NEW MICROBIOLOGICA, **31**, 357-362, 2008

# The characteristics of nasopharyngeal *Streptococcus pneumoniae* in children attending a daycare unit

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**SUMMARY**

*S. pneumoniae* is a component of normal nasopharyngeal flora in children. Nasopharyngeal colonization in children attending daycare units has an important effect on the spread of *S. pneumoniae*. In this study, we aimed to investigate colonization status, antimicrobial susceptibility, and clonal relatedness of the *S. pneumoniae* strains in children attending a daycare unit. One hundred and six nasopharyngeal swabs were collected from 25 children attending a daycare unit in an 8-month period. *S. pneumonia*e was identified by a conventional method. Antibacterial sensitivities of the strains were tested by disc diffusion method. Pulsed field gel electrophoresis (PFGE) was used to analyze the clonal relationship of the strains. A total of 25 (23.5 %) *S. pneumoniae* strains were identified from 106 nasopharyngeal swaps. *S. pneumoniae* growth was detected in at least one culture of the 19 children (colonization rate; 76%). Seven of the 25 strains (28%) showed resistance to penicillin, 5 (20%) were resistant to trimethoprim-sulfomethoxsazole. The other tested antibiotics were almost effective. The clonal relationship among strains was found as 54.5%. The highest rate of strain entry was in the winter months with strains of opaque colonies, which are known to be more pathogenic. However, the spreading rate among the children was the highest in the summer months and the strains detected in these months had transparent colonies with more transmitting characteristics. Therefore, to prevent *S. pneumoniae* infection in closed crowded areas, the summer months should not be overlooked.

*KEY WORDS: Streptococcus pneumoniae,* Nasopharyngeal colonization, Nasopharyngeal pathogen, Care day unit

*Received* November 13, 2007 *Accepted* May 05, 2008

## INTRODUCTION

*Streptococcus pneumoniae* is a component of normal nasopharyngeal flora in children. Nasopharyngeal colonization begins shortly after birth. People usually carry pneumococci without symptoms, nasopharyngeal colonization of which subsequently serves as the focus for pneumococcal infection in children, the elderly, the immune compromised, and individuals with chronic disease. *S. pneumoniae* is the etiology of many diseases including pneumonia, bacteremia, meningitis, otitis media, and sinusitis. One million children die annually from pneumococcal diseases (WHO 2003). Treatment of *S. pneumoniae* infections has been increasingly challenging since bacteria have developed resistance to antibiotics (Fairchok *et al.,* 1996, Li *et al.,* 2001, Memish *et al.,* 2004, Riedel *et al.,* 2007).

Nasopharyngeal carriage of this bacterium is considerably affected by age, season, characteristics of child daycare centers, number of siblings, acute respiratory illness and diet. The possibility of person-to-person transmission is higher among children attending daycare units than children not attending daycare units. Impeding the dis-

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semination of colonization is an important step for prevention of pneumococcal disease (Garcia-Rodriguez *et al.,* 2002). This study was conducted to investigate the characteristics of the nasopharyngeal colonization of *S. pneumoniae* among children attending a daycare unit.

## MATERIALS AND METHODS

### Sample collection and daycare unit characteristics

The study was conducted by the microbiology departments of Kirikkale and Inonu Universities School of Medicine on the children attending a daycare unit in Kirikkale, a city in the Middle Anatolia Region of Turkey where the average temperature is 20-25ºC in summer and minus 2 to plus 2ºC in winter seasons. The samples were collected between November 2004 and June 2005. The daycare unit was for children of 1-6 years of age and on the second floor of an apartment. The flat has a large living room (approximately 60 $m^2$), two bedrooms (approximately 30 $m^2$), one kitchen, and one management room. All the rooms of the flat were clean and well-kept. However, it was housing twenty-one people (sixteen children, two teachers, two workers, and one manager) at one time, and there was no modern air conditioning system.

A letter explaining the aim and details of the study was sent to the parents of each child attending the daycare unit to obtain their consent for nasopharyngeal sample collection from the children. The children whose parents signed the consent form were included in the study.

