Original Article

Rapid Detection of *Haemophilus influenzae* and *Haemophilus parainfluenzae* in Nasopharyngeal Swabs by Multiplex PCR

TIAN Guo Zhong[1,△], ZHANG Li Juan[2,△], WANG Xiao Lei[3], ZHANG Li[4], LI Shu Feng[5], GU Chang Mei[5], SUN Jian[5], and CUI Bu Yun[1,#]

1. State Key Laboratory for Infectious Diseases Control and Prevention, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China; 2.Department of Physiology and Pathophysiology, Basic Medical Sciences School of Capital Medical University, Beijing 100069, China; 3. The Third People's Hospital of Chengdu City, Chengdu 610031, Sichuan, China; 4. Chengdu Children Hospital, Chengdu 610017, Sichuan, China; 5. Feicheng Mining Group Company Health and Epidemic Prevention Station, Feicheng 271608, Shangdong, China

Abstract

Objective To establish multiplex PCR-based assays for detecting *H.influenzae* and *H.parainfluenzae*. And the PCR-based assays were applied to detect the carriage rates of *H.influenzae* and *H.parainfluenzae* in nasopharyngeal swab specimens which were collected from healthy children.

Methods Multiplex primers for species-specific PCR were designed by using DNAstar soft based on the sequences of 16S rRNA genes from genus Haemophilus to detect *H.influenzae* and *H.parainfluenzae.*

Results The sensitivity of the 16S rRNA PCR assay for detecting *H.influenzae* and *H.parainfluenzae* was 97.53% and 100% respectively, and the specificity was 95.89% and 96.63% respectively. Youden's Index on the ability to detect *H.influenzae* and *H.parainfluenzae* was 0.9342 and 0.9663 respectively. 666 nasopharyngeal swab specimens were collected from healthy children. The detection rates of *H.influenzae* and *H.parainfluenzae* were 14.11% and 16.07% respectively by using isolation and culture methods. The detection rates of *H.influenzae* and *H.parainfluenzae* were 43.54% and 57.96% respectively by 16S rRNA PCR assays. The carriage rates of serotypes a, b, c, d, e, f and non-typeable isolates were 0% (0/666), 0.15% (1/666), 1.20% (8/666), 0.15% (1/666), 1.20% (8/666), 1.80% (12/666), 95.50% (636/666) respectively.

Conclusion The multiplex PCR assays were very rapid, reliable and feasible methods for detection of *H.influenzae* and *H.parainfluenzae* in pharyngeal swab specimens which were compared to conventional isolation and culture methods. 95.5% of *H.influenzae* strains in healthy children were nontypeable. The encapsulated or typable strains were mainly three serotypes which was c, e, and f serotype.

Key words: *H.influenzae*; *H.parainfluenzae*; Multiplex PCR

Biomed Environ Sci, 2012; 25(3):367-371 doi:10.3967/0895-3988.2012.03.016 ISSN:0895-3988

www.besjournal.com(full text) CN: 11-2816/Q

INTRODUCTION

The family Pasteurellaceae comprises genera Huemophilus, Pasteurella, and Actinobacillus. The genus Haemophilus includes a number of species that caused a wide variety of infections but share a common morphology and a requirement for blood-derived factors during growth that has given the genus its name. Haemophilus influenzae, a Gram negative bacillus or coccobacillus, is a frequent

[#]Corresponding author: CUI Bu Yun. Tel: 86-10-58900767. Fax: 86-10-58900700. E-mail: cuibuyun@icdc.cn

[△]The first two authors contributed equally to this article.

Biographical note of the first authors: TIAN Guo Zhong, male, born in 1965, Professor, majoring in pathogen biology; ZHANG Li Juan, female, born in 1975, DM, majoring in Pathophysiology.

