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Incidence of *Bacillus cereus* in Food and Environmental Sources in Basrah / Iraq. Antibiotic Resistance Profiles of Recovered Isolates

AL-Hadithi, T. Hadeel^{1,3*} and Ali, K. Entesar*

¹Biology Dept / College of Science / Univ. of Basrah

²Poly techniques / Basrah Medical Institute

³Faculty of Pharmacy / Isra University / Amman / Jordan

*Corresponding author

KEYWORDS

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A B S T R A C T

Two hundred and fifty five samples were collected from food (N= 210), surface water (N=20) and soil (N=25) at Basrah Governate/Iraq. Samples were cultured on the selective medium Mannitol-Egg Yolk Polymyxin B Agar (MYPA). Identification was confirmed by Gram staining, biochemical tests and resistance to penicillin. *B. cereus* was recovered from sixty six samples: Fifty two (24.76%) from food and fourteen (31.1%) from environmental sources. The highest percentage recovery of *B. cereus* was from soil (36%), followed by infant milk (30%) and the least was from white cheese and raw vegetables (20% each). More than 80% of *B. cereus* isolates from all sources were resistant to carbencillin, cephalothin and ampicillin. The least resistance was against erythromycin, gentamycin, chloramphenicol, nalidixic acid and sulfamethoxazole-trimethoprim. Of sixty six *B. cereus* identified in food and environment samples; fifty isolates (75.7%) were found resistant demonstrating eight patterns of resistance ranging from one to all antibiotics.

Introduction

Bacillus cereus is a Gram-positive, spore-forming, motile, aerobic rod that also grows well anaerobically. It is widespread in nature and frequently isolated from soil and growing plants, but it is also well adapted for growth in the intestinal tract of insects and mammals; It produces various toxins, bacteriocins and antibiotics to compete with other microbiota. Because of its ubiquitous

nature, it is easily spread to occur in a wide variety of foods (Arnesen *et al.*, 2008; Carlin *et al.*, 2010; Ceuppens *et al.*, 2013; Organji *et al.*, 2015).

Bacillus cereus has been recognized in Norway as an agent of food poisoning as early as the 1950s (Hauge, 1955), occurs year-round without any particular geographic

distribution (Bean and Griffin, 1990; Logan, *et al.*, 2011). The disease is often associated with the consumption of rice-based dishes, Chinese food, macaroni and cheese made from powdered milk (Smith *et al.*, 2004; Di Pinto *et al.*, 2013). From 1998 to 2008, 1229 foodborne outbreaks caused by *Bacillus cereus*, *Clostridium perfringens*, and *Staphylococcus aureus* were reported in the United States, rice dishes were commonly implicated in *B. cereus* outbreaks (50%) (Bennett *et al.*, 2013). Agarwal (2014) reported that prevalence of *Salmonella*, *B. cereus* and *V. parahemolyticus* was higher in most of the mail-ordered foods than foods from other retail sources. Moreover, almost 50% of different Korean fermented soybean products were found to harbor *B. cereus* (Yim *et al.*, 2015).

Bacillus cereus food poisoning is of two different types: Slow-onset diarrheal type which is an infection caused by ingestion of the vegetative cells or spores, producing protein enterotoxins in the small intestine. It is frequently associated with meat or vegetable-containing foods after cooking. One outbreak of the long-incubation form was traced to a "meals-on-wheels" program in which food was held above room temperature for a prolonged period (Dworkin *et al.*, 2006; Shetty *et al.*, 2009). The second one is rapid-onset emetic type which is food intoxication and is frequently associated with the consumption of rice-based dishes (Jaquette *et al.*, 1998; Ehling-Schulz *et al.*, 2004; Schoeni, 2005; Arnesen *et al.*, 2008; Kim *et al.*, 2010; Heilkenbrinker *et al.*, 2013; Yim *et al.*, 2015).

