Kumar, S. Maharjan, S. Mushtaq, T. Noorie, D. L. Paterson, A. Pearson, C. Perry, R. Pike, B. Rao, U. Ray, J. B. Sarma, M. Sharma, E. Sheridan, M. A. Thirunarayan, J. Turton, S. Upadhyay, M. Warner, W. Welfare, D. M. Livermore, N. Woodford, *Lancet Infect. Dis.* **2010**, *10*, 597.

[5] R. C. Moellering, Jr., *N. Engl. J. Med.* **2010**, *363*, 2377.

[6] Y. Arakawa, M. Murakami, K. Suzuki, H. Ito, R. Wacharotayankun, S. Ohsuka, N. Kato, M. Ohta, *Antimicrob. Agents Chemother.* **1995**, *39*, 1612.

[7] N. O. Concha, C. A. Janson, P. Rowling, S. Pearson, C. A. Cheever, B. P. Clarke, C. Lewis, M. Galleni, J.-M. Frère, D. J. Payne, J. H. Bateson, S. S. Abdel-Meguid, *Biochemistry* **2000**, *39*, 4288.

[8] U. Heinz, H.-W. Adolph, *Cell. Mol. Life Sci.* **2004**, *61*, 2827.

[9] a) A. Carfi, S. Pares, E. Duée, M. Galleni, C. Duez, J.-M. Frère, O. Dideberg, *EMBO J.* **1995**, *14*, 4914; b) A. Carfi, E. Duee, M. Galleni, J.-M. Frère, O. Dideberg, *Acta Crystallogr. D Biol. Crystallogr.* **1998**, *54*, 313; c) N. O. Concha, B. A. Rasmussen, K. Bush, O. Herzberg, *Structure* **1996**, *4*, 823.

[10] a) L. I. Llarrull, M. F. Tioni, A. J. Vila, *J. Am. Chem. Soc.* **2008**, *130*, 15842; b) D. de Seny, U. Heinz, S. Wommer, M. Kiefer, W. Meyer-Klaucke, M. Galleni, J.-M. Frère, R. Bauer, H. W. Adolph, *J. Biol. Chem.* **2001**, *276*, 45065; c) L. I. Llarrull, M. F. Tioni, J. Kowalski, B. Bennett, A. J. Vila, *J. Biol. Chem.* **2007**, *282*, 30586; d) G. R. Periyannan, A. L. Costello, D. L. Tierney, K. W. Yang, B. Bennett, M. W. Crowder, *Biochemistry* **2006**, *45*, 1313; e) E. G. Orellano, J. E. Girardini, J. A. Cricco, E. A. Ceccarelli, A. J. Vila, *Biochemistry* **1998**, *37*, 10173; f) M. Hernandez Valladares, M. Kiefer, U. Heinz, R. P. Soto, W. Meyer-Klaucke, H. F. Nolting, M. Zeppezauer, M. Galleni, J.-M. Frère, G. M. Rossolini, G. Amicosante, H. W. Adolph, *FEBS Lett.* **2000**, *467*, 221; g) R. Bicknell, S. G. Waley, *Biochemistry* **1985**, *24*, 6876; h) R. Bicknell, A. Schaffer, S. G. Waley, D. S. Auld, *Biochemistry* **1986**, *25*, 7208; i) M. W. Crowder, Z. Wang, S. L. Franklin, E. P. Zovinka, S. J. Benkovic, *Biochemistry* **1996**, *35*, 12126.

[11] a) S. Haruta, H. Yamaguchi, E. T. Yamamoto, Y. Eriguchi, M. Nukaga, K. O'Hara, T. Sawai, *Antimicrob. Agents Chemother.* **2000**, *44*, 2304; b) S. Siemann, D. Brewer, A. J. Clarke, G. I. Dmitrienko, G. Lajoie, T. Viswanatha, *Biochim. Biophys. Acta* **2002**, *1571*, 190.

[12] N. Laraki, N. Franceschini, G. M. Rossolini, P. Santucci, C. Meunier, E. de Pauw, G. Amicosante, J. M. Frère, M. Galleni, *Antimicrob. Agents Chemother.* **1999**, *43*, 902.

[13] P. Kuzmič, *Anal. Biochem.* **1996**, *237*, 260.

[14] a) B. L. Vallee, B. Holmquist in *Advances in Inorganic Biochemistry Vol. 2* (Eds.: D. W. Darnall, R. G. Wilkins), Elsevier, New York, **1980**, p. 27; b) B. Holmquist, T. A. Kaden, B. L. Vallee, *Biochemistry* **1975**, *14*, 1454.

Received: May 31, 2011
Published online on July 7, 2011

Jpn. J. Infect. Dis., 63, 2010

Laboratory and Epidemiology Communications

Isolation of *Streptococcus pneumoniae* Serotypes 6C and 6D from the Nasopharyngeal Mucosa of Healthy Japanese Children

Bin Chang*, Taketo Otsuka[1], Atsushi Iwaya[2], Minoru Okazaki[1], Satoko Matsunaga, and Akihito Wada

Department of Bacteriology I, National Institute of Infectious Diseases, Tokyo 162-8640;
[1]Department of Pediatrics, Sado General Hospital, Niigata 952-1209; and
[2]Department of Pediatrics, Ryotsu Hospital, Niigata 952-0007, Japan

Communicated by Yoshichika Arakawa

(Accepted August 24, 2010)

Streptococcus pneumoniae, a primary causative agent of otitis media, pneumonia, bacteremia, and meningitis in children, results in substantial morbidity and mortality in many countries, including Japan (1-3). Of the 93 *S. pneumoniae* serotypes identified to date, serotypes 6C and 6D were recently differentiated from the classical serotypes 6A and 6B, respectively (4-6). Serotype 6C was subsequently reported to be isolated in several countries (5-9), especially as an important replacement serotype after introduction of the 7-valent pneumococcal conjugate vaccine (PCV7) (7,9,10). The naturally occurring *S. pneumoniae* serotype 6D was isolated from the Fiji Islands, Korea, and Poland (4,11,12). In this study, 32 6C and 1 6D *S. pneumoniae* isolates were identified from the nasopharyngeal mucosa of healthy children who had not received PCV7 residing on Sado Island, Niigata Prefecture, by using serological and genetic characterization.

S. pneumoniae, *Haemophilus influenzae*, and other pathogens among children residing on Sado Island, Niigata Prefecture, are monitored as part of the Sado Island, Antimicrobials, Day-care attendance, Older siblings (SADO) Study (13). In SADO study, which was conducted in 2008, pharyngeal swabs obtained from healthy children at check-up periods of 4, 7, 10, and 18 months old (mo) were cultured. Two of the children included had received PCV7. Fifty-two percent of the children at 18 mo had been attending day nursery. All *S. pneumoniae* isolates were serotyped using the conventional Quellung reaction using commercially available pneumococcal antisera (Statens Serum Institut [SSI], Copenhagen, Denmark) and home-made factor antiserum (designated factor 6dh [h indicates home-made]) for serotypes 6C and 6D. The factor 6b antiserum used in this study could react with both serotypes 6A and 6C; the new version of the factor 6b antiserum from SSI only reacts with serotype 6A (14,15). Factor 6dh antiserum was prepared by immunization of rabbits with formaldehyde-fixed serotype 6C whole cells and subsequent absorption of the antiserum with serotype 6A whole cells. In addition to the serological examination, serotypes 6C and 6D of the isolates were confirmed by genetic characterization involving comparison of the *wciN* region of 6A, 6B, 6C, and 6D isolates using PCR with primers 5106 and 3101 (5), and DNA sequencing of the *wciP* gene. The size of the *wciN* PCR products was determined by electrophoresis with 0.8% SeaKem GTG agarose gel (Takara Bio, Otsu, Japan). The DNA sequence of the *wciP* gene was determined using BigDye v1.1 (Applied Biosystems, Foster City, Calif., USA) and 3130*xl* Genetic Analyzer (Applied Biosystems). The antibiotic susceptibility of the isolates was analyzed by the microbroth dilution method according to the Clinical and Laboratory Standards Institute (CLSI M100-S18). Multi-locus sequence typing (MLST) was performed as described by Enright and Spratt (16).

A total of 337 *S. pneumoniae* isolates were obtained in this study. All isolates were initially serotyped using the Quellung reaction, and those that exhibited positive reactions with serogroup 6 antiserum were further tested using factor 6b, 6c, and 6dh antisera. Serotypes 6A and 6C exhibited positive reactions with factor 6b antiserum, whereas serotypes 6B and 6D exhibited positive reactions with factor 6c antiserum. Serotypes 6A and 6B exhibited negative reactions, and serotypes 6C and 6D exhibited positive reactions, with factor 6dh antiserum (Fig. 1). Thirty-two isolates (9.5%) exhibited positive reactions with both factor 6b and 6dh antisera, thus suggesting that they expressed the serotype 6C capsule. Furthermore, 1 isolate (0.3%) exhibited positive reactions with factor 6c and 6dh antisera, thus suggesting that it expressed serotype 6D capsule.

The *wciN* gene of the *S. pneumoniae* isolates was subsequently examined using PCR. The lengths of the PCR products for serotype 6A and 6B isolates found to be 2.0 (Fig. 2, lane 1) and 2.0/2.2 kb (Fig. 2, lanes 2 and 3), respectively. The length of each of the PCR products of the putative serotype 6C and 6D isolates was 1.8 kb (Fig. 2, lanes 4 and 5). The 2.0- and 2.2-kb *wciN* PCR products indicate the presence of capsular polysaccharide (PS) containing galactose, whereas the 1.8-kb PCR product indicates substitution of galactose by glucose (5). The DNA sequences of the *wciP* gene were determined for the isolates (4,5,11). The 138th amino acid residue in WciP for the 6A isolate is serine (AGT),

*Corresponding author: Mailing address: Department of Bacteriology I, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan. Tel: +81-3-5285-1111 ext. 2228, Fax: +81-3-5285-1163, E-mail: b-chang@nih.go.jp

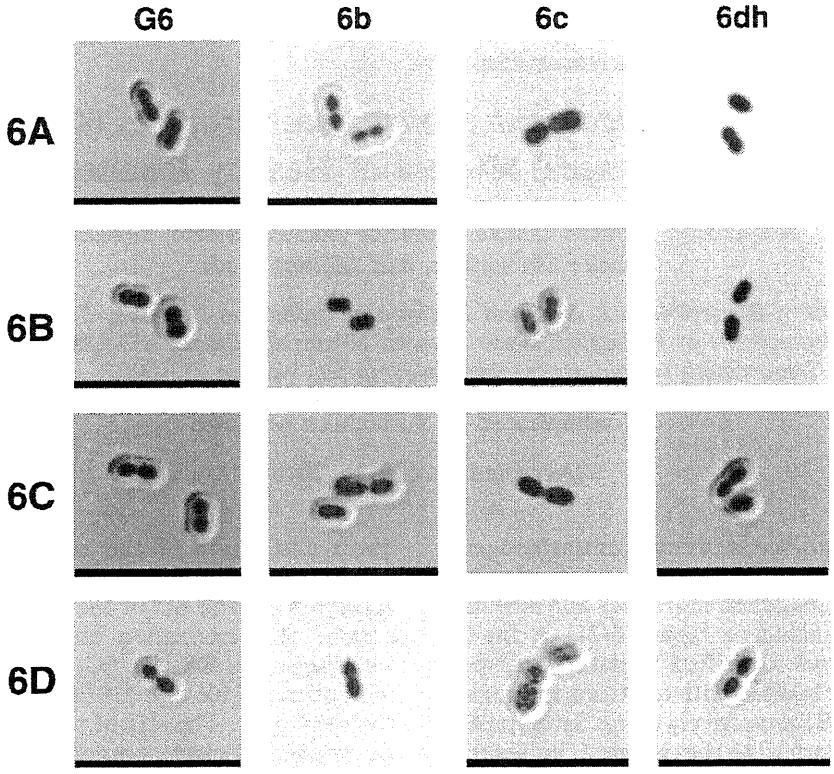

Fig. 1. Quellung reaction of *Streptococcus pneumoniae* serotypes 6A, 6B, 6C, and 6D. *S. pneumoniae* serotypes 6A (SP128) and 6B (KSP120) were isolated from cerebrospinal fluid. *S. pneumoniae* 6C (SP569) and 6D (SP687) were isolated from nasopharyngeal mucosa in this study. The antisera used are indicated on top of each column. G6, antiserum for serogroup 6; 6b, factor antiserum 6b; 6c, factor antiserum 6c; 6dh, home-made factor antiserum 6dh. Serotypes of *S. pneumoniae* are indicated on the left of the photographs. The underlined photographs illustrate positive results.

