VILNIUS UNIVERSITY

Sigita Petraitiene

STREPTOCOCCUS PNEUMONIAE STRAINS IN THE NASOPHARYNX OF PRESCHOOL CHILDREN- SURVEY OF VILNIUS DAY CARE CENTERS ATTENDANTS

Summary of the Doctoral Dissertation
Biomedical Sciences, Medicine (07 B)

Vilnius, 2009

The dissertation was prepared at Vilnius university faculty of medicine in 2005-2009.

Scientific supervisor

Prof. Dr. Habil. Vytautas Usonis (Vilnius university faculty of medicine, Biomedical Sciences, Medicine – 07 B)

Doctoral dissertation will be defended at the Council of Medical Research of Vilnius University:

Chairman

Dr. Augustina Jankauskienė (Vilnius university faculty of medicine, Biomedical Sciences, Medicine – 07 B)

Mememberss:

Doc. Dr. Giedra Levinienė (Kaunas University of Medicine, Biomedical Sciences, Medicine – 07 B)

Doc. Dr. Laimutė Vaidelienė (Kaunas University of Medicine, Biomedical Sciences, Medicine – 07 B)

Dr. Rimantė Čerkauskienė (Vilnius university faculty of medicine, Biomedical Sciences, Medicine – 07 B)

Dr. Violeta Kvedarienė (Vilnius university faculty of medicine, Biomedical Sciences, Medicine – 07 B)

Oponents:

Doc. Dr. Odilija Rudzevičienė (Vilnius university faculty of medicine, Biomedical Sciences, Medicine – 07 B)

Doc. Dr. Jolanta Kudzyte (Kaunas University of Medicine, Biomedical Sciences, Medicine – 07 B)

Doctoral dissertation will be defended at the open session of the Council of Medical Research on the 27th November, 2009, 12 a.m. in the 201 auditorium of Vilnius University Children's Hospital.

Address: 4 Santariškiu St., LT-08661 Vilnius, Lithuania

Summary of the Doctoral dissertation was mailed on the 26th October, 2009.

The dissertation is available at the library of Vilnius University.

Address: 3 Universiteto St, LT-01513 Vilnius, Lithuania

VILNIAUS UNIVERSITETAS

Sigita Petraitienė

STREPTOCOCCUS PNEUMONIAE PADERMĖS VAIKŲ, LANKANČIŲ VILNIAUS IKIMOKYKLINES UGDYMO ĮSTAIGAS, NOSIARYKLĖJE

Daktaro disertacijos santrauka

Biomedicinos mokslai, medicina (07 B)

Vilnius, 2009

Disertacija rengta 2005 – 2009 metais Vilniaus universitete

Mokslinis vadovas:

prof. habil. dr. Vytautas Usonis (Vilniaus universitetas, biomedicinos mokslai, medicina – 07B)

Disertacija ginama Vilniaus universiteto biomedicinos mokslo krypties taryboje:

Pirmininkas

dr. Augustina Jankauskienė (Vilniaus universitetas, biomedicinos mokslai, medicina – 07B)

Nariai:

doc. dr. Giedra Levinienė (Kauno medicinos universitetas, biomedicinos mokslai, medicina – 07B)
doc. dr. Laimutė Vaidelienė (Kauno medicinos universitetas, biomedicinos mokslai, medicina – 07B)
dr. Rimantė Čerkauskienė (Vilniaus universitetas, biomedicinos mokslai, medicina – 07B)
dr. Violeta Kvedarienė (Vilniaus universitetas, biomedicinos mokslai, medicina – 07B)

Oponentai:

doc. dr. Odilija Rudzevičienė (Vilniaus universitetas, biomedicinos mokslai, medicina – 07B)
doc. dr. Jolanta Kudzytė (Kauno medicinos universitetas, biomedicinos mokslai, medicina 07B)

Disertacija bus ginama viešame Medicinos mokslo krypties tarybos posėdyje 2009 metų. lapkričio mėn. 27d., 12 val. Vilniaus universiteto vaikų ligoninėje, Pediatrijos centre, 201 auditorijoje.

Adresas: Santariškių 4, LT –08661 Vilnius, Lietuva.

Disertacijos santrauka išsiuntinėta 2009 m. spalio mėn. 26 d.

Disertaciją galima peržiūrėti Vilniaus universiteto bibliotekoje.

Adresas: Universiteto g. 3, LT-01513 Vilnius, Lietuva

1 INTRODUCTION

Streptococcus pneumoniae, or pneumococcus, is Gram-positive, alpha-hemolytic, bile soluble diplococcus aerotolerant anaerobe and a member of the genus *Streptococcus* (phylum Firmicutes). *Streptococcus pneumoniae (S.pneumoniae)* is known in medical microbiology as the pneumococcus. It has a polysaccharide capsule that acts as a virulence factor for the organism; more than 90 different serotypes are known, and these types differ in virulence, prevalence, and extent of drug resistance.

Streptococcus pneumoniae is a normal inhabitant of the human upper respiratory tract. The serotype distribution among nasopharyngeal carriage isolates varies by country, age-group, origin, type of cohort. Pneumococcal disease will not occur without preceding nasopharyngeal colonization with the homologous strain. In addition, pneumococcal carriage is believed to be an important source of horizontal spread this pathogen within the community. Because the highest frequency of the pneumococcal colonization and the highest crowding index are found in young children, this risk group is thought to be the most important vector for horizontal dissemination of pneumococcal strains within the community. In Europe asymptomatic nasopharyngeal carriage of pneumococcal infection is 30% -60% and in Asia or Africa it is up to 98%. Carriage rate of different serotypes in the same population changes during the time.

S.pneumoniae is a common bacterial agent that causes a wide variety of infections including mucosal infections (e.g. sinusitis and otitis media), pneumonia, arthritis, pericarditis, peritonitis and severe invasive infections such as meningitis and septicemia. Morbidity and mortality due to pneumococcal infections is high, especially in developing countries. The incidence of invasive pneumococcal diseases (IPD) in young children residing in the United States and Europe is 8-75 cases/100000 population/years, whereas the incidence in young children residing in developing countries is several times higher, 100 to >500 cases/100000 population/year. Different serotypes have different ability to cause serious invasive pneumococcal diseases. Only some serotypes cause serious invasive diseases. According to a number of studies there is a significant inverse correlation between invasive disease and carriage prevalence for considered serotypes, which implies that the most invasive serotypes and serogroups are

rarely carried, and that the most frequently carried serotypes and serogroups not allways cause invasive diseases.

