RESEARCH ARTICLE

Comparison of Serotype Prevalence of Pneumococci Isolated from Middle Ear, Lower Respiratory Tract and Invasive Disease Prior to Vaccination in Iceland

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Abstract

Background

Information on pneumococcal serotype distribution before vaccination is a prerequisite for evaluation of vaccine effect. The aim was to investigate the prevalence of pneumococcal serotypes isolated from middle ear (ME), lower respiratory tract (LRT) and from invasive disease (IPD) in Iceland prior to implementation of ten-valent pneumococcal Haemophilus influenzae protein D conjugate vaccine (PHiD-CV-10) into the infant vaccination program (April 2011).

Methods and findings

All isolates cultured 2007–2011 from ME, LRT and IPD identified as pneumococci were serotyped and tested for susceptibility at the Clinical Microbiology Department, Landspítali University Hospital that serves approximately 85% of the Icelandic population. Pneumococcal isolates were 1711 and 1616 (94.4%) were available for serotyping and included. Isolates belonging to PHiD-CV10 serotypes (VTs) were 1052 (65.1%). Isolates from ME were 879 (54.4%), with 639 (72.7%) from 0–1 year old patients and 651 of VTs (74%). Isolates from LRT were 564 (34.9%), with 292 (51.8%) from ≥65 years old patients, and 300 (53.2%) of VTs. IPD isolates were 173 (10.7%), although more evenly distributed according to age than isolates from the other sites most were from adults and the youngest age group, 101 (58.4%) isolates were of VTs. The most common serotype was 19F, 583 (36.1%). Its prevalence was highest in ME, 400 (45.5%), 172 (30.5%) in LRT and 11 isolates (6.4%), in IPD. Penicillin non-susceptible isolates were 651 (40.3%), mainly belonging to VTs, 611 (93.9%), including 535 (82.2%) of 19F.

Conclusions

Multiresistant isolates of serotype 19F were highly prevalent, especially from ME of young children but also from LRT of adults. Serotype 14 was the most common serotype in IPD.
The rate of VTs was high and almost all PNSP were of VTs. There was great difference in vaccine coverage between sampling sites, also reflecting difference in vaccine coverage by age groups.

Introduction

*Streptococcus pneumoniae* (pneumococcus) causes a range of diseases from mild localized infections like acute otitis media to more severe infections like pneumonia, bacteraemia and meningitis [1]. Pneumococci are also commonly carried in the nasopharynx, especially by young children and spread from there to other parts of the body, or to other individuals [2, 3].

Serotype prevalence is dynamic and depends on many variables, which can be related to pneumococcal properties, host factors, antimicrobial use and vaccination in the community. Furthermore, prevalence may differ according to geographic area and time [4–7]. Serotypes with low invasive potential e.g. 6A, 6B, 14 and 19F most commonly cause non-bacteraemic pneumonia and acute otitis media and are frequently found in the nasopharynx of healthy children [8–10]. Serotypes that are considered to be of high invasive potential, e.g. 1, 4, 7F and 9V, cause invasive disease, but are infrequently found in milder infections and very rarely in carriage [9, 11]. However, serotypes of low invasive potential can be a relatively common cause of invasive disease in high risk individuals, such as young children, the elderly and immunocompromised patients [12].

Pneumococcal disease, both in children and older non-vaccinated individuals, has decreased following childhood vaccinations with protein conjugated vaccines (PCV) and replacement from vaccine to non-vaccine serotypes has been documented [8, 13–15]. At the same time the overall levels of pneumococcal carriage in children remain virtually unchanged, but the types carried are non-vaccine serotypes. [16, 17].

It is important to study serotype prevalence prior to implementation of pneumococcal vaccination programs and to monitor post vaccination changes to evaluate the effect of the vaccine on disease and carriage and changes in the serotype prevalence. The aim was to investigate the prevalence of pneumococcal serotypes isolated from middle ear, lower respiratory tract and from invasive disease in Iceland prior to implementation of ten-valent pneumococcal *Haemophilus influenzae* protein D conjugate vaccine (PHiD-CV-10) into the infant vaccination program.

