The microbiome of the maxillary sinus and middle nasal meatus in chronic rhinosinusitis*

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Abstract

Aim: This multicenter study was focused on the identification of the microorganisms inhabiting the maxillary sinus and middle nasal meatus in chronic rhinosinusitis.

Methodology: 112 middle meatus swabs and 112 maxillary sinus aspirates from 103 patients were available for culture.

Results: A total of 244 strains of microorganisms representing more than 50 families were identified in the maxillary sinus and middle nasal meatus (164 and 80, respectively). These included 154 (63.0%) strains of aerobic bacteria from 32 species and 90 (37.0%) strains of anaerobic bacteria from 23 species. Aerobes were more common than anaerobes in both the nasal cavity (78.7% vs. 21.3%) and in the maxillary sinus (55.2% vs. 44.8%). Species of *Streptococci* (28.8%) and *Prevotella* (17.8%) were the most common findings in the maxillary sinus aspirates. *S. pneumonia*, *H. influenza*, and *S. aureus* were relatively rare, and found in only 6.7%, 5.4%, and 8.9% of the samples, respectively.

Conclusions: The results obtained suggest that common upper airway pathogens do not play a major role in the pathogenesis of chronic rhinosinusitis. The microbiome of inflamed sinonasal mucosa is extremely diverse and involves exotic species of bacteria that, to date, have not been considered as potential inhabitants of the paranasal sinuses.

Key words: chronic rhinosinusitis, microbiology, culture dependent techniques, maxillary sinus, middle nasal meatus

Introduction

Chronic rhinosinusitis (CRS) is, in fact, a cluster of heterogeneous disorders that significantly impacts the patients' quality of life and personal health care expenditure. According to the European Position Paper on Rhinosinusitis and Nasal Polyp (EPOS, 2012), the prevalence of CRS is increasing annually. Recent data have demonstrated that CRS affects approximately 5–15% of the population in both Europe and the USA[1-4]. Currently, CRS is diagnosed when specific sinonasal symptoms last for 12 weeks or more. Modern classification divides CRS patients into those who have nasal polyps (CRSwNP) and those who do not (CRSsNP)[4]. Despite major progress that has been made in this field, we still do not have a clear understanding of the pathogenic mechanisms and triggering factors involved in this disease. In recent years, the role of bacteria in the pathogenesis of CRS has been the subject of intense debate, and CRS microbiology has been studied extensively. Bacterial associations found in the paranasal sinuses and nasal cavity in CRS patients contain a large variety of aerobic, facultative anaerobic and anaerobic (both pathogenic and commensal) organisms: *Peptostreptococcus spp., Bacteroides spp., Veillonella spp., Prevotella spp., Fusobacterium spp., Corynebacterium spp., Staphylococcus spp., Streptococcus spp., Haemophilus spp., Helicobacter pylori* and other Gram-positive and Gram-negative bacteria [4-6]. However, recent microbiological

studies have failed to provide a convincing answer to the question of which particular bacterial pathogens, if any, are capable of perpetuating chronic inflammation in the paranasal sinuses. Hypotheses regarding bacterial biofilm formation, intracellular persistence of some specific pathogens, immunological responses to Staphylococcus aureus superantigens or fungi have not accurately explained the existence of so-called "difficult-to-treat" recalcitrant CRS cases that are resistant to antimicrobial therapy [7-11].

Therefore, our prospective multicenter study was designed to investigate the spectrum of aerobic and anaerobic microorganisms inhabiting the middle nasal meatus and maxillary sinuses in CRS patients. The study also investigated resistance of the recovered organisms to various antimicrobial agents.

Materials and methods

The study was conducted during the period from May 2011 to May 2012 at four centers in Moscow (two clinics), St. Petersburg and Smolensk. All microbiological investigations were performed at the same laboratory (Institute of Antimicrobial Chemotherapy at the Smolensk State Medical Academy).

A total of 103 patients of both genders (48 men and 55 women) 16 to 70 years of age with CRSsNP were recruited. Diagnosis of CRS was based on EP3OS guidelines, namely: the presence of two or more symptoms, one of which was either nasal obstruction or nasal discharge (anterior/posterior nasal drip), ±facial pain/pressure, ± reduction or loss of smell lasting for ≥12 weeks [4]. Only cases with opacification of one or both maxillary sinuses on computed tomography (CT) scans performed within the last month were included in the evaluation.

