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Full Length Research Paper

Non-typable *Haemophilus influenzae* (NTHi) is a dominant strain causing invasive diseases in Taiwan

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To determine the relations between *Haemophilus influenzae* (Hi) genotypes (antibiotic resistance and active IgA1 protease) and infectious diseases, strains from infected blood, pus, sputum, bronchial washing and thorax patient samples with invasive diseases were cloned, and assayed for IgA1 protease activity and the enzymic subtype, as well as antibiotic resistance. Clinic samples of patients aged 1 to over 71 with invasive diseases of pneumonia, sinusitis, bacteremia, bronchitis, chronic obstructive of pulmonary diseases (COPD), conjunctivitis or otitis media were analyzed. Results showed that all Hi isolates contained IgA gene, but only 80% contained active IgA1 protease. Majority of Hi isolates (84%) are non-typable *Haemophilus influenzae* (NTHi), suggesting that NTHi had become major population in causing invasive diseases. Protease assays showed that 76% NTHi and 85% *Haemophilus influenzae* (THi) contained active IgA1 protease. Pulse-field agarose gel electrophoresis (PFGE) analysis showed that none of the Hi isolates had identical genome. Phenotypic comparison of bacterial strains showed a weak relation between active IgA1 protease and antibiotic resistance. Deoxyribonucleic acid (DNA) sequencing showed that mutations in silent IgA gene are common in Hi isolates. In conclusion, the antibiotic resistance and active IgA1 protease are two essential but independent phenotypes for NTHi infection and colonization.

Key words: *Haemophilus influenzae* (Hi), IgA1 protease, invasive diseases, non-typable *Haemophilus influenzae* (NTHi), typable *Haemophilus influenzae* (THi).

INTRODUCTION

Haemophilus influenzae (Hi) is known to cause invasive diseases. Hi contains non-typable (NTHi) and typable (THi), which may be classified by polymerase chain reaction (PCR) for bex gene (Falla et al., 1994). Systemic infections in children are primarily caused by Hi possessing the type b capsule (Hib), thus it was listed as an endemic bacterium in clinical assessments; Respiratory infections are primarily caused by NTHi (Murphy and Sethi, 1992; Geme, 1993). The dominant strain causing invasive diseases, however, requires evaluation after the use of vaccines against Hib.

NTHi is a common commensal organism in the human upper respiratory tracts and an important cause of localized respiratory tract disease (Centers for Disease Control and Prevention, 2002), and has become major bacterial population causing invaded diseases in Taiwan during 2004 to 2009. The infection pathway and the pathogenesis of the diseases begin with bacterial colonization of the nasopharynx, a process that involves establishment on the mucosal surface and evasion of local immune mechanisms (Rao et al., 1999). Cleavage of IgA1 on the mucosal surface enables the NTHi to

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Abbreviations: PFGE, Pulse-field agarose gel electrophoresis; **Hi,** *Haemophilus influenza*; **COPD,** chronic obstructive of pulmonary diseases; **THi,** *Haemophilus influenza*; **NTHi,** non-typable *Haemophilus influenza*; **DNA,** deoxyribonucleic acid; **PCR,** polymerase chain reaction; **Kbp,** kilo base pair.

parasitize the mucous membranes of the human host (Kilian et al., 1995; Kett et al., 1986).

It is likely that the resulting Fab fragment of IgA1, which retains its ability to bind the antigen, loses its biologic functions (Mulks et al., 1982; Poulson et al., 1992). Thus, IgA1 protease is believed to be a virulence determinant in bacterial pathogenesis (Kilian et al., 1996). It is not clear, however, the evolutionary significance of IgA gene in Hi. Relations between marker genes and phenotypes of Hi were characterized (Nørskov-Lauritsen et al., 2009), and found association of IS1016 with the hia adhesin gene and biotypes V and I in invasive NTHI (Satola et al., 2008). Antibiotic treatment plays an essential role in managing Hi invasive diseases. For years, ampicillin was the cornerstone of therapy. Amp^R Hi was first reported in the 1970s and during the following decades it steadily increased (Jacobs et al., 2003). Therapeutic options in the treatment of Hi meningitis included chloramphenicol in combination with ampicillin and, more recently, third-generation cephalosporins or carbapenems in the treatment of other invasive diseases (Jacobs et al., 2003). Hi invasive disease is potentially life threatening, antimicrobial susceptibility surveillance studies are therefore required in order to identify the genotype of virulence. We report new characterization results of NTHi in Taiwan. The work includes the identification of domi-nant strain causing invasive diseases, the evolutionary significance of IgA gene, relations between active IgA1 protease and antibiotic resistance, and Amp^R and β-lactamase.