Historically pneumococci have been susceptible in vitro to penicillins, cephalosporins, macrolides (including erythromycin, clarithromycin, azithromycin), clindamycin, rifampicin, vancomycin, and trimethoprim-sulfamethoxazole. In the early 1970s multiresistant *S.pneumoniae* strains as well as resistant to penicillin were detected. In the early 1990s, however, pneumococcal strains resistant to penicillin and other antimicrobial agents emerged throughout the world. Resistance rates to penicillin and macrolides in various countries differ from 60% to 5%.

The continuing morbidity and mortality of pneumococcal infections in the antibiotic era led to the development and licensure of a polyvalent polysaccharide vaccine in the late 1970s. The currently available pneumococcal polysaccharide vaccine, 23PS, is composed of purified capsular polysaccharide antigens of 23 serotypes that represent up to 90% of the serotypes causing invasive pneumococcal infections. But polysaccharide vaccines elicit type-specific antibody responses in most healthy adults and children of 5 years and older, the serologic response to the polysaccharide antigens is generally poor in children younger than 2 years of age. New conjugate vaccines are immunogenic for infants as young as 2 month of age, including B-lymphocyte memory cells resulting in an anamnestic response with subsequent doses, and reduce carriage of vaccine serotypes. A 7-valent type of pneumococcal conjugate vaccine was approved for infant immunization since late 1990s. Great changes in epidemiology of IPD were detected in countries where universal infant immunization started a few years ago. In Lithuania there is no universal vaccination with any of pneumococcal vaccines.

S.pneumoniae is able to stimulate immune responses. In addition to 'innate immunity', the specific, antibody-mediated defence takes action already at the mucosal surfaces. Relatively few data exist on immune responses to the pneumococcus after natural exposure. It was demonstrated previously that contacts with *Streptococcus pneumoniae* induced natural salivary IgA responses against protein and polycaccharide antigens in children and adults. IgA is the predominant and relatively important in host defence immunoglobulin isotype in all mucosal secretions. In addition to IgA, pentameric IgM is likewise actively enriched in most exocrine fluids and is associated with the secretory component. IgG in mucosal secretions has traditionally been regarded

as originating from serum by diffusion. In addition, IgG can be produced locally. This suggests that three isotypes: IgA, IgM and IgG are important in mucosal immunity.

2 THE AIM OF THE STUDY

To evaluate the peculiarities of *S.pneumoniae* nasopharyngeal carriage in children day care

centers attendants in Vilnius city.

3 OBJECTIVES OF THE STUDY

- To determinate the rate of *S.pneumoniae* carriage in nasopharyngx of healthy children day care centers attendants in Vilnius city.
- To determinate the rate of *S.pneumoniae* carriage in nasopharynx of children day care centers attendants in Vilnius city which had frequent respiratory tract infections and were treated with antibiotics.
- To determinate serotypes of detected *S.pneumoniae* strains.
- To determinate susceptibility of detected *S.pneumoniae* strains to antibiotics.
- To collect and to analyse data on previously prescribed antibiotics for *S.pneumoniae* nasopharyngeal carriage in investigated children.
- To evaluate the mucosal immunogenic salivary response to pneumococcal infection in investigated children.

4 MATERIALS AND METHODS OF THE STUDY

The research work was performed in Vilnius University Clinic of Children diseases in the period of 2005-2008.

The part of the research work with healthy children was done in cooperation with scientists from Reykjavik, Iceland. This research work was done during three stages: in 1999, 2001 and 2006. The author of this dissertation took part in preparing documents, taking and analyzing samples of nasopharyngeal swabs, and summarizing data of the last stage of the work. Also the author performed research work with frequently ill children.

Salivary samples were examined in cooperation with Institute of Immunology, Vilnius University.

Nasopharyngeal samples were taken and analysed for the *S.pneumoniae* carriage from the nasopharynx of 672 children.

Nasopharyngeal samples from healthy children were collected in 13 children day care centers. These centers were chosen to represent all parts of Vilnius city. Some days before study parents were asked to fill a special agreement form and data about their children health, previous antimicrobial treatment. Only children with parent's written permission were included into study.

The study was performed during cold year season – in February and March. The same medical staff took part in all three study stages.

During three study stages 1625 healthy children from Vilnius city were investigated. All children were 2-7 years old. The average age was 4,7 (4,7-5,1) years, similar during all study stages. About half of investigated children were boys (1 table).

Tabl 1. **Children age and gender.**

	Total	**Year of the study**		
		1999	**2001**	**2006**
Number of investigated children	1625	508	516	601
Average age (years)	4,7	4,2	4,7	5,1
Girls	738	256	185	297
Boys	887	252	331	304

601 nasopharyngeal samples were taken from healthy children in 2006. The average age was 5,1 years . According to the age children were divided into two groups: 2-4 years old and 5-7 years old (2 table).

Table 2. **Children groups according to age.**

Children groups	**Number of the children**	
	N	**%**
2-4 years old	266	43,6
5-7 years old	335	55,7

Nasopharyngeal samples from frequently ill children were taken in Vilnius University Children Hospital. Only 2-7 years old children who were treated with three and more antibiotic courses during last 6 month were enrolled into the study. 71 children of average age of 4 years were investigated in 2007-2008. Boys were enrolled in the study with prevalence (3 table).

Table 3. **Children groups according to gender and age**

Children groups	N	%	Average age (years)
Girls	30	42,3	3,6
Boys	41	57,7	4,26
All the children	71	100	4,02

All the nasopharyngeal swabs were obtained through a nostril with a flexible swab „Mini-tip culturette“ (Becton Dickinson, Germany) and transported to the bacteriological laboratory of Vilnius University Children Hospital to test for carriage of *Streptococcus pneumoniae*. The nasopharyngeal swabs were cultured on chocolate agar and selective blood agar with 5% CO_2 according to the methods certified by CLSI. Susceptibility to antibiotics was detected also according to CLSI requirement. *S.pneumoniae* strains from healthy children were examined for susceptibility to penicillin, erythromycin, clindamycin, cotrimoxazol, tetracyclin, chloramphenicol – according to Iceland-Lithuanian study protocol. *S.pneumoniae* strains from often ill children were examined for susceptibility to penicillin, azitromycin, ampicillin, cefuroxim, ceftriaxon, cotrimoxazol - according to CLSI standards to clinical practice.

Cell culture was used for identification by standard methods and to obtain pure culture for microtiter plate covering. Cell concentration was estimated by McFarland Turbidity Standards. Pneumococcal strains were serotyped/grouped by Pneumotest-Latex Antisera from Statens Serum Institut (Copenhagen, Denmark). Saliva samples were collected from every subject by cotton swab placed under the cheek for 5 minutes, then centrifuged at 3000 rpm to collect the saliva and stored at -70ºC before the analyses. Saliva samples were thawed only once. Prior to analyzing, samples were incubated at 56ºC for 15 min, centrifuged at 13 000 *g* for 15 min and the supernatant was used for the assay.

