillus cereus introduced from the farm environment, bedding materials, and feces (Svensson et al., 2004; Magnusson et al., 2007). Powdered dairy products including milk powder, infant formula, and processed foods containing milk powder also frequently test positive for Bacillus cereus (Carp-Carare et al., 2000; Reyes et al., 2007; Kumari & Sarkar, 2014). High spore counts have been detected in both raw and pasteurized milk powder (Montanhini et al., 2013). Bacillus cereus is frequently detected in various cheeses (Molvá et al., 2009; Mugadza & Buys, 2017), where it can introduce risk of toxin production during storage depending on temperature, pH, and background microflora (Tirloni et al., 2017).

The reported prevalence of raw milk contamination varied widely between studies. Yíbar et al. (2017) found a prevalence of 3.8%, while Chang et al. (2021) and Mohamed et al. (2016) reported higher rates of 33.3% and 60%, respectively. The highest reported prevalence of 100% was documented by Osama et al. (2020). Studies investigating the prevalence of contamination in milk powder have reported varying rates. Zhang et al. (2017), Pei et al. (2018), and Mohamed et al. (2016) documented relatively lower prevalence of 6.8%, 7.5%, and 15%, respectively. Higher prevalence was noted by Reyes et al. (2007) and Kumari and Sarkar (2014), who reported contamination rates of 46% and 52%. The highest prevalence of 68% was reported in the study by Osama et al. (2020). Studies reporting the prevalence of contamination in cheese products have shown a wide range. Relatively lower rates were reported by Kumari and Sarkar (2014), Berthold-Pluta et al. (2019), and Yíbar et al. (2017), who found prevalence of 4%, 8.6%, and 10.4%, respectively. Higher contamination rates were documented by Montone et al. (2020) at 26.2%, Adame-Gómez et al. (2020) at 29.48%, Spanu et al. (2016) at 33.3%, and Owusu-Kwarteng et al. (2017) at 38.7%. Even higher prevalence was noted by Iurlina et al. (2006) at 50% and Osama et al. (2020) at 95%.

Milk and dairy products have frequently been implicated in Bacillus cereus outbreaks globally due to poor hygiene practices, improper food handling and storage temperatures permitting growth of vegetative cells and toxin production (Bennett et al., 2013; Osimani et al., 2018). Traditional culture methods involving selective media and biochemical tests have been widely used for isolating and identifying Bacillus cereus from foods including dairy samples (Mohamed et al., 2016; Bursóva et al., 2018). Multiple studies demonstrated that VITEK2 for

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confirmation of Bacillus cereus isolates from milk and dairy products (Alanber et al., 2020; Jaber et al., 2021) and various food (Hashhash et al., 2023; Sidiq & Arif, 2023) with probability results indicate good confidence in identification of Bacillus cereus (Jaber et al., 2021; Sidiq & Arif, 2023). However, PCR-based molecular methods targeting virulence genes such as hemolysins (hbl), nonhemolytic enterotoxins (nhe), cytotoxins (cysK) and cereulide toxins (ces) have become the gold standard for detecting and characterizing enterotoxigenic strains of Bacillus cereus with higher sensitivity and specificity compared to phenotypic methods (Molva et al., 2009, Zhang et al., 2016).

The nhe is comprised of three components encoded by an operonic gene cluster present in all Bacillus cereus strains. The key cytotoxicity factor is nheA, while nheB and nheC function as binding components (Ankolekar et al., 2009). The nhe was first characterized following a foodborne outbreak in Norway caused by an isolate lacking the hemolytic BL (hbl) enterotoxin complex, and it is now believed to be the most dominant diarrheal toxin (Stenfors et al., 2008). This study aimed to determine and compare the prevalence of Bacillus cereus contamination in raw milk, milk powder and Ras cheese samples from three cities in Egypt. As well as comparing the methods of either traditional isolation, biochemical identification with VITEK 2, or PCR targeting the nheA gene for detection of Bacillus cereus in the examined samples.

2. MATERIAL AND METHODS

2.1. Collection of samples:
A total of 180 dairy samples comprising raw cow’s milk, milk powder and Ras cheese samples (60 of each) were obtained randomly from different supermarkets across three cities in the El-Gharbia Governorate of Egypt. Sampling was performed between December 2022 to April 2023, with aseptic collection of the samples and immediate transfer to the laboratory under refrigeration at 4°C.