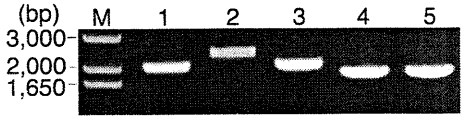

Fig. 2. PCR products of the *wciN* region of *Streptococcus pneumoniae* serogroup 6 isolates. M, 1 kb plus DNA ladder; lane 1, serotype 6A (SP128); lane 2, serotype 6B (KSP123); lane 3, serotype 6B (KSP120); lane 4, serotype 6C (SP569); lane 5, serotype 6D (SP687). The 2.0-kb or 2.2-kb fragments were obtained from serotype 6A (2.0-kb only) and 6B (2.0-kb or 2.2-kb) isolates, whereas the 1.8-kb fragments were obtained from serotype 6C and 6D isolates.

whereas that for the 6B isolate is asparagine (AAT) (17). The former amino acid is responsible for the rhamnose-(1→3)-ribitol linkage in the PS of serotype 6A, whereas the latter is responsible for the rhamnose-(1→4)-ribitol linkage in the PS of serotype 6B. The corresponding amino acids of the putative 6C and 6D isolates were serine and asparagine, respectively. The serological and genetic analyses yielded identical results in that both were consistent with the PS structure [→2)-glucose-(1→3)-glucose-(1→3)-rhamnose-(1→3)-ribitol-(5→phosphate] for 6C and [→2)-glucose-(1→3)-glucose-(1→3)-rhamnose-(1→4)-ribitol-(5→phosphate] for 6D, thus confirming the colonization of *S. pneumoniae* serotype 6C and 6D isolates in the nasopharynx of healthy Japanese children.

The 32 6C *S. pneumoniae* isolates were obtained from a total of 30 children (3 from 4-mo children, 5 from 7-mo children, 13 from 10-mo children, and 11 from 18-mo children); 2 of the isolates were obtained from the same child at 7- and 10-mo, and a furthre 2 isolates, which showed different colony morphologies and different antibiograms, were simultaneously obtained from a child at 18 mo. The *S. pneumoniae* serotype 6D was isolated from an 18-mo child. None of the children who carried the *S. pneumoniae* serotypes 6C or 6D had received PCV7. As for the children's residential area and day nursery attendance, there was no obvious association between the 30 children from whom the *S. pneumoniae* serotype 6C was isolated. The minimum inhibitory concentration (MIC) of penicillin G for the serotype 6C isolates ranged between ≤ 0.015 and 0.25 μg/ml, and that for 26 (81.3%) of the isolates being ≤ 0.06 μg/ml. All of the 6C isolates were susceptible to both cefotaxime (MIC ≤ 1 μg/ml) and meropenem (MIC ≤ 0.25 μg/ml), whereas 30 (93.8%) of them were resistant to erythromycin (MIC ≥ 1 μg/ml). The 6D isolate was susceptible to penicillin G (0.03 μg/ml), cefotaxime (0.25 μg/ml), and meropenem (≤ 0.008 μg/ml) but resistant to erythromycin (≥ 8 μg/ml). MLST analysis revealed that the frequent sequence types (STs) of the serotype 6C isolates were ST2923 (40.6%) and ST2924 (31.3%), whereas the ST of the 6D isolate was ST2924. The MLST analysis showed that the serotype 6C isolates from children on Sado Island comprised multiple clones.

The routine immunization of infants and toddlers in the United States with PCV7 has successfully reduced

the incidence of invasive pneumococcal disease (IPD) in children caused by the vaccine serotypes (18–20). Vaccination of children with PCV7 has also lowered the incidence of IPD among the elderly, a phenomenon known as the herd-immunity effect (18–20). The observed reduction in the incidence of IPD among the nonimmunized population is likely to be due to a change in the nasopharyngeal colonization of *S. pneumoniae* in immunized individuals. There has, however, been a rise in the incidence of IPD caused by non-PCV7 serotypes (known as replacement serotypes), including serotypes 19A, 6C, and others, in the United States (7,9,19, 21–24). As far as 6D is concerned, this serotype was isolated at a high rate (41%) from the nasopharyngeal mucosa of Fijian children, 86% of whom had received at least 1 dose of PCV7, thereby suggesting that serotype 6D may have a selective advantage after immunization with the vaccine (11). In addition, 5 IPD cases due to *S. pneumoniae* serotype 6D were reported in Poland (12). Because serotypes 6C and 6D were recognized after the introduction of PCV7, the surveillance data for infection with these serotypes in the United States and other countries are retrospective (12,18,19). PCV7 was released in Japan in February 2010 and widespread PCV7 vaccination is expected to lead to a similarly large reduction in pneumococcal infections, including IPD, pneumonia, and otitis media, in both the immunized and nonimmunized populations to that observed in other countries. We have initiated a population-based study to monitor the changes in IPD incidence and the serotype distribution among Japanese children, and we are also monitoring the colonized *S. pneumoniae* in the nasopharynx of healthy children. Initial results showed that *S. pneumoniae* serotype 6C was isolated from less than 2% of IPD cases without PCV7 vaccination (unpublished data) but could be isolated from the nasopharyngeal mucosa of 9.5% of the healthy children. PCV7, which includes only serotype-6B conjugate, would not affect the colonization or infection by *S. pneumoniae* serotypes 6C and/or 6D. A prospective surveillance on both colonization and infection by *S. pneumoniae* serotypes 6C, 6D, and others is therefore warranted to obtain an accurate evaluation of the effects of the 7- and 13-valent conjugate vaccines.

Acknowledgments This work was supported by grants from the Ministry of Health, Labour and Welfare of Japan (H21-Shinkou-ippan-002 and H21-Shinkou-ippan-008), the Japanese Society for Pediatric Infectious Diseases, St. Luke's Life Science Institute, and the Mother and Child Health Foundation.

Conflict of interest None to declare.

REFERENCES

1. Austrian, R. and Gold, J. (1964): Pneumococcal bacteremia with special reference to bacteremic pneumococcal pneumonia. Ann. Intern. Med., 60, 759–776.
2. Ishiwada, N., Kurosaki, T., Terashima, I., et al. (2008): The incidence of pediatric invasive pneumococcal disease in Chiba prefecture, Japan (2003–2005). J. Infect., 57, 455–458.
3. Pallarés, R., Liñares, J., Vadillo, M., et al. (1995): Resistance to penicillin and cephalosporin and mortality from severe pneumococcal pneumonia in Barcelona, Spain. N. Engl. J. Med., 333, 474–480.
4. Bratcher, P.E., Kim, K.H., Kang, J.H., et al. (2010): Identification of natural pneumococcal isolates expressing serotype 6D by genetic, biochemical and serological characterization. Microbiology, 156, 555–560.
5. Park, I.H., Park, S., Hollingshead, S.K., et al. (2007): Genetic basis for the new pneumococcal serotype, 6C. Infect. Immun., 75, 4482–4489.
6. Park, I.H., Pritchard, D.C., Cartee, R., et al. (2007): Discovery of a new capsular serotype (6C) within serogroup 6 of *Streptococcus pneumoniae*. J. Clin. Microbiol., 45, 1225–1233.
7. Carvalho, M. da G., Pimenta, F.C., Gertz, R.E., Jr., et al. (2009): PCR-based quantitation and clonal diversity of the current prevalent invasive serogroup 6 pneumococcal serotype, 6C, in the United States in 1999 and 2006 to 2007. J. Clin. Microbiol., 47, 554–559.
8. Jacobs, M.R., Dagan, R., Bajaksouzian, S., et al. (2010): Validation of factor antiserum 6d for serotyping *Streptococcus pneumoniae* serotype 6C. J. Clin. Microbiol., 48, 1456–1457.
9. Jacobs, M.R., Good, C.E., Bajaksouzian, S., et al. (2008): Emergence of *Streptococcus pneumoniae* serotypes 19A, 6C, and 22F and serogroup 15 in Cleveland, Ohio, in relation to introduction of the protein-conjugated pneumococcal vaccine. Clin. Infect. Dis., 47, 1388–1395.
10. Tocheva, A.S., Jefferies, J.M., Christodoulides, M., et al. (2010): Increase in serotype 6C pneumococcal carriage, United Kingdom. Emerg. Infect. Dis., 16, 154–155.
11. Jin, P., Kong, F., Xiao, M., et al. (2009): First report of putative *Streptococcus pneumoniae* serotype 6D among nasopharyngeal isolates from Fijian children. J. Infect. Dis., 200, 1375–1380.
12. Kuch, A., Sadowy, E., Skoczyńska, A., et al. (2010): First report of *Streptococcus pneumoniae* serotype 6D isolates from invasive infections. Vaccine, 28, 6406–6407.
13. Otsuka, T., Ono, T. and Okazaki, M. (2009): Resistance of nasopharyngeal pathogens and antimicrobial prescription rates for children in an area under controlled antimicrobial use. Pediatr. Infect. Dis. J., 28, 128–130.
14. Bratcher, P.E. and Nahm, M.H. (2010): Cross-reactivity of current serogroup 6 factor sera from Statens Serum Institut with the recently described pneumococcal serotype 6D. J. Clin. Microbiol., 48, 3044–3045.
15. Oftadeh, S., Satzke, C. and Gilbert, G.L. (2010): Identification of newly described *Streptococcus pneumoniae* serotype 6D by use of the Quellung reaction and PCR. J. Clin. Microbiol., 48, 3378–3379.
16. Enright, M.C. and Spratt, B.G. (1998): A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. Microbiology, 144, 3049–3060.
17. Mavroidi, A., Godoy, D., Aanensen, D.M., et al. (2004): Evolutionary genetics of the capsular locus of serogroup 6 pneumococci. J. Bacteriol., 186, 8181–8192.
18. Lexau, C.A., Lynfield, R., Danila, R., et al. (2005): Changing epidemiology of invasive pneumococcal disease among older adults in the era of pediatric pneumococcal conjugate vaccine. JAMA, 294, 2043–2051.
19. Pilishvili, T., Lexau, C., Farley, M.M., et al. (2010): Sustained reductions in invasive pneumococcal disease in the era of conjugate vaccine. J. Infect. Dis., 201, 32–41.
20. Whitney, C.G., Farley, M.M., Hadler, J., et al. (2003): Decline in invasive pneumococcal disease after the introduction of protein-polysaccharide conjugate vaccine. N. Engl. J. Med., 348, 1737–1746.
21. Byington, C.L., Samore, M.H., Stoddard, G.J., et al. (2005): Temporal trends of invasive disease due to *Streptococcus pneumoniae* among children in the intermountain west: emergence of nonvaccine serogroups. Clin. Infect. Dis., 41, 21–29.
22. Hicks, L.A., Harrison, L.H., Flannery, B., et al. (2007): Incidence of pneumococcal disease due to non-pneumococcal conjugate vaccine (PCV7) serotypes in the United States during the era of widespread PCV7 vaccination, 1998–2004. J. Infect. Dis., 196, 1346–1354.
23. Pelton, S.I., Huot, H., Finkelstein, J.A., et al. (2007): Emergence of 19A as virulent and multidrug resistant pneumococcus in Massachusetts following universal immunization of infants with pneumococcal conjugate vaccine. Pediatr. Infect. Dis. J., 26, 468–472.
24. Singleton, R.J., Hennessy, T.W., Bulkow, L.R., et al. (2007): Invasive pneumococcal disease caused by nonvaccine serotypes among Alaska native children with high levels of 7-valent pneumococcal conjugate vaccine coverage. JAMA, 297, 1784–1792.

Jpn. J. Infect. Dis., 64, 341-344, 2011

Short Communication

Serotyping and Multilocus Sequence Typing of *Streptococcus pneumoniae* Isolates from the Blood and Posterior Nares of Japanese Children Prior to the Introduction of 7-Valent Pneumococcal Conjugate Vaccine

Tomohiro Oishi[1,2], Akihito Wada[3], Bin Chang[3], Shinichi Toyabe[4], and Makoto Uchiyama[1]

[1]*Department of Pediatrics and*
[4]*Crisis Management Office, Niigata University Medical and Dental Hospital, Niigata 951-8520;*
[2]*Department of Pediatrics, Niigata Prefectural Shibata Hospital, Niigata 957-8588; and*
[3]*Department of Bacteriology I, National Institute of Infectious Diseases, Tokyo 162-8640, Japan*

(Received April 21, 2011. Accepted July 4, 2011)

SUMMARY: In Japan, the 7-valent pneumococcal conjugate vaccine (PCV7) was introduced in 2010. To assess the effects of PCV7 on invasive pneumococcal infection in children, a population-based prospective survey has been conducted in 10 prefectures. As a part of the study, blood and nasopharyngeal isolates from children admitted to the Shibata Hospital, Niigata Prefecture, were analyzed for determining the serotypes, their susceptibilities to antimicrobial agents, and multilocus sequence types. Sixteen blood isolates were obtained from October 2007 to December 2009. Sixty-three nasopharyngeal isolates were obtained from the posterior nares of 118 children with pneumonia from April to September 2008. The coverage rates of the blood and nasopharyngeal isolates for PCV7 were 81.3% and 57.1%, respectively. Although none of these children had received PCV7, serotype 19A isolates were recovered from 12.5% (2/16) of the blood samples and 12.7% (8/63) of the nasopharyngeal samples. The sequence type of a nasopharyngeal isolate of serotype 19A was ST320, and the minimum inhibitory concentration of penicillin G was 4 μg/mL. In addition to the continuous prospective survey of pneumococcal infection, early introduction of the 13-valent conjugate vaccine, in which the 19A conjugate is included, will be necessary in Japan.

