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Detection and Determination of *Bacillus cereus* in Cooked Rice and Some Types of Spices with Ribosomal 16SrRNA gene Selected from Iraqi Public Restaurants

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Abstract

Spices can be considered as one of the sources of bacterial food pollution because of spices multiuse, in addition to the manufacturing processing and use, also they considered as one of food contamination reason due to the quantity of microorganisms like spore forming Bacteria such as *Bacillus cereus*. These road shape bacteria and its spores can move by air fast and easy, *B. cereus* is indicated to Diarrheal syndrome which linked to Non-hemolytic enterotoxin (NHE). Different kinds of 40 samples of spices was tested and 24 samples of cooked rice which considered as traditional food for wide stratum of Iraqi people from public restaurants located in Baghdad city, spices samples continued high number of *B. cereus* in spice (Fennel) in high number about 44×10^5 Cfu g^{-1} in addition to sample of spice (Kebab) 39×10^5 Cfu g^{-1} these numbers are high and unacceptable. As for samples of cooked rice have a fewer numbers of bacteria except one sample had record 4.9×10^3 Cfu g^{-1} in winter. But in summer samples had a higher numbers of B. cereus than winter period, some samples continued 14.4×10^5, 12.3×10^5 and 1.15×10^5 Cfu g^{-1}, these numbers are high and in this case can cause the diarrheal syndrome. Due to morphological and biochemical studies, these isolates are belong to *B. cereus*. After proceeding molecular biology and Polymerase Chain Reaction (PCR) for Ribosomal 16SrRNA gene-specific for these isolates, where tests are positive for 15 isolates of 64 selected isolates from spices and cooked rice, results showed these isolates are toxigenic

Keywords: *Bacillus cereus*, enterotoxin, spices, non-hemolytic enterotoxin

1. Introduction

Bacillus cereus is a group of ubiquitous, facultative anaerobic, spore-forming, and gram-positive, rod shapes (Tallent et al., 2012). Widely, *B. cereus* disturbed in nature and contaminates all agricultural products, also isolated from animal hair, cereal crops, dust, vegetation, fresh water and sediments, although in several cases isolated from fish (ICMSF, 1996). So as to isolated from soils (Tallent et al., 2012). Motile bacteria, found in plant material, hay, raw and processed food, also found frequently in pasteurized milk, causing spoilage because of the production of lipases and proteases (Torkar and Mozina, 2000). Two known types of diseases, Emetic and diarrheal, are caused by produced toxins by *Bacillus cereus*, an emetic caused by single heat –stable toxin, but the diarrheal, are caused by 3 or 4 heat-instable enterotoxins (Wijnands et al., 2002; Ehling-Schulz et al., 2005). It's an emetic cases characterized by vomiting after a few hours from ingested contaminated food. In the diarrhoeal cases, the symptoms appear after 8 to 16 hour from ingestion as diarrhea accompanied with abdominal pain. Generally, both types of food-borne illness are relatively and do not more than 24 houres (Ehling-Schulz and Messelhausser, 2013). Emetic illness frequently is linked with raw foods such as starchy food of plant origin (such as rice, pasta, potatoes, pastries and vermicelli, in 95% of emetic cases, caused by fried or cooked rice which contaminated by *B. cereus* (Jenson and Moir, 2003). Also spices have a number of Bacteria such as *Bacillus* can cause a variety of foodborne illnesses when the spiced food are refrigerated or when leftovers are stored for several days (Jenson and Moir, 2003; Agata et al., 2002). The impact being on public health risks happened when using these spices and herbs as an addition to ready-to-eat foods (Jacobs and Steffen, 2003). This is especially true for those that undergo little further processing after being added to these foods. Studies have shown that overall 3.0% of herbs and spices contained high counts of *B. cereus* (Granum and Lund, 2006). In modern studies, toxins related to illnesses which caused by *B. cereus* characterized at molecular level, where in diarrheal syndrome is linked to non-haemolytic enterotoxin (NHE), hemolytic enterotoxin (HBL) and cytotoxin K (CytK) and that of emetic type results from action of cereulide (Ces) toxin (Desai and Varadaraj, 2009). Consumption of foods that contain more than 10^4 spores or vegetative cells of *B. cereus* per gram may result

in food poisoning (Lopes and Alippi, 2007; Pirttijarvi et al., 1999). Spores can germinate and multiply in humid, low acid foods, from 4-5 °C to 55 °C. However, strains able to multiply below 7 °C, and strains able to multiply above 45 °C, are not a most common. Emetic *B. cereus* is presumably unable to grow and produce their toxin cereulide below 10°C, or in the absence of oxygen (EFSA, 2005). The (ELISA) Enzyme linked immunosorbent Adsorbed, is an immunoreactions which can use in toxins detections, and these types is useful in certain strains of bacteria to detect toxins (Brychta et al., 2009). The genotypic and phenotypic heterogeneities of the genus *Bacillus*, evident already for a long time (Ash et al., 1993). Molecular biology researches confirms the isolates certainly, there is no doubt that these type of researches helps a lot to fix the data in gene banks, thus confirm differentiation among strains of toxic bacteria in food poisoning cases (Banerjee et al., 2011). This study aimed to determine toxigenic *B. cereus* in cooked rice, and associated with common spices to understand which represent as traditional Iraqi food and if can cause harm to the consumers.