2.2. Materials:
Peptone water, Bacillus cereus agar base (Hi MEDIA M833), Polymyxin B selective supplement (FD003), and Sterile egg yolk emulsion (FD045) (HiMedia, India). nheA oligonucleotide primers and DNA polymerase were obtained from Willowfort, UK. Agarose, Tris base, boric acid, and EDTA were purchased from Sigma Aldrich, USA. RedSafe nucleic acid stain was purchased from Intron bio, USA. DNA ladder was purchased from GeneDirex, USA. A reference strain of Bacillus cereus (ATCC 11778) was obtained from the Food Hygiene Department, Animal Health Research Institute, Egypt. This served as a positive control.

2.3. Preparation and primary enrichment of the samples:
Primary enrichment of dairy samples was performed as described in previous studies. Briefly, 1 ml of raw milk sample was inoculated into 9 ml of peptone water and incubated overnight at 37°C (Mohamed et al., 2016). For cheese samples, 25 g of Ras cheese was added to 225 ml of peptone water, homogenized for 2 min using a stomacher, and incubated under the same conditions (Luiz et al., 2017). Similarly, 10 g of milk powder was enriched in 90 ml of peptone water, homogenized, and incubated at 37°C overnight (Heini et al., 2018). This primary enrichment allowed growth and recovery of cells from the various dairy samples.

2.4. Traditional isolation of Bacillus cereus:
To select for spores and inhibit vegetative cells, the enrichments were heat treated at 70°C for 10 minutes in a water bath. After heat shock, samples were inoculated onto Bacillus cereus selective agar base supplemented with polymyxin B and sterile egg yolk emulsion. Bacillus cereus agar base plates were streaked to obtain isolated colonies and incubated at 30°C for 48 hours. The selective conditions allowed growth and phenotypic confirmation of Bacillus cereus isolates based on colony morphology and lecithinase activity (Saleh-Lakha et al., 2017).

2.5. Biochemical identification by VITEK 2 compact system:
Identification of bacterial isolates was performed using the VITEK2 compact system (BioMérieux) as per the manufacturer's instructions (BioMérieux Manual 414532, 2006). In brief, the isolated colonies were suspended in sterile saline and the turbidity adjusted to match a 1.8-2.2 McFarland standard using a densitometer. The standardized bacterial suspension was used to inoculate Bacillus (BCL) identification cards containing miniaturized biochemical substrates tailored to Bacillus species identification. The inoculated cards were introduced into the VITEK2 analyzer’s vacuum filling station along with a cassette mechanism to transfer the suspension into the cards’ microwells. After sealing, the loaded cards were incubated kinetically at 35.5°C in the carousel incubator, with frequent automated optical readings at 15-minute intervals to measure biochemical reactions. The kinetic data obtained was automatically analyzed by the VITEK2 software through comparisons to an onboard database, allowing rapid and standardized identification of Bacillus isolates based on species-specific metabolic patterns. This technique provides a highly reproducible inoculation, incubation and reading system for automated bacterial identification from culture plates.

2.6. Molecular identification for isolates:
2.6.1. DNA extraction:
Genomic DNA extraction from bacterial isolates was performed through a boiling method as described by Zhang et al. (2014). Fresh bacterial isolates were resuspended in 1 ml of sterile water, centrifuged at 8000 rpm for 5 minutes, and washed twice with an equal volume of sterile water. The cell pellets were then resuspended in 1 ml of sterile water, boiled for 15 minutes to lyse the cells, and release genomic DNA, and centrifuged again at 8000 rpm for 5 minutes to remove cell debris. The resulting supernatant, containing the extracted genomic DNA, was used as a template in subsequent procedures. This modified boiling protocol allows for the rapid isolation of genomic DNA from bacterial cultures in a simple and cost-effective manner.

2.6.2. Conventional PCR for detection of enterotoxigenic Bacillus cereus:
To amplify the 755 bp species-specific fragments for enterotoxigenic Bacillus cereus, a pairs of oligonucleotide primers specific for the nheA gene (Table, 1) were used.

