Multiplex PCR reveals a high rate of nasopharyngeal pneumococcal 7-valent conjugate vaccine serotypes co-colonizing indigenous Warao children in Venezuela

Ismar A. Rivera-Olivero,^{1†} Martijn Blommaart,^{2†} Debby Bogaert,³ Peter W. M. Hermans² and Jacobus H. de Waard¹

Correspondence

Jacobus H. de Waard
jacobusdeward@gmail.com

¹Laboratorio de Tuberculosis, Instituto de Biomedicina, Caracas, Venezuela

²Department of Paediatrics, Nijmegen Medical Centre, Radboud University, Nijmegen, The Netherlands

³Department of Epidemiology, Harvard School of Public Health, Boston, MA, USA

Knowledge of co-colonization with multiple pneumococcal serotypes is becoming very important in the light of both serotype replacement and switching as a result of vaccination. Co-colonization has been reported to occur in up to 30 % of carriers, especially in populations with high *Streptococcus pneumoniae* carriage rates. For the determination of co-colonization, single colonies of nasopharyngeal specimens are serotyped with the Quellung method, a costly method with a low sensitivity. Here we explore the use of a multiplex PCR to identify simultaneous carriage of the capsular serotypes targeted by the 7-valent conjugate vaccine. We applied this multiplex PCR to 50 primary cultures from the nasopharyngeal swabs of healthy Warao Amerindian children, a population with a high pneumococcal carriage rate, most of them with vaccine serotypes, and we identified a second serotype in 20 % ($n=10$) of the pneumococci carriers. These results were confirmed by detailed serotyping of multiple colonies isolated from the primary culture with the Quellung method. We conclude that the multiplex PCR is a sensitive, simple and cost-effective method for detecting multiple serotypes in nasopharyngeal cultures, and thus might be useful for the monitoring of pneumococcal colonization over time, especially in the surveillance of nasopharyngeal colonization after conjugate vaccination.

Received 20 September 2008

Accepted 7 January 2009

INTRODUCTION

Streptococcus pneumoniae is the most common cause of invasive bacterial infections in children, including bacteraemia, pneumonia and meningitis. Based on serological properties 90 distinct serotypes of this bacterium can be differentiated. A few studies have shown that children carry more than one pneumococcal serotype at a time, and 2–30 % co-colonization with two or more serotypes has been described (Gratten *et al.*, 1994; Montgomery *et al.*, 1990) with increased rates of multiple pneumococcal carriage in populations with higher carriage rates (Barker *et al.*, 1989; Lloyd-Evans *et al.*, 1996; O'Brien & Nohynek, 2003). However, information on this phenomenon is scarce due to the absence of a sensitive method for detecting multiple-serotype carriage. Serotyping in general is done with the Quellung method (Neufeld, 1902), and detection of co-colonization with this method requires the subculturing

and consequently the serotyping of many c.f.u. It has been calculated that if the less common serotype represents only 5 % of the total pneumococcal population, 59 colonies from each specimen would need to be serotyped to reach a 95 % probability of detecting the second pneumococcal type (Huebner *et al.*, 2000), making this method very laborious, insensitive and expensive, and not practical for use in large nasopharyngeal colonization studies. An improvement of this method, where co-colonization was determined using selection by colony morphology, has been evaluated in a population with high carriage rates. This approach was more efficient but still underestimated the true rate of multiple carriage (Hare *et al.*, 2008). Other, high-throughput methods, have been developed: a colony blot assay (Bogaert *et al.*, 2004) and an immunoblot method (Bronsdon *et al.*, 2004), but both are operator dependent, expensive and time-consuming.

Here we have explored the use of a multiplex PCR for the detection of multiple pneumococcal serotypes in primary nasopharyngeal cultures of samples from Warao

Abbreviation: ACIP, Advisory Committee on Immunization Practices.

†These authors contributed equally to this work.

Amerindian children. This PCR was originally developed for the determination of the pneumococcal capsule types targeted by the currently licensed 7-valent conjugate vaccine (O'Halloran & Cafferkey, 2005). Our Warao children show high rates of nasopharyngeal carriage (up to 70 % of the children are colonized), most of them (62 %) with vaccine serotypes covered by the 7-valent conjugate vaccine (Rivera-Olivero *et al.*, 2007). Because of these high carriage rates for vaccine serotypes we anticipated that these children have a high risk of being colonized with more than one of the serotypes. Hence, the primary cultures of nasopharyngeal samples, especially nasopharyngeal samples that have heavy growth, could be appropriate for the evaluation of the sensitivity of a multiplex PCR for the detection of co-colonization.

