Bacteriological valuations of some powdered infant milk and antibiotic resistance estimation

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Background and objectives: Milk is one of the widely consumed products in the world, highly susceptible to contamination by microorganisms and it is also a suitable medium for the rapid growth and multiplication of bacteria at favorable temperatures, infant formula (baby milk) contain purified cow's milk whey and casein as a protein source, the high nutrient contents of infant formula provide a good growth medium for bacterial pathogens. This study aimed to evaluate the prevalence of bacterial contamination in powder milk sold in local market of Erbil city, and to determine their antibiotic susceptibility .

Method: In a total of 12 samples of commercial dried milk products (infant powdered milk) used by consumers in Erbil state, biochemical and molecular identification were used for identification. A region of 16S *rRNA* gene were amplified by PCR t*o allow species identification,* and the sensitivity of isolates to eight antibiotics was studied by using standard disc diffusion method

Results: Eight samples of infant powdered milk (N = 12) were contaminated with (62.5%) *Staphylococcus* sp and (37.5%) *Lactobacillus* sp . In addition, *Staphylococcus* sp. resistance against AMC and E was between (72-69%) and the lowest resistance was against AMK and TE (25-28%). *Lactobacillus species* resistance was most frequently observed to E and AMC (88-83%), and lowest resistance was against CIP,TE (25-30%)

C**onclusion :** The current study found that infant powdered milk used by consumers in Erbil state were contaminated with *Staphylococcus* sp. and *Lactobacillus* , these isolates exhibited multiple antimicrobial resistance. No significant differences were found between isolates against antibiotic resistances.

Keywords: Powdered infant milk, Stain, Antibiotic, Staphylococci, Lactobacillus.

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Introduction

Milk is the basic dairy product and people use it as basic supplement in food stuff. Liquid fresh milk can be utilized in drinking purpose while after processing cheese, cream, butter, yogurt, buttermilk and other products are also used. It is truly an amazing food when it is non-contaminated with any other microorganisms.[1]

Milk is a highly nutritious food that serves as an excellent growth medium for a wide range of microorganisms. The microbiological quality of milk and dairy products is influenced by the initial flora of raw milk, the processing conditions, and post-heat treatment contamination.[2]

With regard to infant formula (baby milk) which is a manufactured food designed and marketed for feeding to babies and infants under 12 months, contain purified cow's milk whey and casein as a protein source in developing countries has caused higher rates of diarrheal morbidity and mortality, possibly because contaminated water is often used to prepare infant formula and because the high nutrient contents of infant formula provide a good growth medium for bacterial pathogens.[3]

Although, milk is one of the widely consumed products in the world. However, it is also highly susceptible to contamination by microorganisms and it is also a suitable medium for the rapid growth and multiplication of bacteria at favorable temperatures. It is necessary to use great care in the collection and handling of milk samples to prevent any extraneous contamination and to control the growth of organisms during transportation and storage of the milk. the types of bacteria which change the properties of milk and are often produce proteolytic and lipolytic enzymes. They include bacteria such as *Staphylococcus* sp., *Bacillus cereus*, *Pseudomonas aeruginosa*, *Proteus* sp., *Escherichia coli*, *Micrococcus* sp. and *Serratia marcescens.*[4]

Staphylococcus sp. is one of the most dangerous bacteria that lead to food poisoning. It is a pathogenic bacterium which is able to produce enterotoxin in foods. Milk is a typical growth medium for Staphylococcus sp., that may cause problem if it is to be consumed, especially by infant.[5] Bacteria of the genus *Lactobacillus* are an important group of lactic acid bacteria (LAB), which have a predominant habitat in the gastrointestinal tract of humans and animals. They also persist in the external environment and can colonize diverse niches due to their high heat tolerance and ability to survive under adverse environmental conditions.

Therefore, Enterococci occur in extended range of foods, especially those of animal origin, such as fermented sausages and cheeses. It is well known, that Enterococci are an important part of the bacterial population of several cheeses, such as Manchego.[6]

There is no aim of study here.