Materials and Methods

Study population

The Department of Microbiology, Landspitali University Hospital, Iceland, serves as the primary microbiology laboratory for the Reykjavik metropolitan area. Furthermore, the laboratory serves as a reference laboratory for the whole country. Inhabitants from other areas of the country often seek health services, both general and specialist services, in the capital and are included in the study. The population of Iceland was 312,872 in 2007 and 318,452 in 2011. In 2011 202,339 inhabitants lived in the metropolitan area. It is estimated that the departments serve 60% of the countryside, thus in that year it served 272,009 (85.4%) inhabitants. Children younger than 7 years old were then 10.1% of the total population and in the metropolitan area 10.7%. (www.statice.is).

The study was approved by the National Bioethics Committee (license no: 12.010.S1).

Vaccination with PHiD-CV10 was initiated in April 2011 thus only part of the children born in that year had received the first vaccinations.
When analysing the data, the patients were divided into the age groups: 0–1, 2–6, 7–17, 18–64 and ≥65 years old.

**Bacterial isolates**

The study included all clinical isolates from middle ear (ME), lower respiratory tract (LRT) and invasive diseases (blood, cerebrospinal fluid and joint fluid) (IPD) identified as pneumococci during a five year period, 2007–2011, at the Department of Clinical Microbiology, Landspitali University Hospital. Only one isolate of the same phenotype (defined as identical serotype and antibiogram) from the same patient within 30 days was used. When possible, the first isolate indicating the most severe infection was chosen (IPD, LRT and ME respectively). The isolates were stored in tryptose-glycerol broth at -80°C.

**Antimicrobial susceptibility testing**

Antimicrobial susceptibility was performed on all isolates by disk diffusion, against oxacillin, chloramphenicol, erythromycin, trimethoprim-sulfamethoxazole, tetracycline and clindamycin, according to the methods of the Clinical and Laboratory Standards Institute (CLSI) [18, 19] and also interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [20]. Screening for penicillin non-susceptibility was done using 1 μg oxacillin disks. All oxacillin sensitive isolates were defined as sensitive to penicillin, but all resistant isolates were tested for minimum inhibitory concentration to penicillin and ceftriaxone using the E-test (AB-Biodisk) [21]. Isolates with penicillin MIC >0.06mg/L were defined as penicillin non-susceptible.

**Serotyping**

All available isolates were serotyped by conventional methods using the Pneumotest kit and/or latex or coagglutination solutions for single antisera (State Serum Institute, Copenhagen) [22, 23] and/or multiplex PCR [10].

A multiplex PCR panel was designed based on previously published methods [24–27]. The serotypes were selected to include the vaccine serotypes that are included in the PHID-CV10 (VT), the additional serotypes in the 13-valent vaccine and the most common non-vaccine serotypes (NVT) in Iceland, according to our previous studies. The panel consisted of seven multiplex PCR reactions, each with three to four serotype specific primers pairs, in total 27 serogroups/serotypes, and primers for cpsA, for the cps locus, and lytA, for autolysin, that were used for internal positive control. Furthermore, a series of PCR reactions previously described was used to detect serotypes 6A, B,C, D and to separate 6B and its variant 6Bii in all isolates of serogroup 6 [28, 29].

**Statistical analyses**

To study association between groups Pearson’s Chi-square and Fisher’s exact test were used. P value <0.05 was considered significant.

**Results**

In total, 1711 pneumococcal isolates fulfilled the criteria of the study and of those 1616 (94.4%) were available for serotyping and were included. Distribution according to specimen site and age group did not differ between the available and the 95 (5.6%) non-available (not stored, or non-viable) except for the yield of available isolates from IPD that was higher (99.4%) than for the other sampling sites.
The isolates from ME specimens were 879/1616 (54.4%) and most were from young children. Isolates from 0–1 year old children more often originated from ME than other specimens ($p < 0.0001$). The ME isolates were also more often from 0–1 than 2–6 year old children ($p < 0.0001$). LRT isolates were 564/1616 (34.9%) and most were from adults. Isolates from ≥65 year old patients were more commonly from LRT than the other sampling sites ($p < 0.0001$). The isolates from IPD specimens were 173/1616 (10.7%), although more evenly distributed according to age than isolates from the other sites most were from adults and the youngest age group (Table 1).

