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*Full Length Research Paper*

# Identification and quantitation of conjunctival aerobic bacterial flora from healthy residents at different ages in Southwest China

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**To identify and quantitate conjunctival aerobic bacterial flora from healthy residents of three age groups in Chongqing city, Southwest China. Samples taken with moist swabs from the lower fornix of bilateral eyes of 33 children (9.85 ± 0.57 years old), 31 youths (21.23 ± 0.88 years old), and 30 elders (72.97 ± 4.94 years old) respectively were tested for bacterial cultures. Positive cultures were identified and colony forming units (CFUs) were counted. Bacterial quantity was expressed as the number of CFUs divided by the weight difference of each sample transport swab-tube measured before and after the sample collection. The elders showed a higher isolation frequency (93.33%) of conjunctival aerobic bacteria than that of children (45.45%) and youths (50.00%), (P<0.01). *Corynebacterium diphtheroid, Staphylococcus epidermidis* and *Staphylococcus aureus* isolated from the elders all had a greater percentage. The elders also had a larger number of aerobic bacteria species per eye than did children and youths (P<0.01). Children showed a lower quantity of conjunctival aerobic bacterial flora than that of the other two groups (P<0.01). *C. diphtheroid* was the most numerous isolate from all the subjects. The quantity of *C. diphtheroid* became larger when residents grew older. *S. epidermidis* was the second most numerous bacteria in the groups of children and elders, and the elder group had a greater number of *S. epidermidis* than the children group (P<0.01). The number of species and quantity of conjunctival aerobic bacterial flora from the elders were larger than those from children and youths, which should be of concern to an ophthalmologist.**

**Key words:** Conjunctival sac, aerobic bacteria, age, quantitative analysis, microecology.

## INTRODUCTION

The conjunctival sac, a semi-open cavity, is parasitized with microflora throughout our lifetime from when we were born, and the microbiota, which does not always cause abnormal senses or sickness, are called conjunctival normal flora in microecology. Normal floras play an important role in keeping our ocular surface healthy. Disease will be caused when the normal flora lose codependence and co-regulation with the host. Therefore, research on conjunctival flora is significantly important in the field of infectious diseases of the ocular surface, especially in the pathogeneses, prevention and management of chronic ocular surface infections. At present, researches on conjunctival normal flora have mainly been focused on prophylaxis of postoperative endophthalmitis by using topical antibiotics in patients undergoing intraocular surgeries (Herminia et al., 2008; Enrique et al., 2008; Jason et al., 2008; Rubio, 2006). Quantity of conjunctival flora has mainly been at the level of semi-quantitation (Herminia et al., 2008; Enrique et al., 2008; Jason et al., 2008) . In our study, we identified and quantitated conjunctival aerobic bacterial flora from healthy residents of three different age groups (children, youths and elders) in Chongqing city, Southwest China.

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## METHODS

### Subjects

Our research was performed with the approval of the appropriate ethics committee of our hospital. Researches carried out on

humans were in compliance with the Helsinki Declaration. Informed consent was obtained from each volunteer after giving a detailed explanation of the purpose of the study (for each child, consent was obtained from his/her supervisor).

Three age groups of healthy residents in Chongqing city, Southwest China were enrolled in the study. Among them, 33 children volunteers (66 eyes), including 17 boys and 16 girls with an average age of 9.85 ± 0.57 years old, were selected from students in Grade 4 of a primary school. 31youths volunteers (62 eyes), of whom, 15 were male and 16 were female with an average age of 21. 23 ± 0.88 years old, were selected from students of a university. 30 elder volunteers (60 eyes), of whom, 16 were male and 14 were female with an average age of 72.97 ± 4.94 years old, were selected from a district in Chongqing city, Southwest China.

All subjects were free of systemic and local infectious diseases, and none had used antibiotic drugs for four weeks before the conjunctival sample collection, and none had used steroid hormones or immune depressant drugs. Those with ocular trauma or those who had undergone intraocular surgery or wore contact lenses were not included. Slit-lamp microscope examination was taken to exclude those with inflammation in the eyelid, ocular surface or dacryocyst. Subjects with a deformity in eyelid or eyeball, or those with dry eye disease were also excluded. Conjunctival samples of all volunteers were collected from bilateral eyes.

