

# Serotype distribution and antimicrobial resistance in invasive *Streptococcus pneumoniae* isolates in Croatia

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**UNIVERSITY OF ZAGREB**

**SCHOOL OF MEDICINE**

**Iva Butić**

**Serotype distribution and antimicrobial  
resistance in invasive *Streptococcus pneumoniae*  
isolates in Croatia**

**DISSERTATION**

**Zagreb, 2022**

**I dedicate this dissertation to my daughter Zara who showed me a whole  
new magic and power of love.**

This dissertation was made at the Department of Clinical Microbiology of the University Hospital for Infectious Diseases “Dr Fran Mihaljević” in Zagreb, Croatia

Mentor 1: Professor Arjana Tambić Andrašević, MD, PhD

Mentor 2: Professor Waleria Hryniewicz, MD, PhD

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## **LIST OF ABBREVIATIONS:**

AMR – antimicrobial resistance

AOM – acute otitis media

CAP – community-acquired pneumonia

CARS – Croatian Committee for Antibacterial Resistance Surveillance

CDC – Centers for Disease Control and Prevention

COPD – chronic obstructive pulmonary disease

CSF – cerebrospinal fluid

EARS-Net - European Antimicrobial Resistance Surveillance Network

EARSS - European Antimicrobial Resistance Surveillance System

ECDC – European Centre for Disease Prevention and Control

ESCMID – European Society of Clinical Microbiology and Infectious Diseases

EUCAST – European Committee for Antibiotic Susceptibility Testing

IDSA – Infectious Diseases Society of America

IPD – invasive pneumococcal disease

M-phenotype – macrolide phenotype

MIC – minimal inhibitory concentration

MLS<sub>B</sub>-phenotype - macrolide, lincosamide, streptogramin B phenotype

NIP – National Immunization Programme

PBP – penicillin-binding protein

PCR – polymerase chain reaction

PCV - pneumococcal conjugate vaccine

PCV7 – 7-valent pneumococcal conjugate vaccine

PCV10 – 10-valent pneumococcal conjugate vaccine

PCV13 – 13-valent pneumococcal conjugate vaccine

PPV23 – 23-valent pneumococcal polysaccharide vaccine

RCARS - Reference Centre for Antibiotic Resistance Surveillance

WHO – World Health Organisation

## 1. Introduction and background

*Streptococcus pneumoniae* (*S. pneumoniae*, pneumococcus) is one of the major human pathogens from its initial recognition in 1881 up to nowadays. Upon discovery, its crucial part in the pathogenesis of lobar pneumonia was appreciated. *S. pneumoniae* successfully colonises the upper respiratory tract forming a balance with the immune system and playing an important role in the nasopharyngeal microbiota. The main reservoirs of this Gram-positive diplococcus are infants and toddlers. Respiratory droplets are known as the major route of transmission, therefore the highest colonisation rate is detected among children attending the crowded settings, especially during the colder part of the year. Based on different studies, the nasopharyngeal colonisation rate differs among developed and developing countries, 28% and 85%, respectively (1). Up to nowadays more than 100 different serotypes of *S. pneumoniae* have been identified based on the polysaccharide capsular structure. The distribution of these serotypes varies by age, clinical presentation, the severity of the pneumococcal disease, geographic region and time of the year (2–4). Infections caused by *S. pneumoniae* are classified as non-invasive, usually mild mucosal infections and invasive, often life-threatening diseases. Globally, prior to the introduction of the first pneumococcal conjugate vaccine, the majority of invasive pneumococcal infections were caused by a small number of serotypes. The high prevalence and high mortality rate of invasive pneumococcal disease (IPD), together with the increased resistance of *S. pneumoniae* to routinely administered antibiotics, emphasizes the urgent need for the introduction of preventive public health measures. The Centers for Disease Control and Prevention (CDC), as one of the major public health organizations, highlighted the importance of pneumococcal vaccine introduction in the National Immunisation Programme (NIP) in the United States of America (USA) (5). Today, there are two types of vaccines available on the European vaccine market, the 23-valent polysaccharide vaccine for adults and children  $\geq 2$  years of age and two conjugate vaccines, the 10-valent vaccine for children up to 5 years of age and the 13-valent vaccine for all age groups (6, 7). Very recently two new conjugate vaccines were approved by the European Medicines Agency, 15- and 20-valent vaccines (8, 9). All vaccines cover the most prevalent *S. pneumoniae* serotypes that are causing the majority of IPD cases. The vaccines contain the pneumococcal capsular polysaccharides from the most resistant serotypes, which is of great importance for public health, as well. The introduction of pneumococcal vaccines significantly decreased the number of IPD cases, mainly those caused by the serotypes included in the vaccines. Consequently, serotypes not included in the vaccines



emerged, resulting in their increasing incidence of IPD infections despite the overall decrease in the incidence rates of invasive infections.

### 1.1. Epidemiology of *Streptococcus pneumoniae*

*S. pneumoniae* is one of the dominant members of nasopharyngeal microbiota together with numerous  $\alpha$ -haemolytic streptococci and other opportunistic human pathogens such as *Neisseria spp.*, *Haemophilus influenzae*, *Haemophilus parainfluenzae*, *Moraxella catarrhalis* and *Staphylococcus aureus*. In the majority of the healthy populations, mainly small children, *S. pneumoniae* nasopharyngeal colonisation is asymptomatic. Despite continuous interaction between bacterial virulence factors and adequate immune system response, the balance is usually maintained (10). Sometimes this synchronized bacterial community can be disrupted resulting in various presentations of pneumococcal disease, from mild, non-invasive to severe, invasive forms of the disease (11). In the situations where the equilibrium between the host and bacteria is not achieved the organisms will lose their capsule, meaning immunological specificity and become non-encapsulated and usually avirulent. Bacterial adhesion to the host's nasopharyngeal mucosa is an inevitable first step in the pathogenesis of all pneumococcal infections. Additionally, the nasopharyngeal niche is recognized as the main source of *S. pneumoniae* horizontal spread inside the community. Increased horizontal spread of this pathogen is very specific to crowded settings like day care centres, orphanages, hospitals, nursing homes and prisons. Young children, up to 5 years of age, are characterized by the highest percentage of pneumococcal colonisation and are recognized as the main reservoir of this pathogen for the whole population, especially older people. Therefore, the prevention of nasopharyngeal pneumococcal colonisation, especially in children and the elderly, is a crucial part of the pneumococcal disease prevention strategy. Contagious spread of this bacterium from the nasopharyngeal niche can cause mucosal infections such as acute otitis media (AOM), sinusitis and non-bacteremic pneumonia. Clinical presentations of IPD are bacteraemia, sepsis, meningitis and bacteremic pneumonia. Pneumococcal infections are following a pattern typical for most respiratory diseases, with a peak in the number of patients during the winter months and early spring. Two major risk groups for acquiring IPD are children <5 years of age and adults, 65 years and older and those without a spleen (12).

*S. pneumoniae* is the most frequent bacterial pathogen causing community-acquired pneumonia (CAP). These infections range from mild, non-invasive without a bacterial presence in the blood, and invasive form of disease with bacteria invading the bloodstream causing severe

infections (12). Non-bacteremic pneumonia represents the major burden of *S. pneumoniae* infections in adults. As the burden of CAP is based on confirmed bacteremic infection, with the positivity of blood culture 10-20%, the real burden of this disease is significantly underestimated. Based on estimation, CAP in adults requires hospitalization in approximately 30%–50% of patients in the USA and in Europe. In children, lobar pneumonia and bronchopneumonia caused by *S. pneumoniae* were diagnosed in 78% and 13% of the cases respectively (13).

Morbidity and mortality due to IPD are the highest in children <5 years of age and in patients 65 years and older. According to the World Health Organisation (WHO) data for 2008, out of 8.8 million global annual deaths among children <5 years of age, around 529 000 were caused by invasive pneumococcal infections. Children with HIV infection are at a significantly higher risk for acquiring serious pneumococcal disease (14). In 2015, based on WHO data, 294 000 children <5 years of age died due to pneumococcal infections of estimated 5.83 million deaths among children of that age (15). Case fatality rates for sepsis and meningitis in developing countries can be high, up to 20% and 50%, respectively (16). Disease and mortality rates are higher in low-income countries, with the majority of deaths occurring in Africa and Asia. Vaccination played a paramount role in the significant decrease of the high mortality rate providing better protection for this vulnerable population group (17, 18).

#### 1.1.2. Susceptibility to antimicrobial agents

Since the beginning of the 21<sup>st</sup> century, the problem of acquired antimicrobial resistance emerged and the global spread of multidrug-resistant bacteria was recorded all over the globe. Leading health organisations are trying to cope with this multi-sectoral problem which highly affects human health and quality of life. Until the 1970s, all *S. pneumoniae* isolates were susceptible to penicillin, cephalosporins, macrolides, clindamycin and vancomycin. In the early 1990s, many *S. pneumoniae* isolates showed reduced susceptibility to commonly used antibiotics, mainly penicillin and macrolides (19, 20). A significant difference in antimicrobial resistance pattern was recorded among serotypes, patients` age and geographical areas (21). The increasing prevalence of resistant *S. pneumoniae* has been observed in France, Spain and Eastern European countries, while the resistance rate of up to 79%, reported in South Africa, was very concerning (22–24). Nevertheless, some countries reported decreased resistance rates among *S. pneumoniae* isolates (25–27).

Acquired resistance to macrolides was detected among invasive and non-invasive *S. pneumoniae* isolates. In the USA and some European countries, the resistance to macrolides has significantly increased and become more frequent than resistance to penicillin (21, 28). The introduction of the pneumococcal conjugate vaccine rapidly decreased the prevalence of penicillin-resistant pneumococcal serotypes. Since the introduction of the 13-valent pneumococcal conjugate vaccine (PCV13) in the USA, erythromycin resistance has become more common among invasive pneumococcal isolates than resistance to penicillin, largely due to the vaccine removal of penicillin-resistant strains (29).

In some countries, due to the increased resistance to macrolides and  $\beta$ -lactam antibiotics among *S. pneumoniae* strains, new fluoroquinolones became among the first choices for empirical treatment of bacterial respiratory tract infections, mainly pneumonia in adults. Levofloxacin, gatifloxacin and moxifloxacin, also called respiratory quinolones, have enhanced *in vitro* and *in vivo* activity against *S. pneumoniae* isolates. An increasing resistance rate to fluoroquinolones has been reported in Asia and Africa (30, 31). Additionally, in several cases, resistance to fluoroquinolones occurred during antibiotic therapy which resulted in therapeutic failure (32, 33).

*S. pneumoniae*, primarily as the nasopharyngeal colonizer, has emerged as the top-priority invasive human pathogen due to acquired resistance to frequently use antimicrobial agents and the ability to avoid vaccines currently available for the prevention of invasive forms of pneumococcal diseases. Regardless of the availability of vaccines and antibiotics, infections caused by *S. pneumoniae* are still characterized by high morbidity and mortality rates. Empirical therapy must be administered as early as possible to prevent severe forms of diseases, consequential sequelae and death. IPD mortality rate varies widely based on geographical region, age and clinical presentation even in cases where antibiotic therapy was timely initiated (34). Wild type isolates of *S. pneumoniae* are susceptible to  $\beta$ -lactam antibiotics, macrolides, clindamycin, co-trimoxazole, fluoroquinolones (moxifloxacin, levofloxacin and gatifloxacin), tetracycline, vancomycin and linezolid. Nowadays, acquired resistance to penicillin, macrolides, co-trimoxazole, tetracycline and fluoroquinolones is detected. Ceftaroline, a fifth-generation cephalosporin, displays marked activity against *S. pneumoniae*, including antibiotic-resistant strains, especially ceftriaxone resistant (35, 36). Local epidemiological data on the resistance rate in invasive and non-invasive *S. pneumoniae* isolates are extremely important for starting the appropriate empirical treatment. For over 20 years, the Croatian Committee on Antimicrobial Resistance Surveillance has been collecting data on antibiotic resistance among major human pathogens including *S. pneumoniae*. Also, the data on the antimicrobial resistance

in the most important invasive human pathogens are collected through the largest antimicrobial surveillance network in Europe, the European Antimicrobial Resistance Surveillance Network (EARS-Net).

### 1.2.1. Beta-lactam antibiotics

Since penicillin was introduced in human medicine in the 1940`s it was successfully used in the treatment of pneumococcal infections for several decades. Penicillin and its derivatives are excellent and cheap antibiotics with minor side effects. The bactericidal activity of penicillin is based on the inhibition of cell wall synthesis. Resistance to penicillin is based on the modification of the penicillin receptors called penicillin-binding proteins (PBP). Those enzymes are obligate in bacterial metabolic activity (37). Due to acquired target modification, peptidoglycan synthesis in penicillin-resistant *S. pneumoniae* strains will be undisturbed. However, detection of penicillin resistance in these pathogens requires treatment changes (19). Knowledge of the mechanism of penicillin resistance is essential for choosing alternative antibiotic therapy. The rapid spread of penicillin-resistant *S. pneumoniae* isolates was multifactorial, mainly due to antibiotic overuse, attending the crowded settings, the age of the patients and resulted in a higher prevalence of respiratory tract infections. According to EARS-Net data, penicillin resistance in 2019 varied among countries, with the highest rates recorded in Romania and Spain, 19.8% and 17.9%, respectively, while the lowest rates (<2%) were observed in Norway, Germany, UK and Croatia. The percentages of penicillin susceptible, increased exposure *S. pneumoniae* isolates varied among countries. The highest rate was observed in France, followed by Belgium and the United Kingdom.

Penicillin susceptible, increased exposure isolates do not interfere with the mortality rates of patients diagnosed with pneumococcal pneumonia (38). On the other hand, penicillin-resistant isolates have been associated with increased morbidity and mortality in patients with bacteremic pneumonia (39–41).

### 1.2.2. Macrolides

The worldwide resistance to macrolides in *S. pneumoniae* isolates has increased significantly in the past decades, although the difference in the prevalence among countries was recorded (42). Previous antimicrobial therapy is a major risk for acquiring infection with the resistant strain. The percentage of resistance is higher in the paediatric population, day care settings,

nursing homes and hospitals. Resistance is also higher among strains isolated from the middle ear, nasopharynx and respiratory tract samples. Up to now, two major phenotypes of macrolide resistance have been described. Resistance to macrolides, lincosamide and streptogramin B, known as MLS<sub>B</sub> phenotype, was the first mechanism described in the pneumococcal isolates. This resistance phenotype, either constitutive or inducible, is based on target modification due to the activity of ribosomal-methylase encoded by *erm(B)* genes (43, 44). The second mechanism described the M phenotype, is efflux pump-based and encoded by *mefA* genes (45). Beside different mechanisms of macrolide inactivation, these phenotypes have different MIC levels as well. The MLS<sub>B</sub> phenotype, expressing high-level resistance to macrolides, is characterised by erythromycin MIC levels  $\geq 32$  mg/L compared to MIC levels of 1-4 mg/L specific for the M phenotype (46–48). Recently, pneumococcal isolates with both resistance mechanisms are increasing. Also, additional resistance mechanisms have been described in *S. pneumoniae* strains. Resistance to macrolides was associated with mutations in the 23S rRNA or modification of the ribosomal proteins, which requires additional research (49).

### 1.2.3. Fluoroquinolones

The new fluoroquinolones, also known as respiratory quinolones, have enhanced antimicrobial activity for Gram-positive bacteria including *S. pneumoniae*. These antibiotics, including moxifloxacin, levofloxacin and gatifloxacin, are the drug of choice for the treatment of patients at higher risk for infection caused by multidrug-resistant bacteria. Also, due to convenient dosing schemes, these antibiotics were registered for the empirical treatment of patients with CAP. Worldwide, the prevalence of fluoroquinolone-resistant isolates is still low, but increasing in many parts of the world (50, 51). Fluoroquinolone resistance in the USA, based on the CDC's Active Bacterial Surveillance System is <1% (52). In the European Union and European Economic Area countries (EU/EEA), based on the EARS-Net report for 2019, overall resistance to fluoroquinolones was 4.9%. While, the highest resistance rate among European countries was recorded in Italy (5.6%) (42). Information on the recent fluoroquinolone therapy is a contraindication for the starting the empirical treatment for outpatient pneumonia with this antibiotic group (30, 53, 54). In most cases, reduced susceptibility to fluoroquinolones is a result of alteration in *parC* and *gyrA* genes that encode enzymes topoisomerase IV and DNA gyrase (55). Both enzymes contain two subunits, GyrA and GyrB in DNA gyrase and ParC and ParE in topoisomerase IV, encoded by *gyrA*, *gyrB*, *parC* and *parE* genes, respectively (56).

### 1.3. Virulence factors

*S. pneumoniae* is a Gram-positive, lancet-shaped diplococcus whose pathogenesis is based on the activity of many virulence factors. Besides polysaccharide capsule, as the major virulence factor, *S. pneumoniae* expresses several others, such as the pneumolysin (the pore-forming toxin), choline-binding proteins (autolysin, pneumococcal surface protein A, choline-binding protein A), pili, IgA1 protease, neuraminidase, hyaluronidase and adhesins. Enabling and maintaining bacterial survival at the host together with avoiding the host immune system, evoking an inflammatory reaction and producing tissue damage are common to all virulence factors. *S. pneumoniae* transition from colonisation of nasopharyngeal mucosa to non-invasive and invasive infections is a very complex process, not fully clarified yet. To achieve successful colonisation of the nasopharynx, the *S. pneumoniae* needs to maintain its survival without killing the host.

In the nasopharynx, the population of colonising bacteria are organized to maintain balance with the host inflammatory response, mainly neutrophils and macrophages. To initiate an infection, *S. pneumoniae* has to move from the nasopharyngeal niche to primary sterile sites such as the middle ear, lungs, bloodstream, brain, heart or joints (57).

#### 1.3.1. Polysaccharide capsule

The polysaccharide capsule is the pneumococcal principal virulence factor also recognised in other mucosal colonizers such as *Haemophilus influenzae* and *Neisseria meningitidis*. Capsular expression is mandatory for achieving the complete pathogenicity of this bacterium. The production of the capsule requires certain metabolic activity which represents a significant burden to the bacterial cell. The polysaccharide capsule differs in size and may account for half of the bacterial volume or more. Each serotype is characterized by a unique polysaccharide capsular composition. More than 100 different serotypes have been described so far due to capsular polysaccharides diversity, their interaction and position in the capsule (58). The major roles of polysaccharide capsule greatly contribute to the full virulence of this important human pathogen. Anti-phagocytic activity, as a paramount role, is characterized by inhibition of opsonophagocytosis. Meaning, that bacterial cell has the ability to avoid phagocytosis by human phagocytic cell and remain extracellular due to the capsular possibility to inhibit the interaction of the complement and immunoglobulins on the cell wall with the receptors on the surface of the phagocytic cell (25). Capsular polysaccharides play a crucial role in

nasopharyngeal colonisation providing prolonged bacterial adherence to the nasopharyngeal mucosa and avoiding mechanical removal by the mucus. Also, capsular polysaccharides participate in the control of cell autolysis and reduce exposure to antibiotics (59). Capsular polysaccharides are highly immunogenic and responsible for a type-specific antigenicity. Antibodies against capsular polysaccharides induce the process of opsonophagocytosis providing protection against the homologous serotype. Certain serotypes have similar polysaccharide structures which will lead to cross-reaction and cross-protection.