<table>
<thead>
<tr>
<th>Oligonucleotide primers specific for nheA gene</th>
<th>Primer sequence 5<code>→3</code></th>
</tr>
</thead>
</table>
| nheA                                         | F-5'CTGAGATCTAACATGGCGC
R-ACGAAATGTAAATTTGGATGTC  | 755 bp                |

PCR amplification of target DNA was performed in 15 μl reactions containing 7.5 μl of DNA polymerase master mix, 1 μl of a 10 pmol primer mix, 2.5 μl of template DNA, and PCR-grade water for the remaining volume. The thermal cycling protocol consisted of an initial denaturation step at 95°C for 2 minutes followed by 30 cycles of denaturation at
95°C for 15 seconds, primer annealing at 56°C for 20 seconds, and extension at 72°C for 45 seconds. A final extension step at 72°C for 5 minutes was included to ensure complete amplification. To analyze PCR products, 7.5 μl of each amplified sample was separated by electrophoresis on 1.5% agarose gels with RedSafe dye at a constant 100V for 30 minutes. Gels were visualized using a Bio-Rad Gel Doc XR imaging system and amplicons sizes estimated by comparison against a 100 bp DNA ladder (Gainebretièrre et al., 2002).

2.7. Statistical analysis:
In order to report the prevalence, descriptive statistics were utilized. To compare the performance of traditional isolation, VITEK 2 biochemical testing, and PCR, diagnostic test evaluation statistics including sensitivity and specificity were calculated using SPSS software (version 20; spss Inc., Chicago, USA).

2.7.1. Method of calculation of sensitivity and specificity
Sensitivity and specificity were calculated according to the following equations:
- Sensitivity: \( \frac{TP}{TP + FN} \times 100 \)
- Specificity: \( \frac{TN}{TN + FP} \times 100 \)

3. RESULTS

3.1. Traditional isolation of Bacillus cereus from milk and some of its products:
The presumptive identification of Bacillus cereus was based on the observation of blue colonies surrounded by cloudy halos on Bacillus cereus agar plates. The cloudy halos are indicative of lecithinase production, a characteristic phenotype of Bacillus cereus. Traditional microbiological techniques were utilized for isolation and identification of Bacillus cereus. Prevalence data for Bacillus cereus in various dairy products was presented in Table (2). In raw milk samples, 1.66% tested positive for Bacillus cereus. A higher prevalence rate of 24.99% was observed in milk powder samples. Ras cheese samples exhibited an intermediate Bacillus cereus prevalence of 4.99%.

Table 2 Prevalence of Bacillus cereus using traditional isolation (n=60 each)

<table>
<thead>
<tr>
<th>Products</th>
<th>Traditional isolation Positive samples (%)</th>
<th>% According to No. of total samples (n=180)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw milk</td>
<td>1</td>
<td>1.66</td>
</tr>
<tr>
<td>Milk powder</td>
<td>15</td>
<td>24.99</td>
</tr>
<tr>
<td>Ras cheese</td>
<td>3</td>
<td>4.99</td>
</tr>
<tr>
<td>Total (180)</td>
<td>19</td>
<td>10.5</td>
</tr>
</tbody>
</table>

3.2. Biochemical identification
The confirmation of presumptive isolates was achieved through biochemical profiling using the VITEK 2 automated system. The biochemical test results for all isolates were presented in table (3).

Table 3 Prevalence of Bacillus cereus using VITEK 2 biochemical approach (n=60 each)

<table>
<thead>
<tr>
<th>Products</th>
<th>VITEK 2 Positive samples</th>
<th>% VITEK 2 According to No. of total samples (n=180)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw milk</td>
<td>1</td>
<td>8.33</td>
</tr>
<tr>
<td>Milk powder</td>
<td>15</td>
<td>24.99</td>
</tr>
<tr>
<td>Ras cheese</td>
<td>3</td>
<td>8.33</td>
</tr>
<tr>
<td>Total (180)</td>
<td>19</td>
<td></td>
</tr>
</tbody>
</table>

