Comparison of the ocular microbiome on ocular surface between chronic Stevens-Johnson syndrome patients and healthy subjects

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A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Clinical Sciences

Common Course

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การศึกษาเพื่อเปรียบเทียบความแตกต่างของ ocular microbiome ของอาสาสมัครกลุ่มอาการสตีเวนส์จอห์นสันชนิดเรื้อรัง เทียบกับในอาสาสมัครปกติ

น.ส.ธนัชพร กิตติพิบูลย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	Comparison of the ocular microbiome on ocular surface between chronic Stevens-Johnson syndrome patients and healthy subjects
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ธนัชพร กิตติพิบูลย์ : การศึกษาเพื่อเปรียบเทียบความแตกต่างของ ocular microbiome ของอาสาสมัครกลุ่มอาการสตีเวนส์จอห์นสันชนิดเรื้อรัง เทียบกับในอาสาสมัครปกติ. (Comparison of the ocular microbiome on ocular surface between chronic Stevens-Johnson syndrome patients and healthy subjects) อ.ที่ปรึกษาหลัก : รศ. พญ. วิลาวัณย์ พวงศรีเจริญ, อ.ที่ปรึกษาร่วม : ดร.ธนิษฐา ฉัตรสุวรรณ

วัตถุประสงค์ เพื่อศึกษาเปรียบเทียบความแตกต่างของเชื้อจุลชีพประจำถิ่นของผิวดวงตา ระหว่างกลุ่มผู้ป่วยโรคสตีเวนส์จอห์นสันในระยะเรื้อรัง และกลุ่มอาสาสมัครปกติ

วิธีการวิจัย เป็นการศึกษาเชิงวิเคราะห์ แบบไปข้างหน้า ในผู้ป่วยโรคสตีเวนส์จอห์นสันและอาสาสมัครปกติ ที่มีอายุตั้งแต่ 18 ปีขึ้นไป โดยผู้ทำการศึกษาได้เก็บตัวอย่างน้ำตาและผิวดวงตาจากบริเวณเยื่อบุตาของอาสาสมัครทั้ง 2 กลุ่มมาตรวจหาเชื้อจุลชีพประจำถิ่น ด้วยวิธีการเพาะเชื้อและการวิเคราะห์หาลำดับเบสแบบ Next Generation Sequencing (NGS)

ผลการศึกษา จากการตรวจหาเชื้อจุลชีพด้วยวิธีการเพาะเชื้อ พบว่ามีอัตราการเพาะเชื้อขึ้น ในกลุ่มผู้ป่วยโรคสตีเวนส์จอห์นสัน (60%) สูงกว่า กลุ่มอาสาสมัครปกติ (10%) อย่างมีนัยสำคัญทางสถิติ (p-value 0.001) นอกจากนี้การตรวจหาลำดับเบสแบบ NGS ก็ได้แสดงให้เห็นถึงความหลากหลายทางสายพันธุ์ของเชื้อจุลชีพที่สูงกว่า และปริมาณสัดส่วนของเชื้อจุลชีพก่อโรคอันได้แก่ *Pseudomonas* spp., *Staphylococcus* spp., *Streptococcus* spp., *Acinetobacter* spp ที่มากกว่า ในกลุ่มผู้ป่วยโรคสตีเวนส์จอห์นสัน

สรุปผลการศึกษา กลุ่มผู้ป่วยโรคสตีเวนส์จอห์นสันมีการเปลี่ยนแปลงของเชื้อจุลชีพที่บริเวณผิวดวงตาอย่างมาก เมื่อเทียบกับอาสาสมัครปกติ โดยผู้ทำการศึกษาได้ตรวจพบความหลากหลายของเชื้อจุลชีพประจำถิ่นและสัดส่วนของเชื้อจุลชีพก่อโรคที่เพิ่มมากขึ้น ปัจจัยข้างต้นอาจเป็นเหตุกระตุ้นที่ทำให้เกิดการอักเสบหรือติดเชื้อที่บริเวณผิวดวงตาของผู้ป่วยกลุ่มดังกล่าว แพทย์ผู้ให้การรักษาจึงควรให้ความสำคัญในการเปลี่ยนแปลงดังกล่าว และพิจารณาเลือกใช้ยาปฏิชีวนะในการป้องกันและรักษาภาวะติดเชื้อที่ดวงตาในผู้ป่วยกลุ่มนี้อย่างเหมาะสม

สาขาวิชา เวชศาสตร์คลินิก ลายมือชื่อนิสิต .....
ปีการศึกษา 2561 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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KEYWORD: Stevens-Johnson syndrome, ocular microbiome, conventional culture method, molecular biological method, next-generation sequencing, bacterial community

Thanachaporn Kittipibul : Comparison of the ocular microbiome on ocular surface between chronic Stevens-Johnson syndrome patients and healthy subjects. Advisor: Assoc. Prof. VILAVUN PUANGSRICHARERN, M.D. Co-advisor: TANITTHA CHATSUWAN, Ph.D.

Background: Stevens-Johnson syndrome (SJS) usually causes ocular surface disease. 35% of these patients develop chronic ocular sequelae including symblepharon, limbal stem cell deficiency, etc. The sequelae from SJS may also affect the alteration of ocular microbiome.

Subjects: 20 chronic SJS patients and 20 healthy subjects were enrolled for specimen collection from inferior conjunctiva for microbiome analysis by using conventional culture and next-generation sequencing(NGS) methods

Methods: Prospective, age and sex matched analytical study

Results: A significant higher proportion of culture-positive specimen was demonstrated in SJS group (SJS group 60%, healthy 10%, p-value=0.001). In SJS group, we found significantly higher severity score in culture-positive group comparing with culture-negative group (p-value=0.016). In addition, NGS has demonstrated greater diversity of microbial species and higher proportion of pathogenic microorganisms including *Pseudomonas* spp., *Staphylococcus* spp., *Streptococcus* spp., *Acinetobacter* spp. in SJS group. The obvious difference between 2 bacterial communities was displayed by principle coordinate analysis (PCoA).

Conclusions: Ocular surface in SJS is occupied by more diversed microorganisms with increased proportion of opportunistic pathogens. These alterations may cause chronic inflammation and opportunistic infections. Because the marked changes of ocular surface in SJS may cause atypical infection and inflammation, the clinicians should carefully consider appropriate treatment for these patients.

Field of Study:	Clinical Sciences	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....
		Co-advisor's Signature .....