Received: April 13, 2011; Accepted: September 8, 2011

isolate from the upper respiratory tract of healthy individuals. It can be recovered from the nasopharynx of up to 80% of the normal population. The major pathogen, can be separated into encapsulated or typable strains, of which there are seven types (a through f) based on the antigenic structure of the capsular polysaccharide, and unencapsulated or nontypable strains. Type b *H.influenzae* is by far the most virulent organism in this group, commonly causes bloodstream invasion and meningitis in children younger than 2 years. Nontypable strain is frequent causes of respiratory tract disease in infants, children, and adults. *Haemophilus* parainfluenzae causes pneumonia or bacterial ndocarditis. Other *Haemophilus* species cause disease less frequently. *Haemophilus* ducreyi caused chancroid. *Haemophilus aphrophilus* is a member of the normal flora of the mouth and occasionally caused bacterial endocarditis. Haemophilus aegyptius, which causes conjunctivitis and Brazilian purpuric fever, and *Haemophilus haemolyticus* used to be separated on the basis of their ability to agglutinate or lyse red blood cells, but both were now included among the nontypable *H.influenzae* strains[1-4]. The polyribosylribitol phosphate Hib conjugate vaccine was introduced into China in 1998. However, by far no data on active laboratory-based surveillance of invasive *H.influenzae* diseases are available in China. The detection of *H.influenzae* and *H.parainfluenzae* is crucial for predicting levels of disease, reducing incidence rate, and ultimately preventing disease. The standard laboratory tests involving isolation and culture methods are laborious and time consuming, and carriage rates are likely underestimated[5]. Molecular surveillance of these pathogens is required. Here we describe a set of species-specific multiplex PCR assays to detect H.influenzae and H.parainfluenzae in nasopharyngeal swab specimens.

MATERIALS AND METHODS

Nasopharyngeal Swab Samples

Nasopharyngeal swab samples were collected from 666 healthy children aged between one and five years old from Jiangsu, Sichuan, Heilongjiang, and Liaoning provinces in China. Participants had not previously received Hib vaccination. The nasopharyngeal swab samples were immediately grown on selective chocolate agar plates supplemented with 7% defibrinated horse blood and vancomycin 500 mg/L and plates. And were placed under microaerobic conditions (N_2 85%, CO_2 7%, O_2 5%) and incubated at 37 °C for 36 h as described previously[5]. Four Haemophilus-like colonies from per swab were selected for isolation and identification. An isolate was identified as *H. influenzae* or *H.parainfluenzae* on the basis of colony morphology, Gram stain, requirement for hemin and NAD, synthesis of porphyrin, and hemolytic activity, And sequence analysis of the 16S rRNA gene[5-6]. Capsule types (a-f) of *H.influenzae* were determined by using primer pairs for each capsular type. Primer pairs and the PCR reactions were as described by Falla et al.[7]. Mixed culture from each swab sample was scraped from chocolate agar plates by using a sterile cotton swab and resuspended in 1 mL of TE buffer (10 mmol/L Tris HCl, 1 mmol/L EDTA [pH 8.0]). The mixed bacteria was heated at 100 °C for 20 min and then centrifuged for 15 min at 15 000×g[8-9]. 400 µL of the supernatant was removed were used as template DNA for PCR. The genomic DNA of the identified isolates was extracted by using a QIAGEN DNA extraction kit (QIAGEN, Valencia, CA, USA) and was preserved at −20 °C.

Multiplex PCR Assays Design

The nucleic acid sequences of the 16S rRNA genes from the genus Haemophilus, included *H.influenzae, H.parainfluenzae, H.aphrophilus, H.paraphrophilus, H.segnis, H.haemolyticus, H.parahaemolyticus,* and *H.actinomycetemcomitans* were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Species-specific PCR primers were designed by using DNAstar soft. The sequences of all primers were listed in Table 1. *H.influenzae*-specific PCR assays by three sets of primers to detect serotypes a-d and non-typeable, serotype e, and serotype f isolate respectively. The primers for detecting *H. influenzae* based on the coding P6 protein gene assay was also used as previously described by Ketel et al.[3]. The *H.parainfluenzae* isolate was detectd by PCR assay required two sets of primers: H-para-F and H-para-R-1, and H-para-F and H-para-R-2. Universal 16S rRNA gene primers were also used as previously described[10].