METHODS

Clinical specimens. We studied 50 primary cultures of nasopharyngeal samples from a previous colonization study among Warao children (Rivera-Olivero *et al.*, 2007). In this study swabs were plated on selective medium (blood agar with 5 µg gentamicin ml⁻¹) and incubated overnight at 36 °C with 5 % CO₂. The growth from these swabs was removed with STGG (skimmed milk/tryptone/glucose/glycerol) medium (O'Brien & Nohynek, 2003) and stored at -70 °C.

The studied cultures were demonstrated to contain pneumococci of serogroup 6 (*n*=18) (6A *n*=10 and 6B *n*=8), serotype 23F (*n*=15), serotype 19F (*n*=6), serotype 14 (*n*=5), serotype 9V (*n*=4) and serotype 18C (*n*=2). Samples representing serotype 4 could not be included in this study because this serotype has never been detected in the Warao population. Only primary cultures of swabs plated on blood agar plates with gentamicin giving a semi-quantitative growth of 4+ (O'Brien & Nohynek, 2003) (about 200 colonies ml⁻¹) were used for this study.

Extraction of DNA. A total of 10 µl culture sample in STGG storage medium was plated on blood agar with gentamicin and grown overnight at 36 °C in a CO₂-enriched atmosphere. The cells growing on half of a plate were harvested and suspended in 100 µl TE (10 mmol Tris, 1 mmol EDTA, pH=8.0). This dense cell suspension was boiled for 10 min, centrifuged for 10 min at 13 000 g and the supernatant (crude DNA preparation) stored at 4 °C until further use.

Multiplex PCR. PCR was performed in 25 µl volumes containing 12.5 µl PCR mix (Fundaim) and 2.5 µl crude DNA preparation, and 2.5 µl primer mix (containing 1 µM primers 9VR and 9VF, 0.1 µM primers 14F and 14R, 0.1 µM primers 19FF and 19FR, 0.3 µM primers 18CF and 18CR, 0.3 µM primers 4F and 4R, 1.2 µM primers 23FFor and 23FRev, and 0.28 µM primers 6BF and 6BR). Primers and PCR conditions were as described by O'Halloran & Cafferkey (2005). PCR products were analysed by running 10 µl PCR mixture, for 1 h at 10 V cm⁻¹, on a 3 % (w/v) agarose gel (Scientific Trade), containing 0.5 µg ethidium bromide ml⁻¹, together with a 100 bp size marker (Novagen). Bands were visualized and sizes were digitally calculated with a Bio-Rad Fluor-S MultiImager. The amplification products expected for each serotype were as follows: 507 bp for serotype 9V, 430 bp for serotype 4, 354 bp for serotype 18C, 268 bp for serotype 14, 220 bp for serogroup 6, 177 bp for serotype 23F and 130 bp for serotype 19F (O'Halloran & Cafferkey, 2005).

Serotyping. Pneumococci were serotyped by the capsular swelling method (Quellung reaction) and observed microscopically using commercially available antisera (Statens Serum Institut).

RESULTS

To show the functionality of the multiplex PCR for the serotyping of *S. pneumoniae* isolates in our setting, initially, a set of 14 pneumococcal strains previously typed with the Quellung reaction, two strains of each of the 7-valent conjugate vaccine serotypes, were PCR-typed. The results of the PCR were in agreement with the serotypes as determined by the Quellung reaction (data not shown). In addition, several strains of related serotypes (23A and 19A) not included in this multiplex PCR were tested and no PCR products were found. The 6B, 9V and 18C primers of this multiplex PCR will not distinguish between the serotypes of serogroup 6, 9 and 18. When we tested different serotypes of these serogroups a corresponding PCR product was found (data not shown).