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1. Introduction

Background and rationale

Stevens-Johnson Syndrome (SJS) and Toxic epidermal necrolysis (TEN) are conditions that manifest through the exfoliation of epidermis and mucosal tissue. Involvement of total body surface is not more than 10% in SJS while it is more than 30% in TEN. These conditions are usually caused by medications such as sulfonamides, allopurinol, and systemic infections caused by viral infection and mycoplasma.

Incidence of SJS is 1.2-6 out of 1,000,000 persons per year while TEN affects 0.3-1.2 for every 1,000,000 persons per year. Although the incidences of SJS and TEN are low, the mortality rate is so high, that is, 1-5% in SJS and 25-35% [1] in TEN. After exposure to the causative factors, abnormal immune responses are activated.

According to epidermal and mucosal involvement of SJS, ocular structure is commonly affected. Ocular involvement is reported in 25-75% of SJS patients. Ocular symptoms can be categorized between acute and chronic stage. In the acute stage, patients may have conjunctivitis, corneal abrasion, and the like. In the chronic stage, patients may have cicatricial changes which can be seen in 35% of SJS patients. Aside from damage to the anatomical structure, the chronic ocular SJS can also cause abnormal epithelization and vascularization on ocular surface area. These changes affect the immune response with an impact on ocular surface flora. [2, 3]

There are evidences that showed differences in ocular microbiomes between SJS patients compared to healthy subjects. For subjects with healthy eyes, the most common microorganisms that can be found are coagulase-negative staphylococci, *Corynebacterium*, and *Propionibacterium*.[4, 5] On the other hand, SJS patients have an apparent increase in diversity of microorganisms. Luciana, et al [2] found the different compositions of microorganisms which consisted of 55% of Gram-positive cocci, 19% of

Gram-positive bacilli, 25% of Gram-negative bacilli. The incidence of Gram-negative microorganisms is increased in SJS patients. Moreover, the antimicrobial susceptibility in SJS patients shows more tendency of resistance.

In this study, Gram-negative bacilli showed 33% of response in chloramphenicol and *Streptococcus* showed 50% of response in ciprofloxacin. As the intraocular infection is usually related with the colonization of microorganisms in that area, ocular microbiome seems to affect the ocular infection especially post-operative infection. There is high incidence of endophthalmitis following keratoprosthesis implantation in the SJS and OCP patients. [6] Similar findings were found in the amniotic membrane with the living related corneal/limbal conjunctival allograft. Gomes, et al[7] found a high rate of post-operative infection (40%) in SJS with limbal stem cell deficiency (LSCD). Apart from the higher rate of post-operative infection in SJS/TEN patients, the incidences of high virulence microorganisms were also seen. Sotozono,et al [8] found that there are significant association between SJS patients and MRSA-MRSE keratitis.

In conclusion, alteration of ocular immune system and ocular surface microorganisms which are usually found in SJS/TEN patients can cause more severe secondary infection as compared to healthy individuals.

Analyzing methods for detecting microorganisms presents to be another consideration. To identify the definite microorganisms, several methods have been introduced, especially molecular biological methods. Conventional culture methods usually demonstrate a lower number of microorganisms due to the limitation of detecting slow-growing microorganisms [4]. In contrast, molecular biological methods have shown higher rates of microbial detection [9, 10]. The advantage of these methods is the ability to detect slow-growing microorganisms. Two methods commonly used are 16s rRNA sequencing and a newer technique called Next-generation sequencing method (NGS).

NGS method, as compared to the other, has the advantage of large-amount database detection with reduced analytic time[11].

However, there are still some gaps of knowledge about the difference of ocular microbiome between SJS patients and healthy subjects. In addition, the information about ocular microbiome in Thai population is still scarced and inconclusive. Moreover, the changes of antimicrobial susceptibility in SJS patients is another important issue that is still indeterminate. In this light, we aim to conduct the study in Thai subjects to compare the differences of ocular microbiome between different hosts (SJS and healthy) by using NGS methods to analyze the data. Along with ocular microbiome analysis, we aim to study the differences of antimicrobial susceptibility between SJS patients and healthy subjects. For clinical application, this study may provide better understanding of the microbial community, symbiosis and dysbiosis on ocular surface. It may also help us consider other options for better practice, particularly for antimicrobial judgement, that will be beneficial for the prophylaxis treatment of SJS patients.

Objectives

Primary objective

To compare the ocular microbiome between chronic SJS patients and healthy subjects by using next-generation sequencing analysis (NGS)

Secondary objective

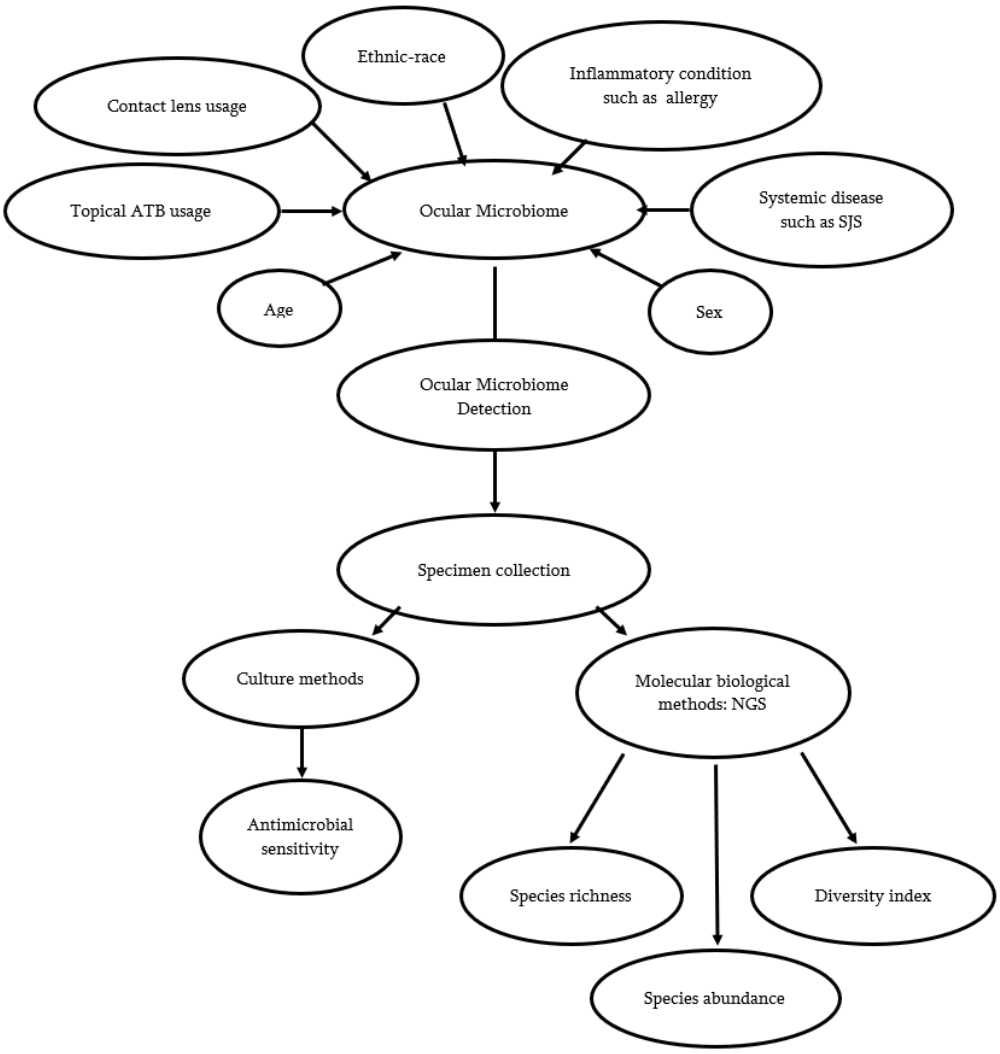
To detect the microorganisms from conventional culture method

