DIFFICULTIES IN SPECIES IDENTIFICATION WITHIN THE GENUS *HAEMOPHILUS* – A PILOT STUDY ADDRESSING A SIGNIFICANT PROBLEM FOR ROUTINE DIAGNOSTICS

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Diagnostic misidentifications of commensalic *Haemophilus haemolyticus* as pathogenic *Haemophilus influenzae* are frequent. This pilot study evaluates whether isolations of *H. haemolyticus* are frequent enough in Germany to cause a relevant diagnostic problem, considering the fact that even *H. influenzae* is a mere colonizer in about 30% of isolations.

In microbiological laboratories of two hospitals located in Northern and Southern Germany, the distribution of *Haemophilus* spp. was analyzed during a six-month-period. Site of infection, sex, and age of the patients was taken into consideration.

A total of 77 *Haemophilus* spp. isolates was acquired and discriminated on species level, comprising: 48 *H. influenzae*, 25 *Haemophilus parainfluenzae*, 3 *H. haemolyticus*, and 1 *Haemophilus parahaemolyticus*. The proportion of *H. haemolyticus* was calculated to range between 1.2% and 16.2 % within the 95% confidence limits. Commensalic *Haemophilus* spp. were isolated from oropharynx-associated sites only. *H. influenzae*, in contrast, was detected in clinically relevant materials like lower respiratory materials and conjunctiva swabs.

Altogether, there was a low proportion of clinical *H. haemolyticus* isolates. Accordingly, the problem of unnecessary antibiotic therapies due to misidentifications of *H. haemolyticus* as *H. influenzae* is quantitatively negligible compared with the risk of confusing *H. influenzae* colonizations with infections.

Keywords: *Haemophilus influenzae*, misidentification, upper respiratory tract infection, epidemiology, Germany, antibiotic therapy

Introduction

Most upper respiratory tract infections (URTI) are caused by viral agents [1–7]. Among the bacterial infections, the Gram-negative rod-shaped bacterium *Haemophilus influenzae* is one of the quantitatively most important pathogens [8, 9]. As shown in our previous study [10], *H. influenzae* is frequently isolated from adult patients with recurrent tonsillitis and retropharyngeal abscesses [10–12], particularly in case of fine-needle aspiration [13]. Combined infections due to *Staphylococcus aureus* and *H. influenzae* were frequently observed [10, 14]. *H. influenzae* type b conjugate vaccines allow for an efficient protection against severe infections by this serotype in case of boosted vaccination [15]; however, there is no general cross-protection at the species level.

In spite of the pathogenic potential of *H. influenzae*, asymptomatic colonization of the upper respiratory tract

(URT) by this microbial species occurs frequently [16, 17]. Colonization rates of the respiratory tract are particularly high in children and decrease with increase in age [17]. Altogether, a total of 30% of healthy volunteers was found to be colonized by *H. influenzae* in the respiratory tract [16].

Other *Haemophilus* spp. that are occasionally isolated from the URT include: *Haemophilus parainfluenzae*, *Haemophilus haemolyticus*, and *Haemophilus parahaemolyticus*. These species are phylogenetically related but virtually apathogenic. Altogether, *Haemophilus* spp. account for about 10% of the culturable bacterial flora of the URT [17]. *H. parainfluenzae* shows saprophytic growth in the URT but hardly ever causes local infections; however, infrequent cases of infectious endocarditis due to *H. parainfluenzae* have been described [18]. Although recent studies suggest occasional etiological relevance of *H. haemolyticus* [19], most studies describe

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this species as a commensal [20–22] without clinical importance.

In case of URTI, a reliable discrimination of *H. influenzae* from other *Haemophilus* spp. in the diagnostic routine is crucial due to the species-depending etiological relevance. However, as recently shown, traditional biochemical differentiation systems like api-NH (bioMérieux, Nürtingen, Germany) or VITEK-NH cards (bioMérieux) fail in 1–10% of the analyses [23–26]. Modern routine-compatible approaches like matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI–TOF–MS) or fluorescence *in situ* hybridization (FISH) do not score better in the discrimination of *H. influenzae* from other *Haemophilus* spp. as recently shown in our study [27].

The differentiation between *H. influenzae* and *H. haemolyticus* is particularly difficult. Most *H. haemolyticus* isolates lead to an alpha-hemolysis on horse, cow, or rabbit blood agar [28–30]. However, this phenomenon is of little practical relevance because sheep blood agar, where *H. haemolyticus* usually grows without hemolysis, is usually used in microbiological routine diagnostics. To make things even more complicated, nonhemolytic *H. haemolyticus* isolates are frequently cultured in microbiological routine diagnostics [22, 31].

