Serotype Prediction for Frequently Isolated Serotypes of *Streptococcus Pneumoniae* by Heteroduplex Analysis and Modification of This Technique to Real-Time Fluorometric Nucleic Acid Detection System

Sıklıkla İzole Edilen *Streptococcus Pneumoniae* Serotipleri İçin Heteroduplex Analizi ile Serotiplendirme ve Bu Yöntemin Gerçek Zamanlı Florometrik Nükleik Asit Saptama Sistemine Uyarlanması

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Geliş Tarihi/*Received:* 07.09.2009
Kabul Tarihi/*Accepted:* 05.06.2010

Presented in part at XXXI. Turkish Microbiology Congress, 19-23 September 2004, Kuşadası, Aydın-Turkey, Oral Presentation No: S 3.9 (Oral Presentation Award).

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ABSTRACT Objective: Several molecular serotype prediction methods have been published in order to simplify the pneumococcal serotyping. The aim of this study is to develop a molecular serotype prediction technique alternative to other molecular approaches. **Material and Methods:** *S. pneumoniae* serotypes 1, 3, 5, 6A, 7F, 8, 9V, 11A, 14, 15B, 18C, 19A, 19F and 23B, that are the most frequently isolated serotypes from infections in Turkey, were included in the study. *S. pneumoniae* cpsA and cpsB genes that are involved in the processing, regulation and export of the capsular polysaccharides, were amplified by polymerase chain reacsion (PCR). Then, heteroduplex analysis was performed to the 1.8 kb PCR products obtained from all serotypes with constant serotype 1. This technique was then modified to real-time fluorometric nucleic acid detection system. **Results:** The PCR products obtained from serotypes 1, 6A, 14 and 19F showed only homoduplex bands while those from serotypes 3, 5, 7F, 8, 9V, 11A, 15B and 23B showed specific heteroduplex bands. Although serotypes 18C and 19A showed a specific heteroduplex banding pattern, they could not be differentiated from each other. When further heteroduplex analysis was performed to serotypes 1, 6A, 14 and 19F with serotype 5 as a constant component, serotype 1 and 6A could be differentiated from the others. Heteroduplex and homoduplex DNA strands could be distinguished by melting curve analysis in real-time fluorometric nucleic acid detection system. **Conclusion:** As a rapid and cost-effective method, real-time heteroduplex analysis may be an alternative to other molecular methods for serotype prediction of pneumococci.

Key Words: *Streptococcus pneumoniae;* serotyping; polymerase chain reaction; heteroduplex analysis

ÖZET Amaç: Literatürde pnömokok serotiplendirmesini daha kullanışlı hale getirmek için birkaç moleküler serotip tahmin yöntemi yayınlanmıştır. Bu çalışmanın amacı bu moleküler yaklaşımlara alternatif oluşturacak farklı bir moleküler serotip tahmin yöntemi geliştirmektir. **Gereç ve Yöntemler:** Türkiye'de enfeksiyonlardan en sıklıkla izole edilen *S. pneumoniae* sertipleri olan 1, 3, 5, 6A, 7F, 8, 9V, 11A, 14, 15B, 18C, 19A, 19F ve 23B, çalışmaya dahil edilmiştir. *S. pneumoniae* kapsül polisakkaritinin işlenmesi, düzenlenmesi ve taşınması ile ilgili genler olan cpsA ve cpsB bölgeleri PCR ile çoğaltılmıştır. Elde edilen 1.8 kb boyundaki PCR ürünleri heteroduplex analizine alınmıştır. Analizde tüm serotiplere ait ürünler sabit serotip 1 ürünü ile çalışılmıştır. Bu yöntem daha sonra gerçek zamanlı florometrik nükleik asit saptama sistemine uyarlanmıştır. **Bulgular:** Serotip 1, 6A, 14 ve 19F'ye ait PCR ürünleri sadece homodupleks bantları oluştururken serotip 3, 5, 7F, 8, 9V, 11A, 15B ve 23B kendilerine özgül heteroduplaks bantları vermişlerdir. Serotip 18C ve 19A da özgül hetrodupleks bantları vermesine rağmen, birbirlerinden ayrımları mümkün olmamıştır. Serotip 1, 6A, 14 ve 19F, serotip 5'in sabit komponent olarak kullanıldığı heteroduplex analizine alındığında serotip 1 ve 6A birbirinden ayrımlanmıştır. Heteroduplex ve homodupleks DNA dizileri, gerçek zamanlı florometrik nükleik asit saptama sisteminde erime eğrisi analizi ile ayrımlanabilmişlerdir. **Sonuç:** Hızlı ve ucuz bir yöntem olarak gerçek zamanlı heteroduplex analizi, pnömokokların moleküler serotip tahmininde diğer moleküler yöntemlere alternatif olabilir.

