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Evaluation of Bacterial Contamination of Powdered Food Products, Pakistan

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Abstract: Food contamination is a serious issue because infectious diseases may spread through food or beverages. Infants and babies are more susceptible to infection by pathogens because of their less well-developed immune system and lack of competing organisms in the gut flora. The aim of this research study was to investigate microbiological safety of a range of powdered food products that are consumed by either infants or adults, who may be immune-compromised. In order to achieve the goal, 21 powdered food products including infant formula milks and powdered protein-based shakes were purchased from retail stores and analyzed for bacterial contamination. Seven out of 21 products were contaminated with different combinations of 9 bacterial isolates belonging to three different types. Isolation and identification was carried out using conventional methods including, culturing, microscopic analysis and biochemical testing. Three bacterial species including two Gram-positive (*Bacillus cereus* and S*taphylococcus aureus*) and one Gram-negative (*Enterobacter sakazakii*) were isolated from seven locally made food products. *B. cereus* was found predominantly in five products. Furthermore, antibiotic susceptibility was determined using various commercially available antibiotic disks.

Keywords: Powdered foods, Bacterial contamination, Food contamination, Infant formula milk.

1. INTRODUCTION

Bacterial contamination of foods is of grave concern as infectious diseases may spread through contaminated food or beverages. Babies and infants have been demonstrated to be more susceptible to infections by pathogens because of low level of immunity and absence of competing organisms in the gut flora (Townsend and Forsythe 2008). Food products consumed during the neonatal, infant and weaning periods are limited, with a few food items representing the main nutrition source. These foods generally represent a rich source of nutrients and contain ingredients from various origins, hence carrying a potential risk of exposure to food borne pathogen (Kim *et al.* 2011). Dried milk products, such as milk powder, IMF and infant cereal products often contain high level of carbohydrates (starch, sucrose or lactose) and minerals which can promote proliferation and enterotoxin production when they are reconstituted and held at ambient temperature for extended periods, potentially even at refrigeration temperature (Jaquette and Beuchat 1998, Reyes *et al.* 2007, Rowan *et al.* 1997). Several studies have reported the contamination of infant food formula by pathogenic microorganism including *Enerobacteriacea , Staphylococcu*s spp, and *Bacillus* spp (Shadlia-Matug *et al.* 2008) .

E. sakazakii is a facultative anaerobic Gram-negative bacillus found in intestinal tract of animals and in the natural environment. Foods could be contaminated with *E. sakazakii* under condition of hygiene mismanagement involving various factors including incorrect temperature and time factor as well as due to contact transmission of microorganisms via hands, insects, small vertebrates, equipments, and should be avoided during production, preparation and storage of food and drinks (Hamilton *et al.* 2003, Kuzina *et al.* 2001, Gakuya *et al.* 2001). *B. cereus* is an endemic, soil-dwelling Gram-positive, rod-shaped bacterium. Because of its cosmopolitan distribution in nature (Wong *et al.* 1988) it has been isolated from rice, spices, meat, egg and dairy products (Johnson 1984). Besides this dried milk products and infant foods have been shown to be frequently contaminated with *B. cereus* (Heinz Becker 1994). *B. cereus* is becoming an important food poisoning organism. (Ahmed *et al.* 1983) has shown its association with diarrhea and emetic types of food poisoning outbreaks in U.S (Ahmed 1983). *B. cereus* is a predominant species of Bacillus isolated from milk at all stage of processing and may reach the level associated with enterotoxin production. *B. cereus* has particular importance in powdered foods as it can form spores which can survive various drying and heat-treatments used in the food industry such as pasteurization (Andersson *et al.* 1995). The spores have been reported to adhere to stainless steel and resist to cleaning procedures in food factories (Tauveron *et al.* 2006) indicating obvious implication as the equipment could contaminate the food and resulting in high numbers of the spores present in the food products. The spores have ability to withstand the pasteurization and storage in a desiccated environment over an extended period time. On rehydrated of powdered foods, the spores may revert to the vegetative state and release of

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the toxins. High growth rate of the cells together with release of toxin may cause food poisoning especially in immune-compromised individuals.