When the multiplex PCR was performed on the DNA preparations of the 50 pneumococcal primary cultures of 7-valent conjugate vaccine serotypes isolated from our study population, in 10 of the samples (20 %) two bands were detected. One band matched the molecular mass that corresponded with the serotype as determined with the Quellung reaction for the isolate. The other band with a molecular mass matching a second serotype suggested the presence of two different vaccine serotypes in these primary cultures. The PCR products of three of these isolates are shown in Fig. 1. False-positive PCR due to cross-contamination was excluded because in every PCR negative controls were included.

To show that the ten children were indeed colonized with two vaccine serotypes, the primary cultures were replated on blood agar and from each culture ten randomly chosen individual colonies were tested with the Quellung reaction. In seven of the ten samples the co-colonization could be proven because one to five colonies out of the ten colonies tested showed the serotype matching the molecular mass of the extra band in the multiplex PCR. For the other three isolates another ten colonies were serotyped and the secondary serotype was found for one. For two primary cultures the serotyping of 20 colonies didn't result in the detection of the minor serotype.

DISCUSSION

Since 2000, according to the recommendations of the Centers for Disease Control and Prevention Advisory Committee on Immunization Practices (ACIP), the heptavalent pneumococcal conjugate vaccine is considered necessary for all children under 2 years of age, and for all high-risk children between the ages of 2 and 5 years (ACIP, 2000). As a result of vaccination, a decrease in carriage of vaccine serotypes and a significant increase of non-vaccine serotypes occurs in immunized children, probably due to replacement of serotypes or unmasking of minority populations of *S. pneumoniae* present in the nasopharynx since multiple serotypes of pneumococcus can simultaneously colonize (Byington *et al.*, 2005; Gray *et al.*, 1980;

Fig. 1. Gel electrophoresis of multiplex PCR amplification products from the primary cultures of swabs from pneumococci carriers who were demonstrated to contain pneumococci with a serotype present in the 7-valent conjugate vaccine. Lanes 1 and 15, 50 bp DNA ladder (Invitrogen); lane 2, culture with serotype 23F and an extra band of serogroup 6; lane 3, culture with serotype 14 only; lane 4, culture with serotype 23F only; lane 5, culture with serogroup 6 only; lane 6, culture with serotype 6A and an extra band of serogroup 9 (9V/9A); lane 7, culture with serotype 14 and an extra band of serotype 23F; lane 8, culture with serotype 23F only; lane 9, culture with serogroup 6 only; lane 10, culture with serogroup 9 (9V/9A) only; lane 11, culture with serotype 23F only; lane 12, culture with serogroup 9 (9V/9A) only; lane 13, negative PCR control; lane 14, culture with serogroup 6 only.

Huang *et al.*, 2005) For the detection of co-colonization, i.e. for studying the unmasking of minority populations, the current conventional serological methodology is insensitive, laborious and expensive. Serotyping of one colony takes as long as 30 min and costs up to £70 (\$100) per strain, and the detection of co-colonization with this method requires the subculturing and consequently the serotyping of dozens of c.f.u., especially if co-colonization rates are low (Huebner *et al.*, 2000).

The multiplex PCR explored in this investigation can detect low co-colonization rates. As shown in Table 1, most co-colonizing serotypes could be detected when 10–20 c.f.u. were tested with the Quellung reaction and thus, this PCR easily replaces the Quellung reaction in terms of feasibility. In addition, the multiplex PCR method permits the processing of many samples simultaneously, and the use of crude DNA extracts, as described in our methodology, obviates the need for more specialized template preparation protocols making the methods even more economical and time-saving. In this study we used only nasopharyngeal samples that had heavy growth (200 c.f.u. ml⁻¹). This was to increase the chance of finding a second serotype. But of course the multiplex PCR can also be

applied to nasopharyngeal cultures with only a few c.f.u. We suggest that a sensitivity study should be carried out using spiked co-cultures at different ratios to determine the sensitivity of the assay. In our hands, this multiplex PCR, spiked with purified DNA of two serotypes at different ratios, detected 1 ng DNA of a serotype 14 strain in a background of approximately 1 µg DNA of a serotype 23F strain.

