Egyptian Journal of Medical Microbiology Volume 32 / No.4 / October 2023 9-16 Online ISSN: 2537-0979

ORIGINAL ARTICLE

Association between Dysbiosis of Key Species of Nasal Microbiome and Allergic Rhinitis in Adult Patients

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Key words:
Allergic rhinitis; Nasal microbiome; Dysbiosis; Corynebacterium

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ABSTRACT

Background: *Nasal cavity of healthy adults is dominated by nasal microbiome as Corynebacterium on the genus level. The interactions between the local microbiota and the human immune system have a significant impact on the frequency of allergic diseases. Each year, there are more cases of inflammatory nasal mucosal diseases including allergic rhinitis (AR). Many of these illnesses still have an unclear cause. Since nasal microbiota have been found to play important role in regulating immune function, dysbiosis of the nasal microbiota may be the cause of AR.* ***Objective:*** *Investigate association between dysbiosis of nasal microbiome (Corynebacterium genus) and AR in adult patients.* ***Methodology:*** *This case control study included 56 subjects (28 in case group and 28 in control group), all were subjected to skin prick test and nasal swab collection for identification and quantitation of Corynebacterium by cultivation and real time PCR.* ***Results:*** *There was a statistically significant lower Corynebacterium colony count and relative expression (RQ) of 16S rRNA gene in AR patients compared to control group. Corynebacterium RQ of 16S rRNA gene was better in assessment of AR severity (sensitivity of 80%, a specificity of 84.6% and 82% accuracy at cut off ≤0.187 fold change) than Corynebacterium colony count (sensitivity of 73.3%, a specificity of 62.5% and 70% accuracy at a cut off value of ≤19.5×10^3 CFU/ml).* ***Conclusion:*** *Patients with decreased Corynebacterium colony count and RQ of 16S rRNA gene have a higher risk for AR.*

INTRODUCTION

Sneezing, postnasal drip, nasal pruritis, and nasal congestion are all signs of allergic rhinitis (AR), an atopic condition. It affects one in six people and is linked to high morbidity, severe productivity loss, and high healthcare expenses[1]. The prevalence of AR is around 15%; however, the prevalence is thought to be as high as 30% based on individuals who have nasal symptoms. The second to fourth decade of life is the age of known peak for AR occurs, after which it gradually declines. One of the most prevalent chronic pediatric illnesses is AR, which has a significant incidence in the population of children[2]. AR is an IgE-mediated illness that develops in genetically vulnerable people after exposure to environmental allergens[3].

The effect of nasal mucosal barrier in addition to the control of the local and distal immune response are both thought to be influenced by the microbiota; the communities of microorganisms that colonize all the surfaces of the human body that exposed to the external environment. The host's microbiome may have local or remote effects on physiological and pathological processes[4]. Like other mucosal areas of the body, the nasal cavity is home to colonies of commensal bacteria that play a key role in maintaining mucosal homeostasis as well as providing defence against infections[5].

In healthy normosmic volunteers the olfactory area's microbiome, 23 bacterial phyla and four archaeal phyla, including *Actinobacteria, Firmicutes, Proteobacteria, and Bacteroidetes* were discovered. *Corynebacterium, Staphylococcus* and *Dolosigranulum* are the most prevalent genus level signatures. They found that people who were hyposmic had significantly different microbiomes than normosmic subjects in terms of community composition and diversity[6].

Microbial dysbiosis, a shift in the composition of the normal microbiota, causes pathological disorders that are harmful to one's health[7]. The interactions between the host immune system and the local microbiota significantly affect the probability of allergic diseases[8]. A lack of symbiotic microbiota, according to some studies, can increase basophil proliferation, increase the number of infiltrating lymphocytes and eosinophils, stimulate allergic inflammation and Th2 cell reactions, and down regulate the number of regulatory T (Treg) and Th17 cells[9].

Each year, there are more cases of inflammatory nasal mucosal diseases like AR. Despite major improvements in their pathogenic mechanism, the exact etiology of many of these illnesses remains unknown[10]. It has been demonstrated that nasal microbiota are included in the regulation of immune function, which suggests that nasal inflammatory diseases may be triggered by a dysbiosis of the nasal microbiota[11]. This study will investigate the association between dysbiosis of nasal microbiome *(Corynebacterium genus)* and AR in adult patients compared to healthy control.