To study the difference of antimicrobial susceptibility between SJS patients and healthy subjects

Hypothesis

Ocular microbiome in SJS patients is different from healthy subjects.

Figure 1 Conceptual framework



Expectable benefits of the study

- Better knowledge about ocular microbiome in both SJS/TEN patients and subjects
- Better ophthalmic care for the SJS/TEN patients who are encountered with risks of intraocular infection and severe ocular infection

Limitations

Possibility of inadequate number of participants.

2. Review of literature

- Venugopal, et al conducted the study in 176 eyes of SJS patients and 124 of normal subjects. They got the specimens from inferior conjunctival fornices and sent for conventional cultures. The high incidences of microbial detection were coagulase-negative staphylococci, *Corynebacterium* and *Staphylococcus aureus*. There were increased tendencies of positive-cultured specimen and antimicrobial resistance in SJS group. [12]
- Frizon, et al collected the specimens from 41 eyes of SJS patients and found high rate of positive-cultured specimens (39 eyes). After analyzing the data, 54% of subjects showed diversed conjunctival flora. [2]
- Ormerod, et al collected the data of microbial keratitis in mucosal scarring disorder patients. They found high incidence of staphylococci and *Pseudomonas aeruginosa* while group B beta-hemolytic streptoccocci was also found in lesser proportion.The interesting information is that group B beta-hemolytic streptoccocci is rarely found in normal subjects, this finding may be caused from abnormal immunity on ocular surface area.[13]
- Sotozono, et al studied about MRSA-MRSE keratitis and found associated factors in these patients. These factors were antibiotics usage, chronic steroid usage, hospitalized patients, corneal-transplanted patients and SJS patients. After analyzing the data, SJS was found to be strong factors for MRSA-MRSE keratitis. [8]
- Jain, et al reviewed the data of causes, signs and symptoms, management and treatment outcome of SJS patients. [1]
- Kang, et al showed the incidence of keratitis in limbal stem cell deficiency. 35% of patients were diagnosed as LSCD from SJS. The onset of infection was at mean time of 1.6 years after diagnosed as LSCD. 80% of microorganisms was

Gram-positive group which respond to vancomycin. The important issue from this study is about the follow-up time which should be long enough to monitor the infections [3]

- Nouri, et al collected the incidence of post-operative endophthalmitis in patients who underwent double-plated keratoprosthesis implantation. They found that the infection was correlated with the pre-operative condition especially for SJS and ocular cicatricial pemphigoid(OCP). [6]
- De Aguiar Moeller, et al evaluated the microorganisms detected from conjunctiva of healthy subjects. The most common microorganisms were coagulase-negative staphylococci and *Corynebacterium,* respectively. [4]
- Dong, et al collected specimens from conjunctiva of normal subjects and brought to analyze by conventional culture method and 16S rRNA sequencing technique. In culture methods, most of detected organisms were *Staphylococcus, Propionibacterium* and *Corynebacterium*. Contrast with 16S rRNA sequencing techniques, most of detected microorganisms were *Pseudomonas, Propionibacterium* and *Bradyrhizobium*. This discrepancy of detection may be from the distinct ability to grow in culture media of each microorganism. Conventional culture method has shown limitation to detect slow-growing microorganisms. [5]
- Egushi, et al compared the microbial detection from several methods such as conventional culture methods, 16S rRNA sequencing and next-generation sequencing methods. They included patients with varied ocular-periocular infection such as dacryocystitis, contact lens induced keratitis, etc. They found that tendency to find more microorganisms were shown by molecular biological methods. For NGS method, it's the newer method that provide large amount of data and take shorter time for operation and analysis. [10]

- Huang, et al studied about core microbiome of ocular surface in normal subjects by using NGS method. They found the composition of core microbiome which was *Corynebacterium, Pseudomonas, Staphylococcus, Acinetobacter, Streptococcus, Millisia, Anaerococcus, Finegoldia, Simonsiella, Veillonella*. [11]
- Wilcox, et al compared microbial detection between conventional culture methods and 16S rRNA sequencing analysis. They found that 16S rRNA sequencing analysis provide more data of microorganisms than culture method.[14]
- Kasai, et al analyzed about gut microbiome by using NGS analysis. The NGS analysis had shown the various information in term of species richness, species abundance. They found that this finding can give comprehensive information of local microbial community. [11]
- Wen, et al conducted the study in 90 healthy subjects who were analyzed about the ocular microbiome through metagenomic shotgun sequencing. The results showed the influences of age and sex that affect types and diversity of ocular microbiome. [15]
- Shin, et al studied the association of contact lens wearing and changes of ocular microbiota. They compared the ocular microbiota between lens wearers and non-lens wearers and found higher abundance of microorganisms in lens wearer group. Moreover in non-lens wearers, gender seemed to effect the ocular microbiota. [16]
- Gomes, et al conducted the study in 10 eyes of 10 patients with limbal stem cell and conjunctival deficiency secondary to Stevens-Johnson syndrome underwent excision of cicatricial tissue followed by amniotic membrane and living related corneal limbal/ conjunctival transplantation and found high rate of post-operative

infection(40%) which was suspected to be from instability of ocular surface and chronic inflammatory processes. [7]

- Gupta, et al found the difference of microbiome between different ethnicity in several human area such as skin, oral, vaginal area, etc. They claimed that changes in human diet, lifestyle and traditions brought about by the Neolithic revolution have been reflected in the microbiota.[17]

3. Material and methods

Research design

Prospective, age and sex matched analytical study

Research methodology

Subjects

SJS groups

Inclusion criteria

Patients who had been diagnosed as SJS for more than 4 weeks after the onset. All paticipants aged 18 years old or more and able to come for check-up at OPD of Ophthalmology, King Chulalongkorn Memorial Hospital. Chronic SJS patients were classified according to severity score based on chronic ocular complications which was previously reported by Sotozono, et al[8] .

