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Molecular investigation of the influence of oral fed *Lactobacillus reuteri* on the gut microbiota of HIV-infected infants – A pilot study

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Abstract

The composition of the gut microbiota has been shown to be important to the immune system. In immune compromised patients (e.g., AIDS), the balance of health promoting bacteria might be disturbed by antibiotic treatment or the disease itself. HIV-induced diarrhea and secondary infections are a hazard especially for babies and infants, and oral treatment with probiotics has been shown to improve diarrhea and gastrointestinal problems. In this placebo-controlled preliminary study with seven patients suffering from diarrhea, the *Lactobacillus* gut flora was investigated before and after oral administration of a probiotic *Lactobacillus reuteri* strain or a placebo product. In the study, three infants were HIV-positive and four were HIV-negative; while four infants were treated in the probiotic group and three the placebo group. Lactic acid bacteria cultured from faecal samples were enumerated and molecular PCR-DGGE analysis was performed to determine the diversity and the persistence of the probiotic *in vivo*. After oral administration of *L. reuteri,* the probiotic was found in all stool samples of the treatment group but not in the placebo group. The *Lactobacillus* diversity was significantly different after administration of the probiotic. Molecular PCR-DGGE seems to be a promising tool for the investigation of the influence of probiotic treatment and differences in the *Lactobacillus* gut communities between patients and healthy persons.

Key words: Probiotics, *L. reuteri*, DGGE, HIV, gut flora.

Introduction

The immune suppressive disease AIDS is one of the biggest health problems. In 2005, e.g., 3.2 million new infections and 2.4 million AIDS deaths were registered in South Africa [1]. Exogenous and endogenous factors like nutrition, drugs and diseases influence the composition of the gut microbiota. A shift in the diversity of the microbiota is often observed in immune suppressed or immune compromised patients, virus-infected patients, people with gut or metabolic diseases or under the influence of antibiotics [2, 3]. In immune suppressed people, the gut microbiota, which plays a big role in the development of the immune system, is disturbed. Pathogenic bacteria and potentially pathogenic food-associated microorganisms may colonize the compromised gut and lead to secondary infections. This often makes treatment with antibiotics necessary. The antibiotic treatment further influences the gut microbiota and may result in antibiotic associated diarrhea [4]. Furthermore, the HI virus may change the gut epithelium [5], make the gut mucosa more permeable and trigger enhanced water elimination [6, 7]. The combination of antibiotic- and virus-associated diarrhea results in an extensive loss of gut bacteria, a lower bacterial diversity and an increased risk of colonization of antibiotic resistant pathogens [7-9]. The mortality caused by secondary infections and HI virus-induced diarrhea is very high, especially for babies and infants [10-13].

Several probiotics have been examined for their ability to prevent traveller's antibiotic- and virus-induced diarrhea. These include *Lactobacillus, Bifidobacterium*, *Streptococcus* and *Saccharomyces* [14-16]. *Lactobacillus* species in particular are known to produce bacteriocins against potential pathogenic bacteria and have an immune relevant and health supporting effect [17, 18]. For example, *L. rhamnosus GG* has been used to treat rotavirus induced diarrhea and could reduce the morbidity by dehydration [16].

Studies using oral fed probiotics (e.g., *Lactobacillus reuterii* or *Saccharomyces cerevisiae*) showed, that HIV and antibiotic associated diarrhea can be alleviated [19], secondary bacterial infections can be prevented [20] and the gut flora can be changed in a positive way [19-22].

This pilot study aimed to test the experimental protocols needed to investigate the gut microbiota of HIV-positive and -negative infants with diarrhea, before and after the oral admission of *L. reuteri,* and compared it to a placebo group (no *L. reuteri* treatment). We used polymerase chain reaction (PCR) and denaturant gradient gel electrophoresis (DGGE) in conjunction with conventional culture methods to investigate changes in the gut microbiota.

Material and Methods

Selection of patients, ethics and study design: Infants entered into the study (n = 7, 2 male, 5 female) were between 4 and 12 months old and were in-patients at the "Red Cross Childrens' Hospital" in Cape Town. All children had diarrhea (> 3 to 8 times liquid stools per day), cough and vomiting. Six infants received Cotrimoxazol, 2 additionally received Amoxil, while 1 did not receive antibiotic treatment. HIV-positive children (n = 3) were included in the study, but they had a CD4+ count over 15%. All children received best supportive care in addition to the probiotic. Written informed consent was given by the parent(s) or caregiver of each child. Pre-test and post-test counselling was given to the parent or caregiver of the children before and after determining their HIV status. The study was approved by the Research and Ethics Scientific Committee of the University of Cape Town. The study was randomised according to Table 1.