METHODOLOGY

Patients:

This case control study included 56 subjects (28 in case group and 28 in control group). They were recruited from the Allergy and Immunology Unit, Medical Microbiology & Immunology Department, Faculty of Medicine, Zagazig University, Egypt. A written informed consents were obtained from the study participants. Approval by IRB research committee of Zagazig Faculty of Medicine was obtained (9154-14-12-2021).

Inclusion criteria; included Adult patients more than 18 years with typical nasal symptoms and positive skin prick test. Exclusion criteria; included patient refusal, patients less than 18 years old and Patients who had immunodeficiency disease and negative skin brick test.

Diagnosis of allergy was confirmed by a history of exposure to allergens, family history for allergic diseases and careful clinical examination for typical nasal symptoms by Total Nasal Symptom Score (TNSS) which is a short questionnaire that asses the severity of main AR symptoms. It is the sum of scores for each of nasal congestion, sneezing, nasal itching, and rhinorrhea at each time point, using a four point scale (0–3) [12].

Score	Symptoms
0= None	No symptoms evident
1= Mild	Symptom present but easily tolerated
2= Moderate	Definite awareness of symptom; bothersome but tolerable
3=Severe	Symptom hard to tolerate; interferes with daily activity

TNSS is calculated by addition of the score for each of the symptoms, the total number is out of 12, ranging from 0 (no symptoms) to 12 (maximum symptom intensity) [13].

Skin prick test (SPT):

Diagnosis of allergy was also confirmed by positive skin test. Allergen extracts for skin test: Different Coca's extracted antigens were used from the Allergy and Immunology Unit, Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University; house dust mites, tobacco leaf, wool, cotton, mixed fungi, hay dust, date palm pollen, rye grass. Saline as a negative control and histamine as positive control were used. Interpretation of the tests after 15 – 20 minutes of application, with a positive result defined as a wheal ≥3 mm diameter. Skin prick test was performed on the volar aspect of the forearm[14].

Sample collection

Two nasal samples were taken from each subject by two nasal swabs, one for culture for *Corynebacterium* counting and the other for DNA extraction and real time PCR. For bacterial counting, immediately after collection, the tip of the swab was removed aseptically and transferred to 1.5 ml nutrient broth and was vigorously shaken then 1ml of the sample was 10-fold serially diluted in sterile saline for five dilutions and 100 µl of each dilution was plated by sterile pipetting on the surface of blood agar plates . After incubation at 37°C for 24 hours aerobically, the *Corynebacterium* was identified by gram stainig and biochemical reactions as Catalase, Oxidase, Urease and Motility tests. The colony-forming unit (CFU) was calculated as the number of colonies of *Corynebacterium* on the blood agar taking into account the respective dilution factors[15].

DNA extraction

DNA was extracted from nasal swabs by (QIAamp® DNA Mini Kit – QIAGEN # 51304) according to the manufacturer instructions. The purified DNA was stored at -20 °C until further analysis.

Real-Time PCR

The real-time PCR assay was performed containing the following components per reaction:

10µL 2x QuantiNova SYBR Green PCR Master Mix (**QIAGEN);** 0.1µL QN ROX Reference Dye; 1µL forward primer; 1µL reverse primer; 5µL DNA of each sample and 2.9 µL Nuclease–Free water to total reaction volume of 20µL. The real-time PCR assay was performed in (Applied Biosystem, Quantstudio™ 5) according to the following cycler conditions; 2 min for PCR initial activation step at 95ºC, then 40 cycles of; 5s for denaturation at 95ºC and 15s for combined annealing/ extension at 60ºC. Primers used in PCR reaction; for *Corynebacterium* 16S rRNA gene are forward: (5′-TGGCTCAGATTGAACGCTGGCGGC-3′) and reverse: (5′-TACCTTGTTACGACTTCACCCCA-3′)[16].

Universal primers for the 16S rRNA gene are forward (5′-AGAGTTTGATCMTGGCTCAG-3′) and reverse (5′-CTGCTGCSYCCCGTAG-3′)[17]. These primers were supplied by (Invitrogen, thermo fisher scientific, ANALYSIS, USA).