Measurement of pneumococcal antibodies in saliva. Microtiter plates (Polysorp; Nunc, Roskilde, Denmark) were coated with pure pneumococcal cell culture $2.4x10^8$ cell/ml PBS (50 µl/well) and incubated without cover overnight at 37ºC temperature in order to get dried. The

following pneumococcal serotypes were used: 3, 6B, 14, 18, 19B, 23. For measurements of antibodies specific to cell wall polysaccharides the plates were coated with 20 μg/ml (50 μl/well) of pneumococcal cell wall polysaccharide mixture (CWPS Multi) from Statens Serum Institut (Copenhagen, Denmark) under the same conditions. All plates were blocked with 2% BSA-PBS for 1 h at 37ºC and then emptied without washing. The following steps were similar for detection of both types of pneumococcal antibodies. Human serum 89-SF from the U.S. Food and Drug Administration was used as reference sera. Saliva samples were diluted at 1:10 in BSA-PBS. Samples were assayed at a single dilution in duplicate, reference sera at eight serial three-fold dilutions in duplicate. Samples were aliquoted (50 μl/well) and incubated for 1.5 h at 37ºC. Mouse monoclonal anti-human IgA, IgM and IgG biotin conjugated antibodies were produced and characterized earlier. Biotinilated antibodies were incubated for 1 hour at 37ºC, followed by streptavidin-horseradish peroxidase conjugate incubation for 40 min at 37ºC (50 μl/well). Bound conjugate was detected calorimetrically by using o-phenylenediamine/H_2O_2 substrate (100 μl/well). Color developed in 15 min (±5 min); the reaction was stopped by addition of 50 μl of 2M H_2SO_4 solution to all wells of the test. Between the steps plates were washed four times with PBS containing 0.1% Tween 20 (PBS-T) (Merck, UK). Absorbance was measured at 490 nm by microtiter plate reader ELx800 (BioTek, USA).

Measurement of total S-IgA, IgA IgM and IgG quantities in saliva. The pears of capture and detection monoclonal antibodies were chosen for sandwich ELISA for quantitative evaluation of appropriate immunoglobulin classes on the basis of developed test systems. Microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with 10 μg/ml of capture monoclonal antibodies overnight at 4ºC temperature. WHO International Standard Immunoglobulins G, A and M, Human Serum Code: 67/086 from NIBSC, UK and purified human secretory IgA (Cat: 55905) from MP Biomedicals, USA were used as reference materials. Saliva samples were diluted in BSA-PBS 1:100 for IgM, 1:400 for S-IgA, 1:2000 for IgG. Detection antibodies, streptavidin-horseradish peroxidase conjugate and substrate used were the same as for measurement of anti-pneumococcal antibodies.

*Data analysis.*The concentration of anti-pneumococcal IgA, IgM and IgG antibodies was calculated on the basis of OD value resulted by subtracting OD value of the saliva sample obtained on plate covered with CWPS Multi from that obtained on plate covered with pneumococcal culture. Concentrations were determined by using a four-parameter logistic-log curve fitting model ("Gen5" software) and expressed in nanograms per millilitre (ng/ml) of saliva. The lowest detected concentration was 0.2 ng/ml for IgA, IgM and IgG for all serotypes.

Considering the absence of reference material for quantitative measurement of antibodies to cell wall polysaccharide cut off value was established. The sample with OD value obtained on plate covered with CWPS Multi ≥ 2 SD of the blank was regarded as positive (cut off value).

The normality assumption was tested by Kolmogoroff-Smirnoff test. Non-parametric statistical methods were used, because of the non-normality of salivary antibody data. Antibody concentrations at two groups (age, sex, carriers-noncarriers) were compared using the Mann-Whitney Rank Sum test. Differences in antibody concentrations between serotype groups were analysed with the Kruskal-Wallis test. The significance of the correlation between the total and specific antibody concentrations was estimated by Spearman's correlation analysis. Differences were considered statistically significant when the p-value was <0.05.

5 RESULTS OF THE STUDY

5.1 Study of healthy children

5.1.1 Rate of *S.pneumoniae* nasopharyngeal carriage in healthy children

In the nasopharyngeal cultures from 601 healthy children 280 different microbial strains were determined.

Results were calculated according to children age. *S.pneumoniae* was found more often in 2-4 years old children if compared with 5-7 year old children. According non-parametric *Mann-Whitney Rank Sum* test it is statistically significant difference between these two age groups (p<0,001), but any statistically significant difference according to gender was not found in this children groups.

Children were attending 13 different children day care centers. Centers for the study were selected to represent all regions of Vilnius city. *S.pneumoniae* in the nasopharynx of children attending day care center “Daigelis” was found extremely rarely. It was the only day care center were children also stay at night time. The most often *S.pneumoniae* carriage was found in day care center “Delfinukas”. “Delfinukas” was the only day care center which had swimming pool (all the children attended swimming pool twice a week). Non-parametric *one-way ANOVA* method showed statistically significant difference in *S.pneumoniae* nasopharyngeal carriage between the children of these two day care centers (p<0,05).

5.1.2 Distribution of different *S.pneuomoniae* serotypes in nasopharynges of healthy children

During the study *S.pneumoniae* strains were serotyped. Two different *S.pneumoniae* serotypes were found in the nasopharynges of 24 children. Definite serotypes for 240 *S.pneumoniae* strains were detected. The rest 40 strains were specified only to serogroup. In all these cases 33 different serotypes/ serogroups were detected. The results of serotyping are summarized in the table 4.

Table 4. **The distribution of different *S.pneumoniae* serotypes among all the found strains.**

Serotype	Number of found strains	Percentage from all the founded strains
19F	37	13,2
23F	37	13,2
6B	22	7,8
6A	21	7,5
3	19	6,8
18C	15	5,4
37	12	4,3
10	10	3,6
14	9	3,2
9V	8	2,8
11	8	2,8
15	7	2,5
16	6	2,1
19A	5	1,8
23A	5	1,8
1	3	1,1
8	2	0,7
23	2	0,7
18B	2	0,7
17	2	0,7
4	1	0,4
9A	1	0,4
7	1	0,4
9N	1	0,4
18A	1	0,4

6	1	0,4
19	1	0,4
23B	1	0,4
Not specified	40	14,3

Six different serotypes (19F, 23F, 6B, 6A, 3and 18C) were found in 58% of all the strains. Other serotypes were found rarely.