The *H.influenzae* was detected by PCR assay need to carry out three reactions, each using a different set of primers. *H.parainfluenzae* was detected by two set of primers in two reactions. PCRs were performed in 20 uL volumes. PCR mixture contained 100 ng of genomic DNA, each primer at 0.5 mmol/L, each deoxynucleoside triphosphate at 100 mmol/L, and 1 U of Taq DNA polymerase in a final volume of 25 µL. The amplification protocol

included 22 cycles of PCR. An initial denaturation step of 5 min at 95 °C was followed by 30 cycles of denaturation at 94 °C for 30 s, a annealing for 30 s (the annealing temperature for each set of primers were seen in Table 1) , and extension at 72 °C for 30 s. followed by a final extension at 72 °C for 10 min. The amplified production was visualized by horizontal gel electrophoresis for 2 h at a constant 100 V in 1.4% agarose. The expected PCR amplification fragment lengths was listed in Table 1. Sixteen isolates could not be identified by using the species-specific primers. They were identified by the results of the PCR amplification were sequenced using the primers 20F and 1500 R[10].

Nucleotide Sequence Accession Numbers

The sequences of the 16S rRNA genes were determined in this study, which were submitted to the NCBI GenBank database with the following accession numbers: HQ680852–HQ680861 and HQ846512-HQ846515.

Table 1. Oligonucleotide Primers for Multiplex and Single Polymerase Chain Reaction Assays

Target Strain	Primer Name	Primer Sequence (5'–3')	Annealing Temperature	PCR Product Length (bp)
H. influenzae type a-d and non-typeable	Hi-in-F	AAAGTGCGGGACTGAGA	52 °C	313
	Hi-in-R	CCGGTGCTTCTTCTGTAT		
H. influenzae type e	Hi-in-e-F	GATGAAAGTGCGGGACTGTA	58 °C	848
	Hi-in-e-R	AACGTTCCCGAAGGCACTCC		
*H. influenza*e type f	Hi-in-f-F	GAAAGGGTGGGACTTTTA	54 °C	848
	Hi-in-f-R	CTAAGTTCCCGAAGGCACATT		
H. parainfluenzae	H-para-F	GATGAAAGTGTGGGACCTTCG	56 °C	848
	H-para-R-1	AGTTCCCGAAGGCACCAATC		
	H-para-R-2	GTTCCCGAAGGCACTCTCA		
H. influenzae (P6)	Hi-F	ACTTTTGGCGGTTACTCTGT	50 °C	273
	Hi-R	TGTGCCTAATTTACCAGCAT		
16S rRNA gene	20F	AGTTTGATCATGGCTCAG	53°C	1500
	1500R	GGTTACCTTGTTACGACTT		

RESULTS

Bacterial Isolates and Nasopharyngeal Swab Samples

In this study, 361 strains were used to evaluate the sensitivity and specificity of PCR assays, which included 162 *H.influenzae* strains (serotypes a-f and non-typeable), 83 *H.parainfluenzae* strains, 42 *H.haemolyticus* strains, each of *H.ducreyi, H.segnis, H.aegyptius, H.actinomycetemcomitans, H.parahaemolyticus, H.paraphrohaemolyticus, H.aphrophilus, H. paraphrophilus, H. pleuropheumoniae,* and *H.haemoglobinophilus*, 56 *N.meningitidis* strains (including serogroups A, B, C, W135, X, Y, Z, 29E, K, and H), 3 *N.lactose* strains, 3 *E.coli* strains, and 2 *B.catarrhalis* strains. These isolates were isolated and identified by hospitals. Identification was confirmed again in our laboratory by API NH system [API Neldent (Analytab Products, Inc., Plainview, N.Y.), and RapID NH] and sequence analysis of the 16S r RNA gene[5,7].

