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Isolation and Identification of *Bacillus cereus* from Infant Food Using 16s rRNA Sequence

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Abstract: Microbial contamination of infant food is a common problem and sometimes life-threatening for new born babies throughout the world. The main aim of our study was to isolate pathogenic bacteria present in baby food using conventional (Biochemical) and Modern technique (16s rRNA gene analysis). About five types of powder (Cereal) were used during our study and collected from different pharmacies of Makkah, Saudi Arabia. Samples were analyze aerobically and anaerobically in order to isolate the bacterial strains. Among five samples only three strains were identified as pathogen using biochemical tests. Furthermore, 16s rDNA analysis confirmed that these strains belong to genus *Bacillus* and was identified as *Bacillus cereus species*, which may be a threat to the baby health. So, our study revealed that molecular techniques such as 16s rDNA is a powerful tool to detect pathogenic bacteria in infant food. Some of the cereal powder needs to be sterilized. Finally, future research should be continued with all other stage of cereal food products to ensure the good quality and safety.

Key word: Infant Food · Bacillus Cereus · 16s rDNA Gene Analysis · Foodborne Diseases · GenBank

INTRODUCTION

Before the digestion of supplementary food materials baby foods are the prime source of kid's nutrition. These specific food materials for kids have a high content of vitamins, proteins as well as other necessary ingredients. Globally, millions of children use such kind of high and valuable food materials per day. Unfortunately, kids have somehow weak immunity and germs in these food packages can cause severe infections to them. Therefore, it is utmost need to make high quality, healthy and sterile foods materials to avoid any discomfort however, sometimes it will be changed with numerous contaminations which instigated illness. Some well-known reasons for these food contaminations are the attack of viruses, parasites & bacteria and even some time chemical adulteration generated a high risk of foodborne diseases globally and consider a growing health issue. *Bacillus cereus* (*B. cereus*) was reported by World Health Organization (WHO) [1] and many researches [2, 3] (Change Ref. S. No. in the list??) as the most sever and common food-born treated foodstuffs bacterium.

B. cereus is rod-shaped, spore former and Gram-positive bacterium which instigated emesis, diarrhea, spoilage and fatal meningitis [4, 5]. Therefore, it should be controlled before diffusion through sterilized and disinfected processed foodstuff by using heat otherwise it is a high health risk issue. Their spores have the ability to tolerate and survive in both high and low temperatures. Most common sources include milk powder, mixed food products, liquid food products and the most specific worries about this bacterium is that it is mostly found in the baby formula manufacturing materials [6, 7]. *B. cereus* was previously reported in the kid's food products [8, 9]. The aim of present study was to detect microbial contaminates in baby food using conventional (Biochemical) and modern techniques (16s rRNA gene analysis).

MATERIAL AND METHODS

Five infant food samples were collected directly from Makkah local markets in Saudi Arabia between Month 3 / 2014 and Month 12/ 2015 as showed in Table 1.

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Isolation of Bacteria: About 0.1g of powder baby food was transferred to 99 ml of distilled water in sterilized conditions. Then after serial dilution samples were transferred to Nutrient Agar plates and were normal incubated for 24 hr. at 37 °C. After that pure colonies were transferred to separate tube containing 5 ml of LB broth and were incubated for 24 hr. at 37 C. These pure colonies were used for biochemical and molecular identification.

Identification

Biochemical Identification: For taxonomic identification, the isolates were subjected to a series of biochemical tests [10] which included nitrate reduction, anaerobic growth, gas production from glucose, Voges-Proskauer (VP), growth at different NaCl concentrations, Nitrate reduction, degradation of starch, casein, gelatin & urea, acid production from arabinose, mannitol, xylose, glucose, lactose and citrate utilization.

16s rDNA gene PCR Amplification: DNA was extracted from all samples by following the company's commands (Mention extraction kit name, company and its catalog No.). For the amplification of 16s rDNA approximately 1-2 iL of DNA was used. For the amplification of 16S rDNA the Forward primer: 5'- CAGCGGTACCAGAGTTTGA TCCTGGCTCAG-3' and Reverse primer: 5'- CTCTCTGCAGTACGGCTACCTTGTTACGACTT-3'were used in the assay. The PCR Program was set according to the procedure as 35 cycles, Amplification, 94 for 4min 58 for 1 min and 72°C for 2min respectively and at the end incubated for 4 °C [11, 12]. The evolutionary spaces were calculated by means of the Maximum Composite Likelihood method of Tamura *et al*. [13] based on the units of the amount of base replacements for every site. *MEGA* version 4 was used for the assembly of the evolutionary analyses and phylogenetic tree [14]. Agarose gel electrophoresis was used to amplify gene resolved and then illuminated under UV.

RESULTS

Biochemical Identification: In this study, 15 strains were isolated from Baby food taken from Jeddah regional market. Three of them were identified as *bacillus cereus*. The samples were designated as S1, S2, S3, S4 and S5 as shown in Table 1. Biochemical and morphological characterization of bacterial isolates indicated that three strains were motile and rod shaped, positive for Gram staining, citrate utilization, gelatin, casein and potato starch hydrolysis. All the three strain were Negative for indole, Urease and Mannitol as shown in Table 2.