5.1.3 Immunogenic response to *S.pneumoniae* infection

Total (S-IgA, IgM, IgG), specific to cell wall polysaccharide (IgA CWPS, IgM CWPS, IgG CWPS) and anti-pneumococcal (IgA Pn, IgM Pn, IgG Pn) antibodies were assessed in saliva samples of 129 non-carriers (pneumococcus-free) and in 107 carriers of *S.pneumoniae*. Anti-pneumococcal antibodies were measured by sandwich ELISA with the use of pure pneumococcal cell culture of 19F, 23F, 6B, 18C, 3, 14 serotypes. The data obtained was not normally distributed; the normality was not achieved by log-transformation in some data sets. Consequently nonparametric statistics was used to compare the distribution of concentration of different isotypes and specificity of antibodies to six most abundant serotypes. Distribution of three isotypes of total immunoglobulins and pneumococcal antibodies (IgA Pn, IgM Pn, IgG Pn) concentrations by six serotypes was statistically significant ($p<0.01$). Data of different distribution of immunoglobulin isotypes according to serotypes are presented in Table 5.

Table 5. **Median immunoglobulin concentrations and distribution of total and anti-pneumococcal antibodies by serotypes.**

Ig class	Median, µg/ml	Pneumococcal serotype						p-value*
		3	6B	14	18C	19F	23F	
S-IgA	134	62	60	20	78	70	4	0.000
IgM	2.3	54	53	40	56	57	13	0.022
IgG	6	62	63	60	33	61	13	0.002
IgA Pn	0.02	46	50	20	67	74	29	0.031
IgM Pn	0.003	23	77	60	11	91	4	0.000
IgG Pn	0.002	54	77	40	56	48	17	0.001

* Kruskal-Wallis test

The numerals in the table show percent of children whose antibody concentration of separate isotype exceeded median. Data was expressed in percentage because of high variation of count of serotype representatives. The highest percentage of children with total immunoglobulin production exceeding median was related to serotypes 19F and 18C, the lowest one to serotype 23F. Similar results were obtained for anti-pneumococcal IgA Pn. In the case of anti-pneumococcal IgM Pn 91% of children possessed enhanced concentration of antibodies to serotype 19F.

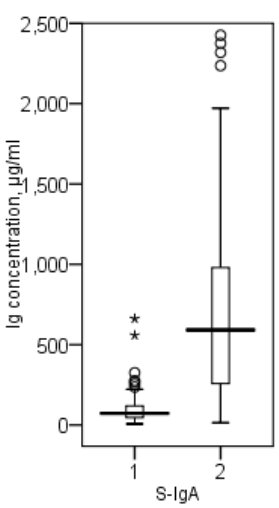
Nonparametric statistics was also used to compare the distribution of different antibody concentration by age. Distribution of total S-IgA between the two age groups (age of 2-4 years and 4-7 years) was statistically significant ($p<0.01$). The amount of S-IgA (Mean $rank_{2\text{-}4\ m.}$ 46, Mean $rank_{4\text{-}7\ m.}$ 75) was higher in older children.

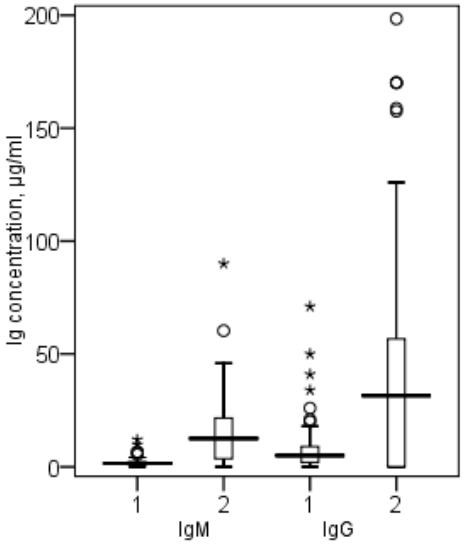
Sandwich ELISA with double monoclonal system was used for immunoglobulin concentration measurement. Corresponding pairs of monoclonal antibodies demonstrated strict specificity to human S-IgA, IgM, IgG and did not show any cross reactions with other immunoglobulin isotypes.

Statistical data of separate immunoglobulin isotype concentrations in saliva samples of healthy preschool age children are presented in table 5 (the exceptional cases with supposed IgA defficiency were excluded). Three children (n=236) were identified as exceptional with zero level of both total and specific imunoglobulins of S-IgA and IgA isotypes. The concentration of total IgM was 3.3, 25, 18 fold higher in comparison with the group median for three cases. The concentration of total IgG was 3, 6.6, 13.2 fold higher respectively. The concentrations of specific antibody of IgM and IgG isotypes were also higher than median. The parents of these children did not declare any complaints during the last 6 months.

Nonparametric Mann-Whitney test revealed statistically significant difference in distribution of total S-IgA, IgM ($p<0.001$) and IgG ($p=0.005$) concentrations between pneumococcal carriers and non-carriers group: the carriers had significantly higher immunoglobulin concentrations in saliva in comparison to pneumococcus-free children (figure1 and figure 2).

Figure 1 and figure 2. Distribution of sIgA, IgM and IgG among *S.pneumoniae* carries and non-carries. 1- *S.pneumoniae* carries. 2- *S.pneumoniae* non-carries.





Sandwich ELISA with the same biotinilated monoclonals as for total Ig detection was used to assess concentration of specific antibody of three isotypes. Plates were covered by pure pneumococcal culture of serotypes 3, 6B, 14, 18C, 19F and 23F. Thus, antibodies detected were polyspecific to structural components of the bacteria. Concentration of anti-pneumococcal IgA Pn, IgM Pn, IgG Pn (table 5) was calculated from the OD of sample after subtraction of OD obtained in saliva reaction with cell wall polysaccharides. Specific IgA Pn antibodies were not detected in saliva of only 1 child, IgM Pn antibodies were not found in 34 children and IgG Pn – in 30 children. It implies that 99% of pneumococcal carriers had specific of IgA Pn class antibodies, 68% of IgM Pn and 70% of IgG Pn. The most abundant class of specific anti-pneumococcal antibodies was IgA Pn (median 40 ng/ml).

Nonparametric Spearman's rank correlation analysis indicated a moderate positive correlation existing between the total and specific immunoglobulins of appropriate isotypes in carriers group (n=105): correlation coefficients for three isotypes of total and anti-pneumococcal immunoglobulins were 0.60±0.02 (p=0.01). The weakest significant correlation was assessed for IgG - IgG CWPS pair (r=0.27, p=0.05). These correlations showed that a rising concentration of total immunoglobulin matched moderately to rising concentration of specific one.

