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Serotype Analysis of *Streptococcus pneumoniae* in Lung and Nasopharyngeal Aspirates from Children in the Gambia by MassTag PCR

Rafal Tokarz,[a] Thomas Briese,[a] Gerard Morris,[b] Readon Ideh,[b] Osaretin Chimah,[b] Bernard Ebruke,[b] Aaloki Desai,[a] Saddef Haq,[a] Stephen Sameroff,[a] Stephen R. C. Howie,[b] W. Ian Lipkin[a]

Center for Infection and Immunity, Mailman School of Public Health, Columbia University, New York, NY, USA[a]; Child Survival Theme, Medical Research Council Unit, the Gambia[b]

***Streptococcus pneumoniae* strains comprise >90 serotypes. Here we describe establishment of a MassTag PCR assay designed to serotype *S. pneumoniae* and demonstrate its utility in tests using 31 paired lung aspirate and nasopharyngeal aspirate samples from children with pneumonia in the Gambia. Serotypes 1, 5, and 14 in were implicated in 90% of lung infections. With 5 exceptions, serotypes found in lung aspirates were also found in nasopharyngeal aspirates.**

Streptococcus pneumoniae is a major cause of pediatric morbidity and mortality worldwide, particularly in developing countries. A frequent colonizer of the nasopharynx, *S. pneumoniae* can cause pneumonia, meningitis, and sepsis and annually results in approximately 800,000 deaths in children under 5 years of age (1). A polysaccharide capsule is the major virulence factor in invasive pneumococcal disease (IPD). The capsule is immunogenic and the chemical composition of capsular polysaccharides varies among strains, resulting in the generation of multiple pneumococcal serotypes. Currently, >90 serotypes are recognized that show various potentials for invasiveness and geographical distributions (2–4). Based on the most common serotypes associated with IPD, a heptavalent pneumococcal conjugate vaccine (PCV7) was licensed in 2000 that included serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F. PCV10, licensed in 2008, added 1, 5, and 7F to the existing vaccine serotypes, and PCV13 added serotypes 3, 6A, and 19A in 2009. The PCV7 vaccination program greatly reduced the incidence of IPD caused by vaccine-targeted serotypes (5, 6). However, IPD remains a substantial problem in the developing world, presumably due to limited vaccine access (6, 7).

Continued surveillance is essential to determine the geographical prevalence of serotypes involved with IPD and to monitor the replacement of vaccine-targeted serotypes with other serotypes. Historically, serological assays such as the Quellung reaction were used for serotype determination. However, such assays require bacterial culture and substantial investments in time, money, supplies, and operator expertise.

MassTag PCR is a multiplex molecular tool that enables rapid, inexpensive detection of bacteria, viruses, fungi, and parasites associated with respiratory, tick, and bloodborne diseases (8–10). It uses a library of distinct low-molecular-mass tags to code microbial gene targets by conjugating each primer species in a multiplex reaction with a distinct mass tag via a photocleavable linkage. After PCR amplification, the identity of the microbial gene target is determined by the presence of its 2 cognate tags, 1 from each primer, detected by mass spectrometry. Here, we adapted this method to develop molecular assays for serotyping *S. pneumoniae* and evaluated the assay on a set of pediatric clinical samples.

MassTag PCR serotyping panels were assembled to detect >90 recognized serotypes. Primers were designed using Primer3 software. The *wzy* gene was chosen as a target, except for serotype 3, where the sequence of *cap3A* gene was used, and serotypes 32A and 32F, where the *wzx* gene was used. In cases where differentiating among individual serotypes proved infeasible, primers targeting multiple serotypes/serogroups were chosen. A total of five panels were assembled, consisting of 8 to 12 primer pairs; together, these differentiate 28 individual serotypes and 25 serogroups (see Table S1 in the supplemental material). Panel sensitivity was tested on serial dilutions of cloned DNA standards with a minimal threshold for detection set at 100 copies for each primer pair in the panel. Panel performance was assessed using a control set of 90 *S. pneumoniae* serotypes obtained from the Centers for Disease Control and Prevention. In specificity tests, all primer sets amplified only their cognate serotype/serogroup target.