MATERIALS AND METHODS

Patients and bacterial strains

Bacterial strains including Hi were clinically isolated from blood, nasal pus, sputum or bronchial washing from patients aged 1 to 94 for the present study. The patients were admitted to the hospital or visited outpatient section of the Chang-Gung Memorial Hospital-Kaohsiung Medical Centre, and all of them with invasive diseases. The bacterial strains were isolated and identified on the basis of laboratory standard methodology (Muray et al., 1999).

Bacterial deoxyribonucleic acid (DNA) extraction

Colonies of Hi strains sub-cultured for 18 to 20 h on chocolate agar medium (Falla et al., 1994) were picked up and suspended in 50 µl of ddH_2O, boiled at 100°C for 3 min. Debris were removed by a centrifugation at 2000×g for 2 min. The supernatants were aspirated and stored at 20°C for further use.

Identification of Hi types by polymerase chain reaction (PCR)

Primers (~20 bp in length) specific for bex A gene were designed (Falla et al., 1994) and used to identify Hi types. PCR reaction contained 1 µM of each paired primers, 1×Taq buffer, 3 µl of dNTP mix (200 µM of each dNTP), 1.5 µl of template DNA and 0.5 U of Taq polymerase (Takara Bio Inc., Japan). Reactions were set to 25 cycles to complete.

IgA1 protease activity assays

Human plasma IgA1-λ (Calbiochem-Novabiochem, USA) dialysed against 50 mM Tris-HCl (pH 7.5) was mixed with bacterial washing in the same buffer and incubated at 37°C. The reaction was stopped with reducing SDS-PAGE buffer, boiled for 3 min and separated in 10% SDS-PAGE. Protein bands were visualized by staining with Coomassie blue.

Pulse-field agarose gel electrophoresis (PFGE)

PFGE was performed according to a modified method (Cerquetti et al., 2000). Hi DNA digested with SmaI (Roche Diagnostics) was separated on 1% agarose gel with Bio-Rad CHEF Mapper apparatus (Hercules, Calif, USA). The initial pulse time of 5 s was increased linearly to 50 s over 19 h at 6 V/cm at 13°C. Gels were then stained with EtBr and the fragment patterns were analysed with MVSP software on the basis of DNA ladders (Boehringer-Mannheim, Mannheim, Germany).

Antimicrobial susceptibility testing

Hi antibiotic susceptibility screening was performed on the standard Haemophilus test medium (Doern et al., 1991) with 5 antibiotics. Hi isolates were sorted as susceptible, intermediate, or resistant according to the criteria (Brown 1988) used by NCCLS. In the case of conflicting susceptibility data results, disk diffusion tests were replicated. The control strains were Hi ATCC49247 and ATCC51907. β-Lactamase activity was studied by the chromogenic cephalosporin test with nitrocefin as the substrate.

RESULTS

Non-typable *Haemophilus influenzae* (NTHi) in infectious *Haemophilus influenzae* (Hi) strains

Among the 225 Hi isolates, 189 were characterized to be NTHi (Table 1). No preferential infection is found between male and female, but it did prefer to infect children aged 10 (25%) and elderly people aged $>$ 61 (40%). Among the patients with invasive diseases, 84% were caused by NTHi, 16% by THi. In addition, 31% of the cloned strains were isolated from patients who suffered from sinusitis and pneumonia. Of the 38 NTHi strains, over 32% were isolated from patients with acute sinusitis. Other invasive diseases, such as bacteremia, were also caused primarily by NTHi (Table 2).