Streptococcus pneumoniae infection is a leading cause of childhood morbidity. In 2005, the World Health Organization estimated that pneumococcal diseases caused 1.6 million deaths, including 0.7–1 million deaths per year in children under 5 years of age (1). The 7-valent pneumococcal conjugate vaccine (PCV7) has been widely used in USA and other countries; this has resulted in a dramatic reduction of invasive pneumococcal disease (IPD) in both immunized children (2–10) and non-immunized adults (2–5)—the "herd immunity" effect. PCV7 has also reduced hospitalization due to all-cause pneumonia in children under the age of 2 years in USA (11). In Japan, PCV7 became available in February 2010. Several surveys on IPD had been conducted in Japan before the introduction of the vaccine (12–16). To characterize invasive pneumococcal infection and respiratory infection/colonization in the same population before the introduction of PCV7, we obtained blood and nasopharyngeal *S. pneumoniae* isolates from hospitalized children, and analyzed the serotypes, their susceptibility to antimicrobial agents, and multilocus sequence types.

All pneumococcal isolates were obtained from children hospitalized at the Department of Pediatrics, Niigata Prefectural Shibata Hospital. This hospital is the only one in the region that has inpatient wards for children. The population of Shibata City in 2011 is 102,758, of which 3,835 are children under 5 years of age. Blood isolates were obtained from October 2007 to December 2009. Posterior nares swabs were obtained from 118 children with pneumonia from April to September 2008. Pneumonia was diagnosed by fever, cough, sputum production, chest radiography examination, blood cell count, and/or elevation of C-reactive protein. The pneumococcal isolates were serotyped by the Quellung reaction with serotype-specific antisera (Statens Serum Institut, Copenhagen, Denmark) and factor serum for serotype 6C, which was made in our laboratory (17). Susceptibilities to antimicrobial agents were determined by the microbroth dilution method using Dry Plate (Eiken Chemical Co., Tokyo, Japan), according to the Clinical and Laboratory Standards Institute M100-S18 guidelines (18). Although the penicillin G susceptibility criteria have changed in M100-S18, the previous criteria in minimum inhibitory concentration (MIC) (penicillin-susceptible *S. pneumoniae* [PSSP], ≤ 0.06 μg/mL; penicillin-intermediate *S. pneumoniae* [PISP], 0.12–1 μg/mL; penicillin-resistant *S. pneumoniae* [PRSP], ≥ 2 μg/mL) were used in this study. To determine the sequence type (ST) of the isolates, multilocus sequence typing (MLST) was performed as described previously by Enright and Spratt (19). STs were determined by the Internet database search at http://spneumoniae.mlst.net/. Informed consent for this

*Corresponding author: Mailing address: Department of Pediatrics, Niigata University Medical and Dental Hospital, Asahimachidori 1-757, Chuo-ku, Niigata 951-8520, Japan. Tel & Fax: +81-25-227-2222/+81-25-227-0778, E-mail: oo0612@hotmail.com

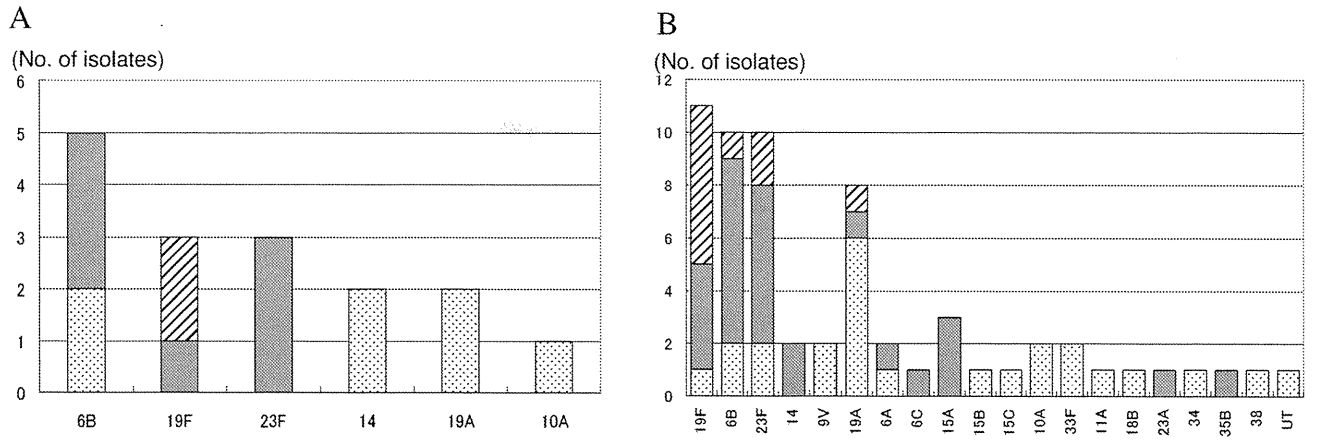

Fig. 1. Relationship between serotype and penicillin G resistance categories for *S. pneumoniae* isolates from (A) blood and (B) posterior nares. Shaded bar, gray bar, and dotted bar represent PRSP (penicillin G MIC ≥ 2 μg/mL), PISP (penicillin G MIC 0.12–1 μg/mL), and PSSP (penicillin G MIC ≤ 0.06 μg/mL), respectively. One serotype 14 isolate, in which MIC could not be determined, is not included in panel B.

study was obtained from parents or guardians, in accordance with the Helsinki Declaration. None of the children had received PCV7 or the 23-valent polysaccharide vaccine.

Sixteen blood isolates were obtained from 16 patients with bacteremia (age, 4 months to 3 years). No meningitis or sepsis was observed in the study period. In addition, a total of 63 nasopharyngeal isolates were obtained. Out of the 63 isolates, 58 (2 isolates of different serotypes were obtained from 2 patients) were from 56 hospitalized patients (age, 1 month to 15 years) who had pneumonia without bacteremia, and 5 isolates were from 5 patients with pneumonia and bacteremia. One-year-old children were most frequently affected by invasive and respiratory infections; these children account for 68.8% and 38.1% of those with bacteremia and pneumonia, respectively. The serotypes of the 16 blood isolates were as follows: type 6B (5 isolates, 31.3%), types 19F and 23F (3 isolates each, 18.8%), types 14 and 19A (2 isolates each, 12.5%), and type 10A (1 isolate, 6.3%) (Fig. 1A). The coverage rates of PCV7 and the 13-valent conjugate vaccine (PCV13) for the blood isolates were 81.3 and 93.8%, respectively. The serotypes of the 63 nasopharyngeal isolates were as follows: type 19F (11 isolates, 17.5%); types 6B and 23F (10 isolates each, 15.9%); type 19A (8 isolates, 12.7%); types 14 and 15A (3 isolates each, 4.8%); types 9V, 6A, 10A, and 33F (2 isolates each, 3.2%); other serotypes (9 serotypes, including 6C, with 1 isolate of each type, 1.6%); and untypeable (1 isolate, 1.6%) (Fig. 1B). Isolates of serotypes 19F and 33F were obtained from 1 patient and those of serotypes 6B and 14 from another patient. Some isolates obtained from both blood and nasopharyngeal samples of a single patient showed the same serotypes: 6B (1 patient), 19F (2 patients), and 23F (2 patients). The coverage rates of PCV7 and PCV13 for the nasopharyngeal isolates were 57.1% and 73.0%, respectively. The blood isolates were tested for susceptibility to penicillin G, and the results were as follows: 7 isolates (43.8%), PSSP; 7 isolates (43.8%), PISP; and 2 isolates (12.5%), PRSP. The relationship between serotypes and penicillin G susceptibility in the blood isolates is shown in Figure 1A. Isolates of serotypes 19F and 23F were PISP or PRSP, while those of serotypes 14, 19A, and 10A were PSSP. The 63 nasopharyngeal isolates were examined for susceptibility to penicillin G also, and 62 isolates showed the following results: 25 isolates (40.3%), PSSP; 27 isolates (43.6%), PISP; and 10 isolates (16.1%), PRSP. Isolates of serotypes 6B, 19F, 23F, and 19A were PRSP (Fig. 1B). The antimicrobial susceptibility of a serotype 14 isolate could not be determined because it did not show growth in Mueller Hinton broth.

The results of the MLST analysis of the blood and nasopharyngeal isolates are shown in Table 1. Blood isolates of serotype 6B comprised ST90 and ST2983, and those of serotype 19F comprised ST236 and ST115. ST2983 and ST115 are double-locus variants (DLVs) of ST90 and ST236, respectively. Isolates of serotypes 23F, 14, and 19A showed had only 1 ST (ST1437, ST13, and ST3111, respectively). Nasopharyngeal isolates showed more variation in STs than blood isolates. For example, isolates of serotype 6B comprised 4 STs: ST90, ST2983, ST902, and ST5864. ST2983 is a DLV of ST90, but ST90, ST902, and ST5864 are not related to each other. Another example was serotype 19A with 3 STs—ST320, ST3111, and ST5842—that are not related to each other. The isolates of serotype 23F comprised 5 STs, but these STs could be grouped under 2 STs (ST242 and ST1437). Two blood isolates and 6 nasopharyngeal isolates of serotype 19F showed resistance to penicillin G at MIC of 2–4 μg/mL; all the isolates were ST236 or ST115. The MIC of penicillin G for a nasopharyngeal serotype 19F isolate with ST257 was 0.03 μg/mL. This was the only PSSP found among isolates of serotype 19F. The MIC of penicillin G for a nasopharyngeal isolate of serotype 19A with ST320 was 4 μg/mL, whereas other serotype 19A isolates with ST3111 or ST5842 were PSSP or PISP (Fig. 1B). The MIC of cefotaxime, meropenem, and panipenem for the isolate of serotype 19A with ST320 was 2 μg/mL, 0.5 μg/mL, and 0.12 μg/mL, respectively.

A population-based survey has been conducted across 10 prefectures of Japan to assess the effect of PCV7 on invasive pneumococcal infection in children. This study was started in 2007, 3 years before the introduction of

Table 1. Serotype and sequence type of blood and posterior nares isolates

Serotype	Blood		Nasopharynx		Remarks[1)]
	Sequence type	No. of isolates	Sequence type	No. of isolates	
6B	ST90, ST2983	3, 2	ST90, ST2983	6, 2	ST2983, DLV of ST90 (*xpt*, *ddl*)
			ST902	1	
			ST5846[2)]	1	
19F	ST236, ST115	2, 1	ST236, ST257	10, 1	ST115, DLV of ST236 (*spi*, *ddl*) ST257, DLV of ST236 (*aroE*, *ddl*)
23F	ST1437	3	ST1437, ST5845[2)]	2, 1	ST5845, SLV of ST1437 (*gdh*, *gki*)
			ST242, ST5841[2)], ST5844[2)]	5, 1, 1	ST5841, DLV of ST242 (*gdh*, *recP*) ST5844, SLV of ST242 (*xpt*)
14	ST13	2	ST13, ST2922	1, 1	ST2922, SLV of ST13 (*xpt*)
			ST5240[3)]	1	
19A	ST3111	2	ST3111	6	
			ST320	1	
			ST5842[2)]	1	
10A	ST6412[2)]	1	ST1263	1	
			ST5236	1	
6A			ST3787	2	
6C			ST5241	1	
9V			ST280	2	
15A			ST63	3	
15B			ST199	1	
15C			ST5843[2)]	1	ST5843, SLV of ST199 (*spi*)
33F			ST5840[2)]	2	
11A			ST99	1	
18B			ST3594	1	
23A			ST5246	1	
34			ST3116	1	
35B			ST558	1	
38			ST393	1	
Untypeable			ST1106	1	

1): SLV/DLV, single-/double-locus variant.
2): newly identified sequence type in this study.
3): MICs could not be determined (see text).