#### Conjunctival sample collection methods

Sample transport swab-tubes were made ourselves by connecting the end of a cotton swab shaft with a gel silica bung, which bunged into the tube so that it could be weighed and carried as a single unit the transport swab- tubes were then sterilized. We measured and recorded the weight of each sample transport swab-tube before and after collection using an electronic balance, of which the precision is $10^{-4}$g. The whole weighing procedure was performed by the operator in the lab room where temperature and humidity were kept similar during the study to ensure the consistency of the experimental conditions. Samples from volunteers' bilateral eyes were collected by the same operator at a room temperature of about 20°C after being disinfected by ultraviolet light. Each volunteer was asked to face upward slightly and both eyes gazed upwards as the skin around the eyelid was cleaned with sterilized saline swabs. The operator then pressed the inferior eyelid downward slightly to expose the lower fornix sufficiently and wrapped up the exposed part of the gel silica bung of the transport tube with a piece of sterilized gauze. The operator then took out the gel silica bung, which was connected to the sterilized moist swab, and put the swab onto lower fornix. The swab was then turned around slightly from one side to the other side of the lower fornix from the outer canthus to the inner canthus, paying attention not to leave out the medial canthus. The swab and the gel silica bung was then inserted into the transport tube immediately after the opening of the tube was disinfected with an alcohol burner. Touching the eyelashes and the skin of the eyelid was avoided to avoid contamination.

#### Bacterial culture, identification and quantitation

Sample transport tubes were weighed again quickly after the collections using the same electronic balance, and the difference in weight of the sample transport tube before and after collection was calculated, which represented the weight of the sample. Samples were transferred onto Colombia blood agar medium (Pangton Medical Equipment Co. Lit, Chongqing, China) by streaking lines with the swab, and the plate was then incubated for 72 h in an atmosphere containing 5% $CO_2$ at 37°C. We viewed the colony growth on agar the first time 18 to 24 h after starting the culture, and then made an observation once per day. The number of colony forming units (CFUs) were counted and recorded, so that quantitation of a bacterial species in an individual's conjunctiva could be expressed as the number of CFUs counted on the agar divided by the weight of sample collected from the conjunctival sac (Quantitation of a bacterial species = CFUs of the bacteria / sample weight). The sample that had no colony growth on the agar 72 h after culture was regarded as negative. We sent the positive cultures to the bacterium-detection room of our hospital to take isolated cultures for identifications using Auto-Microbiology Identification, Vitek II Compact (bioMérieux, France).

#### Statistical treatment

Pearson × 2 test was used to compare positive ratios of aerobic bacteria. Kruskal-Wallis H test and Mann-Whitney U test were used to compare the number of bacterial species per eye, and ANOVA to compare the quantity of some species of aerobic bacteria using the softwear package SPSS13.0.

## RESULTS

### Isolation frequency of conjunctival aerobic bacteria from 188 eyes of three age groups

Conjunctival cultures from 188 eyes of 94 volunteers were analyzed for aerobic bacteria. 62.23% of conjunctiva showed positive results. The elders showed a significant higher isolation frequency (93.33%) than that of children (45.45%) and youths (50.00%) ($P<0.01$), while there was no statistical difference between the children and youth groups ($P>0.05$) (Table 1).