Methods

Powder Milk Samples

In the present study twelve different powder milk samples were purchased from supermarkets and belong to twelfth different companies. The collecting samples of milk powder were dissolved in sterilized distilled water and were streaked on nutrient agar and were incubated at 37°C for 24 h and then examined for bacterial growth (Table 1).

Preparation of solid L.B media

Ten g NaCl, 5g yeast extract, 10 g peptone and 16 g agar were added to ddH_2O, and completed up to 1000 ml, pH was adjusted at 7.2.[7] The mixture was heated till complete dissolving and then autoclaved, after that the media were poured in Petridishes and were leaved to cool down.[8]

Table 1. Name of powder baby milk samples used in study and growth of bacteria

No.	Powdered Infant Milk Name	Growth Status of Bacteria
1	Bediashore	N.G*
2	Aptamil	N.G
3	Mudhish	G
4	Hanna	G
5	Altunsa	G
6	Warda	G
7	Dialack	G
8	Cihan	G
9	Kanny	G
10	Evolack	N.G
11	Bebilight	N.G
12	Nedo	G

Sample preparation

Using aseptic technique, 5 g of powdered milk weighted mixed for 2 min using a vortex, dissolved in 10 ml of pre-warmed sterile distilled water at 40°C, and incubated for 15-20 min in a water bath at the same temperature, serial dilutions were prepared.[8]

Identification of bacterial species

Bacteria were identified by using Biochemical identification (gram stain, oxidase, catalase, motility DNase, coagulase, ureas), .

Molecular identification

For further confirmation the identity of bacteria, the isolates were examined for rRNA gen A region of *16S* rRNA gene were amplified by PCR to allow species identification.

Genomic DNA extraction

Genomic DNA was extracted from pure

cultures using the Presto™ Mini gDNA Bacteria Kit (Geneaid, Taiwan) following the manufacturer's instructions; 100 µL elution buffers were used for extracting. Extracted genomics was stored at –20 °C before running PCR.

The NanoDrop 1000c spectrophotometer was used to evaluate the concentration and purity of DNA in which one µL of the DNA genome was used to define the concentration and purity of DNA samples at 260 nm (Thermo Scientific, USA).

Amplification of *16S* rRNA gene

The genome of Staphylococcus sp. and Lactobacillus sp was first checked for the presence of of 16S rRNA gene see Table 2, A region of 16S rRN were amplified by PCR to allow species identification. PCR amplification was carried out with a final volume of 1 µL of elution in 25 µL. After the addition of 12.5 µL of 2× HotStart Taq Master Mix (RED AMPLICON, Denmark), then one µL of each primer added into the tube, and the volume completed with free nuclease water at 25 µL. DNA fragments were analyzed by gel electrophoresis after PCR amplification. Ten µL of the amplification products were subjected to electrophoresis using 1.2 percent agarose gel (TAE buffer, 100 V, 75 min) with a safe GelRed DNA stain (Biotium, Inc., USA) and 100 bp DNA ladder used as the standard size.

Enumeration of total viable cells CFU

Total viable counts of all samples were enumerated using nutrient agar medium. The plates were incubated at 37°C for 48 hrs. [7] . The bacterial count was expressed as CFU/g milk.[8]

Antibiotic Sensitivity Test

For this study seven antibiotics were used by using Standard disc diffusion method (Amikacin, Amoxicillin, Erythromycin, Cefixime, Ceftriaxone, Ciprofloxacin, and Tetracycline). All results were recorded appropriately and interpreted using the National Committee for Clinical Laboratory Standards (NCCLS) interpretation chart[9] (Table 3).

Results

Identification of bacterial genera

Out of the 12 powdered infant milk samples eight samples were contaminated by bacteria (37.5%) of samples were positive for *Lactobacillus* sp. and 5 (62.5%) positive for *Staphylococcus* sp. , by using bio-

Table 2. Oligonucleotide sequences of primers for *16S* rRNA gene used in this research used for PCR amplification of *Staphylococcus* sp. and *Lactobacillus* sp.