Biochemical identification of *Haemophilus* spp. is frequently used in diagnostic routine conditions. Growth factor-based methods make use of the fact that *H. influenzae* lacks the enzymatic capacity to convert d-aminolevulinic acid (ALA) to protoporphyrin, hence, depends on factor X (heme) for growth [24, 32]. However, these approaches fail to discriminate *H. haemolyticus* from *H. influenzae* [27]. Sophisticated, labor-consuming, and expensive molecular approaches like ribosomal DNA sequence analysis, multilocus sequence analysis, DNA–DNA hybridization, and P6 gene sequencing reliably allow for such discrimination [22, 33]. MALDI–TOF–MS also allows for discrimination but on condition that a new *H. haemolyticus* mass spectrum is manually implemented as we previously showed [27]. However, the mass spectra of both species are quite similar. Accordingly, *H. haemolyticus* isolates will usually be misidentified as *H. influenzae* under routine conditions by a large proportion of diagnostic laboratories.

Consequently, in patients with URTI, confusions of *H. haemolyticus* to be *H. influenzae* will lead to unnecessary antibiotic therapies while the relevant, usually viral causative agent remains undetected. Retrospective analyses suggest that 10–40% presumed *H. influenzae* isolates were indeed *H. haemolyticus* [22, 33]. The present study tries to estimate the quantitative dimension and relevance of this problem in Germany to allow for a substantiated discussion about the need for more sophisticated approaches to discriminate within the *Haemophilus* genus in routine diagnostics: Are isolations of *H. haemolyticus* frequent enough in Germany to cause a relevant diagnostic problem? Or is there a higher risk of confusing mere *H. influenzae* colonization with real *H. influenzae* infections?

Materials and methods

Isolate collection and differentiation

During a six-month sampling period between June and November 2011, clinical *Haemophilus* spp. isolates were collected at the Institutes for Microbiology, Virology, and Hygiene of the University Hospitals Rostock, Germany and Ulm, Germany.

To become included into the analysis, reliable differentiation of the isolates on species level had to be guaranteed as follows. All included isolates were analyzed by two diagnostic MALDI–TOF–MS approaches at the Departments of Microbiology, Virology, and Hygiene of the University Hospitals Hamburg and Rostock, Germany, as well as by FISH at the Bernhard Nocht Institute for Tropical Medicine Hamburg, Germany. In case of contradicting results, partial 16S rRNA gene sequencing was used as the diagnostic gold standard. The test comparison describing the reliability of the different approaches has been previously published, and sequence information was deposited in FASTA format in the supplementary data [27]. Isolates for which individual test results were missing were not included into the study to assure standardized quality.

Isolate- and patient-related data

Isolate-specific information was documented for each included isolate. This information comprised the site of the presumed infection, age, and sex of the patients. Afterwards, patient data on each isolate were anonymized. The clinicians that sent the clinical samples provided all assessed information. Further inquiries on the wards regarding severity or other details of the disease were not performed.

Statistics

Regarding the parameter patient age, mean and median values were calculated for each *Haemophilus* sp.

With respect to the samples sizes, the distribution of the different *Haemophilus* sp. regarding site of infections, patient age, and patient sex was descriptively assessed.

Based on the assessed species distribution, the true proportion of *H. haemolyticus* among the subgroup of *H. haemolyticus*/*H. influenzae* isolates was estimated by calculating the 95% confidence interval using the Clopper–Pearson method [34] that leads to conservative only positive confidence limits [35].

Ethical approval

Ethical clearance for the analysis was obtained from Ethics Committee of the University Hospital Rostock, Germany.

Results

Distribution of Haemophilus spp. in relation to the sites of infection

In total, 60 clinical *Haemophilus* isolates from the University Hospital Rostock and 17 isolates from the University

Hospital Ulm were included into the study [27] (Table 1). Isolates from Rostock comprised: 35 *H. influenzae*, 23 *H. parainfluenzae*, 1 *H. haemolyticus*, and 1 *H. parahaemolyticus*. Isolates from Ulm included: 13 *H. influenzae*, 2 *H. parainfluenzae*, and 2 *H. haemolyticus*.

