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Detection of Microbial Contaminations in Baby Milk Using Microbial and Molecular Techniques

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Abstract: Microbial contamination in Baby milk is a common problem and sometimes life-threatening for new born babies all over the world. Different types of microorganisms can cause milk contamination. Molecular techniques such as RAPD-PCR and 16S rRNA gene sequencing were used to identify various microorganisms present in the milk. The main goal of this study was to exploit the advanced microbial and molecular biology techniques for the detection and diagnosis of microorganisms in milk. Improvements were made in the sample preparation, testing procedures and data analysis to enhance the sensitivity and specificity of detection methods for tracking the pathogens in baby milk. This study has shown that most of baby milk retailed in Saudi Arabia possess useful bacteria such as *Brevibacillus* and *Bacillus subtilis.* However liquid milk sample was found to be contaminated with harmful bacteria, *Acinetobacter baumannii.*

Key word: Bacteria · Molecular techniques · PCR · Baby milk · Microbial contaminations

INTRODUCTION

Foodborne diseases are a major health concern globally. Millions of people become sick and many die after consuming unhygienic food, which prompted WHO Member States to adopt a resolution in 2000 to recognize food safety as a vital public health issue. Food safety requires actions to ensure that the food is safe for human consumption. Food safety policies need to encompass the entire chain i.e. from food production to consumption [1].

Food poisoning syndrome results from ingestion of water and food items contaminated with pathogenic microorganisms including bacteria, viruses, protozoa and fungi or contaminated with the toxins released by the pathogens and various chemicals. It has been estimated that more than 10 million infants and young children in developing countries die before reaching age five every year [2] and 70% of the children die due to infections like acute respiratory infections, diarrhoea, measles and malaria or from malnutrition [3].

Powdered baby milk, powdered infant formula (PIF) and other baby food products have been associated with illness and death in infants due to microbial contamination. Dairy products, cereals, fruits and nuts are the main ingredients which are used in the formulation of baby food. Baby food may get contaminated with pathogenic and non-pathogenic microbes during improper manufacturing process. During the production of baby food and infant formula, inappropriate handling practices can aggravate the contamination problem. The FAO/WHO, Food and Drug Administration and European Commission have set certain permissible levels for total bacterial counts in infant food and formula, however there is not much information on levels of fungi in baby products. Dairy products may get contaminated with fungi during the process of drying and storage. According to the survey by FAO, mycotoxins contaminate 25 percent of agricultural crops worldwide [4].

The 16S rRNA genes sequence and other genetic regions are commonly used in identification of microbes

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in clinical microbiology laboratory [5 -10]. The aim of this study was to apply modern molecular biology techniques for the detection and diagnosis of microbial contamination in baby milk.

MATERIALS AND METHODS

Sample Collection and Preparation: Twenty different types of powder and five liquid baby milk were procured from pharmacies/ Departmental stores in Jeddah, Saudi Arabia. One gm of milk powder was mixed in 50ml of sterilized water. 100µl of the suspension was plated on LB agar plate and 1 ml of the suspension was inoculated in 10 ml of LB broth and incubated at 37°C for 24 hrs. Two types of cultures were prepared one was to identify aerobic and another was to detect anaerobic microorganisms. The bacterial growth was monitored for all the sample preparations. Analysis of various milk samples was performed during years 2014 to 2015. Various microbiology tests such as Gram staining and molecular biology techniques such as 16S rRNA gene sequencing, RAPD PCR were performed to detect and characterize various contaminating microrganisms present in milk samples.

Gram Staining: Gram staining was performed using the standard protocol [11].

Genomic DNA Isolation: Genomic DNA was isolated by using Gene JET Genomic DNA Purification Kit (Thermo Scientific) according to the manufacturer's instructions.

Purification of Genomic DNA from Gram Negative Bacteria: Bacterial culture (2 x 10^9 cells) was harvested in a 1.5 ml eppendorf tube by centrifugation at 5000 × g for 10 min. The supernatant was removed and the pellet was resuspended in 180 µl of Digestion solution. Around 20 µl of Proteinase K solution was added and mixed thoroughly by vortexing. The samples were incubated at 56°C, in a shaking water bath for 30 min. Around 20µl of RNase A solution was added and mixed by vortexing and further incubated at room temperature for 10 min. About 200 µl of Lysis solution was added to the sample and mixed thoroughly by vortexing. About 400 µl of 50% ethanol was added and mixed gently. The lysate was transferred to a Gene JET genomic DNA purification column placed in a collection tube and centrifuged at 6000 × g for 1 min. Column was placed in a fresh 2 ml collection tube and DNA was eluted with 40 µl of Elution buffer.