B. cereus, is typically resistant only to β -lactams (Logan and Turnbull, 2003; Fenselau *et al.*, 2008; Yim *et al.*, 2015). Nevertheless, reports of resistance in *B. cereus* to erythromycin and tetracyclines in the United States and Europe predict the development of further resistance (Jensen *et*

al., 2001, Citron and Appleman, 2006). The increased antimicrobial resistance of *B. cereus* to antibiotics is of growing concern worldwide Interest in *B. cereus* has been growing lately because *B. cereus*-related diseases, in particular food poisonings, are growing in number (Bottone, 2010).

Being a spore forming bacteria, which displays large variability in pathogenicity and ecological lifestyle, is accounted mainly as being a troublesome bacteria to food producers. Spores may be present on various types of raw and cooked foods, and are able to survive high cooking temperatures while the competitive microbiota present in food is inactivated by heat treatment or drying (Ceuppens *et al.*, 2011, Logan, 2011; Ceuppens *et al.*, 2013; Tewari and Abdullah, 2015). A study on the incidence of *B. cereus* in vomitus and stool among inpatients, aged between 3 months and over 40 years, with acute diarrhea in Basrah Governate was conducted by AL-Hadithi and Entisar (2014). The unique properties such as heat resistance, endospore forming ability, toxin production, swarming and biofilm formation in addition to psychrotrophic nature and resistance to antibiotics were also determined on recovered isolates.

The aim of the present study therefore, is to continue the above work to find out incidence of *B. cereus* in various sources of food and environment (surface water and soil) and to determine their antibiotic resistance patterns.

Materials and Methods

Samples

Two hundred and ten samples were collected from food including: Infant milk (N=40), Raw milk (N=25), White cheese (N=40), Cooked rice (N=40), Raw vegetables (N=40) and Raw meat (N=25). Also, samples from environment including: surface water (N=20)

and soil (N=25) were collected from Basrah Governate / Iraq. Milk and water samples were collected in sterile screw capped bottles and solid samples in disposable sterile containers. Samples were transported in cool boxes to the laboratory, and stored at 20°C until they were processed.

Sample processing

Solid food and soil samples

Five gram of each solid food sample and 5gms of soil (Collected from the surface to a depth of about 5 cm using sterile spatulas from different sites in Basrah Governate) were dissolved in 45 ml sterile normal saline in a sterile flask, serially diluted (Mossel *et al.*, 1967); then cultured on the selective and differential medium mannitol egg yolk polymyxin blood agar (MYPA) (Margesin *et al.*, 2009).

Milk and surface water samples

Standard loopfuls from each sample were cultured on MYPA.

Identification

Cultures were incubated for at 30°C for 24 - 48 hours. Colonies not utilizing mannitol, producing phospholipase C, were selected, purified and cultured on nutrient agar and subjected to the following characterization tests: Gram and spore staining, motility, production of acid and citrate and resistance to penicillin G (Harley and Prescott, 1996; Arnesen *et al.*, 2008; Banerjee *et al.*, 2011).

Susceptibility to antimicrobials

Disk-plate method using Muller-Hinton agar was applied to detect antibiotic susceptibility of recovered isolates toward the following antibiotics: Erythromycin (15µg), Gentamycin (10µg), Tetracyclin (30µg), Streptomycin (10µg), Chloramphenicol (30µg), Cephalothin (30µg), Nalidixic acid

(30µg), Ampicillin (10µg), Carbenicillin (10µg), Sulfamethoxazole-trimethoprim (25µg). Single colonies of 18 - 24 h old cultures were transferred to 5 ml of Tryptic Soy Blood agar TSB (Difco) and incubated at 37°C for 6-8 h. A sterile cotton swab dipped into the TSB growth was applied evenly onto pre-dried Mueller-Hinton agar (Difco) plate. After drying for 15 min, antibiotic discs were placed aseptically and the plates were incubated at 37°C for 18-24 h. Inhibition zones were measured in millimeter and compared with standard tables (Whong and Kwaga, 2007; CLSI, 2008).

Results and Discussion

Incidence of *Bacillus cereus* in food

B.cereus was isolated from all types of food, surface water and soil. Out of two hundred and ten food samples, *B. cereus* was isolated from fifty two samples (24.76%). Table 1 illustrate that twelve samples were from infant milk (30%) followed by raw milk and raw meat: seven samples each (28%); ten samples of cooked rice (25%) and finally eight samples of each white cheese and raw vegetables (20%).