Anahtar Kelimeler: *Streptococcus pneumoniae;* serotiplendirme; polimeraz zincir reaksiyonu; heteroduplex analizi

Turkiye Klinikleri J Med Sci 2010;30(5):1487-92

Streptococcus pneumoniae remains an important human pathogen associated with significant morbidity and mortality.[1,2] The prevalence of penicillin resistance among pneumococci is alarmingly increasing worldwide and international spread of a restricted number of multiresistant pneumococcal clones has significantly contributed to this increase.[1,3,4] The emergence of multiple drug-resistance in *S.pneumoniae* has complicated the treatment of pneumococcal infections. The increasing incidence of drug-resistant *S. pneumoniae* worldwide has emphasized the need for epidemiological surveillance of this pathogen.[5] Vaccination is one of the approaches to prevent development of pneumococcal disease as it provides protection against the vaccine types. Continued serotype surveillance is critical in order to monitor vaccine efficacy and the changes in incidence and distribution of colonizing and invasive serotypes.[6,7] Any increase in pneumococcal disease caused by previously uncommon non-vaccine serotypes could necessitate a change in vaccine composition.[8] Surveillance studies in different communities indicate that there is an increase in the carriage of serotypes that are not included in the current 23-valent vaccine.[9,10]

Pneumococcal serogroup and serotype identification are currently performed with capsular swelling (Quellung) reaction. Cross-reactions between serotypes may occur and some strains are non-serotypable.[11] Besides, as prevailing serotypes change, a broader spectrum of sera will be necessary to detect those types, thus the cost increases. A molecular method with the ability to predict pneumococcal serotype could have immense advantages in convenience and cost.

Capsule production in *S. pneumoniae* is largely controlled by capsular polysaccharide synthesis (*cps*) gene clusters.[12] The capsular loci for all 90 serotypes have now been sequenced and published.[13] The *cps* gene cluster contains genes responsible for synthesis of the serotype-specific polysaccharide. At the 5'-end of the cps gene cluster there are four relatively conserved open reading frames, *cpsA (wzg)-cpsB (wzh)-cpsC (wzd)-cpsD (wze)*.[11] In *cps* gene cluster, high-frequency recombinations may occur. The result of these recombinations is serotype switching among isolates within genetic lineages.[14,15] In the region between the 3'-end of *cpsA* and the 5'-end of *cpsB*, there are sites of heterogeneity among serotypes.[15,16] The *cpsA* and *cpsB* genes are involved in the processing, regulation and export of the capsular polysaccharide,[17] but they are not directly involved in capsular biosynthesis. On the other hand, the *cpsA* and *cpsB* genes are physically linked to the serotype-specific genes and thus polymorphism within them would be expected to be tightly linked to capsular type.

Several molecular typing methods using *cps* gene clusters have been developed to predict serotypes and serogroups.[16,18,19,20,21] In this study we described a possible alternative molecular method, a PCR-based heteroduplex analysis in the *cpsA-cpsB* region of pneumococcal capsular gene clusters, and we modified this technique to real-time fluorometric nucleic acid detection system in order to predict serotypes of pneumococci.

MATERIAL AND METHODS

STRAINS

One of each pneumococcal serotypes 1, 3, 5, 6A, 7F, 8, 9V, 11A, 14, 15B, 18C, 19A, 19F and 23B were included in this study. These have been the most frequently isolated serotypes in Hacettepe University Hospitals since 1995.[22] Representative isolates of each serotype from stock cultures were inoculated on to 5% sheep blood agar and incubated overnight at 35^0C in 5-10% CO_2. *S.pneumoniae* identification was performed by optochin susceptibility and bile solubility, as described previously.[23] The serotypes were confirmed by using Pneumotest antisera in the Danish chequerboard typing system according to the instructions of the manufacturer (Statens Seruminstitut, Copenhagen, Denmark).