Functional category	Primers detail			References
	Primer Sequence (5´ – 3´) (Oligonucleotide)	**Amplicon size (bp)**	**Cycling program**	
16S rRNA for *Staphylococcus* sp.	CAC CTT CCG ATA CGG CTA CC GTT GAC TGC CGG TGA CAA AC	372	95°C–30s; 59°C–45s; 72°C–1min; 40 cycles	In this study
16S rRNA for *Lactobacillus* sp.	AGAGTTTGATCCTGGCTCAG GGCTGCTGGCACGTAGTTAG	272	94°C–15s; 55°C–15s; 72°C–1min; 35 cycles	(Balcazar *et al.*, 2007)

Table 3. Antibiotics name, symbol and company

No.	Name of antibiotics	Symbol	Company/ Country
1	Amikacin	AMK	Bioanalysis (Turkey)
2	Amoxicillin	AMO	Bioanalysis (Turkey)
3	Erythromycin	E	Bioanalysis (Turkey)
4	Cefixime	CEF	Bioanalysis (Turkey)
5	Ceftriaxone	CFT	Bioanalysis (Turkey)
6	Ciprofloxacin	CIP	Bioanalysis (Turkey)
7	Tetracycline	TE	Bioanalysis (Turkey)

chemical identification (gram stain, oxidase, catalase, motility DNase, coagulase, ureas), as shown in Table 4. For further confirmation the identity of bacteria, the isolates were examined for the occurrence of the *16S* rRNA gene to characterize and validate the *Staphylococcus* sp. and *Lactobacillus* sp. All of the strains were confirmed as *Staphylococcus* sp. and *Lactobacillus* sp. by the existence of *16S* rRNA), amplicons of the predicted size (372 bp for *Staphylococcus* sp. and, 272 bp for *Lactobacillus* sp, Figure1. This study also found that the number of infant milk contaminated with *Staphylococcus* sp. ,was higher than the *Lactobacillus* sp (% 62.5 of *Staphylococcus* sp (n=8) %37.5 *Lactobacillus* sp (n=8), as shown in Table 5.

The antibiotic resistance

In the current study, seven different antibiotics were used to study the resistance of the isolated bacteria against these antibiotics (Table 6). The Kirby-Bauer disk diffusion test was used in this experiment to determine whether the isolated organisms were resistant to a selection of antimicrobial agents. Antibiotic resistance pattern of *Lactobacillus* sp. and *Staphylococcus* sp. isolates are shown in Table 7. *Staphylococcus* sp. resistance was ranged from 25–72%, and the lowest percent was 25% against amikacin and the highest 72% against AMC. *Lactobacillus species* resistance started from 25–88%, the minimum resistant percentage against ciprofloxacin and the maximum one was erythromycin against *Lactobacillus* sp.

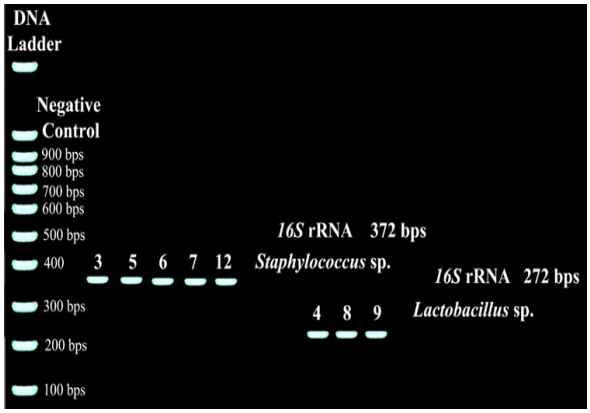

Figure 1. Agarose gel electrophoresis of PCR amplification products for the *16S* rRNA gene of *Staphylococcus* sp. and *Lactobacillus* sp. M: The DNA marker (100 bp ladder), lane NG: negative control, lanes (3, 5, 6, 7, and 12) positive amplification of 372 bp for *16S* rRNA gene in *Staphylococcus* sp., lanes (4, 8, and 9) positive amplification of 272 bp for *16S* rRNA gene in *Lactobacillus* sp.