S. aureus is a Gram-positive, facultative anaerobe, coccus-shaped bacterium, which is commonly carried asymptomatically on human skin, typically around the nose and nails. *S. aureus* is a well-known cause of food poisoning. The higher growth rate of *S. aureus* ($\geq 10^6$) in food products pose hazards because of the ability of organism to produce heat stable enterotoxins (Bergdoll 1991). Low bacterial count in foods is generally not considered as hazardous and thus not a public health concern. *S. aureus* has been demonstrated to survive for extended time in powdered food products. Foods having post-process contamination with the staphylococci also present a potential hazard because of the absence of competitive organisms that might restrict the growth of *S. aureus* and subsequent production of enterotoxin (Ronald and Santos 2001). The focus of this study was to determine whether dried powdered food products are safe enough to be consumed. It has been demonstrated that some bacteria are known to be able to survive the drying processes involved in making these products, as well as being able to survive in the harsh dry environment. The contamination of the powdered foods therefore poses serious health problems especially for neonates, lacking the fully developed immune system and also for immune-compromised individuals. Two products were selected to undertake analysis in this study. These products included Infant formula milk and Protein based drinks. The aim of this study was therefore to investigate bacterial contamination of commercial powder food available in Pakistan and to determine the susceptibility of the isolated bacteria against some antibiotics.

2. MATERIALS AND METHODS

2.1 Bacterial strains and growth conditions

All bacterial strains were isolated from various powdered food products and were initially grown on either Muller Hinton agar (MHA) or Nutrient agar (NA) and incubated at 37°C for 24-48 hrs.

2.2 Preparation and sterilization of growth media

All glassware including Petri plates, test tubes, flask, pipette etc were sterilized in a Hot-air oven at 180°C for approx. 1-2 hrs. Unless otherwise stated, all media (solid and liquid) were prepared according to the manufacturer's instructions. Sterilization was carried out by autoclaving at 121°C 15 psi for 20-30 min. Following sterilization, media were cooled to 45 °C and poured aseptically in sterilized Petri plates and tubes as per requirements of the specific experiments.

2.3 Preparation of Buffered Peptone Water (BPW)

One litre of BPW was prepared by adding 10g of Peptone, 5g sodium chloride, 3.5g of di-sodium phosphate, and 1.5 g of mono-potassium phosphate to distilled H_2O. The solution was sterilized by autoclaving for 15 min at 121 °C. After sterilization, 225 ml of the buffered peptone water were poured into separate sterile bottles for use in future experiments. Peptone is the nitrogen, carbon, vitamin, and mineral sources in Buffered Peptone Water, pH 7.0. Sodium Chloride maintains the osmotic balance. Disodium Phosphate and Mono-potassium Phosphate are the buffering agents in this medium. Formula may be adjusted and/or supplemented as required to meet performance specifications.

2.4 Collection of food samples

A total of 21 samples of powdered food including energy drinks and powder infant formula (PIF) were purchased from retailer across the Hyderabad and Jamshoro.

2.5 Sampling of powdered foods

A 25 g sample of powdered food/milk was reconstituted in 225 ml of sterilized BPW or sterilized distilled water **(Fig. 1)** and the mixture/suspension was left for 30-45 min to enrich the sample. Aliquots of 100 and 200µl of sample were taken with the help of micropipette and spread on to MHA and NA plates and incubated at 37 °C for 24-48 hrs.

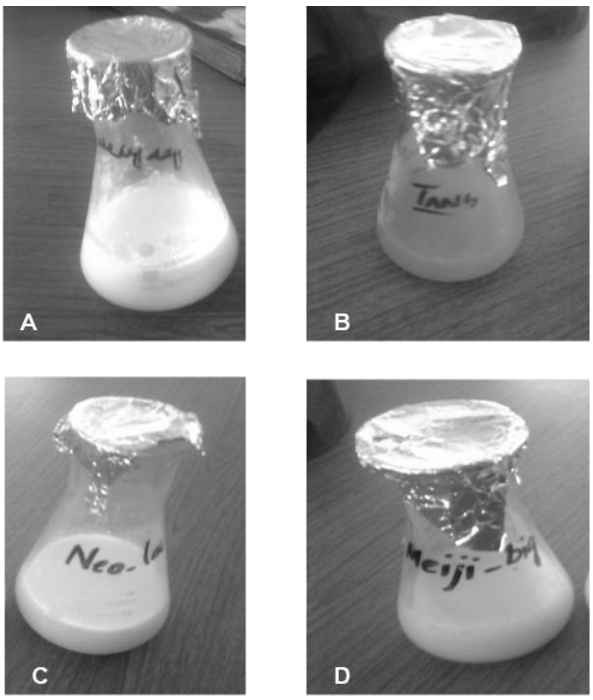


Fig. 1. Preparation of samples of various powdered food products for determining the microbiological safety of these foods