Exclusion criteria were: CRSwNP, antrochoanal polyps, clinical or radiological signs of any kind of fungal maxillary sinus disease, the presence of a foreign body (dental fillings/tooth roots) in the sinus, oro-antral fistula, orbital or intracranial complications from rhinosinusitis, primary or secondary immunodeficiency (AIDS, X-linked agammaglobulinemia, common variable immunodeficiency), hereditary mucociliary clearance disorders (Kartagener and Young syndromes) and cystic fibrosis. Patients receiving systemic or topical antimicrobials within 4 weeks prior to enrollment and those with individual intolerance of topical anesthetics were also excluded. The study protocol was approved by the Interinstitutional Ethics Committee of the Sechenov First Moscow State Medical University.

Sampling

Samples for microbiological examinations were collected as follows: first, cotton pads soaked in a solution of 2% lidocaine mixed with 0.1% epinephrine were applied in the nasal cavity on the side where radiological signs of maxillary sinusitis were more prevalent (or on the side of unilateral disease). Then, a standard thin cotton probe was introduced into the middle nasal meatus with the guidance of a 0° degree endoscope and placed in a test tube containing Amies transport medium with charcoal (Copan, Italy). Care was taken to avoid any contact with the walls of the nasal vestibule when inserting and removing the probe.

Secondly, a needle antral puncture was performed through the inferior nasal meatus approximately 2.5 cm behind the anterior head of the inferior turbinate. Then, a sterile disposable syringe was connected to the needle and 1 cc of the sinus content was aspirated and placed in the test tube with the semi-solid ACT medium (Remel, USA) which insures the preservation and survival of both aerobic and anaerobic microorganisms for 72 hours. If the liquid content of the sinus could not be easily aspirated into the syringe, 2 cc of sterile saline was injected into the sinus and the fluid was aspirated again after 30 seconds.

Culturing

The specimens were inoculated on 5% defibrinated horse blood agar and Columbia chocolate agar (Becton Dickinson, USA) plates for aerobic and facultative anaerobic organisms. The plates were incubated at 37°C in 5% carbon dioxide and examined at 24 and 48 h. For anaerobes, the samples were placed on Wilkins-Chalgren agar (Oxoid, UK) with 5% defibrinated horse blood and Wilkins-Chalgren agar supplemented with G-N Anaerobic Selective Supplement (Oxoid, UK), which contains growth inhibitors of Gram-positive microorganisms (nalidixic acid and vancomycin) and additional anaerobe growth promoters (haemin and menadione). The anaerobic plates were incubated in a BugBox anaerobic chamber (Ruskin Technology, UK) and examined at 48 and 96 h. The microorganisms were identified using time-of-flight mass spectrometry (Microflex Maldi Biotyper 2.0, Bruker Daltonics, Germany).

Susceptibility to antimicrobial agents was determined using the disk diffusion method on Mueller-Hinton medium (BioRAD, France), in accordance with performance standards CLSI 2011, M100-S21 (Clinical and Laboratory Standards Institute, USA). Standard disks with antibiotics (BioRAD, France) were used. Susceptibility of *S. pneumoniae* was determined on Mueller-Hinton medium employing the same method and using disks supplemented with 5% defibrinated sheep blood. Susceptibility of *S. pneumoniae* to penicillin was determined by screening with a 1 mcg disk. The minimum inhibitory concentration of penicillin was checked using gradient test strips with penicillin (E-test) (Table 1) in cases where the identified areas of inhibition were less than the specified value. Susceptibility of *H. influenzae* isolates to ampicillin and amoxicillin was determined using clavulanic acid on a Haemophilus Test Medium Base, with the growth additive Haemophilus Test Medium Supplement (Oxoid, UK) (Table 1). The susceptibility of anaerobic bacteria to antibiotics was not tested because these microorganisms are still very susceptible to basic anti-anaerobic drugs, according to previous publi-

Table 1. Antimicrobials used to determine susceptibility of various pathogens.