### Conjunctival aerobic bacteria isolated from 188 eyes in three age groups and the isolation frequency of each bacterial species

Of the 188 eyes studied, 18 species were found in total. *Staphylococcus epidermidis (*76.07% of positive cultures) was the predominant organism found in the conjunctival samples in the three groups. Second was *Corynebacterium diphtheroid*, whose isolation frequency was 52.14%, next was *bacillus* (30.77%). The numbers of strains isolated in the children, youth and the elder groups were 11, 9, and 12, respectively, of which, five species were isolated in all three groups. Isolation frequency of *S. epidermidis* in the elder group (94.64%) and youth group (83.87%) were higher than that of the children group (33.33%) ($P<0.05$). *C. diphtheroid* isolated from the elders (67.86%) had a greater percentage than that of the youth (45.16%) and children group (30.00%) ($P<0.05$). Isolation frequency of *Staphylococcus aureus* in the elder group (26.79%) and the children group (26.67%) were higher than that of the youth group (3.23%) ($P<0.05$). There was no statistical difference in the isolation frequency of *Micrococcus luteus* and *bacillus* among the three groups ($P>0.05$). *Candida albicans* was isolated from one child's conjunctival sample, and no

**Table 1.** Isolation frequency of conjunctival aerobic bacteria from 188 eyes of the three age groups.

| Group | Positive cultures | Negative cultures | Total cultures | Isolation frequency (%) |
|---|---|---|---|---|
| Children | 30 | 36 | 66 | 45.45[a] |
| Youth | 31 | 31 | 62 | 50.00[a] |
| Elder | 56 | 4 | 60 | 93.33 |
| Total eyes | 117 | 71 | 188 | 62.23 |

[a]P<0.01, compared with the elder group.

**Table 2.** Conjunctival aerobic bacteria isolated from 188 eyes in the three age groups and the isolation frequency of each strain.

| Microorganism | Isolation frequency (%) | | | |
|---|---|---|---|---|
| | Children group | Youths group | Elder group | Total |
| **Aerobic bacteria** | | | | |
| *S.epidermidis* | 33.33 (10/30) | 83.87 (26/31)[a] | 94.64 (53/56)[a] | 76.07 (89/117) |
| *C. diphtheroides* | 30.00 (9/30) | 45.16 (14/31) | 67.86 (38/56)[a b] | 52.14 (61/117) |
| *Bacillus* | 30.00 (9/30) | 19.35 (6/31) | 37.50 (21/56) | 30.77 (36/117) |
| *S.aureus* | 26.67 (8/30)[b] | 3.23 (1/31) | 26.79 (15/56)[b] | 20.51 (24/117) |
| *M. luteus* | 6.67 (2/30) | 9.68 ( 3/31) | 16.07 (9/56) | 11.97 (14/117) |
| *Neisseria* | 6.67 (2/30) | 3.23 (1/31) | 0 | 2.56 (3/117) |
| *Escherichia coli* | 0 | 3.23 (1/31) | 1.79 (1/56) | 1.71 (2/117) |
| *Brevundimonas vesicularis* | 10.00 (3/30) | 0 | 3.57 (2/56) | 4.2 7 (5/117) |
| *Staphylococcus hominis* | 10.00 (3/30) | 0 | 0 | 2.56 (3/117) |
| *Staphylococcus saprophyticus* | 3.33 ( 1/30) | 0 | 0 | 0.85 (1/117) |
| *Streptococcus pneumoniae* | 3.33 (1/30) | 0 | 0 | 0.85(1/117) |
| *Acinetobacter* | 0 | 3.23 (1/31) | 0 | 0.85 (1/117) |
| *Kocuria varians* | 0 | 3.23 (1/31) | 0 | 0.85 (1/117) |
| *Enterococcus faecalis* | 0 | 0 | 7.14 (4/56) | 3.42 (4/117) |
| *Pseudomonas aeruginosa* | 0 | 0 | 1.79 (1/56) | 0.85 (1/117) |
| *Branhamella catarrhalis* | 0 | 0 | 1.79 (1/56) | 0.85 (1/117) |
| *Rhizobium radiobacter* | 0 | 0 | 1.79 (1/56) | 0.85 (1/117) |
| *Kocuria rosea* | 0 | 0 | 1.79 (1/56) | 0.85 (1/117) |
| **Fungus:** | | | | |
| *Candida albicans* | 3.33 ( 1/30 ) | 0 | 0 | 0.85 (1/117) |

[a]P<0.05 compared with the children group, [b]P <0.05 compared with the youth group.

other fungal species had been found (Table 2).

