DIFFICULT IDENTIFICATION OF *HAEMOPHILUS INFLUENZAE*, A TYPICAL CAUSE OF UPPER RESPIRATORY TRACT INFECTIONS, IN THE MICROBIOLOGICAL DIAGNOSTIC ROUTINE

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Haemophilus influenzae is a key pathogen of upper respiratory tract infections. Its reliable discrimination from nonpathogenic *Haemophilus* spp. is necessary because merely colonizing bacteria are frequent at primarily unsterile sites. Due to close phylogenetic relationship, it is not easy to discriminate *H. influenzae* from the colonizer *Haemophilus haemolyticus*. The frequency of *H. haemolyticus* isolations depends on factors like sampling site, patient condition, and geographic region.

Biochemical discrimination has been shown to be nonreliable. Multiplex PCR including marker genes like *sodC*, *fucK*, and *hpd* or sequencing of the 16S rRNA gene, the P6 gene, or multilocus-sequence-typing is more promising. For the diagnostic routine, such techniques are too expensive and laborious. If available, matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry is a routine-compatible option and should be used in the first line. However, the used database should contain well-defined reference spectra, and the spectral difference between *H. influenzae* and *H. haemolyticus* is small. Fluorescence *in-situ* hybridization is an option for less well-equipped laboratories, but the available protocol will not lead to conclusive results in all instances. It can be used as a second line approach. Occasional ambiguous results have to be resolved by alternative molecular methods like 16S rRNA gene sequencing.

Keywords: routine diagnostics, upper respiratory tract infection, *Haemophilus influenzae*, *Haemophilus haemolyticus*, MALDI-TOF-MS, PCR

Introduction

As primarily nonsterile sites like the upper respiratory tract are physiologically colonized by bacterial flora of varying composition, it is difficult to discriminate relevant pathogens from harmless colonizers.

Haemophilus influenzae is a common cause of upper-respiratory tract infections (URTI) [1, 2], including infections of the middle ear [3]. As shown for patients with cystic fibrosis, some persistent *H. influenzae* clones show frequent recurrence despite antimicrobial therapy [4]. Accordingly, chronic inflammation may consequently result.

Although *H. influenzae* is frequently isolated and of great importance for the microbiological routine laboratory, its discrimination from the phylogenetically closely related, predominantly harmless colonizer *Haemophilus haemolyticus* often fails [5, 6]. This review highlights potential ways how this diagnostically important discrimination can be reliably achieved.

H. influenzae vs. *H. haemolyticus* – why is a discrimination needful?

The Gram-negative rod-shaped *H. influenzae* is one of the quantitatively most important bacterial causes of URTI [1, 2], being frequently isolated from adult patients with recurrent tonsillitis and retropharyngeal abscesses [7–9]. *H. influenzae* frequently occurs in coinfections with other pathogens like *Staphylococcus aureus* [7, 10], or – associated in biofilms – with *Streptococcus pneumoniae* [11]. Next to *H. influenzae*-caused URTI, asymptomatic colonization of the upper respiratory tract by this species is frequently observed [12, 13] with colonization rates of 30% in healthy volunteers [12]. *H. influenzae*-colonization frequency is particularly high in children and decreases with increasing age [13].

H. parainfluenzae, *H. haemolyticus*, and *H. parahaemolyticus* [14] are other *Haemophilus* spp. being occa-

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sionally isolated from the upper respiratory tract. They are phylogenetically related with *H. influenzae* but without relevant pathogenic potential. *Haemophilus* spp.-colonization of the upper respiratory tract is frequent, accounting for as much as 10% of the culturable bacterial flora [13]. The saprophytic colonizer *H. parainfluenzae* is no typical cause of local infections of the upper respiratory tract and has been described as a potential cause of infectious endocarditis in few incidences [15]. Most available studies deny a relevant clinical importance of *H. haemolyticus* [5, 16, 17], although recent investigations suggest etiological relevance at least at some occasions [18].

