

Haemophilus influenzae and Haemophilus parainfluenza in Cystic Fibrosis: 15 Years Experience

Rosanne Ebbing², Colin F Robertson^{1,2,3} and Philip J Robinson^{1,2,3*}

¹Department of Respiratory Medicine, The Royal Children's Hospital Melbourne, Australia

²Murdoch Children's Research Institute, The Royal Children's Hospital Melbourne, Australia

³University of Melbourne, Melbourne, Australia

Abstract

Background: *Haemophilus influenzae* and *Haemophilus parainfluenzae* are commonly identified in the lower airways of patients with cystic fibrosis (CF). Little is known of the change in prevalence and antimicrobial susceptibility in this population over time. We examined the epidemiology of both organisms over 15 years in our CF clinic.

Results: 1538 isolates from respiratory specimens of 349 CF patients over 15 years were investigated. Annual prevalence increased significantly for both bacteria, being more pronounced for *H. parainfluenzae*. Average percentage of resistant cultures increased by 46% (*H. Influenzae*) and 61% (*H. Parainfluenzae*). For *H. influenzae*, resistance to ampicillin was 34.4%, co-trimoxazole 21.4%. For *H. parainfluenzae*, resistance to ampicillin was 50.0%, co-trimoxazole 26.8%. Resistance in *H. influenzae* and *H. parainfluenzae* to ampicillin and in *H. parainfluenzae* to amoxicillin/ clavulanic acid and co-trimoxazole increased over the study.

Conclusion: This present study has shown an increased annual prevalence of *H. influenzae* and *H. parainfluenzae* in a large group of CF patients. Resistance to ampicillin significantly increased for *H. influenzae* and *H. parainfluenzae*, but increased resistance to amoxicillin/ clavulanic acid and co-trimoxazole was only significant in *H. parainfluenzae*.

Keywords: Cystic fibrosis; *Haemophilus influenzae*; *Haemophilus parainfluenzae*; Resistance; Ampicillin; Amoxicillin/Clavulanic acid; Co-trimoxazole; Rifampicin; Cefotaxime

Introduction

Cystic fibrosis (CF) is an autosomal recessive, genetic disease characterised by chronic inflammation and infection, which is also associated with accelerated lung damage, increased morbidity and premature death [1]. The airways of patients are predominantly infected by specific pathogens, both *Staphylococcus aureus* and *Pseudomonas aeruginosa* are frequently identified and are often associated with significant inflammation and lung damage [2].

Haemophilus parainfluenzae and *Haemophilus influenzae* are commonly identified in the lower airways in patients with CF with the former being the less aggressive organism in eliciting an inflammatory response. The latter organism is regularly involved in chronic lung infections and acute exacerbations in CF patients and has recently been shown to be associated with lower lung function in infants with CF [3,4]. Although generally not regarded as a pathogen, *H. parainfluenzae* does occasionally cause infections in humans, including pneumonia [5].

The emergence and spread of microbial resistance is a troublesome problem in the treatment of infectious diseases. The wide use of aggressive antibiotic treatments in CF disease has resulted in an increased risk of emergent antibiotic resistance, as demonstrated in the pathogens *S. aureus* and *P. aeruginosa* [6]. The monitoring of bacterial resistance to antimicrobial agents may therefore provide scientific information that would be useful in developing guidelines for the rational use of antibiotics to prevent drug resistance. The risk of developing antibiotic resistance in CF patients is clearly higher than in other patients because they are used more often [7]. Ampicillin is already no longer recommended because of widespread resistance [8]. Due to the potential role of *H. parainfluenzae* and *H. influenzae* in the infectious disease process, epidemiology and susceptibility testing of both bacteria is important to guide antibiotic treatment when required.

Yet, in both *H. influenzae* and *H. parainfluenzae* very little is known of the prevalence and antibiotic resistant trends in CF patients.