DNA EXTRACTION AND PURIFICATION

Randomly selected 3-5 colonies from pure cultures were emulsified in 1 ml tris-EDTA (TE) buffer in a microcentrifuge tube, washed twice in TE buffer

and the pellet was resuspended in 100ml TE buffer. DNA extraction and purification were performed by automatized "MagNa Pure LC DNA Isolation Kit III (Bacteria, Fungi)" system (Roche Diagnostics) according to the manufacturer's instructions.

PCR

The CpsA3 and CpsB2 primers that amplify a 1795 bp fragment of the *cpsA-cpsB* genes were used and the amplification reaction was performed according to a previously published protocol[16] using the "Accurase Taq Polymerase" (Gene Sys Ltd), because of the length of the PCR products in a MJ Research PTC -200 Peltier Thermal Cycler. The PCR products were electrophorized in 0.8% agarose gel and visualized by ethidium bromide staining.

HETERODUPLEX ANALYSIS

In heteroduplex analysis, all *S. pneumoniae* serotypes were analyzed in comparison with serotype 1. To inhibit *Taq* polymerase, 0.5 µl of 0.5 M EDTA was added to each reaction tube. In a separate tube, 25 µl of DNA extract from the isolate in question was mixed with 25 µl of the amplified DNA (1795 bp) from serotype 1 which was used as a constant component, and placed in the thermocycler. DNA strands were denatured at 95°C for 10 min and slowly cooled to 25°C by a 1-min stay at every fifth degree. The entire 50 µl specimen was then mixed with 10 µl of loading buffer and loaded onto a 8% polyacrylamide gel in a mini-gel system (Owl Scientific). Electrophoresis was carried out at 200 V for 4.5 h, followed by staining with ethidium bromide and examination by UV transillumination. Heteroduplex banding patterns were differentiated on the basis of the number and relative positions of bands, compared to the homoduplex band. Further heteroduplex analysis were performed with amplified DNA from serotype 5 as a constant component to differentiate those serotypes for which homoduplex band was obtained with serotype 1. Heteroduplex analysis was repeated three times and three different isolates from same serotype were analyzed in order to confirm the heteroduplex banding patterns. Additionally, for serotypes which have alike heteroduplex patterns, heteroduplex analysis was repeated on same gel in neighbor lanes in order to differentiate them.

REAL-TIME FLUOROMETRIC NUCLEIC ACID DETECTION

Heteroduplex and homoduplex DNA strands were distinguished by melting-curve analysis with Syber Green I at LightCycler system (Roche Diagnostics). Melting-curve analysis were repeated three times for each homoduplex and heteroduplex groups.

RESULTS

On the basis of the heteroduplex analysis with serotype 1 (Figure 1), pneumococcal serotypes tested here were classified into ten groups. The PCR products obtained from serotypes 1, 6A,

14 and 19F showed only a homoduplex band while those from serotypes 3, 5, 7F, 8, 9V, 11A, 15B and 23B showed specific heteroduplex bands. Although serotypes 18C and 19A showed a specific heteroduplex banding pattern, they could not be differentiated from each other (Table 1).

Heteroduplex analysis of any serotypes with same serotype or another member of the same group showed only a homoduplex banding pattern (data not shown).

In order to distinguish the homoduplex group members (1, 6A, 14 anf 19 F) from each other, heteroduplex analysis with amplified DNA from serotype 5 as a constant component was performed (Figure 2).

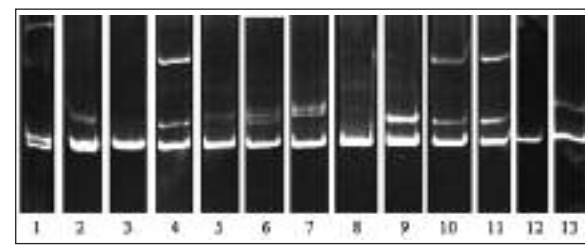

FIGURE 1: Heteroduplex analysis with amplified DNA from serotype 1 as a constant component.
Lanes: 1: Serotype 1 + 3, 2: Serotype 1 + 5, 3: Serotype 1 + 6A, 4: Serotype 1 + 7F, 5: Serotype 1 + 8, 6: Serotype 1 + 9V, 7: Serotype 1 + 11A, 8: Serotype 1 + 14, 9: Serotype 1 + 15B, 10: Serotype 1 + 18C, 11: Serotype 1 + 19A, 12: Serotype 1 + 19F, 13: Serotype 1 + 23B

TABLE 1: Groups of pneumococcal serotypes as defined by heteroduplex analysis.