PCV7. Shibata Hospital participated in this surveillance study from the beginning, and the period of this study corresponds to the period just prior to the introduction of PCV7. The children in Shibata City and the surrounding area did not receive PCV7 in this period. In spite of this, *S. pneumoniae* serotype 19A—well recognized as a major replacement serotype following PCV7 introduction in USA and other countries (2,3,5,7,20)—was isolated in 12.5% and 12.7% of the blood and nasopharyngeal cultures, respectively. These rates are much higher than those observed before PCV7 introduction in USA (2,3,20) and Canada (5) and are comparable to those that had been observed in France (7). In a country-wide survey in Japan, Chiba et al. reported 12 pediatric invasive cases by serotype 19A *S. pneumoniae* (6.2%) out of a total of 193 cases from 2006 to 2007 (15). MLST analysis showed that the ST of a nasopharyngeal isolate of serotype 19A was ST320. *S. pneumoniae* with this serotype and ST has been isolated from multiple regions in Japan (unpublished data) and from many other countries, including USA, Venezuela, Spain, Italy, China, and South Korea (http://spneumoniae.mlst.net/sql/burstspadvanced.asp). The penicillin G MIC of all the ST320 isolates obtained from patients in Japan was 2–4 μg/mL. However, the 2 blood isolates of serotype 19A obtained in this study showed a penicillin G MIC of 0.03 μg/mL and belonged to ST3111. At all of the 7 loci [*aroE, ghd, gki, recP, spi, xpt,* and *ddl*] in the pneumococcal MLST analysis, the alleles differed between ST320 and ST3111 (ST320 [4, 16, 19, 15, 6, 20, 1] and ST3111 [61, 60, 67, 16, 10, 104, 14]). In addition, ST5842 [10, 16, 150, 1, 13, 1, 29], isolated from a swab sample, had allele numbers that were different from those of both ST320 and ST3111. These findings suggest that multiple serotype 19A variants have already spread in children in the Shibata City region; these variants may cause respiratory infections and would cause invasive infections. The invasive infection surveillance in 10 prefectures showed that various STs, including ST320 and ST3111, have been observed in serotype 19A isolates (unpublished data). In Japan, routine immunization with PCV7 has been recently initiated in 2011, and the reduction in the number of invasive and respiratory infection cases caused by the vaccine-serotype *S. pneumoniae* is anticipated, as has been observed in USA and other countries (2–11). PCV13, however, is not yet available in Japan. The domestic phase III study is still on-going in 2011. This situation raises concern about the rapid replacement of the PCV7 serotypte by non-PCV7 serotypes, as has been observed in USA (2,3,20). Replacement by serotype 19A (ST320), in particular, would be serious because of its high

resistance to penicillin and non-susceptible phenotype to meropenem. The prospective survey of pneumococcal infection in both children and adults, together with intensive laboratory analysis, will be necessary for detecting the very early stage of the replacement. We do anticipate the early introduction of PCV13 in Japan.

Acknowledgments This work is supported by grants from the Ministry of Health, Labour and Welfare of Japan (H21-Shinkou-ippan-002, H21-Shinkou-ippan-008, and H22-Iyaku-shitei-028).

Conflict of interest None to declare.

REFERENCES

1. World Health Organization (2007): Pneumococcal conjugate vaccine for childhood immunization—WHO position paper. Wkly. Epidemiol. Rec., 82, 93–104.
2. Hicks, L.A., Harrison, L.H., Flannery, B., et al. (2007): Incidence of pneumococcal disease due to non-pneumococcal conjugate vaccine (PCV7) serotypes in the United States during the era of widespread PCV7 vaccination, 1998–2004. J. Infect. Dis., 196, 1346–1354.
3. Pilishvili, T., Lexau, C., Farley, M.M., et al. (2010): Sustained reductions in invasive pneumococcal disease in era of conjugate vaccine. J. Infect. Dis., 201, 32–41.
4. Roche, P.W., Krause, V., Cook, H., et al. (2008): Invasive pneumococcal disease in Australia, 2006. Commun. Dis. Intell., 32, 18–30.
5. Kellner, J.D., Vanderkooi, O.G., MacDonald, J., et al. (2009): Changing epidemiology of invasive pneumococcal disease in Canada, 1998–2007: update from Calgary-area *Streptococcus pneumoniae* research (CASPER) study. Clin. Infect. Dis., 49, 205–212.
6. Hsu, H.E., Shutt, K.A., Moore, M.R., et al. (2009): Effect of pneumococcal conjugate vaccine on pneumococcal meningitis. N. Engl. J. Med., 360, 244–256.
7. Lepoutre, A., Varon, E., Georges, S., et al. (2008): Impact of infant pneumococcal vaccination on invasive pneumococcal diseases in France, 2001–2006. Euro Surveill., 13(35):pii = 18962.
8. Dubos, F., Marechal, I., Husson, M.O., et al. (2007): Decline in pneumococcal meningitis after the introduction of the heptavalent-pneumococcal conjugate vaccine in northern France. Arch. Dis. Child., 92, 1009–1012.
9. Verstrheim, D.F., Løvoll, Ø., Aaberge, I.S., et al. (2008): Effectiveness of a 2 + 1 dose schedule pneumococcal conjugate vaccination programme on invasive pneumococcal disease among children in Norway. Vaccine, 26, 3277–3281.
10. Rückinger, S., van der Linden, M., Reinert, R.R., et al. (2009): Reduction in the incidence of invasive pneumococcal disease after general vaccination with 7-valent pneumococcal conjugate vaccine in Germany. Vaccine, 27, 4136–4141.
11. Grijalva, C.G, Nuorti, J.P., Arbogast, P.G, et al. (2007): Decline in pneumonia admissions after routine childhood immunisation with pneumococcal conjugate vaccine in the USA: a time-series analysis. Lancet, 369, 1179–1186.
12. Sakata, H. (2005): Study of *Streptococcus pneumoniae* bacteremia in children. J. Jpn. Assoc. Infect. Dis., 79, 1–6 (in Japanese).
13. Nishimura, T., Fukuzawa, M., Yoshida, H., et al. (2008): Clinical epidemiological study of occult pneumococcal bacteremia in a pediatric outpatient setting. J. Jpn. Pediatr. Soc., 112, 973–980 (in Japanese).
14. Sakata, H. (2005): A study of bacterial meningitis in Hokkaido between 1999 and 2003. J. Jpn. Assoc. Infect. Dis., 79, 680–687 (in Japanese).
15. Chiba, N., Morozumi, M., Sunaoshi, K., et al. (2010): Serotype and antibiotic resistance of isolates from patients with invasive pneumococcal disease in Japan. Epidemiol. Infect., 138, 61–68.
16. Sakai, F., Chiba, N., Ono, A., et al. (2010): Molecular epidemiologic characteristics of *Streptococcus pneumoniae* isolates from children with meningitis in Japan from 2007 through 2009. J. Infect. Chemother. DOI: 10.1007/s10156-010-0180-3.
17. Chang, B., Otsuka, T., Iwaya, A., et al. (2010): Isolation of *Streptococcus pneumoniae* serotypes 6C and 6D from the nasopharyngeal mucosa of healthy Japanese children. Jpn. J. Infect. Dis., 63, 381–383.
18. Clinical and Laboratory Standards Institute (2008): Performance standards for antimicrobial susceptibility testing; 18th Informational Supplement. Document M100-S18. Clinical and Laboratory Standards Institute, Wayne, Pa., USA.
19. Enright, M.C. and Spratt, B.G. (2008): A mutilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. Microbiology, 144, 3049–3060.
20. Jacobs, M.R., Good, C.E., Bajaksouzian, S., et al. (2008): Emergence of *Streptococcus pneumoniae* serotypes 19A, 6C, and 22F and serogroup 15 in Cleaveland, Ohio, in relation to introduction of the protein-conjugated pneumococcal vaccine. Clin. Infect. Dis., 47, 1388–1395.

Tabuchi et al. BMC Infectious Diseases 2011, 11:22
http://www.biomedcentral.com/1471-2334/11/22

BMC Infectious Diseases

RESEARCH ARTICLE Open Access

Tuberculosis infection among homeless persons and caregivers in a high-tuberculosis-prevalence area in Japan: a cross-sectional study

Takahiro Tabuchi[1,2], Toshio Takatorige[1,2], Yukio Hirayama[2], Nobuaki Nakata[2], Shigeyoshi Harihara[2], Akira Shimouchi[3], Koshiro Fujita[4], Hiroko Yoshida[4], Yoshitaka Tamura[4], Takayuki Nagai[4], Tomoshige Matsumoto[4], Tetsuya Takashima[4], Hiroyasu Iso[1*]

Abstract

Background: Tuberculosis (TB) is a major public health problem. The Airin district of Osaka City has a large population of homeless persons and caregivers and is estimated to be the largest TB-endemic area in the intermediate-prevalence country, Japan. However, there have been few studies of homeless persons and caregivers. The objective of this study is to detect active TB and to assess the prevalence and risk factors for latent TB infection among homeless persons and caregivers.

Methods: We conducted a cross-sectional study for screening TB infection (active and latent TB infections) using questionnaire, chest X-ray (CXR), newly available assay for latent TB infection (QuantiFERON-TB Gold In-Tube; QFT) and clinical evaluation by physicians at the Osaka Socio-Medical Center Hospital between July 2007 and March 2008. Homeless persons and caregivers, aged 30-74 years old, who had not received CXR examination within one year, were recruited. As for risk factors of latent TB infection, the odds ratios (OR) and 95% confidence intervals (95% CI) for QFT-positivity were calculated using logistic regression model.

Results: Complete responses were available from 436 individuals (263 homeless persons and 173 caregivers). Four active TB cases (1.5%) among homeless persons were found, while there were no cases among caregivers. Out of these four, three had positive QFT results. One hundred and thirty-three (50.6%) homeless persons and 42 (24.3%) caregivers had positive QFT results. In multivariate analysis, QFT-positivity was independently associated with a long time spent in the Airin district: ≥10 years versus <10 years for homeless (OR = 2.53; 95% CI, 1.39-4.61) and for caregivers (OR = 2.32; 95% CI, 1.05-5.13), and the past exposure to TB patients for caregivers (OR = 3.21; 95% CI, 1.30-7.91) but not for homeless persons (OR = 1.51; 95% CI, 0.71-3.21).

Conclusions: Although no active TB was found for caregivers, one-quarter of them had latent TB infection. In addition to homeless persons, caregivers need examinations for latent TB infection as well as active TB and careful follow-up, especially when they have spent a long time in a TB-endemic area and/or have been exposed to TB patients.

Background

Globally, there were an estimated 9.27 million cases of tuberculosis (TB) in 2007, with the larger number of cases of latent TB infection [1]. The Airin district of Osaka City is known as an urban area with a dense population of day-laborers and homeless people in Japan, an intermediate-prevalence country [2]. The prevalence of active TB in the Airin district was approximately 1,000 per 100,000 in the early 2000s, which was the highest in Japan and similar to that in developing countries [1,3,4]. In the Airin district, there are estimated to be 15,000 to 20,000 homeless persons with no medical insurance, accounting for 80% of visitors to a free or low-cost hospital, i.e. Osaka socio-medical center

* Correspondence: iso@pbhel.med.osaka-u.ac.jp
[1]Public Health, Department of Social and Environmental Medicine, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita-shi, Osaka, Japan
Full list of author information is available at the end of the article

hospital [3]. TB screening programs have been carried out at Airin Health Office since 1990 and at the Osaka socio-medical center hospital since 2005. Mobile screening for TB was carried out monthly between 1973 and 2005, and weekly since 2006, by the Osaka City government on the streets of the Airin district [5]. The directly observed therapy short course (DOTS) program for homeless persons has been carried out since 1999. To date, several communities (i.e., NGOs, hospitals and facilities) for accommodation, health and welfare have been organized for homeless persons, following the Stop TB Strategy [5,6]. The prevalence of active TB in Osaka City decreased by 50% from 2000 to 2008. However, there are still estimated to be many unknown active TB cases in the Airin district [5].

Previous studies have identified risk factors for TB, such as immigrants, HIV, poverty, incarceration, smoking and alcohol use [7-10]. Homelessness-related TB remains a widespread problem [7]. An anti-TB strategy targeting homeless people, empowering caregivers and communities, and promoting research has been recommended [6,7]. However, studies on TB infection of homeless persons and their caregivers are scarce [11-13].

The commercially available blood test, QuantiFERON-TB Gold In-Tube (QFT; Cellestis Limited, Carnegie, Australia), is an interferon-gamma release assay (IGRA) in response to *M. tuberculosis*-specific antigens [14], and has been validated [15]. The QFT has excellent specificity and gives us valuable information of latent TB infection, even for a Japanese Bacillus Calmette-Guérin (BCG)-vaccinated population, whereas the accuracy of the tuberculin skin test (TST) is hampered by poor specificity due to the widespread use of BCG vaccination and re-vaccination in Japan [16].

The principal mechanism for the Stop TB Strategy is the detection and treatment of patients with TB [1]. The objectives of this study were to detect active TB cases and to assess the prevalence and risk factors of latent TB infection among homeless persons and their caregivers in a TB-endemic area in an intermediate-prevalence country.

Methods

Participants and Measurements

We conducted a cross-sectional study of homeless persons and their caregivers in the Airin district. Homeless persons were defined as persons who had had no permanent residence for more than one month. Caregivers were defined as persons who worked and supported homeless persons with regard to job arrangement, medical care, food supply, accommodation and clothing in the Airin district. Caregivers who belonged to five NGOs (mainly associated with a homeless shelter, job assistance, anti-alcoholism action, a soup-run and DOTS), as well as staff at the Osaka socio-medical center hospital and two clinics (nurses, social workers, dietitians, counselors and physicians) were enrolled in this study. Through our network of five NGOs, Osaka socio-medical center hospital and two local clinics by the use of posters, handouts and personal communication through a study recruiter, homeless persons and caregivers aged 30-74 years who had not received chest X-ray (CXR) examination within one year were recruited for tuberculosis screening at Osaka socio-medical center hospital between July 2007 and March 2008.