Capsular production is expressed in two phases, known as the opaque and transparent phases. The opaque phase, characterized by extensive capsular production, is associated with invasive isolates while pneumococcal isolates colonizing the nasopharynx are characterised by reduced expression of capsular production, so-called the transparent phase. Also, phase variation has been observed in the pneumococcal biofilm formation with the predominance of the transparent phase while an opaque capsular growth was expressed in virulent planktonic cells (60, 61).

Though, a decrease in capsular production is essential for exposing the numerous underlying bacterial adhesins, necessary for the attachment to the nasopharyngeal epithelium, is an essential step in the bacterial colonization and further invasion. Loss of polysaccharide capsule is associated with the suppression of a single cluster gene, the *cps* locus, regulating the capsular production. Also, mutations or deletions of *cps* genes may result in the emergence of non-encapsulated pneumococcal strains (62).

Although more than 100 different serotypes are detected, they differ in their prevalence, clinical significance, clinical presentation and severity of the disease. The prevalence of the most common serotypes varies among different age groups and geographical regions. Among serotypes causing invasive infection in children the most common serotypes are 1, 5, 6A, 6B, 14, 19A, 19F and 23F (15). The most prevalent invasive serotypes among adults are 1, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 11A, 12F, 14, 18C, 19A, 19F, 22F and 23F. Serotype 3, the most prevalent serotype among adults, is rarely causing invasive infection in children.

### 1.3.2. Pneumolysin

Pneumolysin, a thiol dependent cytolytic toxin, is an important virulence factor specific for *S. pneumoniae*. It binds to cholesterol in the cell membrane and is released by cell lysis. Pneumolysin, for the host cells, is a very potent toxin and due to many activities has an important impact on the severity of the disease. Its primary role, mediating the host cell death, is achieved through the formation of pores in the host cell membrane leading to cell lysis. In

animal models of infection, the lytic activity of pneumolysin was confirmed by its ability to directly cause vascular leakage and oedema. Pneumolysin presence in a systemic circulation during invasive pneumococcal infection can cause damage of the myocardial cells, causing heart failure often leading to death. Its contribution to bacterial virulence is also expressed in process of the biofilm formation and interference with the bacterial removal of the mucous membrane. Due to its ability to interfere with the complement, pneumolysin can reduce innate immune cells phagocytosis. Moreover, pneumolysin, as a pro-inflammatory toxin, releases reactive oxygen and nitrogen species resulting in DNA damage and host tissue damage, consequently (62, 63).

### 1.3.3. Pneumococcal surface proteins

*S. pneumoniae* produces a number of different surface proteins which contribute to pneumococcal pathogenesis. Its virulence activity is expressed via enabling the adherence of bacterium to cell host and interference with the immune system, mainly complement. Four groups of pneumococcal surface proteins are known: choline-binding proteins, lipoproteins, non-classic proteins and neuraminidase.

Nowadays, up to 16 different choline-binding proteins are known and their presence is necessary for the adhesion of *S. pneumoniae* to the host cell. Immunoglobulins are inactivated by blocking the activation of the complement activity. The most important choline-binding proteins are autolysin, pneumococcal surface protein A and pneumococcal surface protein C. Autolysin is an important pneumococcal virulence factor. Its main role, the activation of the cell autolysis, is achieved through the release of pneumolysin from the cytoplasm together with teichoic acid and other cell wall components. Also, autolysin promotes nasopharyngeal colonisation by releasing pneumolysin during cell lysis. Importantly, autolysin has the ability to cause cell lysis in the presence of penicillin and vancomycin. Autolysin is mainly localized intracellularly, but small amounts of this enzyme are localised on the outer cell wall. Pneumococcal bacterial cells are protected from activation of this enzyme during the exponential phase of growth due to interaction between extracellular and intracellular autolysins. The highest concentration of autolysin is recorded during the stationary phase of growth. Lytic activity of this enzyme is visualized as induration of the bacterial colonies on the blood agar after 24-48 hours of incubation.

Pneumococcal surface protein A has the ability to inhibit opsonophagocytosis by binding to complement. Additionally, binding to apolactoferrin inactivates its bactericidal activity.



Pneumococcal surface protein C contributes to pneumococcal virulence by its role in the epithelial cell adhesion and inactivation of complement by binding to complement regulatory protein factor H.

Lipoproteins are responsible for the transportation of different substrates in the cell. So far 50 different lipoproteins have been identified. The most important lipoproteins are pneumococcal iron acquisition, pneumococcal surface adhesion, pneumococcal iron transporter and pneumococcal iron uptake. These metal-binding proteins are mainly responsible for the transport of iron, magnesium and zinc.

Neuraminidase contributes to pneumococcal pathogenesis by enabling its bactericidal activity by removing the sialic acid from the lactoferrin. Its role in nasopharyngeal colonisation has also been observed (64).

#### 1.3.4. Other virulence factors (pili, immunoglobulin A protease, neuraminidase, hydrogen peroxide)

Pili are hair-like formations located on the cell surface of *S. pneumoniae*. Two types of pili, pilus-1 and pilus-2, are identified and both play an important role in the nasopharyngeal colonisation of *S. pneumoniae* to epithelial cells. The production of pili is not observed in all pneumococcal strains. The ability of pili to escape the immune system phagocytosis is also described. Recently discovered is their role in the stimulation of the host inflammatory responses.

*S. pneumoniae* also produces immunoglobulin A protease (IgA protease) responsible for the inactivation of the mucosal innate defence by disintegration of immunoglobulin A.

Hydrogen peroxide, produced by *S. pneumoniae* cell, is responsible for DNA damage and apoptosis of the host cell. Its bactericidal effect is also identified. Hydrogen peroxide induces the innate immune response through the release of pro-inflammatory cytokines (64).

#### 1.3.5. Biofilm formation

Nowadays it is well known that the ability of *S. pneumoniae* cells to form a biofilm is responsible for the bacterial persistence in the majority of pneumococcal infections. For this opportunistic pathogen, it is crucial to maintain at the nasopharyngeal mucosa after initial activity compared to their planktonic variant (65). The formation of biofilm is crucial for *S. pneumoniae* colonization, survival, persistence, as well as disease outcome. By forming the

biofilm, *S. pneumoniae* cells have the ability to evade the host immune response through activation of complement. Also, bacteria in the biofilm are less accessible to antibiotics which is an added advantage for this human pathogen.

#### 1.4. Clinical presentation

##### 1.4.1. Non-invasive pneumococcal diseases

Pneumococcal diseases are classified as non-invasive and invasive forms of infections. Local bacterial spread from nasopharyngeal mucosa to the Eustachian tube or paranasal sinuses can cause AOM or sinusitis. Aspiration of *S. pneumoniae* to a lower respiratory tract in combination with a failure of the local immune defences will cause infection of the alveoli resulting in (lobar) pneumonia. The incubation period for pneumonia is approximately 1 to 3 days.

In children, AOM is the leading reason for visiting a paediatrician and the most common cause for prescribing antibiotics (66). The majority of AOM have a viral etiology but in many countries, antibiotic overuse is very common for this clinical presentation. The most common bacterial pathogens causing AOM in children are *S. pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*. Although pneumococcal conjugate vaccines were designed to provide protection from an invasive form of diseases, the introduction of vaccines resulted in a significant decrease in the incidence of AOM among children in many countries. Moreover, a decrease in antibiotic consumption was observed (67).

Pneumococcal infections, mainly pneumonia, are one of the leading causes of death among children in developing countries. Pneumonia accounts for 15% of all deaths among children under 5 years of age, resulting in 808 694 children`s deaths in 2017 (16). *S. pneumoniae* is the most prevalent bacterial pathogen causing severe pneumonia and pneumonia deaths worldwide. It occurs most frequently in infants and toddlers. The incidence of pneumonia in adults  $\geq 65$  years of age is almost five times higher comparing to young adults. In that vulnerable patient group, *S. pneumoniae* is the principal cause of CAP with substantial morbidity and mortality rate (68–70). In the majority of patients, the infection is localized in lung parenchyma only, without invasion of the bloodstream. The burden of pneumococcal disease in adults is mainly determined by CAP (71, 72). In routine medical practice, around 20% of CAP is confirmed by microbiological diagnostics. The higher sensitivity of microbiological diagnostics, up to 60%, can be accomplished by extensive and expensive diagnostic methods (molecular diagnostic methods) (73). *S. pneumoniae*, due to various virulence factors, successfully manipulates an

impaired immune system causing different forms of disease. Nowadays, the risk factors for acquiring CAP are well known. The most important is older age ( $\geq 65$  years of age), followed by previous viral respiratory tract infections (mainly influenza), alcohol abuse, cigarette smoking, prolonged stay in crowded settings, chronic obstructive pulmonary diseases, absence of splenic function and other disorders characterized by lack of adequate immune response.

Pneumonia related mortality rate is prone to variation depending on the severity of the disease and timely administration of antibiotic treatment. The mean mortality rate is 13.7 to 14.4% (73). In the USA, based on CDC estimation, around 150 000 patients hospitalized with pneumococcal pneumonia occur annually. *S. pneumoniae* accounts for up to 30% of adult CAP. Bacteraemia is recorded in 25–30% of patients diagnosed with pneumococcal pneumonia. Although the overall case-fatality rate is 5–7%, much higher numbers are recorded among adults, 65 years and older. The most common complications of pneumococcal pneumonia are empyema, pericarditis and respiratory failure. Hospital admission of CAP patients ranges from 20 to 35%, depending on different hospitalisation criteria, different access to medical services and different outcomes of completing oral antibiotic treatment. Mortality rates correlate with the severity of the disease. The mortality rate of ambulatory treated CAP patients is low (1 to 5%). While, patients requiring hospitalization or intensive care unit treatment have increased mortality rates ranging from 5.7 to 12% and 37 to 50%, respectively (73).

#### 1.4.2. Invasive pneumococcal diseases

*S. pneumoniae*, mainly due to various virulence factors, host factors, such as impaired immune system and socio-economic factors, can invade normally sterile sites causing life-threatening infections such as bacteremia, sepsis, meningitis and bacteremic pneumonia with or without primary focus. These clinical presentations are a real burden of disease. Prior to the introduction of the 7-valent vaccine in the USA, approximately 65000 patients with IPD in all age groups were diagnosed annually. Also, around 25% of all IPD cases were diagnosed in children  $< 5$  years of age (74). The diagnosis requires the isolation of *S. pneumoniae* from a normally sterile site, like blood, cerebrospinal fluid or pleural aspirate. The diagnostic approach to pneumococcal infection is challenging knowing that blood cultures have high specificity but low sensitivity. The sensitivity of blood culture in diagnosing pneumococcal pneumonia is approximately 10–15%, which is lower compared to other syndromes (16). Isolation of this pathogen from non-primary sterile samples, like sputum, requires differentiation between colonisation and infection. Colonisation of *S. pneumoniae* in patients with chronic obstructive

pulmonary disease (COPD) ranges from 7.5 to 17% while *S. pneumoniae* is isolated in 25% of patients with acute exacerbation of COPD (75–77). Also, antibiotic administration prior to microbiological sampling of clinically important samples significantly decreases the incidence of bacterial cultivation.

The real burden of confirmed pneumococcal CAP is significantly underestimated, based on data from bacteremic infections only. Approximately, for every case of bacteremic CAP, there are three additional cases of non-bacteremic pneumonia. Bacteremia is diagnosed in approximately 25% of patients with pneumococcal pneumonia (71).

*S. pneumoniae* is an important human pathogen causing bacteraemia in immunocompetent patients and those with a compromised immune system. Invasion of bacteria in the blood can occur in complicated cases of pneumococcal pneumonia or without any known primary focus. Also, secondary complications of bacteraemia can occur such as meningitis, arthritis or endocarditis. Pneumococcal meningitis, the most severe clinical presentation of IPD, has the highest incidence rate among adults, especially those  $\geq 65$  years of age. *S. pneumoniae* is the second most common pathogen causing meningitis in children 2-5 years of age. A significant number of neurological sequelae are described in those who survive this severe pneumococcal infection. Even an appropriate and timely administered antimicrobial treatment result in a mortality rate of 20 to 30%. Invasive *S. pneumoniae* isolates are responsible for  $>50\%$  of all bacterial meningitis cases in the USA with 2000 estimated cases of pneumococcal meningitis yearly. Also, some patients with pneumococcal meningitis may have pneumonia (78).

An estimated 5,000 cases of bacteraemia (without pneumonia) occur each year. The overall case-fatality rate for bacteraemia is about 20% but can be as high as 60% among elderly patients. In asplenic patients, bacteraemia can progress to a fulminant infection. Bacteraemia without primary focus or sepsis is the most common invasive clinical presentation of pneumococcal infection among children  $<2$  years of age, accounting for approximately 70% of invasive diseases in this age group. IPD and meningitis in children  $<2$  years of age occur in 75% and 83% of all IPD cases among the paediatric population. Case fatality rates (CFR) for IPD can be high, ranging up to 20% for sepsis and 50% for meningitis in developing countries. Mortality is highest among infants (1). Bacteremic pneumonia, in the same age group, accounts for about 12–16% of IPD cases. In the older population, *S. pneumoniae* takes advantage of a weakened natural defence due to immunosenescence and life habits causing invasive infection with high-risk complications such as empyema, sepsis, septic shock, and acute respiratory distress.

According to the ECDC's "Invasive Pneumococcal Diseases Annual Epidemiological Report for 2018" bacteraemic pneumonia was reported in 43%, septicaemia in 35%, meningitis in 19%, meningitis and septicaemia in 1% of all reported IPD cases and additional 2% had other clinical presentations. Meningitis was the most common clinical presentation in children <1 year and 5–14 years of age, sepsis and bacteraemic pneumonia were equally frequent in 1 to 4-year-olds while bacteraemic pneumonia was the most common clinical presentation among those >15 years of age (21).

In EU/EEA countries the incidence of notified IPD cases was slightly higher compared to previous years, 6.4 cases per 100 000 population of confirmed IPD in 2018. Infants and adults  $\geq 65$  years of age were the most affected age groups as in the previous period. Notification rates differ among countries, ranging from 0.2 to 16.0 IPD cases per 100 000 population. Observed variation may be linked to the differences in healthcare systems, vaccination policies, surveillance and reporting of IPD.

In 2018, the most common serotypes 8, 10A, 3, 19A and 24F were not covered by currently available vaccines for children <5 years of age, with the exception of serotypes 19A and 3 (21).

### 1.5. Treatment

Penicillin remains the first drug of choice for majority of pneumococcal infections. Although, infections caused by *S. pneumoniae* strains penicillin susceptible, increased exposure or resistant ( $\text{MIC} \geq 0.06$  mg/L) are increasing in Europe, the USA and other areas of the world (42, 79, 80). Based on the treatment guidelines and important clinical studies, penicillin, ampicillin or cefuroxime should be adequate for the treatment of patients hospitalized with pneumonia caused by pneumococcal isolates with penicillin MICs  $\leq 2.0$  mg/ml. Also, oral therapy with amoxicillin or cefuroxime-acetyl should be efficacious for initial outpatient management or oral switch therapy in patients with a resolution of symptoms following parenteral treatment. For isolates with penicillin MICs  $> 2.0$  mg/ml, alternate agents, such as cefotaxime or ceftriaxone, if susceptible, are suggested. When the MICs of these drugs are higher, alternate agents such as vancomycin and/or carbapenems may be needed. For immunocompromised or critically ill patients, if *S. pneumoniae* is among the suspected pathogens, empirical therapy including vancomycin should be considered (81, 82).

According to the guidelines of the European Respiratory Society (ERS), the European Society of Clinical Microbiology and Infectious Diseases (ESCMID), the British Thoracic Society, the American Thoracic Society (ATS), the Infectious Diseases Society of America

(IDSA) and the Croatian Society for Infectious Diseases, the drug of choice for ambulatory treatment of CAP is amoxicillin (83–86). For patients allergic to penicillin, the drug of choice is doxycycline, moxifloxacin or levofloxacin. For hospitalized patients with a moderate or severe infection, the treatment includes third-generation cephalosporins, fluoroquinolones and carbapenems (84, 85, 87). The treatment of patients with pneumococcal meningitis has changed significantly during the past two decades.  $\beta$ -lactam antibiotics have proven their efficiency against invasive *S. pneumoniae* serotypes causing meningitis with sensitivity to cefotaxime or ceftriaxone 96 to 98%. Given the emergence of penicillin non-wild type pneumococcal strains, penicillin is not recommended for the empirical therapy of patients with suspected pneumococcal meningitis. The sensitivity of *S. pneumoniae* isolates to vancomycin is 100%. In some countries, depending on the local epidemiology, a combination of vancomycin and third-generation cephalosporins (either cefotaxime or ceftriaxone) should be used. Meropenem is not a good alternative monotherapy for the treatment of infections associated with penicillin- or cephalosporin-resistant *S. pneumoniae* strains, although a fluoroquinolone (namely moxifloxacin) with *in vitro* activity against *S. pneumoniae*, is an option in patients failing standard therapy. However, if used, meropenem should be combined with a third-generation cephalosporins or vancomycin. Major guidelines for the treatment of CAP do not recommend the use of the new fluoroquinolones in patients who are not at risk for having an infection caused by penicillin-resistant pneumococcus.

In accordance with the IDSA/ATS and ERS/ESCMID guidelines for CAP, monotherapy with a fluoroquinolone should be recommended only to outpatients with CAP and co-morbidities, age 65 and older or to those patients who recently underwent antibiotic treatment, or as an alternative to the combination of a beta-lactam antibiotic and a macrolide in patients with mild to moderate disease (83, 84). Furthermore, the fluoroquinolone should be recommended to adults with previous treatment failure, patients with confirmed allergy to penicillin, or patients with *S. pneumoniae* infection caused by penicillin-resistant strain. Croatian guidelines for the treatment of community-acquired pneumonia in adults recommend fluoroquinolones as an alternative monotherapy for patients with pneumococcal infection caused by penicillin-resistant *S. pneumoniae* strains (85). Alarming, recent reports described an increasing trend in the number of pneumococcal isolates with reduced susceptibility to fluoroquinolones (53, 88). Cases of treatment failure in pneumococcal pneumonia caused by fluoroquinolone non-susceptible isolates have emphasized the potential clinical importance of this emerging problem (89).

