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Haemophilus influenzae and *Haemophilus haemolyticus* in tonsillar cultures of adults with acute pharyngotonsillitis☆

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Abstract

Objective: The aim of this study was to evaluate the clinical implication of *Haemophilus haemolyticus*, one of the closest relative of *Haemophilus influenzae*, on acute pharyngotonsillitis.
Methods: We applied polymerase chain reaction (PCR) for 16S ribosomal DNA (rDNA) and IgA protease gene (iga) to distinguish *H. haemolyticus* and *H. influenzae*.
Results: Among the 199 *Haemophilus* spp. isolated from 214 patients with acute pharyngotonsillitis, 52 (24.3%) *H. influenzae* strains and 23 (10.7%) *H. haemolyticus* strains were identified by polymerase chain reaction (PCR) for 16S rDNA and IgA protease gene (iga). All *H. haemolyticus* strains showed hemolysis on horse blood agar and there were no other *Haemophilus* spp., nonhemolytic *H. haemolyticus* and *H. influenzae* variant strains that had absent iga gene. *H. hemolyticus* showed close genetic relationship with *H. influenzae* evaluated by pulsed field gel electrophoresis (PFGE). The cases of acute pharyngotonsillitis showing WBC = 7000/mm^3 or CRP = 8 mg/dl were frequently found among cases with *H. influenzae* rather than cases with *H. haemolyticus*.
Conclusion: H. haemolyticus is a pharyngeal commensal that is isolated frequently from adults with acute pharyngotonsillitis.

Keywords: Acute pharyngotonsillitis; *Haemophilus haemolyticus*; *Haemophilus influenzae*; Clonal dissemination

1. Introduction

Haemophilus species constitute about 10% of the bacterial flora in the human upper respiratory tract [1–3]. Among the eight *Haemophilus* species, *Haemophilus influenzae* (*H. influenzae*) resides as a commensal organism in the human pharynx and often causes infectious diseases in humans [4,5]. The strains lacking a capsule that are referred to as nontypeable *H. influenzae* (NTHi) are the predominant types in the pharynx and are usually associated with localized mucosal infectious diseases such as otitis media, sinusitis, bronchitis, and pharyngotonsillitis [6–9]. Encapsulated type b strains cause invasive disease such as meningitis, septicemia and pneumonia [10,11]. In contrast to the *H. influenzae* as a pathogenic organism, the other *Haemophilus* species are rarely associated with disease [1,3].

Haemophilus haemolyticus (*H. haemolyticus*) is phylogenetically closely related to *H. influenzae* and frequently resides as a commensal organism in the pharynx of healthy adults [12]. Both *H. haemolyticus* and *H. influenzae* require X and V factors for growth, cannot ferment sucrose, share similar G + C contents, and show similar colony and cellular morphology [13]. *H. haemolyticus* has been phenotypically differentiated from *H. influenzae* by their clear β-hemolysis on horse blood agar [14,15]. However, recent studies showed that β-hemolyisis might be a poor indicator for distinguishing *H. haemolyticus* from *H. influenzae* [16–18]. The hemolytic activity of *H. haemolyticus* may sometimes be

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lost during several subcultures. Various collections of apparent *H. influenzae* strains that were identified using standard techniques actually contained significant proportions of nonhemolytic *H. haemolyticus* strains [17]. It is important to discriminate *H. haemolyticus* from *H. influenzae* to fully understand the pathogenesis of these bacteria.

In this study, we identify *H. haemolyticus* separated from *H. influenzae* in the pharynx of adult patients with pharyngotonsillitis by polymerase chain reaction (PCR) and studied genetic relationship between *H. haemolyticus* and *H. influenzae* by pulsed field gel electrophoresis (PFGE). We further evaluated the relationship between inflammatory parameters and these bacteria to understand the clinical implications of acute pharyngotonsillitis.

2. Materials and methods

2.1. Populations

Two hundred and fourteen adult patients with acute pharyngotonsillitis between the ages of 15 and 80 years old irrespective of gender were eligible and enrolled in this study. The diagnostic criteria for acute pharyngotonsillitis included sore throat, history of fever, erythema of pharyngotonsillar mucosa and, if any, tonsillar exudates. Exclusion criteria included cases with complications that require antimicrobial treatments, prior antibiotic prescriptions with in a month, pregnancy, and immune deficiency including immunosuppressive medications. Swabs from tonsils and blood for examining the numbers of white blood cells (WBC) and the levels of C-reactive protein (CRP) were collected at the first visit.