Table 1. Randomisation of the study.

	Probiotic	Placebo	Total
HIV positive	2	1	3
HIV negative	2	2	4
Total	4	3	7

Sample collection: On the first day of hospitalization, stool samples (approx. 5 g) of all infants were taken. *L. reuteri* (10^{10} cells) (BioGaia Biologics, Inc. Raleigh, NC, USA) was supplied to patients joining the probiotic group 12 hours after collection of the first stool sample. *L. reuteri* was suspended in coconut oil and given orally once per day for 3 consecutive days. A second stool sample was collected at the third day. Stool samples were stored at +4°C immediately and processed within 24 hours. The placebo group received only coconut oil.

Cultivation: Fecal samples (1 g) were diluted in anaerobic 0.9% (w/v) NaCl and plated on MRS media (SA Biolabs, South Africa). The samples were incubated at 37°C for 2 days under anaerobic conditions (5% H_2, 10% CO_2 and 85% N_2) in an anaerobic chamber (Forma Scientific). Colonies from all plates were resuspended in 2 ml MRS broth and totally scraped off the plate using a sterile glass spreader. Sterile glycerol (final concentration 25% v/v) was added to the suspension, and the cells were frozen at -70°C for further molecular investigation.

DNA extraction: A freeze-thaw step (liquid nitrogen for 30 seconds and 70°C water bath for one minute) was performed using 200 µl of each frozen cell suspension. The samples were processed using the Genomic DNA Purification Kit (Fermentas Inc., Burlington, Canada), according to the manufacturer's instructions with the following modifications: After Step 1, 200 µl buffer solution AL and 15 µl proteinase K (Qiagen GmbH, Vienna, Austria) were added and incubated at 70°C for 10 min. No additional washing step with 70% ethanol was performed after Step 6. The concentration and quality of the purified DNA was measured using a Nano-Drop UV spectrophotometer (Nano-Drop Technologies, Wilmington, USA) and samples were stored at -20°C for further investigation.

DNA amplification: PCR amplification of the ribosomal 16S rRNA encoding gene was performed using the universal primers 27f and 1492r (*E. coli* numbering) [23]. The reaction was carried out in 25 µl volumes using the 2x PCR Mastermix (Fermentas) and contained 12.5 pmol of each primer, 20 µg/µl BSA (bovine serum albumin; Fermentas) and 1.5 ml DNA extract as a template. PCR was performed in a GeneAmp 9600 PCR-Cycler (Perkin Elmer Inc., Wellesley, USA) using the following conditions: 5 min denaturation at 95°C, 35 thermal cycles of 1 min at 95°C (denaturation), 1 min at 58°C (annealing),1 min at 72°C (extension), followed by a final extension step at 72°C for 5 min. The length of the amplified PCR products (approx. 1500 bp) was determined by electrophoresis using a 2% agarose gel followed by ethidium bromide staining according to standard protocols [24]. The gels were visualized using the Biorad Gel Doc System.

A nested PCR, amplifying a 200 base pair (bp) fragment of the V3 variable region of the 16S rRNA of the eubacterial population was amplified by PCR with primers 341f and 518r as described by Muyzer and co-workers [25]. An improved 40-nucleotide GC clamp was attached to the 52 end of the forward primer, 52-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-32[26]. The reactions were carried out in a final volume of 150 µl, using 12 µl PCR product obtained from the first round of PCR as template. No BSA was added. The PCR products of the nested PCR were concentrated by precipitation in chilled ethanol and resuspension in 15 µl nuclease-free deionised water and 5 µl loading dye (Fermentas). The length of the amplified PCR products (200 bp) was determined as described above and the DNA quantified using the Nano-Drop spectrophotometer.