Exclusion criteria

Patients with history of

- received topical antibiotics within the past 4 weeks.
- had acute ocular or periocular infection within the past 4 weeks.
- underwent ocular or periocular surgery within the past 3 months.
- are using contact lens, or had punctal plug performed previously

Healthy group

Inclusion criteria

All participants were the persons who visited at OPD of department of Ophthalmology, King Chulalongkorn Memorial Hospital for check-up with aged 18 years old or more.

Exclusion criteria

Participants with history of

- chronic ocular allergy, inflammation and any other ocular surface disease
- received topical antibiotics within the past 4 weeks.
- had acute ocular or periocular infection within the past 4 weeks.
- underwent ocular or periocular surgery within the past 3 months.
- are using contact lens, or had punctal plug performed previously

Remarks: The age and sex matching between 2 groups will be done. In terms of age matching, the subjects from each groups will be matched within 10-years range.

Sample size determination

Due to the limitation of the resources and small numbers of chronic SJS patients, the researchers performed the pilot study by including 20 persons per group. We compared the SJS and the healthy subjects in ratios of 1:1.

Recruitment processes

1. In SJS group, we retrieved SJS patients from database. In Healthy subjects, we included the subjects after inclusion of SJS group is completed. According to matching process, the researchers had selected the subjects who had the range of age that match with SJS group. Then the subjects would be asked for participation at the time they visit at OPD.

   All participants had received information about the study and decided whether they want to participate in the study or not. After deciding to participate, the informed consent form would be accomplished with signature.
2. Participants were interviewed for their clinical history to detect any underlying diseases.
3. Participants were examined by slit lamp biomicroscopy.

4. All information was recorded in clinical record forms (CRF). Each patients with chronic SJS were assigned to severity grading score using the previously reported severity score of chronic SJS[8].
5. In the SJS group, the specimens were collected from the eye which had worse clinical symptoms.

   In the healthy group, the specimens were collected from either right or left eye.
6. Before sampling the specimen, topical anesthetic agent (0.5% Tetracaine hydrochloride) was applied on the ocular surface of all participants. After waiting for a 3-5 minutes, the specimens were collected from inferior fornices of all participants by sterile cotton swab. The swabs were swiped from the medial to the lateral part 5-6 times. The sweeping processes were done without touching the lid margin. The specimen collection was done by T.K. .
7. The specimens were placed in a sterile container filled with transport media. All specimens were sent to laboratory room within 2-3 hours after collecting processes were completed. After that the specimens were separated for analyzing by conventional culture methods and next-generation sequencing method.
8. The specimens which provided for molecular biological analysis were lysed and the DNA was extracted by QIAamp® DNA microbiome kit. The optical density was used to estimate the amount of DNA. After achieving adequate amount of DNA, the specimens were sent to analyze by Illumina sequencing instruments.
9. For the results from NGS method, the raw data was analyzed by bioinformatics methods before sending back to the researchers for interpretation and statistical analysis.
10. For conventional culture method, the specimens were placed on culture plates. They would be observed for any growth every 24 hours for 72 hours. If there was any growth, the colony was further identified and determined for antimicrobial susceptibility test.
11. The researchers analyzed all data by statistical methods.

12. The participants had received appropriate care after the procedures of the study were completed.

Data collection

Data recording was accomplished with the clinical record form (CRF)

1. Outcomes

1.1 Primary outcome

Information of ocular microbiome in SJS patients and healthy subjects

1.2 Secondary outcome

Microbial detection from conventional culture method and antimicrobial susceptibility of microorganisms from SJS patients and healthy subjects

2. Measures/instruments

2.1 Molecular biological methods

Next-generation sequencing analysis was done by Illumina Miseq system which had accruracy rate of 99.99%.The maintainace was done by using buffer to flush out DNA after each cycle of process. The preventive maintainance was routinely done 3 times/year.

2.2 Antimicrobial susceptibility testing

Susceptibility testing was performed by the automated susceptibility (Vitek 2XL) method and disk diffusion method using the subsequent antibiotics: ampicillin, amoxycillin-clavulanate, cefazolin, cefotaxime, imipenem, cefoxitin, ceftriazone, ceftazidime, norfloxacin, amikacin, gentamycin, meropenem, trimetroprim-sulfamethoxazole, erythromycin, tetracycline, ciprofloxacin, moxifloxacin, levofloxacin.

3. limitations

Possibility to have inadequate number of participants

4. Ethical consideration

4.1 Respect for person

The researchers had explained the detail of the study and answered the questions of the participants. The participants independently decided whether they want to participate in the study. In case of participation to the study, the information of the participants would be managed with high confidentiality. All recorded data was done by number in order not to identify the participants.

4.2 Beneficence/Non-maleficence

The information from the study gave more comprehension about ocular microbiome in SJS group. This knowledge would help the clinicians to consider more appropriate treatment for patients especially in cases with uncommon presentation.

4.3 Justice

All participants were treated with the same standard treatment according to their clinical conditions. Inclusion and exclusion criteria were applied equally in all participants.

5. Data analysis and statistics

5.1 Analysis of microorganism's species

5.1.1 Species richness

This data demonstrated the types of species in the specimens

5.1.2 Species abundance

This data demonstrated the proportion of each species in the specimen of each participants.

5.2 Analysis of microbiome diversity

5.2.1 Diversity index

This index had compared the diversity of ocular microbiome and analyzed the statistical difference of 2 groups (SJS and healthy)

Independent variables that may affect ocular microbiome would be analyzed by multivariant analysis using R programme.

5.3 Analysis of antimicrobial susceptibility

5.3.1 The percentage of susceptibility will be reported by descriptive analysis

5.3.2 Analysis of the difference of antimicrobial susceptibility was done by Chi-square test. P-value < 0.05 was defined as a statistical significant difference.

