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Isolation of Antibiotic-Resistant *Micrococcus aloeverae* from Milk Powder

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Abstract

The emergence of antibiotic resistance in humans *via* the food chain has become an area of great concern. Antibiotic resistance gene transfer from a food-borne bacteria or probiotic agent to a human gut microbe can lead to a serious food safety issue. Such seemingly harmless bacteria or probiotics can potentially become a threat to human health if they carry mobile genes conferring antibiotic resistance. The recent trend of probiotic application in powdered infant formula and milk products and the presently known contamination of these products by certain opportunistic Enterobacteriaceae bacteria serves as the rationale for evaluating their safety. In this study, 25 milk powder samples and 25 powdered infant formula samples were analyzed for the presence of antibiotic resistant bacteria of family Enterobacteriaceae using an isolation technique. Out of the 50 samples screened, an isolate was detected in one milk powder sample. Its biochemical characteristics, Gram character, antibiotic susceptibility, and motility were analyzed. It was found to be a Gram-positive, non-motile, catalase-positive and oxidase-negative microbe. The isolate was resistant to Rifampicin and Vancomycin, but susceptible towards Streptomycin, Ciprofloxacin, Ceftriaxone and Gentamycin. Molecular characterization by 16S rDNA sequencing identified the isolate as *Micrococcus aloeverae*. The unique occurrence of *Micrococcus aloeverae* in milk powder elicits an area of food safety concern for such samples. To the best of our knowledge, this study is the first to report antibiotic resistance by *Micrococcus aloeverae*. Although it may be a non-pathogenic microbe, its resistance towards antibiotics cannot be ignored. The possibility of *Micrococcus aloeverae* serving as a potential vehicle for transfer of antibiotic resistance genes to human gut microbiota represents a prospective food safety concern.

Keywords: *Micrococcus aloeverae*; Antibiotic resistance; Milk powder; Gene transfer; Food safety

Introduction

Antibiotic resistance is one of the major threats to food security and human health [1]. Bacterial resistance towards antibiotics could endanger the current medicinal advancements in antibiotic therapy [1]. The transmission of antibiotic resistance from an external source to humans can occur *via* a number of routes but the food-borne route is considered to be the most important [2]. Hence, reports of antibiotic resistance in food-borne bacteria and probiotic agents used in the food industry shed a great concern with respect to food safety. The World Health Organization, the Food and Agricultural Organization and the World Animal Health Organization have stated that there is evidence of unfavourable human health consequences due to resistant organisms arising from non-human usage of antimicrobials [3]. Antibiotic genes exist in food animals, fish, plants and vegetables. These sources serve as reservoirs of genes that can be potentially transferred to human pathogens by indirect contact *via* the consumption of contaminated food [4].

Powdered infant formula is not a completely sterile product [5,6]. The pasteurization process during the manufacture of powdered infant formula eliminates any pathogenic bacteria. Thus, reports of contamination in powdered infant formula are most likely attributed to poor hygiene standards post pasteurization [7]. There have been reports of isolation of a pathogenic and opportunistic bacterium of Enterobacteriaceae family in powdered infant formula in Mexico [8], South Africa [9], Jordan [10], India [11], Iraq [12] and Nigeria [13] and in milk powder in Turkey [14]. Likewise, in a study from Iran, an antibiotic resistant opportunistic microbe *Pantoea (Enterobacter) agglomerans* was isolated from a consumed powdered infant formula [15]. Hence, it is necessary to screen such products for investigation of Enterobacteriaceae bacteria.

The use of beneficial probiotic agents in powdered infant formula is becoming increasingly popular [16]. The application of probiotics as antimicrobial agents in powdered infant formula has been suggested as a promising approach to improve the microbiological safety of the product [17]. Similarly, probiotic use in dairy products can serve as an innovative alternative to improve the quality of the product [18]. In a certain study, the viability of probiotic agents added to whole milk powders was evaluated [18]. From the microbiological point of view, the milk powders with added probiotics showed satisfactory results. It was observed that milk powders provide optimum conditions for maintaining the viability of probiotics [18].