Temporal changes in number of pneumococcal isolates were noted, the highest number of isolates was in 2007, 357 (22.1%) and gradually declined to 283 in 2011 (17.5%).

### Prevalence of serotypes

The isolates were of 52 serotypes. The most common was serotype 19F with 583/1616 (36.1%) isolates, followed by 23F with 157/1616 (9.7%) (Table 2). Of all isolates, 1052/1616 (65.1%) were VT-10, 651/1616 (40.3%) were VT-13, 228/1616 (14.2%) were NVT-10, and 152/1616 (9.5%) were NVT-13. Other possibilities might be serotypes 25F/A, or 38, known for changes in the cpsA gene causing negative reactions.

| Type   | ME n (%) | LRT n (%) | IPD n (%) | Total n (%) |
|--------|----------|-----------|-----------|-------------|
| 19F    | 400 (45.5) | 19F 172 (30.5) | 14 28 (16.2) | 19F 583 (36.1) |
| 23F    | 111 (12.6) | 3 49 (8.7) | 19A 17 (9.8) | 23F 157 (9.7) |
| 6A     | 73 (8.3) | 6A 42 (7.4) | 4 14 (8.1) | 6A 122 (7.5) |
| 14     | 61 (6.9) | 23F 40 (7.1) | 9V 13 (7.5) | 14 117 (7.2) |
| 19A    | 61 (6.9) | 6B 32 (5.7) | 7F 12 (6.9) | 6B 101 (6.3) |
| 6B     | 60 (6.8) | 14 28 (5.0) | 19F 11 (6.4) | 19A 99 (6.1) |
| 3      | 28 (3.2) | NT 23 (4.1) | 3 9 (5.2) | 3 86 (5.3) |
| 9V     | 14 (1.6) | 19A 21 (3.7) | 6B 9 (5.2) | 9V 47 (2.9) |
| Other  | 71 (8.1) | Other 157 (27.9) | Other 60 (34.7) | Other 304 (18.8) |
| Total  | 879 (54.4) | Total 564 (34.9) | Total 173 (10.7) | Total 1616 |
| VT-10  | 651 (74.1) | VT 300 (53.2) | VT 101 (58.4) | VT 1052 (65.1) |
| NVT-10 | 228 (25.9) | NVT 264 (46.8) | NVT 72 (41.6) | NVT 564 (34.9) |
| VT-13  | 813 (92.5) | VT-13 412 (73.0) | VT-13 134 (77.5) | VT-13 1359 (84.1) |
| NVT-13 | 66 (7.5) | NVT-13 152 (27.0) | NVT-13 39 (22.5) | NVT-13 257 (34.9) |

n = the number of isolates.

NT = unencapsulated isolates, negative in PCR reactions using cpsA and serotype specific primers, positive in lytA reactions. Other possibilities might be serotypes 25F/A, or 38, known for changes in the cpsA gene causing negative reactions.
belonged to VT-10 and were significantly more common than non-vaccine serotypes (NVT-10) \( (P < 0.0001) \).

The ME isolates were of 34 serotypes and the most common was serotype 19F with 400/879 (45.5%) isolates and significantly more common than in LRT and IPD \( (P < 0.0001) \). The prevalence of the 8 most common serotypes ranged from 45.5–1.6% (Table 2). The proportion of isolates belonging to VTs was highest in ME, 651/879 (74.1%). The LRT isolates were of 45 serotypes and the most common was 19F with 172/564 (30.5%) isolates. The prevalence of the 8 most common serotypes ranged from 30.5–3.7%. The proportion of isolates belonging to VT-10 was lowest in LRT, 300/564 (53.2%). The IPD isolates were of 29 serotypes and the most common serotype was serotype 14 with 28/173 (16.2%) isolates. The prevalence of the 8 most common serotypes ranged from 16.2–4.6% (Table 2). The isolates belonging to VTs were 101/173 (58.4%) (Table 2). Overall, the most common NVTs were 11A, 41 isolates (2.5%) and 33D, 18 (1.1%) isolates.

