JOURNAL OF PURE AND APPLIED MICROBIOLOGY, September 2013. Vol. 7(3), p. 2043-2047

The Genetic Profiling of the Oral Microbiota in Twins of Primary Dentition

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(Received: 17 June 2013; accepted: 21 August 2013)

In this study, we evaluated the oral microbial diversity in caries and caries-free twin children of primary dentition by Polymerase chain reaction-based denaturing gradient gel electrophoresis (PCR-DGGE). Saliva samples were collected from 10 pairs of twin children aged 3-6, including 3 monozygotic (MZ) twin pairs and 7 dizygotic(DZ) twin pairs. Total microbial genomic DNA was isolated from these samples, and a portion of the 16S rRNA gene locus was PCR amplified by using universal primers. We observed the mean species richness of the bacterial population was greater in the caries-free (CF) ones (n=13)(14.00 ± 2.74)than in the early childhood caries (ECC) children (n=7) (11.00 ± 1.56); the difference was statistical significant (P<0.05). The overall diversity of saliva samples as measured by the Shannon index was 1.44 ± 0.37 for the CF children and 1.05 ± 0.36 for the ECC children. More interesting, by means of a cluster analysis, the UPGMA cluster analysis, we find out that there is a high similarity of the oral bacterial community within co-twins, which is much more higher than in the unrelated ones, but no significant difference was seen between MZ and DZ twins. The result suggests that the microbial diversity and complexity of microbiota in the saliva of the ECC children are less than in the CF children. Also, there is a high similarity in the composition of oral microbiota in twin children of primary dentition, and there is no significant difference between the MZ and DZ twins. This may suggest that the genetic make-up of the host may have less impact than the environment factors on the composition of oral microbiota.

Key words: PCR-DGGE, Twin, Primary dentition, Oral microbiota.

Humans have a lifelong close relationship with bacteria[1].The human oral microbioma currently comprises 600-700 taxa, which plays an important role in maintaining the homestasis of oral cavity. Shifts in microbiota composition may predispose humans to disease[2]. But how the variation in microbes occur and what are the influence factors is still largely undetermined.

Monozygotic (MZ) twins share the same genes, whereas dizygotic (DZ) co-twins on average share only half of their genes. Therefore, by assuming that both types of twins have been sampled from the same gene pool and that similar environmental factors act upon them, one can estimate the relative contributions of genetic and environmental influences to observed variation in different features or traits.

Therefore, we collected the saliva sample of MZ and DZ twin pairs in primary dentition to investigate the oral microbial diversity. MZ and DZ twins in different caries status were enrolled in this study, by using one culture-independent method- Polymerase chain reaction-based denaturing gradient gel electrophoresis (PCR-DGGE) analysis, we obtained the genetic profile of

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the twins, and analysis the oral microbial composition.

MATERIALS AND METHODS

Subjects

10 pairs of twin children were enrolled in this study. Among them, there were 3 monozygotic (MZ) twin pairs and 7 dizygotic (DZ) twin pairs. The average age of children was 4.3±1.3 y (age range, 3-6 y), all of them were in primary dentition. Inclusion criteria were raised up together, good general health, no use of antibiotics in the last 3 months, no orthodontic appliances. Parents provided informed consent for their twins. The oral statuses of the children were examined by one calibrated pediatric dentist and a score for decayed, missing, and filled tooth surface (dmfs index) were recorded. 7 pairs of twins were concordant for caries or healthy, 3 pairs of twins were caries/ healthy ones. In all, there are 7 caries children and 13 caries-free children . The mean dmfs score of the caries ones was 2.29±1.38.

Saliva collection

2 ml un-stimulated saliva was obtained from subject by having them spit directly into a saliva collection tube after 2 h of absence from eating or drinking. The saliva samples were immediately transfered on ice to store in -80^{0}C until needed.

Zygocity

Zygosity of the twins has been confirmed by DNA analysis of 16 highly polymorphic genetic STR loci (*D3S1358, *vWA, *FGA, *AMEL, *D8S1179, *D21S11, *D18S51, *D5S818, *D13S317, *D7S820ÿTH01, PE, D16S539, CSF1P0, PD, TPOX,) covering 16 chromosomes from buccal swabs.

DNA extraction

After desolved in the room temperature ,the genome DNA were extracted from the saliva sample using the QIAamp™ DNA Micro Kit (QIAGEN, Hilden, Germany) with small modification by adding additional lysozome solution (3mg/ml, final concentration) to the sample in the first step, the following steps were taken according to the protocol.

DNA quality and quantity were measured by a spectrophotometer at 260nm and 280nm. The final concentration of each DNA sample was adjusted to 10 ng/µL for PCR applications.

