JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2004, p. 3813–3815 Vol. 42, No. 8
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.8.3813–3815.2004

Detection of *Haemophilus influenzae* Type b by Real-Time PCR

Adrian Marty,[1] Oliver Greiner,[1] Philip J. R. Day,[1,2] Sibylle Gunziger,[1] Kathrin Mühlemann,[3] and David Nadal[1]*

Division of Infectious Diseases[1] and Division of Oncology,[2] University Children's Hospital of Zurich, CH-8032 Zurich, and Institute of Medical Microbiology, University of Berne, CH-3010 Berne,[3] Switzerland

Received 22 January 2004/Returned for modification 24 February 2004/Accepted 30 April 2004

A real-time PCR assay targeting the capsulation locus of *Haemophilus influenzae* type b (Hib) was developed. The linear detection range was from 1 to 10^6 microorganisms per reaction mixture. No *H. influenzae* other than Hib or any other control bacteria typically found in the upper respiratory tract was detected.

Haemophilus influenzae type b (Hib) can cause severe disease predominantly in children (25). Nasopharyngeal carriage is of paramount importance in the transmission of Hib. Conjugate Hib vaccines decrease carriage of Hib (2). However, a reemergence of invasive disease caused by Hib (8, 9, 19, 21, 26, 31) has cast doubt on the current vaccination strategies and emphasizes the need for surveillance of Hib (19).

Assessment of Hib carriage using cultures of nasopharyngeal specimens gives an accurate picture of the epidemiological status of the bacteria (1, 3–5, 22, 30). Hib bacteria are identified by biochemical tests, followed by serotyping for capsular antigen, but the latter is troublesome and ambiguous (18). Genotyping and ribotyping are more accurate (7, 17, 18, 23). We established a highly sensitive and specific assay based on real-time PCR (TaqMan) to detect Hib.

The bacterial strains used were five Hib and five nontypeable *H. influenzae* strains already characterized by capsular genotyping and ribotyping (23), *H. influenzae* type a (ATCC 9006), *H. influenzae* type c (gift of Loek van Alphen, Amsterdam, The Netherlands), *H. influenzae* type d (ATCC 9332), *H. influenzae* type e (ATCC 8142), *H. influenzae* type f (ATCC 9833), 15 randomly selected invasive *H. influenzae* strains, *Streptococcus pneumoniae* (ATCC 49619), *S. pneumoniae* serotypes 6A and 6B, *Streptococcus oralis* (ATCC 10557), *Neisseria meningitidis* types B and C, *Neisseria lactamica*, *Moraxella catarrhalis*, *Moraxella lacunata*, and *Moraxella phenylpyruvarica*. Capsular genotyping of *H. influenzae* was performed by the method of Kroll et al. (17) as modified by Mühlemann et al. (23) and serotyping by slide agglutination with antiserum polyvalent for types a to f and specific for type b (Difco Laboratories, Detroit, Mich.) plus antiserum polyvalent for type a, types c to f, and specific for type b (Phadebact; Boule Diagnostics AB, Huddinge, Sweden).

Genomic DNA was extracted as described previously (13). The primers and the fluorogenic minor groove binding probe for region II of the capsulation locus (32) of Hib (GenBank accession no. X78559) were designed using Primer Express software, version 1.5 (Perkin-Elmer, Applied Biosystems, Foster City, Calif.) and synthesized by Microsynth GmbH (Balgach, Switzerland) and Applied Biosystems (OligoFactory, Weiterstadt, Germany), respectively. The oligonucleotide sequences used were as follows: forward primer, 5′-CAAGATA CCTTTGGTCGTCTGCTA-3′ (positions 5481 to 5504); reverse primer, 5′-TAGGCTCGAAGAATGAGAAGTTTTG-3′ (positions 5631 to 5607). The probe was 5′-ATGATATGGG TACATCTGTT-3′ (positions 5563 to 5582). The fluorescent reporter dye at the 5′ end of the probe was 6-carboxyfluorescein (FAM), and the quencher at the 3′ end was 6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine (TAMRA). The principle of real-time PCR utilizing fluorescence resonance energy transfer has been described extensively (13). PCR amplification was performed in 25-μl reaction mixture volumes containing 2× TaqMan universal master mix (Perkin-Elmer Biosystems), 400 nM concentration of each primer, 200 nM probe, and 1 μl of DNA extract. PCR amplification was performed on duplicate samples, using an ABI PRISM 7700 sequence detection system. Standard amplification parameters were 2 min at 50°C and 10 min at 95°C, followed by 40 temperature cycles, with 1 cycle consisting of 15 s at 95°C and 1 min at 60°C. Real-time data were analyzed with Sequence Detection Systems software, version 1.7.