**Antimicrobial susceptibility**

Penicillin non-susceptible pneumococcal isolates (PNSP) were 651/1616 (40.3%) (Table 1). The number of PNSP isolates remained stable during the study period, 128–138 isolates each year, while the number of all isolates gradually decreased from 357–283. Accordingly, the PNSP proportions gradually increased from 36.7% in 2007 to 44.9% in 2011, resulting in significant difference between the first and last year \( (p = 0.035) \). PNSP isolates of VT-10 were 611/651 (93.9%). Similar temporal changes in the rates of VTs of PNSP were seen but with more fluctuations between years.

PNSP isolates were 428/879 (48.7%) of ME, 205/564 (36.3%) of LRT and 18/173 (10.4%) of IPD isolates. PNSP of VTs were significantly more common in ME than in LRT \( (p = 0.0001) \) and also more common than in IPD \( (p = 0.0025) \). The VTs were significantly more common than the NVTs among PNSP in all instances (Table 3).

The PNSP were of 18 serotypes with serotype 19F being the most common, 535/651 (82.2%) isolates. Serotype 19F was the most common PNSP serotype in all specimen groups \( (p < 0.0001) \), most commonly found in ME with 378/428 (88.3%), in LRT 150/205 (73.2%) and in IPD 7/18 (38.9%) of PNSP isolates (Table 3). The majority of the serotype 19F isolates, or 501/535 (93.6%) isolates had identical antibiograms and were multi-resistant. Their penicillin MIC was close to the breakpoint between intermediate and resistant (median MIC 1.0 \( \mu \text{g/mL} \)), thus either defined as intermediate or resistant, resistant to erythromycin, tetracycline and trimethoprim-sulfamethoxazole and sensitive to chloramphenicol and clindamycin as previously described.

### Table 3. Ranking of the 4 most common PNSP serotypes according to sampling site.

| Type | ME n (%) | LRT n (%) | IPD n (%) | Total n (%) |
|------|----------|-----------|-----------|-------------|
| 19F  | 378 (88.3) | 19F 150 (73.2) | 19F 7 (38.9) | 19F 535 (82.2) |
| 14   | 12 (2.8) | NT 13 (6.3) | 9V 2 (11.1) | 6B 32 (4.9) |
| 6B   | 19 (4.4) | 6B 12 (5.9) | 14 2 (11.1) | 14 20 (3.1) |
| 19A  | 7 (1.6) | 9V 10 (4.9) | 23F 2 (11.1) | 9V 14 (2.2) |
| Other| 12 (2.8) | Other 20 (9.8) | Other 5 (27.8) | Other 50 (7.7) |
| Total| 428 (65.7) | Total 205 (31.5) | Total 18 (2.8) | Total 651 |
| VT   | 416 (97.2) | VT 181 (88.3) | VT 14 (77.8) | VT 611 (93.9) |
| NVT  | 10 (2.8) | NVT 24 (11.7) | NVT 4 (22.2) | NVT 40 (6.1) |

n = the number of isolates

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The remaining 34 (6.4%) isolates of PNSP serotype 19F had diverse antibiograms. Isolates of 6B sharing the antibiograms of the previously dominating clone in the country, of the variant 6Bii of serotype 6B [30] were 24 (1.5%).

**Discussion**

Serotype 19F was the dominant serotype, with more than a third of all isolates and more than four of every five PNSP isolates belonging to 19F. It had a predilection for middle ear where most of the samples originated from young children. The serotype was also the dominant PNSP type and almost all the isolates had identical antibiogram. The vast majority of isolates of PNSP belonged to VTs.