Genomic DNA purification from Gram Positive Bacteria: Bacterial culture (2x 10^9 cells) was harvested in a 1.5 ml microcentrifuge tube by centrifugation at 5000 × g for 10 min. The pellet was resuspended in 180 µl of lysis buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1.2% Triton X-100, 20 mg/ml lysozyme) and kept at 37°C for 30 min. Around 20 µl of Proteinase K solution was added and mixed thoroughly by vortexing. The samples were kept at 56°C for 30 min. Around 20 µl of RNase A solution was added, mixed and the mixture was further incubated for 10 min at room temperature. About 400 µl of 50% ethanol was added and mixed thoroughly and the lysate was transferred to a Gene JET Genomic DNA Purification Column placed in a collection tube. The column was centrifuged at 6000 × g for 1 min. Column was placed into a new 2 ml collection tube and DNA was eluted with 40 µl of Elution buffer.

PCR Amplification of 16s rRNA Gene: Purified genomic DNA was used for 16s rRNA gene amplification by using universal primers for 16s rRNA gene i.e. Forward primer: 5'- CAGCGGTACCAGAGTTTGATCCTGGCTCAG -3', Reverse primer: 5'- CTCTCTGCAGTACGGCTACCTTGTTACGACTT-3'. The reaction mixture was prepared in 25 µl volumes by adding 50 ng/µl of genomic DNA, 3.5 µl of 10× PCR buffer (100 mM Tris–HCl, pH 8.3, 250 mM KCl, 1.5 mM $MgCl_2$), 0.1 mM of each dNTPs, 1.5 U of Taq DNA polymerase, 50 pmol of both the primers and distilled water to makeup the total volume. PCR was set at 30 cycles and the amplification was carried out in a thermal cycler by setting the following program; denaturing at 92°C for 2 min, annealing at 42 °C for 30 sec and extension at 72 °C for 4 min [12]. The amplified DNA fragments were resolved using agarose gel electrophoresis and visualized under UV.

RAPD PCR Technique: Four random primers, each consisting of 10 bases, were used for determining the genetic variations in the genomic DNA of microbes present in powder milk samples. Sequences of primers are illustrated in Table 1. All primers were resuspended in sterile Milli-Q water at a concentration of 100 pmol/µl as a stock solution. Working solution of each primer was prepared at 10 pmol/µl. The amplification was performed for 40 cycles in a thermocycler. Each cycle comprised of denaturation at 95°C for 1 min, annealing at 30°C for 1 min and extension at 72°C for 1 min, with initial incubation at 95°C for 5 min at the beginning of the first cycle and post extension step at 72°C for 10 min after the end of the last

Table 1: RAPD primers used for PCR amplification

Primer	Sequence
1	5`-GTGGGCGAC-3`
2	5`-GTCCATCCA-3`
3	5`-ACATCGCCCA-3`
4	5`-AAGGGCGAGT-3`

cycle [13]. PCR products were resolved on agarose gel electrophoresis. The gel was photographed using gel documentation system and size of each DNA fragment was estimated by using DNA molecular weight marker.

RESULTS AND DISCUSSION

Morphological Characterization: The bacterial strains, obtained from various milk samples were stained with Gram stain. Four strains were found to be Gram positive and one was Gram negative as shown in Fig. 1. A and B. Samples no. M1, M2, M7 and M20 were Gram positive bacteria, while sample M5 was found to be Gram negative bacteria.

Molecular Characterization and Identification of Bacterial Species: A good quality genomic DNA was isolated from bacterial isolates without any signs of smearing when observed under UV transilluminator. The yield of genomic DNA varied from 60 to 140 ng/µl. PCR amplification of ribosomal RNA gene was carried out with universal forward and reverse primers of 16S rRNA gene. A fragment of approximately 1500 bp was amplified as seen in Fig. 2. The amplified PCR product was run on 1% agarose gel and viewed under the UV transilluminator.

RAPD-PCR Analysis: RAPD-PCR was performed to characterize and differentiate various bacterial isolates obtained from milk samples. RAPD analysis of the individual DNA samples of the bacterial isolates was done using four different primers that amplified distinct fragments in each bacterial isolates. Finger printing data revealed multiple DNA products with 3 to 10 bands ranging from 250 to 3000 bp.