4. Results

Clinical characteristics

A total 40 conjunctival swabs were collected from 20 eyes of 20 chronic SJS patients and 20 eyes of 20 healthy subjects. We included 10 men (25%) and 30 women (75%) with a mean of age 44.5 in SJS group and 44.2 in healthy group. In SJS group, the most common cause was drug-induced. Most common causative drug was penicillin. The duration of illness from the onset of SJS ranging from 3 months to 30 years. In chronic SJS group, the overall median severity score for all patients was 7.

Table 1: Demographic data

	SJS group (n=20)	Healthy group (n=20)
Age (yrs)		
Mean (range)	44.5 (20-77)	44.2 (24-77)
Median (Min, Max)	43 (20, 81)	42 (24, 77)
Male/Female	5/15	5/15

Table 2: Etiology of SJS patients

Etiology of SJS	SJS patients
Anti-epileptic drugs	
Carbamazepine	2
Phenytoin	2
Antibiotics	
Penicillin	6
Cephalosporin	2
Fluoroquinolone	1
Macrolides	1
Ampicillin/Sulbactam	1
Sulfamethoxazole/Trimethoprim	1
Dapsone	1

Others	
NSAIDs	1
Allopurinol	2

Microbiological examination

Among 20 eyes of chronic SJS group, 12 eyes(60%) had positive cultures for bacteria. Predominant microorganisms detected from conventional culture methods were Gram-positive cocci and Gram-positive bacilli. Eleven different kinds of bacteria were identified. Among these, *Corynebacterium* spp. was found in 4 eyes(20%), *Streptococcus pneumoniae* was found in 2 eyes (10%), *Streptococcus agalactiae* was found in 1 eye (5%), *S. aureus* was found in 1 eye(5%)(Table 4).

Multiple organisms were isolated from 2 patients (10%) including *Corynebacterium striatum with Staphylococcus capitis*(1/20) and *Streptococcus milleri with Corynebacterium jeikeium* (1/20). Among 20 eyes of healthy group, 2 eyes(10%) had positive culture. The isolates identified was *Staphylococcus epidermidis*. We did not find multiple isolates in healthy group.

The analysis of microbial detection between 2 groups was performed and showed a significantly higher rate of bacterial positivity in chronic SJS group(60%) compared with healthy group(10%) (p-value= 0.001, confident interval 24.8-75.2)

Among chronic SJS patients, the severity score affected the results of positive culture. We found the median score of 12 in culture-positive group and the median score of 1 in culture-negative group. Statistical analysis showed a significant difference between the 2 groups with a p-value of 0.016.

Table 3: Results of conventional culture method (culture-positive group)

	SJS group (eyes)	Healthy group (eyes)
No growth	8 (40.0%)	18 (90.0%)
Growth*	12 (60.0%)	2 (10.0%)
Gram-positive bacilli	6	0
Bacillus amyloliquefaciens	1	-
Cellulomonas species	1	-
Corynebacterium species	4	-
Gram-positive cocci	7	2
Staphylococcus aureus	1*	-
Staphylococcus capitis	1	-
Staphylococcus epidermidis	1	2
Streptococcus agalactiae	1	-
Streptococcus milleri	1	-
Streptococcus pneumoniae	2	-
Gram-negative bacilli	1	-
Proteus mirabilis	1**	-

* *S. aureus* showed resistance to benzylpenicillin, ciprofloxacin, moxifloxacin, erythromycin, clindamycin and trimethoprim/sulfamethoxazole.

** *P. mirabilis* showed resistance to tetracycline, trimethoprim/sulfamethoxazole.

Antimicrobial susceptibility

In chronic SJS, the highest percentage of antibiotic resistance is shown in *S. aureus* to benzylpenicillin, ciprofloxacin, moxifloxacin, erythromycin, clindamycin and trimethoprim/sulfamethoxazole followed by *P. mirabilis* which showed resistance to tetracycline and trimethoprim/ sulfamethoxazole.

In the healthy group, *S. epidermidis* was detected in 2 patients and showed resistance for benzylpenicillin and oxacillin. These two isolates were considered the microorganisms as methicillin-resistant *S. epidermidis* (MRSE).

Next-generation sequencing analysis

Illumina sequencing of 16S rRNA genes generated quality filtered reads of 2,838,365 from all specimens. A total of 40 specimens were rarefied. Rarefaction curves derived from the observed OTU number which were flattened around 20,000 reads.

Core microbiome

To identify core microbiome by 100% of core OTUs samples matching, we found Pseudoalteromonadaceae , Vibrionaceae, Burkholderiaceae, Enterobateriaceae in SJS patients while Pseudoalteromonadaceae and Vibrionaceae were identified as core microbiome in healthy subjects.

Taxonomic composition of microbial community on ocular surface

We identified the taxonomic composition of ocular surface microbiome by 16S rRNA sequencing at phylum and genus level. In SJS group, 15 bacterial phyla were identified. Predominant phyla consisted of Proteobacteria (34.80%), Firmicutes (23.80%), Bacteroidetes (13.10%), Tenericutes (11.9%), Actinobacteria (9.8%) and etc. The significant difference of relative abundance between 2 groups was shown *Corynebacterium, Acrobacter, Streptococcus, Lactobacillus, Bacillus, Bifidobacterium, Bacteroides, Pseudomonas, Acinetobacter, Staphylococcus, Pseudoalteromonas, Clostridium*, etc. Data was analyzed by Welch t-test. A p-value less than 0.05 was considered as having a statistically significant difference(Figure 2). When comparing with healthy subjects, the relative proportion of *Lactobacillus, Bacteroides, Pseudomonas, Staphylococcus, Streptococcus, Bacillus, Acinetobacter* were higher in SJS group.

Figure 2 : Significant difference of taxa abundance

This figure represents the significant difference of taxonomic level of each genus comparing between SJS patients and healthy subjects. We analyzed the mean proportion of each genus by Welch's t-test. The p-value less than 0.05 was defined as statistically significant difference.

Column A is the genus which has significant higher proportion in SJS group.

Column B is the genus which has significant higher proportion in healthy group.

Figure 2 *Significant difference of taxa abundances*

The alpha-diversity

We used rarefaction curve which is derived from observed OTU number to assess the species richness in each single specimen. The rarefaction curve have approached asymptotes. The higher diversity was demonstrated in SJS patients. (Figure 3). To analyze the species richness by diversity index, Shannon index showed a statistically significantly higher abundance in SJS patients (p-value 0.0063).

