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Full Length Research Paper

An improving DNA isolation method for identification of anaerobic bacteria in human colostrum and faeces samples

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The efficiency, with which the lysis of 20 anaerobic bacterial samples [8 different bacterial species isolated from 4 colostrum and 4 infants (which are fed only by breast milk and from 3 to 30-day-old) faeces] were lysed, was tested using a reference or an improving method that involves higher levels of lysozyme (Sigma-Aldrich) and proteinase K solution (Sigma-Aldrich). Eight species were lysed with the efficiency of > 87% by the reference methods. Also, the lytic efficiency for *Bifidobacterium bifidum* BB-12 was > 90%. Accordingly, since the DNA samples isolated by the improved methods can reflect nearly the true genomic information in the microbial flora, the improved methods should be appropriate for genomic analyses, that is, not only for bacteria in the human intestine, but also for bacteria in other human samples, such as colostrum. The study reports that the extraction method used to produce DNA of suitable quantity and quality for subsequent PCR is based on detection applications. The procedures are reliable and reproducible, in typically planning a success rate of over 90%. In summary, it develops a fast and reliable genomic DNA extraction protocol for anaerobic bacteria.

Key words: Lactobacilli, DNA isolation.

INTRODUCTION

Lots of bacteria are presented in human bodies as parasites. The microflora in the human intestinal tract is an extremely complex yet relatively stable ecological community that is populated by an excess of 10^{11} bacterial cells/g of content and contains more than 400 bacterial species in the colon (Hammes et al., 1990). These are balanced in the normal flora of healthy host. Proper stability of the intestinal system flora depends on the beneficial and harmful microorganisms which are presented in the flora (Cakir, 2003). While these beneficial microrganisms aid the digestion of nutrient substances, they also conserve them from effects of pathogen microorganisms by some substances they revealled while digesting nutrient. These are called “probiotic microorganisms grup”. Some lactic acid bacteria (LAB) genus and species are in the group of probiotic microorganisms. Lactic acid bacteria (LAB) are used commonly in milk product which is very important in human development and health (Tannock et al., 1999, 2000; Shinoda et al., 2001). These bacteria are classified into 4 groups, and they are *Lactobacillus*, *Streptococcus*, *Pediococcus* and *Leuconostoc* (Yeniel, 2006).

Anaerobic bacteria are bacteria that do not live or grow in the presence of oxygen, although *Lactobacillus* spp. have been shown to constitute less than 1% of the total bacterial community (Sghir et al., 2000). The genus *Lactobacillus* contains diverse assemblage of gram-positive, facultative anaerobic or microaerophilic bacteria, catalase-negative, non-sporulating, rod-shaped organisms, with a DNA base composition of less than 53% G + C and it includes more than 25 species (Collins et al., 1991; Hammes et al., 1995; Jackson et al., 2002). They are a major part of the lactic acid bacteria group, named as such because most of its members convert lactose and other sugars to lactic acid. *Lactobacillus* is fairly common in nature. They are found in animal and human intestine, fermentative milk products, mouth cavity, and skin. The majority of these *Lactobacillus* species inhabit the intestinal tract. The growth temperature can change from 5 to 55°C, whereas the optimum pH growth is from 5.5 to 5.8. They need complex nutrients and vitamins for their growth and the species are used in the production

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of vegetables, fermentative meat and milk (Tannock, 1999).

Although *Bifidobacterium* is a genus of Gram-positive, non-motile, non-spore forming acidophilic, they are microorganisms that have different tolerance to oxygen. The growth temperature can change from 37 to 38°C, whereas the optimum pH growth is from 6.5 to 7.0. (Gomes and Malcata, 1999; Scardovi, 1986; Holt et al., 1993). Bifidobacteria are one of the major genera of bacteria that make up the gut flora, that is, the bacteria that reside in the colon (Guarner and Malagelada, 2003). The development of the gut microbiota is profoundly influenced by the infant diet within the first few days. As a result, the microbiota of infants receiving exclusive breast-feeding becomes dominated by bifidobacteria during the first week and, simultaneously, there is a decrease in the enterobacteria counts (Yoshioka et al., 1983). In contrast, breast milk seems to constitute a good source of maternal lactobacilli to the infant gut (Heikkilä and Saris, 2003; Matsumiya et al., 2002; Martín et al., 2003).