## 1.6. Prevention

Pneumococcal vaccination is a principal preventive health care measure which significantly minimizes the burden of pneumococcal disease, not only among vaccinated individuals but in the overall population, as well. Vaccination against invasive pneumococcal disease is a routine part of infant and childhood immunization programmes globally. Furthermore, this preventive measure is indicated for adults in a risk group for acquiring severe, invasive forms of pneumococcal diseases. The surface capsular polysaccharides of *S. pneumoniae* produce serotype-specific immune responses. Although more than 100 pneumococcal serotypes have been identified nowadays, all pneumococcal vaccines include the predominant most virulent serotypes.

Today, two types of pneumococcal vaccines, polysaccharide and conjugate, are currently licenced for use in humans. Both vaccines are characterized by different immunogenicity, efficacy, target populations and configuration.

Pneumococcal polysaccharide vaccine (PPV23) was licensed in the USA in 1983 for immunisation of adults and children  $\geq 2$  years of age. Vaccine comprises 23 different pneumococcal capsular polysaccharides (serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F). These serotypes altogether provide protection for 85% to 90% of IPD cases. In some high-impact countries, PPV23 is included in the vaccination programme for adults  $\geq 65$  years of age and people with co-morbidities focusing on the prevention of pneumonia and bacteraemia (90, 91). Two significant limitations are specific to the capsular polysaccharide-based vaccine. Firstly, humoral immunity is achieved through direct activation of B-cells, without the participation of T-helper cells resulting in a very low immunogenicity detected in children  $< 2$  years of age comparing to the conjugate vaccines (92). Secondly, although a significant number of serotypes are already included in the vaccine, still not all are covered.

The first pneumococcal conjugate vaccine, 7-valent (PCV, including serotypes 4, 6B, 9V, 14, 18C, 19F and 23F), was licensed in 2000 in the USA. The main characteristic and the difference of 7-valent PCV compared to PPV23 are conjugation of capsular polysaccharides to diphtheria toxin. This major difference provides higher immunogenicity with a more vigorous immune response. Conjugated vaccines, characterized by activation of both, T- and B-cells, provoke the production of antibodies, induction of immunological memory and long-term immunity. Since 2006, WHO recommended the pneumococcal conjugate vaccine for routine childhood

vaccination schedule. By 2012 conjugate vaccine was introduced in many WHO member states and European countries, 44% and 49% respectively (93).

In Europe, since 2009, two pneumococcal conjugate vaccines (PCV) are available, a 10-valent vaccine (PCV10, including PCV7 serotypes plus serotypes 1, 5 and 7F) and a 13-valent vaccine, (PCV13, including PCV10 serotypes plus serotypes 3, 6A and 19A) (6, 7). PCV10 is registered for infants (>6 weeks) and young children, up to 5 years of age. The recommendation for the PCV13 vaccine changed over time. In February 2010, FDA licensed the vaccine for the prevention of IPD and AOM in infants and young children. Additionally, in December 2011, PCV13 was recommended for the prevention of pneumonia and IPD in people aged  $\geq 50$  years (94). Finally, based on the results of the CAPITA study, in 2015, the license was extended to adults 18 to 49 years of age (95). In the USA and recently in Europe, two additional PCVs are registered, 15-valent (including PCV13 plus serotypes 22F and 33F) and 20-valent (including PCV15 serotypes plus serotypes 8, 10A, 11A, 12F and 15B). Prior to the introduction of PCV, 6–11 serotypes accounted for  $\geq 70\%$  of all invasive pneumococcal diseases occurring in children worldwide (96). All conjugate vaccines markedly improved immune response in neonates and young children. Also, high effectiveness was manifested in the prevention of life-threatening infections in children caused by the vaccine serotypes. PCV use in infants was recommended by the WHO which resulted in the successful introduction of these vaccines in the childhood immunization program of many industrialized and non-industrialized countries. All conjugate vaccines experienced the same post-vaccination effect in the population, an increase of IPD cases caused by non-vaccine serotypes. PCV10 and PCV13 resulted in an increase of pneumococcal infections caused by 19A and 35B (not represented either in 10- or in 13- valent vaccines), respectively (80, 97). However, the introduction of conjugate vaccines resulted in the overall reduction of IPD cases. Additionally, a significant reduction in antibiotic consumption was recorded in many countries after the introduction of the conjugate vaccine in the childhood vaccination schedule (66, 98, 99). Another huge advantage of conjugate vaccines is indirect so-called herd immunity. In the nasopharyngeal mucosa of vaccinated children, vaccine serotypes were replaced with those not included in vaccines, resulting in the stop in the horizontal spread of *S. pneumoniae* vaccine serotypes among children and consequently their spread to adults (100,101). The incidence of IPD significantly decreased among adults, especially in the vulnerable group,  $\geq 65$  years of age (102). Prior to the PCV7 vaccination in Europe and the USA, the mean incidence of IPD cases in children <2 years of age was 44.4 cases per 100 000 population and 167 cases per 100 000 population, respectively (17, 103).



CDC reported data on the reduction of IPD due to vaccination. Interestingly, in 1998 IPD cases linked to vaccine serotypes decreased by 98% in children <5 years of age compared to data for 2018. The overall IPD incidence declined from 61 cases per 100 000 population in 1998 to 24 cases per 100 000 population in 2018.

All EU Member State countries, except Malta and Estonia, have introduced conjugate vaccines to their childhood immunization schedule (104). In six countries (Bulgaria, Croatia, France, Hungary, Poland and Slovakia) vaccination is mandatory. Based on the latest IPD annual report of the European Centre for Disease Prevention and Control (ECDC), infections caused by non-vaccine serotypes have slowly increased over time (105). In 2018, a high percentage of IPD cases were caused by non-PCV serotypes, with 75% of IPD cases in children <5 years of age and 71% of IPD in adults  $\geq 65$  years of age (21).

Pneumococcal diseases are a major public health problem globally, and therefore the introduction of vaccines for infants, children and older adults is a priority in many countries. So far, the majority of European countries have conjugate vaccines in their NIP.

Information on the distribution of serotypes among invasive *S. pneumoniae* strains is extremely important for the prevention of IPD providing the information valuable for choosing the most appropriate pneumococcal vaccine. Since 1987, the PPV23 was the only vaccine available for immunization of adults. In Europe and the USA, the PCV13 was additionally approved for adults  $\geq 65$  years of age in 2011 and all adult groups in 2015. In Croatia, PPV23 was registered for adults and children  $\geq 2$  years in 2014, while registration dates for PCV13 were the same for all EU countries. Both vaccines have high potential coverage of invasive pneumococcal isolates together with high coverage of the resistant isolates. The Croatian Institute of Public Health revised the recommendations for pneumococcal vaccination of adults in January 2021. Immunocompetent adults are advised to be vaccinated with PPV23 only, while both vaccines are recommended for immunocompromised and asplenic patients, starting with PCV13 as the first dose. Pneumococcal conjugate vaccines have been associated with a reduction in antibiotic consumption which, in the long term, contributes to a lower antimicrobial selection pressure (106).

## 2. Hypothesis

1. Serotype distribution of invasive *Streptococcus pneumoniae* isolates is depending on the age of the patient and is changing over time.
2. Most of the invasive *Streptococcus pneumoniae* isolates in Croatia belong to serotypes that are covered by currently available vaccines.
3. Resistance to quinolones in invasive *Streptococcus pneumoniae* isolates is still low in Croatia, but non-susceptibility to penicillin and resistance to macrolides is high.

### **3. Aims and purpose of the research**

#### 3.1. General aim:

To observe changes in the characteristics (serotypes, antimicrobial resistance) of invasive pneumococcal strains depending on the time period and patient age and to determine the extent of coverage by currently available vaccines.

#### 3.2. Specific aims:

1. To determine the serotype distribution of invasive *Streptococcus pneumoniae* isolates
2. To determine the antimicrobial susceptibility of invasive *Streptococcus pneumoniae* isolates
3. To determine the mechanisms of resistance to macrolides and quinolones among invasive *Streptococcus pneumoniae* isolates

## 4. Materials and methods

### 4.1. Strain collection

Invasive *S. pneumoniae* strains were collected throughout Croatia through the network of microbiological laboratories engaged in the national antibiotic resistance surveillance organised as the Croatian Committee for Antibacterial Resistance Surveillance (CARS). The collection of invasive strains was initiated by the European Antimicrobial Resistance Surveillance System (EARSS) project that evolved into the European Antimicrobial Surveillance Network (EARS-Net) program at the European Centre for Disease Prevention and Control (ECDC). The Reference Centre for Antibiotic Resistance Surveillance (RCARS) at the University Hospital for Infectious Diseases “Dr. Fran Mihaljević” provides laboratory support for the national antibiotic resistance surveillance that among other things includes resistance surveillance of invasive and non-invasive isolates of *S. pneumoniae*. Consecutive, non-copy, invasive pneumococcal isolates, collected from 2005 to 2019, were analysed. The strain collection is very representative of the Croatian population as the microbiological laboratory network coverage is more than 95%.

### 4.2. Culture and identification of *Streptococcus pneumoniae*

*S. pneumoniae* strains isolated from primary sterile body sites (blood, cerebrospinal fluid) were first cultured and tested in the local microbiological laboratories and then delivered to the RCARS in a transport medium (Stuart or Aimes, Copan, Italy). When received at the RCARS the isolates were subcultured on Columbia blood agar (Oxoid, USA), incubated at  $35\pm 1^\circ\text{C}$  in an atmosphere with 5%  $\text{CO}_2$  for 18-24h. The identification of *S. pneumoniae* isolates was confirmed by Gram staining, colony morphology including the presence of  $\alpha$ -haemolysis on the Columbia blood agar, a positive optochin disc test and a positive bile-solubility test.

#### 4.2.1. Colony morphology

The colony morphology of *S. pneumoniae* greatly depends on the production of the capsule. Large, mucoid colonies were observed in strains with an abundant capsule. On the blood agar, these colonies are greyish, very mucoid and measure several millimetres in diameter. Usually,

much smaller colonies on the blood agar have a typical appearance compared to those pneumococcal strains that are less heavily encapsulated.

#### 4.2.2. Optochin disc test

Optochin (ethylhydrocupreine hydrochloride) disc (Oxoid, Basingstoke, England) is routinely used as a screening disc for the identification of *S. pneumoniae* strains. This quinine derivative can differentiate *S. pneumoniae* from other  $\alpha$ -hemolytic streptococci based on its power to selectively inhibit the growth of *S. pneumoniae* colonies. The sensitivity of the test is very high, > 95% (107).

The optochin disc test was performed by the disk diffusion method on the Columbia blood-agar medium. A few single colonies of the tested bacterium were inoculated on blood agar and an optochin-impregnated filter paper disc was placed on that streaked part and incubated for 18-24h. *S. pneumoniae* ATCC 49619 was used as a control strain. After overnight incubation, the growths of *S. pneumoniae* colonies were observed around the optochin disc. If tested bacterial colonies produced an inhibition zone of  $\geq 14$  mm, around a 6-mm disc, screening test was positive, meaning the tested bacterium was identified as *S. pneumoniae*. If the inhibition zone was <14 mm, the screening test was negative which required additional testing.

Nowadays, less than 5% of *S. pneumoniae* strains are resistant to optochin, therefore, the identification must be done with another, more sensitive test (108). Usually, final confirmation is done with a bile solubility test.

#### 4.2.3. Bile-solubility test

The bile-solubility test is the most commonly used confirmation test for the identification of *S. pneumoniae*. Bacterial strains negative on the optochin screening disc require an additional confirmation test. The bile solubility test is based on the ability of the bile salt to activate autolytic enzymes of *S. pneumoniae* and additionally accelerate bacterial lytic activity (109).

The test is very easy to perform, either in a tube or on the agar plate.

In the tube test, a bile-salt solution (sodium-deoxycholate, Sigma-Aldrich, USA), 2% sodium deoxycholate, was added in a heavily prepared inoculum of tested bacterium and incubated at  $35\pm 1^\circ\text{C}$  for three hours. *S. pneumoniae* ATCC 49619 was used as a control strain. The activation of the autolytic enzyme was manifested with the clearing of turbidity of the bacterial inoculum compared to a control tube without bile salt added. On the plate test, a few drops of

the bile-salt solution were placed on  $\alpha$ -haemolytic colonies and incubated at  $35\pm 1^\circ\text{C}$  for 30 minutes. If bacterial colonies were not present after incubation, meaning the  $\alpha$ -hemolytic zone on the blood agar was clear of bacterial colonies, the activation of *S. pneumoniae* autolytic enzymes had occurred confirming identification of *S. pneumoniae*.

As only 86% of *S. pneumoniae* strains lyse completely in the presence of bile salts, additional tests might be needed occasionally.

#### 4.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility of *S. pneumoniae* strains was tested by the disk diffusion and gradient test methods. In pneumococcal strains positive on the oxacillin screening disc, minimal inhibitory concentration (MIC) for penicillin and ceftriaxone was determined by gradient test (E-test, Biomerieux, France). Antimicrobial susceptibility testing followed the CLSI recommendation from 2005 to 2010 and the EUCAST recommendation from 2011 to 2019 (110). *S. pneumoniae* ATCC 49619 was used as a control strain. MIC for erythromycin was determined for the strains resistant or susceptible, increased exposure to macrolides. In *S. pneumoniae* strains with reduced susceptibility to norfloxacin (inhibition zone  $<12$  mm), the susceptibility to moxifloxacin was determined by the disk diffusion method.

##### 4.3.1. Disk diffusion method

The disk diffusion method is a qualitative test. The methodology is based on the diffusion of antibiotics from the disc in the agar previously inoculated with a suspension of the tested bacterium. The filter paper discs are impregnated with defined antibiotic concentrations. The effective antibiotics will inhibit the bacterial growth around the filter paper disc forming the inhibition zone (110). The size of the inhibition, expressed in millimetres, is proportional to the efficacy of an antibiotic. Susceptibility to oxacillin, erythromycin, norfloxacin and moxifloxacin was determined by the disk diffusion method. Three categories of susceptibility to antibiotics are declared: susceptible, resistant or susceptible, increased exposure (111). Measured inhibition zone diameter was compared with the CLSI (from 2005 to 2011) and the EUCAST breakpoint values (from 2011 to 2019) and the corresponding susceptibility category was reported. The EUCAST breakpoint tables are available on the EUCAST official website, free of charge, [www.eucast.org](http://www.eucast.org).

#### 4.3.2. Determination of minimal inhibitory concentration

MIC of *S. pneumoniae* strain was determined using the gradient diffusion test method (E-test, Biomerieux, France). The gradient test was placed on a previously inoculated (within 15 minutes) Mueller Hinton-Fastidious (MH-F) medium (Oxoid, USA) with 0.5 McFarland bacterial suspension and incubated at 35±1°C for 18-24 hours. Reading of MIC values was done according to the manufacturer's recommendations. After overnight incubation on the MH-F agar plate, an ellipsoid inhibition zone or bacterial overgrowth appeared. The MIC value was read at the point of complete inhibition of the bacterial growth. Interpretation of the MIC value was done according to the CLSI and EUCAST breakpoint tables.

#### 4.4. Serotyping of *Streptococcus pneumoniae*

The capsular swelling test, (the Quellung reaction, also known as Neufeld reaction) remained the gold standard method for serotyping of *S. pneumoniae* isolates through the years (112). The method is based on the reaction between the capsular polysaccharides (antigen) and antibodies, which are present in specific pneumococcal antisera (commercially available as pooled, group, or serotype-specific). In the reference and research laboratories worldwide, this method is used for serotyping of several important human pathogens such as *S. pneumoniae*, *N. meningitidis*, *H. influenzae*, *Escherichia coli* and *Salmonella* spp. The Quellung reaction is simple to perform method where the antigen-antibody reactions are observed microscopically. The binding of the capsular polysaccharide antigen with a type-specific antibody, contained in the serotyping antiserum, will be reported as a positive Quellung reaction (113).

The latex agglutination method is based on the reaction of capsular polysaccharide (antigen) and latex beads coated with a specific capsular antibody. If positive, the latex beads will clump together (agglutinate).

Each *S. pneumoniae* strain was serotyped by latex agglutination method (Statens Serum Institut, Copenhagen, Denmark) and/or Quellung reaction (Statens Serum Institut, Copenhagen, Denmark)

#### 4.5. Detection of resistance mechanisms

In invasive isolates of *S. pneumoniae* non-susceptible to erythromycin, by disk diffusion and gradient test methods, and in those resistant to moxifloxacin by disk diffusion, the mechanism of resistance was determined using molecular methods.

##### 4.5.1. Macrolides

The presence of macrolide resistance genes, *ermB* and *mefA*, was detected by polymerase chain reaction (PCR) and subsequent agarose gel electrophoresis (48,114). For bacterial DNA isolation, 1.0 McFarland bacterial suspensions were incubated at 99°C for 10 minutes and then centrifuged at 20 000 g for 5 minutes. Crude DNA from the supernatant was used for PCR. All primers were used in a final concentration of 0.2 µM, with 0.45 units of Hot Start Polymerase Apta+ (Jena Bioscience, Germany). PCR reaction was performed on Veriti™ Thermal Cycler (Thermo Fisher Scientific, USA) with 30 seconds annealing step (Table 1). PCR product visualisation was performed by 1% agarose gel electrophoresis using SYBR® safe DNA gel stain and GeneRuler 100 bp Plus DNA Ladder (both Thermo Fisher Scientific, USA). PCR product sizes were 617 base pairs (bp) for *ermB* and 1759 bp for *mefA*.

Table 1. Primers used for the detection of macrolides resistance genes

Gene	Primer	Sequence	Annealing temperature	Reference
<i>ermB</i>	ermB_F	5'-CGA GTG AAA AAG TAC TCA ACC-3'	55°C	(48, 114)
	ermB_R	5'-GGC GTG TTT CAT TGC TTG ATG-3'	59°C	
<i>mefA</i>	mefA_F	5'-GCGTTTAAGATAAGCTGGCA-3'	57°C	
	mefA_R	5'-CCTGCACCATTGCTCCTAC-3'	59°C	



#### 4.5.2. Fluoroquinolones

Detection of fluoroquinolone resistance mechanism was performed by sequence analysis of quinolone resistance determining regions (QRDRs) of *gyrA* and *parC* genes. Crude bacterial DNA isolation, PCR and gel electrophoresis were performed in the same way as for macrolide resistance genes (Table 2) (115). Amplicons were cleaned up using DNA Clean & Concentrator™-5 (ZYMO Research, USA) according to the manufacturer's instructions. Sequencing reactions were done using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA) and sequencing products were cleaned up using BigDye XTerminator™ Purification Kit (Thermo Fisher Scientific, USA). Capillary electrophoresis was performed on a 3500 Genetic Analyzer (Thermo Fisher Scientific, USA). Obtained sequences were analysed using DNA Baser sequence assembly software (Heracle BioSoft S.R.L., Romania).