Homoduplex	Heteroduplex 1	Heteroduplex 2	Heteroduplex 3	Heteroduplex 4
Serotype 1	Serotype 3	Serotype 5	Serotype 7F	Serotype 8
Serotype 6A				
Serotype 14				
Serotype 19F				
Heteroduplex 5	**Heteroduplex 6**	**Heteroduplex 7**	**Heteroduplex 8**	**Heteroduplex 9**
Serotype 9V	Serotype 11A	Serotype 15B	Serotype 18C	Serotype 23B
			Serotype 19A	

As shown in Figure 2, serogroup 6 could be distinguished from others by giving only one homoduplex band with serotype 5.

When amplified DNA of different isolates from same serotypes (similar heteroduplex band patterns with serotype 1 or 5) were mixed for additional heteroduplex analysis, only homoduplex band patterns were detected.

Every heteroduplex analysis step contains an electrophoresis procedure which takes approximately five hours. In order to shorten these procedures, heteroduplex analysis was modified to a real-time fluorometric nucleic acid detection system. After mixing the amplified products, heteroduplex analysis was carried out in the LightCycler instrument. Melting curve analysis of all heteroduplex groups was performed and melting points of each group were determined. There was no correlation between heteroduplex groups and their melting points, somelting paints cannot be used to distinguish serotypes. On the other hand at least 2^{O}C difference was detected between melting points of heteroduplex and homoduplex groups (data not shown).

Partial modification of heteroduplex analysis to real-time fluorometric nucleic acid detection system has shorten the electrophoresis step from five hours to 30 minutes.

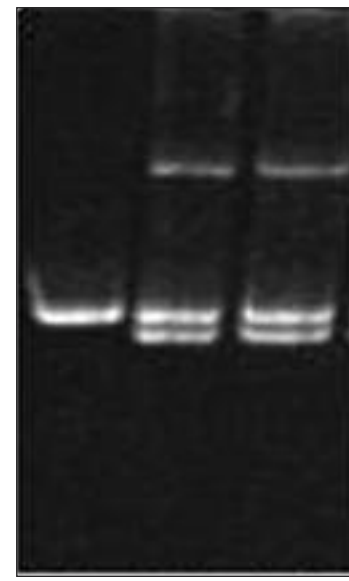

FIGURE 2: Heteroduplex analysis with amplified DNA from serotype 5 as a constant component.
Lanes: 1: Serotype 5 + 6A, 2: Serotype 5 + 14, 3: Serotype 5 + 19F

DISCUSSION

The first four genes of the *cps* locus (*cpsA* to *cpsD*) are common to all pneumococcal serotypes.[13] The phylogenetic tree generated from partial *cpsA-cpsB* sequences of 140 *S. pneumoniae* sequence types reveals that there are instances where the sequences of strains of the same serotype or serogroup are quite divergent.[24] In serotype 3, the *cpsA- D* genes are present but three of them are frameshifted. The presence of a cryptic 33F *cps* gene cluster that also possess the *cpsA-D* genes is present in two serotype 37 strains.[13] On the other hand, the region between *cpsA* position 951 to *cpsB* position 302 (approximately 800 bp) has been demonstrated as the most heterogeneous region among pneumococcal serotypes. [11] Therefore, molecular serotype prediction methods have been focused on this region. Several restriction fragment length polymorphism (RFLP), DNA sequencing and multiplex PCR based methods have been evaluated.[11,16,18-20,24-26] RFLP is a relatively

easy-to-use method, but it needs two electrophoresis steps and does not properly work unless all polymorphisms and mutations within the *cps* locus of serotypes are revealed. DNA sequencing is an end-point method for detection of heterogeneities. However, its performance depends on the technical capabilities of the laboratory. Multiplex PCR is a straightforward technique and easily established in laboratories that are equipped for DNA template preparation and PCR. The main pitfall of this technique is the coverage of restricted numbers of serotypes.