In our study we determined that co-colonization is an important phenomenon that should be taken into account in carriage studies, and perhaps also when evaluating invasive isolates, especially in populations with high carriage rates. We determined that 20 % of the children were co-colonized with a second 7-valent conjugate vaccine serotype, a rate comparable with a study by Gratten *et al.* (1989), who showed that about 30 % of carriage-positive children from Papua New Guinea harboured more than one type of *S. pneumoniae*. In that study all known *S. pneumoniae* serotypes were taken into account when screening for multiple colonization. In our pilot study, applying a multiplex PCR, we only screened for the co-presence of the seven serotypes present in the conjugate vaccine. Expansion of our multiplex PCR for other serotypes frequently found in this population potentially could reveal higher degrees of co-colonization. Recently several other more comprehensive multiplex PCR assays were published with the possibility of expanding the method to cover the detection of more capsular types (Kong *et al.*, 2006; Pai *et al.*, 2006). Our limited assay has the potential to miss potentially predominant serotypes as

Table 1. Detection of the co-colonizing minor serotype in the ten primary cultures that showed two bands in the multiplex PCR compatible with the presence of two serotypes

From the primary culture 10 or 20 (*) c.f.u. were serotyped by the Quellung method and the number of colonies with the minor serotype was determined. In two samples (sample 1 and 9) the co-colonizing serotype could not be detected from the serotyping of 20 individual colonies.

Sample	Dominant serotype or serogroup	Minor serotype or serogroup	Colonies with the minor serotype
1*	23F	14	Not found in 20 colonies tested
2	14	23F	1 out of 10
3	14	23F	1 out of 10
4	23F	6A	2 out of 10
5	23F	6B	5 out of 10
6	6A	9V	1 out of 10
7	23F	18C	1 out of 10
8	6A	23F	1 out of 10
9*	23F	Serogroup 18	Not found in 20 colonies tested
10*	18C	6A	1 out of 20

only 62 % of the strains colonizing our children are vaccine serotypes covered by the 7-valent conjugate vaccine.

We conclude that the use of multiplex PCR can further broaden our understanding of the dynamics of pneumococcal carriage, including carriage of multiple serotypes, and the effect of vaccination on carriage, and also in transmission, as well as surveillance, of invasive disease and the ecology of co-colonization. In conclusion the multiplex PCR applied to a 'multiple colonies primary clinical isolate' provides a basis for further studies of the 'in vivo' interactions of the different serotypes of *S. pneumoniae*, investigating the symbiosis of serotypes and the dynamics of (co)-colonization. We suggest that PCR applied directly to the nasopharyngeal swab should be explored as well. In our approach minority strains may autolyse under the plating conditions. Also, there is potential for intraspecies bactericidal competition between strains growing on plates (Dawid *et al.*, 2007). This aspect has not been explored by us.

ACKNOWLEDGEMENTS

This investigation was financed by a LOCTI research grant from Shell Venezuela CA. We thank Enza Spadola BSc, Departamento de Bacteriología, Instituto Nacional de Higiene 'Rafael Rangel', Caracas, Venezuela, for technical support in serotyping by the Quellung method.