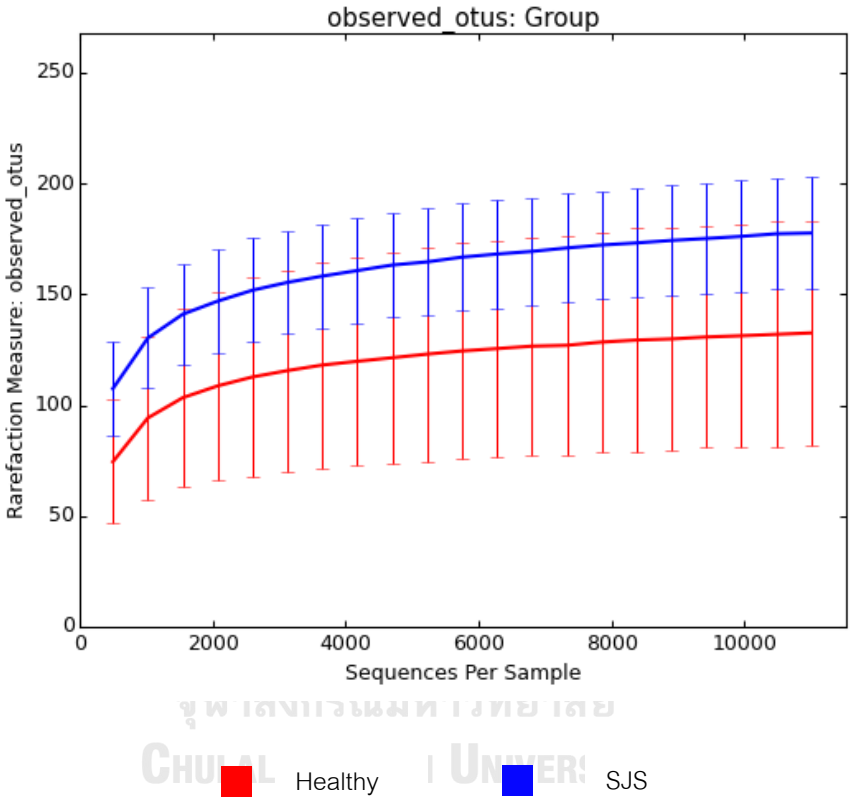


Figure 3 *Rarefaction curves*

This figure demonstrates the number of OTUs which calculated from individual samples. This curve shows the plots of the number of species in SJS and healthy groups. Greater amount of OTUs were observed in SJS. group .

The beta-diversity

To determine differences between the 2 groups, we applied 3D principal coordinate analysis (PCoA) by using weighted UniFrac distances to represent scale of difference between groups. The significant dissimilarity between 2 groups is demonstrated by the definite separation of 2 clusters on PCoA plots.(Figure 4)

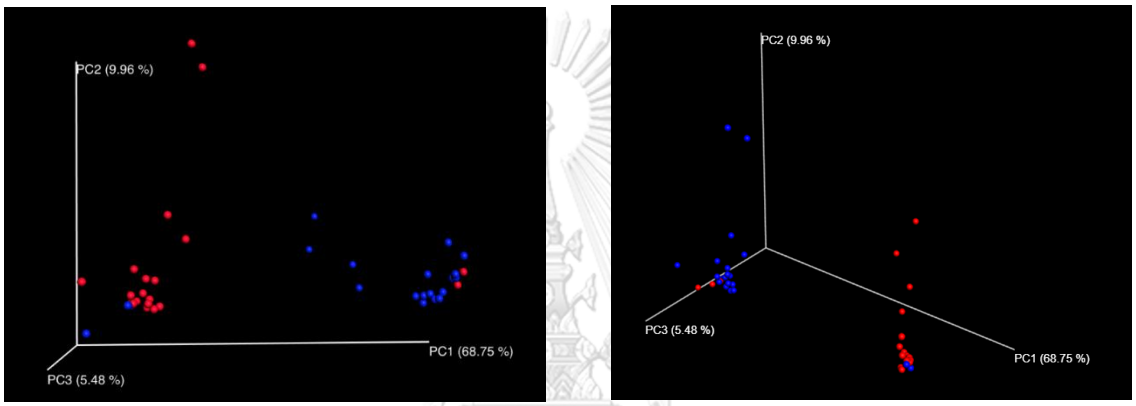


Figure 4 *Principal Coordinate Analysis (PCoA)*

This figure shows beta diversity in conjunctival samples between SJS patients and healthy subjects. Weighted UniFrac distances were used to evaluate diversity between groups. PCoA plots show the clusters of ocular microbiota in SJS (blue) and healthy (red) groups.

These figures show 2 dimensions of PCoA. The displays demonstrate the obvious separation between SJS and healthy groups.

5. Discussion, conclusion and suggestions

Knowledge in the human microbiome has been discovered and reported, using complex analysis of genome technology. The human microbiome is believed to maintain equilibrium of the specific organ which it has colonized. A previous study from Wikoff et al [18] reported the association between microbiota and gut metabolism in mammalian model. On the ocular surface, alterations of microbiome were demonstrated in several conditions such as aging changes [15], geographic location, nutritional status, contact lens wear[16], dry eye syndrome [19], allergy and chronic inflammation [20]. However, most of the studies used conventional culture method while few used the molecular method. At present, there is still limited information about the ocular microbiome which is analyzed by molecular method. To our knowledge, there is no study that compares the ocular microbiome between SJS and healthy groups. Our study aimed to report the ocular microbiome in SJS and healthy by using conventional culture method and NGS analysis.

In this study, the result from conventional culture method showed *Corynebacterium* spp. and *Streptococcus* spp as prominent species in SJS patients which was similar to previous studies. Venugopal, et al[12] from India reported *Corynebacterium* spp. as the prominent species in SJS patients. Frizon, et al [2] also found coagulase-negative staphylococci and *Corynebacterium* spp. as the prominent species in SJS group. We also found a higher incidence of culture-positive specimens in SJS group comparing with healthy subjects (p-value = 0.001) as has been shown in the study from Venugopal, et al. A greater variation of bacterial species was found in SJS group which is consistent with previous studies[12].

Comparing the severity scores in chronic SJS group, significantly higher score was shown in culture-positive SJS groups (p-value=0.016). According to the severity score of chronic ocular SJS proposed by Sotozono, et al [21], they reported the correlation between grading score and visual outcome. In our study, we found the

association between high grading score and higher rate of culture-positive specimens. Another interesting finding is the presence of *S. agalactiae* in one patient who had a high severity score of 26. Since *S. agalactiae* resides as commensal bacteria of gastrointestional and genitourinary tract, the presence of this organism on the ocular surface is considered to be atypical colonization. These results imply that the higher severity score may affect greater changes of ocular surface microbiome with higher possibility to detect atypical bacterial species. We did not find multidrug resistance strains (i.e.methicillin-resistant *S. aureus* or MRSA) which was different from previous studies from India and Japan, which reported high rate of antibiotic resistance in viridans streptococci [12] and significant high incidence of methicillin-resistant *Staphylococcus* infection[8]. The variation of antibiotic resistant patterns in each country may be related to different bacterial distribution, patterns of antimicrobial use and antimicrobial resistance patterns in each country [22].