The lack of reference material prevented quantitative measurements of the level of specific antibodies to CWPS. Cut off value established allowed to classify children to CWPS-positive (OD≥cut off) and CWPS-negative (OD<cut off). The level of antibodies to CWPS was

expressed by the percent of CWPS-positive children. Full amount anti-CWPS antibody (three isotypes as one) posessed 45% of children from non-carriers group and 61% from carriers group.

5.1.4 The influence of consumed antibiotics to *S.pneumoniae* nasopharyngeal carriage

Data collected on antibiotics consumption were analysed. Children's parents were asked to indicate antibiotics used by their children during 6 month before the study. On the last month before the study 136 children were taking antibiotics (22% of all the children who participated in the study). Parent of 86 children only new the name of antibiotic. Nine children were treated with two different antibiotics (table 6).

Table 6. **Rate of antibiotics consumption of 86 children during the last month before the study.**

Antibiotic	**Number of treated children**	**Percentage off all the treated children**
Amoxicillin	34	35,8
Amoxicillin/ac. clavulanicum	15	15,8
Ampicillin/sulbaktam	6	6,3
Azithromicin	14	14,7
Clarithromycin	17	17,9
Cefadroxil	8	8,4
Cefuroxim	1	1,1
Total	95	100

The children mostly were treated with antibiotics from penicillin group - 58%. Less frequently children were treated with macrolides of new generation: azithromicin (14,7%) and clarithromycin (18%). *S.pneumoniae* nasopharyngeal carriage rate among these 86 children was 27%.

All the study participants children were divided into four groups according to the period when antibiotics were used. One group of the children did not used antibiotics during all 6 month before the study. They were called “not users”. Second group used antibiotics only during the last month before the study. They were called “used the last month”. Third group of the children used three courses and more of antibiotics but the last time they took antibiotics was one month before the study. They were called “used 1-6 months”. And the fourth group of the children used antibiotics during the last month

before the sturdy and during the period of previously 5 months before the study. These children were called “used all 6 months”. According one way ANOVA method, significant difference was found between “not users” and “used the last month” (table 7).

Table 7. **Comparison of different children groups according to antibiotics consumption (the higher rank disparity and higher Q shows the higher difference between the two groups).**

Comparison of different children's groups according antibiotics consumption *	**Rank's disparity**	**Q**	**P**
not users *vs* used the last month	69,606	3,427	P<0,05
not users *vs* used all 6 months	51,301	1,961	P>0,05
not users *vs* used 1-6 months	8,841	0,422	P>0,05
used 1-6 months *vs* used the last month.	60,765	2,3	P>0,05
used 1-6 months *vs* used all 6 month	42,461	1,364	P>0,05
used all 6 months *vs* used the last month	18,304	0,596	P>0,05

*****Higher rate of *S.pneumoniae* nasopharyngeal carriage *vs* lower rate of *S.pneumoniae* nasopharyngeal carriage.

Additionally we analysed the influence of antibiotic consumption to nasopharyngeal carriage of different *S.pneumoniae* serotypes. The results of the analysis are displayed in table 8.

Table 8. **The influence of antibiotic consumption to nasopharyngeal carriage of different *S.pneumoniae* serotypes.**

***S.pneumoniae* serotypes**	**Children groups according to antibiotic consumption**			
	Not users	**Used the last month**	**Used 1-6 month**	**Used all 6 month**
23F	27	0	6	1
19F	22	4	5	4
6A	14	0	4	0
6B	15	2	1	1
3	10	3	1	1
10	8	0	0	0
14	3	2	2	1
37	8	0	0	0
9V	8	0	0	0
18C	8	2	3	1
Other	34	6	7	3

Invasive *S.pneumoniae* serotypes (23F, 19F, 6A, 6B, 18C) were found after frequent antibiotic usage.

5.1.5 *S.pneumoniae* susceptibility to antibiotics

The susceptibility to antibiotics was detected for all *S.pneumoniae* strains.

Susceptibility to macrolides group was detected according to susceptibility to erythromycin (?) 9% out of all *S.pneumoniae* strains were resistant to erythromycin. Only a few serotypes – 23F, 6B (table 9) were mostly resistant to antibiotics.

Table 9. **Susceptibility to erythromycin data of most often found *S.pneumoniae* serotypes.**

Susceptibility	**Absolute strains number**	**Diameter, average, mm**	**Standard Deviation**	**Standard Bias**	**Max**	**Min**	**Mediana**	**25% quantilium**	**75 % quantiliu**
23F									
Resistant	9	13,89	1,05	0,35	15	12	14	13	15
Middle resistancy	7	16,29	0,49	0,18	17	16	16	16	16,75
Susceptible	20	29,60	2,33	0,52	34	25	29	28,5	31
19F									
Susceptible	37	30,43	2,12	0,35	35	26	30	29	31
6A									
Susceptible	19	30,21	2,04	0,47	34	27	30	29	32
6B									
Resistant	3	11	1	0,58	12	10	11	10,25	11,75
Susceptible	17	30	1,37	0,33	33	27	30	29,75	30,25
„G“ serogroup									
Susceptible	10	29,30	1,64	0,52	32	26	30	28	30
10									
Susceptible	8	30,88	1,81	0,64	33	29	30	29,5	33
14									
Susceptible	8	31,13	1,13	0,40	33	30	31	30	32
37									

Susceptible	8	35,63	1,92	0,68	38	33	35,5	34	37,5
"little" serogroup									
Resistant	5	14	1	0,447	15	13	14	13	15
Middle resistancy	1	17	--	--	17	17	17	17	17
Susceptible	10	34,3	2,751	0,87	38	29	35	32	35
3									
Susceptible	16	32,56	2,71	0,68	38	28	33	30	34,5
9V									
Susceptible	8	30,13	2,59	0,92	34	27	29,5	28,5	32
18C									
Susceptible	14	31,36	1,22	0,33	34	30	31	30	32

The period of antibiotic consumption influenced on susceptibility to erythromycin. Non parametric one-way ANOVA method was detected that mostly resistant *S.pneumoniae* strains were found in the nasopharynx of the children who used antibiotics during the last month before the study (table 10).

Table 10. ***S.pneumoniae*** **susceptibility of to erythromycin depends on antibiotic consumption.**

Comparison of different children groups according to antibiotics consumption*	**Rank's disparity**	**Q**	**P**
not users *vs* used the last month	70,089	3,451	P<0,05
not users *vs* used all 6 months	53,11	2,03	P>0,05
not users *vs* used 1-6 months	11,343	0,542	P>0,05
used 1-6 months *vs* used the last month.	58,745	2,224	P>0,05
used 1-6 months *vs* used all 6 months	41,767	1,341	P>0,05
used all 6 months *vs* used the last month	16,978	0,553	P>0,05

* Susceptible to erythromycins *vs* resistant to erythromycin

9% out of all *S.pneumoniae* were strains resistant to penicillin. Analysis of different serotypes was performed. Results are shown in table 11.