The sensitivity and detection range of the assay were determined by generation of standard curves for five reference Hib strains: bacteria cultured at 37°C for 24 h were displaced from the blood chocolate agar plates using saline (0.9% NaCl), diluted with saline to a McFarland standard of 0.5 (10^8 microorganisms/ml), and subjected to 10-fold serial dilutions in saline. One milliliter of each dilution was used for DNA extraction, followed by in vitro gene amplification. The calculated cycle threshold (C_T) values were plotted against the numbers of microorganisms per microliter (13). The assay detected each of the strains (Table 1) linearly from 1 to 10^6 organisms per reaction mixture, with C_T values (mean ± standard deviation) of 35.1 ± 0.8, 31.6 ± 0.3, 28.5 ± 0.6, 23.7 ± 0.8, 19.6 ± 0.8, 17.1 ± 0.6, and 15.3 ± 0.5, respectively. The intra- and interassay variability of C_T values employing replicates from the same DNA extraction per dilution or replicates from DNA extracted from different dilution series was <1 (data not shown). To determine the specificity of the assay, bacteria other than Hib (10^6 organisms of each strain per reaction mixture) were tested. Nontypeable *H. influenzae*, *H. influenzae* type a, types c to f (Table 1), and any other control bacteria were not detected. PCR of a dilution series of Hib was not

* Corresponding author. Mailing address: Division of Infectious Diseases, University Children's Hospital of Zurich, Steinwiesstr. 75, CH-8032 Zurich, Switzerland. Phone: 41-1-266-7562. Fax: 41-1-266-7157. E-mail: david.nadal@kispi.unizh.ch.

TABLE 1. Comparison of capsular genotyping, serotyping by agglutination, and detection of Hib by real-time PCR

Organism	Strain	Origin of isolate[a]	Capsular genotyping[b]	Capsular serotyping[b]		Real-time PCR for Hib
				Difco antisera	Phadebact antisera	
Reference *H. influenzae* strains[c] (n = 10)	734		b	b	b	Positive
	737		b	b	b	Positive
	760		b	b	b	Positive
	777		b	b	b	Positive
	784		b	b	b	Positive
	774		nt	b	nt	Negative
	776		nt	b	nt	Negative
	783		nt	b	nt	Negative
	778		nt	nt	nt	Negative
	788		nt	nt	nt	Negative
Control *H. influenzae* strains	ATCC 9006		a	Non-b	Non-b	Negative
			c[d]	Non-b	Non-b	Negative
	ATCC 9332		d	Non-b	Non-b	Negative
	ATCC 8142		e	Non-b	Non-b	Negative
	ATCC 9833		f	Non-b	Non-b	Negative
Invasive *H. influenzae* isolates (n = 15)	158	BC	b	b	b	Positive
	163	JA	b	b	b	Positive
	339	CSF	b	b	b	Positive
	426	JA	b	b	b	Positive
	505	BC	b	b	b	Positive
	559	BC	b	b	b	Positive
	572	BC	b	b	b	Positive
	639	BC	b	b	b	Positive
	671	JA	b	b	b	Positive
	990	BC	b	b	b	Positive
	229	BC	nt	b	nt	Negative
	509	CSF	nt	b	nt	Negative
	644	BC	nt	b	nt	Negative
	380	CSF	nt	nt	nt	Negative
	386	BC	nt	nt	nt	Negative

[a] BC, blood culture; JA, joint aspirate; CSF, cerebrospinal fluid.
[b] nt, nontypeable.
[c] Reference *H. influenzae* strains from reference 23.
[d] A control *H. influenzae* type c strain was kindly provided by Loek van Alphen, Amsterdam, The Netherlands.

inhibited by spiked DNA from nasopharyngeal secretions from four patients shown to be devoid of *Haemophilus* spp. by culturing.