2. Materials and Methods

2.1. Spices samples

A different types of spices were collected from local Iraqi markets at Baghdad city, samples were rehydrated in sterilized glass bottles by adding sterilized distilled water, 500 µl of spice suspension to 500 µl of Ethanol C_2H_6O 100% and preparing serial dilutions after 30 minutes of C_2H_6O exposure, the samples was cultured on Luria Bertani agar.

2.2. Rice samples

Along study period for two seasons' winter and summer of 2014, 48 samples are collected randomly from different public restaurants at Baghdad city. Then 10 grams of cocked rice weighted and put in continuers of normal saline, 500 µl of suspension taken and mixed with 500 µl of Ethanol C_2H_6O 100% for 30 minutes, and preparing serial dilutions after 30 minutes of C_2H_6O exposure, the samples was cultured on Luria Bertani agar.

2.3. Chemical and biochemical tests

All isolates of *Bacillus* spp. from Spices and Cooked Rice were tested for catalase, motility, production of lecithinase, reduction of nitrate, the Voges–Proskauer reaction, tyrosinase activity, mannitol and arabinose utilization, anaerobic utilization of glucose, starch and gelatin hydrolysis, according to standard protocols (Gordon, 1981; Lancette and Harmon, 1980; Pirttijarvi, 2000). Isolates were tested for hydrolysis of lecithin after incubation in egg-yolk nutrient agar, to detect production of phosphate acetylcholine hydrolase (Van Netten and Kramer, 1992), and for haemolytic activity and production of a discontinuous haemolytic pattern on blood agar plates (Beecher and Wong, 1994). When necessary, API 50 CHB strips (Biomerieux ux®) were used for further identification

2.4. Extraction of chromosomal DNA from Bacillus

A fresh colony of *Bacillus* spp. isolates is inoculated in 7 ml of L. B. with a specific antibiotic then incubated overnight at 37 °C. After incubation, bacterial cells of *Bacillus* spp. centrifuged at 7000 rpm for five minutes, the obtained pellets was washed with five ml of Lysis Buffer consisting of 50 mM of EDTA and 0.1 M NaCL then centrifuged again and resuspended cells with 1 ml of Lysis Buffer to which added 0.250 ml of lysozyme, after that the mixture above is incubated for 10 min. at 37 °C. Then we added 0.075 ml of 20% Sarkosyl, stirred by vigorously and centrifuged at 1000 rpm at 4 °C for 1 min. then moved to precipitation of DNA by adding 0.1 volume of sodium acetate and 2.5 volumes of $2(CH_3)O$. Subsequently, the precipitated DNA was washed with $2(CH_3)O$ 70%, and centrifuged again in same conditions above, the obtained pellets re-suspended in 500 µl of sterilized deionized water. As previously described analysis is performed by electrophoresis in a 1.6% Agarose gel

2.5. Amplification and analysis of 16SrRNA by PCR

Primers U1 and U2 described by (Ash et al., 1993), which used for PCR amplification of the 16SrRNA (Lynn et al., 2013), from *B. cereus* Isolates of both spices and Cooked Rice. They are derived from conserved regions amplifying a DNA fragment of about 1.1 kb, of 16SrRNA genes from *Bacillus* spp. and closely related genera. PCR mixtures and amplifications were carried out as described previously (Alippi et al., 2002).

3. Results and Discussion

Spices are known to be contaminated with different microorganisms. Fennel and Kabab spices carried a high number of bacteria as 44.6×10^5 and 39.2×10^5 Cfu g^{-1} Curry, to a lesser extent, may also be infected [25,26,27,10]. Most of present bacteria in spices are aerobic spore-former kind of spices and number of bacteria was recorded as shown in Table 1.

Table 1: Kinds of spices and No. of *B. cereus*

Common name	No. of bacteria Cfu mg^{-1}
Cubeb	2.0×10^5
Cloves	0.42×10^5
Nutmeg	1.4×10^5
Black pepper	14.0×10^5
Cinnamon	1.2×10^5
Fennel	44.6×10^5
Cumin	12.1×10^5
Chili pepper	11.3×10^5
Turmeric	2.7×10^5
Curry	22.5×10^5
Biryani spices	0.22×10^5
Shawerma spices	0.21×10^5
Kabab spices	39.2×10^5
Bastirma spices	0.38×10^5
Dolma spices	0.6×10^5

One sample was chosen at my house as a control have no growth of bacteria, but when cooked with oil and salt after incubation 1.6×10^2 Cfu g^{-1} of bacteria, here may be boiled rice may will not reach growing of spores, and the counts of bacteria are shown in Table 2.