Sensitivity and Specificity of 16S rRNA PCR Assays

The sensitivity of the Hi-16S rRNA PCR assay to identify *H.influenzae* was 97.53% (158/162). The specificity of the Hi-16S rRNA PCR assay was 95.89% (210/219). The serotype a–d and non-typeable strains were amplified specifically using primers Hi-in-F and Hi-in-R, produced a 313 bp DNA fragment. The strain was not considered to be *H. influenzae* when the PCR band was <313 bp, or multiple bands were amplified. *H.influenzae* serotype e was amplified specifically by primers Hi-in-e-F and Hi-in-e-R. *H.influenzae* serotype f was amplified specifically using primers Hi-in-f-F and Hi-in-f-R. The amplification product for both serotype e and f was 848 bp. The sensitivity of the Hi-para-16S rRNA PCR assay to identify *H.parainfluenzae* was 100% (83/83). *H.parainfluenzae* was amplified specifically using primers Hi-para-F and Hi-para-R-1 or Hi-para-R-2. The Hi-para-16S rRNA-PCR produced a single band of 848 bp. The specificity of the Hi-para-16S rRNA PCR

assay was 96.63% (287/297). The false positive rate of the Hi-16S rRNA and Hi-para-16S rRNA PCR assays was 4.11% (9/219) and 3.37% (10/297) respectively. The false negative rate of the Hi-16S rRNA and Hi-para-16S rRNA PCR assays was 2.47% (4/162) and 0% (0/83) respectively. The Youden Index to determine the ability of the 16S rRNA PCR assays to detect *H.influenzae* and *H.parainfluenzae* was calculated as 0.9342 and 0.9663 respectively (Table 2).

P6- PCR Assays and API NH system

The Hi-F and Hi-R primers for PCR to identify*H.influenzae* based on the *H.influenzae* coding P6 protein gene were performed[3]. The resultes suggested that these primers were not very specific for *H.influenzae*. It also produced same PCR product for both *H.parainfluenzae* and *H.haemolyticus*. The sensitivity and specificity of the Hi-P6-PCR assay was 97.53% (158/162) and 86.30% (189/219) respectively. The false positive and false negative rate was 13.70% (30/219) and 2.47% (4/162) respectively. The Youden Index for the Hi-P6-PCR assay was 0.8383. The sensitivity and specificity of APINH system for identifying *H.influenzae* was 95.06% (154/162) and 88.58% (194/219) respectively. The false positive and false negative rate was 11.42% (25/219) and 4.94% (8/162) respectively. The Youden Index for APINH system for identifying *H.influenzae* was 0.8364 (Table 2).

Table 2. Comparative Results for Sensitivity and Specificity for 16S rRNA PCR, Hi-P6 PCR and APINH Assays

Experimental Assays	Sensitivity	Specificity	False Positive Rate	False Negative Rate	Youden Index
Hi-16S rRNA PCR Assay	97.53% (158/162)	95.89% (210/219)	4.11% (9/219)	2.47% (4/162)	0.9342
Hi-P6 PCR Assay	97.53% (158/162)	86.30% (189/219)	13.70% (30/219)	2.47% (4/162)	0.8383
APINH Assay	95.06% (154/162)	88.58% (194/219)	11.42% (25/219)	4.94% (8/162)	0.8364
Hi-para 16S rRNA PCR Assay	100.00% (83/83)	96.63% (287/297)	3.37% (10/297)	0.00% (0/83)	0.9663