The daycare unit was visited 6 times in an 8 month period. Nasopharyngeal swabs collected from children were plated on tryptic soy agar (Difco) supplemented with 5% sheep-blood and incubated in a 5% $CO_2$ atmosphere at 37°C. *S. pneumonia*e was identified by type of hemolysis, colony morphology, sensitivity to optokin, and dissolution in bile.

### Drug susceptibility and molecular typing of the strains

The antimicrobial susceptibilities of the *S. pneumoniae* strains to 1 mcg oxacilin (bioanalyse), erythromycin (15 µg), trimethoprim-sulfamethoxsazole (1,25-23,75 µg), clindamycin (2 µg), ofloxacin (5 µg), vancomycin (30 µg), rifampicin (5 µg), and tetracycline (30 µg) were tested by disc diffusion method according to the criteria of the Clinical and Laboratory Standards Institute (CLSI).

Molecular typing of the strains was performed in the Molecular Microbiology Laboratory of Inonu University Medical Faculty, Malatya/Turkey. PFGE typing was performed on 22 strains. *S. pneumoniae* colonies grown on sheep blood agar overnight at 37°C with 5% of $CO_2$ atmosphere were suspended in 500 ml cell suspension buffer (1 M NaCl, 10 mM Tris-HCl [pH:8.0]) and the optical density was adjusted to 1.0 absorbance (l=560) in a UV/Vis. spectrophotometer (Boeco, Germany). Isolation and deproteinization of the genomic DNA were done based on the protocol of Lefevre *et al.* (Lefevre *et al.*, 1993) with a minor modification. Genomic DNA in the plugs was restricted by 50 U of *Sma*I (Promega, Madison, Wis., USA) for 2 h at 37°C in a water bath. DNA fragments were separated on 1% pulse field certified agarose (Bio-Rad Laboratories, Nazareth, Belgium) gels run in 0.5X Tris-borate-EDTA buffer (44,5 mM Tris, 44.5 mM Boric Acid, 1mM EDTA [pH:8.0]) by using a CHEF-DR II system (Bio-Rad Laboratories, Nazareth, Belgium). The electrophoresis conditions were 14°C at 6 V/$cm^2$. The parameters in block 1 were an initial pulse time of 1s increased to 30s for 17h, and in block 2, 5s of the initial pulse increased to 9s for 6 h. The gel was stained with ethidium bromide (5 mg/ml) and photographed under UV light. According to the interpretative criteria of Tenover *et al.* (Tenover *et al.*, 1995), the isolates were classified as indistinguishable (cluster), closely related, possibly related or different.

## RESULTS

We collected 106 samples from 25 children and identified 25 *S. pneumoniae* strains (23.5 %). No *S. pneumoniae* growth was detected in the cultures of six children. Six swabs were taken from each of the three of these six children, while only three swabs could be obtained from each of the other three children since they left the daycare unit in March 2005. *S. pneumoniae* growth was detected in at least one culture of the 19 children (colonization rate; 76%). Sixteen of these

strains formed opaque colonies on the culture medium. Nine of the 25 strains formed transparent colony morphology, and they were isolated in May and June. These strains were strains no 1, 4, 5, 6, 7, 8, 9, 10, and 11, and they were susceptible to all of the antimicrobials. The information on the isolates was detailed in Table 1.