REFERENCES

- ACIP (2000). Preventing pneumococcal disease among infants and young children. Recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Recomm Rep* **49**, 1–38.
- Barker, J., Gratten, M., Riley, I., Lehmann, D., Montgomery, J., Kajoi, M., Gratten, H., Smith, D., Marshall, T. F. & Alpers, M. P. (1989). Pneumonia in children in the Eastern Highlands of Papua New Guinea: a bacteriologic study of patients selected by standard clinical criteria. *J Infect Dis* **159**, 348–352.
- Bogaert, D., Veenhoven, R. H., Sluijter, M., Sanders, E. A., de Groot, R. & Hermans, P. W. (2004). Colony blot assay: a useful method to detect multiple pneumococcal serotypes within clinical specimens. *FEMS Immunol Med Microbiol* **41**, 259–264.
- Bronsdon, M. A., O'Brien, K. L., Facklam, R. R., Whitney, C. G., Schwartz, B. & Carlone, G. M. (2004). Immunoblot method to detect *Streptococcus pneumoniae* and identify multiple serotypes from nasopharyngeal secretions. *J Clin Microbiol* **42**, 1596–1600.
- Byington, C. L., Samore, M. H., Stoddard, G. J., Barlow, S., Daly, J., Korgenski, K., Firth, S., Glover, D., Jensen, J. & other authors (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.
- Dawid, S., Roche, A. M. & Weiser, J. N. (2007). The *blp* bacteriocins of *Streptococcus pneumoniae* mediated intraspecies competition both in vitro and in vivo. *Infect Immun* **75**, 443–451.
- Gratten, M., Montgomery, J., Gerega, G., Gratten, H., Siwi, H., Poli, A. & Koki, G. (1989). Multiple colonization of the upper respiratory tract of Papua New Guinea children with *Haemophilus influenzae* and *Streptococcus pneumoniae*. *Southeast Asian J Trop Med Public Health* **20**, 501–509.
- Gratten, M., Manning, K., Dixon, J., Morey, F., Torzillo, P., Hanna, J., Erlich, J., Asche, V. & Riley, I. (1994). Upper airway carriage by *Haemophilus influenzae* and *Streptococcus pneumoniae* in Australian aboriginal children hospitalised with acute lower respiratory infection. *Southeast Asian J Trop Med Public Health* **25**, 123–131.
- Gray, B. M., Converse, G. M., III & Dillon, H. C., Jr (1980). Epidemiologic studies of *Streptococcus pneumoniae* in infants: acquisition, carriage, and infection during the first 24 months of life. *J Infect Dis* **142**, 923–933.
- Hare, K. M., Morris, P., Smith-Vaughan, H. & Leach, A. J. (2008). Random colony selection versus colony morphology for detection of multiple pneumococcal serotypes in nasopharyngeal swabs. *Pediatr Infect Dis J* **27**, 178–180.
- Huang, S. S., Platt, R., Rifas-Shiman, S. L., Pelton, S. I., Goldmann, D. & Finkelstein, J. A. (2005). Post-PCV7 changes in colonizing pneumococcal serotypes in 16 Massachusetts communities, 2001 and 2004. *Pediatrics* **116**, e408–e413.
- Huebner, R. E., Dagan, R., Porath, N., Wasas, A. D. & Klugman, K. P. (2000). Lack of utility of serotyping multiple colonies for detection of simultaneous nasopharyngeal carriage of different pneumococcal serotypes. *Pediatr Infect Dis J* **19**, 1017–1020.
- Kong, F., Brown, M., Sabananthan, A., Zeng, X. & Gilbert, G. L. (2006). Multiplex PCR-based reverse line blot hybridization assay to identify 23 *Streptococcus pneumoniae* polysaccharide vaccine serotypes. *J Clin Microbiol* **44**, 1887–1891.
- Lloyd-Evans, N., O'Dempsey, T. J., Baldeh, I., Secka, O., Demba, E., Todd, J. E., McArdle, T. F., Banya, W. S. & Greenwood, B. M. (1996). Nasopharyngeal carriage of pneumococci in Gambian children and in their families. *Pediatr Infect Dis J* **15**, 866–871.
- Montgomery, J. M., Lehmann, D., Smith, T., Michael, A., Joseph, B., Lupiwa, T., Coakley, C., Spooner, V., Best, B. & other authors (1990). Bacterial colonization of the upper respiratory tract and its association with acute lower respiratory tract infections in Highland children of Papua New Guinea. *Rev Infect Dis* **12** (Suppl. 8), S1006–S1016.
- Neufeld, F. (1902). Über die Agglutination der Pneumokokken und über die Theorie der Agglutination. *Z Hyg Infektionskr* **1**, 54–72.
- O'Brien, K. L. & Nohynek, H. (2003). Report from a WHO Working Group: standard method for detecting upper respiratory carriage of *Streptococcus pneumoniae*. *Pediatr Infect Dis J* **22**, e1–e11.
- O'Halloran, D. M. & Cafferkey, M. T. (2005). Multiplex PCR for identification of seven *Streptococcus pneumoniae* serotypes targeted by a 7-valent conjugate vaccine. *J Clin Microbiol* **43**, 3487–3490.
- Pai, R., Gertz, R. E. & Beall, B. (2006). Sequential multiplex PCR approach for determining capsular serotypes of *Streptococcus pneumoniae* isolates. *J Clin Microbiol* **44**, 124–131.
- Rivera-Olivero, I. A., Bogaert, D., Bello, T., del Nogal, B., Sluijter, M., Hermans, P. W. & de Waard, J. H. (2007). Pneumococcal carriage among indigenous Warao children in Venezuela: serotypes, susceptibility patterns, and molecular epidemiology. *Clin Infect Dis* **45**, 1427–1434.