The presumed sites of infection of these 77 *Haemophilus* spp. were as follows: the lower airways (44 samples),

Table 1. Summarized presentation of the clinical *Haemophilus* isolates with details on clinical material, patient age, patient gender, and origin

Species	Clinical material	Patient age (in years)	Patient gender	Origin
<i>H. haemolyticus</i>	Lower airways	65	Male	Ulm
<i>H. haemolyticus</i>	Lower airways	61	Female	Ulm
<i>H. haemolyticus</i>	Oropharynx	9	Female	Rostock
<i>H. influenzae</i>	Lower airways	70	Female	Rostock
<i>H. influenzae</i>	Lower airways	55	Male	Rostock
<i>H. influenzae</i>	Lower airways	34	Male	Rostock
<i>H. influenzae</i>	Lower airways	67	Male	Rostock
<i>H. influenzae</i>	Nasopharynx	9	Female	Rostock
<i>H. influenzae</i>	Nasopharynx	0.67	Female	Rostock
<i>H. influenzae</i>	Nasopharynx	1	Female	Rostock
<i>H. influenzae</i>	Nasopharynx	1	Male	Rostock
<i>H. influenzae</i>	Lower airways	54	Male	Rostock
<i>H. influenzae</i>	Conjunctiva	6	Male	Ulm
<i>H. influenzae</i>	Conjunctiva	6	Male	Ulm
<i>H. influenzae</i>	Lower airways	44	Male	Ulm
<i>H. influenzae</i>	Oropharynx	14	Male	Ulm
<i>H. influenzae</i>	Oropharynx	23	Female	Ulm
<i>H. influenzae</i>	Oropharynx	14	Male	Ulm
<i>H. influenzae</i>	Conjunctiva	65	Female	Ulm
<i>H. influenzae</i>	Lower airways	45	Male	Ulm
<i>H. influenzae</i>	Nasopharynx	3	Male	Rostock
<i>H. influenzae</i>	Lower airways	51	Male	Rostock
<i>H. influenzae</i>	Lower airways	60	Male	Rostock
<i>H. influenzae</i>	Nasopharynx	2	Male	Rostock
<i>H. influenzae</i>	Oropharynx	2	Male	Rostock
<i>H. influenzae</i>	Lower airways	17	Male	Rostock
<i>H. influenzae</i>	Nasopharynx	1	Female	Rostock
<i>H. influenzae</i>	Conjunctiva	0.07	Female	Rostock
<i>H. influenzae</i>	Lower airways	48	Female	Rostock
<i>H. influenzae</i>	Lower airways	2	Female	Rostock
<i>H. influenzae</i>	Nasopharynx	10	Male	Rostock
<i>H. influenzae</i>	Nasopharynx	2	Male	Rostock
<i>H. influenzae</i>	External ear	1	Male	Rostock
<i>H. influenzae</i>	Lower airways	62	Female	Rostock
<i>H. influenzae</i>	Nasopharynx	2	Male	Rostock
<i>H. influenzae</i>	Lower airways	32	Male	Rostock
<i>H. influenzae</i>	Lower airways	52	Female	Rostock
<i>H. influenzae</i>	Lower airways	59	Male	Rostock

Table 1. (cont.)

Species	Clinical material	Patient age (in years)	Patient gender	Origin
<i>H. influenzae</i>	Lower airways	48	Male	Rostock
<i>H. influenzae</i>	Nasopharynx	0.83	Male	Rostock
<i>H. influenzae</i>	Lower airways	59	Female	Ulm
<i>H. influenzae</i>	Conjunctiva	5	Male	Ulm
<i>H. influenzae</i>	Nasopharynx	0.06	Female	Ulm
<i>H. influenzae</i>	Lower airways	54	Male	Ulm
<i>H. influenzae</i>	Conjunctiva	37	Male	Ulm
<i>H. influenzae</i>	Nasopharynx	2	Female	Rostock
<i>H. influenzae</i>	Paranasal sinus	64	Male	Rostock
<i>H. influenzae</i>	Lower airways	22	Female	Rostock
<i>H. influenzae</i>	External ear	1	Female	Rostock
<i>H. influenzae</i>	Nasopharynx	2	Female	Rostock
<i>H. influenzae</i>	Lower airways	62	Male	Rostock
<i>H. parahaemolyticus</i>	Oropharynx	6	Male	Rostock
<i>H. parainfluenzae</i>	Lower airways	30	Female	Rostock
<i>H. parainfluenzae</i>	Lower airways	77	Female	Rostock
<i>H. parainfluenzae</i>	Lower airways	73	Male	Rostock
<i>H. parainfluenzae</i>	Lower airways	29	Male	Rostock
<i>H. parainfluenzae</i>	Middle ear	Not assessed	Not assessed	Rostock
<i>H. parainfluenzae</i>	Lower airways	58	Male	Rostock
<i>H. parainfluenzae</i>	Lower airways	67	Female	Rostock
<i>H. parainfluenzae</i>	Lower airways	25	Male	Rostock
<i>H. parainfluenzae</i>	Lower airways	28	Male	Rostock
<i>H. parainfluenzae</i>	Lower airways	2	Female	Rostock
<i>H. parainfluenzae</i>	Lower airways	61	Male	Rostock
<i>H. parainfluenzae</i>	Lower airways	61	Male	Rostock
<i>H. parainfluenzae</i>	Lower airways	61	Male	Rostock
<i>H. parainfluenzae</i>	Lower airways	82	Female	Rostock
<i>H. parainfluenzae</i>	Oropharynx	46	Male	Rostock
<i>H. parainfluenzae</i>	Lower airways	74	Male	Rostock
<i>H. parainfluenzae</i>	Lower airways	76	Male	Ulm
<i>H. parainfluenzae</i>	Oropharynx	38	Female	Ulm
<i>H. parainfluenzae</i>	Oropharynx	13	Female	Rostock
<i>H. parainfluenzae</i>	Lower airways	80	Male	Rostock
<i>H. parainfluenzae</i>	Lower airways	71	Male	Rostock
<i>H. parainfluenzae</i>	Lower airways	71	Male	Rostock
<i>H. parainfluenzae</i>	Lower airways	60	Male	Rostock
<i>H. parainfluenzae</i>	Lower airways	60	Male	Rostock
<i>H. parainfluenzae</i>	Lower airways	60	Male	Rostock