3.3. Molecular identification by PCR
Molecular identification of enterotoxigenic Bacillus cereus isolates was performed through PCR targeting of the nheA gene, which encodes for the non-hemolytic enterotoxin. A 755bp amplicon indicates presence of the nhe A gene. As shown in Figure (1), PCR analysis demonstrated the expected 755bp band in a subset of the Bacillus cereus isolates, confirming enterotoxigenicity. The prevalence of enterotoxigenic Bacillus cereus amongst the dairy isolates is summarized in Table (4). PCR-based screening for virulence factors provides a rapid technique for assessing the pathogenic potential of bacterial isolates. The inclusion of nheA screening expands the characterization of Bacillus cereus isolates beyond basic prevalence data, offering insight into the occurrence of enterotoxigenic strains across the dairy samples tested.

3.4. Sensitivity and specificity of different techniques in relation to PCR as a gold standard.
The diagnostic performance of traditional isolation, VITEK 2 biochemical identification, and PCR targeting the nheA gene for detection of enterotoxigenic Bacillus cereus was compared, with PCR as a gold standard reference method. Calculated sensitivity and specificity values for each testing methodology are presented in Table (5). Traditional isolation technique exhibited 100% sensitivity and specificity relative to PCR, with complete agreement between the two methods. All 19 PCR-confirmed enterotoxigenic isolates were correctly identified through traditional culture-based analysis. The VITEK 2 automated system demonstrated slightly lower diagnostic sensitivity of 79% compared to PCR, failing to detect 4 of the 19 enterotoxigenic isolates. However, the VITEK 2 maintained perfect specificity, generating no false positives relative to PCR. Overall, while the VITEK 2 provides rapid biochemical confirmation of Bacillus cereus, traditional isolation paired with PCR molecular screening proved optimal for reliable detection of enterotoxigenic strains among the tested dairy isolates.

Table 4 Prevalence of Bacillus cereus using PCR molecular approach (n=60 each)

<table>
<thead>
<tr>
<th>Products</th>
<th>Traditional isolation Positive samples (%)</th>
<th>PCR Positive samples (%)</th>
<th>% PCR Positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw milk</td>
<td>1</td>
<td>1</td>
<td>1.66</td>
</tr>
<tr>
<td>Milk powder</td>
<td>15</td>
<td>15</td>
<td>24.99</td>
</tr>
<tr>
<td>Ras cheese</td>
<td>3</td>
<td>3</td>
<td>4.99</td>
</tr>
<tr>
<td>Total (180)</td>
<td>19</td>
<td>19</td>
<td>10.5</td>
</tr>
</tbody>
</table>

Fig 1 Agarose gel electrophoresis PCR assay showed amplified fragment of 755bp with enterotoxigenic Bacillus cereus nheA gene specific primer. Lane M: molecular marker (100bp).

Table 5 Sensitivity and specificity of different techniques in relation to PCR as a gold standard (n=180)

<table>
<thead>
<tr>
<th>Techniques</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PP-True Positive</th>
<th>FP-False Positive</th>
<th>TN-True Negative</th>
<th>FN-FALSE NEGATIVE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Traditional isolation</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>VITEK 2</td>
<td>79%</td>
<td>100%</td>
<td>79%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>
4. DISCUSSION

The prevalence of Bacillus cereus in dairy products is a relevant concern for quality assurance and food safety. Bacillus cereus is an established causative agent of foodborne illness outbreaks, attributable to its virulence factors and associated enterotoxins (Tirloni et al., 2022).

The prevalence of Bacillus cereus in raw milk was investigated using traditional isolation on selective media and PCR, resulting in a prevalence rate of 1.66%. Interestingly, VITEK2 did not detect any Bacillus cereus in the raw milk samples (Table 2.3.4). The 1.66% prevalence rate mirrors the low prevalence of ≤3.8% noted across studies in locales like Egypt and Turkey (Mohamed et al., 2016; Yíbar et al., 2017). However, strikingly higher prevalence of 33.3% (50/150) from China (Chang et al., 2021) and up to 100% (25/25) has been documented in raw milk from Egypt (Osama et al., 2020). This variation in prevalence could be attributed to seasonal fluctuations in spore counts, as past researchers have demonstrated heightened raw milk contamination during warmer spring and summer periods (Lanverne persisien, 1997; Sverdotto et al., 2004; Bartoszewicz et al., 2008). The nature of Bacillus cereus permits multiplication during warm weather, which could promote seasonal spikes.