PCR-DGGE analysis

Amplification of bacterial 16S ribosomal RNA genes by PCR was carried out as described previously[3,4]. Briefly, the universal primer set, Bac1 (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GAC TAC GTG CCA GCA GCC-3') and Bac2 (5'-GGA CTA CCA GGG TAT CTA ATC C-3') was used to amplify an approximately 300-base-pair (bp) fragment of the 16s ribosomal RNA gene. Each 50 µl PCR contains 100 ng purified genomic DNA, 40 pmol of each primer, 200 µM of each dNTP, 4.0 mM MgCl2, 5 µl 10× PCR buffer, and 2.5 U Taq DNA polymerase (Invitrogen). Cycling conditions were 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min, with a final extension period of 5 min at 72°C. PCR products were evaluated by electrophoresis in 1% agarose gels that were run at 60 V for 60 min, and the sizes of all amplicons were confirmed according to a molecular size standard.

Polyacrylamide gels at an 8% concentration were prepared with a denaturing urea/formamide gradient between 40% [containing 2.8 M urea and 16% (vol/vol) formamide] and 70% [containing 4.9 M urea and 28% (vol/vol) formamide]. Approximately 200 ng of the PCR product were applied per well. The gels were submerged in 1× TAE buffer (40 mM Tris base, 40 mM glacial acetic acid, 1 mM ethylenediamine-tetraacetic acid) and the PCR products were separated by electrophoresis for 16 hrs at 58°C using a fixed voltage of 60 V in the Bio-Rad DCode System (Bio-Rad Laboratories, Inc., Hercules, CA). After electrophoresis, gels were rinsed and stained for 15 min in 1× TAE buffer containing 0.5 µg/ml ethidium bromide, followed by 10 min of de-staining in 1× TAE buffer. DGGE profile images were digitally recorded using the Image Lab Documentation system (Bio-Rad Laboratories).

Statistical analysis

The similarities of PCR-DGGE DNA profiles were analyzed with Quantity One® 1-D Analysis software (version 4. 6.2; Bio-Rad Laboratory, Hercules, CA, USA). Dendrograms were constructed by the unweighted pair group method, using arithmetic averages (UPGMA). The differences in microbial diversity were assessed by comparing the DGGE profiles between the MZ-

DZ groups and CF-ECC groups respectively. The statistical analysis of the CF and ECC children were performed with the student's t test. The analyses were performed with the SPSS software version 13.0 (Statistical Package for the Social Sciences; SSPS Inc, Chicago, IL).

RESULTS

DGGE profile of amplified bacterial 16S rRNA gene segment

DGGE profiles were obtained from the saliva samples of the 10 co-twins, among whom the caries status 3.1. The DGGE profile of the twins The DGGE profile of the twins showed that the number of distinct bands (amplicons) ranged from 9 to 18, with a mean ± SD of 12.10 ± 5.66 for each individual sample. On average, the total number of detectable bands was significantly higher in the CF group (14.00 ± 2.74) than in the ECC group (11.00 ± 1.56), and that difference was statistically significant ($P < 0.005$ by the Student's t test) . Also is the comparison of the Shannon index, which represents a measure of the richness and evenness of microbial diversity in a given sample. The diversity of bacteria in the CF group (H= 1.44 ± 0.37) was greater than that in ECC group (H = 1.05 ± 0.36). The difference was also statistically significant ($P < 0.05$ by student's t test). (Fig. 1).

Dendrogram using UPGMA

Fig. 2 demonstrate the result of the UPGMA analysis. Samples are depicted in the columns. Co-twins, who participated in the study, are marked by the same colour, each colour representing different family. Unrelated individuals have no colour. In the UPGMA tree, each co-twin displayed a significant clustering of profile than unrelated individuals. However, there is no statistical difference in microbial similarity between the MZ and DZ twins. The result of the UPGMA analysis also showed that the ECC and CF group did not display any significant clustering compare to the MZ/DZ group.