16s rDNA gene Analysis for Molecular Identification of Strains: About 15 bacterial strains were isolated from enrichment cultures that were maintained at 30°C for 24 hours. Then DNA was isolated using extraction kit and bacterial strains were identified molecularly using 16s rDNA gene amplifiy as shown in Fig. 1 as *Bacillus cereus abc1, Bacillus cereus abc2* and *bacillus cereus abc38*. The phylogenetic tree of these three 16s rDNA gene sequences (KX405589, KX405594 and KX405592) against 24 different *bacillus cereus* sp. strains from Gen Bank (http://www.ncbi.nlm.nih.gov) using *MEGA* version 4 is shown in Fig. 2. The optimal tree with the sum of branch length = 47.28402346 is shown. The data showed that these three *Bacillus* species 16s rRNA sequences lay in the same cluster with many of *Bacillus cereus* sp strains from GenBank, reflecting high genetic similarity with these strains.

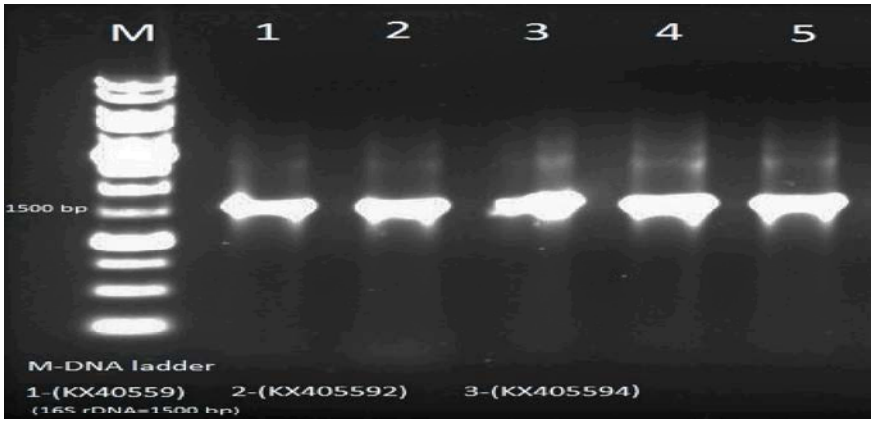

Fig. 1: PCR amplification of 16S rRNA gene of bacterial isolates from infant Ffood, M: DNA lader and lanes 1 to 5 samples.

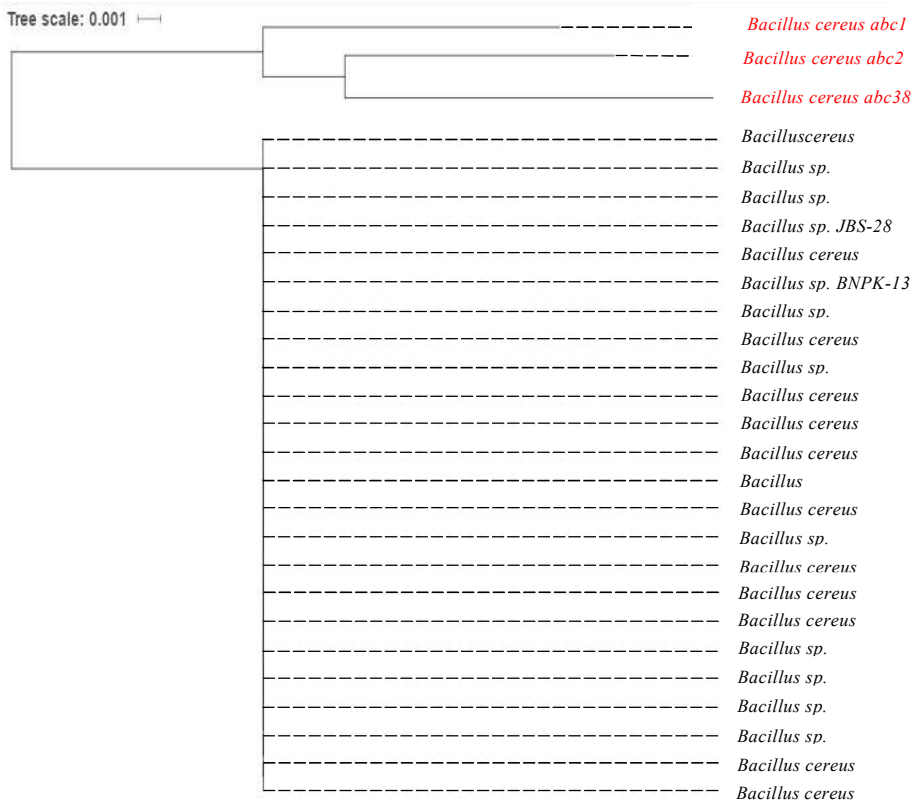

Fig. 2: Comparative phylogenetic analysis of the *Bacillus cereus abc1* (KX405589), *Bacillus cereus abc2* (KX405594) and *bacillus cereus abc38* (KX405592) from GenBank.