Correct identification of anaerobic bacterial and bacteria DNA isolates from human specimens is of high importance in research and clinical laboratories. Isolation and studies of anaerobic bacterial phenotypic and genotypic characteristics are the traditional methods of identifying anaerobic bacteria. Phenotypic methods include morphology, culture requirements, biochemical and microbial reactions. Since anaerobic bacteria can not tolerate oxygen, these methods are difficult to perform. It is also difficult to identify the slow growing bacteria, which indeed many anaerobic bacteria are. Bacteria, which exhibit deviant microbiologic characteristics, are also in the group of bacteria that are difficult to identify. The alkali lysis DNA isolation technique is becoming a method regularly used for identifying or extracting anaerobic bacteria. In this study, we isolated Lactobacilli from the colostrum and feces of a three-day-old and one-month-old breast-fed infant and identified Lactobacilli by API 50 CHL, after which alkali lysis DNA isolation technique was performed on it. We used this method for the detection and identification of the predominant bacterial species in 150 volunteers. We aimed high with the DNA isolation technique according to traditional methods.

MATERIALS AND METHODS

This study was done from September 2008 to June 2009 in Konya Dr. Faruk Sukan Birth and Children Hospital and Konya Province Control Laboratory. 100 colostrum samples and 50 infants (which were fed only by breast milk from 3 to 30-day-old) faecal samples were obtained in sterile small jars from Konya Dr. Faruk Sukan Maternity and Children Hospital. These samples were carried within two hours to Konya Province Control Laboratory and were study there.

Media and culture condition

Colostrum and infant (from 3 day-old to 1 month-old) samples were plated in MRS (Man Rogosa Sharpe, Merck) Agar with 0.25% (w/v) L-cysteine (Sigma-Aldrich L-cysteine). The culture process is that 1 ml colostrum samples were plated homogeneously to the solid medium with a sterile baget, while 1 ml faecal samples were also plated onto solid medium with a sterile baget after diluting them in a proportion of 1/10 within MRD (Maximum recovery diluent, Merck). These plates were then placed in jars. Anaerobic medium was prepared using GENbox anaerobic kit (Bio-merieux, Marcy l'Etoile, France), but pure water was not added to the medium. The kit obtained the anaerobic medium of jar by absorbing oxygen and releasing carbon dioxide. Then it was incubated at 37°C for 48 h. The growing colonies in plates in the end of 48 h were identified by the API 50 CHL system. From these colonies, stock cultures were prepared parallelly by passaging. Afterwards, the ependorf tups were prepared, including 85% nutrient broth. The colonies from strain cultures of stock bacteria were plated in these tups, while 15% glycerine were added to the tups and were conserved in a deep-freezer for -20°C.

API 50 CHL system

Fermentation of carbohydrates was determined using API 50 CHL (API 50 CH is a standardized system), associated with 50 biochemical tests for the study of carbohydrate metabolism in microorganisms. API 50 CH was used in conjunction with API 50 CHL medium for the identification of *Lactobacillus* and related genera strips according to the manufacturer's instructions (Biomerieux, Marcy l' Etoile, France) (Ghanbari et al., 2009). The incubator was calibrated at 35°C optimum temperature, after which the incubation box (in shape tray) and cover of the API 50 CHL system were prepared. For composure of an atmosphere moist, 10 ml distilled water was disposed within the box incubator and the strip was installed in the incubation box. Bacteria colonies, which indicate growth on the MRS Agar, were prepared within API 50 CHL system in API 50 CHL medium (5 ml), in concentration 2 McFarland. It was incubated again in the event of little concentration and was diluted by serum physiologic in the event of intense concentration. The executed suspension was distributed properly to the strip. Wells were filled by suspension via line mark, and mineral oil was added. These were incubated in the anaerobic medium for 48 h incubation at 35°C.