2.6 Identification and characterization of the isolates.

Identification and characterisation of the isolates was carried out using standard methods (Cheesbrough, 1985). Briefly, size, shape, colour, elevation and margins of the bacterial colonies were observed following growth of cultures on Nutrient or Muller Hinton agar and incubation for 24-48 hrs at 37°C. The shape and arrangement of the cells were observed by the Microscope. A number of biochemical tests including catalase and coagulase tests were performed for all isolates as per standard protocols. A loop of sterile distilled water was placed onto a microscope slide and part of a fresh colony was mixed with it using a sterile loop. A cover slip was then placed on top of the microscope slide. One drop of immersion oil was then put on the cover slip. Using a 100× oil immersion lens, the motility of the organism, morphology and other characteristic features, such as cell arrangement or any pigmentation, were recorded**.**

2.7 Antibiotic susceptibility test

Antibiotic susceptibility tests were conducted following the method of Kirby-Bauer disk diffusion method (Bauer *et al.* 1966). Mueller-Hinton agar was used as the growth medium in all the antibiotic disk (Oxoid, UK) susceptibility tests. The zones of growth inhibition were measured to the nearest millimeters using a ruler. The zone margin was considered to be the area showing no obvious, visible growth that can be detected with the unaided eye. The sizes of the zones of inhibition were interpreted by referring to British Society for Antimicrobial Chemotherapy (BSAC) standards and were reported as being susceptible, or resistant to the agents that were tested. The zones of inhibition diameters were measured and compared to the expected diameters according to BSAC (2011) guidelines **(Table -1).**

Table 1.Antibiotic discs used in this study

S.No	Antibiotic	Code	Potency	Mode
1	Cefotaxime	CTX	30 µg	Cell wall inhibitor
2	Imipenum	IPM	10 µg	Cell wall inhibitor
3	Amikacin	AK	30 µg	Protein Inhibitor
4	Gentamicin	CN	10 µg	Protein Inhibitor
5	Naldixic Acid	NA	30 µg	Nucleic Acid inhibitor

3. RESULTS AND DISCUSSIONS

3.1 Isolation of pure culture of bacteria from powdered foods

The present study evaluates the bacterial contamination of powdered food products. It has been observed that the infants and adults consuming these powdered products have the potential to be in some way immune-compromised. For example, newborn's have very little immunity, which may disappear if they are not being breastfed. This is because there is no passive immunity being transferred to the new born baby. Therefore babies being fed infant formula from birth may be at risk of illnesses from organisms that are in their food. Immuno-compromised individuals taking protein based drinks may also be at higher risk. Especially, elderly individuals are also reported to be potentially at risk. This is because as they get older, their immune system begins to decline, leaving them more vulnerable to pathogens that would not normally be a problem for healthy adults.

A total of 21 different powdered food products including infant formula milk and protein based energy shakes were purchased from retail stores across Hyderabad and Jamshoro, Sindh, Pakistan. Initial screening of the food products demonstrated that seven products were contaminated with either one or two types of bacteria. The sampling was carried out under aseptic conditions and experiments were repeated three times for each sample to avoid the false positive results of contamination. To obtain pure cultures of the isolates discrete, single colonies of the bacterial isolates formed on sampling media were carefully picked and inoculted on diagnostic media .The pure culture of the some of the isolates are presented in (**Fig. 2)**. which were further subjected to Gram staining followed by microscopy to determine cell morphology, and biochemical tests to identify the organisms to the species level.

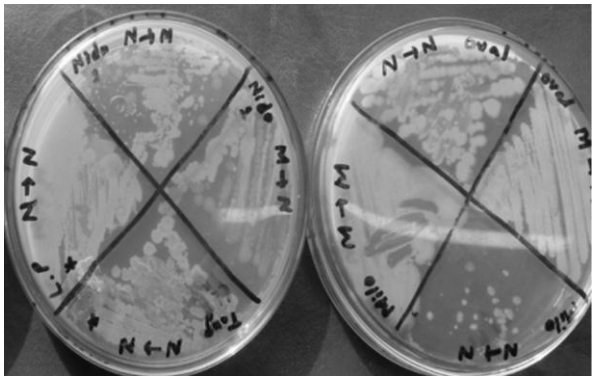


Fig. 2. Isolation of pure culture of the bacterial contaminants on selective media plates

3.2 Identification and biochemical characterization

In an effort to identify bacterial isolates, the culture plates were observed for colonial characteristics such as color, shape and surface and elevation. The cell characteristics were noted following cultivation on NA or MHA at 37°C overnight and morphological

characteristics pertaining to color and shape were noted. The results of microscopic analysis are presented in the Table 2. All isolates were tested for enzyme production and their ability to ferment different types of sugars.