Table 2. Primers for detection of fluoroquinolone resistance genes

Gene	Primer	Sequence	Annealing temperature	Reference
<i>gyrA</i>	gyrA_F	5'-TG TTCACCGTCGCATTCTCT-3'	55°C	(115)
	gyrA_R	5'-ATACCAGTTGCTCCATTAACC-3'	55°C	
<i>parC</i>	parC_F	5'-CGGTTCAACGCCGTATTCTT-3'	59°C	
	parC_R	5'-ATCCCAGTCGAACCATTGAC-3'	57°C	

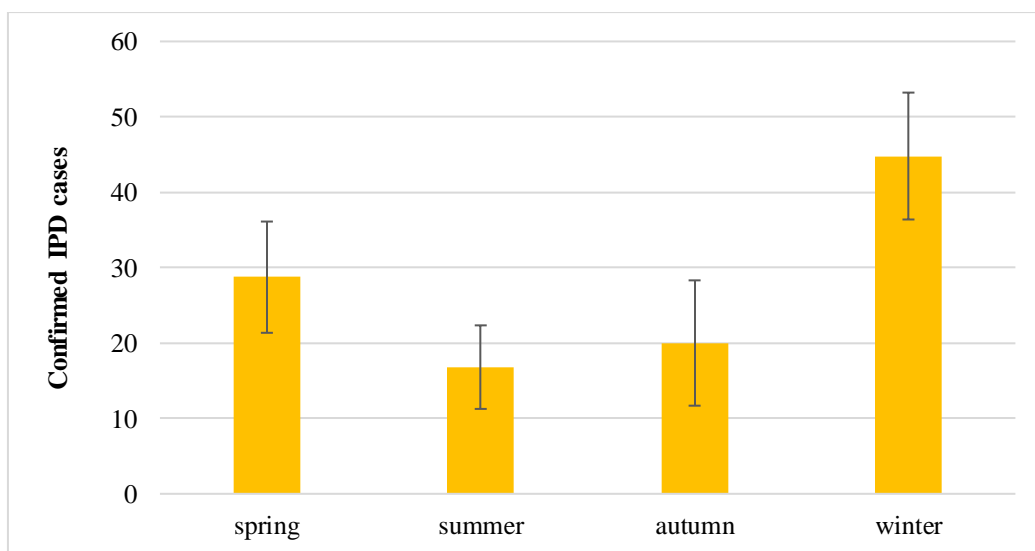
#### 4.6. Statistical analysis

Comparisons of serotype frequencies among underlying symptoms and between age groups were performed by Fisher's exact test or the chi-squared test, as appropriate. To assess the age difference in patients infected with a certain serotype, a two-sided Mann-Whitney U test was used. Repeated measures analysis of variance (repeated measures ANOVA) was used in order to compare IPD incidence among age categories and to assess the seasonality of IPD cases during the 15 years (2005-2019). Statistical analysis was performed using SPSS 19.0 (Chicago,

IL),  $p$  values less than 0.05 were considered significant. Parsons correlation coefficient was calculated in order to assess if the number of overall confirmed IPD cases linked to a particular serotype has changed throughout the study period in a trend-like fashion.

## 5. Results

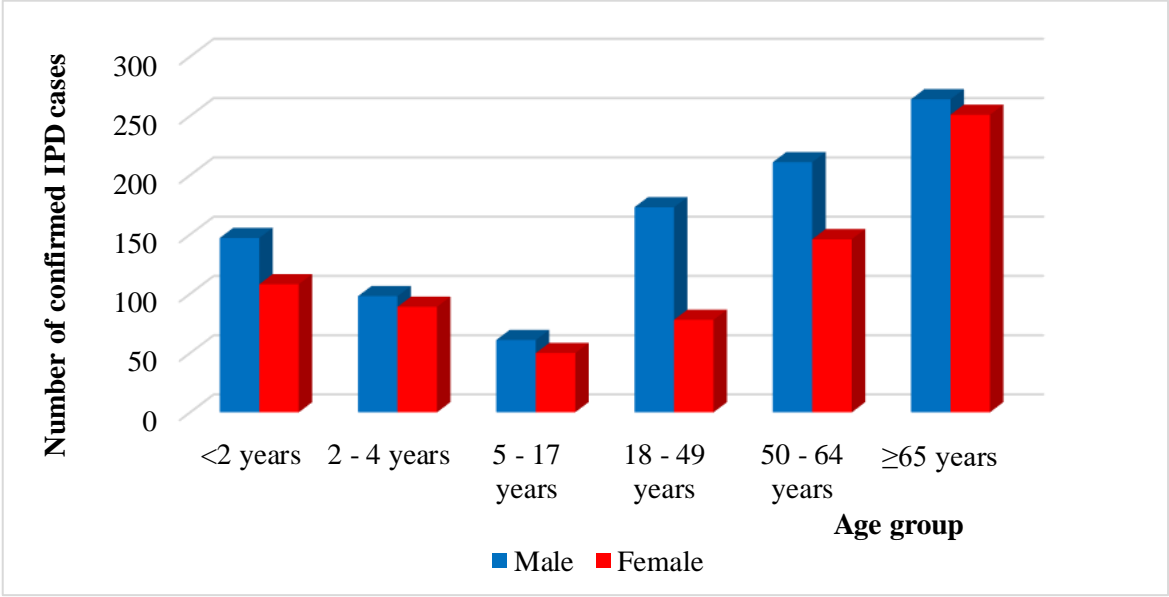
A total of 1854 non-copy, consecutive, invasive isolates of *S. pneumoniae* were collected during the fifteen-year period. All data were analysed anonymously. The overall incidence of IPD was 2.92 confirmed cases per 100 000 population (ANOVA,  $P < 0.05$ , 95% CI: 2.65-3.19). During the study period, the number of invasive infections caused by *S. pneumoniae* showed a seasonality pattern typical for the majority of respiratory pathogens. The lowest numbers of patients were observed during summer, increased during autumn and reached a seasonal peak through the winter months (Figure 1).



**Figure 1.** Seasonal distribution of confirmed IPD cases, 2005 – 2019

A total of 1666 isolates were available for further analysis while 188 isolates were excluded from the study either due to insufficient patient and/or clinical data or due to no growth when subcultured from  $-80^{\circ}\text{C}$ . The median age for the analysed study population was 51 (< 6 months of age to 97 years). The median age for children <18 years of age and adults  $\geq 18$  years of age was 2 (<6 months of age to 17 years) and 62 (18 to 97 years), respectively. All isolates were obtained from primary sterile sites with a predominance of strains isolated from the blood samples (1488 isolates or 89.3%), followed by 163 isolates (9.8%) from CSF samples and 15 isolates from both samples taken from the same patient (0.9%). Overall male to female ratio was 1.34:1. A total of 543 invasive *S. pneumoniae* strains (32.6%) were isolated from children <18 years of age. Almost half of the paediatric strains collected (255 strains or 47%) were isolated from children <2 years of age followed by 187 strains (34.4%) isolated from children

2 to 4 years of age while the lowest number of strains (101 or 18.6%) was isolated from children 5 to 17 years of age. Among 1123 invasive strains isolated from adults, the highest number of *S. pneumoniae* strains, 515 or 45.9%, was isolated from patients 65 years and older. The lowest number of strains was collected among adults, 18 to 49 years of age (251 strains or 22.3%) while 357 strains were isolated from adults, 50 to 64 years of age (31.8%). The highest numbers of strains were isolated in children <5 years (444/1666 or 26.5%) and adults 65 and older (515/1666 or 30.9%) (Figure 2).



**Figure 2.** Distribution of confirmed IPD cases by age and gender

Among isolates in which demographic data were available, the highest incidence of IPD was observed in two major risk groups, children <5 years of age and adults 65 years and older (Table 3).

**Table 3.** Incidence of IPD cases per 100 000 population by age groups

Age group (years)	0 - 4	5 - 9	10 - 19	20 - 49	50 - 64	≥65	Overall collection
IPD incidence / 100000 population	14.54	2.46	0.45	0.93	2.67	4.47	2.92
95% CI (min-max)	12.49 -16.59	2.09 - 2.82	0.24 - 0.65	0.77 - 1.1	2.33 - 3.02	3.78 - 5.17	2.65 - 3.19

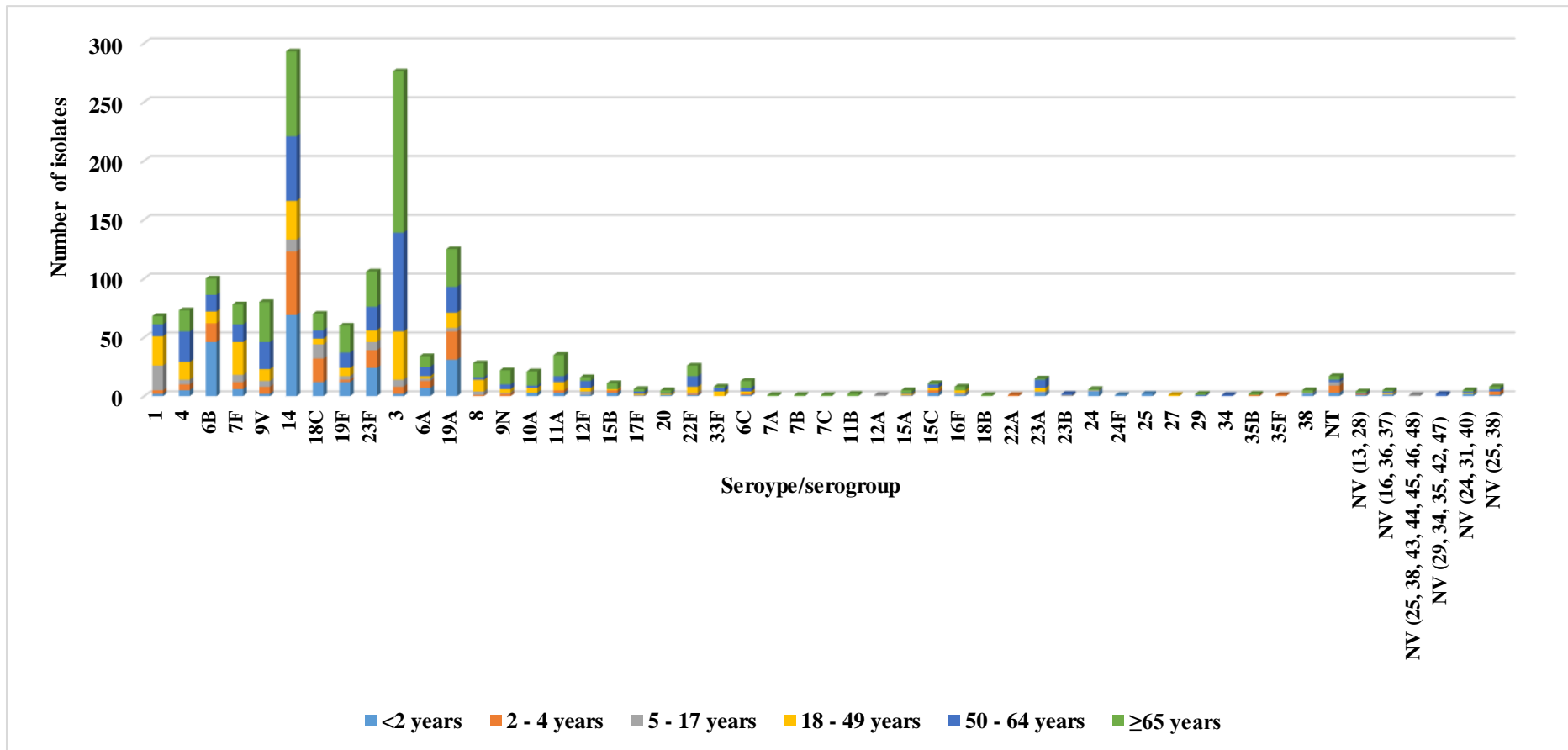
Serotype was determined in 1624 isolates (97.3%), while 17 isolates were non-typeable. For 25 invasive isolates, serotyping was finished at the non-vaccine group level according to the Staten Serum Institute scheme for serotyping of *S. pneumoniae* (116). Among fifty-one different *S. pneumoniae* serotypes identified, only a few were represented with higher frequency. In the whole study collection, the six most prevalent serotypes were 14 (293 isolates or 17.8%), 3 (276 isolates or 16.8%), 19A (125 isolates or 7.6%), 23F (106 isolates or 6.5%), 6B (100 isolates or 6%) and 9V (80 isolates or 5%) comprising 60% of all invasive *S. pneumoniae* isolates (Table 4., Figure 3).

**Table 4.** Ranking of serotype prevalence among different age group

	All	<2 years	2 - 4 years	5 - 17 years	18 - 49 years	50 - 64 years	≥65 Years
<b>14</b> <sup>a,d</sup>	1	1	1	3	2	2	2
<b>3</b> <sup>c,d</sup>	2	12	6	5	1	1	1
<b>19A</b> <sup>c,d</sup>	3	3	2	8	6	5	4
<b>23 F</b> <sup>a,d</sup>	4	4	5	4	7	6	5
<b>6B</b> <sup>a,d</sup>	5	2	4	11	7	8	9
<b>9V</b> <sup>a,d</sup>	6	12	6	6	7	4	3
<b>7F</b> <sup>b,d</sup>	7	8	6	5	3	7	8
<b>4</b> <sup>a,d</sup>	8	9	7	7	5	3	7
<b>18C</b> <sup>a,d</sup>	9	5	3	2	9	13	9
<b>1</b> <sup>b,d</sup>	10	12	8	1	4	10	11
<b>19F</b> <sup>a,d</sup>	11	6	9	8	8	9	6
<b>11A</b> <sup>d</sup>	12	11	9	11	8	15	7
<b>6A</b> <sup>c</sup>	13	7	6	9	12	12	10
<b>8</b> <sup>d</sup>	14	14	10	8	7	18	10
<b>22F</b> <sup>d</sup>	15	13	10	10	9	11	10
<b>9N</b> <sup>d</sup>	16	14	9	10	11	16	10
<b>10A</b> <sup>d</sup>	17	11	11	11	10	18	10
<b>NT</b>	18	11	6	8	14	18	14
<b>12F</b> <sup>d</sup>	19	13	11	8	11	14	14
<b>23A</b>	20	11	11	10	11	13	16
<b>6C</b>	21	13	10	11	12	17	12
<b>15B</b> <sup>d</sup>	22	11	9	11	13	20	13
<b>15C</b>	22	11	9	11	12	17	16
<b>33F</b> <sup>d</sup>	23	14	11	11	10	17	16
<b>16F</b>	23	13	11	9	12	20	14

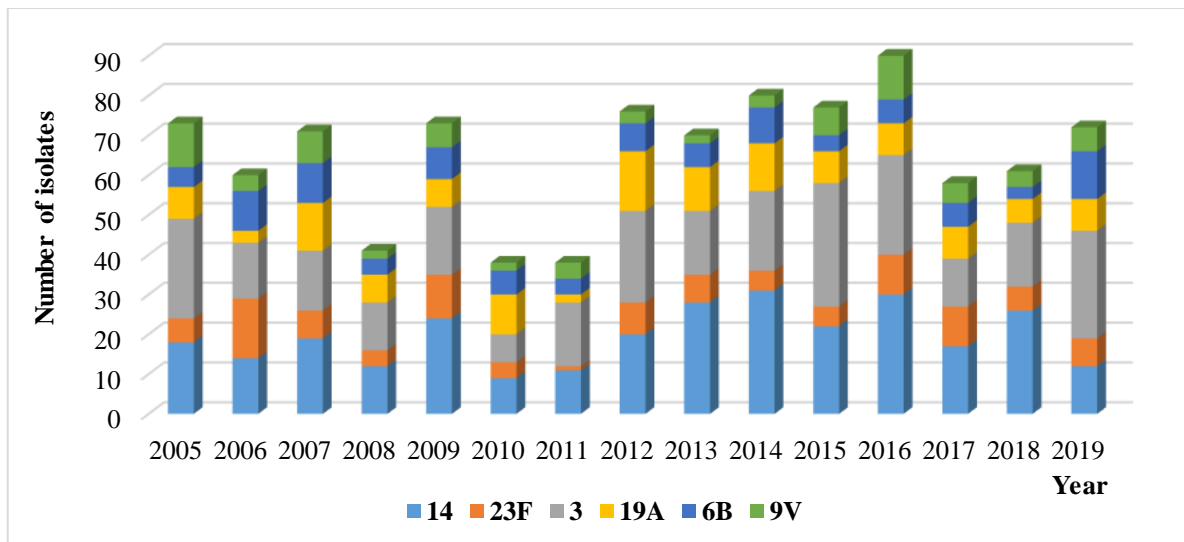
	All	<2 years	2 - 4 years	5 - 17 years	18 - 49 years	50 - 64 years	≥65 Years
<b>NV (25, 38)</b>	<b>23</b>	<b>13</b>	<b>8</b>	<b>11</b>	<b>14</b>	<b>18</b>	<b>15</b>
<b>17F<sup>d</sup></b>	24	14	11	10	13	18	15
<b>24</b>	24	10	11	10	14	20	16
<b>20<sup>d</sup></b>	25	13	11	11	13	19	15
<b>15A</b>	25	14	11	10	13	19	15
<b>38</b>	25	12	11	10	14	20	15
<b>NV (16, 36, 37)</b>	25	13	1	10	13	19	16
<b>NV (24, 31, 40)</b>	25	12	11	11	13	19	16
<b>NV (13, 28)</b>	26	13	10	11	14	19	16
<b>11B</b>	27	14	11	11	14	20	15
<b>23B</b>	27	14	11	10	14	19	17
<b>25</b>	27	12	11	11	14	20	17
<b>29</b>	27	14	11	11	14	19	16
<b>35B</b>	27	14	10	11	14	20	16
<b>NV (29, 34, 35, 42, 47)</b>	27	14	11	11	14	18	17
<b>7A</b>	28	14	11	11	14	20	16
<b>7B</b>	28	14	11	11	14	20	16
<b>7C</b>	28	14	11	11	14	20	16
<b>12A</b>	28	14	11	10	14	20	17
<b>18B</b>	28	14	11	11	14	20	16
<b>22A</b>	28	14	10	11	14	20	17
<b>24F</b>	28	13	11	11	14	20	17
<b>27</b>	28	14	11	11	13	20	17
<b>34</b>	28	14	11	11	14	19	17
<b>35F</b>	28	14	10	11	14	20	17
<b>NV (25, 38, 43, 44, 45, 46, 48)</b>	28	14	11	11	14	20	17

\*NT – non-typeable, \*\*NV – non-vaccine group; a – PCV7 serotypes; b – 3 additional serotypes to PCV7 = PCV10; c – 3 additional serotypes to PCV10 = PCV13; d – PPV23 serotypes



**Figure 3.** Serotype distribution of *Streptococcus pneumoniae* isolates by age groups

No correlation was observed in the total number of IPD cases throughout the study period ( $r=0.17$ ,  $P 0.52$ ). Among all serotyped *S. pneumoniae* isolates, a strong negative correlation was observed only in serotype 1 ( $r= -0.739$ ,  $P <0.001$ ). A statistically significant difference in the incidence rates of the six most common serotypes by years was not observed (Figure 4).