The universal gene was used as normalizing to calculate the relative gene expression as follow:

1- The control group was considered as calibrator, while other group considered as test group in both target and reference genes.
2- The threshold cycle numbers (Ct) of the target gene were normalized to the Ct of reference (ref) gene, in both the test group and the control group by using the following equations:
   - ΔCt (test) equal difference between Ct (target in test group) and Ct (ref. in test group)
   - ΔCt (calibrator) equal difference between Ct (target in control) and Ct (ref. in control)
3- The ΔCt of the test gene were normalized to the ΔCt of the calibrator and ΔΔCt was calculated as difference between ΔCt (test) and ΔCt (calibrator).
4- Finally, the fold change of relative gene expression (RQ) was calculated by the following equation: Fold change = $(2^{-\Delta\Delta Ct})$ [18].

Statistical analysis

All data were collected, tabulated and statistically analyzed using IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp. Released 2015. Quantitative data were expressed as the mean ± standard deviation (SD), median and range, and qualitative data were expressed as number and percentage. The t-test was used to compare between two group of normally distributed variables. Mann Whitnney U test was used to compare between two group of not normally distributed variables. Percent of categorical variables were compared using Chi-square test or Fisher exact when appropriate. All tests were two sided. p-value < 0.05 was considered statistically significant, p-value ≥ 0.05 was considered statistically insignificant.

RESULTS

This study included 56 subjects 28 among case group and 28 among control group.The mean±SD of ages of patients and control groups were 37.4±11.3 and 34.6±9.8, respectively. Percentages of males and females in patient group were 46.4% and 53.6% , respectively and in control group were 60.7% and 39.3%, respectively. Percentage of rural and urban subjects in patient group were 57.1% and 42.9% while in control group were 50% and 50%, respectively. There was no statistically significant difference between AR patients and control groups in demographic characters regarding age, sex and residence (p= 0.32, 0.28 and 0.59), respectively. TNSS mean ±SD in AR patients was 8.6±2.82 with range of 4-12 while in control group was 0.43±0.504 with range of 0-1, there was statistically significant difference between AR patients and control groups regarding total nasal symptoms score (p=0.0001). Regarding severity of symptoms, in AR patients, 10.7% was mild, 35.7% was moderate and 53.6% was severe. Skin prick test was positive in 28 (100%) of AR patients and negative in all control group. Skin prick wheal diameters ranged between 5-15mm with mean ±SD (10.75 ±3.12) in AR patients. *Corynebacterium* colonies were identified as catalase positive, oxidase negative, urease negative, and non motile. On blood agar were greyish-white, circular and slightly raised colonies with non haemolytic appearance as shown in figure 1a. *Corynebacterium* was identified microscopically as Gram-positive rods, non-spore forming often with clubbed ends, with V palisades or Chinese letters appearance as shown in figure 1b.

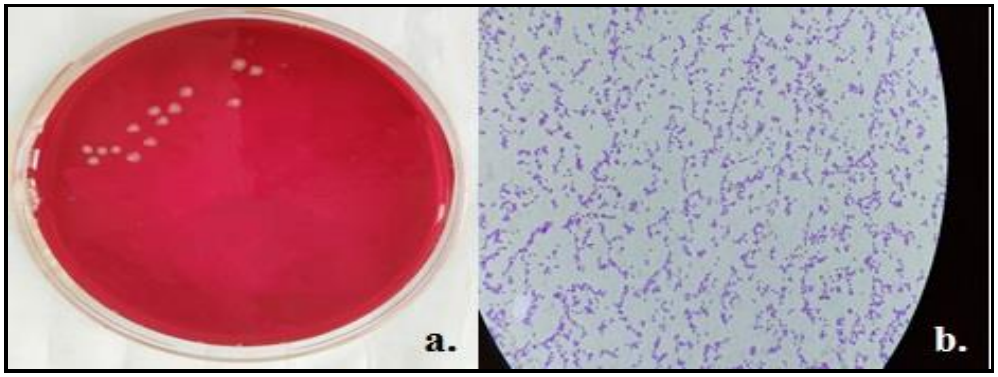

Fig. 1: a. Grayish white colonies of *Corynebacterium* on blood agar. b. Gram positive rods of *Corynebacterium*.

There was a statistically significant lower *Corynebacterium* colony count in AR patients compared to the control group as shown in table 1 and figure 2, and ΔCt value of 16S rRNA gene expression of *Corynebacterium* in AR patients was higher than that of *Corynebacterium* in control group as shown in table 1 and figure 3. Consequently, RQ of 16S rRNA gene of *Corynebacterium* was significantly lower in AR patients than in control group as shown in table 1.