Although probiotic use in powdered infant formula and milk powders may be beneficial, the safety of these food products should be assessed and the safety of the probiotic strain should be evaluated for their ability of acquiring and transferring resistant determinants [19]. In certain bacteria and probiotics, specific antibiotic resistant determinants present on mobile genes exist and they serve as reservoirs of resistance for potential food or gut pathogens [20]. Transfer of antibiotic resistance genes from probiotic *Lactobacillus spp.* to *Enterococcus faecalis* has been demonstrated [21]. Transmissible antibiotic resistance genes in *Bifidobacterium spp.* have also been observed [21]. Such genes conferring antibiotic resistance can be

transferred to other bacteria by conjugative plasmids, transposons, integrons and insertional elements [16].

Hence, it is imperative to screen powdered infant formula and milk powders for antibiotic resistant Enterobacteriaceae bacteria. The aim of this study was to evaluate powdered infant formula and milk powder samples for the presence of antibiotic resistant bacteria of family Enterobacteriaceae.

Materials and Methods

Sample collection

A total number of 50 samples, comprising 25 powdered infant formula samples of an international brand and 25 milk powder samples of international and local brands were procured from markets in Mumbai city in Maharashtra, India. The typical composition of the powdered infant formula samples used in this study include milk solids, sucrose, maltodextrin, dried glucose syrup, soyabean oil, corn oil, vitamins and minerals. The milk powders contained milk solids, sugar and stabilizer. The media used were obtained from HiMedia Laboratories (India).

Isolation

The samples were evaluated using the isolation technique described by the United States Food and Drug Administration [22]. Pre-enrichment was carried out by adding 10 g of sample to 90 ml of Buffered Peptone water. Following incubation at 37°C for 24 h, 1ml of pre-enrichment culture was inoculated into 9 ml of Enterobacteriaceae Enrichment Broth and incubated at 37°C for 24 h. In the selection step, 1 ml of incubated Enterobacteriaceae Enrichment Broth was inoculated into sterile plates of Violet Red Bile Glucose Agar. These plates were incubated at 37°C for 24 h. Yellow colonies from the Violet Red Bile Glucose Agar plates were picked and streaked onto sterile Tryptone Soya Agar plates in duplicates. The plates were incubated at 37°C for 24 h. Yellow colonies that appeared on Tryptone Soya Agar were selected for further identification. The isolate was maintained in Tryptone Soya Agar slants and further subcultured at an interval of 2 weeks to ensure viability.

Biochemical analyses

Biochemical tests including the indole production, methyl red, Voges-Proskauer, citrate utilization, catalase, oxidase and Triple Sugar Iron Agar tests were carried out to analyze the biochemical characteristics of the isolate.

Motility test

Sterile motility agar slants were prepared using Motility Test medium. The culture was stabbed into the medium and incubated at 37°C for 24 h.

Antibiotic susceptibility test

Antibiotic Susceptibility Test was performed using the Kirby-Bauer Disk Diffusion technique on Mueller-Hinton Agar, as recommended by the Clinical and Laboratory Standards Institute [23]. Six antibiotics, which include Streptomycin (10 µg/disc), Ciprofloxacin (5 µg/disc), Ceftriaxone (30 µg/disc), Gentamycin (10 µg/disc), Rifampicin (5 µg/disc) and Vancomycin (30 µg/disc), were used to carry out the test. The inoculum of the isolate was prepared using sterile Buffered Peptone water and standardized by adjusting its density to match the 0.5 McFarland turbidity standard. Using a sterile cotton swab, the inoculum was spread onto the entire surface of the agar. These inoculated plates were allowed to stabilize for 5 min, after which the antibiotic discs were laid aseptically on the surface. Sterile discs of 6 millimeter diameter were prepared using Whatman paper no. 1 and they served as 'Blank' controls. The plates were incubated at 37°C for 18 h. The zones of inhibition were measured and recorded. The results were interpreted in accordance to the criteria set by the Clinical and Laboratory Standards Institute [23].

Gram staining

The Gram character of the isolate was assessed by the Gram staining technique [24]. A primary Crystal violet stain was applied to a heat-fixed smear of the bacterial culture for 1 min. The slide was washed with water, followed by application of the mordant (Gram's iodine) for 1 min. The slide was washed with water, followed by decolorizing it with alcohol. A counter-stain Safranin was applied to the smear for 1 min. The slide was washed with water and then observed under oil-immersion objective.

Bile salt tolerance test

The ability of any Gram-positive isolate to grow on media such as Enterobacteriaceae Enrichment Broth and Violet Red Bile Glucose Agar containing bile salts suggests that it exhibits bile salt tolerance. If a Gram-positive isolate was detected, it was tested for bile salt tolerance by inoculating the isolate in the Bile Salt Agar, followed by incubation at 37°C for 24 h.