DNA isolation protocol

The isolated *Lactobacilli* strain was subcultured in the MRS agar with 0.25% L-cysteine at 37°C for 48 h. Bacterial colonies were added in 1 ml Tris–EDTA buffer by loop. From the mixture, 1.5 µl was taken and was centrifuged at 5000 ×rpm for 5 min, after which the supernatant was removed by micropipet and 10 µl Tris–EDTA buffer (pH 8.0) was mixed and added to the pellet. Again, it was centrifuged at 5000 ×rpm for 5 min and the supernatant was removed, after which 567 µl TE (Tris–EDTA) buffer and lysozyme (1 µl) (Sigma-Aldrich) were added and mixed by the micropipet. The mixture was then incubated at 37°C for 30 min in water bath. The cells were lysed by an addition of 3 µl of 10% sodium dodecyl sulfate and 3 µl of proteinase K solution (Sigma-Aldrich), followed by a 15-min incubation at 37°C. Subsequently, 1 µl of 5 M NaCl and CTAB 80 µl were added and the mixture was incubated at 65°C for 10 min. The water phase was extracted once more with chloroform: isoamyl alcohol (24:1) 780 µl and the mixture was centrifuged at 10000 ×rpm for 5 min. The supernatant was taken in order to clear the tube, before phenol-chloroform-isoamyl alcohol (25:24:1) was added to the supernatant and the mixture was centrifuged at 10000×rpm for 5 min. Isopropanol (0.6 volume) was added to the supernatant that was taken to clear the tube and the mixture was centrifuged at 10000 × rpm for 5 min, after which the supernatant

Table 1. Bacterial reference strains used in the study.

Strain	Medium	Source
Lactobacillus acidophilus	MRS Agar	Colostrum
Lactobacillus acidophilus-3	MRS Agar	Colostrum
Lactobacillus brevis	MRS Agar	Infant faeces
Lactobacillus brevis	MRS Agar	Colostrum
Lactobacillus casei	MRS Agar	Colostrum
Lactobacillus fermentum	MRS Agar	Infant faeces
Lactobacillus plantarum	MRS Agar	Infant faeces
Lactobacillus plantarum	MRS Agar	Colostrum
Lactobacillus reuteri	MRS Agar	Infant faeces
Lactobacillus rhamnosus	MRS Agar	Infant faeces

was removed. The pellet was washed with 10 µl of 70% ethanol and centrifuged at 5000 ×rpm for 2 min. The pellets were air dried and finally the DNA was suspended in 100 µl of TE buffer. Purified DNA samples were stored at -20°C until they were used. The melted 1% agarose by 1XTAE buffer was given up to cool down and was then poured into the horizontal gel tank installed comb in smooth ground. The solidified agarose gel was placed in the electrophoretic tank and the tank was filled to gel level (about 1 to 3 mm) by 1XTAE buffer. For visualizing the DNA extracts, 5 µl of each extract and 3 µL of bromophenol blue was electrophoresed on 1% agarose gel in 1X TAE buffer, which were then stained with ethidium bromide and examined under UV transilluminator.

RESULTS

API 50 CHL identification from bacteria

Strains of bacteria from colostrum and faecal samples were identified in order to determine the fermentation of carbohydrates. Identification tables were prepared (+/-) according to colour change in evaluation of the results of API strips reaction. The numerical profiles of strains were identified by adding positive values to the indicative table. Species designation was identified and evaluated by software identification apiweb^{TM}. The bacteria were isolated from 37 of the 150 samples, 17 of the 100 colostrum and 17 of the 50 faeces used in this study. From these, different species according to researches were indicated in Table 1.

DNA isolation from bacteria recovered from faeces and colostrum

The bacterial DNA samples were prepared by the alkali lysis DNA isolation protocol. To evaluate the quantity and quality of the DNA, the DNAs were selected as representatives of the infant samples. The DNA isolated from the samples of faeces and colostrum by the modified method was electrophoresed on a 1% agarose gel in 1XTAE buffer, and the DNA concentration was measured in a spectrophotometer at 260 nm. The total amount of DNA isolated from faeces depends on the number of bacteria in the faecal samples and the efficiency of lysis. These DNA concentration are 2 to 3 times higher than those obtained using the reference methods for both samples sufficient for the preparation of genomic libraries. In addition, the ratio of the optical density of the DNA at 260 to 280 nm was over 1.9 with the modified methods. As shown in Figures 1 and 2, the DNA samples isolated using the improved or modified methods were of sufficiently higher molecular weight DNA and were observed in the gel. Thus, the alkali lysis DNA isolation methods are useful for the isolation of the DNA to be used in the preparation of genomic libraries from faeces infant and colostrum samples (Figures 1 and 2). In this study, we presented an alkali lysis method that has high DNA efficiency with high molecular weight and was obtained in sufficient quantity as to allow the contraction of genomic libraries with large insertions.