CXR, QFT, questionnaire and clinical evaluation by physicians were performed. If a participant had the symptom of sputum, smear testing and culture of sputum were performed for active TB diagnosis. A physician's interview using a standardized questionnaire covered the following data: age, sex, past history of TB, use of immunosuppressive drugs, past exposure to patients diagnosed with tuberculosis, current smoking and drinking status (more than 10 g of ethanol almost every day, yes or no), present symptoms of cough and/or sputum, general fatigue, elevated body temperature (≥37.0°C) and years spent living and/or working in the Airin district. Past exposure to tuberculosis patients was defined as self-reported exposure through living and/or working with TB patients in a shared space before the patients had been diagnosed with active TB.

CXR findings were categorized into two groups, normal and abnormal, based on our standard method [4]. Further classification was not performed since TB shadows vary and could take any kind of shape [17]. Quality control was performed by double-checks on each radiograph by another TB specialist. Active TB case was defined as an individual with symptoms compatible with TB plus detection of nucleic acid from mycobacterium tuberculosis complex from a clinical specimen, or as a patient with tuberculosis clinically suspected by an expert physician plus a response to anti-tuberculosis treatment [18].

QFT was performed and interpreted according to the manufacturer's instructions, with an interferon-γ response for the tuberculosis antigen tube minus Nil of ≥0.35 IU/ml defined as a positive result. QFT results were considered indeterminate if the subject did not respond to the mitogen-positive control tube with at least 0.5 IU/ml of interferon-γ [19,20].

Written informed consent was provided by each participant and each received 500 yen (about five US dollars) as an incentive to participate. The ethical committees of Osaka socio-medical center hospital and Osaka University approved this study.

Statistical Analysis

The proportions of basic characteristics and TB-related findings were descriptively shown among homeless persons and caregivers. The prevalence and 95% confidence

intervals (95% CI) for active TB were calculated using method based on the F-distribution.

The odds ratios (OR) and 95% CI for QFT-positivity were calculated using logistic regression model. We assessed how the positive QFT prevalence varied according to the potential factors such as length of time spent living and/or working in the Airin district and past exposure to TB patients, using the dichotomized categories to avoid statistical instability derived from small sample size. We investigated the multivariate model among homeless persons and caregivers, who did not have active TB disease or indeterminate QFT result.

Because of the failure in convergence, we removed the variables that had <5% cases with exposure or non-exposure category from the age-adjusted and multivariate logistic model [21]. Thereby, female and elevated body temperature for homeless, cough and/or sputum, elevated body temperature, past history of TB and abnormal chest X-ray finding for caregivers were excluded from the analyses.

Probability values for statistical tests were two-tailed and $p < 0.05$ was regarded as statistically significant. The SAS statistical software package (version 9.1; SAS Institute Inc., Cary, NC, USA) was used for all analyses.

Results

A total of 448 persons were enrolled in the study. However, of these 448 participants, four homeless persons refused to give a blood sample, and eight homeless persons left the waiting room before having a CXR and/or completing a questionnaire. Thus, complete results of CXR, QFT and questionnaire were available for 436 participants (263 homeless persons and 173 caregivers).

Table 1 shows the basic characteristics and TB-related findings for homeless persons and caregivers. The homeless persons included only two women and exhibited higher values for almost all variables of interest than caregivers. Four active TB cases were found among the homeless persons: the prevalence of 1.52% (95% CI, 0.42-3.85), while there were no cases among caregivers. One-half of the homeless persons (50.6%) and one-quarter of the caregivers (24.3%) had a positive QFT result.

Table 2 shows the characteristics of active TB cases. Out of the four active TB cases, all four subjects were homeless, male, more than 60 years of age, had abnormal CXR findings, had lived or worked for more than 9 years (mean 13 years) in the Airin district and had symptoms of cough and/or sputum. Three subjects had positive QFT results. None of them had acknowledged past exposure to TB patients.

Table 3 shows age-adjusted and multivariate OR (95% CI) of QFT-positivity according to potential risk factors among homeless persons and caregivers, who did not have active TB disease or indeterminate QFT result. Both homeless persons and caregivers who had spent more than ten years in the Airin district had significantly higher positive QFT result than those who had spent less than ten years in both age-adjusted and multivariate models. When we used a cutoff point of half of the period, namely, five years, homeless persons who had spent more than five years had significantly higher positive QFT result (multivariate odds ratio, 2.93; 95% CI, 1.43-6.01) but caregivers did not (multivariate odds ratio, 1.93; 95% CI, 0.84-4.45). Current drinker was a significant predictive factor for QFT-positivity among homeless persons, but not among caregivers in both age-adjusted and multivariate models. The caregivers who had acknowledged past exposure to TB patients had significantly higher QFT-positivity than those who did not in both age-adjusted and multivariate models, while the homeless persons did not in the multivariate model.

Past history of TB and abnormal chest X-ray finding were not associated significantly with positive QFT result among homeless persons in the multivariate model, and the analysis was not carried out for caregivers owing to the small number with a past history or abnormal chest X-ray finding.

Discussion

Active TB prevalence

We showed that homeless people in the Airin district remain at high risk for active TB, and that the cross-sectional estimate of the prevalence of active TB was a rate of 1.52% (95% CI, 0.42-3.85) among homeless people in the Airin district, Osaka, Japan. A similar high prevalence of active TB was reported among homeless day-laborers (2.2%) in the same district from a previous study in 2003-2005 [4]. According to annual TB patients' registry database, the prevalence of active TB among residents in the Airin district was 653 per 100,000 (0.65%) in 2007, and the homeless people accounted for 79.3% of TB patients in that district [22]. These estimates of active TB prevalence were markedly higher than those of the total Japanese population and the Osaka City population in 2007, namely, 19 per 100,000 (0.019%) and 53 per 100,000 (0.053%), respectively [1,22].

High prevalence of active TB among the homeless population was reported from other countries. In New York City, McAdam et al. reported the high active TB prevalence of 1,502 per 100,000 among homeless persons in 1992 [23]. In London, Story et al. showed the high prevalence of 788 per 100,000 among homeless people in 2003 [24]. TB is concentrated in the homeless population in developed countries and TB transmission may occur from homeless people to other populations [25].

Table 1 Basic characteristics and TB-related findings of the study participants

		Homeless persons (n = 263)	Caregivers (n = 173)
Basic characteristics			
Sex, female	n (%)	2 (0.8)	94 (54.3)
Age, years	mean median (range)	58.0 59 (30-74)	48.2 48 (30-71)
30-49	n (%)	40 (15.2)	97 (56.1)
50-59	n (%)	108 (41.1)	52 (30.1)
60-74	n (%)	115 (43.7)	24 (13.9)
Current smoker	n (%)	189 (73.0)	63 (37.1)
Current drinker	n (%)	134 (51.9)	54 (32.0)
Length of time spent living and/or working in the Airin district, years	mean median (range)	13.5 10 (0.1-58)	10.3 5 (0.1-64)
<5 years	n (%)	63 (25.9)	73 (43.2)
5-10 years	n (%)	46 (18.9)	33 (19.5)
≥10 years	n (%)	134 (55.1)	63 (37.3)
TB-related findings			
Past history of TB	n (%)	33 (12.6)	6 (3.5)
Past exposure to TB patients	n (%)	50 (19.8)	32 (18.7)
Cough and/or sputum	n (%)	64 (24.6)	6 (3.5)
General fatigue	n (%)	51 (19.6)	9 (5.2)
Elevated body temperature	n (%)	9 (3.5)	1 (0.6)
Abnormal chest X-ray finding	n (%)	68 (25.9)	7 (4.1)
Active TB case	n (%)	4 (1.5)	0 (0.0)
Positive QFT result	n (%)	133 (50.6)	42 (24.3)
Indeterminate QFT result	n (%)	4 (1.5)	1 (0.6)

Abbreviations: TB, tuberculosis; QFT, QuantiFERON-TB Gold In-Tube.

The number of missing values was 20 for length of time spent in the Airin district, 10 for past exposure to TB patients, 4 for smoking, 5 for drinking and 3 for cough and/or sputum, general fatigue and elevated body temperature among homeless persons, and 4 for length of time spent in the Airin district, 2 for past exposure to TB patients, 3 for smoking and 4 for drinking among caregivers.

None of the participants had a history of extra-pulmonary TB or used immunosuppressive agents.

The trends for active TB prevalence among homeless people varied considerably by location. In New York City, the prevalence had a substantial decline, that is, 1,502 per 100,000 in 1992 to 171 per 100,000 in 2004 [23]. In the Airin district, where approximately 80% of residents were estimated to be homeless people, the prevalence declined substantially from 1400 per 100,000 in 2000 to 680 in 2005, and declined further to a small extent to 653 per 100,000 in 2007 [22].

The substantial decline in the active TB prevalence in New York City and the moderate decline in the Airin district among homeless people were attributable

Table 2 Characteristics of active TB cases

	Case 1	Case 2	Case 3	Case 4	Total*
Homeless or caregivers	Homeless	Homeless	Homeless	Homeless	4
Sex	Male	Male	Male	Male	4
Age	62	63	66	66	Mean 64.3
Current smoker	-	+	+	-	2
Current drinker	-	+	-	+	2
Length of time spent living and/or working in the Airin district, years	10	9	20	13	Mean 13.0
Past history of TB	-	+	-	-	1
Past exposure to TB patients	-	-	-	-	0
Cough and/or sputum	+	+	+	+	4
General fatigue	+	-	+	+	3
Elevated body temperature	-	-	-	-	0
Abnormal chest X-ray finding	+	+	+	+	4
QFT-positivity	-	+	+	+	3

Abbreviations: TB, tuberculosis; QFT, QuantiFERON-TB Gold In-Tube.

*The number of positive for each factor or mean value for continuous variables among four active TB cases.

Table 3 Age-adjusted and multivariate odds ratios (OR) and 95% confidence intervals (95% CI) of QFT-positivity according to potential risk factors among homeless persons and caregivers, after excluding the subjects with active TB disease or indeterminate QFT results

		Homeless persons (n = 255)				Caregivers (n = 172)			
		No. of subjects	No.(%) of QFT-positivity	Age-adjusted OR (95% CI)	MultivariateOR (95% CI)†	No. of subjects	No.(%) of QFT-positivity	Age-adjusted OR (95% CI)	MultivariateOR (95% CI)†
Sex	Male	253	130 (51.4)	NA	NA	79	20 (25.3)	1.0	1.0
	Female	2	0 (0.0)	NA	NA	93	22 (23.7)	0.95 (0.46-1.93)	0.91 (0.39-2.11)
Current smoker	No	66	33 (50.0)	1.0	1.0	106	27 (25.5)	1.0	1.0
	Yes	185	93 (50.3)	1.19 (0.66-2.14)	1.23 (0.63-2.40)	63	13 (20.6)	0.81 (0.38-1.75)	0.72 (0.29-1.74)
Current drinker	No	121	56 (46.3)	1.0	1.0	114	26 (22.8)	1.0	1.0
	Yes	129	70 (54.3)	1.74 (1.02-2.96)*	1.84 (1.01-3.37)*	54	13 (24.1)	1.23 (0.56-2.69)	1.15 (0.48-2.74)
Length of time spent living and/or working in the Airin district, year	<10 years	107	38 (35.5)	1.0	1.0	105	18 (17.1)	1.0	1.0
	≥10 years	128	78 (60.9)	2.52 (1.46-4.35)*	2.53 (1.39-4.61)*	63	24 (38.1)	2.60 (1.24-5.42)*	2.32 (1.05-5.13)*
Past exposure to TB patients	No	196	96 (49.0)	1.0	1.0	138	27 (19.6)	1.0	1.0
	Yes	50	31 (62.0)	2.05 (1.05-3.99)*	1.51 (0.71-3.21)	32	14 (43.8)	3.63 (1.55-8.47)*	3.21 (1.30-7.91)*
Cough and/or sputum	No	194	100 (51.6)	1.0	1.0	166	42 (25.3)	NA	NA
	Yes	59	29 (49.2)	0.91 (0.50-1.67)	0.64 (0.32-1.32)	6	0 (0.0)	NA	NA
General fatigue	No	207	102 (49.3)	1.0	1.0	163	40 (24.5)	1.0	1.0
	Yes	46	27 (58.7)	1.66 (0.84-3.29)	1.47 (0.66-3.28)	9	2 (22.2)	0.96 (0.19-4.93)	1.06 (0.19-5.96)
Elevated body temperature	No	244	124 (50.8)	NA	NA	171	42 (24.6)	NA	NA
	Yes	9	5 (55.6)	NA	NA	1	0 (0.0)	NA	NA
Past history of TB	No	225	109 (48.4)	1.0	1.0	166	37 (22.3)	NA	NA
	Yes	30	21 (70.0)	2.23 (0.95-5.21)	1.51 (0.43-5.31)	6	5 (83.3)	NA	NA
Abnormal chest X-ray finding	No	193	86 (44.6)	1.0	1.0	165	37 (22.4)	NA	NA
	Yes	62	44 (71.0)	2.62 (1.39-4.93)*	1.90 (0.78-4.60)	7	5 (71.4)	NA	NA

*$p < 0.05$.

Abbreviations: TB, tuberculosis; NA, not applicable.

†Multivariate-adjusted for the listed factors and age.

to intensive population-based TB screening and DOTS [5,26].