Table 4. Biochemical identification of *Staphylococcus* sp. and *Lactobacillus* sp.

Tests	Reactions or Results of Bacterial Genera	
	Staphylococcus sp.	*Lactobacillus* sp.
Gram stain	Gram positive coccus	Gram positive rod
Motility	–	–
Oxidase	–	–
Catalase	+	–
DNase	+	–
Coagulase	+	–
Urease	–	–

Table 5. Presence of bacterial genera from infant milk powder.

Powdered Infant Milk	Presence of Bacterial genus	
	Staphylococcus sp.	*Lactobacillus* sp.
Mudhish	+	–
Hanna	–	+
Altunsa	+	–
Warda	+	–
Dialack	+	–
Cihan	–	+
Kanny	–	+
Nedo	+	–

Table 5. Total viable bacteria count for infant milk powder.

Powdered Infant Milk Name	Total viable bacteria count CFU
Mudhish	7
Hanna	4
Altunsa	1
Warda	8
Dialack	2
Cihan	2
Kanny	5
Nedo	1

Table 6. Resistance percentage of adult samples isolates.

No.	Antibiotics	Bacterial Resistant %	
		Staphylococcus sp.	*Lactobacillus* sp.
1	Amikacin	25	49
2	Amoxicillin	72	83
3	Erythromycin	69	88
4	Cefixime	48	42
5	Ceftriaxone	47	55
6	Ciprofloxacin	33	25
7	Tetracycline	28	30

Discussion

The results of this study shows that out of the 12 powdered infant milk samples eight samples were contaminated by bacteria (37.5%) of samples were positive for *Lactobacillus* sp. and (62.5%) positive for *Staphylococcus* sp. This result agree with the results obtained by Abdalla et al. (2014).[8] They reported that over 42% of the milk samples contained with *Staphylococcus* spp.[10,11] They reported also that milk samples collected from a baby food factory contaminated by *Staphylococcus aureus.*

Staphylococci are among the most significant pathogens that cause wide spectrum of diseases in both humans and animals. *S. aureus* is capable of producing enterotoxins which are resistant to most cooking temperature.[12] Moreover, Reves et al. (2007)[13] who isolated Bacillus mycoieds from dried milk which constituted (11.11%) from infant powdered milk and (23.1%) from adults.

Another aspect of our finding is the resistance of *Staphylococcus* sp. and *Lactobacillus species* against antibiotics (Table 7). *Staphylococcus* sp. resistance against AMC and E was between (72-69%), CIP, CFT, (48 -49%) and the lowest resistance was against AMK and TE (25-28%). Moreover, *Lactobacillus species* resistance was most frequently observed to E and AMC (88-83%), followed by AMK, CEF, CFT (49-42-55%) and lowest resistance was against CIP, TE (25-30%). It is interesting to note that no differences were found in resistance of *Staphylococcus* sp. and *Lactobacillus* against antibiotic.

This results in agreement with Abdulla et al. (2014)[8] who found antibiotic resistance of bacteria *S. aureus* isolated from both infant formula and adult's powdered milk, in which the resistance against AMC and TE was between (45-60%), E (30-40%), CFT, CIP (25-30%), AMK and MEP (0-5%). A study by Wang et al. (2014)[14] found that *S. aureus* resistance was extremely observed to E (75.9%), followed by CIP (51.9%), TE (18.5%).

Conclusions

The current study found that infant powdered milk used by consumers in Erbil state were contaminated with *Staphylococcus* sp. and *Lactobacillus*. These isolates exhibited multiple antimicrobial resistance. No significant differences were found between isolates against antibiotic resistances.

Conflicts of interest

The authors report no conflicts of interest.

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