Incidence of *Bacillus cereus* in Environmental sources

Of forty five environment samples, *B. cereus* was identified in fourteen samples (31.1%): nine from soil (36%) and five from water (25%) (Table 1).

Resistance of *B. cereus* to Antibiotics

Food Isolates

More than 70% of isolates from all food sources were resistant to ampicillin; the highest resistance was reported by isolates from fresh milk and raw meat (100%) followed by those of infant milk and dairy product (90 and 85% respectively). More

than 80% of *B. cereus* isolates from infant milk, fresh milk, cheese and raw meat were resistant to cephalothin and carbencillin. The highest resistance to tetracycline and streptomycin was revealed by fresh milk (85%) and raw meat (70%) and the least resistance toward streptomycin was exhibited by infant milk isolates (less than 10%). Nevertheless, the least resistance among food isolates was reported against erythromycin, gentamycin, nalidixic acid, chloramphenicol, and Sulamehox azotrimethoprim (less than 30% of isolates were resistant).

Environment Isolates

Isolates of *B. cereus* from soil and surface water demonstrated almost similar mode of resistance against antibiotic under test. More than 80% were resistant to carbencillin, ampicillin and cephalothin with the least resistance against erythromycin, gentamycin and chloramphenicol (10 & 20% respectively). However, soil isolates showed greater resistance to tetracycline and streptomycin (60%) as compared to surface water isolates (40%).

Patterns of Antibiotic Resistance of *Bacillus cereus* Isolates

Out of sixty six *B. cereus* identified in food and environment samples; fifty isolates (75.7%) were found resistant to at least one antibiotic. Table (2) illustrates that isolates of *B. cereus* from different sources, and even from the same source, are varied in their resistance ranging from resistance to all antibiotics to only one antibiotic. Eight patterns of resistance were expressed, three of which were resistant to the same number of antibiotics though against different types of antibiotics. Among resistant isolates from food sources (N=39), infant milk demonstrated ten multidrug resistant isolates

(≈25%); resistance was ranging from 3 to 10 antibiotics.

All food and environmental sources, apart from vegetables, demonstrated isolates resistant to all antibiotics under study (Pattern No.1, 7, 14%) and apart from soil and raw vegetables; ten isolates from all sources demonstrated isolates resistant to five antibiotics (Pattern No.4, 20%).

Incidence of *Bacillus cereus* in food and Environmental sources

Since 1950, many outbreaks from a variety of foods including meat and vegetable soups, cooked meat and poultry, fish, milk and ice cream were described in Europe. In 1969, the first well-characterized *B. cereus* outbreak in the USA was documented (Gilbert, 1979; Griffiths and Schraft, 2002). Since 1971, a number of *B. cereus* poisonings of a different type, called the vomiting type, were reported (Logan *et al.*, 2011; Tewari and Abdullah, 2015).

MYPA medium is indicative for existence of *B. cereus* even for ratios as challenging as one cell of *Bacillus cereus* to 10⁶ cells of other organisms (Downes and Ito, 2001; Arnesen *et al.*, 2008).

In a study conducted by Organji *et al.* (2015), 110 food samples were collected from Saudi Arabia and Egypt and screened for incidence of *B. cereus*, 19 samples were positive (17.3%) which is lower than that reported by the present study (24.76 %); despite that these three countries (Saudi Arabia, Egypt and Basrah / Iraq) have almost similar warm weather. However, results of the present study is in accordance with Anthony (1992) who reported that contaminated food and warm weather (15°C or higher) support incidence of *B. cereus*. On the other hand, higher incidence of *B. cereus*

was reported by Kumari and Sarkar (2014) in cheese, ice cream, milk powder, and milk (33%–55%). Besides, Reyesa *et al.*, (2007) reported higher prevalence of *B. cereus*: 175 of 381 (45.9%) in dried milk products used by Chilean School Feeding program as compared with the present study (30% 28% and 20% from infant, raw milk and cheese respectively). However, the highest contamination percentages with *B. cereus* (90%) was reported in raw milk samples in Ankaraas (Gundogan and Avci, 2014). On the other hand, Fossi *et al.*, (2016) reported much lower incidence of *B. cereus* in raw milk (8.22%) and in milk powder (13.33%). The frequent occurrence of *B. cereus* in foodstuffs with high fat content makes these products risky.