Investigation of probiotic property

If an isolate was found to be bile salt resistant, it was further investigated for probiotic properties, such as survival in an acidic pH medium and the ability to inhibit other bacteria. This was done by inoculation of isolate into Nutrient Broth, whose pH was adjusted to 3, in order to mimic the acidic pH of the stomach. Two other Nutrient Broth tubes containing inoculated broth at pH 7 and sterile 'Blank' broth were also maintained for comparison. The tubes were incubated at 37°C for 72 h. Inoculation of the isolate was carried out in Tryptone Soya Agar plates containing other test microorganisms to check whether it is capable of inhibiting their growth. The plates were incubated at 37°C for 24 h.

Molecular characterization by 16S rDNA sequencing

The isolate was sent to geneOmbio Technologies Private Limited in Pune, Maharashtra (India) for molecular characterization. Bacterial genomic DNA was isolated using Qiagen DNeasy kit. Bacterial 16S region gene was amplified using a standard PCR reaction. The primer pair 27F and 1492R was used in a PCR reaction with an annealing temperature of 57°C. After amplification, the products were purified by using Invitrogen PCR product purification kit (Life technologies, USA) and were directly sequenced using an ABI PRISM BigDye Terminator V3.1 kit (Applied Biosystems, USA). The sequences were analyzed using Sequencing Analysis 5.2 software. BLAST (Basic Local Alignment Search Tool) analysis was performed at the BlastN site at National Center for Biotechnology Information (NCBI) server.

Results and Discussion

The present study had originally investigated the presence of antibiotic resistant Enterobacteriaceae bacteria in 25 powdered infant formula and 25 milk powder samples. However, after screening all 50 samples, a non-Enterobacteriaceae antibiotic resistant microorganism was isolated from only one local brand of milk powder sample. The microorganism was found to be Gram-positive and non-motile in nature. It produced negative results for all four tests of the IMViC (Indole, Methyl red, Voges-Proskauer, Citrate) series.

It was observed to be catalase-positive and oxidase-negative. It was capable of fermenting sucrose, lactose and D-glucose with the production of acid and gas. It was capable of growing on Bile Salt Agar, thereby displaying bile salt resistance. Enterobacteriaceae Enrichment Broth and Violet Red Bile Glucose Agar contain bile salts which inhibit growth of Gram-positive bacteria. The bile salt tolerant nature of this Gram-positive isolate justifies its ability to survive and grow in such media. The isolate exhibited resistance towards Rifampicin and Vancomycin but was susceptible towards Streptomycin, Ciprofloxacin, Ceftriaxone and Gentamycin. It did not exhibit potential probiotics properties other than bile salt tolerance. The isolate was finally identified as *Micrococcus aloeverae via* molecular characterization by 16S rDNA sequencing (Table 2). The distinct occurrence of this antibiotic resistant microbe in milk powder sparks a food safety concern. To the best of our knowledge, this study is the first to report antibiotic resistance by *Micrococcus aloeverae*. The Antibiotic susceptibility pattern of *Micrococcus aloeverae* is represented in Table 1.

Antibiotic	Disc content (µg/disc)	Zone of inhibition (mm)	Interpretation
Streptomycin	10	18	Susceptible
Ceftriaxone	30	28	Susceptible
Ciprofloxacin	5	25	Susceptible
Gentamycin	10	17	Susceptible
Rifampicin	5	8	Resistant
Vancomycin	30	7	Resistant

Table 1: Antibiotic Susceptibility Pattern of isolated *Micrococcus aloeverae*

Description	Max Score	Total Score	Query Cover	E Value	Identity	NCBI Accession Number
Micrococcus aloeverae strain AE-6 16S ribosomal RNA, partial sequence	1254	1254	100%	0	100%	NR_134088.1

Table 2: BLAST Results

In this study, *Micrococcus aloeverae* displayed growth as yellow, circular, convex colonies on Tryptone Soya Agar. This type of growth by *M. aloeverae* on Tryptone Soya Agar is consistent with a previous study reported from India [25]. The results of motility, catalase, oxidase, citrate utilization and D-glucose fermentation obtained for *M. aloeverae* are consistent with the same study [25]. The results for indole production, methyl red test and Voges-Proskauer test are in agreement with a study reported from India for *Micrococcus spp.* [26].