Genotyping of infectious *Haemophilus influenzae* (Hi) strains

Genome analysis based on the location of special sequence (CCCGGG.) showed that genomic constituents of Hi strains (Figure 2) were heterogeneity. All the clones under the study contain SmaI-restricted fragments extending from 10 to 120 kilo base pair (Kbp), demonstrating different CCCGGG sites at different loci. As expected, different isolates of the intra-type Hi strains

Table 1. Sources of *Haemophilus influenzae* (Hi) strains by patient's gender, age, sex and diagnosis.

Patient's feature	Number of patient (%)			
	NTHi group (n = 189)		THi group (n = 36)	
Gender				
Male	100	(53.0)	15	(43.0)
Female	90	(47.0)	20	(57.0)
Age (year) [mean ± SD]	38.3 ± 30.2		47.1 ± 33.2	
1 – 10	55	(28.0)	5	(14.0)
11 – 20	20	(11.0)	10	(29.0)
21 – 30	15	(8.0)	0	(0.0)
31 – 40	5	(3.0)	0	(0.0)
41 – 50	10	(5.0)	0	(0.0)
51 – 60	20	(11.0)	0	(0.0)
61 – 70	30	(16.0)	15	(43.0)
> 71	35	(18.0)	5	(14.0)
Diagnosis				
Pneumonia	60	(31.6)	10	(28.6)
Sinusitis	45	(34.2)	5	(14.2)
Bacteremia	30	(15.8)	10	(28.6)
Bronchitis	15	(7.9)	0	(0.0)
COPD	10	(5.3)	10	(28.6)
Conjunctivitis	5	(2.6)	0	(0.0)
Otitis media	5	(2.6)	0	(0.0)

SD, Standard deviation; n, number.

Table 2. Serotypes of Hi isolates and the sources of clinical samples.

Serotype	Number of isolate				
	Blood (n = 75)	Thorax (n = 5)	Pus (n = 80)	Sputum (n = 40)	Bronchial washing (n = 25)
Hia	5	0	5	0	0
Hib	5	0	0	0	0
Hic	20	0	0	0	0
NTHi	45	5	75	40	25

had narrower range of heterogeneity than inter-type, whereas NTHi isolates had wide range of heterogeneity.

Types of IgA1 protease

All isolated Hi strains contained a copy of IgA gene in their genome, but only 80% showed protease activity. Approximately 76% of NTHi and 85% of THi strains contained the protease activity (Table 3). There are only 2 types (types 1 and 3) of IgA1 protease identified in all Hi isolates (Figure 1).

Antimicrobial susceptibility of infectious *Haemophilus influenzae* (Hi) strains

Susceptibility tests of all the Hi isolates to 5 antimicrobial agents showed that Amp^R was the most frequently detected phenotype (Table 4). All of the Amp^R Hi isolates were β-lactamase producers. Although both Hib and NTHi strains demonstrated high rate of Amp^R, the difference of higher NTHi Amp^R rate (58%) than Hib (14.3%) is significant (P = 0.046).

DISCUSSION

Non-typable *Haemophilus influenzae* (NTHi) is dominant in infectious *Haemophilus influenzae* (Hi)

Hi is responsible for a variety of localized respiratory tract infections and invasive diseases (that is, meningitis, septicemia, epiglottitis, and septic arthritis), which are associated with virulent Hib, however, other serotypes or

Table 3. The types of IgA1 protease with serotype of *Haemophilus influenzae* (Hi) strains from clinical samples.

Source	Type	Number	IgA1 protease	
			Type 1	Type 3
Blood (n = 75)	NTHi	45	15	20
	Hia	5	5	0
	Hib	5	5	0
	Hic	20	10	5
Pus (n = 16)	NTHi	75	15	30
	Hia	5	0	1
Sputum (n = 8)	NTHi	40	15	25
Bronchial washing (n = 5)	NTHi	25	15	10
Thorax (n = 1)	NTHi	5	5	0