In this present study the prevalence and antibiotic resistance of *H. influenzae* and *H. parainfluenzae* were studied in a longitudinal manner over 15 years in a single CF community.

Methods and Data Collection

Subjects and samples

This is a retrospective study of CF patients followed at the Cystic Fibrosis Clinic at the Royal Children's Hospital (RCH), Melbourne, Australia from 1 January 1998 to 26 September 2012. All included patients had been previously diagnosed with CF based on recognised criteria (positive sweat test and/or presence of 2 known CF-gene mutations). Specimens, including cough swabs, sputum or bronchoalveolar lavage, were collected at outpatient visits or during admission to the hospital over time. We examined the annual prevalence and resistance of *H. influenzae* and *H. parainfluenzae* specimens by retrieving data from the hospital's computerised microbiological database over the previous 15 years. This database contains results from microbiological analysis of specimens obtained from all paediatric patients attending this hospital. More than 70,000 specimens were interrogated to identify

*Corresponding author: P.J. Robinson, Department of Respiratory Medicine, The Royal Children's Hospital Melbourne, Australia 3052, Tel: 61-3-93455818; E-mail: Phil.robinson@rch.org.au

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the CF specimens and the specific subgroup of *H. influenzae* and *H. parainfluenzae* isolates. We used the hospital's computer based clinical database and the CF units own online CF data registry to identify the isolates obtained from patients with CF.

Over the 15 years of the study *H. influenzae* and *H. parainfluenzae* specimens were routinely tested for their susceptibility to a group of core antibiotics: ampicillin, amoxicillin/ clavulanic acid, co-trimoxazole, rifampicin and cefotaxime. We defined prevalence as patients whose airway specimens were shown to culture either *H. influenzae* and/or *H. parainfluenzae* at least once per calendar year. We defined isolates as resistant if it was resistant to one or more of these antibiotics. Isolates were excluded if two isolates occurred in one patient on the same date with the same resistance pattern but had been obtained through bronchoalveolar lavage from different sides of the lung. In 50 such cases we only used the first isolate in the analysis. Eye, ear, nose and throat specimens were excluded.

Antibiotic sensitivity testing

Over the period of the study, methodology for determination of antibiotic resistance changed from agar dilution, to disk diffusion and finally to VITEK (microbroth). In each case the Clinical and Laboratory Standards Institute (CLSI) standard has been used to interpret the susceptibility.

Statistical analysis

Results are reported using descriptive statistics and figures and presented as percentage of annual specimens. A Chi-square test for trend was used to determine if there was a linear increase or decrease in prevalence and resistance. We also investigated resistance within each separate antibiotic over the study period. We performed a regression analysis to determine the estimated average annual change in resistance over the 15 years. The Spearman's Rank Correlation test was used to identify and test the strength of the relationship between multi-drug resistance and the 15 year time period. We used a Chi-square test to examine an association between resistance and age.

Results

Epidemiology

In total, 1538 isolates from CF patients were analysed, 518 were positive for *H. influenzae* and 1020 for *H. parainfluenzae*, from 349 different patients (192 males and 157 females). From each patient, 1 to 25 isolates were obtained (mean, 4.73 isolates per patient). Both *H. influenzae* and *H. parainfluenzae* were co-cultured from 163 individuals. The ages of the patients growing *H. influenzae* isolates ranged from 1 month to 20.4 years (mean, 7.6yrs) and the ages of the patients growing *H. parainfluenzae* isolates ranged from 2 months to 21.0 years (mean, 8.6yrs). We found an average annual prevalence

of 12% in *H. influenzae* and 20% in *H. parainfluenzae* over 15 years. Over this period, the annual prevalence increased significantly in both pathogens (*H. influenzae* $p \leq 0.001$, *H. parainfluenzae* $p \leq 0.0001$). The prevalence of *H. influenzae* did not increase as much over the years as the prevalence of *H. parainfluenzae*. From 2008 onwards the increase in annual prevalence in *H. parainfluenzae* was particularly evident (Table 1 and Figure 1).