Starchy foods, such as rice are commonly associated with *B. cereus* emetic (vomiting) toxin which stimulate the production and accumulation of the stable toxin cereulide causing gastrointestinal illness (Schoeni and Wong, 2005).

B.cereus was identified in 7of 25 (28%) raw meat samples; which is comparable to that reported by Tewari *et al.*, (2015) who reported 27.8 % overall incidence of *B. cereus* in raw meat collected from different part of northern India. This is possibly attributed to the ambient temperature food storage which might favour the germination of endospores.

The mild acidification (pH 5.0) in different vegetable substrates, inhibit *B. cereus* growth for at least 60 days (Valero *et al.*, 2003). Accordingly, the lowest percentage of *B. cereus* was reported in raw vegetable (20%)

Soil demonstrated the highest percentage recovery of *B. cereus* (36%) which is eventually constitutes the primary environmental reservoir for *B. cereus*

(Ceuppens *et al.*, 2013). Nevertheless, its occurrence in aquatic environments is the concern of only a limited number of studies; although it represents a possible source for food contamination (Østensvik *et al.*, 2004). Five of 20 surface water samples (20%) demonstrated *B. cereus*.

In the long run, soil and water are interrelated environments in addition to climatic events. During rain events, leachates collected after transfer through the soil ultimately reached the groundwater and were loaded (Brillarred *et al.*, 2015).

Antibiotic Susceptibility of Recovered *B. cereus* isolates

The production of β -lactamases is one of potential virulence factors that make the producing strains resistant even to the 3rd generation of cephalosporins (Cormican and Jones, 1995). More than 80% and 70% of *B. cereus* isolates from all food sources were resistant to carbencillin and ampicillin respectively.

This ratio is comparable to that detected among isolates from stool and vomitus samples of inpatients in Basrah / Iraq (Al_Hadithi and Entisar, 2014). β -Lactamase was readily characterized from the *B. cereus* 5/B line (ATCC 13061) by mass spectrometry and two-dimensional gel electrophoresis (Fenselau *et al.*, 2008).

Multidrug resistant isolates from food, soil and surface water were highly prevailed in the present study which is in accordance with the study of Kumari and Sarkar, (2014) who found that all 144 isolates of *B. cereus* group were multidrug (at least five antibiotics) resistant against 14 different antibiotics commonly used against foodborne diseases.

Table.1 Incidence of *Bacillus cereus* in various food sources, water and soil

Source of Samples	No. of Samples	No. of <i>B. cereus</i> (%)
Food:	210	52 (24.76)
Infant Milk	40	12 (30)
Raw Milk	25	7 (28)
White Cheese	40	8 (20)
Cooked Rice	40	10 (25)
Raw Vegetables	40	8 (20)
Raw Meat	25	7 (28)
Environment:	45	14 (31.1)
Water	20	5 (25)
Soil	25	9 (36)