Analyses of core microbiome of SJS and healthy groups showed Pseudoalteromonadaceae and Vibrionaceae as 100% of core OTUs in both groups. Besides these families, we also found Burkholderiaceae and Enterobacteriaceae as 100% of the core OTUs in SJS group. The difference of core microbiome may be another explanation for severe atypical infection in SJS patients. In addition, the analysis of bacterial species revealed an increased proportion of *Acinetobacter* spp., *Bacteroides* spp., *Corynebacterium* spp., *Pseudomonas* spp., *Staphylococcus* spp., *Streptococcus* spp., etc in chronic SJS patients, whereas the healthy group demonstrated higher proportions of *Acrobacter* spp., *Clostridium* spp., *Fusibacter* spp., etc. Gram-positive bacteria was found to dominate the ocular surface when detected by culture method. In contrast, the 16S rRNA gene sequencing showed higher diversity of bacterial species and revealed that the Gram-negative bacteria (i.e. Proteobacteria) was predominate. The more extensive species in NGS analysis may

indicate the better performance to detect small amounts of microbes, slow-growing and non-cultivable microbial species[23].

Frizon, et al reported the increase of bacterial diversity in SJS group[2] . In this study, several analyses were performed to compare the α-diversity in SJS and healthy group. We created the rarefaction curve and found a higher proportion of observed OTUs in SJS patients comparing with healthy subjects. In addition, the Shannon index showed the significant higher diversity in SJS patients compared to healthy group (p-value 0.006). These analyses indicated higher species abundance in SJS group. To compare the differences of microbial community, we performed the PCoA. This analysis showed well clustered data within the groups of SJS and healthy. The assessment by Unifrac based distance demonstrated an obvious difference in microbial community between SJS and healthy groups.

The markedly changes of ocular surface microbiome in chronic SJS patients may be an important causes of chronic-recurrent ocular surface inflammation[24]. Moreover, severe ocular infections which usually presented in SJS patients, may be the result of the microbiome changes. In order to prevent and control infection in SJS patients, clinicians should strongly consider appropriate antibiotics for these patients.

The limitations of this study are small sample size because some information may not be able to display the significant difference between groups. In addition, according to the differences of bacterial distribution between countries, these results may be mainly applicable to Thai population. To analyze another variables which may affect ocular microbiome (i.e.the duration of SJS), further study in a larger group should be conducted.

In summary, the ocular surface microbiome of SJS and healthy subjects was clearly demonstrated by NGS analysis in this study. We found a higher diversity of microbiome with an increased proportion of some opportunistic pathogens in SJS

group. In addition, significantly higher rates of culture-positive specimens were demonstrated in SJS group. The association between culture-positive specimen and the severe grading score of chronic SJS was also demonstrated. Further studies should be pursued to identify whether or not these alterations may be the underlying cause of persistent inflammation and recurrent infections which lead to worsened visual prognosis in SJS patients.

6. Supplements

Case Record Form

Baseline Informations

Age……..

Sex ☐Male ☐Female

Groups of participants

☐Healthy ☐SJS/TEN

☐Mild ☐Moderate ☐Severe

Causes of disease

☐ Medications

Specified……

☐ Infections

Specified……

☐ Others……

Duration of the disease

……..year(s)…….month(s)…….day(s)

Underlying disease

☐DM ☐HT ☐HLP

☐Allergy ☐Others……

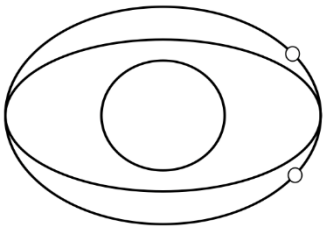
Current medications

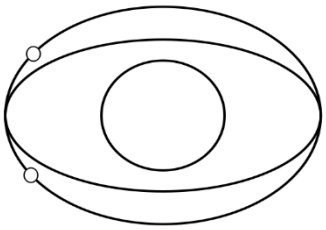
☐Antibiotics

☐Topical ☐Systemic

☐Others……..

Slit lamp examination





	OD	OS
Visual acuity		
Cornea		
Loss of palisades of Vogt	☐ 0 ☐ 1 ☐ 2 ☐ 3	☐ 0 ☐ 1 ☐ 2 ☐ 3
Epithelial defect	☐ 0 ☐ 1 ☐ 2 ☐ 3	☐ 0 ☐ 1 ☐ 2 ☐ 3
Conjunctivalization	☐ 0 ☐ 1 ☐ 2 ☐ 3	☐ 0 ☐ 1 ☐ 2 ☐ 3
Neovascularization	☐ 0 ☐ 1 ☐ 2 ☐ 3	☐ 0 ☐ 1 ☐ 2 ☐ 3
Opacification	☐ 0 ☐ 1 ☐ 2 ☐ 3	☐ 0 ☐ 1 ☐ 2 ☐ 3
Keratinization	☐ 0 ☐ 1 ☐ 2 ☐ 3	☐ 0 ☐ 1 ☐ 2 ☐ 3
SPK	☐ 0 ☐ 1 ☐ 2 ☐ 3	☐ 0 ☐ 1 ☐ 2 ☐ 3
Conjunctiva		
Hyperemia	☐ 0 ☐ 1 ☐ 2 ☐ 3	☐ 0 ☐ 1 ☐ 2 ☐ 3
Symblepharon	☐ 0 ☐ 1 ☐ 2 ☐ 3	☐ 0 ☐ 1 ☐ 2 ☐ 3
Eyelid		
Trichiasis	☐ 0 ☐ 1 ☐ 2 ☐ 3	☐ 0 ☐ 1 ☐ 2 ☐ 3
Mucocutaneous junction	☐ 0 ☐ 1 ☐ 2 ☐ 3	☐ 0 ☐ 1 ☐ 2 ☐ 3
Meibomian gland	☐ 0 ☐ 1 ☐ 2 ☐ 3	☐ 0 ☐ 1 ☐ 2 ☐ 3
Punctum	☐ 0 ☐ 1 ☐ 2 ☐ 3	☐ 0 ☐ 1 ☐ 2 ☐ 3
Total score		
Severity		