2.2. Identification of bacteria

Standard laboratory methods were performed to identify pathogenic bacteria according to the Manual of Clinical Microbiology (8th edition) [3]. Briefly, swabs from tonsillar crypts were cultured on sheep blood agar, chocolate agar and MacConkey agar plates overnight at 37 °C in 5% CO_2. *H. influenzae*, *H. haemolyticus* and nonhemolytic *H. haemolyticus* strains were identified and confirmed in this study by typical colony morphology on chocolate agar, Gram's staining, X and V factor requirement, no reaction in the porphyrin test, and the catalase test. Hemolysis, used to differentiate hemolytic *H. haemolyticus* strains, was assessed as zones of β-hemolysis surrounding individual colonies grown on horse blood agar (Nippon Becton Dickinson Company Ltd., Tokyo, Japan). Antimicrobial susceptibilities were determined by standard microbroth dilution method according to the Clinical and Laboratory Standard Institutes (CLSI) [18].

2.3. Polymerase chain reaction (PCR)

PCR primers were used to amplify 16S ribosomal DNA (rDNA) and IgA protease gene (iga) to distinguish *H. haemolyticus* and *H. influenzae* [16,17]. The nucleotide sequence of the forward primers for the 16S rDNA was 16S-F: 5′-CTCAGATTGAACGCTGGCGGC-3′ and the sequences for the reverse primers were 16S-Nor: 5′-TGACATCCTAAGAAGAGC-3′ for *H. influenzae* and 16S-Pro: 5′-TGACATCCAT/GG/AGG/AAT/CT/CT/CT/A-3′ for *H. haemolyticus*. The nucleotide sequence of the forward primer for the conserved β-core region of IgA protease gene (iga) was BF-1F: 5′-GCAGAATTCAAAG-CACAATTTGTTGCA-3′ and the sequence of reverse primer was BF-1R: 5′-TTATTACGTTAATTCAA-CAGGCTT-3′.

PCR amplification was performed with 50 μl reaction mixtures that comprised of 10 pmol of each relevant oligonucleotide primer, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP), and 1 U of Taq DNA polymerase (Takara Shuzo Co. Ltd., Ohtsu, Japan). The mixture was subjected to 30 amplification cycles, including 1 min at 94 °C template for denaturation, 1 min at 51–55 °C (depending on primer specificity) for primer annealing, and 1 min at 72 °C for chain extension. The PCR amplification was confirmed by gel electrophoresis on 1% agarose.

2.4. Distinguishing H. haemolyticus and H. influenzae isolates

Based on the bacteriological characteristics, the presence of the iga gene and amplifications of 16S rDNA, we defined the criteria to distinguish *H. influenzae* and *H. haemolyticus*. *H. influenzae* isolates were defined as NAD and hemin dependent, nonhemolytic on horse blood agar, and positive for both 16S-Nor and the conserved iga gene. *H. haemolyticus* isolates were defined as NAD and hemin dependent but demonstrated clear hemolysis on horse blood agar and positive for 16S-Pro but lacked the iga gene. Since *H. haemolyticus* isolates sometimes lose their hemolytic activity during subculture, NAD and hemin dependent, nonhemolytic isolates that were positive for 16S-Pro but lacked the iga gene were phylogenetically closely related to *H. haemolyticus* and were designated as nonhemolytic *H. haemolyticus*. The nonhemolytic isolates that were positive for 16S-Nor and lacking the iga gene were designated as *H. influenzae* variant strains.

2.5. Pulsed field gel electrophoresis (PFGE) genotyping

The restriction fragment polymorphisms of *Sma*I-digested chromosomal DNA from *H. influenzae* isolates were evaluated by PFGE analysis. Briefly, a colony of *H. haemolyticus* isolates was grown at 37 °C for 6 h in Brain