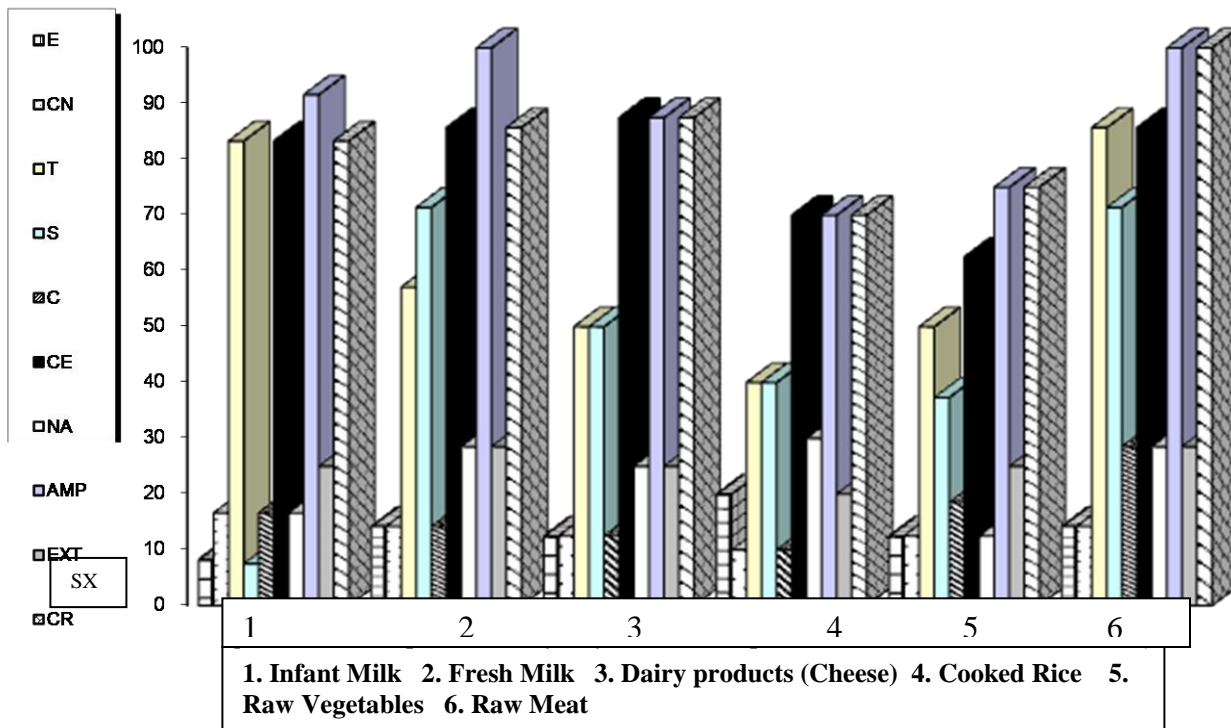
Table.2 Patterns of Antibiotic Resistance of *Bacillus cereus* isolates from food and Environmental sources

Pattern	Antibiotics isolates	<i>Bacillus cereus</i> Isolates
1	Resistant to all antibiotics	**SO6, W4, MIF 2, DP7, RM1, R1, ME1
2A	*CN, T, S, CE, NA, AMP, CR, SXT (8)	MIF5, VE 4
2B	E, T, S, CE, NA, AMP, CR, SXT (8)	R10
3	T, S, CE, NA, AMP, CR, SXT (7)	SO3, RM6, DP2
4	T, S, CE, AMP, CR (5)	W2, MIF4 & 9, RM 3&4, DP3, R7, ME 2, 4 &6
5A	AMP, CE, T, CR (4)	SO 2 & 9, MIF3 & 12, DP5, VE8, ME8
5B	AMP, CE, S, CR (4)	SO 5 & 7, MIF1& 6
6A	AMP, T, CR (3)	SO8, VE 6
6B	AMP, CE, CR (3)	W3 &5, RM7, DP6 & 8, R8, VE 5
6C	AMP, S, SXT (3)	MIF11 &7
7	AMP, CR (2)	ME7, R3
8	CE (1)	VE 3, R 2&4

* Gentamycin (CN), Tetracyclin(T), Streptomycin (S), Cephalothin (CE), Nalidixic acid (NA), Ampicillin (AMP), Carbencillin (CR), Sulfamethoxazole-trimethoprim (SXT), Erythromycin (E), Chloramphenicol (C)