TABLE 1 - *Characteristics of the 25 S. pneumoniae strains.*

| *No of children* | *No. of strain* | *Isolation dates* | *Colony types* | *PFGE types* |
|---|---|---|---|---|
| 1 | 1 | 21.06.2005 | Transparent | Unique |
| 1 | 14 | 07.11.2005 | Opaque | Unique |
| 3 | 23 | 07.02.2005 | Opaque | Exclude |
| 5 | 25 | 17.11.2005 | Opaque | Exclude |
| 7 | 20 | 07.02.2005 | Opaque | Unique |
| 10 | 22 | 07.02.2005 | Opaque | Unique |
| 11 | 24 | 07.02.2005 | Opaque | Exclude |
| 12 | 11 | 03.05.2005 | Transparent | C |
| 12 | 16 | 21.06.2005 | Opaque | Unique |
| 13 | 18 | 07.11.2005 | Opaque | D |
| 14 | 15 | 22.03.2005 | Opaque | Unique |
| 15 | 13 | 7.2..2005 | Opaque | Unique |
| 16 | 8 | 03.05.2005 | Transparent | B |
| 16 | 21 | 07.02.2005 | Opaque | Unique |
| 17 | 5 | 03.05.2005 | Transparent | Unique |
| 18 | 7 | 21.06.2005 | Transparent | B |
| 18 | 10 | 03.05.2005 | Transparent | B |
| 18 | 17 | 3.1..2005 | Opaque | Unique |
| 19 | 19 | 07.02.2005 | Opaque | D |
| 20 | 3 | 07.02.2005 | Opaque | A |
| 20 | 12 | 21.06.2005 | Opaque | A |
| 21 | 6 | 21.06.2005 | Transparent | B |
| 22 | 4 | 03.05.2005 | Transparent | C |
| 23 | 2 | 21.06.2005 | Opaque | A |
| 25 | 9 | 21.06.2005 | Transparent | B |

Seven (28%) of the 25 strains (strain no 13, 15, 17, 18, 19, 21, 22) were resistant to penicillin. five strains (strain no 15, 18, 19, 21, 22) to trimethoprim-sulfamethoxsazole, two strains (strain no 18, 21) to tetracycline, one strain (strain no 22) to erythromycin, and one strain (strain no 22) to ofloxacin. Five strains were resistant to at least two antibiotics. (The resistant category comprised isolates exhibiting either intermediate- or high-level resistance). No strain was resistant to clindamycin, vancomycin or rifampicin. Interestingly all resistant strains had opaque colonies.

Molecular typing was performed on 22 strains since 3 out of 25 strains did not reproduce when strains were passaged for PFGE. Twelve out of 22 strains were clustered in four PFGE genotypes (clustering rate was 54.5%). Three strains (2, 3, and 12) were in genotype A, five (strains: 6, 7, 8, 9, and 10) in genotype B, two (strains: 4, 11) in genotype C, and two (strains:18, 22) in genotype D. There was a correlation between drug resistance pattern and PFGE types of genotype D. They were resistant to trimethoprim-sulfamethoxsazole and penicillin.

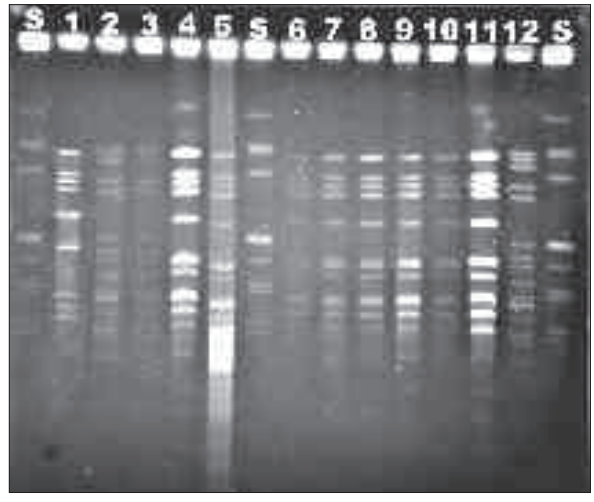


FIGURE 1 - *Representative PFGE results of the 12 S. pneumoniae strains. Lines S; a S. pneumonia clinical isolate running three lines for using normalization of the different gels. Lines 1, 5 unique; lines 2, 3 and 12 were PFGE type A; lines 6, 7, 8, 9, and 10 were PFGE type B; lines 4 and 11 were PFGE types C.*