Resistance

The average resistance (defined as resistance to at least one of the five antibiotics) over 15 years, was higher in *H. parainfluenzae* isolates (61%) and patients (53%) compared to *H. influenzae* isolates (46%) and patients (42%) (Table 2). There was a significant increase of resistance in *H. influenzae* and *H. parainfluenzae* (Figures 2 and 3) (*H. influenzae* $p \leq 0.0001$, *H. parainfluenzae* $p \leq 0.001$) over the period of the study. In addition, we showed an estimated average annual rise in resistance of 2.2% and 1.7% in *H. influenzae* and *H. parainfluenzae*, respectively over the period of our study. This corresponds to a roughly 30% rise in resistance in *H. influenzae* and 25% in *H. parainfluenzae* over the 15 years of our study period.

Resistance to ampicillin and co-trimoxazole was the most frequently detected resistance in both pathogens. Among the 518 *H. influenzae* isolates, 34.4% were resistant to ampicillin and among the 1020 *H. parainfluenzae* isolates 50.0%. A significant increase of resistance over time was found in *H. influenzae* and in *H. parainfluenzae* to ampicillin (*H. influenzae* $p \leq 0.0001$, *H. parainfluenzae* $p \leq 0.01$). Interestingly, *H. influenzae* showed only an average resistance to amoxicillin/ clavulanic acid of 1.7% (9 isolates) over 15 years without a linear pattern. This is in contrast to a higher resistance of 12.5% found in *H. parainfluenzae* with a significant increasing trend ($p \leq 0.01$). There was also significant increase in resistance in *H. parainfluenzae* to co-trimoxazole ($p \leq 0.001$) but not for *H. influenzae*.

H. influenzae isolates had a 0.4% and 0.2% resistance to rifampicin and cefotaxime and *H. parainfluenzae* isolates had a 1.5% and 3.6% resistance respectively. In *H. influenzae* there were only two resistant isolates in 15 years to rifampicin and one to cefotaxime. Overall, resistance occurred more frequently among *H. parainfluenzae* isolates to all individual antibiotics (Table 3). In 15 years, 46.1% of the *H. influenzae* isolates and 30.2% of the *H. parainfluenzae* isolates were fully sensitive.

Multi-drug resistance

We defined multi-drug resistance as isolates being resistant to three or more of the tested antibiotics. Until 2002 in *H. parainfluenzae* and 2005 in *H. influenzae*, no multi-drug resistance was observed. There was a slight increase in this resistance in both bacteria after these time points (Figure 4). There was a higher frequency of multi-drug resistance in *H. parainfluenzae* than in *H. influenzae* and a more pronounced increase over the years ($p \leq 0.0001$).

Year	'98	'99	'00	'01	'02	'03	'04	'05	'06	'07	'08	'09	'10	'11	'12	total
Total patients, n	136	212	199	198	206	195	221	235	222	232	256	259	284	308	277	3440
Total specimens, n	808	1215	1023	987	1255	1304	1515	1605	1566	1707	1897	2237	3184	3516	2690	26509
<i>H. influenzae</i> patients, (isolates)	11(12)	24(29)	16(20)	17(20)	26(28)	21(24)	18(22)	33(40)	26(28)	21(25)	30(37)	42(55)	43(62)	51(62)	43(54)	413(518)
Prevalence <i>H. influenzae</i> , %	8	11	8	9	13	11	8	14	12	9	12	16	15	17	16	12
<i>H. parainfluenzae</i> patients, (isolates)	5(5)	14(14)	4(4)	16(8)	15(15)	27(32)	34(36)	37(47)	34(38)	28(37)	32(48)	66(93)	124(231)	131(225)	112(177)	679(1020)
Prevalence <i>H. parainfluenzae</i> , %	4	7	2	8	7	14	15	16	15	12	13	25	44	43	40	20

Table 1: Annual prevalence of *H. influenzae* and *H. parainfluenzae* among patients with CF between 19, 98 and 2012.