I *Staphylococcus aureus* and *Staphylococcus spp.* Resistance to methicillin, assessed on cefoxitin disks	Cefoxitin, 30 mcg Erythromycin, 15 mcg Clindamycin 2 mcg Gentamicin, 10 mcg Ciprofloxacin 5 mcg Co-trimoxazole, 1.25/23.75 mcg
II *Enterobacteriaceae*	Ampicillin, 10 mcg Amoxicillin/clavulanate, 20/10 mcg Cefotaxime, 30 mcg Gentamicin, 10 mcg Ciprofloxacin 5 mcg Trimethoprim/sulfamethoxazole, 1.25/23.75 mcg Meropenem, 10 mcg Imipenem 10 mcg
III *Pseudomonas aeruginosa* and other *non-fermenting Gram-negative bacteria*	Ceftazidime, 30 mcg Gentamicin, 10 mcg Amikacin, 30 mcg Ciprofloxacin, 5 mcg Meropenem, 10 mcg Imipenem 10 mcg
VI *Stenotrophomonas maltophilia*	Trimethoprim/sulfamethoxazole, 1.25/23.75 mcg Levofloxacin, 5 mcg
VII *Haemophilus influenzae* (*H. parainfluenzae*)	Ampicillin, 10 mcg Amoxicillin/clavulanate, 20/10 mcg
VIII *Streptococcus pneumoniae*	Oxacillin, 1 mcg Erythromycin, 15 mcg Clindamycin, 2 mcg Trimethoprim/sulfamethoxazole, 1.25/23.75 mcg

Notes:

1. A 1 mcg oxacillin disk was used to screen for the susceptibility of S.pneumoniae to penicillin.

2. Isolates of S.pneumoniae with an area of oxacillin growth inhibition ≥20 mm are assumed to be susceptible to penicillin (MIC ≤0.06 mcg/ml), amoxicillin, cefuroxime, ceftriaxone, cefotaxime, ertapenem, meropenem and imipenem. The E-test was used to determine the susceptibility to penicillin of pneumococcal isolates with an area of growth inhibition <19 mm.

cations[12,13]. The susceptibility of fastidious microorganisms (*Streptococci* and *HACEK group*) was not investigated for the same reason.

Results

A total of 224 samples (112 aspirates from the maxillary sinuses and 112 swabs from the middle nasal meatus) were obtained from 103 CRS patients (in 9 cases of bilateral maxillary sinusitis,

specimens were taken from both sides). Forty (35.7%) patients showed no bacteria growth in either location (maxillary sinus and middle nasal meatus). 55 (49.1%) of maxillary sinus specimens were negative. Bacteria were isolated from the swabs taken from the middle meatus, but not from the sinus aspirates in 15 (13.4%) cases. In contrast, 18 (16.0%) of the patients showed microbial growth in the maxillary sinus content, but no bacteria were isolated from the middle meatus swabs.

Isolates

A total of 244 isolates representing more than 50 families were recovered, namely 164 isolates from 57 maxillary sinus cases (2.9/case) and 80 from the middle meatus cases (1.1/case). The number of isolates per specimen varied from one to seven. Altogether, 154 (63.0%) strains of aerobic bacteria from 32 species and 90 (37.0%) strains of anaerobic bacteria from 23 species were identified. The greatest diversity was observed among bacteria from the *Streptococcus* family: a total of 12 species were identified. Aerobic bacteria inhabited both the maxillary sinus cavity and the middle nasal meatus; however, the proportion of aerobic and anaerobic microorganisms was different: 55.2% vs. 44.8% in the maxillary sinus and 78.7% vs. 21.3% in the middle meatus, respectively (Figure 1).

Single isolates were identified more frequently in the middle meatus samples (39 or 63.1% of the samples), whereas microbial associations were recovered more often from the maxillary sinus aspirates (38 or 65.5% of the samples) (Figure 2).

Identical bacterial strains were isolated from the maxillary sinus and middle nasal meatus in only 10(8.9%) cases. Among these, a single strain of *S. pneumonia, H. influenzae, S. aureus*, or *M. morganii* was recovered from 7 samples, an association of 2 isolates (*S. aureus* + *Propionibacterium spp*. and *S. pneumonia* + *M. morganii*) from two samples, and 3 different microorganisms (*S. constellatus* + *Prevotella baroniae* + *Fusobacterium nucleatum*) were isolated both in maxillary sinus and middle meatus in one case. Partial correlation, when the same bacteria were recovered from the middle meatus and maxillary sinus, but as a part of various microbial associations, occurred in 21 (18.8%) patients. Among the others, *S. aureus* (5 samples, 4.5%) and *S. constellatus* (5 samples, 4.5%) were present in both sites more often than other organisms. The full list of microorganisms isolated from the maxillary sinus and middle meatus is presented in Table 2.