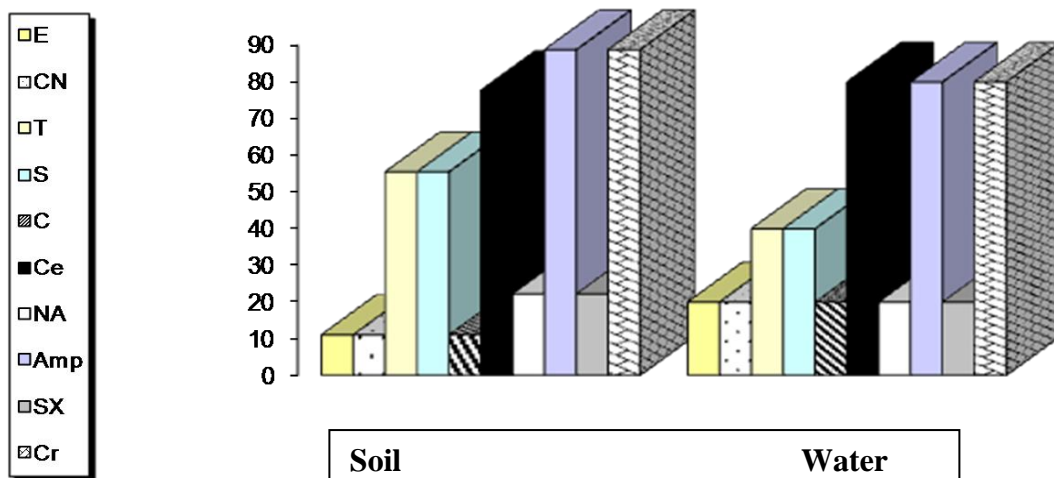
** SO: Soil, W: Surface Water, MIF: Infant Milk, DP: Dairy product (Cheese),R M: Raw milk, R: Cooked Rice, ME: Raw meat, VE: Raw Vegetables

Fig.1 Antibiotic resistance of *B. cereus* isolates from food sources



Erythromycin (E), Gentamycin (CN), Tetracyclin(T), Streptomycin (S), Chloramphenicol (C), Cephalothin (CE), Nalidixic acid (NA), Ampicillin (AMP), Sulfamethoxazole-trimethoprim (SXT), Carbencillin (CR)

Fig.2 Antibiotic resistance of *B. cereus* isolates from environmental sources (soil and water)



Erythromycin (E), Gentamycin (CN), Tetracyclin(T), Streptomycin (S), Chloramphenicol (C), Cephalothin (CE), Nalidixic acid (NA), Ampicillin (AMP), Sulamehox azote-trimethoprim (SXT), Carbencillin (CR)

The highest resistance to tetracycline and streptomycin was expressed by fresh milk and raw meat (85% and 70% respectively); this agrees with the report of Logan and Turnbull (2003) who predicted the development of further resistance of *B. cereus* to erythromycin and tetracyclines in the United States. However, Gundogan and Avci (2014) found that *B. cereus* isolated from raw milk, white cheese and ice cream from three different dairy-processing plants in Ankara were sensitive to cefotaxime, chloramphenicol, ciprofloxacin erythromycin, gentamicin and tetracycline. Nevertheless, the least resistance against streptomycin was recorded by infant milk isolates (less than 10%). The present study revealed that less than 30% of isolates were resistant to Sulfamethoxazole-trimethoprim. This manner of resistance is comparable with that reported in other warm regions (Whong and Kwaga, 2007; Banerjee, *et al.*, 2011; Organji *et al.*, 2015). However, Vicki *et al.*, (2007) reported that all isolates of *B. cereus* show resistance when a lower temperature and longer incubation time during testing was employed. Hereby, we give emphasis to examination of the antibiotic resistance profiles of the isolates that may serve as a major tool in evaluating both the hygienic conditions employed during food collection and preparation and the health hazards that humans may encounter when infected by antibiotic resistant strains (Sadashiv and Kaliwal, 2014).

Conclusion

Because *B. cereus* is underestimated in epidemiological and laboratory analysis as a cause of food illness; we found it is valuable to investigate their incidence in various food and environmental sources: soil and surface water. Besides, understanding range of antibiotic susceptibility of *B. cereus* isolates from these sources might lead us to practical

therapy to shorten the morbidity in acute stage of diarrhoeal disease caused by this bacteria.

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