the oropharynx (nine samples, three of them from known mucoviscidosis patients), the nasopharynx (14 samples), the conjunctiva (six samples), the external ear (two samples), the middle ear (one sample), and the paranasal sinus (one sample) (Table 2).

The chances of isolating *H. influenzae* or other *Hae-mophilus* sp. strains with low pathogenic potential from

the lower airways and other directly oropharynx-associated sites including the middle ear were nearly equal. All four *H. haemolyticus* or *H. parahaemolyticus* isolates were isolated either from the lower airways or the oropharynx. In contrast, only *H. influenzae* was isolated from the nasopharynx, the paranasal sinus, the external ear, and the conjunctiva.

Table 2. Distribution of *Haemophilus* isolates regarding clinical material, patient age, and patient sex

	<i>Haemophilus</i> spp.	<i>H. influenzae</i>	<i>H. parainfluenzae</i>	<i>H. haemolyticus</i>	<i>H. parahaemolyticus</i>
Distribution of presumed sites of infection					
Lower airways	44	21 (48%)	21 (48%)	2 (5%)	–
Nasopharynx	14	14 (100%)	–	–	–
Oropharynx	9	4 (including 3/3 mucoviscidosis patients) (44%)	3 (33%)	1 (11%)	1 (11%)
Conjunctiva	6	6 (100%)	–	–	–
External ear	2	2 (100%)	–	–	–
Middle ear	1	–	1 (100%)	–	–
Paranasal sinus	1	1 (100%)	–	–	–
Total	77 (100%)	48 (62%)	25 (32%)	3 (4%)	1 (1%)
Distribution of patient age					
Median age (years)	37.5 (0.058–82)	15.5 (0.058–70)	60.5 (2–82)	61 (9–65)	6 (–)
Mean age (years) ($\pm$ standard deviation)	35.7 ($\pm$ 27.7)	26.5 ($\pm$ 25.5)	54.3 ($\pm$ 22.5)	45 ($\pm$ 31.2)	6
Distribution of patient sex					
Male	49 (64%)	30 (62.5%)	17 (70.8%)	1 (33.3%)	1 (100%)
Female	27 (36%)	18 (37.5%)	7 (29.2%)	2 (66.6%)	0 (0%)

Distribution of Haemophilus spp. in relation to sex and age

In all instances except one, information on patients' age and sex was extractable from the accompanying forms. The remaining single isolate, a *H. parainfluenzae* isolate, was collected at the University of Rostock from an anonymous patient who visited the University of Rostock hospital complaining of an URT infection in the course of the study [36].

The 76 patients comprised 49 men and 27 women. 62.5% (30/48) of all *H. influenzae* isolates originate from male and, therefore, 37.5% (18/48) from female patients. A comparable male–female ratio was observed for *H. parainfluenzae*. There, 70.8% (17/25) of the isolates were cultured from men and 29.2% (7/25) from women. Thus, the sex-specific distributions of *H. influenzae* and *H. parainfluenzae* were similar to the overall sex distribution of the study participants. The three cultured *H. haemolyticus* isolates were from two men and one woman, while the single *H. parahaemolyticus* isolate was obtained from a man. Table 2 shows a summary of the distribution of *Haemophilus* isolates from men and women.