## The number of conjunctival aerobic bacterial strains isolated per eye in the three age groups

We found that in the children and youth conjunctival sacs, there was usually only one kind of bacterial strain, or two strains coexisting. These were the two main living patterns. While in the unilateral conjunctival sac of the members of the elder group, we often observed several aerobic bacterial species living together, the most we found were six. Analyzing these with nonparametric statistics, we found that the elder group had a larger number of strains per eye than did children and youth groups (P <0.01), while there was no statistically difference between children and youths groups (P > 0.05) (Table 3).

## Conjunctival aerobic bacteria isolated from bilateral eyes of 94 volunteers in the three age groups

As shown in Table 4, in members of elder group, there was always at least one unilateral eye showing positive culture, and the isolation frequency from bilateral eyes was predominately higher in the elder group than that from just one unilateral eye (P <0.01). The isolation

**Table 3.** The number of conjunctival aerobic bacterial strains isolated per eye in the three age groups.

| Group | The number of bacterial species isolated from each unilateral eye | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 strain | 2 strains | 3 strains | 4 strains | 5 strains | 6 strains | Total |
| Children | 17 | 8 | 4 | 1 | 0 | 0 | 30 |
| Youths | 15 | 11 | 3 | 2 | 0 | 0 | 31 |
| Elder | 11 | 16 | 16 | 10 | 2 | 1 | 56 |

**Table 4.** Conjunctival aerobic bacteria isolated from bilateral eyes of 94 volunteers in the three age groups.

| Group | Isolation frequency (%) | | |
|---|---|---|---|
| | Unilateral positive | Bilateral positive | Bilateral negative |
| Children | 66.67 (22/33) | 12.12 (4/33)[a] | 21.21 (7/33) |
| Youths | 67.74 (21/31) | 16.13 (5/31)[a] | 16.13 (5/31) |
| Elder | 13.33 (4/30) | 86.67 (26/30) | 0 |

[a]P<0.01, compared with the elder group.

**Table 5.** Weight of conjunctival samples from the three age groups (mg).

| Group | Minimum | Maximum | n | $\bar{x} \pm s$ |
|---|---|---|---|---|
| Children | 1.90 | 9.10 | 66 | 3.99±1.34 |
| Youth | 1.90 | 8.50 | 62 | 4.34±1.56 [a] |
| Elder | 2.70 | 8.70 | 60 | 4.30±1.19 [a] |

[a]P<0.05, compared with the children group.

frequency from bilateral eyes was predominately higher than that of children and youth groups. In the children and youth groups, positive culture of conjunctival aerobic bacteria was mainly isolated from a single unilateral eye, and there was no significant difference between the children and youth groups (P > 0.05).

## Quantitation of conjunctival aerobic bacteria isolated from the three age groups

The results of conjunctival sample weights of the three age groups indicated that conjunctival sample weights of the elder group and youth group were more than that of the children group (P<0.05) (Table 5). We got the quantitation of conjunctival aerobic bacteria by the formula calculation mentioned above (Quantitation of a bacterial strain = CFUs of the bacteria / sample weight). Quantitation in the children group ranged from 1.85 X $10^2$ to 8.52 X $10^4$ CFU/g, the youth group ranged from 1.61 X $10^2$ to 2.40 X $10^5$ CFU/g, and the elder group ranged from 1.14 X $10^2$ to 3.63 X $10^5$ CFU/g. We then analyzed the data, expressed as log values, by One- way ANOVA and found that children showed a significant lower quantity of conjunctival aerobic bacterial flora than that of the other two groups (P<0.01). *C. diphtheroid* was the most numerous isolate from all subjects, the quantity of *C. diphtheroid* became larger as the residents grew older. The quantity of *C. diphtheroid* in the elder group was more than that of youth group, *C. diphtheroid* in the youth group was more than that of the children group. *S. epidermidis* was the second most numerous bacteria in the groups of elder and children, and the elder group had a greater number than that of the children group (P<0.01) . There was no statistical difference in quantity of *bacillus* , *S. aureus* or *M. luteus* among the three age groups (*P*>0.05). Table 6 represents the quantitation of conjunctival aerobic bacteria of five species isolated from the three age groups.