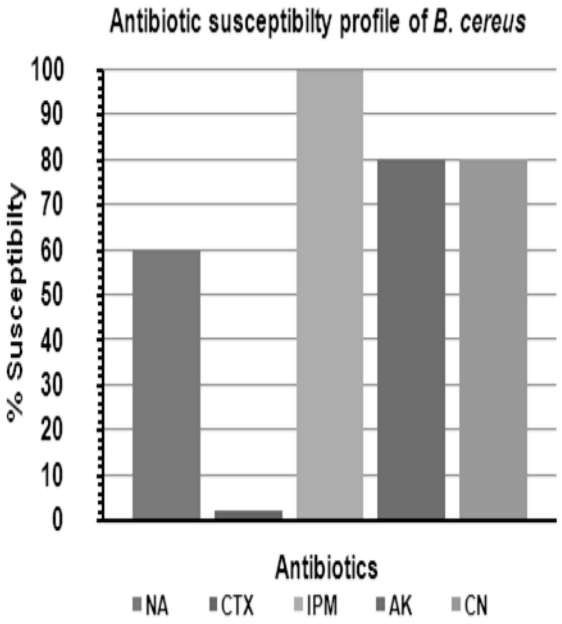
B. cereus was most commonly isolated organism, which was present in 5 products. Microscopic analysis demonstrated that the all organisms isolated from these 5 products, had the features that are characteristic to *B. cereus* (i.e. Gram-positive, motile, spore-forming rods). The member of *Bacillus* genus appears to be very closely related such as *B. cereus* and *B. thuringiensis* cannot be differentiated on the basis of biochemical characteristics. *B. anthracis* and *B. cereus* also have close genetic links which makes the identification a difficult process. PCR-based methods could be useful to discriminate among the isolates. Furthermore, this study also demonstrates that 2 food products were contaminated with *S. aureus.* The lower frequency of contamination by this bacterium in the food is not surprising as *S. aureus* is not a spore former hence cannot survive like *B. cereus* and *C. perfringens*. However *S. aureus* has been shown to survive for longer periods of time in dry, desiccated environments, which may pose the risks for consumers of these powdered foods. *S. aureus* is readily killed by pasteurization temperatures, despite the presence of the organisms in the products suggests either faulty heat processes or post- process contamination due to the organism on the skin of food handlers, that have gone on to contaminate the products **(Table-2).**

Table 2. Gram staining and morphological characteristics (color, shape, arrangement) of the isolates

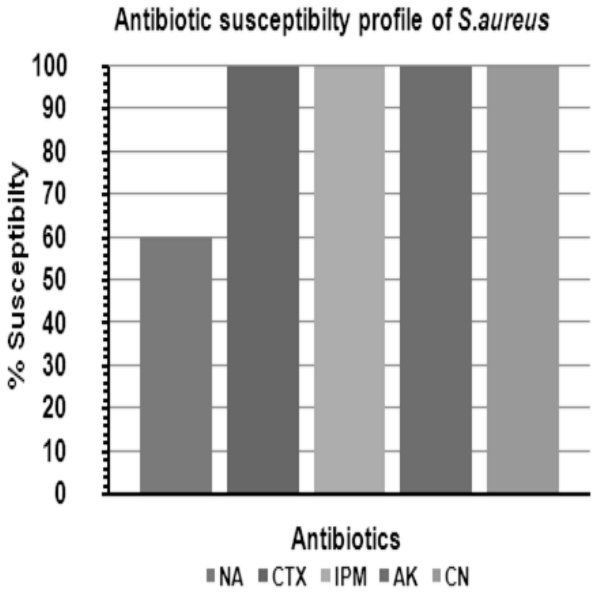
Isolates ID	Probable Organism	MORPHOLOGICAL CHARACTERS		
		Gram Reaction	Colour	Shape
SA1	*B. cereus*	Gram-positive	Purple	Rods
SA2	*B. cereus*	Gram-positive	purple	Rods
SB3	*B. cereus*	Gram-positive	purple	Rods
SD5	*S. aureus*	Gram-positive	Purple	Coccus
SE6	*B. cereus*	Gram-positive	Purple	Rods
SF7	*B. cereus*	Gram-positive	Purple	Rods
SG8	*E. sakazakii*	Gram-negative	Pink	Rods
SH9	*E. sakazakii*	Gram-negative	Pink	Rods
SJ10	*S. aureus*	Gram-positive	purple	Coccus