Denaturant gradient gel electrophoresis (DGGE): DGGE was done by using the Bio-Rad D-CODE system (Biorad Loboratories Inc., Hercules, CA, USA). The *L. reuteri* strain SD2112 used for oral application was used as a marker strain for all subsequent DGGE gels. The perpendicular gel contained a denaturing gradient of 40 to 60% and was formed according to the manufacturer's instructions. Parallel DGGE was performed essentially as described previously [25, 27]. PCR fragments were separated by using 8% (wt/vol) polyacrylamide (acrylamide-bisacrylamide [37.5:1]; Sigma-Aldrich) containing a 40 to 60% linear gradient of denaturants (urea and formamide) increasing in the direction of electrophoresis. Gradients were formed by using a Bio-Rad Gradient Former Model 385. PCR samples were applied to gels in aliquots of 10 µl per lane. The running buffer used was 0.5 × TAE buffer (20 mM Tris, 10 mM acetic acid, 25 mM EDTA [pH 8.0]). Electrophoresis was performed at a constant temperature of 60°C at 40 V for 16 hours. Gels were stained with ethidium bromide for 15 min and visualized using the Gel Doc system (Bio-Rad).

Reamplification of dominant bands: Dominant bands on the DGGE gels were punched out using a sterile pipette tip. The DNA was eluted in ultra pure PCR water (Fermentas) and reamplified using the primers 341f and 518r. The reaction was carried out in a 50 µl volume using the same conditions as used for the nested PCR. The products were purified using the PCR Purification Kit (Qiagen). Length, quality and quantity of the re-amplified PCR products were tested as described above. The band of the marker strain (*L. reuteri* type strain) was processed in the same way.

Purified PCR products (10 µl) were sequenced using the DYEnamic ET Dye terminator cycle sequencing kit for MegaBACE (Molecular Dynamics) based on dideoxynucleotide chain termination chemistry [28]. Reactions were performed according to

manufacturers' instructions and sequenced on a GeneAmp PCR system 9700 (Perkin Elmer, Applied Biosystems) using appropriate primers at concentrations of 5 µM and primer specific hybridization temperatures. Sequencing reaction products were analysed using an automated capillary DNA sequencing system, the MegaBASE 500 sequencer v2.4 (Molecular Dynamic, Amersham Pharmacia Biotech, Amersham Bioscience). Sequences were analysed using Lynnon Biosoft DNAMAN software v 4.13 and the NCBI BLAST program [29].

Results

A total of 20 dominant bands were sequenced. Of these, 17 were found to belong to the genus *Lactobacillus*, while 3 sequences were related to *Veillonella atypica*, *Enterococcus faecalis* and *Streptococcus bovis*. *Lactobacillus fermentum*, *Lactobacillus johnsonii* and *Lactobacillus gasseri* were the most dominant bands among the samples.

L. reuteri was found in all samples taken from patients of the probiotic group after administration of *L. reuteri*. The bands (Fig. 1: Bands 4, 8, and 14) had the same position in the DGGE gel as the *L. reuteri* type strain SD2112 was used as a standard (Fig. 1, Band 20). No *L. reuteri* band could be detected in stool samples before probiotic treatment or after admission of the placebo (Fig. 1, Lanes 2, 4 and 10). DGGE analysis showed a definite change in the bandpattern in all of the patients receiving probiotics after treatment as compared to before (Lanes 6, 8, 12 and 14).

Discussion

The aim of this pilot study was to determine whether conventional culture methods combined with DGGE analysis could be used to monitor the persistence of an administered probiotic in the gut of patients with diarrhea.

Culturing before the molecular investigation ensures that mainly viable *Lactobacillus* ssp. were investigated. Sequenced *L.reuteri* DGGE-bands from samples of patients three days after probiotic admission suggest that this probiotic strain colonized the gut. This confirms the findings of Shornikova *et al.* [30], who showed similar results after administration of *L. reuteri* to children with acute diarrhea, mostly caused by rotavirus infection. *L. reuteri* was virtually absent from the placebo group. In their study, the benefits of the probiotic treatment were evident within 24 h and reduced the length of the diarrhea episode significantly.

Using universal primers, all bacteria grown on the MRS media were amplified by PCR using universal 16S rRNA gene primers. Most of the selected colonies belonged to the *Lactobacillus* genus, however, three other species were also detected. Although MRS medium has been claimed to be specific for *Lactobacillus* spp. [31], it also supports the growth of *V. atypica* [32] and closely related species like *Enterococcus* spp. [33] and *Streptococcus* spp.[34].

The sequenced *Lactobacillus* species have also been found in the microbial gut community in other studies [35, 36]. *L. johnsonii, L. fermentum* and *L. gasseri* are strains which are commonly found in healthy human faecal material [35, 37-39]. In recent studies, *L. reuteri* has rarely been reported in faecal samples using either culture-based or molecular techniques [36], but oral uptake of this probiotic strain seems to result in colonisation of the gut and subsequent modulation of the gut flora [40]. This result agrees with several other feeding studies using *L. reuteri* as a probiotic [30, 41-44].