**Figure 4.** Serotype distribution of the top six *Streptococcus pneumoniae* isolates by years

In children <18 years of age, the six most frequently isolated serotypes were 14 (133/24.5%), 6B (62/11.4%), 19A (58/10.7%), 23F (46/8.5%), 18C (44/8.1%) and 1 (26/4.8%) comprising 68 % of all paediatric strain collection. Serotype 14 predominated among infants (<2 years of age) and toddlers (2 to 4 years of age), while in older children, 5 to 17 years of age, a predominance of serotype 1 was observed. The six most prevalent serotypes among adults were 3 (262/50.9%), 14 (160/31%), 19A (67/13%), 9V (65/57.8%), 23F (60/11.6%) and 7F (60/11.6%) comprising 60% of all invasive strains collected in adults. The total predominance of serotype 3 was observed among all adult age groups reaching the highest numbers among patients 65 years and older (137/515 or 26.6%) (Table 5).



**Table 5.** Percentage of the six most prevalent serotypes of *S. pneumoniae* from confirmed IPD cases, by age group.

Age group (years)	<2 (n=255)	2 – 4 (n=187)	5 – 17 (n=101)	18 – 49 (n=251)	50 – 64 (n=357)	≥65 (n=515)
Six most common serotypes by age group (%)	14 (27)	14 (28.9)	1 (20.8)	3 (16.3)	3 (23.5)	3 (26.6)
	6B (18)	19A (12.8)	18C (11.9)	14 (13)	14 (15.4)	14 (14)
	19A (12)	18C (10.7)	14 (10)	7F (11)	4 (7.3)	9V (6.6)
	23F (9.4)	6B (8.6)	23F (7)	1 (10)	19A (6)	19A (6)
	18C (4.7)	23F (8)	3 (6)	4 (6)	9V (5.9)	23F (5.8)
	19F (4.7)	9V (3)	7F (6)	23F (4)	23F (8)	19F (4.6)

Among children <18 years of age, a statistically significant predominance of serotypes 14, 6B, 19A, 23F and 18C was observed while serotypes 3, 9V, 4 and 8 were statistically more common among adults (Table 6.).

**Table 6.** Serotypes tested for significant differences in their distribution among children and adults and differences in age distribution among patients with IPD caused by a particular serotype compared to patients with IPD caused by other serotypes

Serotype	Total number of patients	Distribution by age		P (Fisher's exact test)	Distribution by serotype		P (Mann-Whitney U-Test)
		% Children (n = 543)	% Adults (n = 1123)		Median age in years (IQR)*	P	
14	293	<b>24,49</b>	14,25	<b>&lt;0,01</b>	<b>35(62,5)</b>	<b>53(62)</b>	<b>&lt;0,01</b>
3	276	2,58	<b>23,33</b>	<b>&lt;0,01</b>	<b>64(22)</b>	<b>45(63)</b>	<b>&lt;0,01</b>
19A	125	<b>10,68</b>	5,97	<b>&lt;0,01</b>	<b>35(63)</b>	<b>52(64)</b>	<b>&lt;0,01</b>
23F	106	<b>8,47</b>	5,34	<b>&lt;0,05</b>	46,5(64)	52(64)	0,14
6B	100	<b>11,42</b>	3,38	<b>&lt;0,01</b>	<b>2(53)</b>	<b>52(63)</b>	<b>&lt;0,01</b>
9V	78	2,39	<b>5,79</b>	<b>&lt;0,01</b>	<b>62(31)</b>	<b>51(64)</b>	<b>&lt;0,01</b>
7F	78	3,31	5,34	0,064	46(37)	52(64)	0,57
18C	70	<b>8,10</b>	2,32	<b>&lt;0,01</b>	<b>5,5(57)</b>	<b>52(64)</b>	<b>&lt;0,01</b>
4	69	2,58	<b>5,25</b>	<b>&lt;0,05</b>	58(33)	51(64)	0,36
1	68	4,79	3,74	0,355	<b>33,5(41,5)</b>	<b>52(64)</b>	<b>&lt;0,05</b>
19F	60	3,13	3,83	0,57	58(59)	51(64)	0,52
6A	34	2,76	1,69	0,19	50,5(64)	51(64)	0,32
8	28	0,74	<b>2,14</b>	<b>&lt;0,05</b>	50(42,5)	51(64)	0,095

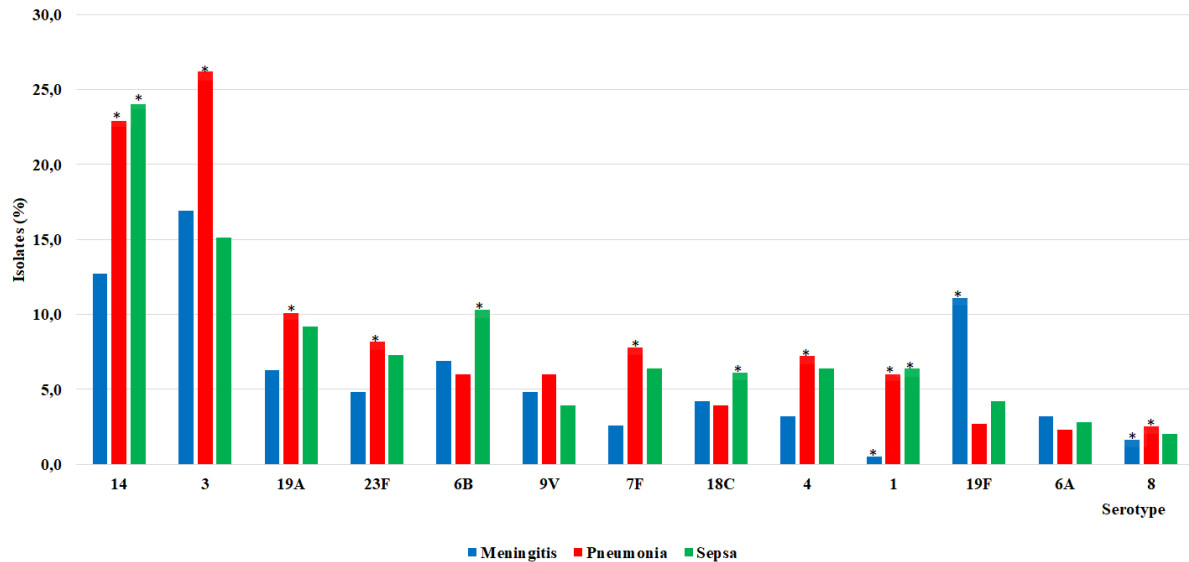
\* IQR – interquartile range

Throughout the study period, the most prevalent clinical presentation was bacteremic pneumonia, diagnosed in 633 patients (38%), followed by sepsis and meningitis in 432 (26%) and 195 patients (11.7%), respectively. The information regarding the clinical presentation was unknown for 263 patients (15.7%). Additional information concerning the patients' co-morbidities and life habits, recognized as risk factors for acquiring IPD, were not accessible. Pneumonia was the most common clinical presentation among adults, reported in 496 patients (44.2%) The most common clinical presentation in children was sepsis with 223 reported cases (41%). Serotype 3 was the most common serotype causing pneumonia and meningitis, while serotype 14 was most frequently isolated in septic patients (Table 7.).

**Table 7.** Distribution of the five most prevalent *S. pneumoniae* serotypes in confirmed IPD cases: by clinical presentation and by age groups

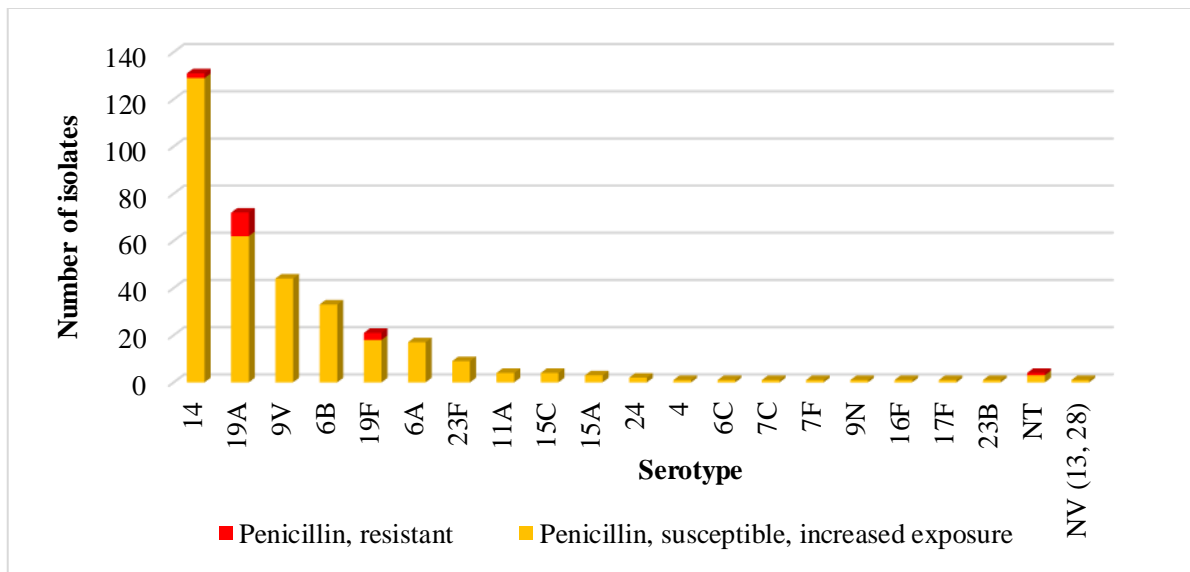
Clinical presentation	Pneumonia			Sepsis			Meningitis		
	all groups (n=633)	<5 years (n=130)	≥65 years (n=220)	all groups (n=432)	<5 years (n=193)	≥65 years (n=88)	all groups (n=195)	<5 years (n=25)	≥65 years (n=60)
Five most common serotypes by clinical presentation (% all cases/clinical presentation)	3 (20%)	14 (25.4%)	3 (29%)	14 (20.6%)	14 (28%)	3 (29.5%)	3 (17%)	6B (34.8%)	3 (21.7%)
	14 (17.5%)	19A (13.8%)	14 (16.4%)	3 (12.6%)	6B (15.9%)	14 (14.8%)	14 (12.8%)	14 (18%)	19F (18.3%)
	19A (7.7%)	6B (13.8%)	23F (8.6%)	6B (8.6%)	19A (11.6%)	9V (8%)	19F (10.8%)	19A (13%)	14 (8.3%)
	7F (6%)	23F (8%)	19A (6%)	19A (7.7%)	23F (9%)	19A (6.8%)	19A (6.2%)	7F (8,7%)	9V (6.7%)
	4 (5.5%)	18C (3.8%)	4 (5.5%)	23F (6.3%)	18C (8%)	7F (4.5%)	9V (4.6%)	6A (4.3%)	11A (6.7%)

Several serotypes had statistically significant higher contribution in some clinical presentations (Figure 5).



**Figure 5.** Serotype distribution based on the clinical presentation. The symbol \* indicates the statistically significant contribution of the serotype in clinical presentation.

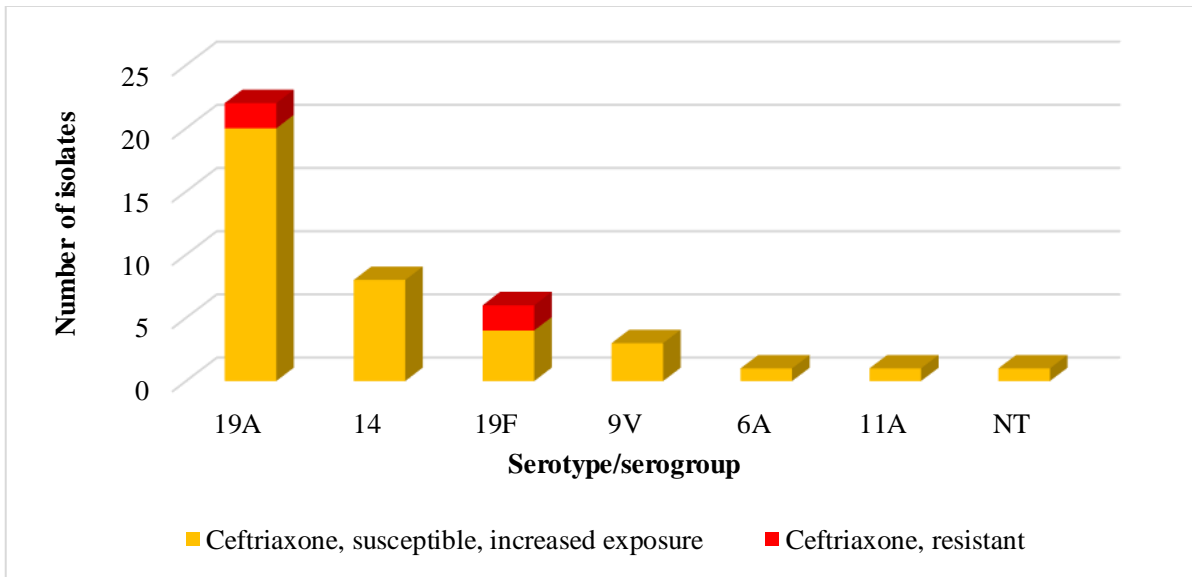
The susceptibility to penicillin was tested in 99.6% of all isolates. A total of 338 *S. pneumoniae* isolates (20%) were penicillin susceptible, increased exposure, mostly due to serotypes 14 (129 isolates or 38%), 19A (62 isolates or 18.3%) and 9V (44 isolates or 13%) accounting for 60% of all penicillin non-wild type isolates. The resistance to penicillin was less than 1% (Figure 6). Among 16 invasive isolates with penicillin MIC value >2 mg/L, 10 isolates belonged to serotype 19A, while three and two isolates belonged to serotypes 19F and 14, respectively. One isolate was non-typeable.



**Figure 6.** Serotype distribution of penicillin non-wild type *S. pneumoniae* isolates

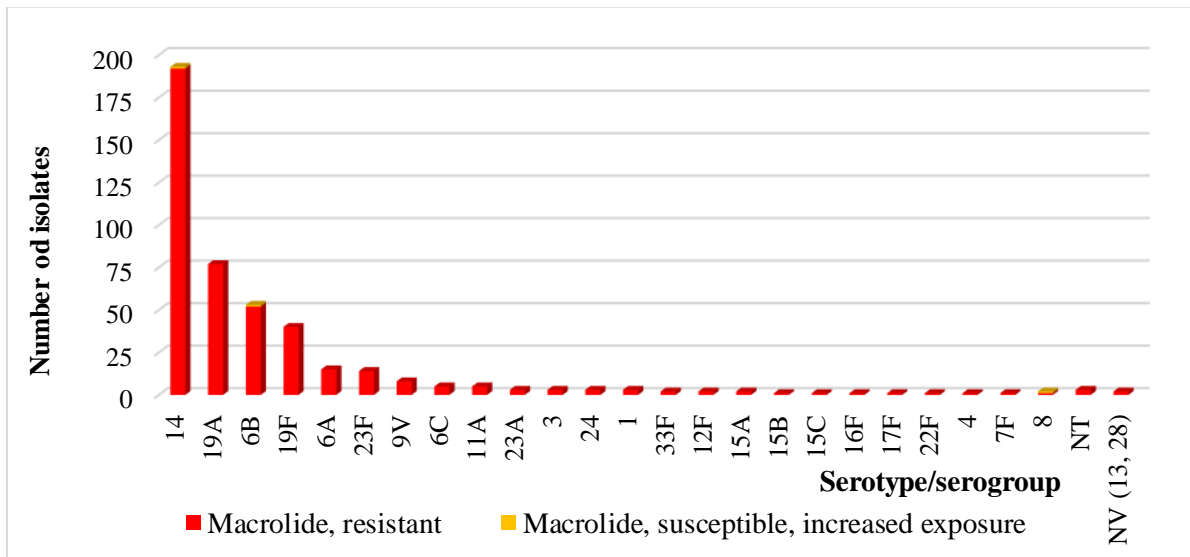
The percentage of penicillin non-wild type varied among age groups. In the whole paediatric population, penicillin susceptible, increased exposure isolates were detected in 24% of all isolates, with the highest rate of 28% among children <5 years of age. The resistance to penicillin was detected in 2% of isolates, mainly due to *S. pneumoniae* strains isolated in children up to 5 years of age. The lower percentage of penicillin susceptible, increased exposure isolates, 18%, was recorded in adults. The percentage of penicillin non-wild type isolates varied among adult groups. The lowest percentage, 14%, was observed in young adults, 18 to 49 years, while the highest percentage of 19% was observed among adults >50 years of age. The resistance to penicillin remained below 1% among all strains isolated in adults.

Antimicrobial susceptibility to third-generation cephalosporins, specifically ceftriaxone, was tested in 99.6% of isolates (1659/1666). Susceptibility to ceftriaxone was very high, 97.6% of all tested isolates. Only 2.2% of isolates (38/1659) were reported susceptible, increased exposure mainly due to serotypes 19A (20 isolates) and 14 (8 isolates), comprising 73.6% of all ceftriaxone susceptible, increased exposure isolates. Resistance to ceftriaxone was detected in four isolates only, belonging to serotypes 19A and 19F, two isolates each (Figure 7).