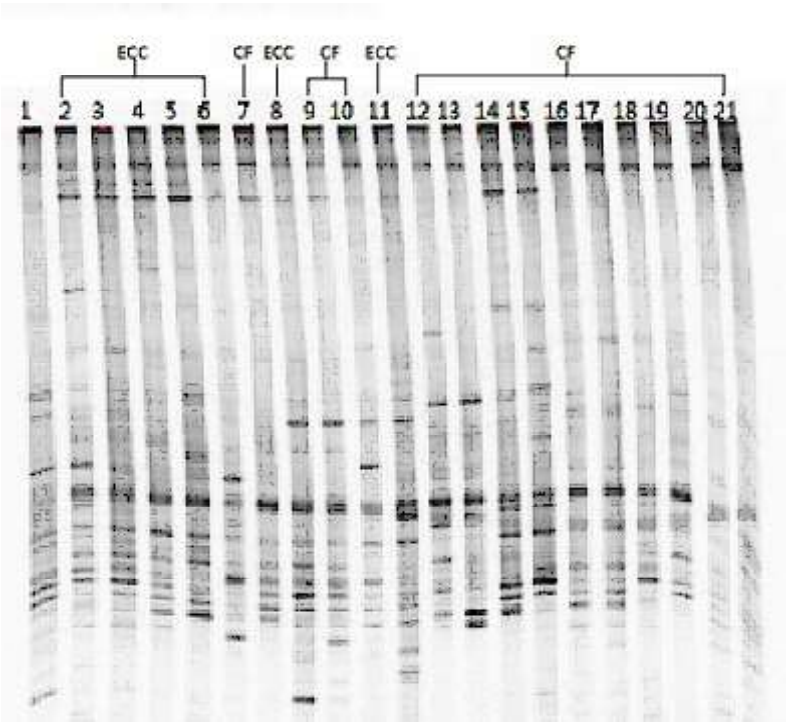

Fig. 1. DGGE profile of the twins in primary dentition, lane 2-3,4-5,6-7,8-9,10-11,12-13,14-15,16-17,18-19,20-21 represent 10 co-twins. Co-twin 2-3, 4-5 are caries pairs, co-twin 6-7, 8-9, 10-11 are caries/healthy pairs, co-twin 12-13, 14-15, 16-17, 18-19, 20-21 are caries-free ones. No significant change can be observed between the CF and ECC ones

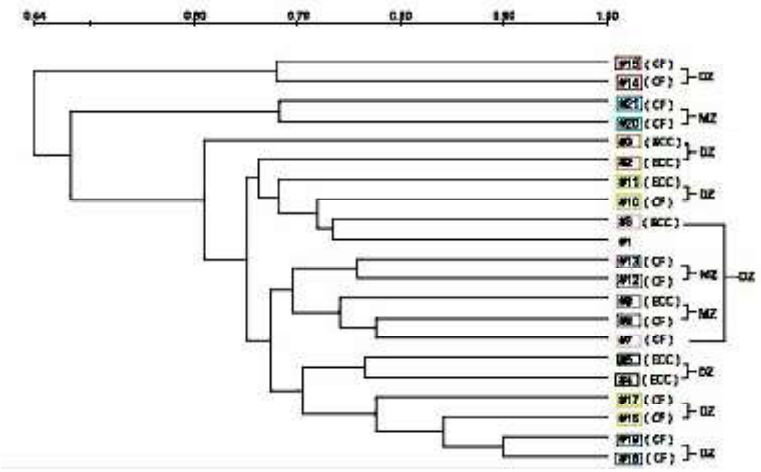

Fig. 2. The UPGMA analyse of the children in primary dentition,# 2-#3,#4-#5,#6-#7,#8-#9,#10-#11,#12-#13,#14-#15,#16-#17,#18-#19,#20-#21 represent 10 co-twins. co-twins are marked by the same colour, each colour representing different family.#1 was a unrelated one1. from the UPGMA tree we can clear see that almost every pair of co-twins represented a high level of similarity in the composition of saliva microbiota, except for twin pair #6—#7. No significant difference can be seen between the DZ and MZ twins

DISCUSSION

PCR-DGGE is a culture- independent molecular method which could yield a distinctive fingerprint representing each of the various bacterial phylotypes or species on a single gradient gel. Microbial profiles from different individuals can be compared with a variety of measurement tool. This approach has been widely used by environmental ecologist to survey entire bacterial communities without cultiva- tion[6,7] and to analyze gastrointestinal microbial ecosystems[4,5].

In this study, DGGE profiling of the bacterial populations in the saliva of ECC children displayed significantly less diversity than that for the CF children. This finding suggests that the caries-associated microbiota becomes less diverse. The results of this study are consistent with previous study with adults and S-ECC children[3,5]. One possible explanation for the lesser microbial diversity in ECC saliva sample is that ECC ones contain higher proportions of acidogenic and aciduric bacteria than caries-free ones, as some research papers have reported[8,9]. Another explanation for the decrease in diversity is that caries lesions create more retentive niches for cariogenic microorganisms, which increase their total numbers of cariogenic bacteria but subsequently decrease the overall richness of the bacterial community[10,11,12].

Also, in this study, the results demonstrate that the DGGE profiles of each co-twin pair formed significant group-specific cluster, co-twins share a higher similarity in saliva microbial profiles than among unrelated individuals. The delineation of distinct clusters was reflected by the number of bands detected (richness), the intensity, and the migration distribution of the PCR 16S amplicons. Moreover, the MZ twin pairs are statistically not more similar than DZ twin pairs. This may suggest that the genetic make-up of the host may have less impact than the environment factors on the composition of oral microbiota.

Though evidences from previous twin studies show that genetic factors contribute to the salivary levels of certain species of bacteria[13], and genetic factors including HLA genes have been reported to influence salivary mutans streptococci colonization and caries susceptibility in human and animal models[14,15] some present studies have demonstrated that the shared environment may serve as the main determinant of microbial populations other than the genetic factor[16], our research get the same conclusion. This may give us a clue to the prevention of oral infectious disease.

In summary, we observed a significant variation in the DGGE profiles between the ECC and CF groups. The microbial diversity and the complexity of the microbial biota in saliva were less in children with ECC than in CF children. Also ,the results of our study demonstrated that there is a much more higher similarity in co-twins rather than in the unrelated others, yet the difference in the similarity between MZ and DZ twin pairs is not significant. The data are most consistent with shared environment serving as the main determinant of microbial populations.

ACKNOWLEDGEMENTS

This study was supported by pre-project of Chinese National Programs for Fundamental Research and Development (973 Program) (2011DFA30940)

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