Table 1: Colony count and real-time 16S rRNA gene expression of *Corynebacterium* in AR patients and control groups

	AR patients	Control group	P
***Corynebacterium* colony count ($\times 10^3$ CFU/ml)**			
Mean ±SD	18.57±2.69	32.32±1.33	0.0001*
Median (Range)	18.5 (14-23)	32 (30-35)	
***Corynebacterium* ΔCt 16S rRNA gene**			
Mean ±SD	20.1±1.33	17.54±0.458	0.0001*
Median (Range)	19.98 (18.07-22.33)	17.34 (16.86-18.65)	
***Corynebacterium* RQ 16S rRNA gene**			
Mean ±SD	0.26±0.204	1.05±0.29	0.0001*
Median (Range)	0.19 (0.04- 0.69)	1.15 (0.64-1.6)	

AR: allergic rhinitis CFU/ml : colony forming unit per milliliter, SD: standard deviation,
RQ: relative expression, *p<0.05 significant.

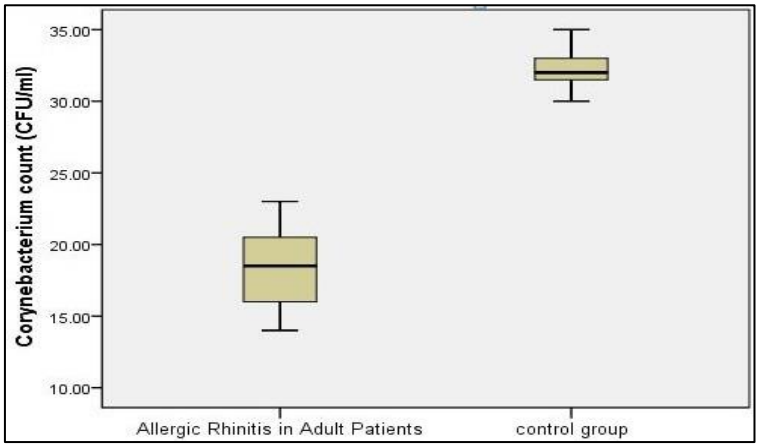

Fig. 2: *Corynebacterium* colony count in AR patients and control groups.

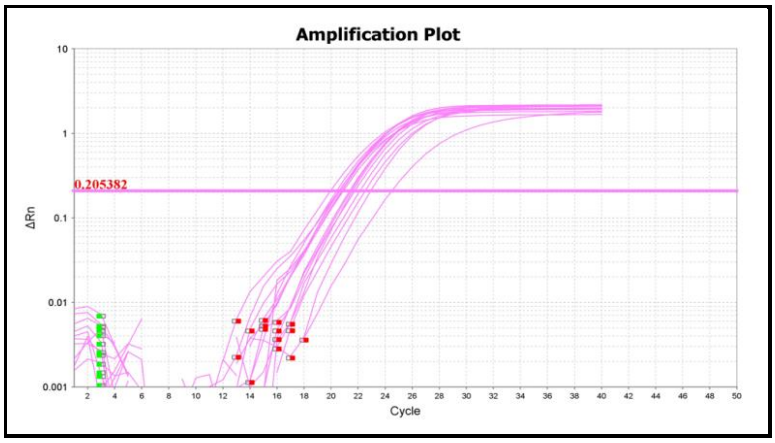

Fig. 3: Amplification plot of *Corynebacterium 16S rRNA* gene.

There was a statistically significant positive correlation between TNSS and SPT wheal diameter in AR patients as shown in table 2 and figure 4. There was statistically significant negative correlation between colony count of *Corynebacterium* in AR patients and their SPT wheal diameter**.** There was a statistically significant negative correlation between RQ of 16S rRNA gene of *Corynebacterium* in AR patients and their SPT wheal diameter. There was a statistically significant positive correlation between colony count and RQ of 16S rRNA gene of *Corynebacterium* in AR patients as shown in table 2.

Table 2: Correlations between study variables in AR patients

Correlation Variables		r value	Significance
TNSS	SPT wheal diameter	0.788	0.0001**
Corynebacterium colony count	SPT wheal diameter	-0.457	0.015*
Corynebacterium RQ	SPT wheal diameter	-0.789	0.0001**
Corynebacterium colony count	*Corynebacterium* RQ	0.63	0.0001**

TNSS: Total nasal symptom score, SPT: skin prick test, RQ: relative expression, (r) correlation coefficient,
**Correlation is significant at the 0.01 level (2-tailed), * Correlation is significant at the 0.05 level (2-tailed).