Heart Infusion broth (Difco Laboratories, Detroit, MI) with hemin and NAD supplements. The cells were harvested by centrifugation at 4000 × *g* at 4 °C for 5 min, washed with phosphate buffered saline (PBS), and suspended in washing buffer (50 mM Tris–HCl, pH 7.5). An equal volume of 2.0% low-melting-point agarose (FMC BioProducts, Rockland, MA) was added to 50 μl of each cell suspension for plug preparation. The mixture was poured into disposable 100 μl scale plug molds (Bio-Rad, Laboratories, Hercules, CA) and chilled at 4 °C for 20 min. After incubation with 2 ml of lysis buffer (0.25 M EDTA, 1% SDS, 10 mM Tris–HCl, pH 9.5, and 0.5 mg/ml of proteinase K) over night at 50 °C, sample plugs were rinsed with 2 ml of washing buffer three times for 30 min. One-third of each plug was sliced off. The restriction of genomic DNA was carried out after equilibration of the sliced plugs with appropriate restriction buffer, then each slice was incubated with 80 U of *Sma*I (Takara Shuzo, Co. Ltd.) for 20 h at 30 °C. The reaction was stopped by an equal volume of 0.5 M EDTA (pH 8.0). Electrophoresis was performed with a GenePath PFGE apparatus (Bio-Rad Laboratories) in 0.5× TBE buffer (1× TBE buffer: 0.1 M Tris–HCl, 0.1 M boric acid, 2 mM EDTA, and pH 8.0). A 48.5 kb bacteriophage lambda DNA ladder (FMC BioProducts) was used as a molecular size marker. The gels were stained with ethidium bromide for 20 min and were photographed under UV light at 302 nm. Cluster analysis was performed for the PFGE band patterns using Fingerprinting software (Bio-Rad Laboratories). The patterns were confirmed by visual inspection. The PFGE band patterns were designated unique on the basis of ≥7 band differences, in accordance with the criteria of Tenover et al. [19] for determining genotypic differences.

2.6. Statistical analyses

Chi-square test was used to assess statistical differences in the distributions of *H. influenzae* or *H. haemolyticus* among the acute pharyngotonsillitis cases. Statistical analyses were performed with Prism 4 (GraphPad Software, La Jolla, CA). A p value of <0.05 was considered statistically significant. The odds ratio (OR) and 95% confidential interval (CI) were calculated.

3. Results

3.1. Identification of H. haemolyticus and H. influenzae

A total 199 (37.6%) *Haemophilus* spp. was identified from 529 clinical isolates among 214 patients with acute pharyngotonsillitis. *H. haemolyticus* was distinguished from *H. influenzae* among *Haemophilus* spp. clinical isolates by three independent PCR sets. The iga gene was identified in only *H. influenzae*. The two sets of PCR for 16S rDNA distinguished *H. influenzae* from *H. haemolyticus*. The 199 *Haemophilus* spp. were classified into 52 (24.3%) *H. influenzae*, 23 (10.7%) *H. haemolyticus*, 116 (54.2%) *H. parainfluenzae*, 7 (3.3%) *H. parahaemolyticus* and one other *Haemophilus* spp. Two isolates of *H. influenzae* were β-lactamase producing strains. All *H. haemolyticus* strains showed hemolysis on horse blood agar. There were no other *Haemophilus* spp., nonhemolytic *H. haemolyticus* and *H. influenzae* variant strains that absent iga gene.

Table 1
The minimal inhibitory concentrations of 5 antimicrobial agents to *H. influenzae* and *H. haemolyticus* evaluated by microbroth dilution methods.

	Range	MIC_{50}	MIC_{90}
H. influenzae			
LVX	<0.06	<0.06	<0.06
CDR	<0.06–0.25	<0.06	0.125
CFN	<0.06–2	<0.06	0.5
CAM	2–16	8	16
AMX	0.06–>64	0.25	8
AMP	0.06–>64	0.25	4
H. haemolyticus			
LVX	<0.06–2	<0.06	<0.06
CDR	<0.06	<0.06	<0.06
CFN	<0.06	<0.06	<0.06
CAM	2–16	8	16
AMX	<0.06–1	0.125	0.5

Note: LVX: levofloxacin; CDR: cefditoren pivoxil; CFN: cefdinir; CAM: clarithromycin; AMX: amoxicillin; AMP: ampicillin.

3.2. Antimicrobial susceptibilities

The minimal inhibitory concentrations (MICs) of 5 antimicrobial agents to *H. influenzae* and *H. haemolyticus* were evaluated by microbroth dilution methods (Table 1). *H. haemolyticus* strains were susceptible to most of the antimicrobial reagents except CAM compared to those of *H. influenzae*. The MIC_{90} of *H. influenzae* to AMX was 16 times higher than those of *H. haemolyticus*.