Heteroduplex analysis is a conformational technique used to detect mutations in PCR-amplified DNA products. As with other conformational techniques, mutations are identified by specific gel banding patterns. However, no indication of the position or nature of the polymorphism is provided. The migration of heteroduplex DNA in polyacrylamide gel electrophoresis is different from that of homoduplex DNA because of an altered three-dimensional structure. Once a correlation between gel banding patterns and mutations is established, this method may be applied to genotyping.[27,28] In this study heteroduplex analysis of *cps A* and *cpsB* genes of *S. pneumoniae* serotype 1 with serotypes 3, 5, 6A, 7F, 8, 9V, 11A, 14, 15B, 18C, 19A, 19F, and 23B was performed. These have been the most frequently isolated serotypes of pneumococci from children and adult patients in our hospital since 1995. Only serotypes 6B, 7B, 11A, 15B, 18C and 23 F of serogroups 6, 7, 11, 15, 18 and 23 are covered by the 23-valent vaccine which is used in Turkey. Therefore serotype prevalance data is important for countries such as Turkey in order to evaluate the protection effectiveness of pneumococcal vaccination. A major rise in pneumococcal disease caused by previously unusual serotypes could necessitate a change in vaccine composition. [16] On the other hand, there is a great need for a more efficient and cost-effective test for detecting serotypes of *S. pneumoniae,* and the heteroduplex analysis can be a valuable tool for pneumococcal surveillance and serotype prediction.

According to our results, amplified DNA from an isolate with unknown serotype can be tested using heteroduplex analysis with amplified DNA from serotype 1 as a constant component. If a homoduplex band is detected, heteroduplex analysis with serotype 5 must be performed. If of heteroduplex bands are detected with serotype 1 or 5, a heteroduplex analysis with a serotype which has similar bands pattern will show only homoduplex band, so serotype prediction can be performed.

In this study, heteroduplex analysis revealed nine groups. Group 1 included the serotypes that showed no heteroduplex bands when combined with serotype 1 (three serotypes). The heteroduplex patterns seem to allow discrimination of both the level of serogroup and serotype, an advantage over RFLP which discriminates only up to serogroup level. The main disadvantage of this technique is the requirement of two electrophoresis steps. To avoid electrophoresis and thus to shorten the duration of the test, we modified the technique to real-time fluorometric nucleic acid detection system. To our knowledge, this is the first molecular typing study with heteroduplex analysis that is performed in real-time detection system. In this technique, complementary chains of different serotypes were used as probes. Melting curve analysis between homoduplex and heteroduplex DNA chains showed more than 2°C melting peak differences. It is known that the shifts greater than 1°C from the characteristic (homoduplex in this situation) derivative melting curve profile arises a suspicion of mutation.[29] By real-time assay the electrophoresis step is skipped, thus the the time needed for the method is shortened remarkably. Real-time heteroduplex analysis may be a rapid and cost-effective alternative to other molecular methods for serotype prediction of pneumococci. This technique may easily be established in laboratories that are equipped for real-time PCR, however it never guarantees 100% accuracy.

The most significant shortcoming of this study is that the method has not been tested on other pneumococcal serotypes and any "unknown" isolates. Pneumococcal serotypes that are tested here

are the most frequently isolated serotypes from patients in Turkey and heteroduplex analysis performance is pretty well with these serotypes. Relatively rarely isolated serotypes can easily be tested in order to validate this method for these serotypes. In this study, heteroduplex analysis was repeated three times and three different isolates from same serotype were analysed in order to confirm the heteroduplex banding patterns. Further studies with "unknown" isolates are needed before using this method in clinical practice.

Heteroduplex analysis seems to have an advantageous potential to screen serotype directly from clinical samples without necessitating prior isolation of the organism. We would therefore define the heteroduplex analysis described here as a valuable tool for serotype prediction of pneumococci. Further serotype discrimination, testing more "unknown" isolates, adaptation of amplification step to real-time instrument and direct serotype prediction from clinical samples are our next goals for evaluation of this technique.

Acknowledgment

This study was supported by Hacettepe University, Scientific Research Unit Grant No: 01.02.101.027.

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