The median age of the participating patients was 37.5 (0.058–82) years, and the mean age was 35.7 ($\pm$ 27.7) years (standard deviation (SD) in brackets). *H. haemolyticus* was isolated from a nine-year-old child and two patients aged 61 and 65 years; *H. parahaemolyticus* was isolated from a six-year-old child. *H. influenzae* strains were predominantly isolated from young patients: median age of 15.5 (0.058–70 years), whereas *H. parainfluenzae* isolates were more frequent in the elderly: median age of 60.5 (2–82) years (Table 2).

Proportion of H. haemolyticus among the subgroup of H. haemolyticus/H. influenzae isolates

Three *H. haemolyticus* isolates and 48 *H. influenzae* isolates were cultured. *H. haemolyticus* isolates were identified in a proportion of three out of 51 (5.88%) of the *H. haemolyticus/H. influenzae* subgroup. With respect to the total number of samples, the 95% confidence interval was 5.9% $\pm$ 6.4%. The application of the Clopper–Pearson method identified a range between 1.2% and 16.2% within the 95% confidence limits.

Discussion

In this study, we have used an expensive but reliable technique to identify clinical *Haemophilus* spp. isolates from two German University Hospitals and to determine their distribution on species level. As expected, due to the sample size, no obvious age and sex distribution patterns in the isolation frequency were observed with the exception of *H. influenzae*, which was frequently isolated from young patients and *H. parainfluenzae* that was frequently isolated from the elderly. This result is in line with the previously observed phenomenon of decreasing colonization rates of the upper respiratory tract with *H. influenzae* with increasing age [17]. However, the practical relevance regarding therapeutic decisions is low because the discrimination of *H. influenzae* and *H. parainfluenzae* is highly reliable in routine diagnostics [27].

H. haemolyticus and *H. parahaemolyticus* were too infrequently isolated to draw any conclusions regarding their distribution in the population. This can be attributed to the

sample size and/or the geographical regions of the study. Importantly, the results that we obtained during this study reveal two very interesting observations. Firstly, the observation that all *H. parainfluenzae*, *H. haemolyticus*, and *H. parahaemolyticus* were isolated from materials directly associated to the oropharynx. Secondly, the observation that *H. influenza* was isolated from the nasopharynx, the paranasal sinus, the external ear, and the conjunctiva-associated materials, indicates its clinical relevance. Our data is in line with previous investigations that *H. influenzae* accounts for less than 2% of the *Haemophilus* burden in the pharynx [17, 37]. However, we strongly suggest further studies to be performed from larger population sizes probably nationwide to confirm or exclude site-specific differences in the colonization frequency with different *Haemophilus* spp. and to confine colonization with species of low pathogenic potential on sites in the oropharynx. Our pilot study about 6 months delivers preliminary data only.

Nevertheless, the data suggest that colonization or infection due to *H. haemolyticus* is rare at least in severely ill patients who are treated in German University Hospitals. Previously published rates of 10–40% from other geographic regions [22, 33] were not confirmed by our data that suggested a ratio of $5.9\% \pm 6.4\%$. In contrast, a previous analysis of healthy volunteers suggested a colonization rate of 30% with the facultative pathogenic species *H. influenzae* [16]. Even when considering that the *H. haemolyticus* proportion among those isolates from healthy individuals could be as high as 10%, there is a considerable and at least two- to threefold higher risk of misidentifying an *H. influenzae* colonization as an *H. influenzae* infection than an *H. haemolyticus* colonization as an *H. influenzae* infection in German URTI patients. In contrast, a reliable discrimination based on sophisticated, expensive, and laborious molecular procedures like ribosomal DNA sequence analysis, multilocus sequence analysis, DNA–DNA hybridization, and P6 gene sequencing [22, 33] is hardly realistic in the microbiological routine laboratory at present due to its labor efforts and costs.

Accordingly, the diagnosis of *H. influenzae* in case of URTI has to be interpreted with care, both due to the risk of misidentifying *H. haemolyticus* as *H. influenzae* [22, 27, 33] and due to the high overall colonization rate with *H. influenzae* [16]. Therefore, this microbiological diagnosis can only be considered as one piece of information for narrowing the differential diagnosis of URTI and, consecutively, finding reasons for the administration of antibiotic drugs.

The lately developed and readily available *H. haemolyticus* mass spectrum [27] that is based on data from the reference strain *H. haemolyticus* ATCC 33390 is recently the most reliable to discriminate *H. haemolyticus* and *H. influenzae*. However, the mass spectrum differences between these species are marginal [27] and more evaluations with higher numbers of well-characterized isolates are needed to estimate the diagnostic accuracy of this procedure.

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Potential conflicts of interest

All authors report no potential conflicts of interest.

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