Prevalence of latent TB infection

We found high prevalence of QFT-positivity for homeless persons (50.6%) and for caregivers (24.3%) at the ages of 30-74 years. When we restricted the sample to those of ages 40-69 years, the respective proportions were 50.4% for homeless persons and 30.8% for caregivers. These prevalences were far higher than that of the general Japanese population aged 40-69 years, which is estimated to be 7.1% [27].

Latent TB infection among vulnerable persons including homeless people

Garfein et al. investigated latent TB infection among 280 homeless persons in a Mexican city with the highest TB prevalence using an IGRA and found the prevalence of QFT-positivity was 51.8% [28], which was nearly equal to the prevalence in our study. In addition to homeless people, high-risk individuals for latent TB infection can be detected by the IGRA. For example, the prevalence of IGRA-positivity was reported to be 29.8% among immigrants, mostly from Latin America, in Italy [29], 33.6% among drug users in Houston, USA [30], and 53.9% among immigrants with close contact to sputum smear-positive TB patients in Netherlands [31].

Latent TB infection among caregivers

A study in Italy showed that the prevalence of latent TB infection (positive QFT) was 55.5% among caregivers working at a homeless shelter [11], which showed higher latent TB prevalence than the caregivers in our study. However, compared with healthcare workers for latent TB infection, the caregivers of our study had higher prevalence of latent TB infection. The prevalence of latent TB infection among healthcare workers in low- to intermediate-prevalence countries including Japan ranged from 1% to 19% [15,32-35], which was much lower than that of the caregivers in our study. Mirtskhulava et al. reported an extremely high prevalence of latent TB infection (60.0%) among healthcare workers, probably because they had frequent contact with TB patients and also high prevalence of TB in the community [36].

Putative risk factors for latent TB infection

Another aim of our study was to determine putative risk factors for latent TB infection. QFT-positivity was associated with past exposure to TB patients among caregivers, but not among homeless people. Caregivers usually know when and how they have been in contact with people with TB, whereas homeless persons often ignored or did not notice this [37]. Homeless people who drank almost every day had higher QFT-positivity than those who did not. Habitual drinkers may be more likely to have contact with other drinkers and had a higher risk of being infected [38]. The past history of TB was not significantly associated with a positive QFT result among homeless persons, which might be related to the waning of immune responses in the time course of TB infection [27]. The duration of living and/or working in Airin district was associated with QFT-positivity among both homeless people and caregivers, but this was the case only for ≥ 5 years among homeless people.

The risk of QFT-positivity was found to increase with increasing length of time spent in the Airin district, independently of acknowledgement of exposure to TB patients. Even caregivers without known exposure to TB patients in this study had approximately twofold higher QFT-positivity (19.6%, Table 3) than healthcare workers in Japan (9.9%) [16], indicating that they may be at high risk for TB infection. As mentioned above, caregivers at a homeless shelter had high latent TB prevalence even in a low-prevalence country [11]. Thus, anti-TB measures for caregivers should be strengthened to ensure their safety.

Limitations

This study has several potential limitations. The setting and selection of homeless persons in our study may limit the ability to generalize our results to the entire Airin district. Our sample of homeless persons may be at high risk to have been in contact with TB patients. The information on homelessness, past history of TB, past exposure to TB patients and length of time spent living and/or working in the Airin district was self-reported. Homeless people may be less likely to recall such information accurately because they are less health-conscious. The cross-sectional nature of the data limits the degree to which we can assign causality, especially with respect to temporality. However, it might be plausible that the exposure, such as past exposure to TB patients and length of time spent in the Airin district, may precede TB infection or disease. Information regarding TB-related factors such as HIV infection, drug abuse and history of incarceration was not included in the data collection. However, it is well known that TB/HIV co-infection is quite low in Japan [39]. In the present study, TST was not carried out because we wanted to avoid the refusal of study participation by homeless persons, and there is poor agreement between TST and QFT results caused by the effect of BCG vaccination in Japan [16].

Conclusions

We found that the prevalence of latent TB infection was approximately 50% for homeless people and 25% for

caregivers, and a long duration spent by both groups in the Airin district in Osaka, Japan, was associated with latent TB infection. Although no active TB was found for caregivers, one-quarter of them had latent TB infection. In addition to homeless persons, caregivers need examinations for latent TB infection as well as active TB and careful follow-up, especially when they have spent a long time in a high TB prevalence area and/or have been exposed to TB patients.

Acknowledgements
This study was supported by a grant-in-aid for social medical research (2007-2008) from Osaka socio-medical center hospital, without any involvement in the design, data collection, data analysis or data interpretation of this study. The authors wish to thank all personnel of the Osaka socio-medical center hospital and many NGOs for providing data used in this study. The authors are also grateful to Drs. S. Yamamoto, T. Ohira, K. Danno and Y. Toyoda for their assistance in the data collection and analyses.

Author details
[1]Public Health, Department of Social and Environmental Medicine, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita-shi, Osaka, Japan. [2]Osaka Socio-Medical Center Hospital, 1-3-44 Haginochaya, Nishinari-ku, Osaka-shi, Osaka, Japan. [3]Osaka City Public Health Office, 1-2-7-1000, Asahimachi, Abeno-ku, Osaka-shi, Osaka, Japan. [4]Osaka Prefectural Hospital Organization Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, 3-7-1 Habikino, Habikino-shi, Osaka, Japan.

Authors' contributions
T. Takatorige, YH, NN, SH, AS, KF, HY and T. Takashima participated in the planning of the study. T. Takatorige and T. Takashima coordinated the study and took overall responsibility for the delivery of the work. T. Tabuchi, YH, KF and HY had responsibility for data collection. T. Tabuchi conducted the analysis, with statistical support from HI, YT, TN and TM. T. Tabuchi, HI and T. Takashima participated in writing the paper. All authors participated in the interpretation of the study and read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Received: 15 July 2010 Accepted: 21 January 2011
Published: 21 January 2011

References

1. World Health Organization: *Global tuberculosis control: epidemiology, strategy, financing, WHO Report 2009* Geneva, Switzerland: WHO; 2009, WHO/HTM/TB/2009.411.
2. Shimao T: **Tuberculosis and its control-lessons from the past and future prospect.** *Kekkaku* 2005, **80(6)**:481-489.
3. Nakata N, Inori F, Nakamura F, Harihara S, Hirayama Y, Suzuki A, Shimouchi A, Takatorige T: **Significance of tuberculosis screening of outpatients in areas with high prevalence of tuberculosis.** *Kekkaku* 2007, **82**:455-458.
4. Takatorige T, Ohsaka T, Yamamoto S, Nishimori T, Fujikawa T, Kuroda K, Iso H: **Tuberculosis and its control measures for homeless people: implementation of chest X-ray examination for three successive years.** *Kekkaku* 2007, **82**:19-25.
5. Shimouchi A: **Fight against urban tuberculosis problems and program effects in Osaka City.** *Kekkaku* 2009, **84**:727-735.
6. Raviglione MC, Uplekar MW: **WHO's new Stop TB Strategy.** *Lancet* 2006, **367**:952-955.
7. Potter B, Rindfleisch K, Kraus CK: **Management of active tuberculosis.** *Am Fam Physician* 2005, **72**:2225-2232.
8. van Zyl Smit RN, Pai M, Yew WW, Leung CC, Zumla A, Bateman ED, Dheda K: **Global lung health: the colliding epidemics of tuberculosis, tobacco smoking, HIV and COPD.** *Eur Respir J* 2010, **35**:27-33.
9. Holtgrave DR, Crosby RA: **Social determinants of tuberculosis case rates in the United States.** *Am J Prev Med* 2004, **26**:159-162.
10. Lonnroth K, Jaramillo E, Williams BG, Dye C, Raviglione M: **Drivers of tuberculosis epidemics: the role of risk factors and social determinants.** *Soc Sci Med* 2009, **68**:2240-2246.
11. Martini A, Tomao P, Di Renzi S, Vonesch N, Rubino L, Signorini S, Tomei F: **QuantiFERON-TB Gold test in homeless shelter staff: preliminary results.** *G Ital Med Lav Ergon* 2007, **29**:767-769.
12. de Vries G, van Hest RA: **From contact investigation to tuberculosis screening of drug addicts and homeless persons in Rotterdam.** *Eur J Public Health* 2006, **16**:133-136.
13. Dewan PK, Grinsdale J, Liska S, Wong E, Fallstad R, Kawamura LM: **Feasibility, acceptability, and cost of tuberculosis testing by whole-blood interferon-gamma assay.** *BMC Infect Dis* 2006, **6**:47.
14. Andersen P, Munk ME, Pollock JM, Doherty TM: **Specific immune-based diagnosis of tuberculosis.** *Lancet* 2000, **356**:1099-1104.
15. Harada N, Higuchi K, Yoshiyama T, Kawabe Y, Fujita A, Sasaki Y, Horiba M, Mitarai S, Yonemaru M, Ogata H, Ariga H, Kurashima A, Wada A, Takamori M, Yamagishi F, Suzuki K, Mori T, Ishikawa N: **Comparison of the sensitivity and specificity of two whole blood interferon-gamma assays for M. tuberculosis infection.** *J Infect* 2008, **56**:348-353.
16. Harada N, Nakajima Y, Higuchi K, Sekiya Y, Rothel J, Mori T: **Screening for tuberculosis infection using whole-blood interferon-gamma and Mantoux testing among Japanese healthcare workers.** *Infect Control Hosp Epidemiol* 2006, **27**:442-448.
17. Andreu J, Caceres J, Pallisa E, Martinez-Rodriguez M: **Radiological manifestations of pulmonary tuberculosis.** *Eur J Radiol* 2004, **51**:139-149.
18. **Diagnostic Standards and Classification of Tuberculosis in Adults and Children. This official statement of the American Thoracic Society and the Centers for Disease Control and Prevention was adopted by the ATS Board of Directors, July 1999. This statement was endorsed by the Council of the Infectious Disease Society of America, September 1999.** *Am J Respir Crit Care Med* 2000, **161(4 Pt 1)**:1376-1395.
19. **QuantiFERON-TB Gold In-Tube package insert.** [http://www.cellestis.com/IRM/Company/ShowPage.aspx?CPID=1255].
20. Mazurek GH, Jereb J, Lobue P, Iademarco MF, Metchock B, Vernon A: **Guidelines for using the QuantiFERON-TB Gold test for detecting Mycobacterium tuberculosis infection, United States.** *MMWR Recomm Rep* 2005, **54**:49-55.
21. Katz MH: *Multivariable Analysis: a practical guide for clinicians.* Second edition. Cambridge University Press; 2006.
22. Osaka city public health center: *Tuberculosis of Osaka city* Osaka; 2008.
23. McAdam JM, Bucher SJ, Brickner PW, Vincent RL, Lascher S: **Latent tuberculosis and active tuberculosis disease rates among the homeless, New York, New York, USA, 1992-2006.** *Emerg Infect Dis* 2009, **15**:1109-1111.
24. Story A, Murad S, Roberts W, Verheyen M, Hayward AC: **Tuberculosis in London: the importance of homelessness, problem drug use and prison.** *Thorax* 2007, **62**:667-671.
25. Kameda K: **Thinking about tuberculosis in Osaka City.** *Kekkaku* 2000, **75**:717-724.
26. Bureau of Tuberculosis Control, New York City Department of Health and Mental Hygiene: *Clinical Policies and Protocols* , 4 2008.
27. Mori T, Harada N, Higuchi K, Sekiya Y, Uchimura K, Shimao T: **Waning of the specific interferon-gamma response after years of tuberculosis infection.** *Int J Tuberc Lung Dis* 2007, **11**:1021-1025.
28. Garfein RS, Laniado-Laborin R, Rodwell TC, Lozada R, Deiss R, Burgos JL, Cuevas-Mota J, Cerecer P, Moser K, Volker ML, Strathdee SA: **Latent tuberculosis among persons at risk for infection with HIV, Tijuana, Mexico.** *Emerg Infect Dis* 2010, **16**:757-763.
29. Orlando G, Merli S, Cordier L, Mazza F, Casazza G, Villa AM, Codecasa L, Negri E, Cargnel A, Ferrarese M, Rizzardini G: **Interferon-gamma releasing assay versus tuberculin skin testing for latent tuberculosis infection in targeted screening programs for high risk immigrants.** *Infection* 2010, **38(3)**:195-204.
30. Grimes CZ, Hwang LY, Williams ML, Austin CM, Graviss EA: **Tuberculosis infection in drug users: interferon-gamma release assay performance.** *Int J Tuberc Lung Dis* 2007, **11**:1183-1189.
31. Kik SV, Franken WP, Arend SM, Mensen M, Cobelens FG, Kamphorst M, van Dissel JT, Borgdorff MW, Verver S: **Interferon-gamma release assays in**