The prevalence of Bacillus cereus in milk powder was assessed using traditional isolation, VITEK2, and PCR, revealing a prevalence rate of 24.99% (Table 2.3.4). These results were in line with the prevalence rate of 27% (54/200) in Taiwan reported by Wong et al. (1988). Conversely, studies by Kumari and Sarkar (2014) and Osama et al. (2020) had reported exceedingly high prevalence rates of 52% (18/35) in India and 68% (17/25) in Egypt. On the other hand, Mohamed et al. (2016) reported a substantially lower prevalence of 15% (3/20) in milk powder from Egypt. Evidence suggests ubiquity of implying cross-contamination during processing and packaging as a risk factor (Liu et al., 2018). Sporulation may also highly promote heat-resistant survival spores during the drying process (Sadiq et al., 2016). Large-scale factory production resulted in more obvious variation in the microbial content (Wang et al., 2018). Finally, the consumer becomes under risk from Bacillus cereus toxins resulting from extended storage of milk powder at improper temperatures (Bursóvá et al., 2018).

In Ras cheese, the prevalence of Bacillus cereus was found to be 4.99% using traditional isolation and PCR, while VITEK2 could not detect any contamination (Table 2.3.4). These results are consistent with the prevalence reported by Yíbar et al. (2017) in Turkey, which was 10.4% (11/106). Additionally, Berthold-Pluta et al. (2019) reported a prevalence of 8.6% (3/35) in fresh acid cheese in Poland. In contrast, Iurlina et al. (2006) reported a remarkably higher prevalence of 50% (15/30) in Argentine cheese, and Berthold-Pluta et al. (2019) reported 52.5% (42/80) in mold cheese in Poland. Thearsen (2016) reported a lower prevalence of 8.6% (3/35) in milk powder at improper temperatures (Bursóvá et al., 2018).

Comparing diagnostic performances provides valuable insights into the utility of various Bacillus cereus detection techniques. In the present study, traditional selective plating demonstrated 100% sensitivity and specificity relative to PCR targeting of enterotoxin genes (Fig.1) and (Table, 5). However, the labor and time intensive nature of selective culture may limit utility for high-throughput screening. Still, isolation provides critical strain availability for downstream characterization and toxin analysis. The automated VITEK 2 system offers rapid turnaround for presumptive identification (Alanber et al., 2020; Jaber et al., 2021).

However, the 79% sensitivity (Table, 5) found here echoes studies citing limitations in differentiating Bacillus cereus from closely related species via VITEK 2 biochemical panels (Navas et al., 2014; Hashhash et al., 2023). While 100% specificity aligns with past findings and affirms the utility of VITEK 2 BCL card as a reliable first-line screen (Halket et al., 2010; Alanber et al., 2020; Wen et al., 2022; Sidiqi & Aref, 2023). PCR-based detection of toxin-encoding genes provides a sensitive and specific means of identifying enterotoxigenic strains capable of causing food poisoning. Thus, the 10.5% prevalence rate for potentially toxigenic Bacillus cereus may overestimate the true risk. Ultimately, PCR provides sensitive screening for potentially toxigenic strains (Guinebretière et al., 2002; Tirloni et al., 2022).

5. CONCLUSIONS

In conclusion, this study revealed concerning levels of contamination with enterotoxigenic Bacillus cereus in dairy products from El-Dharbi Governance, Egypt. Traditional culture techniques, biochemical identification, and PCR targeting the nheA gene were used to analyze raw milk, milk powder and Ras cheese samples collected from December 2022 to April 2023. Overall, 10.5% of the 180 dairy samples tested positive for enterotoxigenic Bacillus cereus by PCR, with the highest prevalence found in milk powder at 24.99%. Compared to PCR detection of the nheA virulence gene, traditional isolation and biochemical identification with VITEK 2 showed reduced sensitivity, indicating these methods underestimate the levels of potentially enterotoxigenic Bacillus cereus. However, traditional culture remains essential to obtain bacterial isolates for further characterization.

6. REFERENCES