Age and resistance

The highest frequency of resistance was found among 0-5 year old CF patients. There was a significant association between age and resistance in *H. parainfluenzae* between 2009 and 2012 as well as in 2006 (Table 4). No similar association was found for *H. influenzae*. No association was found between gender and resistance.

Discussion

To our knowledge this is the first study demonstrating a change in prevalence and resistance in *Haemophilus influenzae* and *Haemophilus parainfluenzae* isolates in a group of CF patients. In addition we describe here the prevalence and resistance over a period of time significantly longer than any similar previous study.

In our cohort of patients, the prevalence of *H. influenzae* on average was 12% over 15 years. A higher prevalence of 17.3% among CF patients over 10 years has been reported by Razvi et al. [9]. In 2000 Saiman reported a prevalence of 19% in their study, however in CF patients younger than one year this was 38% [10]. Our overall prevalence is lower, but looking at the recent years of our study (2008 onwards) the percentages are similar to what has been published previously. Our prevalence in the last 5 years was 15% compared to 21.3% among CF patients over 5 years previously reported by Cardines et al. [11].

Over the past decade, a decreased prevalence of *P. aeruginosa* and *B. cepacia* has been reported in many CF treatment centres, while the prevalence of *H. influenzae*, MRSA, *S. maltophilia* and *A. xylosoxidans* increased [9]. Similar to Razvi's observation, our current study has shown an increased prevalence of *H. influenzae* over time. Razvi et al. showed an increase in prevalence of *H. influenzae* in the period from 1995 to 2005 from 22.2% to 34.1% among CF patients 2-5 years old [9]. The last published analyses before Razvi's study, were performed in 1990, where a predominance of *P. aeruginosa* (60.7%) was described in a group which also showed a prevalence rate of *H. influenzae* which was significantly lower (5.5%) [12]. This data would suggest then that the number of *H. influenzae* isolates in CF groups is increasing over this period. This increase is worrisome since recently it has been showed that infection of CF patients with *H. influenzae* in infancy is associated with a significant lower FEV0.5 (-18.2%) [4]. We are unaware of any previous reports describing this trend in CF patients, with *H. parainfluenzae*. Although *H. parainfluenzae* has been considered a

less aggressive organism, further investigations are needed to clarify the consequence of the presence of *H. parainfluenzae* in association to worsening of the lung function.

Resistance in these bacteria was shown to increase significantly over the period of our study. We defined resistance if the isolate was resistant to one of the five studied antibiotics. In contrast, other studies have looked at each antibiotic separately. In these studies, including CF patients, it was demonstrated that 13.9%-29.2% of the *H. influenzae* isolates were resistant to ampicillin [13,14]. Zhonghua et al. showed a 7.7% resistance rate to ampicillin in 2000 and 14.55% in 2002 [15]. This latter study however had been performed among non-CF children in China. Those resistance rates are in contrast to the results of our study where 34.36% was resistant. The increasing trend we found in resistance to ampicillin is similar to that reported by Jansen et al. in their study of non-CF children [16]. The resistance rate to ampicillin described by Orden Martinez et al. was lower (18.5%) in *H. parainfluenzae* than the 50% resistance found in our current study [17]. Again Orden Martinez did not confine their study group to children with CF and in addition both upper and lower airway specimens were included. The proportion of *H. influenzae* isolates resistant to amoxicillin/ clavulanic acid in our study is similar to previous reports. Zhonghua et al. [15] and Roman et al. described a 100% susceptibility rate similar to our finding of 98.3% [14]. The study by Zhonghua et al. was not confined to patients with CF. We found a higher resistance in *H. parainfluenzae* to amoxicillin/ clavulanic acid (12.45%) than that reported by Felmingham (0%) and Orden Martinez in their separate studies [13,17]. Both studies were performed in Europe and included all types of infection in any patient caused by these pathogens.