In normally sterile sites, colonizers like *H. haemolyticus* are virtually absent [5, 19]. If URTI is suspected, however, sampled materials usually comprise nonsterile fluids or swabs from colonized mucous membranes. Therefore, a reliable discrimination of potentially pathogenic *H. influenzae* from other *Haemophilus* spp. is essential due to the species-depending etiological relevance.

The biochemical approach

Traditional microbiological laboratories basically use biochemical approaches for the identification of bacterial isolates on species level. Growth factor-based methods make use of the fact that *H. influenzae* lacks the enzymatic capacity to convert δ -aminolevulinic acid (ALA) to protoporphyrin, hence, depends on factor X (heme) for growth [20, 21]. For the identification of *H. influenzae*, in particular, X- and V-factor (nicotinic acid diamine, NAD) dependence is usually assessed [20, 22] but does not allow for the discrimination of *H. haemolyticus* from *H. influenzae*.

A majority of *H. haemolyticus* strains causes α -hemolysis on horse, cow, or rabbit blood agar [22–24], which *H. influenzae* cannot do. Unfortunately, *H. haemolyticus* does not show this phenomenon on sheep blood agar, which is most frequently used in microbiological routine diagnostics, considerably reducing the practical relevance. Furthermore, also nonhemolytically growing *H. haemolyticus* isolates have been observed [6, 17] and even primarily hemolytic *H. haemolyticus* isolates can lose this phenotype during passage [25]. Accordingly, the discriminative power of α -hemolysis should not be overestimated and *H. haemolyticus* isolates will usually be misidentified as *H. influenzae* un-

der routine conditions by a large proportion of diagnostic laboratories.

Many laboratories use automated biochemical differentiation systems, which, however, do not score much better. As recently shown, traditional differentiation systems for the identification of *Haemophilus* spp. like api-NH (bioMérieux, Nürtingen, Germany) or VITEK-NH cards (bioMérieux) show misidentifications in 1–10% of the samples [21, 26–28].

PCR- and hybridization-based approaches

Species discrimination by PCR requires the selective amplification of target genes that are present in the target organism but absent in the nontarget organism. The identification of such targets is challenging for phylogenetically closely related species like *H. influenzae* and *H. haemolyticus*.

Nucleic acid sequences that are used as marker genes for *H. influenzae* comprise fuculose kinase (*fucK*), protein D (*hpd*), and IgA protease (*iga*) as well as the adherence and penetration gene (*hap*). In contrast, the [Cu, Zn]-superoxide dismutase gene (*sodC*) has been used as a marker for *H. haemolyticus*. Enzymatically active [Cu, Zn]-superoxide dismutase was shown to be absent in *H. influenzae* [29]. However, no target shows acceptable specificity alone by itself but only in combination [6, 30–33]. Suggested primer sequences are provided in Table 1.

In a study with 78 *H. influenzae* strains and 44 *H. haemolyticus* strains, sensitivity and specificity of *hpd* PCR were 88.5% and 97.7%, of *fucK* PCR 64.1% and 100%, and of a combined approach of *hpd* and *fucK* PCR 92.3% and 97.7%, respectively, for the identification of *H. influenzae* [32]. Another study confirmed the discriminatory power of *hap*, *fucK*, and *sodC* while the reliability of *iga* detection showed limitations. In this study, 10 out of 11 *H. influenzae* strains were genotypically *iga* positive but also 20 out of 31 strains distinct from this cluster [34]. This latter result was confirmed by another study, stating excellent specificity of *hpd*-PCR but specificity problems of the *iga*-based approach [30].