Clinical samples were collected as part of a study of pediatric pneumonia in sub-Saharan Africa. Paired lung fluid and nasopharyngeal aspirates (NPA) were obtained from children with severe pneumonia in the Gambia between March 2007 and June 2008 (11). Lung aspirate samples were collected according to strict protocols by percutaneous transthoracic lung aspiration from children who met defined eligibility criteria, including informed consent (11), and no associated adverse events were observed. All samples were collected before the introduction of PCV7 into routine use in late 2009. DNA was extracted using the EasyMag extraction platform (bioMérieux). A total of 31 paired lung aspirate and NPA samples were screened: 28 lung aspirate samples, previously confirmed positive by *S. pneumoniae*-specific *lytA* PCR, and 3 lung aspirate samples negative for *S. pneumoniae* (12). MassTag PCR identified the *S. pneumoniae* serotype in 27 of 28 (96%) lung aspirate samples (Table 1). Serotypes 14 (10 samples), 1 (8 samples), and 5 (7 samples) were predominant, and serogroups 6 (6A/

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Address correspondence to Rafal Tokarz, rt2249@columbia.edu.
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TABLE 1 List of serotypes detected by MassTag PCR[a]

	Serotype/serogroup(s)					
		Nasopharyngeal				
Sample	Lung aspirate	1st	2nd	3rd	4th	Predominant
L1	14	**14**	19F			19F
L2	14	**14**	19A	5	6	14*
L3	6	**6**				
L4	1	**1**	9A/9V	19F		9A/9V
L5	1	**1**	6	23F	19F	1*
L6	5	**5**				
L7	5	**5**	14			5*
L8	14	**14**	9A/9V			14*
L9	1	**1**	6	19F		19F
L10	1	**1**				
L11	1	**1**	15A/15F			1*
L12	14	**14**				
L13	5	**5**	6			5*
L14	14	**14**	15A/15F			14*
L15	5	**5**				
L16	14					
L17	14	**14**	13			14*
L18	1	**14**				
L19	14	6	19F			
L20	9A/9V	**9A/9V**	5	14		9A/9V*
L21	14	**14**				
L22	14	**14**				
L23	1	4				
L24	5	**5**	9A/9V			5*
L25	5	**5**				
L26	1	17				
L27						
L28	5	**5**				
L29	NEGATIVE	14				
L30	NEGATIVE	9N/9L				
L31	NEGATIVE	6	14			

[a] Lung aspirates L29 to L31 were *S. pneumoniae* negative and served as controls. Serotypes in nasopharyngeal aspirates concurrent with lung aspirates are shown in bold. *, predominant serotype in NPA also detected in the lung aspirate.

6B/6C) and 9A/9V were identified in the remaining 2 samples. None of the lung aspirates contained more than one serotype per sample. The same serotypes identified in 23 of the lung aspirates were also detected in the paired NPAs. Multiple serotypes were present in half of the NPAs (14 of 28), where in some instances up to four serotypes were detected. SYBR green quantitative PCR using the respective non-mass-tagged primer sets was performed to ascertain if the serotype load in the NPA correlated with the serotype identified in the corresponding lung aspirate. In 10 of 13 samples (77%) where the same serotype was present in both sample types, the serotype with the highest load in the NPA represented the serotype detected in the lung aspirate (see Table S2 in the supplemental material).

Overall, serotype 14 was the most common (detected in 13 of 31 NPA samples). One paired set of lung aspirate and NPA samples could not be characterized and may represent a nonencapsulated strain similar to the ones often encountered in *S. pneumoniae* carriage studies (13). All serotype assignments obtained by MassTag PCR were confirmed by singleplex PCR and dideoxy sequencing.

Our results indicate the utility of MassTag PCR for molecular serotyping of *S. pneumoniae*. The assays detected all known serotypes in a semiautomated, high-throughput format. Current gel electrophoresis-based multiplex PCR serotyping systems identify approximately half of the recognized serotypes and require up to 7 sequential reactions (14). Although microarray approaches provide good coverage of serotypes, they are of significantly lower throughput at higher costs and are more labor-intensive (15). The highest sensitivity of serotype detection is achieved with singleplex real-time PCR assays; however, multiplexing of real-time PCR assays requires running excessive numbers of panels (16).

The availability of paired lung aspirate and NPA samples provided a unique opportunity for comparison of serotypes detected in the NPA which could be attributed to carriage to serotypes identified in lung aspirates, where they would more likely represent the etiologic agent. The same serotype present in the lung aspirates was also detectable in the majority of the NPAs, although often combined with other serotypes. In both sample types, the predominant serotypes detected were 14, 1, and 5; together, these three serotypes were detected in 90% of lung infections, providing strong evidence for inclusion of these serotypes in any vaccination program in sub-Saharan Africa. Our findings are in agreement with reports implicating these serotypes with some of the highest proportions of IPD in young children globally (7). Assuming effective immunity following vaccination, the use of PCV7 or PCV13 in a vaccination program would have prevented 41% or 100% of the cases reported in our cohort, respectively.

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