3.3. PFGE genotypes of H. haemolyticus and H. influenzae

To assess the genetic relationship between *H. haemolyticus* and *H. influenzae* isolates recovered from pharyngeal swabs obtained from patients with acute pharyngotonsillitis, the PFGE genotypes of genomic DNA were determined for 48 *H. influenzae* and 28 *H. haemolytics* isolates (Fig. 1). Strains isolated from adult acute pharyngotonsillitis were distributed in 5 distinct clusters. *H. influenzae* isolates demonstrated 2 distinct clusters (A1 and B1). *H. haemolyticus* isolates showed one distinct cluster (C). Interestingly, clusters A2 and B2 included strains of both species, demonstrating the similar PFGE patterns between *H. influenzae* and *H. haemolyticus*. Among 26 isolates in cluster A2, 11 were *H. influenzae* and 15 were *H. haemolyticus*. Among 21 isolates in cluster B2, 14 were *H. influenzae* and 7 were *H. haemolyticus*.

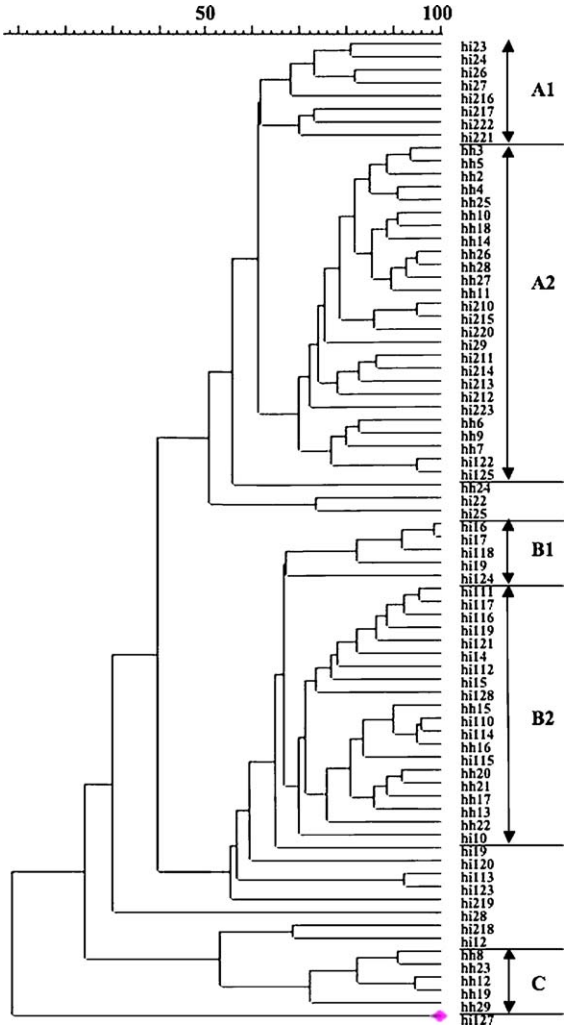

Fig. 1. Dendrogram by PFGE analysis of total geneomic DNA of *H. haemolyticus* and *H. influenzae* with *Sma*I digestion (unweighted pair group methods with arithmetic means). Dice coefficients are shown above the dendrogram. Isolates with ≥80% relatedness on the dendrogram are considered highly genetically related. Totally 5 clusters were found in *H. influenzae* and *H. haemolyticus* strains (A1, A2, B1, B2 and C). *H. influenzae* isolates demonstrate 2 distinct clusters (A1 and B1), while *H. haemolyticus* isolates show one distinct cluster (C). Clusters A2 and B2 included in both species.

3.4. Differences in clinical outcomes of acute pharyngotonsillitis

To evaluate the possible involvement of *H. haemolyticus* in acute pharyngotonsillitis, the numbers of WBC in peripheral blood and the levels of CRP in serum of the patients were compared according to the culture results for *H. influenzae* or *H. haemolyticus*. Depending on the mean − 0.5 S.D. of WBC (7857 mm^3) and mean + 0.5 S.D. of CRP (8.1 mg/dl) among samples from patients without both *H. influenzae* and *H. haemolyticus*, we set 7000 mm^3 for WBC and 8.0 mg/dl for CRP as reference values to analyze clinical importance of *H. influenzae* and *H. haemolyticus*.