Micrococcus aloeverae was found to be bile salt resistant in the present study. Similar findings of *M. aloeverae* tolerance towards high salt conditions were reported in a study from India [25]. Bile salt resistance is a strain-specific trait which varies within species or genus [27]. Other Gram-positive bacteria that have exhibited bile salt resistance include *Bacillus cereus* and *Bacillus licheniformis* [13] and *Listeria monocytogenes* [28]. Bile salt resistance has also been seen in Gram-positive probiotics such as *Lactobacillus fermentum* [29] and *Bifidobacterium spp.* [30].

In 2014, *Micrococcus aloeverae* had been proposed as a novel species isolated from the inner fleshy leaves of *Aloe barbadensis* (Aloe vera) [25]. It was described as a non-motile, non-endospore forming endophytic bacterium. Members of this genus are distributed widely and have been isolated from multifarious habitats such as soil [31], human skin [32], air [33], activated sludge [34], inner tissues of plants [35], cold samples [36] and dairy industry waste [37]. The unique occurrence of *Micrococcus aloeverae* in milk powder in the present study sheds light upon the microbiological quality of such products. Certain possibilities could plausibly explain this occurrence. For instance, lack of hygiene at the milk powder processing unit may have led to its entry into milk powder. A study citing the prevalence of a pathogen in milk powder factories establishes a link between the external and internal environment of the milk powder factory, since the pathogen was isolated both from an employee's shoe and the soil outside the factory [38]. The pathogen in the soil may have gained access into the factory *via* the employee's shoe. Similarly, it can be suggested that *M. aloeverae* could have gained entry into the milk powder factory *via* a similar route. In a Draft Screening Assessment of *Micrococcus luteus* [39], it was stated that *M. luteus* can survive for long periods in adverse conditions such as dryness and also form biofilm formations on stainless steel surfaces. *Micrococcus aloeverae* is genetically similar to *Micrococcus luteus* [25], thereby suggesting that it may exhibit a similar behaviour to thrive in dry conditions such as milk powders and form biofilm formations on stainless steel surfaces of equipments used in the milk powder factory.

The result of this study presents a prospective food safety issue since antibiotic resistant *Micrococcus aloeverae* in milk powder is being reported for the first time. *Micrococcus* species are considered to be harmless and non-pathogenic microorganisms [40]. Reports of fatal infections caused by *Micrococcus aloeverae* have not been reported till date. Although *M. aloeverae* may be non-pathogenic, its resistance towards antibiotics cannot be ignored. It could serve as a potential vehicle for transfer of antibiotic resistance genes to human gut microbiota. Extrachromosomal genetic elements conferring antibiotic resistance have been observed to be present in some members of the genus *Micrococcus* [40]. *Micrococcus luteus* plasmids conferring Streptomycin resistance were found to be transferable and replicable in *Escherichia coli* and *Streptomyces* [41,42]. A strain of *Micrococcus luteus* isolated from an acne-prone skin was found to possess an erythromycin resistance genetic element that could be transferred *via*

conjugation to *Enterococcus faecali* [43]. *Micrococcus spp.* isolated poultry litter was observed to have multiple antibiotic resistance genes which were acquired by *Escherichia coli via* horizontal gene transfer [44]. The characterization of an inducible macrolide resistance gene in *Micrococcus luteus* isolated from human skin has been reported [45]. Hence, the observation of antibiotic resistance in *Micrococcus aloeverae* in this study may be of clinical importance because it could be potentially transferred to other bacteria *via* horizontal gene transfer if mobile antibiotic resistance genes are present.

Conclusion

The present study, to the best of our knowledge, is the first to report antibiotic resistance exhibited by *Micrococcus aloeverae*. Bacterial plasmids may encode a variety of genetic determinants that allow the bacteria to survive efficiently in an adverse environment [46]. The possibility of *Micrococcus aloeverae* containing antibiotic resistance genetic elements or plasmids could perhaps explain its persistence in a dry environment like milk powder. Further investigations into the characteristics of *Micrococcus aloeverae* that enabled it to persist in milk powder, the route of contamination and the amplification and identification of the gene responsible for its resistance are areas that promote research. In a current global scenario wherein reports of multiple antibiotic resistant bacteria are rampant, it is paramount to investigate whether *Micrococcus aloeverae* harbors transferable antibiotic resistance genes and its potential to act as a vehicle for transfer of antibiotic resistance.

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