As well as in samples of cooked rice in this study, along study period from 26/01 to 16/03/2014, for samples contaminated with few number of *B. cereus* with one sample somehow contaminated with high number of *B. cereus*, and for same reason one sample was chosen as control the boiled one

Table 2: No. of *B. cereus* cells in cooked rice samples collected along study period from 26/01−16/03/2014

Sampling date	Sampling place	Type of sample	No. of bacteria Cfu g^{-1}
26/01/2014	House	Uncooked rice	N. G*
26/01/2014	House	Cooked rice	1.6×10^2
26/01/2014	Al Radhwanieya public restaurant	Cooked rice	N. G
26/01/2014	Al Bayaa near wholesale markets	Cooked rice	4×10^2
26/01/2014	Restaurant at Al Bayaa near the garage	Cooked rice	2×10^2
29/01/2014	Bab Al Muadham near the garage	Cooked rice	N. G
02/02/2014	Bab Al Sharqi near the public garage	Cooked rice	$3x10^2$
22/02/2014	Jihad Neighborhood restaurant market	Cooked rice	N. G
22/02/2014	Al Eskan	Cooked rice	N.G
22/02/2014	Police tunnel (Nafakh alshurta)	Cooked rice	23×10^2
09/03/2014	Near Al Andalus Plaza	Cooked rice and vermicelli	3.2×10^2
09/03/2014	Al Karrada Dakhel near Abbas Mustafa mosque	Cooked rice	1.2×10^2
09/03/2014	Al Karrada Dakhel near old Alawrzde	Cooked rice and vermicelli	0.8×10^2
09/03/2014	Al Karrada Kharej	Cooked rice	4.6×10^3
10/03/2014	Jihad Neighborhood on the public road	Cooked rice	N. G
16/03/2014	Jihad Neighborhood near market	Cooked rice and vermicelli	1×10^2

*No growth

have no growth of bacteria, but the same sample that cooked with salt and oil, we count 10×10^2 Cfu g^{-1}, as shown in Table 3, thus mean, there were two reasons can effect growing of bacteria beside of temperature, salts and oil reached spores growing of bacteria, some highest numbers was because of adding spices to the food and ready food, in this case the first reason of food poisoning is belong to human beavers in food cooking and preparing.

On the other hand, for second period of study from 08/06 – 16/06 2014, due to worming and humidity, many samples of cooked rice contaminated heavily and carry number of bacteria 14.4×10^5 Cfu g^{-1} in sample of Al Eskan, 12.3×10^5 Cfu g^{-1} in sample of Police Tunnel (Nafak Alshurta) and 1.15×10^4 Cfu g^{-1} in the sample of Al Karrada Kharej as shown in Table 3.

Among all tested isolated species in this study, only a few of them have associated with food poisoning. Indeed, *B. cerus* recognized as the etiological agent of food poisoning outbreaks in Europe as far back as 1960 (Jenson and Moir, 2003). Black peeper contaminated with aerobic spore formers enormously increased the microbial count of meat like sausage. Aerobic spore formers also detected in Black peeper powder (Agata et al., 2002). According to (Paananen et al., 2002). The recorded results in Nafak Al shurta (Police tunnel), sample of Al eskan and Al Karrada Kharej. Samples, are near to make Emetic syndrome due to the recorded number of *B. cereus.* These results are unacceptable because will make food poisoning due to high number of *B. cereus*.

3.1. Bacterial morphology, chemical and biochemical tests

Morphological studies of vegetative cells and microscopic examination of spores also physiological and biochemical tests of the *Bacillus* spp. All isolates from spices and cooked rice are Gram positive, and catalase test is positive reaction, and hydrolyzed gelatin, and didn't produced acid from arabinose. Variable results obtained for lecithinase activity, V–P reaction, mannitol utilization, starch hydrolysis, tyrosinase activity and reduction of nitrate to nitrites. Haemolytic activity shown by 95% of the isolates, of these, 11% produced a discontinuous haemolytic pattern, which usually correlated with the presence of haemolysin BL (Beecher and Wong, 1994). All the isolates had ellipsoidal spores that occupied a central position without distention of the sporangium. None of the isolates presents a parasporal inclusions. A number of physiological and biochemical properties used to differentiate the *Bacillus* sp. recorded by other research groups (Seenappa and Kempton, 2008). They found a variation in biochemical as well as fermentative reactions particularly

Table 3: No. of *B. cereus* cells in cooked rice samples collected along study period from 08/06–16/06/2014