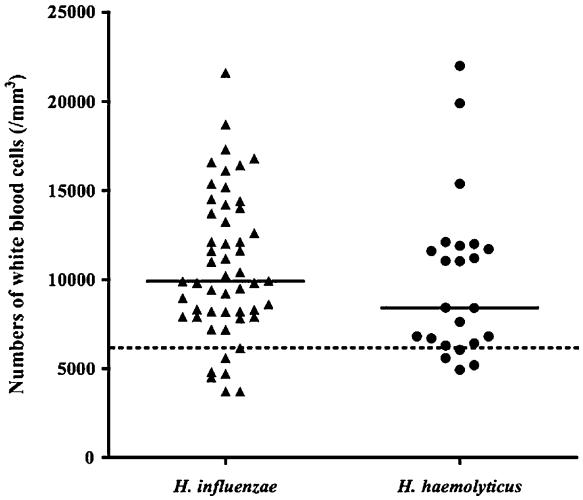

Fig. 2. White blood cell counts in patients with *H. haemolyticus* or *H. influenzae*. Closed triangles depict cases with *H. haemolyticus* and open circle depict the cases with *H. influenzae*. The horizontal lines show the mean WBC counts in patients groups with *H. influenzae* or *H. haemolyticus*. Dot line designates the WBC counts at 7000/mm^3.

The mean ± S.D. numbers of WBC of patients with *H. haemolyticus* and *H. influenzae* were 9961 ± 937.1 and 10,730 ± 561, respectively. There were no significant differences between the mean numbers of WBC in patients with *H. haemolyticus* and *H. influenzae*. However, 14 (60.9%) of 23 patients with *H. haemolyticus* showed WBC ≥ 7000 mm^3 while 45 (86.5%) of 52 patients with *H. influenzae* showed WBC ≥ 7000/mm^3. The ratio of the cases showing WBC ≥ 7000/mm^3 was significantly lower in patients with *H. haemolyticus* than in the patients with *H. influenzae* ($p < 0.05$, OR 0.24, 95% CI 0.08–0.77) (Fig. 2).

The mean ± S.D. levels of CRP of patients with *H. haemolyticus* and *H. influenzae* were 3.57 ± 0.59 and 4.60 ± 0.80, respectively. There was no difference between the mean levels of CRP between patients with *H. haemolyticus* and *H. influenzae*. No patient with *H. haemolyticus* showed CRP ≥ 8.0 mg/dl while 9 (17.3%) of 52 patients with *H. influenzae* showed CRP ≥ 8.0 mg/dl. The ratio of patients showing CRP ≥ 8.0 mg/dl was significantly higher among cases with *H. influenzae* than in cases with *H. haemolyticus* ($p < 0.05$, OR 0.10, 95% CI 0.01–1.75) (Fig. 3).

4. Discussion

H. haemolyticus is frequently identified from the healthy human pharynx and is closely related to *H. influenzae* [16,17,20–22]. However, the clinical implication of *H. haemolyticus* has not been well documented yet. In this study, we evaluate the clinical implication of *H. haemolyticus* in to acute pharyngotonsillitis separate from *H. influenzae*. We first performed three sets of PCR analysis targeting 16S rDNA and IgA protease gene (iga) to

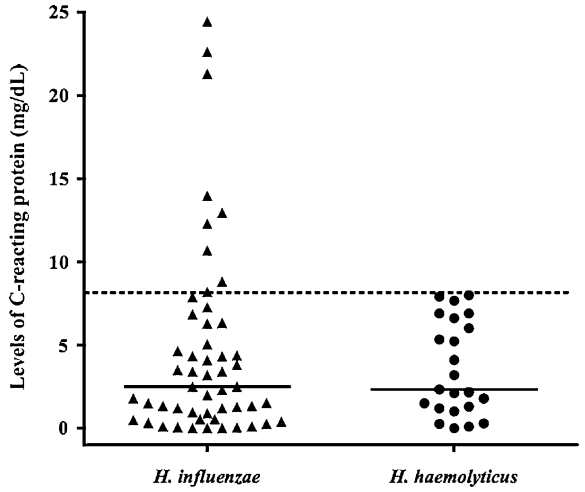

Fig. 3. The CRP levels of patients with *H. haemolyticus* or *H. influenzae*. Closed triangles depict cases with *H. haemolyticus* and open circle depict the cases with *H. influenzae*. The horizontal lines show the mean CRP levels in patients groups with *H. influenzae* or *H. haemolyticus*. Dot line designates the CRP levels at 8.0 mg/dl.