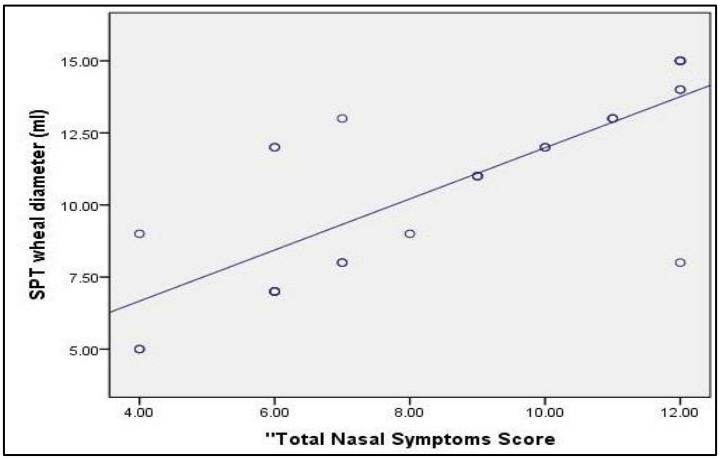

Fig. 4: Scatter dots for TNSS with SPT wheal diameter in AR patients.

Table 3: Performance of C*orynebacterium* colony count / *Corynebacterium* RQ to detect severity of AR.

	Cut off Value	Sensitivity	Specificity	PPV	NPV	Accuracy	AUC (95%CI)	p
***Corynebacterium* colony count ($\times 10^3$ CFU/ml)**	≤19.5	73.3%	62.5%	68.8%	66.7%	70%	0.72 (0.524-0.922)	0.045*
***Corynebacterium* RQ (Fold change)**	≤0.187	80%	84.6%	85.7%	78.6%	82%	0.87 (0.72-1)	0.0001 *

CFU/ml : colony forming unit per milliliter, RQ: Relative expression, PPV: Positive predictive value,
NPV: Negative predictive value, AUC: area under curve, CI: confidence interval,*p=significant.

Corynebacterium colony count was a severity marker of AR and revealed a sensitivity of 73.3%, a specificity of 62.5%.and 70% accuracy at a cut off value of ≤19.5 $\times 10^3$ CFU/ml. This indicated that *Corynebacterium* colony count at calculated cut off were fair in assessment of AR severity. *Corynebacterium* RQ was a severity marker of AR and revealed a sensitivity of 80%, a specificity of 84.6%.and 82% accuracy at a cut off value of ≤0.187 fold change. This indicated that *Corynebacterium* RQ at cut off ≤0.187 fold change was better in assessment of AR severity as shown in table 3.

DISCUSSION

Allergic rhinitis (AR), a systemic airway inflammatory disorder, characterized by sneezing, nasal congestion, itching, and rhinorrhea, is brought on by immunoglobulin E (IgE) mediated responses to inhaled allergens. Mucosal inflammation is a component of these immune responses, and Th2 are responsible for this. Because it frequently causes severe morbidity and economic expanse, AR has a negative effect on quality of life[19]. AR appears to be the outcome of environmental factors working on a genetically predisposed background. AR is commonly associated with conjunctivitis and/or asthma[20].

Many microorganisms, including commensal bacteria that work in symbiotic relationship to preserve the stability of their microenvironment, are found in great numbers in the human nasal mucosa. Nevertheless, reactive nasal inflammatory illnesses like AR can disturb the nasal microbiota (dysbiosis) and have a significant negative impact on health of human by increasing the occurrence of chronic respiratory problems (e.g., asthma, and chronic obstructive pulmonary disease) [21].

The nasal microbiome may play a significant role in the protection of the AR barrier and the immunological regulation of localized responses, despite the fact that there are still few studies examining the association between nasal microbial composition and the initiation and course of allergic inflammation[22,23].

Our results agree with the study which reported that there were no significant differences between AR patients and healthy controls in any of the socio-demographic characteristics as age, sex and residential area[24].

Our study showed that, in AR patients, TNSS ranged between 4-12 with mean ± SD (8.6±2.82), while in control group, TNSS ranged between 0-1 with mean ± SD (0.43±0.504). A statistically significant difference was found between the AR patients and control groups regarding TNSS (p=0.0001), this is consistent with the study that reported that TNSS was significantly higher in patients with AR compared with healthy individuals indicating high discriminant validity, where mean ± SD of TNSS in AR patients was 4.28±2.46 and in healthy individuals was 0.27±0.91(p<0.01)[12]. Also, when another study used TNSS to assess severity of AR , it demonstrated that TNSS mean ± SD was 10.6 ± 2.65[25].