Table 11. **Susceptibility to penicillin data of most frequently found *S.pneumoniae* serotypes.**

Susceptibility	**Absolute strains number**	**Diameter, average, mm**	**Standard Deviation.**	**Standard Bias**	**Max**	**Min**	**Mediana**	**25% quantilium**	**75% quantilium**
23F									
Resistant	2	6	0	0	6	6	6	6	6
Susceptible	34	25,265	1,928	0,331	31	21	25	24	26
19F									
Susceptible	37	26,703	1,956	0,322	30	22	27	25	28
6A									
Resistant	2	8	0	0	8	8	8	8	8
Susceptible	17	28,941	2,727	0,661	33	25	29	26,75	30,25
6B									
Resistant	3	6	0	0	6	6	6	6	6
Susceptible	17	27,294	1,929	0,468	32	24	27	26	28
G serogroup									
Susceptible	10	26,3	1,418	0,448	28	25	26	25	28
10									
Susceptible	8	26,5	2,39	0,845	32	24	26	25,5	26,5
14									
Susceptible	8	26,875	2,748	0,972	30	24	26	24,5	30
37									
Susceptible	8	29,125	2,9	1,025	33	23	29,5	28,5	30,5
„Little " serogroup									
Resistant	13	9,462	1,941	0,538	12	6	10	8	10,25
Susceptible	3	23,667	1,155	0,667	25	23	23	23	24,5

3									
Susceptible	16	27,625	1,857	0,464	30	25	28	26	29,5
9V									
Resistant	3	6	0	0	6	6	6	6	6
Susceptible	5	27	2,55	1,14	29	23	28	25,25	29
18C									
Susceptible	14	25,786	3,62	0,967	30	20	27	23	29

According to the results, invasive serotypes 23F, 9V, 6A more often were resistant to penicillin. *S.pneumoniae* strains found in the nasopharynx of children who used antibiotics during the last month before the study most frequently were resistant.

During the study *S.pneumoniae* susceptibility to chloramphenicolum, clindamycinum, cotimoxazolum, tetracyclinum was detected. Results are shown in table 12.

Table 12. ***S.pneumoniae*** **susceptibility to antibiotics (percentage)**

Susceptibility	**Chloramphenicolum**	**Clindamycinum**	**Cotrimoxazolum**	**Tetracyclinum**
Resistant	17,83	0,78	35,27	26,36
Moderately susceptible	-	0,78	14,73	1,94
Susceptible	82,17	98,45	50,00	71,71

5.2 *S.pneumoniae* nasopharyngeal carriage studies during three different study years

The studies of *S.pneumoniae* nasopharyngeal carriage in Vilnius city were performed three times: in 1999, 2001 and 2006 . All three studies were performed during the same year period – in February and March. Children of the same day care centers took part in all three studies.

Totally 1625 children were examined. On average *S.pneumoniae* strains were found in the nasopharynx of every second child. The results are shown in table13.

Table 13. ***S.pneumoniae*** **nasopharyngeal carriage rates during the three study periods in Vilnius city.**

Study year	**1999**	**2001**	**2006**
Number of examined children	508	516	601
S.pneumoniae nasopharyngeal carriage (percentage)	51	55	43

Results of nasopharyngeal carriage of 6 most frequently found serotypes were compared (table 14).

Table 14. **Nasopharyngeal carriage of 6 most frequently found *S.pneumoniae* serotypes during the first and third study periods.**

Serotype	1999 year		2006 year	
	Absolute number	Percentage value from all found serotypes	Absolute number	Percentage value from all found serotypes
3	36	13	19	6
6B	31	11	20	8
23F	25	9	45	14
15	25	9	6	3
19F	22	8	34	14
6A	22	8	20	7
other	117	42	133	48

Susceptibility to antibiotics was compared. Susceptibility to penicillin and erythromycin changed during the study years. The results are shown in table 15.

Table 15. ***S.pneumoniae*** **resistance to antibiotics (percentage)**

Antibiotics	Study year		
	1999	2001	2006
Penicillin	6%	10%	9%
Erithromycin	4,7%	6%	9%
Tetracyclinum	27%	37%	28%
Chloramphenicolum	7%	19%	18%
Cotrimoxazolum	60%	52%	50%

The consumption of antibiotics during the last 6 months before the study may explain the results of *S.pneumoniae* susceptibility to antibiotics. In 1999 year the erythromycin and amoxicillin for treatment were prescribed most frequently. New generation of macrolides were not prescribed for treatment at that time. In 2006 amoxicillin and new generation of macrolides were mostly used for the treatment of children (table 16).

Table 16. **The most frequently used antibiotics for the treatment of respiratory infections in the children study participants (from most frequently used to rarely used).**

1999 year	**2001 year**	**2006 year**
Erythromycin	Clarithromycin	Amoxicillin
Amoxicillin	Amoxicillin/ac. clav.	Amoxicillin/ac. clav.
Amoxicillin/ac.clav.	Erythromycin	Clarithromycin
Cothrimoxazol	Amoxicillin	Azithromicin
Penicillin	Cephalosporins	Cephalosporins
Cephalosporins	Azithromycin	Ampicillin/sulbakct.

5.3 *S.pneumoniae* nasopharyngeal carriage in the nasopharynges of frequently ill children

In the study 71 children frequently ill with respiratory tract infections were examined. Study children were detected according special questionnaire. Children who were treated with three or more antibiotics during the last 6 months before the study were examined. *S.pneumoniae* was found in the nasopharynx of 22 children (31%).

The significant statistical difference according to gender was not found. More frequently *S.pneumoniae* was found in the nasopharynx of younger children. In 3 years and younger children *S.pneumoniae* nasopharyngeal carriage was 45%. Nasopharyngeal carriage in 4 years old and older children was 24%.

19 families had more than one child of preschool age. *S.pneumoniae* nasopharyngeal carriage among children from these families was 32%. *S.pneumoniae* nasopharyngeal carriage among 46 children who had no preschool age siblings was 28%. There is no significant statistical difference between *S.pneumoniae* nasopharyngeal carriage in these two children groups, only the tendency for higher *S.pneumoniae* nasopharyngeal carriage among children who have preschool age siblings was observed.

Data on consumption of antibiotics during the last 6 month before the study were analyzed. The results are shown in table 17.