Susceptibility

Only one microorganism, namely *S. pneumoniae*, which is resistant to several antibiotics (penicillin, macrolides and lincosamides) was identified in this study. A few sporadic strains of *pneumococci* (4 samples) with acquired resistance to macrolides were also found. All the isolates of *H. influenza* were free of penicillinases and were susceptible to the routine administration of aminopenicillins (both with and without beta-lactamase

Figure 1. Anaerobic and aerobic microorganisms in the maxillary sinus and middle nasal meatus cultures.
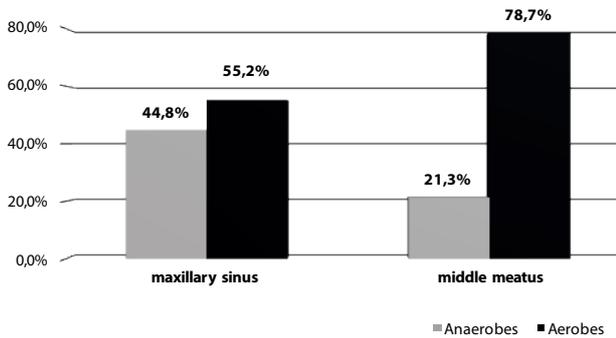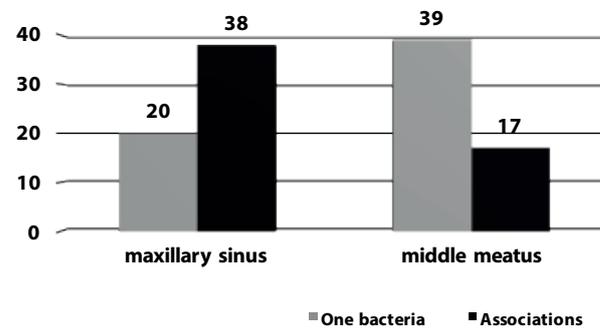

Figure 2. Single isolates and microbial associations in the maxillary sinus and middle nasal meatus cultures (y-axis = number of specimens).

inhibitors), which is typical for Russia. Among the Enterobacteriaceae identified throughout this study, some were resistant to aminopenicillins (including beta-lactamase inhibitors), but this was a natural, not acquired resistance. Among the non-fermenting bacteria (*Pseudomonas spp., Achromobacter spp*. and *Stenotrophomonas spp*.), resistant strains were also identified, but resistance was also natural.

Discussion

The role of microorganisms in the pathogenesis of CRS, undisputed for decades, has been vigorously debated in recent years. There is no good evidence supporting the classic bacterial theory, and CRS is no longer considered to be a consequence and result of recurrent episodes of acute inflammation. The mere fact that in unilateral CRS, the same aerob ic and anaerobic bacteria can be cultured from the content of a diseased sinus and also from a healthy sinus on the opposite side casts doubt on the key role of bacteria in the etiology of the disease [14]. Recent studies have shown that complex polymicrobial communities constantly inhabit both healthy and inflamed paranasal sinus mucosa. It is important to note that the organisms residing in both healthy and diseased paranasal sinuses are often resistant to culture [15].

It has been speculated that CRS develops when the aerobic microorganisms that are constantly present in healthy paranasal sinuses are gradually replaced by anaerobes, such as *Peptostreptococcus spp., Bacteroides spp., Veillonella spp., Prevotella spp., Fusobacterium spp. Corynebacterium spp*., etc., due to decreased oxygen partial pressure and a shift in pH into the acidic range [16,17]. Unlike acute rhinosinusitis, where the "eternal trio" of upper airway pathogens (*S. pneumoniae, H. influenza*, and *M. catharralis*) plays the main role, causative pathogens of CRS have not been defined. Comparative microbiome profiling of a cohort of CRS patients and healthy subjects demonstrated reduced bacterial diversity in the CRS patients, with specific depletion of lactic acid bacteria and a relative increase in *Corynebacterium*

tuberculostearicum [18]. In contrast, a recent study by Boase et al. has shown that the abundance of microorganisms associated with the frequent recovery of anaerobes is significantly greater in CRS patients as compared to controls. This study confirmed that *S. aureus* and *Propionibacterium acnes* were the most common organisms residing in the paranasal sinuses in CRS and healthy controls, respectively [15].

Many previous investigations addressed CRS microbiology using conventional culture-dependent techniques. Samples were usually taken from the maxillary and frontal sinuses and the ethmoidal bulla during or after endoscopic sinus surgery. Under these conditions, the most common pathogens identified were aerobic bacteria – *S. aureus* (~40%) and *coagulase-negative staphylococci* (~30%) [19,20].