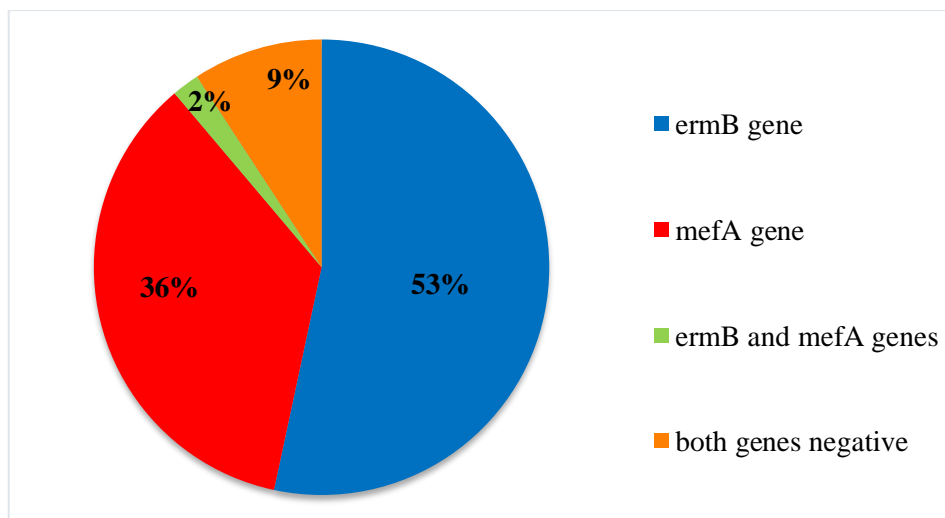
**Figure 7.** Serotype distribution of ceftriaxone non-wild type *S. pneumoniae* isolates

The susceptibility to macrolides was tested in 93.2% of all *S. pneumoniae* isolates. Macrolide resistance detected in 29.4% of all tested isolates (441/1553) mostly occurred in serotypes 14 (192 isolates or 43.5%), 19A (77 isolates or 17.4%) and 6B (52 isolates or 11.8%), comprising 73% of all macrolide-resistant isolates (Figure 8).



**Figure 8.** Serotype distribution of macrolide non-wild type *Streptococcus pneumoniae* isolates

Higher resistance to macrolides, 40%, was observed among children. The resistance rate varied significantly depending on the child`s age. The highest percentage of macrolide resistance, 46%, was detected in children up to 5 years, while in older children, 5 to 17 years, the resistance rate was significantly lower, detected in 15% of isolates, only. The highest macrolide resistance of 46% in children up to 5 years of age was associated with serotypes 14 (87 isolates), 19A (39 isolates) and 6B (32 isolates), comprising 83.6% of all macrolide-resistant isolates in that age group. The lowest macrolide resistance of 22.8% was recorded among the adult population. The presence of macrolide resistance genes was tested in 358 out of 441 erythromycin-resistant isolates (81.2%). Resistance genes were detected in 325/358 (90.8%) isolates: 191 isolates (53.4%) were *ermB* positive, 127 isolates (35.5%) were *mefA* positive while both genes were detected in seven isolates (2%). A total of 33 strains (9.2%) were tested negative for the presence of *ermB* and *mefA* genes (Figure 9). *S. pneumoniae* isolates containing the *ermB* gene mostly belong to serotypes 14 (61/191) and 19A (45/191) while isolates containing the *mefA* gene were mainly associated with serotype 14 (100/127 or 79%).



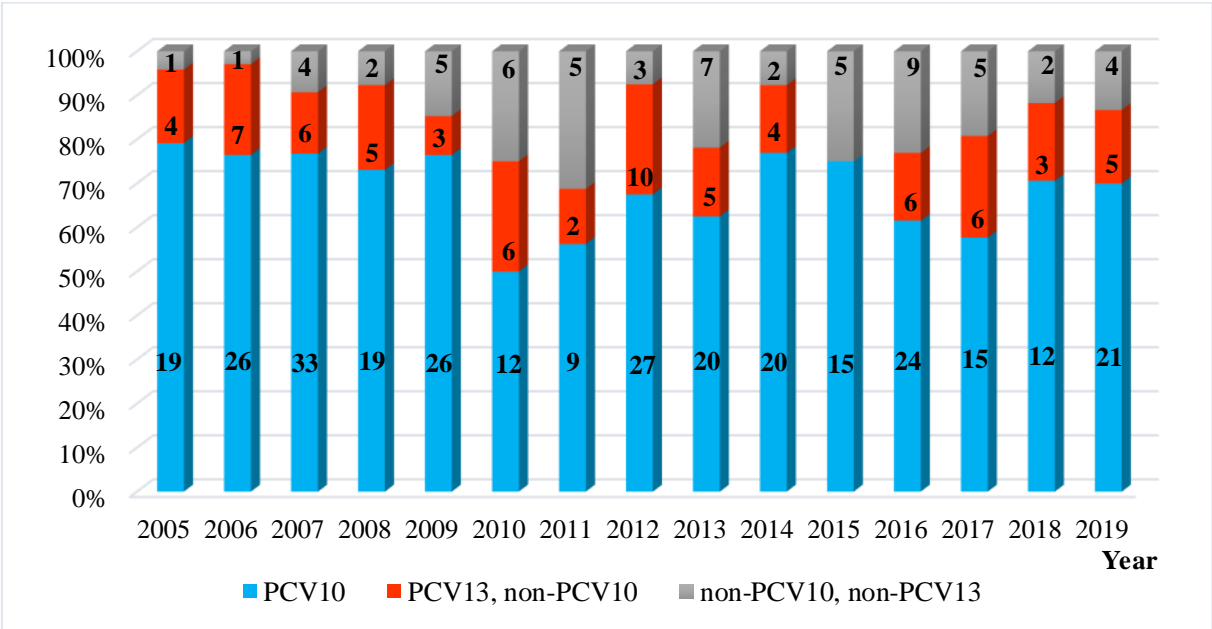
**Figure 9.** Mechanisms of macrolide resistance in *S. pneumoniae* isolates, detection of *ermB* and *mefA* genes

A total of 214 invasive isolates (13%) presented with a dual resistance profile, penicillin non-wild type and macrolide-resistant. Those pneumococcal strains mostly belonged to serotypes 19A (70 isolates or 32.7%) and 14 (67 isolates or 31.3%). Dual resistance was more common among *S. pneumoniae* strains isolated from children <5 years of age (89 isolates or 41.6%).

Four invasive isolates of *S. pneumoniae*, resistant to moxifloxacin by disk diffusion method, were further analysed for mutations in *gyrA* and *parC* genes. These isolates, isolated in adults, belonged to serotypes 23F, 22F, 9V and 19F. All four isolates had mutations in *parC* resulting in amino acid change (Ser79Phe in two isolates, Arg95Lys and Lys137Asn each in one isolate). Only one isolate had a mutation in *gyrA*, resulting in a change of serine to phenylalanine at position 81.

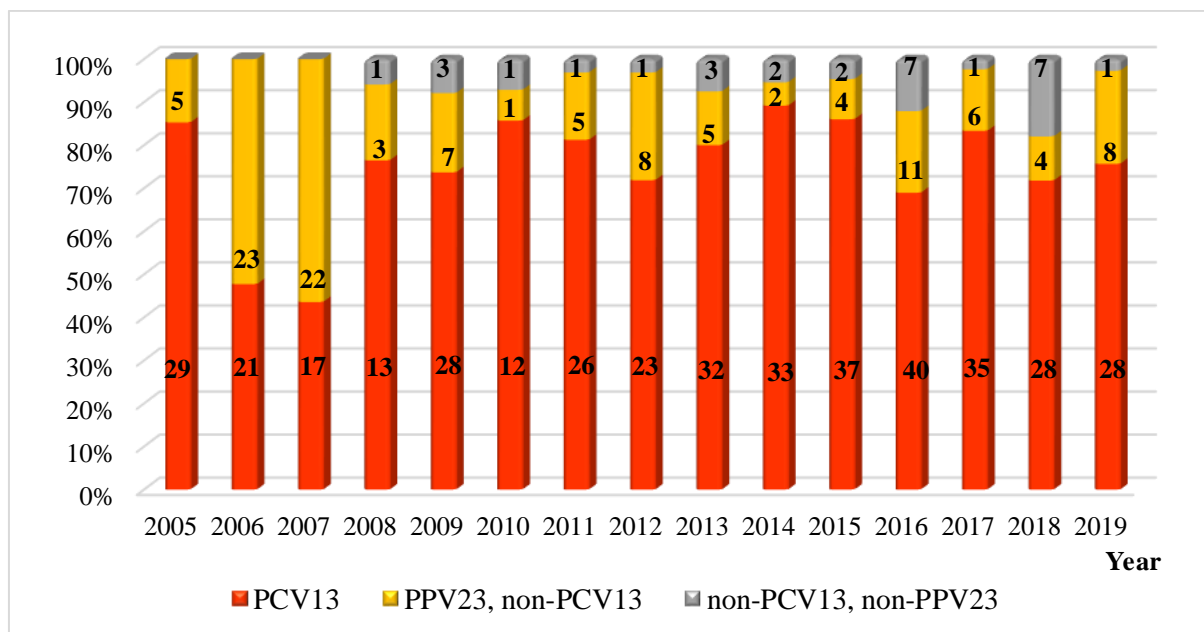
The PCV10 vaccine is recommended for vaccination of infants (>6 weeks of age) and children up to 5 years of age. During the study period, the coverage of a 10-valent conjugate vaccine for this risk group was lower compared to a 13-valent vaccine, 69 % and 86.2%, respectively.

During the study period, the vaccine coverage of PCV13 and PPV23 was 74.5% (1225/1643) and 90.6% (1506/1643), respectively. The PCV13 vaccine coverage varied among age groups. For all children, PCV13 coverage was 84.7%, while in young children up to 5 years of age it was a bit higher, 86.2%. During the study period, in children up to 5 years of age, the numbers of non-vaccine serotypes showed no significant variation without predominance of any particular serotype. In 2016, the highest number (9/39 isolates) of non-vaccine serotypes was detected (Figure 10.)



**Figure 10.** Confirmed cases of IPD in children <5 years of age: serotype distribution by PCV10 and PCV13 serotype and year, 2005–2019. Numbers in the columns indicate the numbers of isolates.

A lower percentage of PCV13 vaccine coverage, 80%, was observed in adults. This vaccine coverage in adults did not vary significantly depending on the age. In adults  $\geq 65$  years of age, PCV13 and PPV23 vaccine coverage were 79% (407/515) and 93.6% (482/515), respectively. The number of non-vaccine serotypes among adults showed minimal variation during the fifteen-year study period. In 2005, 2006 and 2007 all isolates were covered by PPV23 and PCV13. The highest number of non-vaccine serotypes, 7 out of 81 isolates, was recorded in 2018. A predominant non-vaccine serotype was not observed in the whole strain collection (Figure 11).



**Figure 11.** Confirmed cases of IPD in adults  $\geq 65$  years of age: serotype distribution by PCV13 and PPV23 serotype and year, 2005–2019. Numbers in the columns indicate the numbers of isolates.

The overall vaccine coverage of PCV13 and PPV23 for isolates causing pneumonia was 83.7% (530/633) and 92.5% (533/576), respectively. High vaccine coverage was observed for isolates causing sepsis. The PCV13 and PPV23 had coverage of 91.5% and 96.3%, respectively. For meningitis isolates, PCV13 and PPV23 vaccine coverage was 77% and 90%, respectively (Table 8.).



**Table 8.** The coverage of PCV13 and PPV23 vaccines of all populations and two major risk groups for the most common clinical presentations of IPD

Clinical presentation/ Vaccine coverage	Pneumonia			Sepsis			Meningitis		
	all groups (n=633)	<5 years (n=130)	≥65 years (n=220)	all groups (n=428)	<5 years (n=189)	≥65 years (n=88)	all groups (n=190)	<5 years (n=23)	≥65 years (n=60)
PCV13 serotype cases (%)	530 (83.7)	104 (80)	188 (85.4)	366 (92.6)	173 (91.5)	69 (78.4)	146 (76.8)	19 (82.6)	47 (78)
PPV23 serotype cases (%)	597 (94.3)	115 (88.5)	212 (96.4)	401 (93.6)	177 (93.6)	83 (93.2)	167 (87.9)	19 (82.6)	56 (90)

In the fifteen-year study period, the coverage of PCV10, PCV13 and PPV23 vaccines for penicillin non-wild type isolates was 58% (76/131), 93% (329/354) and 92% (254/276), respectively. Currently available vaccines showed no significant differences in the coverage of penicillin non-wild type pneumococcal isolates between children and adults. The percentage of macrolide resistant strains covered by PCV10, PCV13 and PPV23 was 70% (134/172), 92% (410/444) and 94% (307/326), respectively. No variation in the vaccine coverage was observed among different age groups.

## 6. Discussion

In the fifteen-year study period, from 2005 to 2019, an analysis was done on the confirmed IPD cases in Croatia. The main purpose of this study was to analyse the serotype distribution and antimicrobial resistance pattern of invasive *S. pneumoniae* isolates, together with the potential of available preventive measures through coverage of currently recommended and registered vaccines. Pneumococcal infections have shown seasonal character similar to other respiratory pathogens reaching the highest numbers in the coldest period of the year. The overall incidence of confirmed IPD in this study was 2.92 cases per 100 000 population. Croatian data reported to the ECDC's Invasive Pneumococcal Disease Network and published in the Annual Epidemiological Report for 2018 indicate that IPD cases in Croatia are underreported. In 2018, 21 patients were noted in the IPD surveillance network, resulting in a very low incidence of 0.5 cases per 100 000 population (21). A similar incidence rate was observed in previous years, as well. In the period from 2012 to 2016, the incidence ranged from 0.4 to 0.6 cases per 100 000 population (117). In France, in the pre-vaccination period from 2001 to 2002, the overall incidence was 9.3 cases per 100 000 population (118). According to the ECDC Epidemiological Report "Vaccine-preventable diseases – invasive bacterial diseases", the overall incidence of IPD in the pre-vaccination period in Slovenia, from 2008 to 2012, ranged from 10.15 to 12.45 cases per 100 000 population (119). Besides underreporting, the study results might still be underrepresenting the true incidence of confirmed IPD cases due to the low level of blood culture sampling in some Croatian centres (120). Also, most of the blood cultures are sampled in patients with a severe clinical presentation of community-acquired pneumonia who require hospital treatment (121).

In the study population, a predominance of male patients was observed in all age groups. Distribution by age of all confirmed IPD cases in the study period forms a specific "U shape" curve. The highest incidence of confirmed IPD cases was recorded in children <5 years of age with 14.54 cases per 100 000 population and adults  $\geq 65$  years of age with 4.47 cases per 100 000 population. In Finland, in the pre-vaccination four-year period, from 1985 to 1989, the incidence was 24.2 cases per 100 000 population among children <5 years of age (119). In Denmark, during nineteen years in the pre-vaccination period, the mean incidence was 13.4 cases per 100 000 population among children <7 years of age (122). In 2018, in the majority of the EU/EEA countries, the incidence of reported IPD cases in the post-vaccination period showed significant variations, ranging from 0.2 to 16 cases per 100 000 population. The highest incidence was recorded in the United Kingdom and Denmark, while southern and eastern

European countries reported smaller numbers of IPD cases resulting in a very low incidence rate (21). The incidence of confirmed IPD cases differs among countries mainly due to different healthcare systems, surveillance programmes, diagnostic algorithms (proper blood culture sampling), vaccine implementations and clinical presentations of infection (124).

To date, more than 100 serotypes of *S. pneumoniae* surrounded by unique polysaccharide capsule have been identified. Prior to the introduction of conjugate vaccines, invasive pneumococcal infections were mainly limited to several serotypes only. In Europe and North America, six to eleven serotypes accounted for >70% of all IPD cases in children <5 years of age (125–128). Information regarding serotype distribution in a certain population, especially among risk groups, together with antimicrobial resistance profile is of utmost importance for an individual and the public health also. The difference in the prevalent serotype distribution among continents and countries has been recorded (124, 129–132). Also, the difference in serotype predominance has been observed among different age groups. Countries differ in the type of pneumococcal vaccine introduced and the moment of its introduction in the vaccination schedule, which can also contribute to the prevalence of certain serotypes (23, 133–135). Also, a natural fluctuation among different serotypes of *S. pneumoniae* can occur even without vaccine impact as described in the surveillance studies done in Belgium and Germany prior to the conjugate vaccine introduction in the childhood vaccination program (126, 137). In this study serotype distribution in the pre-vaccination period in Croatia was analysed. The significant natural shifts in the distribution of the six dominant serotypes over the fifteen-year period were not observed. Only serotype 1 (overall ranked 10) showed a descending trend.

In this retrospective study, the six most frequently isolated serotypes, 14, 3, 19A, 23F, 6B and 7F accounted for 60% of all invasive *S. pneumoniae* isolates. A difference in serotype prevalence was observed among children and adults. In children <18 years of age, the most frequently isolated serotypes were 14, 6B, 19A, 23F and 18C, while among adults, the prevalence of serotypes 3, 14, 19A, 23F and 4 was recorded. Serotype 14 ranked first in children <5 years of age, serotype 1 ranked first among older children, 5 to <18 years of age, while in adults, especially those  $\geq 65$  years of age serotype 3 was the most predominant. Guzvinec et al. analysed the invasive pneumococcal strain collection isolated in Croatian children <5 years of age for three years (2001, 2005 and 2006). The most common serotypes were the same as in this study (14, 6B, 23F, 18C, 1 and 19A) (138).

Prior to the introduction of the 10-valent conjugate vaccine in Croatian NIP in June 2019, all registered pneumococcal vaccines (10-, 13- and 23 valent vaccines) were recommended for risk groups only, such as children and adults with co-morbidities. Serotype distribution and serotype

prevalence in the fifteen-year period in Croatia are similar to many countries prior to the introduction of the 7-valent conjugate vaccine with a predominance of the so-called paediatric serotypes; 14, 6B, 23F and 18C (118).

After the introduction of the 7-valent conjugate vaccine in childhood immunization schedules, many countries shared similar experiences with a significant and rapid decrease in the overall incidence of IPD cases. The highest decrease in vaccine-serotype IPD cases was observed one year after the introduction of the 7-valent conjugate vaccine in children and this remained the same for the seven-year surveillance period (139). After the introduction of the conjugate vaccine in the USA in 2000, a dramatic decrease in IPD cases caused by PCV7 serotypes was recorded in children <5 years of age (140, 141). Also, vaccination of children had a positive impact on the reduction of pneumococcal infections in non-vaccinated adults, especially those  $\geq 65$  years of age, through reduction of horizontal transmission and the herd immunity. The first conjugate vaccine formulation was based on a selection of serotypes predominating among the paediatric population in North America resulting in the highest decline of IPD cases in that geographic region, more than 80% (132, 142, 143). The introduction of the 7-valent conjugate vaccine led to an overall decline of IPD cases in children <5 years of age in some European countries such as the UK, Norway and Wales (144–147). Low initial PCV7 intake among the paediatric population in France and Spain resulted in the slower decline of vaccine-serotype IPD cases compared to the USA (118,148). Also, some countries reported a decline in vaccine-serotype IPD cases accompanied by simultaneous replacement of serotypes not included in the PCV7 vaccine, mostly serotypes 19A and 7F (149–155). Interestingly, a high prevalence of these serotypes, without vaccine pressure, was observed in Croatia. Perhaps this natural fluctuation occurred due to vaccine introduction in the NIP in the neighbouring countries.