Based on the fluorescence of the DNA smear in the ethdium bromide stained gel, the alkali lysis method produced the greatest yield, but the fragment size was extremely small as compared to the other DNA preparations measured against the molecular weight markers. Depending on the application of the DNA, lower yields of large sized fragments may be preferred. Genomic DNA solutions from lactobacilli by alkali lysis method was of adequate purity and yield for PCR application. As a result of these applications, the expected 100 bp fragments using the lactobacilli bacteria specific primer pair were detected. When the genomic DNA was extracted from the lactobacilli, the DNA solutions by alkali lysis method were of sufficient purity (A_{260}/A_{280} 1.7 to 1.8). Therefore, the lactobacilli DNA extraction procedure is sufficiently efficient and it yields an adequate amount of genomic DNA with a sufficient level of repeatability. A DNA extaction method has been developed and applied succesfully to the detection of lactobacilli. This study has shown that DNA can be extracted efficiently from the processed samples using different protocols. The study reported that the extraction method used to produce DNA of suitable quantity and quality for subsequent PCR was

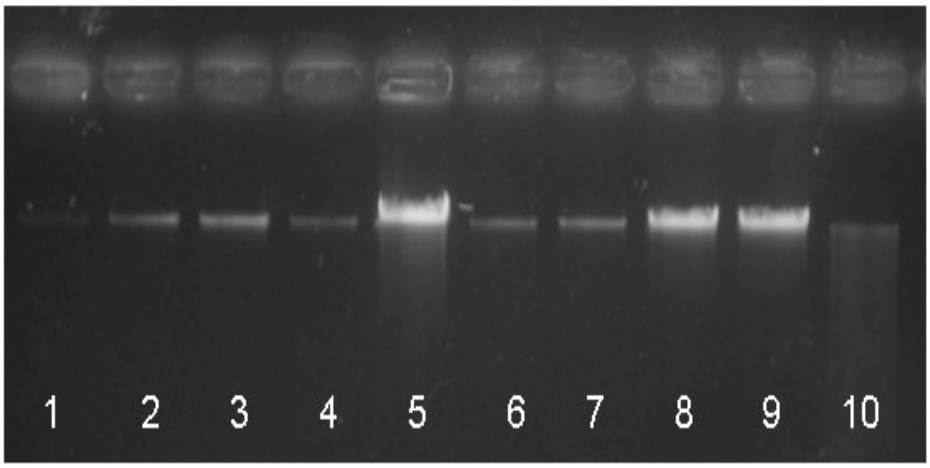

Figure 1. Screening of agarose gel electrophoresis of anaerobic bacteria DNA or *Lactobacillus* isolated from faeces infants and colostrum samples.

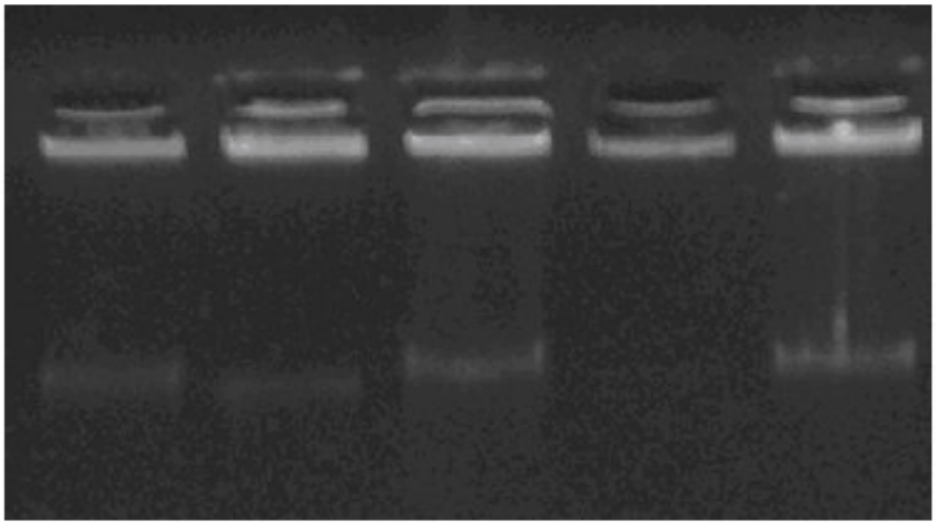

Figure 2. Screening of agarose gel electrophoresis of anaerobic bacteria DNA or *Bifidobacterium*.