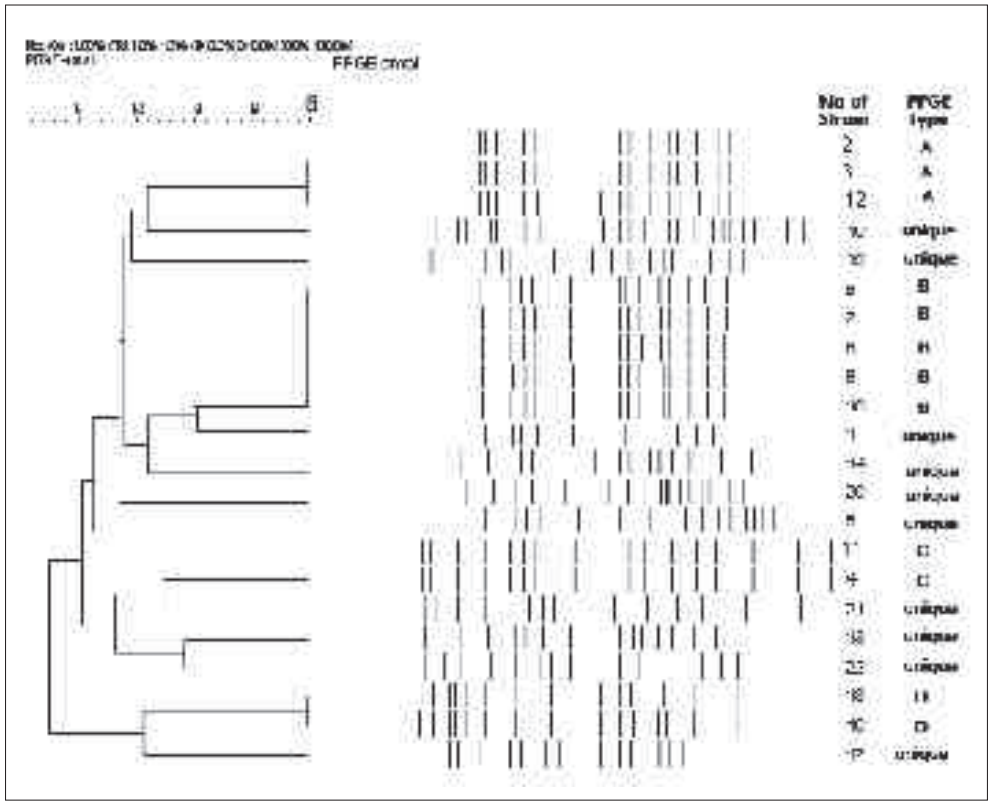

FIGURE 2 - *A dendrogram showing the clonal relationships among the 22 S. pneumoniae strains typed by PFGE.*

Nine of the clustered strains were isolated in May and June 2005; the clustering rate in this period was 9/12 (75%). While only three of the clustered strains were in the strains isolated from November 2004 to the end of March 2005, the clustering rate was 3/13 (23%) in this period. The clustering rates of the strains having opaque and transparent colonies were 29.4% (5/17 strains) and 87.5% (7/8 strains) respectively. Ten strains showed unique (different) genotypes (Table 1). Representative PFGE typing results of the 12 strains and a dendrogram showing clonal relatedness of all typing strains are shown in Figure 1 and Figure 2, respectively.

## DISCUSSION

Children who are within closed environments have higher rates of nasopharyngeal *S. pneumoniae* colonization than children living in their own homes with their families (Sulikowska *et al.,* 2004**,** Gazi *et al.,* 2004). Of the 25 children included in the study, 19 (76%) were colonized by *S.pneumoniae* at least once. In a study from Japan, the samples of 6 children attending daycare units were obtained once a month for 15 months and *S.pneumoniae* colonization was detected in 5 children (Yano *et al.,* 1999). The literature reveals no other studies with a follow-up conducted in daycare units. However, there are some studies involving a single screening in daycare units and schools. Immergluck *et al.* from the USA and Zemlickova *et al.* from the Czech Republic reported rates of 17-38 % for nasopharyngeal colonization (Immergluck *et al.,* 2004, Zemlickova *et al.,* 2006 ). Similarly, Gazi *et al.* from Turkey detected a rate of 23% in schoolchildren 6-14 years of age (Gazi *et al.,* 2004).