In contrast, hybridization-based diagnostic approaches comprising *iga* variable region hybridization to dotblots and library-on-a-slide microarrays show good correlation to multigene sequence-based phylogenetic trees [31], but also, *hap*, *fucK*, and *sodC* amplicons were successfully detected by hybridization blot analysis [6]. Even traditional, very laborious DNA–DNA hybridization [35–37] ap-

Table 1. Primers for the amplification of target genes *sodC*, *fucK* and *hpd* that were found to be suitable for the discrimination of *H. influenzae* from *H. haemolyticus* (according to Ref. [38])

Target	Forward primer	Reverse primer
<i>sodC</i>	5'-CAY-SAA-AAY-CCA-AGC-TG-3'	5'-CAY-MCG-YGS-GCC-GSC-RCC-RCC-3'
<i>fucK</i>	5'-ACC-ACT-TTC-GGC-GTG-GAT-GG-3'	5'-AAG-ATT-TCC-CAG-GTG-CCA-GA-3'
<i>hpd</i>	5'-GGT-TAA-ATA-TGC-CGA-TGG-TGT-TG-3'	5'-TGC-ATC-TTT-ACG-CAC-GGT-GTA-3'

Table 2. Primers for partial 16S rRNA gene sequencing to discriminate *H. influenzae* from *H. haemolyticus* (according to Refs. [5] and [38])

Target	Forward primer	Reverse primer
16S rRNA	5'-GCA-GGT-TCC-CTA-CGG-TTA-3'	5'-CTC-AGA-TTG-AAC-GCT-GGC-GGC-3'

proaches have been used for the diagnostic discrimination of *H. influenzae* and *H. haemolyticus* [5].

In up-to-date epidemiological assessments, easy-to-apply multiplex PCR-approaches are preferably used [38].

Sequence-based approaches

The most frequently used [38] procedure to discriminate *H. influenzae* from *H. haemolyticus* by sequence analysis is partial 16S rRNA gene sequencing [5, 38]. Suitable primers are listed in Table 2. However, 16S rRNA sequence-based discrimination within the *Haemophilus* genus is affected by a high number of polymorphic positions due to intragenomic 16S rRNA operon heterogeneity [34].

A more laborious discriminatory approach is multilocus sequence typing targeting the genes of adenylate kinase (*adk*), ATP synthase F1 subunit γ (*atpG*), fumarate reductase iron-sulfur protein (*frdB*), fuculokinase (*fucK*), malate dehydrogenase (*mdh*), glucose-6-phosphate isom-

erase (*pgi*), and recA protein (*recA*) [5, 34, 39]. Suitable primers are listed in Table 3 [39].

The conserved P6 gene, coding for an outer membrane protein, is another target, which has been sequenced for the successful discrimination of *H. influenzae* from *H. haemolyticus* [5] (Table 4). The P6 sequence comparison of 6 *H. influenzae* strains and 12 *H. haemolyticus* strains showed sequence identity in all *H. influenzae* strains. The sequences of the analyzed 12 *H. haemolyticus* strains were identical as well and differed by 4 of 153 amino acids from that of the P6 genes of *H. influenzae* [5].

Fluorescence *in-situ* hybridization – a rapid molecular tool

Fluorescence *in-situ* hybridization is a rapid and easy-to-perform molecular tool for the identification of bacterial pathogens. Short fluorescence-labeled oligonucleotide

Table 3. Suitable primers for multilocus sequence typing analysis of *Haemophilus* spp. [39]

Target	Forward primer	Reverse primer
<i>adk</i>	5'-GGT-GCA-CCG-GGT-GCA-GGT-AA-3'	5'-CCT-AAG-ATT-TTA-TCT-AAC-TC-3'
<i>atpG</i>	5'-ATG-GCA-GGT-GCA-AAA-GAG-AT-3'	5'-TTG-TAC-AAC-AGG-CTT-TTG-CG-3'
<i>frdB</i>	5'-CTT-ATC-GTT-GGT-CTT-GCC-GT-3'	5'-TTG-GCA-CTT-TCC-ACT-TTT-CC-3'
<i>fucK</i>	5'-ACC-ACT-TTC-GGC-GTG-GAT-GG-3'	5'-AAG-ATT-TCC-CAG-GTG-CCA-GA-3'
<i>mdh</i>	5'-TCA-TTG-TAT-GAT-ATT-GCC-CC-3'	5'-ACT-TCT-GTA-CCT-GCA-TTT-TG-3'
<i>pgi</i>	5'-GGT-GAA-AAA-ATC-AAT-CGT-AC-3'	5'-ATT-GAA-AGA-CCA-ATA-GCT-GA-3'
<i>recA</i>	5'-ATG-GCA-ACT-CAA-GAA-GAA-AA-3'	5'-TTA-CCA-AAC-ATC-ACG-CCT-AT-3'