Detection of H.influenza and H.parainfluenzae in Nasopharyngeal Swab Samples

The detection rate of *H.influenzae* in 666 nasopharyngeal swab samples was 14.11% (94/666) by isolation and identification procedures[5]. Only two strains were identified as *H.influenzae* type c and type d respectively. The detection rate of *H.parainfluenzae* was 16.07% (107/666) by isolation and identification procedures[5]. The detection rate of *H.influenzae* was 44.14% (294/666) by Hi-16S rRNA PCR assay in mixed bacterial DNA of 666 nasopharyngeal swab samples. One sample was identified as type b, eight samples were type c, one sample was type d, eight samples were type e, and 12 samples were type f by PCR assays as previously described[7]. The detection rate of *H. parainfluenzae* was 61.26% (408/666) by Hi-para-16S rRNA PCR assays in mixed bacterial DNA of 666 nasopharyngeal swab samples. The actual carriage rate of *H.influenzae* and *H.parainfluenzae* was calculated to be 43.54% (290/666) and 57.96% (386/666) respectively when the false positive and false negative PCR amplification was taken into consideration. The carriage rate of serotypes a, b, c, d, e, f, and non-typeable isolates was 0% (0/666), 0.15% (1/666), 1.20% (8/666), 0.15% (1/666), 1.20% (8/666), 1.80% (12/666), 95.50% (636/666) respectively.

DISCUSSION

Species belonging to the genus *Haemophilus* were a class of widely parasitic flora in the human upper respiratory tract. To assess the true carriage rate of *H.influenzae* and *H.parainfluenzae* in nasopharyngeal swab samples require all strains to be isolated and identified in culture medium. These procedures were laborious and time-consuming. It was maybe under-estimated on the carriage rate when all strains were not correctly cultured and identified. The *Haemophilus* 16S rRNA gene sequence was highly specific to the genus. And existed multiple copies in the genomic DNA of many species (*H. influenzae* has 6 copies). Therefore, 16S rRNA PCR assays showed good sensitivity and specificity[7,9,11-12].

The methods for identifying *H.influenzae*, such as sub-loop-mediated isothermal amplification[13], multilocus sequence analysis[14], 16S rRNA gene sequence analysis[6,9,15], and APINH system, were not suitable for detecting *H.influenzae* and *H.parainfluenzae* in sputum and nasopharyngeal swabs because they required pure bacterial isolates. The multiplex 16S rRNA PCR assay was developed to improve the detection and identification of

H.influenzae and *H.parainfluenzae* compared to traditional laboratory methods. Comprehensive evaluation indices (Youden Index) confirmed that the multiplex 16S rRNA PCR assay was superior to previous methods for identifying *H.influenzae* and *H.parainfluenzae*.

The carriage rate of *H.influenzae* have been reported to be in the range of 11.0% to 54.8% in children aged between one month and five years[17]. In this study, we found that the carriage rate of *H.influenzae* was 14.11% by standard identification procedures. But It was 43.54% by multiplex 16S rRNA PCR assays. *H.influenzae,* followed by *S.pneumoniae* and *K.pneumonia,* was one of the most common pathogenic bacteria in children with lower respiratory tract infections in the Chinese cities of Shanghai and Chengdu[16,18]. Non-typeable *H.influenzae* was also responsible for pediatric pneumonia[2]. In this study, 95.5% of *H.influenzae* strains in healthy children were nontypeable. The result likely explained the previous finding that non-typeable *H.influenzae* strain was major cause of childhood infection in China[2]. The carriage rate of serotype b has previously been found to range from 0-7%[17]. We found that the carriage rate (0.15%) of serotype b in healthy Chinese children was much lower than previously reported. Serotype f was found to be the major serotype in our test subjects. These findings were important for preventing diseases and monitoring the Hib vaccine program in China.

ACKNOWLEDGEMENTS

We thank all the participants, staff and research fellows from the Jiangsu, Sichuan, Heilongjiang, Liaoning provinces, and China CDC in China for their contributions to this study.