3.4 Antibiotic susceptibility patterns of the isolates

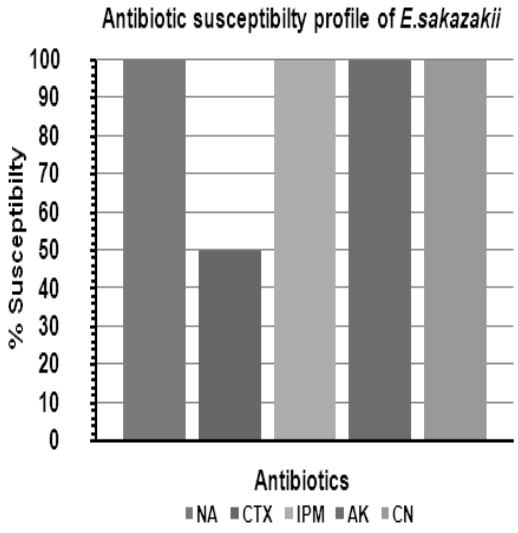
The susceptibility patterns of all bacterial isolates against various antibiotics (Table 1) are shown in **(Fig. 3A, 3B, and 3C)** *B. cereus* demonstrated a mixed pattern of susceptibility against all antibiotics tested except CTX. All isolates of *B. cereus* were 100% resistant to this antibiotic. *B. cereus* and *S. aureus* both showed 60% sensitivity against NA. All isolates of *S. aureus* were susceptible to four antibiotics. *E. sakazakii* was observed to show 50% susceptibility against CTX. The lowest susceptibility was observed for all the isolates to CTX and NA. IMP, AK, and CN generally exhibited good activity against the isolates. All the isolates were approximately 50% resistant to at least one antibiotic.



(A)



(B)



(C)

Fig. 3. The antibiotic susceptibility patterns of (A) *B. cereus* isolated from powdered food. (B) *S. aureus* isolated from powdered food. (C) *E. sakazakii* isolated from powdered food. *E. sakazakii* was also found to be susceptible to majority of antibiotics tested.

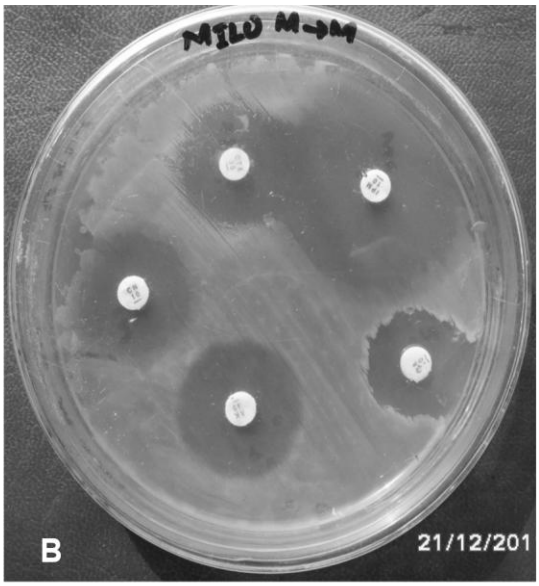


Fig. 4 Antibiotic susceptibility testing, Bacterial isolates including *B. cereus, S. aureus,* and *E. sakazakaii* were grown on MHA and various antibiotics were tested for determining resistance pattern.

5. CONCLUSION

Out of the 21 products tested, 7 (locally made products) were found to have been contaminated with different combinations of the three bacterial pathogens including *S. aureus*, *B. cereus*, and *E. sakazakaii*. Among the products tested, the imported quality foods including PIF milk were microbiologically safe and no contamination of bacteria was detected. From the results, four of the products were found to have been contaminated with one isolate tested for, and three products were contaminated with two isolates. *B. cereus* was observed to have contaminated most of the products. One product was found to have been contaminated with both *B. cereus* and *S. aureus*. The problems seem to increase in case where both *B. cereus* and *S. aureus* are found in the same product. When powdered foods are reconstituted and left at room temperature, both organisms, although may not be at high numbers to cause food poisoning, however, if both produce toxins together a synergistic effect may take place, thus, even less numbers of organism together may cause food poisoning. Also, the data showed that one product was contaminated with *B. Cereus* and *E. sakazakii*, which shows a very poor microbiological quality assurance. It is also worth noting that all the products that were contaminated with more than one of the pathogens always had *B. cereus* in it. The presence of *B. cereus* (endospore forming bacterium) in powered food could be potentially alarming for the health of individuals who consume such type of products because on rehydration these bacteria can turn into their vegetative form and release toxin and cause diseases in the host. The risk of contamination by *B. cereus* should be taken into account and it is suggested that safety criteria for the presence of *B. cereus* in infant foods should be established. The data also suggest that new safety criteria, along with hygienic control measures and consumer education strategies, be established to improve the level of safety of powdered food products.

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