The real-time PCR assay detected all 10 of the invasive *H. influenzae* isolates identified by capsular genotyping as Hib strains, but none of those identified as non-Hib strains. Serotyping also identified all Hib strains, but the Difco antisera identified three reference and three invasive *H. influenzae* strains as Hib, all identified as non-Hib strains by genotyping (Table 1).

PCR assays detecting Hib reported so far are either conventional endpoint or multiplex assays (10, 15, 16, 29). The assay reported here is the first to be based on real-time (TaqMan) technology. The linear detection range of microorganisms, sensitivity, and intra- and interexperimental reproducibility were similar to those from real-time PCR assays for the detection of *S. pneumoniae* (13), *N. meningitidis* types B and C (11), *M. catarrhalis* (12), *Mycoplasma pneumoniae* (14), *Porphyromonas gingivalis* (20), *Borrelia burgdorferi* (24), and *Mycobacterium tuberculosis* (6). Moreover, the assay was highly specific for Hib, since there was no amplification of DNA from a plethora of other bacteria or of DNA from *S. pneumoniae*, including serotypes 6A and 6B that are known to cross-react with antibodies to the Hib capsule (27, 28). Note that real-time PCR assay results for Hib were in full agreement with capsular genotyping results, whereas the results of serotyping with Difco antisera were not. A recent study revealed that 68% of *H. influenzae* isolates identified as Hib by serotyping did not contain the correlating capsule type genes (18). Finally, the efficiency of PCR was not inhibited by components of nasopharyngeal secretions.

The assay can be completed within 1 day, is faster than conventional culturing and identification, and therefore provides a potential reliable diagnostic tool for Hib and is able to assess nasopharyngeal carriage of Hib.

We thank Martin Altwegg and Jacqueline Lüthy for superb advice and Kristin Ladell for helpful comments on the manuscript.

This study was partly supported by the Swiss National Foundation (grant 31-55553.98), the Müller-Grocholski-Stiftung, and an unrestricted grant from AstraZeneca Switzerland.

REFERENCES

1. **Adegbola, R. A., E. K. Mulholland, O. Secka, S. Jaffar, and B. M. Greenwood.** 1998. Vaccination with a *Haemophilus influenzae* type b conjugate vaccine reduces oropharyngeal carriage of *H. influenzae* type b among Gambian children. J. Infect. Dis. **177:**1758–1761.
2. **Barbour, M. L.** 1996. Conjugate vaccines and the carriage of *Haemophilus influenzae* type b. Emerg. Infect. Dis. **2:**176–182.
3. **Barbour, M. L., R. Booy, D. W. Crook, H. Griffiths, H. M. Chapel, E. R. Moxon, and D. Mayon-White.** 1993. *Haemophilus influenzae* type b carriage