## DISCUSSION

Microecology is a frontier branch of science that has developed rapidly in recent years. Microecology is based on research into the constitution of our body's microecosystem. The conjunctival sac is parasitized with microflora that is changing dynamically through our

**Table 6.** Quantitation of conjunctival aerobic bacteria of 5 species isolated from the three age groups (log CFU/g, $\bar{x} \pm S$).

| Group | Microorganism | | | | |
|---|---|---|---|---|---|
| | ***S. epidermidis*** | ***C. diphtheroides*** | ***Bacillus*** | ***S. aureus*** | ***M. luteus*** |
| Children | 2.89 ± 0.53 | 3.20 ± 0.62 | 2.57 ± 0.29 | 2.74 ± 0.50 | 2.43 ± 0.21 |
| Youths | 3.34 ± 0.67 | 3.89 ± 0.73[a] | 2.44 ± 0.14 | 3.52 ± 0.00 | 3.74 ± 1.34 |
| Elder | 3.51 ± 0.68[a] | 4.36 ± 0.53[b] | 2.68 ± 0.39 | 2.89 ± 0.51 | 2.63 ± 0.47 |

[a]P<0.01, compared with the children group, [b]P<0.05, compared with the youth group.

lifetime because of its long- term exposure to the environment. This diverse and large number of conjunctival normal flora have been regarded as important organisms in the field of microecology for ocular surface research. These bacteria are part of the defense mechanism of the eye in preventing colonization by more pathogenic microorganisms. Diseases will be caused when normal flora lose balance with host in quality or quantity. A detailed understanding of the composition and dynamic changes of normal flora in physiological conditions may help in understanding abnormality and in rebuilding a balanced healthy state using measures of microecosystem adjustment. From the viewpoint of microecology, we can judge whether a microecosystem balance is physiological by defining the position, qualitation and quantitation of the flora. At present, research on conjunctival normal flora has mainly been focused on identification and has been at the level of semi-quantitation. In this study, we have identified and quantitated conjunctival aerobic bacterial flora to get experimental data on the microecology of the ocular surface.

Conjunctival cultures from 188 eyes of 94 volunteers were analyzed for aerobic bacteria, and 62.23% of normal conjunctiva has shown positive results. The first three predominant organisms were *S. epidermidis* (76.07%), *C. diphtheroid* (52.14%) and *bacillus* (30.77%). The results are similar to recent reports, which have shown an isolation frequency of conjunctival aerobic bacteria ranging from 43.2 to 84.6%. (Herminia et al., 2008; Enrique et al., 2008; Jason et al., 2008). The predominant organism in the recent report was coagulase negative *staphylococcus*, and followed by *Corynebacterium* or *Staphylococcus aureue* (Herminia et al., 2008; Enrique et al., 2008; Jason et al., 2008). We have found more *bacillus* than these recent reports. *Bacillus* is a kind of saprogen that has a widespread existence in soil, effluent water and dirt and could be found in the normal conjunctival sac. The higher isolation frequency in our study suggests that bacillus may play an important role in keeping the balance of the microecosystem as a member of normal conjunctival flora. The role that bacillus plays in the normal conjunctival flora should be studied in future research.