Although we found an increasing trend in the resistance among *H. influenzae* and *H. parainfluenzae* to co-trimoxazole (overall: 21.4%, 26.8% respectively) it did not reach the resistance levels reported in other studies, albeit these studies were performed in groups not limited to patients with CF (45.9% resistance in *H. influenzae*) [14]. The low percentage of *H. influenzae* isolates resistant to cefotaxime (0.2%) was similar to those reported by Zhonghua et al., Roman et al. and Orden Martinez et al. [14,15,17]. The study by Orden Martinez also showed a low resistance to cefotaxime in *H. parainfluenzae* similar with what we found. Resistance to rifampicin in *H. influenzae* is low in other studies including CF patients (Roman et al.) or without CF patients (Zhonghua et al.) findings similar our own. In contrast, Orden Martinez found a higher resistance to rifampicin in both *H. parainfluenzae* (26.7%) than in *H. influenzae* (4.8%).

Historically sensitivity to rifampicin has been included in our laboratory's testing panel for these organisms. In clinical use however this drug would virtually never been used to treat these bacteria and indeed it could be argued that it would be more useful clinically to be able to consider resistance patterns to more clinically relevant drugs such as imipenem, ciprofloxacin or even azithromycin. Such data is not available however from our laboratory and our rifampicin data is purely included for historical reflection.

The observations of increasing resistance and multi-drug resistance may be suggestive of the impact of prophylactic antibiotic use. The RCH CF unit commenced using anti-staphylococcus antibiotic prophylaxis,

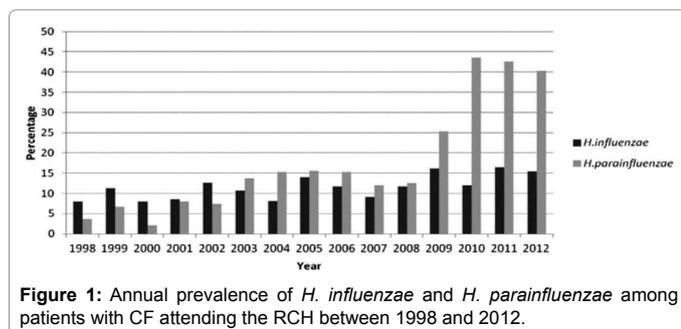


Figure 1: Annual prevalence of *H. influenzae* and *H. parainfluenzae* among patients with CF attending the RCH between 1998 and 2012.

Year	'98	'99	'00	'01	'02	'03	'04	'05	'06	'07	'08	'09	'10	'11	'12	Average
<i>H. Influenza</i> % isolates	8	14	50	30	43	29	41	55	50	32	51	62	62	48	46	46
<i>H. Parainfluenzae</i> %isolates	0	36	0	50	60	56	53	57	66	59	52	61	63	67	63	61
<i>H. Influenza</i> % Patients	9	17	44	29	38	33	44	58	54	29	50	64	63	47	51	42
<i>H. Parainfluenzae</i> % Patients	0	36	0	50	60	63	56	59	65	64	66	67	71	77	68	53

Table 2: Resistance to one or more of the 5 antibiotics in *H. influenzae* and *H. parainfluenzae* isolates and patients with CF between 1998 and 2012.

mainly amoxicillin/ clavulanic acid, in 2005. We did not find an increase in resistance in *H. influenzae* as a related consequence. The increase in resistance in *H. parainfluenzae* and multi-drug resistance could be associated with the introduction of prophylaxis, but further studies are required to define this.

A remarkably strong association was found between the youngest group of children and higher frequency of resistance in *H. parainfluenzae*. This finding is consistent with previous findings by Scheifele et al. who described a more frequent resistance to ampicillin in children from 3-4 years old [18]. It is interesting because the expectation is higher resistance rates in those who had longer exposure to antimicrobial agents, or the older patients. However more factors may play a role, such as hypermutable strains.