and immunity four years after receiving the *Haemophilus influenzae* oligosaccharide-CRM197 (HbOC) conjugate vaccine. Pediatr. Infect. Dis. J. **12:** 478–484.
4. **Barbour, M. L., D. Mayon-White, R. E. Coles, D. W. M. Crook, and E. R. Moxon.** 1995. The impact of conjugate vaccine on carriage of *Haemophilus influenzae* type b. J. Infect. Dis. **171:**93–98.
5. **Dabernat, H., M. A. Plisson-Saune, C. Delmas, M. Seguy, G. Faucon, R. Pelissier, H. Carsenti, C. Pradier, M. Roussel-Delvallez, J. Leroy, M. J. Dupont, F. De Bels, and P. Dellamonica.** 2003. *Haemophilus influenzae* carriage in children attending French day care centers: a molecular epidemiological study. J. Clin. Microbiol. **41:**1664–1672.
6. **Desjardin, L. E., Y. Chen, M. D. Perkins, L. Teixeira, M. D. Cave, and K. D. Eisenach.** 1998. Comparison of the ABI 7700 system (TaqMan) and competitive PCR for quantification of IS*6110* DNA in sputum during treatment of tuberculosis. J. Clin. Microbiol. **36:**1964–1968.
7. **Falla, T. J., D. W. Crook, E. C. Anderson, J. I. Ward, M. Santosham, J. Eskola, and E. R. Moxon.** 1995. Characterization of capsular genes in *Haemophilus influenzae* isolates from *H. influenzae* type b vaccine recipients. J. Infect. Dis. **171:**1075–1076.
8. **Galil, K., R. Singleton, O. Levine, M. Fitzgerald, L. Bulkow, M. Getty, B. Perkins, and A. Parkinson.** 1999. Reemergence of invasive *Haemophilus influenzae* type b disease in a well-vaccinated population in remote Alaska. J. Infect. Dis. **179:**101–106.
9. **Garner, D., and V. Weston.** 2003. Effectiveness of vaccination for *Haemophilus influenzae* type b. Lancet **361:**395–396.
10. **Gonin, P., M. Lorange, and G. Delage.** 2000. Performance of a multiplex PCR for the determination of *Haemophilus influenzae* capsular types in the clinical microbiology laboratory. Diagn. Microbiol. Infect. Dis. **37:**1–4.
11. **Greiner, O., C. Berger, P. J. Day, G. Meier, C. M. Tang, and D. Nadal.** 2002. Rates of detection of *Neisseria meningitidis* in tonsils differ in relation to local incidence of invasive disease. J. Clin. Microbiol. **40:**3917–3921.
12. **Greiner, O., P. J. Day, M. Altwegg, and D. Nadal.** 2003. Quantitative detection of *Moraxella catarrhalis* in nasopharyngeal secretions by real-time PCR. J. Clin. Microbiol. **41:**1386–1390.
13. **Greiner, O., P. J. R. Day, P. P. Bosshard, F. Imeri, M. Altwegg, and D. Nadal.** 2001. Quantitative detection of *Streptococcus pneumoniae* in nasopharyngeal secretions by real-time PCR. J. Clin. Microbiol. **39:**3129–3134.
14. **Hardegger, D., D. Nadal, W. Bossart, M. Altwegg, and F. Dutly.** 2000. Rapid detection of *Mycoplasma pneumoniae* in clinical samples by real-time PCR. J. Microbiol. Methods **41:**45–51.
15. **Hassan-King, M., R. Adegbola, I. Baldeh, K. Mulholland, C. Omosigho, A. Oparaugo, S. Usen, A. Palmer, G. Schneider, O. Secka, M. Weber, and B. Greenwood.** 1998. A polymerase chain reaction for the diagnosis of *Haemophilus influenzae* type b disease in children and its evaluation during a vaccine trial. Pediatr. Infect. Dis. J. **17:**309–312.
16. **Hassan-King, M., I. Baldeh, R. Adegbola, C. Omosigho, S. O. Usen, A. Oparaugo, and B. M. Greenwood.** 1996. Detection of *Haemophilus influenzae* and *Streptococcus pneumoniae* DNA in blood culture by a single PCR assay. J. Clin. Microbiol. **34:**2030–2032.
17. **Kroll, J., S. Ely, and E. R. Moxon.** 1991. Capsular typing of *Haemophilus influenzae* with DNA probe. Mol. Cell. Probes **5:**375–379.
18. **LaClaire, L. L., M. L. Tondella, D. S. Beall, C. A. Noble, P. L. Raghunathan, N. E. Rosenstein, and T. Popovic.** 2003. Identification of *Haemophilus influenzae* serotypes by standard slide agglutination serotyping and PCR-based capsule typing. J. Clin. Microbiol. **41:**393–396.