Sampling date	Sampling place	Type of sample	No. of bacteria Cfu g^{-1}
08/06/2014	House	Non cooked rice	N. G*
08/06/2014	House	Cooked rice	10×10^2
08/06/2014	Al Bayaa near wholesale markets	Cooked rice	5×10^2
08/06/2014	Restaurant at Al Bayaa near the garage	Cooked rice	2.5×10^2
08/06/2014	Bab Al Muadham near the garage	Cooked rice	7.5×10^2
08/06/2014	Bab Al Sharqi near the public garage	Cooked rice	2.5×10^2
08/06/2014	Jihad neighborhood restaurant in market	Cooked rice	2.5×10^2
08/06/2014	Al Eskan	Cooked rice	14.4×10^5
08/06/2014	Police tunnel (Nafakh Alshurta)	Cooked rice	12.3×10^5
15/06/2014	Near Al Andalus Plaza	Cooked rice and vermicelli	8×10^2
15/06/2014	Al Karrada Dakhel near Abbas Mustafa mosque	Cooked rice	3×10^2
15/06/2014	Al Karrada Dakhel near old Alawrzde	Cooked rice and vermicelli	2×10^2
15/06/2014	Al Karrada Kharej	Cooked rice	1.15×10^4
15/06/2014	Jihad neighborhood on the public road	Cooked rice	2.5×10^2
15/06/2014	Jihad neighborhood near market	Cooked rice and vermicelli	3×10^2
16/03/2014	Jihad neighborhood near market	Cooked rice and vermicelli	1×10^2

*No growth

with maltose and millibiose. Distinct type of bimolecular being produced by them and perhaps in the present study, variation was observed with xylose and other sugars. This would clearly indicate that bacillus having specific phenotypic line. According to the above described methods, an isolated *Bacillus* strains, are identified for each strain separately with Ribosomal 16SrRNA gene-specific PCR, which allowed for seven strains (indicated in Figure 1) to be identified

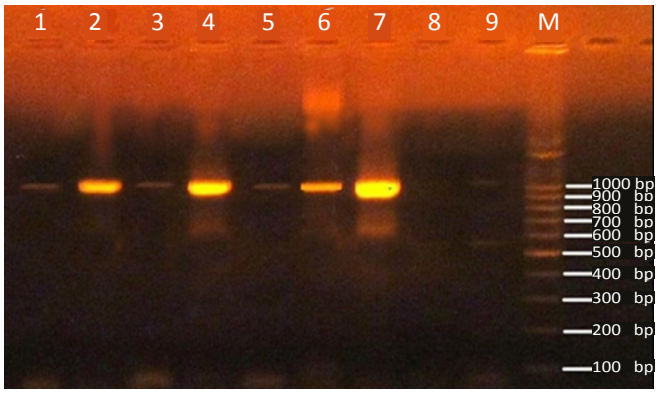

Figure 1: Representative PCR products showing amplicons of six B. cereus isolates. Lanes 1 molecular size marker 100. M=marker

subsequently as positive. Of the 64 isolates, 15 isolates are chosen for molecular characterization. Ten isolates were further characterized by PCR using U1 and U2 primer to identify up to species level. A characteristic banding pattern compare with molecular marker determined as *B. cereus* (Brychta et al., 2009; Beecher and Wong, 1994). From the group of *Bacillus* spp. now all can change the routine work in diagnosis and detection also go on by new methods of bio-molecular to reach subtyping and risk-related strain profiling, due to the extreme intra-species diversity found in the genus *Bacillus*, DNA-based identification and typing methods are gaining increasing importance in routine diagnostics (Ehling-Schulz et al., 2005), our results is near to several studies like (Ombui et al., 2008), which he described that the size marker from 475 – 500 bp. depending on primers were synthesized by Invitrogen ® Co. USA at 2008 compared with our results which depending on primers were synthesized by Promega ® Co. USA at 2014. As shown in Figure 1. The emetic endogenous gene of non-hemolytic enterotoxin (Nhe), described that gene size marker is 500 bp. the gene is same in *B. cereus* isolates from spices and rice, that mean, it may arrived in rice from spices by adding as flavors in rice during cooking or come from spiced chicken or meat due to spores behaviors which come grow in low temperature ≥6 and worm temperature ≤55 °C in manufactured and packed food (Pirttijarvi, 2000).

4. Conclusion and recommendations

Raw spices may contaminated with microbial pathogens, sometimes hold high numbers of bacteria, there are treatment and options of testing are available to reduce risks and to ensure food safety plans, always must be include information of ingredients, control of supply chain auditing suppliers, and planning for supply chain interruptions. Manufacturers

of food must consider these programs to determine using of treated from untreated spice. For rice must treat it before using due to number of meals which consuming by individuals of populations, all of this is taken into account to design monitoring system with control system regime which can help to reduce food poisoning risk.

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