Phylogenetic Analysis: The amplified PCR products of 16S rRNA gene from bacterial isolates were purified and sequenced. A BLASTN analysis was performed through GenBank (http://www.ncbi.nlm.nih.gov) showed that all the bacterial isolates were members of two different genera. Among them 4 bacteria belonged to the group Bacillus and 1 bacteria belonged to the Acinetobacter group. The identified sequences of isolated strains were submitted to NCBI (National Centre for Biotechnology Information) and the accession numbers are mentioned in Table 2. The 16S rRNA based phylogenetic analysis of all bacterial isolates is shown in Fig. 4.

There are no specific regulations regarding the labeling and claims that can be made by manufacturers of food products in several countries. Infant formulas currently available in the market do not reveal the probiotics used for the product preparation. All the baby products analysed in our study declared only the species contained in the powder (useful bacteria) and liquid (Pathogenic), while the bacterial loads were not mentioned on the label. In our study we observed that most of the tested infant formulas were properly labeled and the viable cells count remained stable until the product expiry

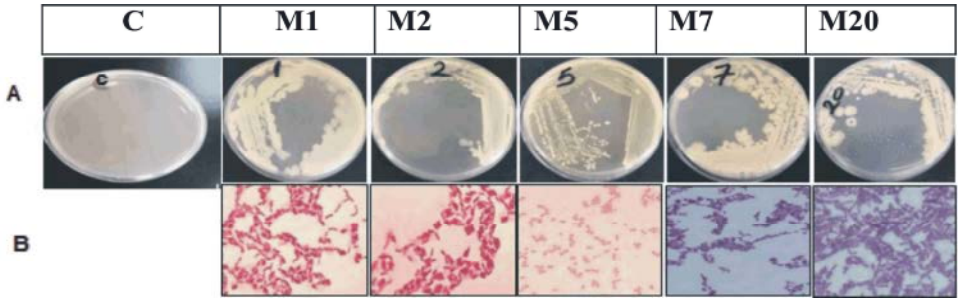

Fig. 1: (A and B) Gram staining of various bacterial isolates obtained from milk samples.

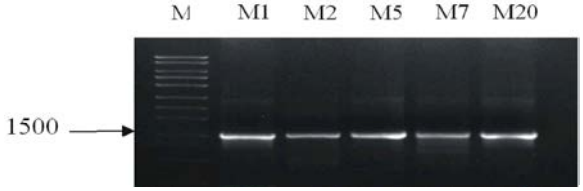

Fig. 2: PCR amplification of 16S rRNA gene of bacterial isolates from milk samples.

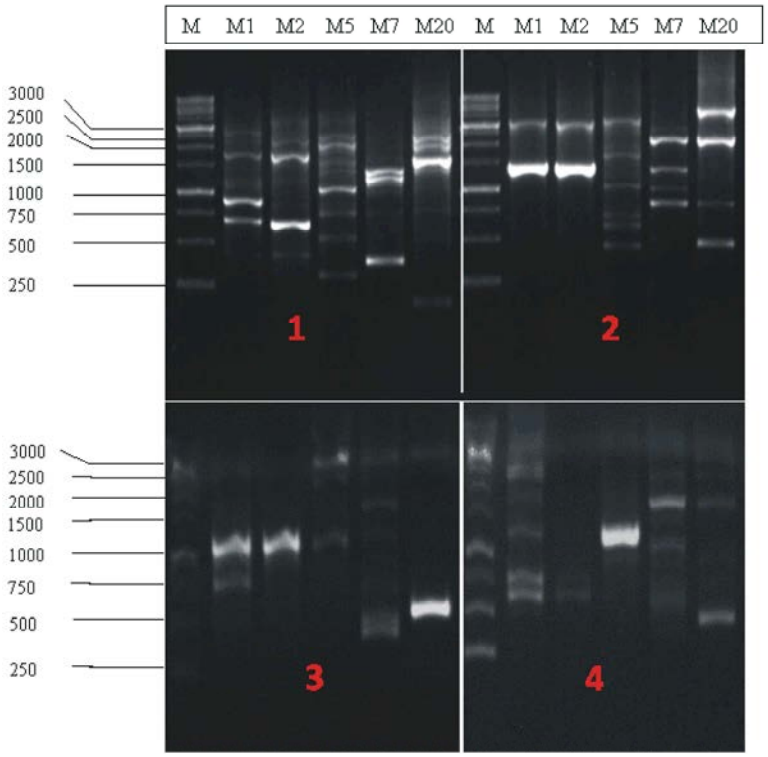

Fig. 3: RAPD profiles of five bacteria isolated from milk samples were subjected to PCR amplification by using four different primers. M1, M2, M5, M7 and M20 represent Milk samples, M represents 1 Kb DNA ladder.