19. **Lucher, L. A., M. Reeves, T. Hennessy, O. S. Levine, T. Popovic, N. Rosenstein, and A. J. Parkinson.** 2002. Reemergence, in southwestern Alaska, of invasive *Haemophilus influenzae* type b disease due to strains indistinguishable from those isolated from vaccinated children. J. Infect. Dis. **186:**958–965.
20. **Lyons, S. R., A. L. Griffen, and E. J. Leys.** 2000. Quantitative real-time PCR for *Porphyromonas gingivalis* and total bacteria. J. Clin. Microbiol. **38:**2362–2365.
21. **McVernon, J., N. Andrews, M. P. E. Slack, and M. E. Ramsay.** 2003. Risk of vaccine failure after *Haemophilus influenzae* type b (Hib) combination vaccines with acellular pertussis. Lancet **361:**1521–1523.
22. **Mohle-Boetani, J. C., G. Ajello, E. Breneman, K. A. Deaver, C. Harvey, B. D. Plikaytis, M. M. Farley, D. S. Stephens, and J. D. Wenger.** 1993. Carriage of *Haemophilus influenzae* type b in children after widespread vaccination with conjugate *Haemophilus influenzae* type b vaccines. Pediatr. Infect. Dis. J. **12:**589–593.
23. **Mühlemann, K., M. Balz, S. Aebi, and K. Schopfer.** 1996. Molecular characteristics of *Haemophilus influenzae* causing invasive disease during the period of vaccination in Switzerland: analysis of strains isolated between 1986 and 1993. J. Clin. Microbiol. **34:**560–563.
24. **Pahl, A., U. Kuhlbrandt, K. Brune, M. Rollinghoff, and A. Gessner.** 1999. Quantitative detection of *Borrelia burgdorferi* by real-time PCR. J. Clin. Microbiol. **37:**1958–1963.
25. **Peltola, H.** 2000. Worldwide *Haemophilus influenzae* type b disease at the beginning of the 21st century: global analysis of the disease burden 25 years after the use of the polysaccharide vaccine and a decade after the advent of conjugates. Clin. Microbiol. Rev. **13:**302–317.
26. **Ramsay, M. E., J. McVernon, N. J. Andrews, P. T. Heath, and M. P. Slack.** 2003. Estimating *Haemophilus influenzae* type b vaccine effectiveness in England and Wales by use of the screening method. J. Infect. Dis. **188:**481–485.
27. **Sarnaik, S., J. Kaplan, G. Schiffman, D. Bryla, J. B. Robbins, and R. Schneerson.** 1990. Studies on *Pneumococcus* vaccine alone or mixed with DTP and on *Pneumococcus* type 6B and *Haemophilus influenzae* type b capsular polysaccharide-tetanus toxoid conjugates in two- to five-year-old children with sickle cell anemia. Pediatr. Infect. Dis. J. **9:**181–186.
28. **Schneerson, R., J. B. Robbins, C. Chu, A. Sutton, W. Vann, J. C. Vickers, W. T. London, B. Curfman, M. C. Hardegree, J. Shiloach, and S. C. Rastoga.** 1984. Serum antibody responses of juvenile and infant rhesus monkeys injected with *Haemophilus influenzae* type b and pneumococcus type 6A capsular polysaccharide-protein conjugates. Infect. Immun. **45:**582–591.
29. **Shoma, S., M. Rahman, and M. Yasmin.** 2001. Rapid detection of *Haemophilus influenzae* type b in Bangladeshi children with pneumonia and meningitis by PCR and analysis of antimicrobial resistance. J. Health Popul. Nutr. **19:**268–274.
30. **Takala, A. K., M. Santosham, J. Almeido-Hill, M. Wolff, W. Newcomer, R. Reid, H. Kayhty, E. Esko, and P. H. Makela.** 1993. Vaccination with *Haemophilus influenzae* type b meningococcal protein conjugate vaccine reduces oropharyngeal carriage of *Haemophilus influenzae* type b among American Indian children. Pediatr. Infect. Dis. J. **12:**593–599.
31. **Trotter, C. L., J. McVernon, N. J. Andrews, M. Burrage, and M. E. Ramsay.** 2003. Antibody to *Haemophilus influenzae* type b after routine and catch-up vaccination. Lancet **361:**1523–1524.
32. **Van Eldere, J., L. Brophy, B. Loynds, P. Celis, I. Hancock, S. Carman, J. S. Kroll, and E. R. Moxon.** 1995. Region II of the *Haemophilus influenzae* type be capsulation locus is involved in serotype-specific polysaccharide synthesis. Mol. Microbiol. **15:**107–118.