REFERENCES

1. Nwaohiri N, Urban C, Gluck J, et al. Tricuspid valve endocarditis caused by *Haemophilus parainfluenzae*: a case report and review of the literature. Diagn Microbiol Infect Dis, 2009; 64, 216-9.
2. Tian GZ, Zhang L, Li MC, et al. Genotypic characteristics of *Haemophilus influenzae* isolates from pediatric pneumonia patients in Chengdu city, Sichuan, China. J Microbiol, 2009; 47, 494-7.
3. van Ketel RJ, de Wever B, van Alphen L. Detection of *Haemophilus influenzae* in cerebrospinal fluids by polymerase chain amplification DNA amplification. J Med Microbiol, 1990; 33, 271-6.
4. Harrison LH, Simonsen V, Waldman EA. Emergence and disappearance of a virulent clone of *Haemophilus influenzae* biogroup aegyptius, cause of Brazilian purpuric fever. Clin Microbiol Rev, 2008; 21(4): 594-605.
5. Murray PR, Baron EJ, Jorgensen JH, et al. Manual of Clinical Microbiology (8nd Edition). ASM Press, Washington, 2003; p623-5.
6. Sacchi CT, Alber D, Dull P, et al. High level of sequence diversity in the 16S rRNA genes of *Haemophilus influenzae* isolates is useful for molecular subtyping. J Clin Microbiol, 2005; 43, 3734-42.
7. Falla TJ, Crook DW, Brophy LN, et al. PCR for capsular typing of *Haemophilus influenzae*. J Clin Microbiol, 1994; 32: 2382-6.
8. Quentin R, Ruimy R, Rosenau A, et al. Genetic identification of cryptic genospecies of Haemophilus causing urogenital and neonatal infections by PCR using specific primers targeting genes coding for 16S rRNA. J Clin Microbiol, 1996; 34, 1380-5.
9. Benson C, Gantt S, Zerr DM, et al. Use of 16S ribosomal DNA polymerase chain amplification to identify *Haemophilus influenzae* type b as the etiology of pericarditis in an infant. Pediatr Infect Dis J, 2005; 24, 287-8.
10. Wiklund T, Madsen L, Bruun MS, et al. Detection of *Flavobacterium psychrophilum* from fish tissue and water samples by PCR amplification. J Appl Microbiol, 2000; 88, 299-307.
11. Ezaki T. RNA amplification from low numbers of bacteria in human blood to solve low sensitivity problem of conventional PCR amplification. Rinsho Byori, 2006; 54, 1055-8.
12. Das I, DeGiovanni JV, Gray J. Endocarditis caused by *Haemophilus parainfluenzae* identified by 16S ribosomal RNA sequencing. J Clin Pathol, 1997; 50, 72-4.
13. Torigoe H, Seki M, Yamashita Y, et al. Detection of *Haemophilus influenzae* by loop-mediated isothermal amplification (LAMP) of the outer membrane protein P6 gene. Jpn J Infect Dis, 2007; 60, 55-8.
14. Meats E, Feil EJ, Stringer S, et al. Characterization of encapsulated and noncapsulated *Haemophilus influenzae* and determination of phylogenetic relationships by multilocus sequence typing. J Clin Microbiol, 2003; 41, 1623-36.
15. Xu J, Crowe M, Millar BC, et al. Non-culturable *Haemophilus influenzae* meningitis identified by 16S rDNA PCR and sequencing. Ir J Med Sci, 2004; 173, 57-9.
16. Wang DG, Li Y, Zhao Q, et al. Laboratory study on the test of *Haemophilus influenzae* and *Haemophilus parainfluenzae* on children in-patient from pediatrics. Chinese J Microecology, 2006; 18, 302-3.
17. Muge OA, Zeynep B, Cem A. Carriage rate of *Haemophilus influenzae* among preschool children in Turkey. Jpn J Infect Dis, 2007; 60, 179-82.
18. Huang C, Wang XL, Zhang L, et al. Distribution and drug resistance of pathogenic bacteria in children with lower respiratory tract infection from Chengdu Children's Hospital between 2001 and 2006. Zhong guo Dang Dai Er Ke Za Zhi, 2008; 10, 17-20.