It had previously been confirmed that age has an effect on the conjunctival normal flora (Rubio, 2006; Singer et al., 1988). Zhang and Ye (2005) gave evidence that age has a significant correlation with conjunctival bacterial isolation frequency by retrospectively looking at references of bacterial culture from the conjunctival sac and doing a Meta- analysis of relevant investigations from the last 13 years in China. Rubio (2006) performed a retrospective case series study of 4432 patients, who underwent cataract surgery and were divided into seven groups according to age. The aim of this study was to ascertain the effect of old age on conjunctival bacteria frequency, and they found that patients aged 75 to 96 years had a greater frequency of *Corynebacteria*, *S. aureus*, *Streptococcus sp*. (except *Streptococcus pneumonia*), Gram-negative cocci and Gram-negative rods (except *Haemophilus sp*.) and other bacterial categories than those aged 3 to 74 years. Singer et al. (1988) has proven that the percentage of eyes from which bacteria could be isolated was similar in both groups (23% in younger subjects and 21% in adults). *Streptococcus sp*. was cultured from 14.9% of the children's eyes as opposed to only 2.2% from adults. Our study got similar results to Rubio (2006) and Singer et al. (1988). We found that the isolation frequency from bilateral eyes and the number of species per eye of conjunctival aerobic bacterial flora from the elders were greater than that from children and youths. This may be due to elders having a lower resistance from decreased immune functions, reduction of lacrimal secretions, reductions in some kinds of antibodies, complement proteins and enzymes in tears (Christopher et al., 2003). Therefore, we should be more concerned with elder patients, especially those who have diabetes, ocular chronic infection, and those that are preoperative or postoperative.

Bacterial quantitation is an important aspect of the research into normal flora. At present, studies of conjunctival aerobic bacteria have mainly been at the level of semi-quantitation (Herminia et al., 2008; Enrique et al., 2008; Jason et al., 2008) that is, the quantity of bacteria is expressed as the number of CFUs directly. Gerald and Robert (1981) dissolved the alginate swabs after collection in a two-step procedure in TC eagle's medium and 2.5% sodium hexametaphosphate solution. Aliquots of suspended bacteria from the dissolved swab were plated on blood agar. After incubation, bacterial colonies present were enumerated and identified. In

Abhay et al. (2008), the number of bacterial colony-forming units on the plates was counted with a grid technique and expressed as log values. Then total number of CFUs per eye and the mean percentage reduction in CFUs were calculated after instillation of topical moxifloxacin hydrochloride ophthalmic solution 0.5%. In our study, the quantitation of a strain from an individual's conjunctiva was expressed as the number of CFUs counted on the agar divided by the weight of sample collected from individual's conjunctival sac. The weight of sample was the increase in transport tube weight after collection. This calculation is in accordance with the definition that the normal flora quantitation is the mean number of colony forming units per gram or per milliliter of sample. The weight of the conjunctival sample is influenced by factors such as the size, shape and dryness of the collecting swabs, the size of conjunctival cavity, and the volunteer's co-operation. These factors will affect the quantity of conjunctival aerobic bacterial flora obtained. However, conjunctival samples collected with moist cotton swabs differ from samples of stool and urine, of which, sample weight can be measured directly. To the best of our knowledge, quantitation of conjunctival flora has not been previously reported. To solve the problem, we made sample transport swab-tubes ourselves and measured the weight of each sample transport swab-tube using an electronic balance. The weight of the sample transport tube before and after collection was calculated, which represented the weight of the conjunctival sample. Measures were taken to ensure that the precise increase in transport tube-weight was obtained. The swab exposure time was as short as we could, and the whole weighing procedure was performed by the same operator. The lab room temperature and humidity were kept similar, to keep consistency of the experiment conditions. In our study, the weight of the conjunctival samples differ among the three groups, however, the average weight of conjunctival sample in each group was very similar.

Our results showed that conjunctival bacterial quantitation ranged from 102 to 105 CFU/g, which is approximately equivalent to the surface of the skin quantity of conjunctival aerobic bacterial flora than that of youth and elder groups. *C. diphtheroid* was the most numerous isolate from all subjects, more than *Staphyloccus epidermid,* which had the highest isolation frequency, indicating that the bacterial quantitation and isolation frequency are not at equal pace with each other. Excessive changes of conjunctival bacterial strains or their quantitation will destroy the microecosystem balance and cause disease. Elderly residents had more conjunctival aerobic bacteria than younger residents. This greater number of bacteria could increase the risk of intraocular surgery contamination. The sample size in this article is small and further studies using large sample size are needed.

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