In our study, the rates of *S. pneumoniae* colonization for various months were as follows: 18% (3 of 16 children) in November; 6.6% (1 of 5 children) in January; 44.4% (8 of 18 children) in February; 6.2% (1 of 16 children) in March; 27.7% (5 of 18 children) in May; 36.8% (7 of 19 children) in June. Two peak levels - in February and June - were observed. The peak in *S. pneumoniae* infections observed in February supports the general information that it is more frequently observed in winter months (Sulikowska *et al.,* 2004).

In this study, PFGE was evaluated in 22 strains and 4 (28.5%) of the 14 genotypes were observed in 12 children. It was striking that the strains isolated in the winter period generally had more distinct genotypes. The clustering rate of this period was significantly lower than that of summer (23% versus 75%, respectively). One of the 8 strains (strain no 19- genotype D) isolated in February 2005 had the same genotype as that of strain no 18 (genotype D) in the 13$^{th}$ child, isolated in November 2004. Because two strains died out, their genotypes could not be studied. The genotypes of the other 5 strains were different from that of the strains isolated in earlier months and 4 of these strains did not infect the other children in the following months. But one of them (Strain no 3-genotype A), first isolated in February 2005, was isolated again from the nasopharynx of the same child and another child in June 2005. The new strain entry was most common in February. Five strains were isolated in May and three new genotypes (genotype B, C, and a unique type) entered the daycare unite in this period. All of them had transparent colonies with higher adherence capability (Hammerschmidt *et al.,* 2005, Ring *et al.,* 1998, Weiser *et al.,* 1999). Genotype B was isolated from two children at the same time in May and infected three other children in June. Although genotype C was isolated from two children in May, this was not detected in the following months. The second highest isolation rate was in June (seven strains).

The genotypes of 3 (strains no 6, 7, and 9) of the seven strains isolated in June were genotype B with transparent colonization detected in May. Two (strains no 12 and 2) of these seven strains had genotype A with opaque colonization detected in February. Only two new unique genotypes entered the daycare unit in June, one of which had transparent colonization and the other had opaque colonization.

The infection rate with the old strains was higher in June than in the winter months. May and June are the months in which daytime temperatures reach 20-26°C in Kirikkale. The peak in June seems to contradict the general belief that pneumococcal infections are usually more common in the winter months. Similar to our results, Sogsrad *et al.* reported higher rates of colonization in August than in the winter months (Sogsrad *et al.,* 2006). In their study, they detected colonization in 3 of the 4 day care units in summer. Marchisio *et al.* found higher rates of colonization of *S. pneumonia* in spring than autumn (Marchisio *et al.,* 2001). Öztuna *et al.* found that *S. pneumoniae* adherence was lower in rats exposed to cold than in the rats kept at room temperature (Öztuna *et al.,* 2006). In Kırıkkale, the mean temperatures in the months of May and June are similar to room temperature. The high rate of transmission found in our study during a summer months may have been due to poor ventilation of the daycare unit and the temperature in that period.

We observed that colonization of transparent strains were more common among children in summer. The less pathogenic but more easily transmitted transparent strains can cause infection peaks among children in closed settings. Nevertheless, since the earlier studies focused on winter months (Gazi *et al.,* 2004**,** Inostroza *et al.,* 1998, Marchisio *et al.,* 2001, Sá-Leão *et al.,* 2000, Zemlickova *et al.,* 2006), the information on summer peaks has been limited.

To sum up, in our study involving 25 children attending a daycare unit with insufficient ventilation, colonization of *S. pneumoniae* was observed at least once in 19 children (76%) within 8 months. The highest rate of strain entry was in the winter months with strains of opaque colonization, which are known to be more invasive (Briles *et al.,* 2005). However, the spreading-rate (clustering rate) among the children was highest in the summer months and the strains detected in these months had transparent colonization with more transmitting characteristics. Therefore, to prevent *S. pneumoniae* infection in closed crowded areas, the summer months should not be overlooked.

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