Table 17. **Consumption of antibiotics by study children in the last 6 months before the study**

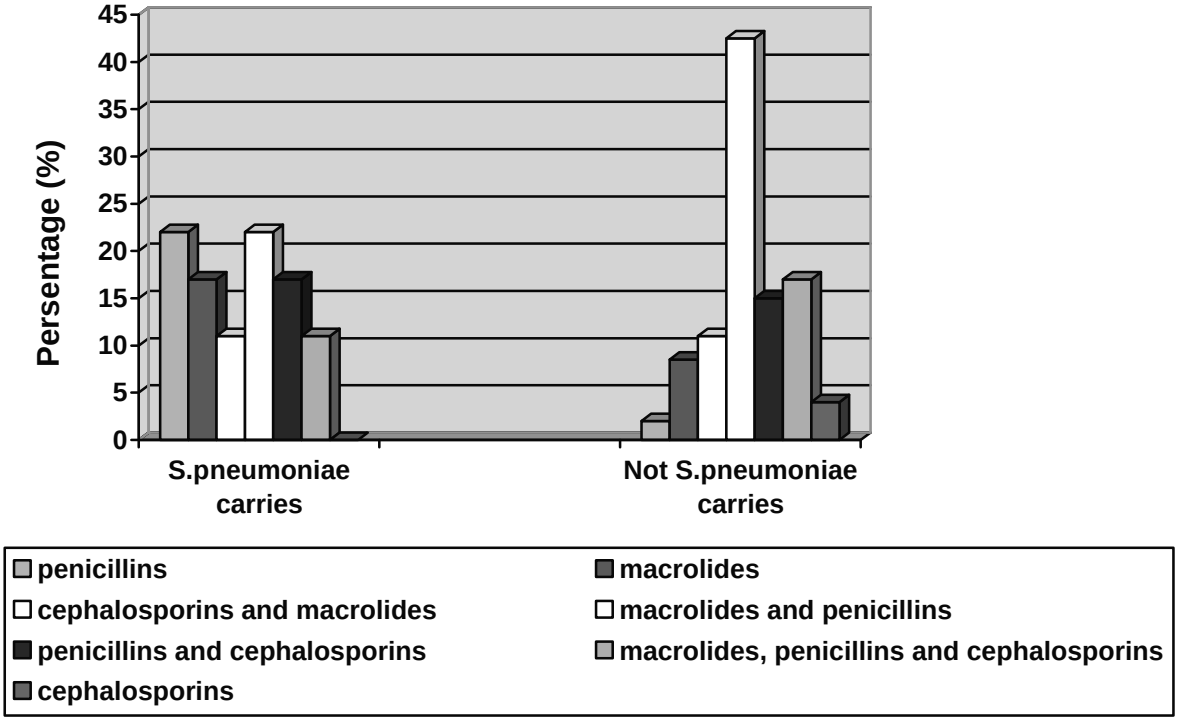
Antibiotics	**Number of children**	
	Antibiotics, used during the last month before study	**Antibiotics, used during the 1-6 months before study**
Amoxicillin	3	26
Amoxicillin/ac. clav.	8	21
Penicillin	1	6
Ampicillin/sulbact.	2	7
Cefuroxim	11	15
Clarithromycin	11	41

Azitromicin	5	26
Cefadroxil	–	10
Erythromycin	–	1

According to our data, 39 children were treated with one or more antibiotics during the last month before the study. *S.pneumoniae* nasopharyngeal carriage among these children was 18%. And *S.pneumoniae* nasopharyngeal carriage was 46,4% among 28 children who received antibacterial treatment 3 and more times during 6 month before the study but the last time they were treated more than a month ago. So, after antibacterial treatment *S.pneumoniae* nasopharyngeal carriage is lower only for very short period – about one month.

The influence of different antibacterial medicine groups for *S.pneumoniae* nasopharyngeal carriage was analyzed. The results are shown in diagram 1.

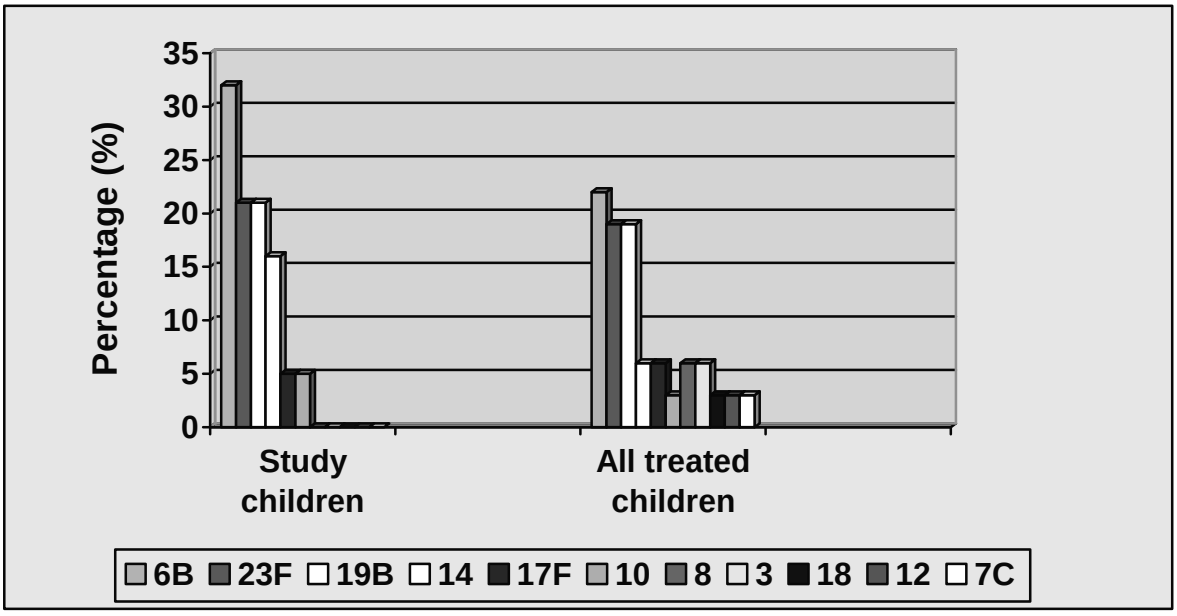
Diagram 1. **Antibiotic consumption among children *S.pneumoniae* nasopharyngeal carriers and not carriers (percentage).**



S.pneumoniae susceptibility to antibiotics was detected. Resistant to penicillin were found 2 (10%) of all *S.pneumoniae* strains. And 4 (18%) *S.penumoniae* strains were resistant to macrolides.