Serotype replacement started to occur approximately two years after the vaccine introduction and continued to increase in the five following years of the surveillance (139). In many countries, the introduction of the 7-valent conjugate vaccine resulted in a significant increase of multidrug-resistant serotype 19A (156–159). In France, Belgium and Spain, in addition to serotype 19A, a significant increase in serotypes 1 and 7F was also recorded (127, 145, 148). However, in the Scandinavian countries, the predominance of serotype 1 was observed prior to the introduction of the conjugate vaccine in the childhood immunization programmes (160, 161). An increase of IPD cases caused by non-conjugate vaccine serotypes 8, 9N, 15A and 23B was recorded in countries such as France, Norway, Finland, the USA and Australia (21,162). At the same time, in some countries, the vaccine-induced serotype shift was insignificant (163, 164).

In many countries, the introduction of the 10- or 13-valent conjugate vaccines in the childhood vaccination programme did not result in a similar change in the prevalence of non-vaccine serotypes as observed with the 7-valent conjugate vaccine and serotype 19A (165, 166).

However, some countries did record a change in the prevalence of particular non-vaccine serotypes in the PCV10 and PCV13 post-vaccination period. In France after the introduction of the PCV13 vaccine, the increase of serotypes 24F and 12F was recorded, 20% and 10%, respectively among children up to 2 years of age. Also, a significant decline in the vaccine serotype IPD cases among adults was observed following by an increase of non-vaccine serotypes without observed predominance of particular serotype(s) (118). In a study done by Hanquet et al. serotype replacement in PCV10 and PCV13 post-vaccination period was analysed in 10 European countries. The highest increase in the incidence of IPD cases caused by non-PCV vaccine serotypes was observed in children <5 years of age followed by adults 65 years and older. In the eight-year study period, no significant difference in the overall incidence and trend of IPD was observed in countries using PCV10 or PCV13, although different distribution of serotypes was recorded (165). In PCV10 countries, a significant increase of serotype 19A, observed in the last three years of the study, is worrisome due to the invasiveness of this serotype combined with acquired resistance to antibiotics (168, 169).

Cui et al. analysed 59 studies on the post-vaccination distribution of non-conjugate vaccine serotypes of *S. pneumoniae* isolated in children and adults in Europe, North and South America, the Pacific Asia Region and African Mediterranean countries. Although countries differ based on the vaccine introduced in the childhood and adult vaccination schedules, the prevalence of non-conjugate vaccine serotypes was recorded among all age groups. The most frequent non-conjugate vaccine serotypes in children <7 years of age were 15B, 22F, 15A and 23A while serotypes 22F, 11A, 10A and 38 were the most frequently isolated in adults ≥65 years of age. Additionally, serotypes 12F and 8 predominated in a large population group, 15-64 years of age. Some of these non-conjugate vaccine serotypes, such as 11A, 22F, 15B and 10A, are included in the 23-valent polysaccharide vaccine (170). Our results did not observe the prevalence of non-conjugate vaccine serotypes among children. Serotypes 11A, 22F and 8 were more frequently isolated among adults compared to other non-conjugate vaccine serotypes. The prevalence among vaccine-conjugate serotypes shows significant variations amongst geographical regions in the post-vaccination period. In Europe and South America, the predominance of serotypes 14 and 1 were recorded, while serotypes 6B, 14 and 19F were the most common in African Mediterranean countries. Overall, the most common serotypes among

adults were 1, 4, 14 and 19A while serotype 3 was the most common among adults in Europe, North America and Asia (170)

In this study serotype 3 was the most dominant serotype in the pre-vaccination period in Croatia among adults, 18 years and older, reaching the highest numbers among adults  $\geq 65$  years of age. These patients are at higher risk for acquiring IPD mainly due to the impaired immune system due to ageing, acquired co-morbidities and life habits such as smoking and alcohol abuse. Therefore, vaccination is strongly recommended for this vulnerable group (171). A high prevalence of serotype 3 among adults was detected in other European countries such as Poland and France (129, 172). In France and in the USA, the prevalence of serotype 3 remained high in the PCV13 post-vaccination period (162, 173). This is probably due to the low serotype-specific immunogenicity of the 13-valent vaccine and/or high colonisation rate among this population group (42). In the PCV13 post-vaccination period in Canada, a decrease in IPD cases due to serotypes 19A and 7F was observed but this was followed by an increase of IPD cases associated with serotype 3 two years later. The vaccination of the children resulted in the herd immunity among adults. The decrease in IPD cases was primarily due to serotypes 19A and 7F while the prevalence of serotype 3 remained high and increasing, especially among adults  $\geq 65$  years of age (174).

According to this study, dominant invasive serotypes among the paediatric population were 14, 6B, 19A, 23F, 18C and 1 which indicates a high coverage potential by currently registered vaccines in Croatia. For children up to 5 years of age, the 10-valent conjugate vaccine showed a lower coverage rate compared to the 13-valent vaccine, 69% and 86.2% respectively. The difference in coverage rate was mainly due to the high prevalence of serotype 19A not included in the 10-valent vaccine.

The overall vaccine coverage of PCV13 and PPC23 during the fifteen-year study period was 75.4% (1225/1643) and 90.6% (1506/1643), respectively.

The PCV13 vaccine coverage varied among age groups. For all children, PCV13 coverage was 84.7%, while in young children up to 5 years of age it was slightly higher, 86.2%. During the study period non-PCV10 and non-PCV13 vaccine serotypes showed no significant variation in children up to 5 years of age. The predominance of some particular serotypes was not observed. The highest number (9 isolates) non-vaccine serotypes were detected in children in 2016. A lower percentage of PCV13 vaccine coverage (80%) was observed among adults without difference among age groups. In adults  $\geq 65$  years of age, PCV13 and PPV23 vaccine coverage was 79% (408/515) and 93.9% (483/515), respectively. The numbers of non-PCV13 and non-PPV23 vaccine serotypes among adults showed minimal variation during the study period. In

the study period, predominant non-PCV13 and non-PPV23 vaccine serotypes were not detected.

In 13 European countries, a moderate decrease in overall IPD cases was observed among adults  $\geq 65$  years of age, after five years of continuous vaccination of children with 10- or 13-valent conjugate vaccine. A significant decrease in vaccine-serotypes IPD cases due to indirect vaccine effect was reduced by an increase of IPD cases caused by non-vaccine conjugate serotypes (102). Although high conjugate vaccine uptake in children resulted in herd immunity among unvaccinated children and adults, the burden of IPD among adults persisted to be high (175).

Recommendations for pneumococcal vaccination of adults differ among Western European countries. They are mainly based on age groups, groups at risk for acquiring IPD, type of vaccine recommended for the particular group and reimbursement policy. In 16 Western European countries (Austria, Belgium, Denmark, Finland, France, Germany, Greece, Ireland, Italy, Luxembourg, The Netherlands, Norway, Portugal, Spain, Sweden, and the United Kingdom) vaccine recommendation is based mainly on age and risk factors. Although PPV23 has a long tradition of usage and is widely implemented, many of these countries have introduced the PCV13 vaccine for adults alone or in combination with PPV23 (176). The Croatian Institute of Public Health, in the recently updated recommendations for pneumococcal vaccination of adults (January 2021), recommends PPV23 for immunocompetent adults such as in Finland, Norway, Sweden, Ireland, Luxemburg, the UK and the majority of Spanish regions. Both vaccines are recommended for immunocompromised and asplenic patients, due to the higher risk of acquiring IPD, with PCV13 being the first dose (176, 177). Both vaccines schedule is recommended in Austria, Belgium and in some Italian regions, also. One vaccine schedule, including PCV13 only, is recommended in Germany, Denmark, Greece and some parts of Italy (174). Based on the results of this comprehensive analysis among Croatian adults, both vaccines, PCV13 and PPV23, provide high protection, especially for adults  $\geq 65$  years of age, with coverage rates of 79% and 93.9%, respectively. Higher PPV23 coverage is associated with 9 additional serotypes comparing to PCV13.

Pneumococcal conjugate vaccines have a direct impact on pneumococcal nasopharyngeal carriage resulting in colonization of non-conjugate vaccine serotypes and changes in nasopharyngeal microbiota (178–181). Several studies analysed this indirect effect of conjugate vaccines on nasopharyngeal microbiota and also the carriage of non-pneumococcal organisms. An increase in the carriage of non-typeable *Haemophilus influenzae* and *Staphylococcus aureus* was observed which resulted in an increase of AOM caused by these pathogens. Also, in the

post PCV7 vaccine period increase of nasopharyngeal colonization with serotype 19A resulted in a decrease of AOM caused by non-typeable *Haemophilus influenzae* (182–185). Pneumococcal conjugate vaccines have an impact on nasopharyngeal microbiota dysbiosis. A study done in the Netherlands showed a significant increase in the nasopharyngeal colonization of anaerobic bacterial species among vaccinated children, with a higher colonization rate observed after 12 months of the post-vaccination period compared to 24 months after vaccination (186).

In the study period, pneumonia was the most frequent clinical presentation of IPD, accounting for 38% of all IPD cases, similar to other EU/EEA countries. The five most prevalent pneumonia-related serotypes (14, 3, 19A, 7F and 4), included in the 13- and 23- valent vaccine, accounted for 57% of all pneumonia isolates. Pneumonia was the most prevalent infection among adults, reported in 44.2% of IPD cases and accounting for 78% of all pneumonia cases during the study period.

Nowadays it is known that some *S. pneumoniae* serotypes are more likely to cause specific invasive pneumococcal infections. A systematic review by Gentile A et al., which included 60 studies on IPD among children in Latin America and the Caribbean, reported serotype 14 as the most common cause of pneumococcal pneumonia in children, followed by serotypes 1 and 5 (187). In Spain, serotype 14 remained the most common cause of pneumonia among adults in the post PCV7 vaccine period followed by non-vaccine serotypes 1 and 5 (188). Similar results were reported in a study done in the UK where serotypes 14, 1, 8, 3 and 19A were the most common serotypes causing pneumococcal pneumonia (189). Analysis done on this pre-vaccination strain collection reported serotypes 14, 3, 19A, 23F, 4, 1, 19F and 8 as statistically significant serotypes causing pneumococcal pneumonia. Serotype 3 was recognised as the most common cause of empyema. Information on empyema was not available in our patient/isolate report form. As serotype 3 was the most prevalent serotype causing pneumonia among adults, more likely is that some patients had empyema as a complication of a severe pneumonia.

The overall prevalence of sepsis in the study population was 26%. Sepsis was the most prevalent clinical presentation of IPD in children up to 5 years of age. A total of 41% of children were diagnosed with sepsis representing 32% of all reported sepsis cases. The most common serotypes reported as sepsis-specific were serotypes 1, 3 and 19A.

Statistical analysis of this strain collection showed that serotypes 1, 19F and 8 more often cause meningitis. Similar serotypes were observed as `meningitis specific serotypes` in studies that mainly analysed the effect of conjugate vaccine on the incidence of meningitis. In England and Wales, the introduction of PCV7 resulted in a higher decrease in meningitis cases compared to



the post-PCV13 period. Also, an overall increase of meningitis cases was mainly caused by non-conjugate vaccine serotypes (190–192). Wahl et al. analysed the burden of *S. pneumoniae* and *Haemophilus influenzae* type B in the era of conjugate vaccine, from 2000 to 2015. Global annual meningitis incidence in children <5 years of age was high, up to 13 cases per 100 000 population, and varied among countries. A decrease in the mortality rate due to IPD was significant (191). However, the overall decline in vaccine serotype IPD cases was recorded. A reduction in pneumococcal pneumonia cases was higher compared to pneumococcal meningitis cases, 35.4% and 18.5%, respectively (70). In the post 7- and 10-valent vaccine period in France, pneumococcal meningitis cases were linked to non-13-valent vaccine serotypes, mainly serotype 24F, followed by serotypes 12F, 15B and 22F (194, 195)

Resistance to antibiotics among *S. pneumoniae* isolates was first reported in Australia in the late 60s (196) and continued to occur sporadically during the 70s (44, 197). The worldwide emergence of multidrug-resistant pneumococcal isolates, in the late 80s and in 90s, resulted in reported isolates in North and South America, Southern and Eastern Europe, Asia and Africa (198, 199). Since 1999 data on the antibiotic resistance among invasive *S. pneumoniae* isolates in the majority of European countries are available through the largest antimicrobial surveillance network, the EARS-Net, previous the EARSS. In 2019, most European countries reported <5% of penicillin-resistant pneumococcal isolates, while in Scandinavian countries, Ireland, Czech Republic and Lithuania detected overall penicillin resistance was less than 1%. In Romania and Spain, the resistance to penicillin was detected in 19.2% and 17.8% of isolates, respectively. Higher percentages of penicillin susceptible, increased exposure isolates were reported in France (25.2%), Poland (15.5%) and Romania (19.8%)(42). The prevalence of penicillin non-wild type isolates, including penicillin susceptible, increased exposure and penicillin-resistant, shows geographical variation. Higher rates are observed in southern and eastern European countries, mainly due to penicillin susceptible, increased exposure isolates, while in northern European countries the prevalence of these isolates remains stable, below 5%. Also, oscillation in the prevalence of penicillin non-wild type was observed over time. Ten-year surveillance data, from 1992 to 2001, showed a significant increase of penicillin non-wild type isolates reported in Spain (from 24.9% to 30.2%), France (from 7.7% to 35.8%) and the USA (5.6% to 20.4%) while the highest rates were recorded in South Africa (74%) and Asian countries (63%) (74, 75). In 2009, the highest percentage of penicillin non-wild type isolates were reported in Bulgaria (37%), Poland (29.8%), Romania (29.4%) and France (26.4%), mostly due to penicillin, susceptible increased exposure isolates. Only in Poland the percentage of penicillin-resistant isolates among penicillin non-wild type strains was very high, 29.4%

(202). During the 21<sup>st</sup> century, in some European countries (France, Spain and Poland) a decrease in penicillin non-wild type isolates was recorded.

Since 1998, the data on the susceptibility of *S. pneumoniae* strains have been collected continuously through the activity of the Croatian Committee for Antibiotic Resistance Surveillance. During the study period, penicillin, susceptible increased exposure isolates varied, ranging from 20%, the lowest rate in 2010 to the highest rate of 31% in 2013. Resistance to penicillin, for the same period, ranged from 2% to 5% (203–214). Additionally, since 2001 the data on antimicrobial resistance of invasive *S. pneumoniae* isolates have continuously been collected through the EARS-Net. Among invasive pneumococcal isolates, the percentage of penicillin susceptible, increased exposure and penicillin-resistant strains recorded oscillation, ranging from 18% to 27% and 1% to 7%, respectively (214).

In the study collection, wild type *S. pneumoniae* isolates, meaning penicillin susceptible, were detected in 79% of all invasive isolates. Penicillin non-wild type isolates, including penicillin susceptible, increased exposure and penicillin-resistant, were detected in 20% and 1%, respectively. *S. pneumoniae* isolates with structurally changed penicillin targets, PBPs, mostly occurred in serotypes 14, 19A and 9V accounting for 60% of all penicillin non-wild type isolates. Worldwide, the pneumococcal strains with acquired resistance, mainly to penicillin and macrolides, belong to serotypes 6B, 6A, 9V, 14, 15A, 19F, 19A and 23F. The percentage of penicillin non-wild type isolates varied among different age groups, being the highest among children. In penicillin susceptible, increased exposure *S. pneumoniae* isolates, penicillin remains the treatment option for non-meningitis infections. Meningitis infections must be treated with a sensitive antibiotic(s) exclusively (46). Increased level of penicillin non-wild type pneumococcal isolates is the result of beta-lactam antibiotics overuse. The surveillance data of antibiotic consumption in Croatia in 2019 reported amoxicillin plus clavulanic acid as the most prescribed antibiotic in outpatient settings, followed by amoxicillin and cefuroxime-axetil (214).

The worldwide increase of penicillin non-wild type *S. pneumoniae* strains did not have a significant impact on ceftriaxone susceptibility. However, the resistance to penicillin and ceftriaxone is based on the modification of PBPs. Both antibiotics are members of the  $\beta$ -lactam antibiotic class and they share an affinity for different PBPs. In our study, pneumococcal isolates with acquired resistance to third-generation cephalosporins, namely ceftriaxone, was very low. Among 2.2% ceftriaxone non-wild type isolates, a predominance of susceptible, increased exposure isolates was observed. Resistance was detected in four isolates only. Due to a very high susceptibility rate (97.6%), and good pharmacokinetic and pharmacodynamic

characteristics, ceftriaxone is the first option for empirical therapy in patients diagnosed with bacterial meningitis.

The introduction of the 7-valent vaccine resulted in a significant decrease in IPD cases caused by penicillin non-wild type pneumococcal strains in many countries (14, 59, 60). Simultaneously, a decrease in macrolide-resistant pneumococcal isolates among children was observed rapidly upon the introduction of the conjugate vaccine (142). Lower incidence of vaccine serotype IPD cases resulted in a significantly lower antibiotic consumption also (215,216). In the post-vaccination period in Spain, macrolide resistance among *S. pneumoniae* strains isolated among adults remained stable (26).

The treatment of IPD in the outpatient setting is mainly focusing on the treatment of pneumococcal pneumonia. Based on the ESCMID and the IDSA treatment guidelines for outpatient pneumonia, amoxicillin is the first choice antibiotic due to its good *in vitro* activity and good *in vivo* effectiveness (49). Amoxicillin is also listed as the first drug of choice in the Croatian treatment guidelines for CAP (85). These recommendations are based on the antimicrobial resistance surveillance data for all *S. pneumoniae* isolates (invasive and non-invasive) in Croatia. In 2019, the susceptibility of *S. pneumoniae* isolates to amoxicillin in Croatia was 86%, confirming this antibiotic as a good choice for empirical therapy (47).

Macrolide resistance observed in 29.4% of all strains in this study collection was mostly associated with serotypes 14, 19A and 6B accounting for 73% of all macrolide-resistant isolates. A difference in resistance rates was observed among age groups, being the highest in children up to 5 years of age (46%). Macrolide resistance is also detected in *S. pneumoniae* strains isolated among adults. Although macrolide resistance rate in adults (22.8%) was lower than in children, macrolides still cannot be recommended for empirical treatment in adults either as the resistance rate is higher than 20%. Overall macrolide resistance of invasive isolates showed oscillation during the study period, ranging from 28% to 40%.

*S. pneumoniae* strains with combined non-susceptibility to penicillin and macrolide resistance were increasing during the 1990s (217). In some countries, such as France, Spain and Ireland, a significant increase in macrolide resistance among pneumococcal isolates was observed, 37.2%, 17% and 15%, respectively (200). In the southern and eastern Mediterranean region, up to 5% of isolates presented with this resistance profile (218). According to the ECDC Annual EARS-Net Report in 2019, the overall isolates with combined resistance profile, penicillin non-wild type and macrolide-resistant, recorded a decreasing trend from 2015 (8.5%) to 2019 (7.2%) (42). In this study, 13% of *S. pneumoniae* isolates presented with a combined resistance profile which is higher than the European average.