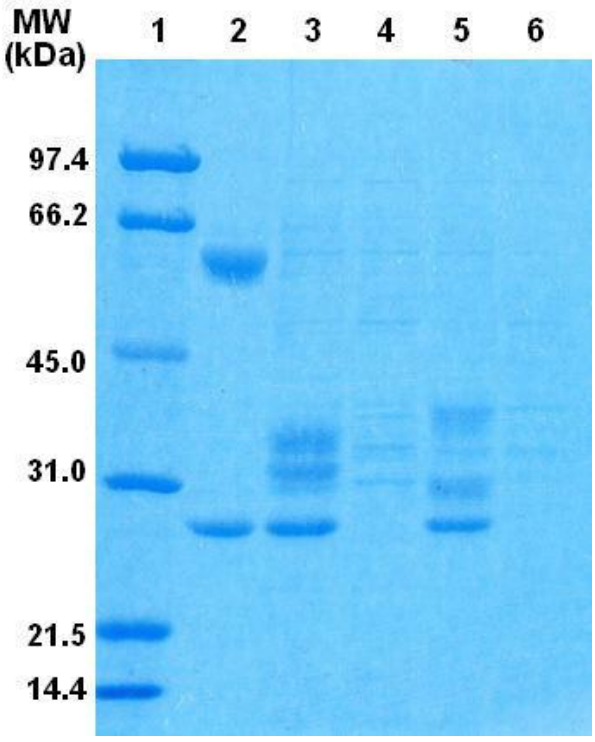

Figure 1. SDS-PAGE of human IgA1-λ digested with NTHi extracts. Reactions contained 3 ∝g of human IgA1-λ only (lane 1), 3 ∝g of human IgA1-λ and NTHi 465 extract (lane 2), NTHi 465 extract only (lane 3), 3 ∝g of human IgA1-λ and NTHi 500 extract (lane 4) and NTHi 500 clone only (lane 5). Incubation was carried out at 37°C and stopped with SDS-PAGE buffer before separation on 10% SDS-PAGE. Protein bands were visualized by staining with Coomassie blue.

non encapsulated strains have also been found responsible (Adams et al., 1993; Slack et al., 1998). This study showed that NTHi is dominant among infectious Hi, particularly in children and elderly patients. The extensive use of Hib vaccine may produce increasingly invasive Hi diseases (Peltola et al., 2005; McVernon et al., 2003), due to spontaneous capsule-deficient mutants of serotype b (b− strains), since these strains are not susceptible to antibodies elicited by the vaccine. We showed that carriage rates of NTHi in all patients are high, but concurrent colonization with Hib is also present. Taking away the rates of concurrent colonization with Hib and other THi, NTHi is still a dominant strain causing invasive diseases (Table 1).

Heterogeneity is common among *Haemophilus influenzae* (Hi) strains

The invasive Hi isolates analyzed by PFGE showed considerable heterogeneity (Figure 2) among NTHi and THi strains. The results also demonstrated that nucleotide mutations had occurred frequently in different types of THi and even among the different isolates of the same type Hi. More variations may occur in different isolates of NTHi than some strains between NTHi and THi (Figure 2). Cross analysis of DNA variation with particular invasive disease, however, showed no close relation to each other.

Active IgA1 protease is common among *Haemophilus influenzae* (Hi) strains

PCR results for IgA gene showed that all the Hi strains isolated from patients with invasive diseases contained a copy, but 20% of them failed to express. The most frequent mutations are point mutations to introduce stop codons and single nucleotide deletion to cause frame shift. This may suggest a trend of evolutionary significance that more potent pathogenic bacteria contain the enzymic activity, and more importantly IgA1 protease may help the invading bacterium to survive. Human IgA1-λ was used to assay for cleavage types of IgA1 protease.

Table 4. Antimicrobial susceptibility of 225 invasive *Haemophilus influenzae* (Hi) strains to 5 antimicrobial agents.