Our study largely included CRS patients who were immediate candidates for surgical treatment, i.e., most were free of acute exacerbation. The study protocol allowed for sampling material not only from the middle nasal meatus, but also aspirates from the maxillary sinus. This means that at the time of sampling, the preceding surgical intervention did not influence the typical anaerobic conditions existing in the inflamed sinus. Our findings illustrate that *streptococci* (47 or 28.8% of 164 isolates) and anaerobic organisms of the *Prevotella* genus (29 or 17.8% of 164 isolates) were the most frequent findings in the samples from the maxillary sinuses of CRS patients.

It is well known that *streptococci* routinely reside mucous membranes in the nasal cavity, pharynx, gastrointestinal tract and other hollow organs under healthy conditions. Importantly, it should be noted that a true pathogen,- *S. pneumoniae*, was identified in only 11 (9.8%) maxillary sinus aspirates, i.e., in 6.7% of the total number of bacterial strains, and 23.4% of *streptococci* strains recovered in this study. The other 18 *streptococci* strains (16.0% of maxillary sinus aspirates, and 11.0% of the total number of isolates) belonged to the *Streptococcus mitis* group. These included S.oralis (7 isolates), *S. mitis, S. sanguinis, S. parasanguinis, S. gordonii, S. peroris* (2 isolates each) and *S. infantis*.

Table 2. Microorganisms recovered from the maxillary sinus and middle nasal meatus in CRS patients.

Isolates	Maxillary sinus	Middle nasal meatus	Isolates	Maxillary sinus	Middle nasal meatus
Streptococcus constellatus	14	7	*Stenotrophomas maltophilia*	1	0
Streptococcus spp.	22	7	*Prevotella melaninogen.*	11	3
Enterococcus faecalis	1	0	*Prevotella nigrescens*	4	1
Granulicatella adiacens	4	0	*Prevotella baroniae*	4	1
Rothia muciloginosa	2	1	*Prevotella bucca*	4	1
Streptococcus pneumoniae	11	9	*Prevotella maculoca*	2	0
Haemopilus parainfluenzae	2	0	*Prevotella spp.*	4	0
Haemophilus influenzae	6	4	*Propionibacterium acnes*	4	3
Aggregatibacter aphroph.	1	0	*Finegoldia magna*	2	1
Eikenella corrodens	1	0	*Parvimonas micra*	7	2
Staphylococcus aureus	11	17	*Fusobacterium naviforme*	5	0
Staphylococcus epidermidis	2	32	*Fusobacterium nucleatum*	9	3
Citrobacter spp.	1	1	*Fusobacterium vinsentii*	1	0
Klebsiella pneumoniae	1	2	*Fusobacterium necrof.*	1	0
Enterobacter aerogenes	3	4	*Gemella sanginis*	1	0
Enterobacter cloacae	0	1	*Veillonella dispar*	2	0
Escherichia coli	3	4	*Veillonella parvula*	8	0
Serratia marcescens	0	1	*Slackia exigua*	1	1
Morganella morganii	2	2	*Actinomyces spp.*	2	1
Pseudomonas aeruginosa	3	2	*Bacteroides spp.*	1	0
Achromobacter xylosox.	2	1	**Total number of isolates**	**164**	**80**

Aggregatibacter aphrophilus (formerly *Haemophilus aphrophilus*) and *Eikenella corrodens* compose the *HACEK* group of microorganisms, which acquires its name from the generic names of the Gram-negative aerobic microorganisms present in the microbiome of the oral cavity. They are believed to be able to form biofilms and to cause endocarditis and other inflammatory complications. Furthermore, the growth of *Granulicatella adiacens* was revealed in 4 (3.6%) maxillary sinus aspirates. These organisms, like others identified in this study, have never been reported as CRS pathogens or maxillary sinus inhabitants before. Against a prevailing background of the *Prevotella* microorganisms, other anaerobic bacteria, such as *Peptostreptococcus spp., Bacteroides spp.* and *Corynebacterium spp.* were quite rare.

Representatives of only two genera of anaerobic Gram-positive cocci were identified: *Parvimonas micra* and *Finegoldia magna*. It is remarkable that fungi (*Candida guilliermondii*) were cultured only once from the middle nasal meatus swabs and in no case from the maxillary sinus aspirates, which generally corresponds to some previous reports [19,20].

The role of intracellular pathogens has been discussed from time to time in terms of their potential ability to contribute to the development of acute and chronic rhinosinusitis. There is limited evidence that *Chlamydia* and *Mycoplasma* can be detected in the paranasal sinuses mucosa in approximately 30% of cases [21], but their role in the pathogenesis of CRS remains unclear. Therefore, the present study was not aimed at the identification

of the intracellular pathogens.