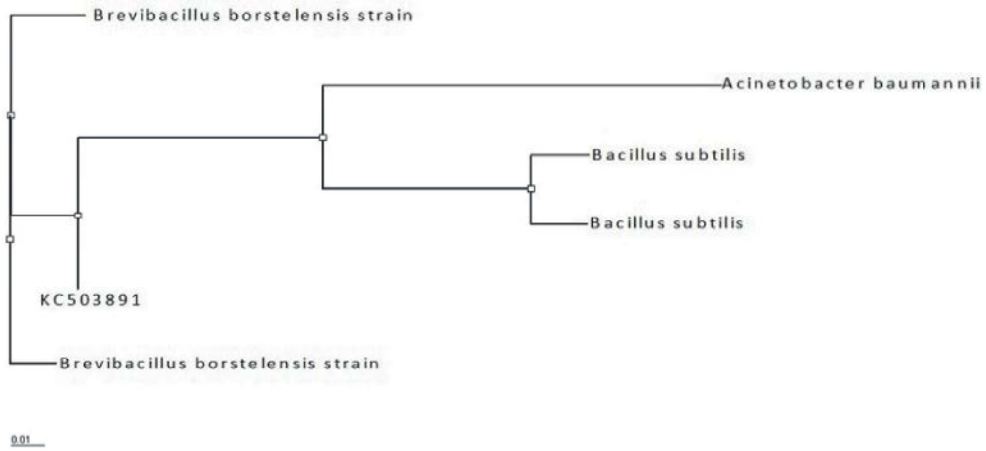

Fig. 4: Phylogenetic analysis of bacterial isolates from milk samples based on the partial nucleotide sequences of 16S rDNA gene. The tree was constructed using the neighbour-joining method.

Table 2: Identification of bacterial strains on the basis of 16S rRNA gene sequence

Sample ID	Strain	Accession Number
M1	*Brevibacillus* sp ES-SL-1	KT630862.1
M2	*Brevibacillus* sp ES-SL-1	KT630863.1
M5	*Acinetobacter baumannii* ZW85-1	KT630864.1
M7	*Bacillus subtilis strain* ASDT1A	KT630865.1
M20	*Bacillus sp* VRC08	KT630866.1

date. It has been shown that the drying process affects the viability of microbes. *Bifidobacteria* are found to be more susceptible to this kind of stress and require anaerobic condition to survive [14]. The packaging and storage conditions for baby food products should be evaluated and carefully monitored as moisture, oxygen, light and high temperatures may affect the shelf-life of dried milk products [6, 10, 14].

Iversen and Forsy investigated 82 powdered infant formulas and detected *Enterobacter sakazakii, Klebsiella pneumonia, Enterobacter cloacae* and *Citrobacter freundii,* which have been shown to be associated with neonatal infections including Necrotizing Enterocolitis (NEC) [15]. The existence of these pathogenic microbes in baby food products should be prevented and carefully monitored. All the baby milk products analysed in this study did not contain any bacterial contamination except one i.e *Acinetobacter baumannii*. Moreover, all infant formulas were found to contain sufficient amount of bacteria, as suggested by the Joint FAO/WHO Expert Meeting reports on probiotics [3]. One important criterion for selecting bacterial strains for use in the food industry is the absence of plasmids harboring antibiotic resistance gene. Various antibiotic resistant microbes are introduced in the human gut through the ingested food and these microbes can potentially transfer antibiotic resistance genes to pathogenic bacteria localized in the intestinal tract. Most probiotics, such as lactobacilli and bifidobacteria, are regular inhabitants of the human gut, hence the presence of antibiotic resistance genes in these strains should be carefully monitored [16].

CONCLUSIONS AND RECOMMENDATIONS

The presence of bacterial strains in baby milk, powder and liquid milk lend weight to this suggestion that most of the identified bacteria are useful and only one of them from liquid milk contained harmful bacteria, which may affect baby health. This study has shown that most of baby milk retailed in Saudi Arabia possess useful bacteria and hence newborn will be less susceptible to infection. But in case of liquid milk sample taken from hospital, pathogenic bacteria was detected which may cause infection in babies after consumption.

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