SPT is a standard in vivo screening method for the evaluation of IgE mediated hypersensitivity. The immediate type 1 hypersensitivity diagnosis, targeted immunotherapy and sensitization patterns screening in epidemiological researches are the three most significant applications of SPT[26].

In our study SPT was positive in 28 (100%) of AR patients and negative in all control group. Skin prick wheal diameters ranged between 5-15mm with mean ±SD (10.75 ±3.12) in AR patients. On the other hand, SPT reaction was reported positive in 74 (68.5%) of patients with AR and SPT wheal diameter ranged between 3-11 mm with mean of 6.8, SPT-negative patients can be attributed to non-IgE mediated pathophysiological reasons or low level IgE mediated allergic reactivity (below the SPT reaction threshold)[27].

Our research is in line with findings showing the AR group had a sharp decline in the *Corynebacterium* abundance and it was higher in healthy subjects[28]. It was reported by another study on nasal microbiome that the AR group has relative lower abundance of *Haemophilus* and *Lactobacillus* than that of the control group, that furtherly support our point of view[29].

These findings differ from those of another study, which found that the abundance of *Corynebacterium* in AR patient nasal mucosa is significantly higher than that in the nasal mucosa of healthy people[30]. These differences may due to different geographical distribution and different sample size.

Symbiotic bacteria influence allergic disease susceptibility, and their absence can lead to an increase in basophil proliferation, an increase in the number of infiltrating lymphocytes and eosinophils, a strengthening of Th2 cell reactivity, and allergic inflammation[31]. The *Corynebacterium* genus, found in greater abundance in control group, has been negatively correlated with atopic markers such as IL-6, IL-7 and IL-21 as well as with total eosinophil counts[32].

In our study, there was a statistically significant direct or positive correlation between TNSS and SPT wheal diameter in AR patients. It was consistent with other studies which reported that all five nasal symptoms (sneezing, runny nose, itchy nose, congestion, and postnasal drip) were significantly and positively correlated with the size of the wheals in AR patients sensitized to *Dermatophagoides farina*; house dust mite (HDM)[33]. Also, another study reported that AR participants with larger SPT wheals achieved a higher TNSS[34].

Additional study agree that patients with all four symptoms of AR (coryza, itching, nasal congestion, and sneezing) had significantly more positive SPT compared to those with fewer symptoms (p=0.03)[35].

On the other hand, others found that SPT wheal diameter had a weaker correlation with the severity of AR symptoms. It had been reported that this result might be because the skin is not the primary organ involved in aeroallergen diseases, and nasal provocation testing (NPT) can increase histamine in early phase and eosinophil cationic protein (ECP) in late phase, whereas SPT only reflects the early phase response[36]. Also, very large reactions of SPT are not necessarily associated with more severe disease was reported[37].

Corynebacterium colony count at calculated cut off value of ≤19.5 $\times 10^3$ was fair in assessment of AR severity and *Corynebacterium* RQ at cut off value ≤0.187 fold change was good in assessment of AR severity. So, qPCR is more sensitive than culture in assessment of AR severity. Even though only a small portion of the microbiota can be cultured using microbiological culture up to this point, advances in culture independent methods like qPCR have increased our understanding of the complexity of this microenvironment. Such methods have illustrated the wide variety and composition of the microbiota as well as the relationships between different diseases, dysbacteriosis, and the dysregulation of the microbiota.. These most recent methods for identifying and enumerating uncultivable microorganisms are quick, accurate, and comfortable high throughput[38].

With the emergence of high-throughput sequencing techniques, it has been possible to predict the diversity and abundance of the microbial community in the nasal mucosa and to shed light on the role of the microbiome in health[39]. Some studies have demonstrated findings through the use of such sequencing techniques that points to adults nasal microbiota dysbiosis in relation to allergic airway inflammation[40].

CONCLUSION

Patients with decreased *Corynebacterium* colony count and relative expression of 16S rRNA gene have a higher risk for AR and higher SPT reactions. According to this study, we conclude that; possiple association between nasal microbiome dysbiosis and the pathogenesis of allergic rhinitis and nasal microbiota exert an impact on disease progression and exacerbation.

Conflict of Interest:

The authors declare that they have no financial support. The authors declare no conflicts of interest for research, authorship and publication of this article

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