The diversity of the gut flora observed by DGGE after the probiotic treatment appears to be different from that observed before the administration of the probiotics. Orally given *L. reuteri* is able to change the *Lactobacillus* flora in the gut [45]. Reuterin, an antimicrobial substance active against other Gram-positive strains produced by *L. reuteri* might be involved in this change[46]. However, since all patients in the current study received best supportive care in the form of antibiotics in addition to the probiotic, these changes could be due to the medication. A controlled study in the absence of antibiotic administration would resolve this.

The selection of a sub-population of faecal bacteria by cultivation of the stool samples on MRS medium might have resulted in an altered profile of the existing flora present in the gut, as the majority of bacterial species cannot be grown under laboratory conditions [47]. The extraction of total bacterial DNA

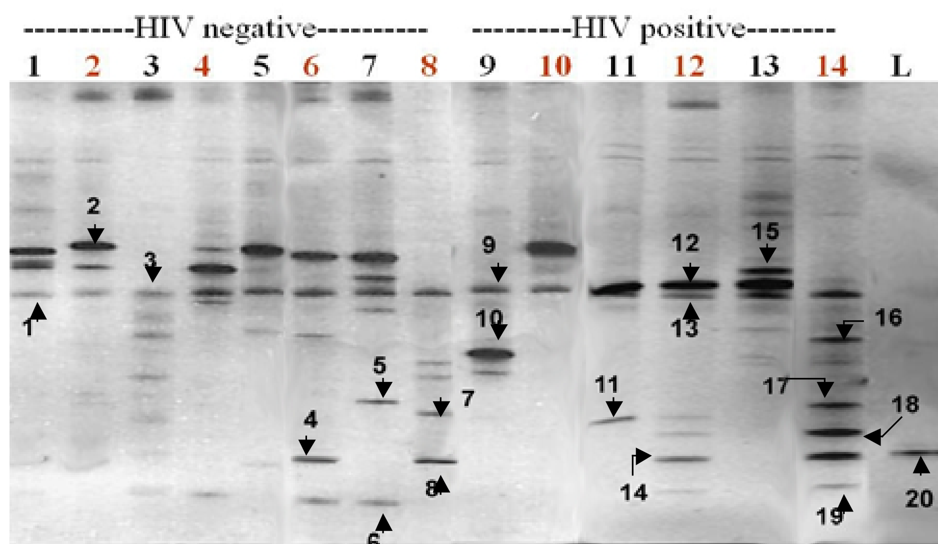

Figure 1. DGGE of lactic acid bacteria cultured from stool samples of HIV- negative (Lane 1-8) and HIV- positive (Lane 9-14) infants before (Lane 1, 3, 5, 7, 9, 11, 13) and after (Lane 2, 4, 6, 8, 10, 12, 14) admission of *L. reuteri* or placebo, respectively. *L. reuteri* group (Lane 5 and 6, 7 and 8, 11 and 12, 13 and 14), placebo group (1 and 2, 3 and 4, 9 and 10) and *L. reuteri* type strain (Lane L).

directly from the stool samples (without cultivation) would be useful to show the real diversity of the flora existing in the gut of these patients and might identify new and uncultivated species, but a differentiation between viable and dead bacteria would be difficult[48].

Conclusions

Molecular PCR-DGGE methods have been shown to be a good method for investigation of the diversity of bacterial gut communities and for determining the influence of antibiotics, disease and probiotics administration on the microbiota. Cultural methods are limited especially in screening qualitative differences in the diversity of large number of samples. This pilot study shows that DGGE analysis of faecal samples is a useful tool in detecting persistence of a probiotic in patients suffering from diarrhea. Direct DNA amplification from stool samples would give a more accurate reflection of the microbiota in the gut. The *L. reuteri* probiotic may also exert an influence on the gut flora under immune suppressed conditions in the presence of antibiotic treatment, and warrants further research. A larger sample size would be needed to investigate differences in the gut flora of HIV patients and the influence of probiotics under these conditions. In addition, the ethical concerns regarding administration of live organisms to immune-compromised patients and the difficulties in sampling patients from a low socio-economic environment after discharge from hospital need to be taken into consideration in designing a controlled study of this nature.

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