immigrant contacts and effect of remote exposure to Mycobacterium tuberculosis. *Int J Tuberc Lung Dis* 2009, **13**:820-828.
32. Alvarez-Leon EE, Espinosa-Vega E, Santana-Rodriguez E, Molina-Cabrillana JM, Perez-Arellano JL, Caminero JA, Serrano-Aguilar P: **Screening for tuberculosis infection in spanish healthcare workers: Comparison of the QuantiFERON-TB gold in-tube test with the tuberculin skin test.** *Infect Control Hosp Epidemiol* 2009, **30**:876-883.
33. Nienhaus A, Loddenkemper R, Hauer B, Wolf N, Diel R: **Latent tuberculosis infection in healthcare workers–evaluation of an Interferon-gamma release assay.** *Pneumologie* 2007, **61**:219-223.
34. Tripodi D, Brunet-Courtois B, Nael V, Audrain M, Chailleux E, Germaud P, Naudin F, Muller JY, Bourrut-Lacouture M, Durand-Perdriel MH, Gordeeff C, Guillaumin G, Houdebine M, Raffi F, Boutoille D, Biron C, Potel G, Roedlich C, Geraut C, Schablon A, Nienhaus A: **Evaluation of the tuberculin skin test and the interferon-gamma release assay for TB screening in French healthcare workers.** *J Occup Med Toxicol* 2009, **4**:30.
35. Soborg B, Andersen AB, Larsen HK, Weldingh K, Andersen P, Kofoed K, Ravn P: **Detecting a low prevalence of latent tuberculosis among health care workers in Denmark detected by M. tuberculosis specific IFN-gamma whole-blood test.** *Scand J Infect Dis* 2007, **39**:554-559.
36. Mirtskhulava V, Kempker R, Shields KL, Leonard MK, Tsertsvadze T, del Rio C, Salakaia A, Blumberg HM: **Prevalence and risk factors for latent tuberculosis infection among health care workers in Georgia.** *Int J Tuberc Lung Dis* 2008, **12**:513-519.
37. Marks SM, Taylor Z, Qualls NL, Shrestha-Kuwahara RJ, Wilce MA, Nguyen CH: **Outcomes of contact investigations of infectious tuberculosis patients.** *Am J Respir Crit Care Med* 2000, **162**:2033-2038.
38. Rehm J, Samokhvalov AV, Neuman MG, Room R, Parry C, Lonnroth K, Patra J, Poznyak V, Popova S: **The association between alcohol use, alcohol use disorders and tuberculosis (TB). A systematic review.** *BMC Public Health* 2009, **9**:450.
39. Yamada N, Nagai H: **The current and future situations of TB/HIV (co-infection of tuberculosis and HIV) in Japan.** *Kekkaku* 2009, **84**:203-211.

Pre-publication history
The pre-publication history for this paper can be accessed here:
http://www.biomedcentral.com/1471-2334/11/22/prepub

doi:10.1186/1471-2334-11-22
Cite this article as: Tabuchi *et al.*: Tuberculosis infection among homeless persons and caregivers in a high-tuberculosis-prevalence area in Japan: a cross-sectional study. *BMC Infectious Diseases* 2011 **11**:22.

Infection, Genetics and Evolution 10 (2010) 1066–1074

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Resolving lineage assignation on *Mycobacterium tuberculosis* clinical isolates classified by spoligotyping with a new high-throughput 3R SNPs based method

Edgar Abadia [a], Jian Zhang [a], Tiago dos Vultos [b], Viviana Ritacco [c], Kristin Kremer [d], Elif Aktas [e], Tomoshige Matsumoto [f], Guislaine Refregier [a], Dick van Soolingen [d], Brigitte Gicquel [b], Christophe Sola [a,b,*]

[a] *Institute of Genetics and Microbiology, UMR8621, IGEPE Team, Universud, CNRS Université Paris-Sud 11, France*
[b] *Unité de Génétique Mycobactérienne, Institut Pasteur, Paris, France*
[c] *Instituto Nacional de Enfermedades Infecciosas ANLIS Carlos Malbrán, Vélez Sarsfield 563, 1281 Buenos Aires, Argentina*
[d] *National Institute for Public Health and the Environment, Bilthoven, The Netherlands*
[e] *Department of Clinical Microbiology, Zonguldak Karaelmas University Hospital, Zonguldak, Turkey*
[f] *Osaka Prefectural Hospital Organization Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, Japan*

ARTICLE INFO

Article history:
Received 19 May 2010
Received in revised form 1 July 2010
Accepted 2 July 2010
Available online 17 July 2010

Keywords:
Mycobacterium tuberculosis complex
CRISPR
Spoligotyping
Population studies
Phylogeny
Evolution
SNPs
Multiplex
High-throughput

ABSTRACT

We developed a new multiplexed-PCR assay to accurately classify *Mycobacterium tuberculosis* complex (MTC) isolates at the sublineage level by single nucleotide polymorphisms (SNPs). This method relies on 7 SNPs located in different genes of the MTC strains (*recC*, *rec0*, *recR*, *ligB*, *ligC*, *alkA*, and *mgtC*). Most of these genes are involved in replication, repair and recombination (3R) functions of *M. tuberculosis* strains, four of the mutations are synonymous, and thus neutral. Genes were chosen as a first empirical approach to assess the congruence between spoligotyping-based phylogeographical classification and SNP typing.

This scheme efficiently classifies most of MTC phylogeographical groups: (1) confirming and identifying new sublineage-specific SNPs, (2) unraveling phylogenetical relationships between spoligotyping-defined MTC sublineages, (3) appropriately assigning sublineages to some spoligotypes and reassigning sublineages to other mis-labeled spoligotype signatures. This study opens the way to a more meaningful taxonomic, evolutionary and epidemiological classification. It also allows evaluation of spoligotype-signature significance towards a more comprehensive understanding of the evolutionary mechanisms of the clustered regularly interspaced short palindromic repeat (CRISPR) locus in MTC.

1. Introduction

Despite the availability of effective antituberculosis chemotherapy for over 50 years (Styblo and Bumgarner, 1991), TB remains a major global health problem since the World Health Organization declared tuberculosis (TB) a global emergency in 1994 (Nakajima, 1993). The spread of multi-drug resistant tuberculosis (MDR-TB) and more recently of extremely drug resistant tuberculosis (XDR-TB) (Ralph et al., 2009), makes the implementation of public health measures, and molecular epidemiological investigations using rapid and high-throughput molecular methods an important point to follow TB transmission.

Current genotyping techniques used to study the epidemiology of *Mycobacterium tuberculosis* complex (MTC) clinical isolates are based on repetitive genetic elements: Clustered Repetitive Interspersed Short Palindromic Repeat (CRISPR) loci through the spoligotyping technique (Groenen et al., 1993; Kamerbeek et al., 1997; van Embden et al., 2000; Sorek et al., 2008) and Mycobacterial Interspersed Repetitive Units-Variable Number of Tandem repeats (MIRU-VNTR) (Frothingham and Meeker-O'Connell, 1998; Supply et al., 2000, 2001, 2006; Le Fleche et al., 2002; Skuce et al., 2002). Indeed, these markers have proven to be highly useful for epidemiological, population structural and evolutionary studies to distinguish between MTC clinical isolates (Abadia et al., 2009; Allix-Beguec et al., 2008a; Baranov et al., 2009; Brudey et al., 2006; Helal et al., 2009; Rohani et al., 2009; Stavrum et al., 2009) and have been used as the alternative to the classical IS*6110*-RFLP method (van Embden et al., 1993). These methods have in addition received recent technological improvements enabling fast and large-scale analyses to be performed (Cowan et al., 2004; Mazars et al., 2001). The classical spoligotyping procedure (Kamerbeek et al., 1997) relies on a reverse-line blot hybridization, a procedure that takes one full day of work to produce 43 profiles without interpretation; with the automatization now, 96 profiles can be

* Corresponding author at: Institute of Genetics and Microbiology, UMR8621, IGEPE Team, Universud, CNRS Université Paris-Sud 11, Campus d'Orsay, F-91405 Orsay Cedex, France; Tel.: +33 01 69 15 46 48; fax: +33 01 69 15 66 78.
E-mail address: christophe.sola@u-psud.fr (C. Sola).

1567-1348/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.meegid.2010.07.006

obtained in half a day (Cowan et al., 2004; Zhang et al., 2010). This new technology relies on microbeads of different spectrum signatures to which capture probes are coupled depending on the targets (Cowan et al., 2004).

For phylogenetical evolutionary studies there is a concern using the fast evolving loci due to the presence of convergent evolutionary events within CRISPRs (Warren et al., 2002) as well as within MIRU-VNTR loci (Hanekom et al., 2008). To define phylogenetic associations unambiguously, genetic markers need to be unique and, ideally, irreversible (Comas et al., 2009).

In *M. tuberculosis*, these markers are large sequence polymorphisms (LSPs) (Hirsh et al., 2004; Mostowy et al., 2002) and single nucleotide polymorphisms (SNPs) (Alland et al., 2003; Dos Vultos et al., 2008; Filliol et al., 2006; Gutacker et al., 2006; Hershberg et al., 2008). LSPs are powerful markers in MTC because horizontal DNA transfer is extremely rare (Supply et al., 2003), but genetic distances based on genomic deletions are difficult to interpret in phylogenies (Gagneux et al., 2006). SNPs are less mutable than other forms of polymorphisms, making them unlikely to converge (Schork et al., 2000) so they are most appropriate markers for phylogenetic studies. In MTC strains structural genes exhibit rare polymorphism (Achtman, 2008; Kapur et al., 1994; Musser et al., 2000; Sreevatsan et al., 1997). However, recently Dos Vultos et al. (2008) have found higher polymorphisms in several genes involved in replication, recombination and repair functions (3R genes). For the first time, we provide a starting point of a new SNP typing of *M. tuberculosis* complex clinical isolates based mainly on 3R genes. We implemented this schedule on a high-throughput platform using a direct hybridization assay (Dunbar, 2006), that was used before for spoligotyping analyses (Cowan et al., 2004; Zhang et al., 2010).

We think that this could be the first step to provide a reliable high-throughput 3R SNP-based method for population structural studies and for further phylogenetical studies on *M. tuberculosis* complex clinical isolates.

2. Materials and methods

2.1. Chemicals, buffers and microbeads

All main materials and reagents required for microbead-based flow cytometry techniques were the same as described before (Zhang et al., 2010).

2.2. Oligonucleotides

To design capture probes and primers we used a demo of PrimerPlex (http://www.premierbiosoft.com/primerplex/index.html) and Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). All capture oligonucleotides (Eurogentec, Liège, Belgium) were manufactured and coupled as described before (Zhang et al., 2010). In this study we are targeting 7 genes that are polymorphic within *M. tuberculosis* sublineages, 6 of them from the 3R system described in Dos Vultos et al. (2008) and one (*mgtC*) reported by Alix et al. (2006). The primers and capture probes used here are listed in Tables 1 and 2, respectively.

2.3. Sequences analysis

Gene sequences were downloaded from http://genolist.pasteur.fr/TubercuList/. The DNAsp package (Rozas et al., 2003), Multalin (Corpet, 1988) and BioEdit sequence alignment editor (Hall, 1999) were used to locate SNPs on gene sequences and to align the gene and probe sequences in the design and/or verification of pre-designed capture probes.

Table 1
Genes, lineage or sublineage association, genomic position of targeted SNPs (up) and primers designed for the multiplex-PCR (down).

Gene[a]	Lineage or sublineage association	SNP targeted[b]
alkA	Bovine	807
recO	EAI	606
ligB	LAM	1212
recR	T2-related	94
mgtC	Haarlem	545
recC	X	1491
ligC	TUR-T3-Osaka	809

Primers		
Gene	Sequence (5′–3′)	bp
alkA-F	CACGCTACGGCTCCCATG	18
alkA-R[c]	CCTTCGTCGATACCTGTTGGG	21
recO-F	TGTTGGACGCCTATCTGCTG	20
recO-R[c]	CCGTTCCAGATGCCATTGC	19
ligB-F	GGCTGGCTGAAGGTCAAG	18
ligB-R[c]	CATGGCGTCGGTCATTCC	18
recR-F	GGACCTGATTGACGAACTCG	20
recR-R[c]	GCCTGGATGTCTTTGGGTTC	20
mgtC-F	TCGTCGCTGTCCATCTCC	18
mgtC-R[c]	CACCAACCGCTCTAGCTTG	19
recC-F	CGCCGAACTGCTCACCATC	19
recC-R[c]	GCCACGCTTGGGAATCCTC	19
ligC-F	CGCGTCGGTCGGCGTGAT	18
ligC-R[c]	CGGGTCGCAGCGGCACGA	18

[a] Genes from the 3R system (Dos Vultos et al., 2008), except *mgtC* (Alix et al., 2006).
[b] nt position related to the gene.
[c] Reverse primers are biotin labeled.