Table 1. Information about the samples used for bacterial study

ID	Product Name	Age	Country	Expiry Date
S1	N. C. wheat	6 month	SAUDI ARABIA	10/04/2016
S2	M. C. wheat	4 month	SAUDI ARABIA	04/11/2015
S3	N. C. Wheat, oat, red fruits	12 month	SAUDI ARABIA	18/8/2015
S4	O. rice	6 month	SAUDI ARABIA	06/12/2015
S5	B. 8 cereal AC	5 month	SAUDI ARABIA	09/04/2016

Table 2: Results of biochemical analysis of *Bacillus* sp. isolates from baby food

S. No	Biochemical test	S1	S2	S3
1	Gram staining	positive	positive	positive
2	shape	Rod	Rod	Rod
3	Motility	positive	positive	positive
4	Citrate utilization	positive	positive	positive
5	Gelatin hydrolysis	positive	positive	positive
6	Casein hydrolysis	positive	positive	positive
7	Potato starch hydrolysis	positive	positive	positive
8	Indole	negative	negative	negative
9	Voges-proskauer	positive	positive	positive
10	Nitrate reduction	positive	positive	positive
11	Urease	negative	negative	negative
12	Glucose	positive	positive	positive
13	Mannitol	negative	negative	negative
14	Xylose	negative	negative	negative
15	Arabinose	negative	negative	negative
16	Maltose	positive	positive	positive
17	Sucrose	positive	positive	positive
18	Lactose	negative	negative	negative
19	sorbitol	negative	negative	negative

DISCUSSIONS

Pathogens usually contaminated the dehydrated foods materials used by fragile consumer, comprising dried infant formulations and dried nutritive foods. The pathogenic bacteria contaminated the powdered infant formula (PIF) up to a degree that it might cause severe sickness and even death in infants [15]. Incorrect handling practices, improper hygienic control and current industrial methodologies associated with the dangerous microbes could causes severe illness in the kids. In the era 2004 and 2006, a report was displayed by FAO/WHO Expert Meetings, consider *B. cereus* the main microorganisms among other PIF contamination [15]. Due to the presence of *Bacillus* spp., its growth after rehydration in hot water is dangerous to health. Hence, EFSA (Put full name and then put it as a Ref.??)strictly recommended to keeping *B. cereus* spores as low as possible during processing of dried dietary foods and dried infant formulation as well as hygiene standards should be followed during the preparation to lessen the time scale amongst its synthesis and usage. Although, the *B. cereus*-positive foodstuff indicated lower intensities of contamination as compared to the quantity needed for infection or toxins generation, such exploration addressed the health problems concerning *B. cereus* in processed children's milk. The non-hemolytic enterotoxin (NHE) and cytotoxin hemolysin BL (HBL) as well as cytotoxin K are presently measured the *B. cereus,* etiological agents which caused diarrhea [16]. Similarly, PCR information reflects that numerous microorganisms are the possible diarrheal enterotoxin generators. Owing to the enhanced tendency and offense of *B. cereus* contaminations in kids with feeble immune system [17, 18]. Our PCR results addressed an adequate control and plan is urgent to implement, so as to protect children with weak immune system. The Commission Regulation (EC) no. 1441/2007 on biological standards for foods recommended a detailed investigation of *B. cereus* for hygiene bases and presence of enterotoxin, for the aim to evaluate the risk of children condition. Besides, uncovering the *Bacillus* sp., *B. mycoides*, *B. licheniformis* and *B. subtilis* are also the leading species which causes health issue in baby milk powdered. While, the other injurious *Bacillus* spp., *B. pumilus, B. subtilis* and *B. licheniformis* inspected very poorly (EFSA) [18] and several reports indicated, *B. licheniformis* and *B. subtilis* as the potential source of food poisonous [19, 20]. *B pumilus*, *B. subtilis*, *B. megaterium* and *B. licheniformis* have remained described to instigate emetic toxins and enterotoxins in foodborne sickness [19]. *B. licheniformis* in infant products are particularly described for the death of children. *B. licheniformis* produced cyclic lipopeptide Lichenysin A which tolerates heat [19]. Moreover, *B. subtilis* and *B. licheniformis* species produced surfactant, which is a strong surfactant having structural resemblance to cereulide and Lichenysin A [19, 21]. Thus, other pathogenic *Bacillus* spp. may possibly be controlled having some structural index similar to *B. cereus*. These discoveries highlight the detection of other *Bacillus* spp. in PIF materials, for the purpose to obtain epidemiological information, which helped in the establishing of precise microbiological principles that would laid down the safety and hygiene of reconstituted dry milk formula. Keeping a suitable storage condition is key to re-constitute dry milk formulation is very necessary due to the frequent use of temperature during manufacturing of dried milk products. An urgent investigation is also needed of the infant food products at maternity nurseries and hospital on the above based investigation.

CONCLUSIONS

Our study reveals that Molecular techniques such as 16s rRNA is a powerful tool to detect pathogenic bacteria in infant food and could be used by industries in order to identify these pathogenic strains. Some of the cereal powder needs to be sterilized. Finally, future research should be continue with all other stage of cereal food products to ensure the good quality and safety.

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