Some authors explain the existence of recalcitrant forms of CRS resistant to maximal medical and surgical therapy by intracellular persistence of *S. aureus* in the mucosa of the nasal cavity and paranasal sinuses [9]. However, data on the presence of *S. aureus* in the nasal cavity and paranasal sinuses vary and largely depend on the sampling method and culturing technique. For example, one study examined middle nasal meatus microbiology and recovered *S. aureus* in 36% of the specimens from CRS patients, and in 39% of those from healthy controls [22]. Another study identified *S. aureus* in the swabs from the inferior turbinate in 50% of CRS patients and 33% of healthy individuals[23]. We were able to detect this organism in 17 (13.9%) middle meatus swabs and 11 (9.0%) sinus aspirates. Hence, the unique role of persistent *S. aureus* infection in the pathogenesis of CRS-sNP remains doubtful and is not supported by the results of our study.

Another factor that may potentially contribute to chronification of inflammation in the paranasal sinuses is the emergence of antibiotic-resistant bacteria: It has been reported that 58–83% of the isolates from CRS patients in the USA in 2005-2006 were lactamase-producing bacteria [24,25]. The results of our study suggest that the problem of bacterial resistance in our country is not as relevant as it is in Western Europe and America. Of the 244 strains isolated from the middle nasal meatus and maxillary sinuses in our CRS patients, there was only one with multiple resistance (*S. pneumoniae*), and four strains of *pneumococci* resistant to macrolides. The isolates of *H. influenzae* did not have penicillinases and were susceptible to conventional antibiotics used for the treatment of rhinosinusitis.

The overall results of this multicenter study allow for the conclusion that the common bacterial pathogens of acute rhinosinusitis and *S. aureus* seem unlikely candidates to give rise to the scenario of chronic inflammation of the paranasal sinuses mucosa. The spectrum of microorganisms residing the middle nasal meatus and maxillary sinus is extremely diverse and includes a huge number of both aerobic and anaerobic bacteria. There is an obvious tendency indicating that under conditions of chronic inflammation, anaerobic bacteria present much more frequently in the maxillary sinuses than in the nasal cavity. The other tendency illustrated by the study is that the microbial community inhabiting the maxillary sinus is more diverse than in the middle nasal meatus. Therefore, it is unlikely that any specific microorganism is responsible for the development of chronic inflammation in the paranasal sinuses. Quite the opposite, a more realistic hypothesis would seem to be that chronic inflammation, driven by an immunological response to some unknown stimuli, might create conditions for persistence of organisms in the paranasal sinuses.

More studies are needed to elucidate the role of bacteria in the pathogenesis of CRS, in particular by comparing the density and composition of the microbial community residing in the nasal cavity and paranasal sinuses under conditions of acute exacerbation and at the time of remission.

Conclusion

The spectrum of microorganisms inhabiting the maxillary sinus and middle nasal meatus in CRS patients is extremely diverse. It includes both typical bacterial pathogens capable of inducing acute exacerbation of rhinosinusitis (*S. pneumoniae, H. influenzae* and *S. aureus*) and many other "exotic" species, which have not been previously considered as a possible cause of CRS.

In this study, it was relatively rare for common upper airways pathogens - *S. pneumoniae* and *H. influenzae* – to be cultured in the maxillary sinus aspirates (9.8% and 5.4%, respectively). This is also true for *S. aureus*, which was detected in 9.8% of the maxillary sinus aspirates and 15.2% of the swabs from the middle nasal meatus. It is unlikely that these pathogens would play a major role in the pathogenesis of CRS. Increasingly, a limited number of resistant bacterial pathogens isolated from the maxillary sinus suggests that acquired bacterial resistance cannot be considered to be a significant factor causing chronic inflammation of the paranasal sinuses mucosa, at least in the Russian population of CRS patients.

Authorship contribution

OAI: collected clinical material, wrote initial manuscript; SAK: collected clinical material, reviewed the manuscript; RSK: microbiological work-up, reviewed the manuscript; OIK: microbiological work-up, reviewed the manuscript; IVO: collected clinical material, reviewed the manuscript; ONS: collected clinical material; GZP: collected clinical material, reviewed the manuscript; ASL: constructed the study design, collected clinical material, reviewed and prepared final version of the manuscript.

Conflict of interest

None

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