Table 4. Primers targeting the P6 gene for amplification with consecutive sequencing to discriminate *H. influenzae* from *H. haemolyticus* (according to Ref. [5])

Target	Forward primer	Reverse primer
P6	5'-ATG-AAC-AAA-TTT-GTT-AAA-TCA-3'	5'-TTA-GTA-CGC-TAA-CAC-TGC-3'

Table 5. FISH-probes for the discrimination of *H. influenzae*, *H. haemolyticus*, and *H. parainfluenzae* [40]

Target organism	FISH probe	Probe sequence
<i>H. influenzae</i>	Hain 16S1251	5'-TCG-CAGCTT-CGC-TTC-CCT-3'
	Hain 16S1253	5'-CGC-AGC-TTC-GCT-TCC-3'
<i>H. haemolyticus</i>	Haha 16S1252	5'-TCG-CAG-YTT-CGC-CAC-CCT-3'
	Haha 16S1242	5'-TCG-CCA-CCC-TCT-GTA-TAC-G-3'
<i>H. parainfluenzae</i>	Hapa 16S444	5'-ACT-AAA-TGC-CTT-CCT-CGC-TAC-C-3'

probes bind specifically at their target sequences within the cells, usually at 16S RNA or 23S RNA molecules, which are available in high copy numbers in vital bacteria. A DNA probe set for the discrimination of *H. influenzae*, *H. haemolyticus*, and *H. parainfluenzae* has been recently described for use on a multiwell slide and evaluated [40] (Table 5). The set comprises two probes for *H. influenzae*, two probes for *H. haemolyticus*, and one probe for *H. parainfluenzae*. Due to the close sequence homology of ribosomal RNA of the phylogenetically closely related species *H. influenzae* and *H. haemolyticus*, both respective species-specific probes have to show specific binding and all other probes must not show binding to allow for a reliable identification. Applied in this way, FISH allowed for 85% correct identifications, 15% noninterpretable results, and no misidentifications in a recent study comprising 84 *Haemophilus* spp. strains [40].

Matrix-assisted laser-desorption–ionization time-of-flight mass spectrometry (MALDI–TOF–MS)

Matrix-assisted laser-desorption–ionization time-of-flight mass spectrometry (MALDI–TOF–MS) is increasingly available in microbiological laboratories, at least in well-equipped laboratories in Western industrialized countries. Suitability of MALDI–TOF–MS for the discrimination of *H. influenzae* from *H. haemolyticus* has been described in 2013 [40, 41] and confirmed in 2014 [42]. Nevertheless, the availability of a well-defined reference spectrum for *H. haemolyticus* in the applied database is an important prerequisite for this. If such a spectrum is not available, it can be designed using well-characterized reference strains as described [40].

Of note, the spectra of *H. haemolyticus* and *H. influenzae* are very similar and there is considerable intraspecies variability. Availability of specific *H. influenzae* and *H. haemolyticus* reference spectra will lead to best matches with the species-specific spectrum. However, the spectral distance of *H. haemolyticus* spectra and *H. influenzae* spectra does not allow for a broad safety margin for the identification. In a recent analysis, 6 out of 50 *H. influenzae* strains also matched with a *H. haemolyticus* spectrum with a score that might theoretically lead to misidentifications [40], although the scores with the *H. influenzae* reference spectrum were higher; so, in fact, no misidentifications were observed.