Table 2. Ocular surface grading score for evaluating ocular sequelae from SJS/TEN

		0	1	2	3
Corneal	Loss of Palisades of Vogt (POV)	No	Loss < 1/2	Loss > 1/2	Total loss
	Epithelial defect	No	< 1/4 of corneal surface	1/4-1/2 of corneal surface	>1/2 of corneal surface
	Conjunctivalization	No	< 1/4 of corneal surface	1/4-1/2 of corneal surface	>1/2 of corneal surface
	Neovascularization	No	At peripheral cornea	Extend to pupil margin	Extend to central cornea
	Opacification	Clear iris details	Partial obscured iris	Poor iris details, visible pupil margin	Complete obscuration
	Keratinization	No	< 1/4 of corneal surface	1/4-1/2 of corneal surface	>1/2 of corneal suface
	Severity of superficial punctate keratopathy (SPK)	A1D1	A1D2 A2D1	A1D3 A2D2 A3D1	A2D3 A3D2 A3D3
Conjunctival	Hyperemia	No	Mild Sectoral engorge	Moderate Diffuse engorge	Severe Significant engorge
	Symblepharon	No	Only conjunctiva	<1/2 corneal surface	>1/2 corneal surface
Eyelid	Trichiasis	No	<1/4 of lid margin	1/4-1/2 of lid margin	>1/2 of lid margin
	Mucocutaneous junction involvement	Normal	Mild irregularity	Moderate irregularity	Severe irregularity
	Meibomian gland involvement	Clear oily fluid expressed	Yellow-white oily fluid	Thick cheesy material	Inability to express fluid
	Punctal involvement	Normal patent puncta	Iathrogenic punctal occlusion	Sup.or inf. Puncta occluded by scar	Sup. and inf. Puncta occluded by scar

A1: stain <1/3 of cornea A2: stain 1/3-2/3 of cornea A3: stain >2/3 of cornea

D1: sparse stained density D2: moderate stained density D3: high stained density

Next-Generation sequencing methods

Types	1	2	3	4	5	6	7	8	9	10
Microorganisms (name)										

Conventional culture methods

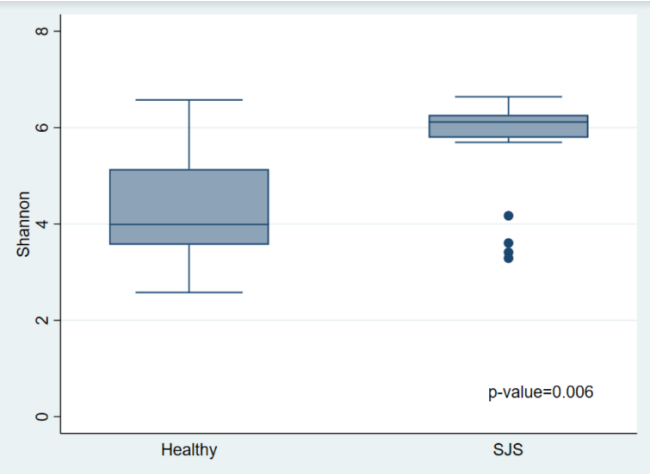
Types	1	2	3	4	5	6	7	8	9	10
Microorganisms (name)										

Antimicrobial susceptibility (report)

……

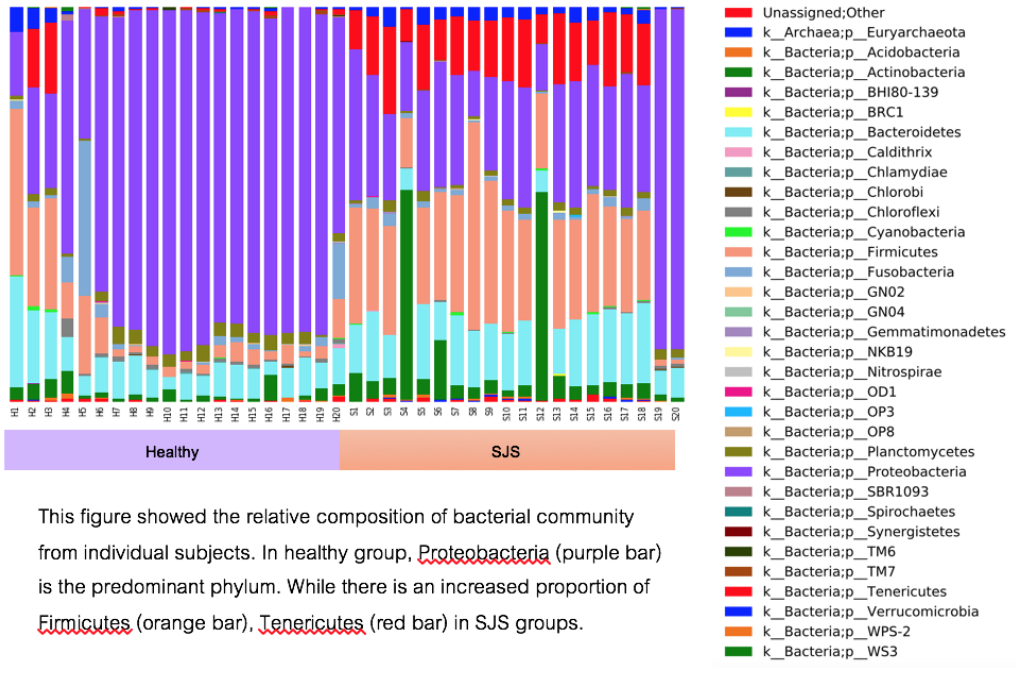
……

Figure 5: Diversity index (Shannon index)



This figure demonstrated a significant difference of species richness between SJS patients and healthy group . Higher diversity was shown in SJS group. Mann-Whitney test was used for analysis. P-value < 0.05 was defined as statistically significant difference.

Figure 6: Species abundance



This figure showed the relative composition of bacterial community from individual subjects. In healthy group, Proteobacteria (purple bar) is the predominant phylum. While there is an increased proportion of Firmicutes (orange bar), Tenericutes (red bar) in SJS groups.

Table 4: Severity grading score in SJS patients

SJS	Severity grading	SJS	Severity grading
S1	0	S11	26
S2	2	S12	9
S3	0	S13	16
S4	16	S14	7
S5	0	S15	1
S6	13	S16	4
S7	0	S17	0
S8	27	S18	7
S9	10	S19	14
S10	19	S20	3

This table provided the information of severity score based on chronic ocular complications of chronic SJS patients in our study [8]. The score was ranging from 0-27.

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