In 2019, the EARS-Net Report on macrolide-resistant *S. pneumoniae* invasive isolates showed a wide range of resistance rates amongst geographical regions. Similarly, as for the penicillin, the lowest macrolide resistance rates were recorded in northern European countries; less than 5% in Denmark and the Netherlands and less than 10% in Norway, Sweden, UK and Germany. The highest resistance rates were reported in Southern European countries; Bulgaria (30.4%), Croatia (29.9%) and Malta (28%).

Same as in this study, macrolide resistance among pneumococcal isolates showed huge oscillation over time in Europe, as well. In the EARS-Net Report for 2005, the highest percentages of macrolide-resistant isolates were detected in France (40.5%), Belgium (31.3%), Romania (30.8%), Hungary (30.2%) and Italy (26.7%) while the lowest resistance rates were detected in Sweden (5.4%) and Denmark (5.6%). Ten years later, in 2015, the EARS-Net data showed a significant decrease in macrolide-resistant pneumococcal isolates in countries with previously reported high resistance rates. In France, the resistance decreased from 40.5% to 24.4%, in Belgium from 31.3% to 18.6% and in Hungary from 30.2% to 10.9%. In Romania, the resistance rate remained very high, while a significant increase in macrolide-resistant isolates was recorded in Slovakia and Poland, 32.6% and 30.2%, respectively (219). Reported macrolide resistance among invasive *S. pneumoniae* isolates in Croatia showed large oscillations during the study period. In 2005, macrolide resistance was reported in 17.9% of all pneumococcal isolates and slowly decreased in 2006 and 2007. A significant increase in the resistance rate occurred again in 2008, 27.7%, reaching its peak in 2017 with 36.2% of all pneumococcal isolates resistant to macrolides. In the last two years, the resistance rate is showing a modest decreasing trend, resulting in 30% resistant isolates (220).

In our study, more than half of all macrolide-resistant isolates (53.4%) belonged to the MLS<sub>B</sub>-phenotype and were mostly associated with serotypes 14 and 19A. This resistance phenotype is common in most European countries (Poland, France, Belgium, Spain, Italy and Russia), Asian countries (China and Vietnam) and in South Africa (221–226). However, the predominance of *S. pneumoniae* isolates with acquired M-phenotype is more common in the USA, Germany, Norway, Canada and UK (47, 227–230). In our study, 35.5% of all macrolide-resistant isolates expressed the M-phenotype of macrolide resistance (mainly serotype 14). Only seven isolates carried two resistance genes, *ermB* and *mefA* (serotypes 14, 19A and 19F). These isolates are of high concern in some countries such as the USA, Russia and South Asian countries (231, 232). A total of 10% macrolide-resistant isolates were negative for both macrolide resistance genes, *ermB* and *mefA* class, suggesting some other acquired resistance mechanisms. Rare resistance mechanisms described among Croatian isolates previously

included structural modification of the ribosomal proteins and ribosomal RNA mutation. (49, 233).

In many countries, the resistance to macrolides occurred mainly due to the overuse of these antibiotics, which is recognized as the major factor contributing to the increasing antimicrobial resistance. The analysis of macrolide resistance in 26 European countries confirmed that outpatient macrolide consumption has a huge impact on macrolide resistance. In some countries, macrolide overuse resulted in a significant increase in IPD cases caused by serotype 19A (234, 235). The highest macrolide consumption was recorded in Greece and the lowest in Latvia. Consequently, macrolide resistance in Greece is high and in Latvia low (236).

In this strain collection, serotype 19A showed high resistance to penicillin and macrolides, similar to other European countries such as France, Belgium and Spain (145). In Belgium, as in our country, a high prevalence of serotype 19A was observed without vaccine impact. Also, a sharp increase of serotype 19A was observed after the 13-valent conjugate vaccine replacement by the 10-valent vaccine in the childhood immunization schedule in the period from 2017 to 2018 (237). As the 10-valent vaccine was introduced in Croatian NIP in June 2019, it will be important to follow the impact of this vaccine on the dynamics of multidrug-resistant non-vaccine serotypes, such as 19A in the post-vaccination period.

During the fifteen-year surveillance of invasive *Streptococcus pneumoniae* isolates, fluoroquinolone resistance (moxifloxacin and levofloxacin) remained very low (<1%). In this study, only four strains isolated in adults were resistant to moxifloxacin. Increased fluoroquinolone usage in the outpatient setting in Croatia (8%/2019) did not affect the resistance rate among invasive isolates (83,115). In many countries, a low level of fluoroquinolone resistance, below 2%, remained stable despite the introduction of PCVs and consequential serotype replacement (49, 238).

This comprehensive analysis of invasive *S. pneumoniae* isolates collected during fifteen consecutive years provides valuable information on the clinical manifestation of IPD, distribution of serotypes and antimicrobial resistance. Analysed results represent a significant contribution to a better understanding of the epidemiology of IPD in Croatia and the real burden of the disease. The majority of analysed isolates were collected in the pre-vaccination period so the information on the potential coverage by available vaccines should be used for future healthcare preventive strategies. The information regarding antimicrobial susceptibility of invasive isolates, together with available comprehensive data on the susceptibility of non-invasive isolates should be used for creating treatment guidelines. Prevention of IPD among the Croatian population should be highly prioritized among healthcare decision-makers.

Continuous and sustained surveillance of IPD, including serotype distribution, antimicrobial resistance and vaccine coverage, needs to be mandatory in order to provide better insight into the epidemiological situation of this important public health-threatening disease. Maybe in the near future, the introduction of new vaccines based on other pneumococcal virulence factors, besides polysaccharide capsule, will provide better control over serotype dynamics and minimise serotype replacement. Also, better compliance with the prudent antibiotic use policy will lower the antibiotic pressure and result in the expected decrease in the antibiotic resistance rate.

## 7. Conclusions:

1. Invasive pneumococcal disease (IPD) in Croatia mostly affects children <5 years of age and adults 65 years and older.
2. Bacteremic pneumonia is the most common clinical presentation of IPD among adults while sepsis is the most common clinical presentation among the paediatric population.
3. The most prevalent serotype causing IPD in adults, serotype 3, is rarely causing invasive infection in children.
4. Non-susceptibility to penicillin and resistance to macrolides is high and resistance to quinolones is low.
5. Penicillin in an adjusted dose is a good drug for the treatment of non-meningitis IPD caused by penicillin susceptible, increased exposure *S. pneumoniae* strains.
6. High susceptibility to third-generation cephalosporins, namely ceftriaxone, suggest this antibiotic as a drug of choice for the treatment of pneumococcal meningitis.
7. MLS<sub>B</sub> phenotype of macrolide resistance, determined by *ermB* gene, predominates over M phenotype determined by *mefA* gene.
8. Empirical therapy of pneumococcal infections, especially in children, should not include macrolides due to their high resistance rate.
9. Pneumococcal vaccines currently available in Croatia have high coverage for the target population and invasive *S. pneumoniae* strains with acquired resistance to the antibiotic.

## 8. Abstract

### **Serotype distribution and antimicrobial resistance in invasive *Streptococcus pneumoniae* isolates in Croatia, Iva Butić, 2022**

**Aims:** The general aim was to observe the characteristics (serotypes, antimicrobial resistance) of invasive *S. pneumoniae* strains depending on the time period and patient age and to determine the extent of coverage by currently available vaccines. Specific aims were to determine the serotype distribution and antimicrobial susceptibility of invasive *S. pneumoniae* and to determine the resistance mechanisms in macrolide and quinolone-resistant isolates.

**Materials and methods:** Invasive pneumococcal strains were collected through the microbiological laboratories engaged in the national antibiotic resistance surveillance organised as the Croatian Committee for Antibacterial Resistance Surveillance. Capsular typing was performed by the capsular swelling method (the Quellung reaction). *In vitro* susceptibility testing was performed according to the CLSI and the EUCAST guidelines. The presence of macrolide and fluoroquinolone resistance genes was detected by PCR.

**Results:** The overall incidence of invasive pneumococcal disease (IPD) was 2.92 confirmed cases per 100 000 population. Analysis was done in 1666 out of 1854 consecutively collected, non-copy isolates. A total of 32.6% strains were isolated in children <18 years and 30.9% in adults  $\geq 65$  years of age. The most prevalent serotypes among children were 14, 6B, 19A, 23F, 18C and 1, while serotypes 3, 14, 19A, 9V, 23F and 7F were the most prevalent among adults. The trend in the serotype distribution was observed only for serotype 1, which was decreasing. Pneumonia was the most common clinical presentation, in the overall population and among adults. Penicillin non-wild type strains (22%) and macrolide-resistant strains (29.4%), mostly belonged to serotypes 14 and 19A. Resistance to fluoroquinolones was detected in 4 isolates only. The coverage by 10-, 13- and 23-valent vaccines was 69%, 74.5% and 90.6%, respectively, for the target population.

**Conclusions:** The incidence of IPD and serotype distribution varied with patient age. All available vaccines have high coverage for the target population and strains with acquired resistance to the antibiotic.



## 9. Abstract

### **Distribucija serotipova i antimikrobna rezistencija u invazivnih izolata *Streptococcus pneumoniae* u Hrvatskoj, Iva Butić, 2022**

**Ciljevi istraživanja:** Analizirati karakteristike (serotipovi, antimikrobna rezistencija) invazivnih sojeva *Streptococcus pneumoniae*, utvrditi eventualne promjene ovisno o vremenskom razdoblju i dobi bolesnika, te opseg pokrivenosti trenutno dostupnim cjepivima. Specifični ciljevi su obuhvaćali distribuciju serotipova i antimikrobnu osjetljivost invazivnih pneumokoka, te mehanizme rezistencije među izolatima rezistentnima na makrolide i kinolone.

**Materijali i metode:** Invazivni sojevi *Streptococcus pneumoniae* su prikupljeni unutar mreže mikrobioloških laboratorija u sklopu Odbora za praćenje rezistencija bakterija na antibiotike u Hrvatskoj. Serotipizacija je učinjena metodom bubrenja kapsule (the Quellung reaction). Ispitivanje *in vitro* osjetljivosti na antibiotike je provedeno u skladu s američkim (CLSI) i europskim (EUCAST) laboratorijskim standardima. Metodom reakcije lančane polimeraze je ispitivana prisutnost gena rezistencije na makrolide i fluorokinolone.

**Rezultati:** Ukupna incidencija invazivne pneumokokne bolesti (IPB) je 2.92 dokazanih infekcija na 100 000 stanovnika. Analizom je obuhvaćeno 1666 od ukupno 1854 uzastopno prikupljenih izolata. Među pacijentima s dokazanom IPB 32.6% su bili djeca <18 godina i 30.9% odrasli ≥65 godina. Najčešće zastupljeni serotipovi među djecom su 14, 6B, 19A, 23F, 18C i 1, a među odraslima 3, 14, 19A, 9V, 23F i 7F. Promjena u distribuciji serotipova u promatranom razdoblju uočena je samo za serotip 1 koji je pokazao silazni trend. Pneumonija je bila najčešća klinička prezentacija u cijeloj populaciji i među odraslima. Pneumokoki smanjene osjetljivost na penicilin (22%) i rezistentni na makrolide (29.4%), većinom su pripadali serotipovima 14 i 19A. Rezistencija na fluorokinolone je dokaza u samo četiri izolata. Pokrivenost 10-, 13- i 23-valentnim cjepivima je iznosila 69%, 74.5% i 90.6% za ciljne dobne skupine.

**Zaključci:** Incidencija IPB i distribucija serotipova ovisi o dobi bolesnika. Sva dostupna cjepiva imaju visoku pokrivenost za ciljnu populaciju u Hrvatskoj i sojeve sa stečenom rezistancijom na antibiotike.

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## 11. Biography

Iva Butić was born in Zadar in 1976. She graduated from the University of Zagreb, School of Medicine in 2000. She became a specialist in clinical microbiology in January 2008. Since December 2008, she has been working at the Department of Clinical Microbiology at the University Hospital for Infectious Diseases "Dr. Fran Mihaljević" in Zagreb. From the beginning of her professional career she has shown interest in the problem of antimicrobial resistance and was engaged in the activities of the Reference Centre for Antibiotic Resistance Surveillance. She actively participated in the courses and symposia organized by the Croatian Committee for Antibiotic Resistance Surveillance. From 2014 to 2019 she worked as a consultant for the World Health Organization, Regional Office for Europe on issues related to antimicrobial resistance. She had an active role in the organization of laboratory courses for microbiologists in the Eastern European and the Central Asian countries. In October 2017, she was elected assistant at the Department of Clinical Microbiology at the University of Zagreb, School of Dental Medicine. In 2020 she was awarded as the best teacher based on the students' evaluation of the teachers. Since July 2019, she has been working as a lecturer at the University of Applied Health Sciences in Zagreb at the Department of Medical Microbiology with Parasitology. Her scientific and professional publications are mainly focused on the topic of antimicrobial resistance.

Iva Butić je rođena u Zadru 1976. godine. Diplomirala je na Medicinskom fakultetu Sveučilišta u Zagrebu 2000. godine. Specijalist kliničke mikrobiologije postala je u siječnju 2008. godine. Od prosinca 2008. godine zaposlena je u Zavodu za kliničku mikrobiologiju Klinike za infektivne bolesti "Dr. Fran Mihaljević". Od samog početka svog profesionalnog rada zainteresirana je za problem antimikrobne rezistencije, te je angažirana u radu Referentnog centra za praćenje rezistencije bakterija na antibiotike. Aktivno je sudjelovala na tečajevima i simpozijima u organizaciji Odbora za praćenje rezistencije bakterija na antibiotike. Od 2014. do 2019. bila je angažirana kao konzultant za antimikrobnu rezistenciju Europskog ureda Svjetske zdravstvene organizacije u sklopu koje je sudjelovala u organizaciji laboratorijskih tečajeva za mikrobiologe u zemljama istočne Europe i srednje Azije. Od listopada 2017. godine zaposlena je kao asistent na Katedri za kliničku mikrobiologiju Stomatološkog fakulteta Sveučilišta u Zagrebu. Nagradu za najboljeg nastavnika prema ocjeni studenata dobila je

2020.godine. U srpnju 2019. postaje predavač na Zdravstvenom Veleučilištu u Zagrebu na Katedri za medicinsku mikrobiologiju s parazitologijom. U znanstvenim i stručnim publikacijama najviše je usmjerena na temu antimikrobne rezistencije.

# Supplements

## EARS-Net Isolate Record Form for *Streptococcus pneumoniae*

Antimicrobial resistance (EARS-Net)

REPORTING & ANALYSIS

### Annex I. Isolate Record Form *S. pneumoniae*

To be filled out by laboratory

**Instructions:** Please send data of the first **blood and/or cerebrospinal fluid (CSF)** - isolate of every patient with an invasive *S. pneumoniae* infection. Send data on resistant and susceptible isolates; use 1 form per isolate.

#### Laboratory Data

Laboratory Code "LaboratoryCode" \* CC000 \_\_\_\_\_

#### Isolate Data

Isolate sample number "IsolateId" max. 12 characters \_\_\_\_\_

Isolate source "Specimen"  tick box  Blood  CSF

Date of sample collection "DateUsedForStatistics" dd-mm-yyyy \_\_-\_\_-\_\_\_\_

#### Patient Data

Patient Name and Surname \_\_\_\_\_

Patient ID / Code max. 12 characters (OIB) \_\_\_\_\_

Gender  tick box  Male  Female  Unknown

Year of birth \_\_\_\_\_

#### Diagnosis

tick box  UTI  GI infection  abdominal infection  skin and soft tissue  bone and joint  prim. sepsis  endocarditis

CNS infection  respiratory infection  other  unknown

#### Hospital Data

Code of hospital "Hospital Id" [LaboratoryCode-sequence number] A/B/C/D \_\_\_\_\_

Origin of patient "PatientType" = Location type  tick box  Admitted  Outpatient  Other  Unknown

Date of admission "DateOfHospitalisation" dd-mm-yyyy \_\_-\_\_-\_\_\_\_

Hospital Department "HospitalUnitType" = Department

tick box  Internal Medicine  Pediatrics/neonatal  Pediatrics/neonatal ICU  Surgery  Haematology/oncology

Ob/Gyn  ICU  Emergency  Urology  Infectious diseases  Other  Unknown

#### Antibiotic susceptibility testing (S/I/R, zone and/or MIC)

Antibiotic	Antibiotic SIR (final interpretation result of all different susceptibility test performed) <i>Fill in S, I or R</i>	Zone diameter (ResultZoneValue) <i>(mm)</i>	Zone diameter interpretation (ResultZoneSIR) <i>Fill in S, I or R</i>	MIC (ResultMICValue) <i>(mg/l)</i>	MIC interpretation (ResultMICSIR) <i>Fill in S, I or R</i>	E-test (ResultEtestValue) <i>(mg/l)</i>	E-test interpretation (ResultEtestSIR) <i>Fill in S, I or R</i>
<input type="checkbox"/> Oxacillin Disk load 1µg	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	-----	<input type="checkbox"/>	-----	<input type="checkbox"/>
<input type="checkbox"/> Penicillin	<input type="checkbox"/>			-----	<input type="checkbox"/>	-----	<input type="checkbox"/>
<input type="checkbox"/> Erythromycin	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	-----	<input type="checkbox"/>	-----	<input type="checkbox"/>
<input type="checkbox"/> Cefotaxime AND/OR	<input type="checkbox"/>			-----	<input type="checkbox"/>	-----	<input type="checkbox"/>
<input type="checkbox"/> Ceftriaxone	<input type="checkbox"/>			-----	<input type="checkbox"/>	-----	<input type="checkbox"/>
<input type="checkbox"/> Norfloxacin Disk load 10µg	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	-----	<input type="checkbox"/>	-----	<input type="checkbox"/>
<input type="checkbox"/> Ciprofloxacin AND/OR	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	-----	<input type="checkbox"/>	-----	<input type="checkbox"/>
<input type="checkbox"/> Ofloxacin AND/OR	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	-----	<input type="checkbox"/>	-----	<input type="checkbox"/>
<input type="checkbox"/> Levofloxacin	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	-----	<input type="checkbox"/>	-----	<input type="checkbox"/>
<input type="checkbox"/> Moxifloxacin	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	-----	<input type="checkbox"/>	-----	<input type="checkbox"/>

\* The national co-ordinators provide the laboratory code, consisting of a Country Code (CC) followed by 3 numbers.

\*\* Consists of the laboratory code, followed by a sequence number identifying the hospital.

Send this form to: Klinika za infektivne bolesti "Dr. Fran Mihaljević", Mirogojska 8, Zagreb,  
tel: 01 2826 648, fax: 01/2826 280, E-mail: slucic@bfm.hr