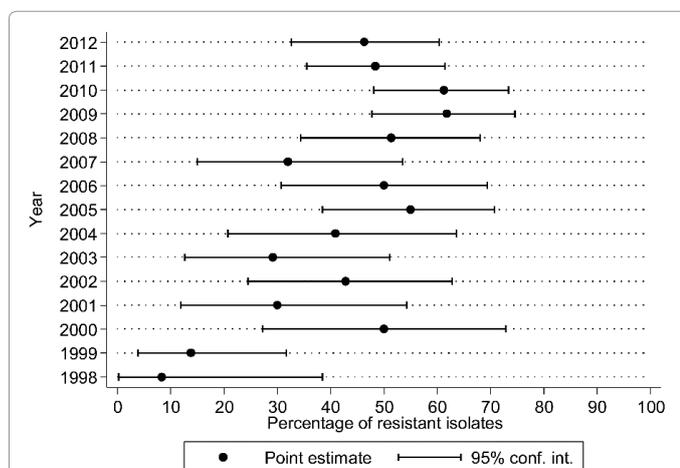


Figure 2: Percentage of resistant *H. influenzae* isolates among patients with CF attending the RCH between 1998 and 2012 with 95% confidence interval (CI).

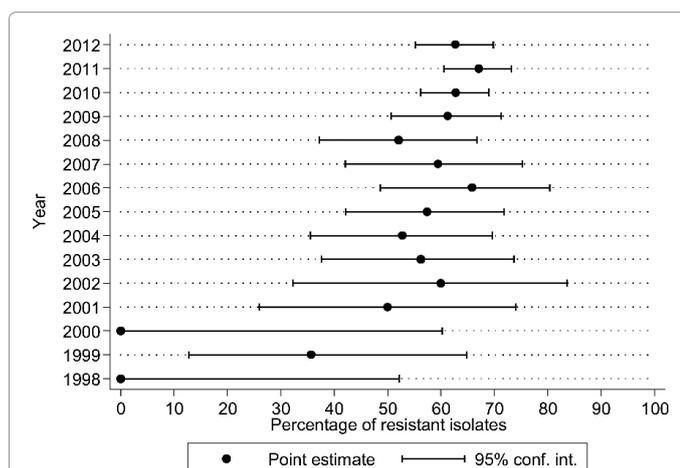


Figure 3: Percentage of resistant *H. parainfluenzae* isolates among patients with CF attending the RCH between 1998 and 2012 with 95% CI.

	<i>H. influenzae</i>	<i>H. parainfluenzae</i>
Ampicillin, %	34.4	50
Amox/ clav acid, %	1.7	12.5
Co-trimoxazole, %	21.4	26.8
Rifampicin, %	0.4	1.5
Cefotaxime, %	0.2	3.6

Table 3: Average resistance in *H. influenzae* and *H. parainfluenzae* isolates among patients with CF between 1998 and 2012.

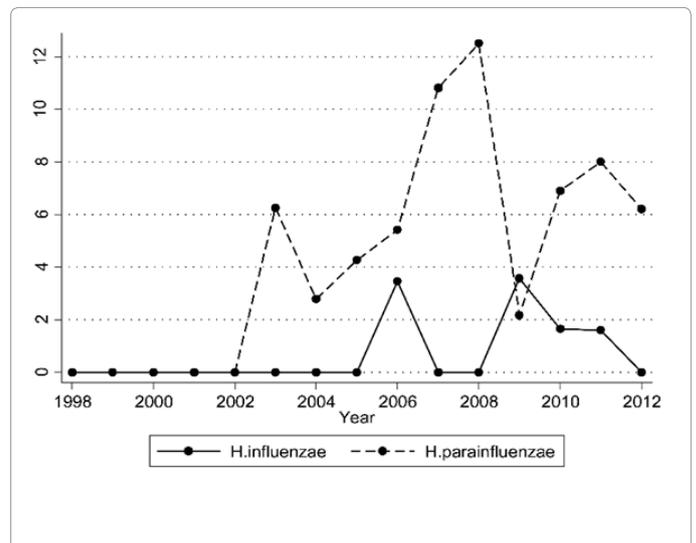


Figure 4: Multi-drug resistance within *H. influenzae* and *H. parainfluenzae* isolates among patients with CF attending the RCH between 1998 and 2012.