based on detection applications. The procedures were reliable and reproducible, in typically planning a success rate of over 90%. In summary, a fast and reliable genomic DNA extraction protocol was developed for anaerobic bacteria. In the future, PCR and Real-Time PCR of afcA gene would be performed for *Bifidobacterium bifidum*. In this study, it was thought that the probiotic products of molecular methods contributed more to baby nutrients and were highly reliable than the microbiological methods.

DISCUSSION

Growth in 20 of the 100 (20%) colostrum samples and in 17 of the 50 (34%) faeces samples was observed as a result of an investigation of the 100 colostrum and 50 infant (from 3 to 30-day-old) samples. Román-Méndez et al. (2009) plated saliva samples which were collected from 42 French and 121 Mexican children (6 to 12 years old) into MRS Agar. While *L. rhamnosus* and *L. acidophilus* were identified by the PCR, other lactobacilli were identified by API 50 CH system. Lactobacilli were investigated by RAPD-PCR technique, although RAPD-PCR and API 50 CH test results indicated similarity. French children isolated only *L. rhamnosus*, while Mexican children isolated *L. rhamnosus* and *L. acidophilus*. They suggested that the reason for this difference was the fermentative milk products used in nutrient and probiotics (Román-Méndez et al., 2009). In

this study, *L. rhamnosus* from faecal (from 3 to 30-day-old) and *L. acidophilus* from colostrum were found by API 50 CHL system for identification. For the mouth, which is the outer part of the digestion system, it is expected that there are many species in the mouth and saliva samples. For human milk, which is sterile in normal condition, growth can occur in the case of contamination from the outside. It is thought that much isolation of *Lactobacilli* from faecal infant (from 3 to 30-day-old) depends on arriving to the digestion system with variety nutrients, especially milk and milk product and excretoring by faecal. Ahrne et al. (2005) isolated lactobacilli from only 21% in 23 of the 112 samples from 1-week-old infants (Ahrne et al., 2005). The results of this study indicated more intensiveness of lactobacilli in a proportion of 34% for 50 infants (from 3 to 30-day-old).

The biochemical tests (API 50 CHL) that were employed commonly were an important application in practice in terms of bacteria, but they were preferred under differences, like sugar fermentation and by reason of non-stability of results. Generally, molecular techniques are composed to identify and categorize the close relatives of the species bacteria not observed within themselves. API 50 CHL, in addition to the traditional method, was used from the existent sources of bacteria in aerobic or anaerobic flora as a result of isolation. If species cannot be identified, new alternatives can compose characterization of the correct LAB in a fast form, using molecular techniques based on PCR. Since intestinal bacteria play an important role in human physiology, numerous papers have been published using traditional culture and molecular methods to detect and identify them in colostrum and fecal samples. Culture methods are time consuming and many intestinal bacteria are not easily cultured or isolated from the complex mixture. However, we performed a modified method for the detection and identification of 8 different bacterial species, and isolated and identified 4 different species from colostrum and feces as well.

Fujimoto et al. (2008) used the DNA isolate from human feces to isolate bacterial DNA by Stool Mini Kit (Qiagen, Valencia, CA, USA) in order to raise the quality of the DNA (Fujimoto et al., 2008). Zoetendal et al. (1998) used, in DNA isolation, five units of DNase-free RNase (Promega), and isolated paralelly RNA too. Busconi et al. (2008) studied the evaluation of biodiversity of lactic acid bacteria microbiota, and they added lysozyme and RNAase to DNA extraction (Busconi et al., 2008), but in this study, we added lysozyme and proteinase K solution to appropriate samples of faeces and colostrum by the modified method of DNA isolation.

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