Antibiotics	*Haemophilus influenzae* (Hi)											
	NTHi group (n = 189)						THi group (n = 36)					
	Susceptible (%)		Intermediate (%)		Resistant (%)		Susceptible (%)		Intermediate (%)		Resistant (%)	
Ampicillin	42.0	P 36.8 NP 5.2	0.0	P 0.0 NP 0.0	58.0	[c]P 23.8 [d]NP 34.2	57.0	P 42.8 NP 14.2	0.0	P 0.0 NP 0.0	43	P 43.0 NP 0.0
AMC[a]	92.0	P 57.8 NP 34.2	0.0	P 0.0 NP 0.0	8.0	P 2.6 NP 5.4	85.7	P 71.4 NP 14.3	0.0	P 0.0 NP 0.0	14.3	P 14.3 NP 0.0
Ceftriaxone	97.4	P 57.9 NP 39.5	0.0	P 0.0 NP 0.0	2.6	P 2.6 NP 0.0	100.0	P 85.7 NP 14.3	0.0	P 0.0 NP 0.0	0.0	P 0.0 NP 0.0
[b]SXT	42.1	P 26.3 NP 15.8	0.0	P 0.0 NP 0.0	57.9	P 34.2 NP 23.7	42.9	P 42.9 NP 0.0	0.0	P 0.0 NP 0.0	57.1	P 42.8 NP 14.3
Tetracycline	58.0	P 42.2 NP 15.8	5.2	P 2.6 NP 2.6	36.8	P 15.8 NP 21.0	100.0	P 85.7 NP 14.3	0.0	P 0.0 NP 0.0	0.0	P 0.0 NP 0.0

[a]AMC, Amoxicillin/clavulanic acid; [b]SXT, sufamethoxazole-trimethoprim; [c]P, IgA1 protease activity; [d]NP, no IgA1 protease activity.

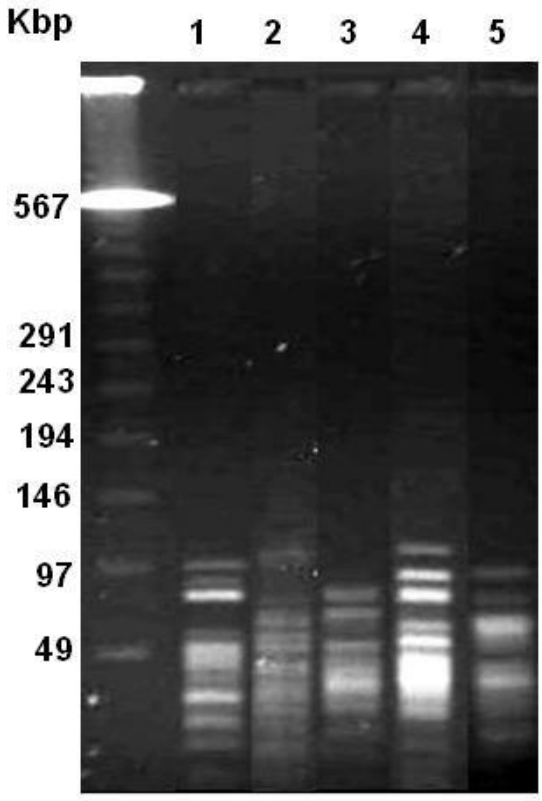

Figure 2. PFGE patterns of genomic DNA from Hi isolates. Chromosomal DNA was digested with restriction endonuclease *Sma*I. Lane 1, Hia; Lane 2, Hib; Lane 3, Hic; Lanes 4, and 5, NTHi strains; M, λ-ladder pulsed-field gel marker.

Results (Table 3 and Figure 1) showed that there were only 2 distinguishable types of IgA1 protease (types 1 and 3) present in both NTHi and THi strains although suggestion of 3 types in NTHi had been made (Mulks et al., 1982). About 78% of the invasive NTHi from blood samples and 60% from pus contain the enzyme. Interestingly, all the isolates from respiratory tracks contain the enzyme, indicating that IgA1 protease activity plays a role in infections and colonization.

Amp^R depends on the presence of β-lactamase

All strains under the current study that are Amp^R contain β-lactamase activity, whereas sss10% of AmpS isolates contain the enzyme, suggesting that AmpR depends largely on the presence of β-lactamase. Earlier studies had suggested that β-lactamase negative and AmpR were relatively uncommon among non encapsulated strains (Doern et al., 1991; 1997; Seki et al., 1999). In this study however, more frequent Amp^R isolates among NTHi than among the Hib was significantly associated.

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