2.4. SNPs typing PCR protocol

For direct hybridization of multiplexed-PCR assays, product length is recommended to be between 150 and 300 bp. We amplified segments around 200 bp of *alkA* (291 bp), *recO* (298 bp), *ligB* (174 bp), *recR* (255 bp), *mgtC* (272 bp) and *recC* (272 bp) to analyse the correlation between SNPs and major MTC lineages. To increase signals/cut-off ratio, PCR-multiplex was firstly divided into 3 sets, set1: *ligB*, *recR*, *mgtC* and *recC*; set2: *alkA* and *recO* and set3: *ligB* and *ligC*; however, running a single 7-Plex PCR protocol now provides similar results (see Supplemental Table 1). PCR assays were performed in 25 μL volumes of the following mixture: PCR buffer (100 mM Tris–HCl, pH 8.3, 500 mM KCl), 0.2 mM each dNTP, a final quantity of primers in a solution of 25 pmol of each one, 1.0 U Taq and 2 μL of DNA. The following PCR program was run: 5 min at 95 °C, followed by a touch-down PCR of 30 s at 95 °C,

Table 2
Sequence of the capture probes used to target the SNPs in this study.

Probes			
Probe name	Sequence 5′–3′	bp	Targeted site (nt)
alkA-wt	CGCGACCTGATGACGGC	17	807 ct**g** (leu) to
alkA-mut	CGCGACCTAATGACGGC	17	807 ct**a** (leu)
recO-wt	GTACGACGGCGATTGGGA	18	606 gg**c** (gly) to
recO-mut	GTACGACGGTGATTGGGA	18	606 gg**t** (gly)
ligB-wt	GGCAAGCTCTCCAATATTCACC	22	1212 tc**c** (ser) to
ligB-mut	GGCAAGCTCTCGAATATTCACC	22	1212 tc**g** (ser)
RecR-wt	CTTCCACCTGTTGTCGGTAGA	21	94 **t**tg (leu) to
RecR-mut	CTTCCACCTGCTGTCGGTAGA	21	94 **c**tg (leu)
mgtC-wt	GGGGTATACGCACGGGGC	18	545 cg**c** (arg) to
mgtC-mut	GGGGTATACACACGGGGC	18	545 ca**c** (his)
RecC-wt	GTGGCGGTTCGGACTCGA	18	1491 tt**c** (phe) to
RecC-mut	GTGGCGGTTGGGACTCGA	18	1491 tt**g** (leu)
ligC-wt	GACCACCCATGGAACTGGGCC	21	809 t**g**g (trp) to
ligC-mut	GACCACCCATTGAACTGGGCC	21	809 t**t**g (leu)

All capture probes have at 5′ a C-12 terminal linker. We are targeting both alleles, the wild type and the mutant for each site.

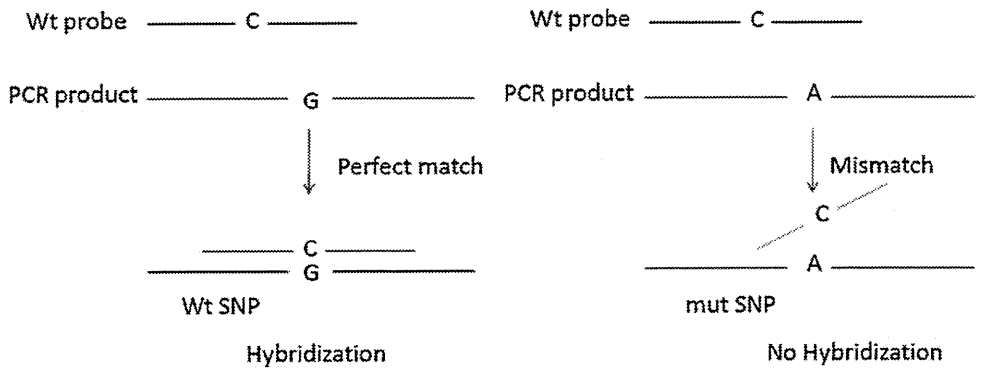

Fig. 1. Allele-specific hybridization, image modified from (Kwok, 2001).

30 s at 65 °C (−1 °C per step), 1 min at 72 °C 10 times and 30 s at 95 °C, 30 s at 56 °C, 1 min at 72 °C for 29 more cycles with a final extension step of 10 min at 72 °C. The PCR amplifications were checked during first tests by agarose gel electrophoresis.

2.5. Hybridization

The procedure detecting the two possible alleles relies on an allele-specific hybridization (Kwok, 2001). Fig. 1 shows how the SNPs were discriminated based on this approach. If the PCR product harbors the wild type (wt) SNP it will match to the wt probe that is complementary to the sequence. This hybridized product is very stable because there is a 100% complementarity. If the PCR product has the mutated (mut) SNP allele, the hybridization is not thermodynamically stable due to the nucleotide mismatch and it will be lost. For this kind of procedure the mismatch base should be placed in the middle of the probe. The hybridization procedure on microbeads was done as described previously (Zhang et al., 2010) except for the second part of the hybridization that was performed at 52 °C for 20 min.

2.6. Mycobacterium isolates, origin, DNA extraction

327 DNA samples (a summary of the lineages being targeted can be found in Supplemental Table 2) were genotyped by our novel SNP typing scheme using a high-throughput microbead-based method, as reported previously (Cowan et al., 2004; Zhang et al., 2010). The clinical isolates for this study were chosen to cover the main lineages of *M. tuberculosis* complex clinical isolates described before by spoligotyping (Filliol et al., 2002; Brudey et al., 2006). They were selected from an international quality control study on membrane-based spoligotyping vs. high-throughput-based spoligotyping (Abadia et al., unpublished results) as well as from the 2004–2008 collection of the TB National Reference Laboratory in the Netherlands, from Zonguldak hospital, Turkey and from a Medical Center in Japan. More specifically, 59 samples were from Buenos Aires – Argentina (Servicio de Micobacterias, Instituto Nacional de Enfermedades Infecciosas, ANLIS "Carlos G. Malbran", Buenos Aires, Argentina), 109 samples came from Bilthoven – The Netherlands (National Institute for Public Health and the Environment – RIVM), 120 DNA samples from the Faculty of Medicine, Department of Microbiology and Clinical Microbiology, Zonguldak Karaelmas University (Zonguldak – Turkey), 40 DNA samples from Japan (Osaka Prefectural Medical Center for Respiratory and Allergic Diseases). The DNA from these samples was extracted either by a simple thermolyzate or by the classical cetyltrimethylammonium bromide (CTAB) procedure.

2.7. Data analysis

Standard MTC lineage assignation (*i.e.* according to spoligotype pattern) was reported either according to spolDB4 database (Brudey et al., 2006) or using the SPOTCLUST algorithm (Vitol et al., 2006) online access http://cgi2.cs.rpi.edu/~bennek/Run.html).

To infer lineages according to SNPs, the presence of allele putative-lineages associated (Dos Vultos et al., 2008) was reported. Hybridization signals for the mutated or wild type SNP alleles, collected as RFI (Relative Fluorescence Intensity) were transformed in a binary code (presence/absence of each allele) using a signal/noise cut-off value of 2.

3. Results

The aim of this study was: (1) to test some associations between spoligotype-defined lineages and a set of potentially lineage-specific SNPs mainly located on 3R genes (Alix et al., 2006; Dos Vultos et al., 2008) on a representative set of samples; (2) to develop a new multiplexed high-throughput assay for this purpose; (3) to assign a lineage to those clinical isolates for which spoligotype signatures were uninformative, the so-called "U" clinical isolates in SpolDB4 (Brudey et al., 2006); and finally (4) to confirm previous lineage associations or correct possible mis-assignations. We thus aimed to further contribute to the study of the MTC molecular evolution.

We searched on the 3R gene database (Dos Vultos et al., 2008) for SNPs that were polymorphic between main MTC lineages and we found that SNPs on: *alkA* (807 ctg [leu] to cta [leu]), *recO* (606 ggc [gly] to ggt [gly]), *ligB* (1212 tcc [ser] to tcg [ser]), *recC* (1491 ttc [phe] to ttg [leu]), *recR* (94 ttg [leu] ctg [leu]), *ligC* (809 tgg [trp] to ttg [leu]) apparently were associated with *M. bovis*, East African (EAI), the Latin-American Mediterranean (LAM), the X, the T2 and the LAM7_TUR/T3_Osaka *M. tuberculosis* lineages, respectively. We added to this set of genes the SNP on *mgtC* 545 cgc [arg]) to cac [his] because it was described previously as being associated with the Haarlem lineage (Alix et al., 2006) and recently had been tested by Chuang et al. (2008). The mgtC protein is a common virulence factor to several intracellular pathogens (Buchmeier et al., 2000).

Our scheme thus targets 7 lineages: 5 large lineages covering a total of 31 sublineages, and two specific sublineages. Other SNPs belonging to other panel of genes could also be added in a near future to our assay. We have developed a single multiplexing reaction including this set of 7 genes to test the previous finding of 3R SNP MTC lineage association in a representative set of samples. Central-Asian (CAS) specific and East-Asian (Beijing) specific SNPs are not reported at this stage of work but are also in progress.

To validate our new SNP typing method, we used 327 DNA samples of *M. tuberculosis* clinical isolates, all having been previously spoligotyped on microbeads using the high-throughput technique (Zhang et al., 2010). These samples were representative of a highly diverse MTC population within the total worldwide genetic diversity of MTC: they covered unclassified strains, as well as 32 sublineages over the 62 that have been named in SpolDB4, representing all main lineages infecting humans including some that were not targeted by our SNP scheme (Beijing, CAS, EAI, LAM, bovis, Haarlem, X, S and T). Only *Mycobacterium africanum* and *Mycobacterium canettii* and Manu lineages were not represented. Out of the 32 sublineages that were represented in our data set, 21 were targeted by our scheme.

Among the seven mutations tested within our scheme we never detected two mutations simultaneously in any of the 327 typed strains, leading either to SNP-specific assignment or to SNP-nonspecific assignment. In addition, none of the isolates assigned to lineages Beijing, CAS, S and T4 did show any of the mutation present in our SNP scheme as expected. This highlights the specificity of the SNP we chose, and confirms the absence of convergence events in this collection. For sixteen sublineages (76%, covering 73 different isolates) targeted by our scheme and present in our data set, complete congruence between spoligotyping-based classification and SNP-based assignation was observed, indicating a high global fit between the two classifications: 6/6 *M. bovis*, 55 EAI (EAI1_SOM, EAI2_MANILLA, EAI5, EAI3), 28/38 H and 14/14 from sublineages H1 and H2, 23/37 LAM and 23/23 from sublineages LAM2, LAM3, LAM5, LAM9, 2/2 X (X1, X3), 23/23 T3-Osaka. For five sublineages that were targeted by the scheme, part or all of the assignations were different between the two classifications: 4/18 clinical isolates from H3, 6/6 from H4, 28/34 from T2, 7/7 from LAM3-S convergent, 40/40 LAM7_TUR (Table 3 for an overview; spoligotype patterns and SNP genotypes in Supplemental Table 3). One T5 sublineage (T5_RUS1) and 6 T1-Tuscany, and one T1 however carried the LAM-specific SNP, 2 other T1 isolates carried the H-specific mutation, and 2 other T1 isolates the T3-Osaka specific SNP, confirming that T-clade is poorly defined.

More specifically, not all of the clinical isolates labeled as Haarlem lineage (H1 to H4) according to their spoligotype signatures in SpolDB4 ($n = 38$) harbored the SNP Haarlem associated on *mgtC*$^{545\ (cgc\text{-}cac)}$ (Alix et al., 2006). All H1 spoligo-signatures ($n = 11$) and all H2 spoligo-signatures ($n = 3$) had this specific mutation whereas 14/18 of H3 spoligo-signature and none of the H4 ($n = 6$) had. Among the four H3 clinical isolates that did not harbor the *mgtC* mutation, two belonged to SIT316, one to

Table 3
SNP lineage assignation on a subset of analyzed strains and comparison with SpolDB4 linage classification to show in some cases the lineage confirmation and in others a lineage re-assignation.

SIT	Spoligotype	Lineage[a]		No. of strains[e]
		SpolDB4	SNP	
35		H4	-	2
262		H4	-	3
777		H4	-	1
760		H3	-	1
134		H3	X	1
316		H3	T2	2
47[b]		H1	H	1
50[b]		H3	H	9
41		LAM7_TUR	TUR- T3- Osa	6
367		LAM7_TUR	TUR- T3- Osa	1
4		LAM3- S/conv	-	7
42[b]		LAM9	LAM	10
33[b]		LAM3	LAM	4
93[b]		LAM5	LAM	1
17[b]		LAM2	LAM	2
52		T2	T2[c]	31[e]
254		T5_RUS1	LAM	1
736		T2	-	1
627		T3-OSA	TUR- T3- Osa	40
78		T1-T2	TUR- T3- Osa	2
53**		T1	TUR- T3- Osa[d]	25[d]
159		T1 (Tuscany v.)	LAM	7
370		T1	LAM	1
53		T1	H	1
120		T1	H	1

[a]Family assignation according to spoligo-signatures and according to SNP identification.
[b]Reference strains for each lineage.
[c]Just 6/31 had the mutation on *recR*$^{94\ (ttg\text{-}ctg)}$ T2-related.
[d]2/25 strains SIT 53 had the *ligC*^{809} SNP.
[e]Number of strains related to the frequency of the spoligotype pattern.
To read and interpret the spoligotype, spacers are placed in order from 1 to 43 (left to right).
"-": Means no detectable SNP.