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Official title of the study: Pneumococcal nasopharyngeal and oropharyngeal carriage in adults older than 50 years of age in outpatient health care facility in Novi Sad

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STUDY PROTOCOL

Objectives and hypothesis

Primary objective:

- To analyze overall serotype distribution of *Streptococcus pneumoniae* nasopharyngeal and oropharyngeal carriage among adults older than 50 years of age in the city of Novi Sad

Secondary objectives:

- To determine serotype on NP and OP carriage with pneumococcal serotypes among adults older than 50 years of age in the city of Novi Sad according to specific clinical indications set for the PPV or PCV

Clinical hypotheses.

- *Streptococcus pneumoniae nasopharyngeal and oropharyngeal carriage among adults older than 50 years of age in the city of Novi Sad is most probably present in less than 10% of the total population.*
- *Streptococcus pneumoniae serotypes covered by PPV or PCV are represented by more than 60% and 50% of the serotypes in overall distribution among tested subjects.*

Study design

Research center(s):

The research will be carried out by the:

1. Institute of Public Health of Vojvodina
   - Centre for Disease Control and Prevention and the Centre for Microbiology
2. Health Care Centre of Novi Sad
   - Department of General Medicine.

Patients

A prospective study will be conducted. We will sample the population based on voluntary participation. The target group of the research will be all adults who will visit their elected physicians in the Health Care Centre of Novi Sad (outpatient care facility). Only adults older than 50 years of age from Novi Sad with 341,624 (2011 Census) inhabitants will be recruited in this research.
All patients older than 50 years of age will be interviewed by physicians at the admission. Nasopharyngeal and oropharyngeal swabs will be obtained and transported to the Institute of Public Health for the laboratory analyses. This research represents a baseline measurement of NP carriage of pneumococci in adults older than 50 years of age. It will contribute to the strengthening of the surveillance and allow us to compare the situation on possible serotype replacement once after PPV or PCV are introduced into this age group and higher immunization coverage among high risk groups is achieved.

Inclusion criteria

Adults older than 50 years of age visiting the Health Care Centre with or without signs of upper respiratory tract infection during the study period.

Exclusion criteria

Living outside of Novi Sad, use of oral antibiotics in the two weeks prior to taking the swab and presence of a confirmed immunodeficiency (hematological malignancies, inherited immunodeficiency, HIV, post-splenectomy status, cancer chemotherapy).

Ethics approval

The study protocol will be approved by the Ethics Committee of the Medical faculty of Novi Sad.

Sample processing
To confirm the NP and OP carriage, sampling will include NP and OP swabs. Swabs will be collected by the general practitioners in the Health Care Centre of Novi Sad and will transport, within 24h of collection, to the Centre for Microbiology of Institute of Public Health of Vojvodina, Novi Sad. The sterile cotton-tipped wire swabs will be inserted into the anterior nares, gently rubbed on the NP swab wall and immediately placed in transport medium (Copan Venturi Transystem, Brescia, Italy).

Laboratory procedure
Within the laboratory NP swabs will be analyzed by 200 µl of swab-inoculated STGG media will be transferred to 5.0 ml Todd Hewitt broth containing 0,5% yeast extract (THY) and 1 ml of rabbit serum and incubated at 35-37 °C for six hours. Cultured broth will be plated on sheep blood agar and incubated in 5% CO_2 at 35-37 °C. After 18-24 hours of incubation, plates will be examined for the appearance of alpha-haemolytic colonies resembling streptococci. Positive samples will be cultured, and optochin disc and bile solubility test will be performed.

Molecular serotyping

DNA Extraction
To obtain DNA extracts for PCR reactions, an overnight growth of blood agar plate will be suspended in in 300 µl of 0.85% NaCl, heated to 70 °C for 15 min, spinned for 2 min and supernatant will be removed. Pellet will be suspended in 50 µl TE buffer with an addition of 10 µl mutanolysin and 8 µl of hyaluronidase, kept at 37°C for 30 min (to overnight), heated for 10 min at 100°C and spinned for 4 min. No more than 2,5 µl of supernatant will be used as DNA template. Extracts will be stored at -20°C until PCR testing (9).
Identification of S. pneumoniae
Identification of *S. pneumoniae* will be achieved by amplifying the *lytA* gene using primers and probes recommended by CDC and Quanta Biosciences PerfeCTa1 qPCR ToughMix1, Low RoxTM (Quanta Biosciences, Beverly, USA) (10). LytA positive samples will be further analyzed for serotype identification.
Serotype identification
Conventional multiplex PCR assays will be performed as a series of multiplex reactions, using CDC recommended schemes and primers for pneumococcal serotype deduction and 2X PCR Buffer - QIAGEN Multiplex PCR Kit (Qiagen, Hilden, Germany). The PCR products will be analyzed on 2% NuSieve agarose gels (Cambrex Bio Science, Inc., Rockland, ME), stained with ethidium bromide. Gel images will be recorded BioDocAnalyze system (Analytik Jena, Jena, Germany).
Real Time PCR will be performed optionally in case of any difficulties with conventional PCR. IT will be performed, as a series of 21 monoplex assays encompassing 21 serotypes using CDC recommended schemes based on geographic prevalence of serotypes. The assays are multiplexed in a sequential triplex format (three targets/serotypes in one reaction), each detecting targets on FAM, Rox (or Cy5) and Hex channels. Reactions will be performed on ABI 7500 Instruments (Thermo Fisher Scientific, Waltham, USA), using specific primers and probes and Invitrogen-Platinum Quantitive PCR SuperMix (Thermo Fisher Scientific, Waltham, USA).
Susceptibility testing
Susceptibility of Spn isolates to Optochin, Oxacilin, Norfloxacin, Erytromycin, Clindamycin, Tetracycline and Trimetroprim-sulfometoxazole will be determined by the Diffusion method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST, www.eucast.org).
This baseline assessment is taking into consideration to present descriptive only current results, but they are not separately taken into account for comparative analysis.
The results of laboratory testing, conducted for each patient, will forwarded to elected physicians who indicated sampling of patient material.