The ME isolates were from patients with perforated tympanic membranes or with tympanic tubes, therefore mainly representing acute otitis media which is most common in children. Accordingly, it must be taken into account that when we compare isolates from the different sites, it not only highlights differences between different diseases but also different age groups. Almost half of the isolates belonged to serotype 19F that was the most common and two thirds of all isolates of serotype 19F originated from ME. The vast majority of the 19F isolates were multi-resistant and shared identical antibiograms with the previously described single and double locus variant of the Taiwan 19F-14 clone [7]. Other isolates belonged to serotypes commonly found in otitis media and carriage and isolates with high invasive potential were rarely seen [11, 12]. Besides the exceptionally high rate of 19F our findings were in concordance with other studies [9, 31–36]. The reason for this high rate might be related to a relatively high antimicrobial usage in the country, making the environment favourable for this multiresistant piliated clone [37]. The high prevalence of VTs and PNSP in ME compared to LRT and IPD can mostly be explained with the exceptionally high rate of 19F in ME samples.

The LRT isolates were mainly isolated from sputum samples and bronchoalveolar lavage fluids, specimens that are rarely obtained from children. Therefore, these isolates are mainly from adults, especially the elderly. The age distribution reflects high risk groups for pneumococcal pneumonia except for young children. Close to a third of the isolates belonged to serotype 19F that was the most common, but isolates of high invasive potential were rarely seen. The rate of isolates of VTs was lower in LRT than in the other specimen groups. Most likely this reflects the high diversity of the serotypes found in LRT isolates, lower rate of 19F than in ME and compared to IPD low rate of isolates of high invasive serotypes that are VTs. More than a third of the isolates were PNSP, most of serotype 19F.

About half the IPD isolates were from the youngest and the oldest age groups. The two most common serotypes were 14 and 19A. This is of interest as these serotypes are generally considered to be of a low or moderate invasive potential [12, 38]. The reason for this remains unclear. It may be related to clonal properties as different clones of serotype 14 have been reported to be more prevalent in IPD than carriage and vice versa [12]. Following vaccination with the 7-valent conjugative vaccine serotype 19A was commonly reported in IPD [39, 40]. It is possible that the effect of vaccination in neighbouring countries may have had an indirect effect on serotype distribution in IPD in this country. Another possible explanation for the relatively common IPD by these less invasive serotypes is the patient's age. Young children and older individuals have less competent immune system. In addition, older people may more often be immunocompromised due to co-morbidity or medical therapy. This weak immune system may certainly render the individuals more prone to infections, even with less invasive serotypes [41]. The next three serotypes in the rank, serotypes 4, 7F and 9V are considered highly invasive [4, 11, 42, 43]. The age distribution of the patients diagnosed with the highly invasive serotypes tended to be more evenly distributed which is in concordance with our
explanations and other studies [5, 12]. Serotypes that are commonly carried by children, or known for causing milder disease, were a relatively common cause for IPD [8–10].

The PNSP rates were high and mostly seen in serotypes that are frequent colonizers and more often in ME than in other infections. This is in concordance with other studies showing that antimicrobial resistance is more likely to be found in milder infections than in invasive disease, with the exception of individuals at high risk [44]. The outpatient usage of antimicrobials in Iceland is relatively high and brings selective pressure for resistant clones that along with the properties of successful clones can influence the rates of serotypes [7, 37]. However, the vast majority of PNSP were of VTs and vaccination is therefore likely to reduce the PNSP rates substantially.

In summary, the rates of serotype 19F were very high, especially in isolates from ME that were mostly from young children. 19F was also the dominant serotype in isolates from LRT that were mainly from adults and the elderly. The vast majority of the 19F isolates were multi-resistant, with identical antibiograms. The most common serotype in IPD was serotype 14. The rate of vaccine serotypes was highest in isolates from middle ear and lowest in isolates from lower respiratory tract, where serotype diversity was the most. Penicillin non-susceptible isolates were almost solely of vaccine serotypes. There was great difference in vaccine coverage between sampling sites, also reflecting difference in vaccine coverage by age groups. These results provide important information on serotype distribution and antibiotic resistance prior to immunization and will serve as a basis for evaluation of the effect of infant pneumococcal vaccination.

Supporting Information
S1 Table. This is the full supporting information that includes impersonalized relevant information of specimen acquisition and results.
(XLSX)

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