S.pneumoniae serotyping was performed. Serotypes 6B, 23F, 19B, 14 were predominant over other serotypes. Serotyping results are shown in diagram 2. In the diagram the results of *S.pneumoniae* serotyping of our study are compared with results of all found S.pneumoniae serotypes from ill 2-7 years old children in the Vilnius university children hospital during the same period.

Diagram 2. ***S.pneumoniae*** **serotypes from the study children in comparison with all found** ***S.pneumoniae*** **serotypes in Vilnius University Children hospital during the same period (percentage).**



6 CONCLUSIONS

- Carriage rate of *S.pneumoniae* in the nasopharynx of healthy children is 43%.
- Carriage rate of *S.pneumoniae* in the nasopharynx of frequently ill children is 31%.
- In both groups invasive *S.pneumoniae* serotypes are predominant.
- Resistance to penicillin is constant 9%. Resistance to macrolides is rising up – from 4% to 18 %. In can be associated with increasing consumption of new generation macrolides.
- Repeated use of antibiotics does not protect from nasopgaryngeal carriage of *S.pneumoniae.*
- The mucosal immune response depends on *S.pneumoniae* serotype and children age.

7 PRACTICAL RECOMMENDATIONS

- *S.pneumoniae* infection is frequent among preschool children in Vilnius city. The first choice antibiotic treatment for suspected pneumococcal infection in Vilnius city must be penicillin group antibiotics.
- Frequent antibiotic consumption children must be avoided as it stimulates the origin of resistant *S.pneumoniae* strains and does not protect against nasopharyngeal carriage of them.
- Pneumococcal vaccines along with strict antibiotic policy is basic for prophylaxis of pneumococcal infection.

8 LIST OF THE AUTHOR'S PUBLICATIONS

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- Petraitiene S, Bernatoniene G, Murauskaite G, Usonis V. *S.pneumoniae* paplitimas dažnai kvėpavimo organų ligomis sergančių ikimokyklinio amžiaus vaikų nosiaryklėje. Visuomenės sveikata2008;4(43):26-30.
- Petraitiene S, Bernatoniene G, Murauskaite G, Erlendsdottir H, Bernatoniene J, Usonis V. *S.pneumoniae* nešiojimas 2-7metų Vilniaus vaikų nosiaryklėje. Vaikų pulmonologija ir alergologija2009;XII(1):4119-30.

9 SUMMARY IN LITHUANIAN

S.pneumoniae yra vienas dažniausiai sutinkamų bakterinių patogenų, sukeliančių ligas mažiems vaikams. *S.pneumoniae*, ypatingai atskirų jo serotipų, paplitimas, jautrumas antibakteriniams preparatams yra skirtingas įvairiose šalyse ir nuolat kinta. Pagrindinis *S.pneumoniae* infekcijos šaltinis – sveiki ikimokyklinio amžiaus vaikai, nešiojantys pneumokoką nosiaryklėje. Šio tyrimo tikslas – išanalizuoti *S.pneumoniae* nešiojimo nosiaryklėje dažnumą tarp sveikų ir dažnai sergančių kvėpavimo takų ligomis vaikų Vilniuje, nustatyti vyraujančius *S.pneumoniae* serotipus ir jų jautrumą antibakteriniams preparatams. Taip pat nustatyti dažno antibakterinių preparatų vartojimo įtaką pneumokokų nešiojimui nosiaryklėje, atskirų serotipų paplitimui, jautrumui antibakteriniams preparatams. Įvertinti gleivinių imuninį atsaką į pneumokokinę infekciją, pagal seilėse esančius imunoglobulinus.

Išvados:

1. Nustatytas dažnas - apie 40% *S.pneumoniae* nešiojimas tirtų vaikų nosiaryklėse, vyrauja invazines ligas sukeliantys *S.pneumoniae* serotipai.
2. Jautrumas penicilino grupės antibakteriniams preparatams išlieka nekintantis ir sudaro 90% visų tirtų *S.pneumoniae* padermių. Jautrumas makrolidų grupės antibakteriniams preparatams palaipsniui mažėja, nuo 96% iki 82 %; tai susiję su dažnėjančiu naujos kartos makrolidų vartojimu.
3. Daugkartinis antibakterinių preparatų vartojimas neapsaugo nuo *S.pneumoniae* nešiojimo nosiaryklėje.
4. Vaikų gleivinių imuninis atsakas skirtingas atskiriems *S.pneumoniae* serotipams, priklauso nuo vaiko amžiaus.

Praktinės rekomendacijos. *Streptococcus pneumoniae* infekcija yra dažna Vilnius ikimokyklinio amžiaus vaikų tarpe. Pirmo pasirinkimo antibakteriniai preparatai gydant pneumokokinę infekciją, turėtų būti penicilino grupės antibiotikai. Dažnas antibakterinių preparatų vartojimas nesumažina *S.pneumoniae* nešiojimą vaikų nosiaryklėje, sąlygoja atsparių antibiotikams padermių atsiradimą, todėl reikia vengti nereikalingo antibakterinių preparatų vartojimo.

10 ABBREVIATIONS

S.pneumoniae (Streptococcus pneumoniae) – pneumococcus

IPL – invasive pneumococcal diseases

Ig – immunoglobulin

IgA – immunoglobulin A

IgG – immunoglobulin G

IgM – immunoglobulin M

S-IgA – secretory immunoglobulin A

CLSI - Clinical and Laboratory Standarts Institute

CWPS - Cell Wall Polysacharide

WHO – World Health Organisation

11 CURRICULUM VITAE

Name:	Sigita
Surname:	Petraitiene
Address:	Nemenčinės pl. 10-32, Vilnius
E-mail:	siga.petraitiene@gmail.com
Date of birth:	April 10, 1972
Birthplace:	Vilnius, Lithuania

Current Position: Doctor of Pediatrics,
Children pulmonologist

PhD student in Vilnius University, Faculty of Medicine,
Clinic of Children Diseases

Medical education:

1992 - 1998	Medical studies in Vilnius University, Faculty of Medicine
1998 - 1999	Primary medical residency in Vilnius University, Faculty of Medicine
1999 - 2002	Residency in pediatrics in Vilnius University, Faculty of Medicine, Clinic of Children Diseases
2002 – 2004	Residency in pediatric pulmonology in Vilnius University, Faculty of Medicine, Clinic of Children Diseases
2005 - 2009	PhD studies in Vilnius University, Faculty of Medicine, Clinic of Children Diseases

Positions in professional societies:

Member of the Lithuanian Society of Pediatrics
Member of Lithuanian pulmonologist Society
Member of European Respiratory Society
Member of European Society for Paediatric Infectious Diseases