	0-5 y	>5-10 y	>10-15 y	>15 y	p-value
2012	69	71	47	43	0.02
2011	82	70	45	54	<0.0001
2010	82	55	58	53	0.003
2009	79	55	45	62	0.04
2006	90	85	38	0	0.005

Table 4: Percentage of resistance in 5 separate years in *H. parainfluenzae* isolates among patients with CF from different age strata.

Our study had several limitations. First of all, regional patients may not be fully represented in this study because on occasion their specimens were analysed by local laboratories. Although we do not believe this is likely to affect our overall findings, our unit has recently reported a difference in *P. aeruginosa* isolates from regional versus Melbourne metropolitan patients [18].

Secondly, the Chi-square tests for trend must be interpreted with caution as they may be picking up minor trends because of the big sample size. However, we do think also these minor changes are important, because it can be the beginning of a trend. Although there is no linear trend visible in the resistance in *H. influenzae* to co-trimoxazole, comparing the last 4 years to the previous ones resistance appears to be increasing.

It is not always clear whether a pathogen is truly emerging or whether the increasing prevalence is related to changes in the CF patient population or enhanced laboratory detection using new techniques and increased surveillance. As shown in (Table 1), we have also analysed the increased number of *H. influenzae* and *H. parainfluenzae* isolates per year and the total number of isolates taken. This showed a similar trend as the increase in prevalence over the study period.

Finally, although the type of susceptibility test changed during the period of the study, the laboratory that conducted them is nationally accredited for these tests, and that during the period of the changeover from one test to another, the new test was standardised against the previous one, using national and international standards, including those of the National Committee for Clinical Laboratory Standards (NCCLS) and its successor the Clinical and Laboratory Standards

Institute (CLSI), to ensure that the results of the new and old methods were comparable to each other. While it is possible that different rates of antibiotic sensitivity may reflect changes in these methods several studies examining these different methods against a range of antibiotics and a range of organisms have generally found excellent correlation between tests with agreement approaching 95% in most studies [19,20]. In each case we have used the CLSI to interpret the susceptibility. We are confident that at all time best standard practices have been employed and thus we do not see that these changes would be likely to influence our outcomes.

Our study also provides us with a platform to consider further studies examining this epidemiology and its relationship to not only other bacterial infections of relevance in Cystic Fibrosis but also the clinical relevance of these findings and the effect of treatment. Studies examining the possible relationship between airway infection with organisms such as *Staphylococcus aureus* or *Pseudomonas aeruginosa* and *H. influenzae* and the temporal relationship between these infections would be of interest.

In summary, using a historical database, we assessed 1538 airway specimens collected from 349 CF patients over a 15-year period for the presence of *Haemophilus* species. A significant trend in the increasing annual prevalence of both *H. influenzae* and *H. parainfluenzae* was observed. Resistance to ampicillin significantly increased during the observation period for both species. In addition, resistance to amoxicillin/ clavulanic acid and co-trimoxazole in *H. parainfluenzae* also significantly increased, in contrast to a continued very low level of resistance in *H. influenzae*. While rifampicin and cefotaxime remain highly effective against *H. influenzae* and *H. parainfluenzae* isolates, our present study has shown an increasing risk of emergent antibiotic resistance to the organisms which has not previously been reported in such detail from the CF population.

Conflict of Interest Statement

No author has any financial and/or personal relationship with other people or organisations that could have inappropriately influenced their role in this work.

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