Study procedure
Adults will be recruited during their health examination at Health Care Centre of Novi Sad by their elected physician.
After providing a verbal and written explanation of the research aim (Appendix 1), informed consent (Appendix 2) will be obtained from subjects before enrolment. Personal and confidential information obtained from participants will be removed, except for demographic information, including date of sampling,

age, gender, data on previous upper respiratory tract infection, number of children (siblings) aged 0–10 years residing in the household of participants with pneumococcal and influenza immunization history (because influenza immunization may prevent pneumococcal superinfections), smoking habits of participants and their household members and the information about home residence (Survey Questionnaire- Appendix 3).
Samples will be provided as one NP swab per study subject. Every participant will be assigned only once. Confirmed case is every laboratory tested participant in which, after testing (PCR or ELISA), a positive result is obtained.

Statistical Analysis and Sample Size Justification

Collection of the data
Characteristics of all respondents acquired by questionnaire from Novi Sad, the test results of laboratory testing of samples of participants and the final characterization of Spn serotypes will be entered in specially designed database. Personal information of participants will be removed.
Investigator of the research along with statistician will analyze the collected data.

Sample size justification
In accordance with the sample size calculation for a study estimating a population prevalence (11), between 350 and 500 samples are planned to be collected. If more than 500 will be eligible they will all be included.We presume to detect pneumococci in up to 10% PCR-positive to Spn. Total number of adults older than 50 years of age is around 120,000, and therefore we expect to collect the sample up to 0.4% of the total targeted population.

Expected results
After carefully implementation of the research, we expected that Spn carriage among adults older than 50 years of age will be present in less than 10% of the total population. In addition, we presume that Spn serotypes covered by PPV or PCV will represent more than 60% and 50% of the serotypes in overall distribution among tested subjects.

Statistical Methods
We will examine associations between risk factors and NP and OP carriage of pneumococci in participants aged older than 50 years. The following factors will be examined as possible risk factors in covered population: age, gender, data on previous upper respiratory tract infection, number of children (siblings) aged 0–10 years residing in the household of participants with pneumococcal and influenza immunization history, smoking habits of participants and their household members and the information about home residence. These factors will be analyzed by univariate and multivariate analyses (if appropriate). Also, the prevalence of NP and OP carriage as the proportion of participants whose nasopharyngeal cultures were positive for Spn and corresponding 95% confidence intervals will be estimated. All descriptive analyses will be performed using the SPSS Statistics software Version 21.0 (IBM Corp., Armonk, NY, USA).

Appendix 1. Information leaflet for subjects

Pneumococcal nasopharyngeal carriage in adults older than 50 years of age in outpatient health care facility in Novi Sad

Streptococcus pneumoniae (pneumococcus) is a cause of morbidity and mortality in people of all ages throughout the world. Children are asymptomatic carriers of *S. pneumoniae.* In children, *S. pneumoniae* is the most common cause of bacterial otitis media, pneumonia and bacteremia. In persons aged >65 years, the annual incidence ranged from 24 to 85 cases/100 000 population. In Europe and the United States, *S. pneumoniae* was estimated to cause approximately 30–50% of community-acquired pneumonias (CAPs) requiring hospitalization in adults. Problms start in persons older than 50 years of age who suffer from various chronic conditions.

It is known that mortality rates and the burden of pneumococcal pneumonia, as with all invasive pneumococcal disease are serotype - specific.

To treat diseases caused by *S. pneumoniae* and to reduce the number of patients with this infection, scientists need to determine the serotypes circulating *S. pneumoniae*. For this purpose, funded project supports research serotyping of *S. pneumoniae* in Novi Sad.

We invite you to participate in this research. In this way you can help find ways to protect others from infection.

Your participation requires only little of your time. After discussing the details of your participation with your doctor, you must sign a document in which you declare that you agree to participate in the research. There will be no need for any additional medical procedures for this research. During your visit, doctor will take nasopharyngeal swab from you for diagnostic tests.

Your agreement for participation in this study is completely voluntary. You will not receive any compensation for your participation, but your contributions will be extremely valuable for science and for other people in society.

If sufficient, your sample will be used for research of other bacterial organisms that may be significant and are not described in the project. Your data will remain confidential. Even if you agree to participate now, you can always change your mind in the future and to cancel your participation at any time.

Thank you in Advance!

Principal Investigator
Prof. Vladimir Petrović M.D. Ph.D

Appendix 2. Informed Consent of the subject

I hereby stat that am familiarized with the goals and methodology of the research, and also not to expect any kind of personal matherial gain

I am aware that I can exclude my child from the research at any time and also if I decide to do so there will be no consequences on my childs treatment.

Full name and surname

_____

Signature

_____

Date:_____

(DD/MM/YY)