distinguish *H. haemolyticus* from *H. influenzae* in addition to determining β-hemolysis on blood agar plates [16,17,19,20]. The primer sets yield an unequivocal result in approximately 90% of strains and more than 90% of *H. influenzae* and less than 10% of *H. haemolyticus* isolates possess the iga gene [23–27]. Based on these PCR amplifications and determination of β-hemolysis, we accurately identify strains of *H. haemolyticus* from strains of *H. influenzae*. Murphy et al. reported that about 40% of the *H. influenzae* isolates collected from the sputum of patients with chronic obstructive pulmonary disease (COPD) and more than 20% of a collection of *H. influenzae* strains obtained from the throats of healthy children attending day care were *H. haemolyticus* [17,28–30]. In contrast to this surprising misidentification of *H. haemolyticus*, there was no misidentification, no nonhemolytic *H. haemolyticus* and no *H. influenzae* variant strains in the present study. We speculate that the interesting absence of nonhemolytic strains of *H. haemolyticus* among the 28 strains of well documented *H. haemolyticus* in the present study is due to the differences in *H. haemolyticus* isolated in Japan compared to those isolated in North America.

The PFGE patterns for these two species were not always distinct. In two clusters, *H. haemolyticus* showed similar PFGE patterns to those of *H. influenzae*. Therefore, if a laboratory performs PFGE on *H. influenzae* isolates and some are actually *H. haemophilus*, the PFGE may still imply some relatedness and therefore confuse the epidemiological study for which the PFGE was performed. *Haemophilus* species are naturally transformable organisms and competent for uptake of DNA [31–33]. Genetic recombination occurs among the species that make up the complex flora of the human pharynx [34–37]. *H. haemolyticus* and *H. influenzae* may potentially take up DNA including antibiotic resistance markers through natural transformation. A disturbing increase in β-lactamase nonproducing ampicillin resistant *H. influnezae* (BLNAR) is occurring in Europe and Japan [38–41]. In contrast to this alarming increasing in BLNAR, the antimicrobial susceptibilities of *H. haemolyticus* still remains unclear. The presence of *H. haemolyticus* on the respiratory mucosa may serve as a source of genetic material for horizontal gene transfer to *H. influenzae* and enhance the pool of genes available to *H. influenzae*. *H. haemolyticus* has important implications with regard to the problem of antibiotic resistance. All *H. haemolyticus* strains in this study are currently highly susceptible to most of the cephems but showed relatively reduced susceptibilities to AMC and resistance to CAM. It is important to monitor antibiotic resistance patterns and to investigate other *Haemophilus* species as a potential reservoir in the human respiratory tract for antibiotic resistance determinants in *H. influenzae*.

In the last approach to evaluating the clinical implications of *H. haemolyticus*, the mean values of WBC and CRP were not different between cases with *H. haemolyticus* or *H. influenzae*. However, cases showing WBC $\geq$ 7000/mm^3 or CRP $\geq$ 8 mg/dl were frequently found among cases with *H. influenzae* compared to cases with *H. haemolyticus*. Murphy et al. reported that the acquisition of a new strain of *H. influenzae* is associated with exacerbations of COPD while acquisition of a new strain of *H. haemolyticus* was not associated with the onset of clinical symptoms of exacerbations [29,30]. Although most acute pharyngotonsillitis has been thought to be caused by viruses and appears to be self-limiting, is also often identified from tonsillar swabs [42–48]. The differences in WBC counts and serum CRP levels between selected patients with only either *H. influenzae* or *H. haemolyticus* raise the intriguing possibility that *H. influenzae* may cause acute pharyngeal tonsillitis in a subset of patients. On the other hand, the absence of signs of systemic inflammation in patients with *H. haemolyticus* suggests that *H. haemolyticus* is not a cause of acute pharyngotonsillitis but, rather, is a commensal in the pharynx.

In conclusion, accurate identification of bacterial pathogens in crypt swab samples is central to creating rational guidelines for the management of acute pharyngotonsillitis.

Conflict of interest

The authors have no conflicts of interest.

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