Frequency of *H. haemolyticus* in diagnostic samples

The frequency of clinical *H. haemolyticus* isolates differs considerably depending on the site of sampling, preexisting patient conditions, and the geographical region [43].

Between 4% and 27% of throat culture strains that were initially identified as *H. influenzae* turned out to be *H. haemolyticus* by confirmatory testing in recent studies [5, 17, 44]. In sputum of COPD patients, up to 39.5% *H. haemolyticus* isolates were observed, at least if no association with acute exacerbations was guaranteed [5, 45]. Frequency of *H. haemolyticus* isolates in respiratory samples of cystic fibrosis patients ranged from 0.5% [4] to 10% [38] in recent studies.

In studies with mixed upper-respiratory tract specimens, low frequencies of *H. haemolyticus* isolations between 0% [38] and 0.4% [6] were repeatedly observed in patients without cystic fibrosis. Of note, *H. haemolyticus* is predominantly present in the oropharynx [46]. In a recent study, *H. haemolyticus* isolations were completely restricted to oropharynx-associated sampling sites [14].

Geographical distribution is particularly difficult to assess because sophisticated laboratory equipment to reliably discriminate *H. haemolyticus* from *H. influenzae* is not available everywhere. Accordingly, available data are restricted to Western industrialized countries. In Australia, very low frequencies of *H. haemolyticus* isolations were observed in patients with respiratory disease with 10% isolations in cystic fibrosis patients and 0% in patients without cystic fibrosis [38]. Of note, a higher isolation rate of 20% *H. haemolyticus* strains was observed by assessing naso-oro-pharyngeal swabs from healthy Australians [33]. In oropharyngeal swabs of Australian indigenous children with bronchiectasis, even more *H. haemolyticus* strains than *H. influenzae* strains were observed [46]. A very low detection rate of only 0.5% *H. haemolyticus* in cystic fibrosis patients was described for Denmark [4]. For mixed upper respiratory patient samples from Germany, a frequency of *H. haemolyticus* isolations between 1.2% and 16.2% was calculated [14]. From non-industrialized, resource-limited settings, surveillance data are widely missing.

Conclusions

The reliable discrimination of *H. influenzae* and *H. haemolyticus* remains challenging for the microbiological routine diagnostic laboratory. Biochemical assays have been shown to lack reliability [6, 17, 20–28]. Complex multiplex PCR-approaches [38] or sequence-based approaches [5] are hardly realistic for the routine setting because such procedures are both expensive and demanding regarding hands-on time of technical laboratory assistants. Accordingly, such techniques will remain restricted to study settings.

If this technology is available and suitable reference spectra are included in the database, MALDI–TOF–MS has the potential to provide diagnostic results with sufficient reliability for the diagnostic routine situation [40–42]. For less well-equipped laboratories, FISH might be a cost-efficient and easy-to-perform alternative but will not provide conclusive results in all instances [40].

For the discrimination of *H. influenzae* and *H. haemolyticus* in the diagnostic routine, MALDI-TOF-MS analysis from colony material should be chosen in the first line. The availability of *H. haemolyticus* reference spectra in the database has to be ensured. If it is not included, it can be designed using a commercially available *H. haemolyticus* reference strain as described [40]. For laboratories without a MALDI-TOF-MS device, FISH from colony material can be applied as a method of second choice. Although it allows for reliable results in the most instances [40], other molecular approaches like *sodC*, *fucK*, and *hpd* PCR, P6 gene sequencing or 16S rRNA gene sequencing should be available to resolve occasional ambiguous results. Especially, 16S rRNA gene sequencing is nowadays established nowadays in many routine laboratories. It is, however, too laborious, time-consuming, and expensive to be applied as a method of first or second choice. The discriminative power of biochemical approaches is low. Thus, their solitary application for the discrimination of *H. influenzae* and *H. haemolyticus* should, if possible, be avoided.

Declaration of interest

The authors declare that there are no conflicts of interest.

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