A 20-months surveillance of non-invasive *Streptococcus pneumoniae* infections in Belgium to evaluate national vaccination strategy

Protocol Version 1.2

05/12/2019

0 Summary

Population	3,600 Belgian patients with community acquired pneumonia or acute otitis media, leading to positive *S. pneumoniae* from non-sterile clinical specimens.
Intervention	N/A
Comparison	Serotype distribution according to vaccination programs (campaigns)
Outcome	Primary: to establish insight in serotype dynamics for non-invasive pneumococcal diseases in Belgium (prospectively and retrospectively), in association with changing vaccination programs (campaigns). Secondary: surveillance of emerging serotypes, clones and drug resistances. Study of the genetic divergences between invasive and non-invasive pneumococcal diseases isolates.
Study Type	Observational

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1 Study Rationale

1.1 Pneumococcal vaccination programs reduced burden of invasive disease

Streptococcus pneumoniae is a leading cause of morbidity and mortality worldwide among children <2 years of age and in the elderly [1]. The pathogenicity of this bacteria can be divided into 2 forms: invasive (IPD) and non-invasive pneumococcal disease (NIPD). Whereas the dangerous IPD type is responsible for meningitis and bacteraemia (septicaemia), the less severe NIPD can cause upper and lower respiratory tract infections such as community acquired pneumonia (CAP) and otitis media (OM). Of note, this subdivision between IPD and NIPD pneumococcal diseases is somewhat arbitrary since a substantial proportion (10-20%) of CAP pneumonia caused by *S. pneumoniae* is associated with contemporary bacteraemia (especially in elderly and debilitated patients). The distinction between PID and NIPD relates to the laboratory diagnosis and anatomical sites from which pneumococci are isolated. In PID, *S. pneumoniae* is detected from normally sterile body fluids (blood, cerebrospinal fluid (CSF), pleural, peritoneal or articular fluids). In NIPD, pneumococcal isolates are typically isolated from superficial body sites such as ear, nose, sputum or other lower respiratory tract sites (bronchial, endotracheal aspirates, bronchoalveolar lavage,...). There are over 90 pneumococcal serotypes, classified by an immunologically distinct capsule encoded by the capsular polysaccharide (*cps*) biosynthesis locus of the genome. The capsule is a major virulence factor and the basis for the currently licensed vaccines targeted toward the paediatric and elderly communities.

In the course of the decades, various pneumococcal conjugate vaccines were successively introduced, with the 7-valent PCV7 vaccine gradually being replaced by the 10-valent PCV10 and the 13-valent PCV13 [4]. These vaccination programs have led to drastic changes in the global epidemiology of pneumococcal diseases. Spawned surveillance data unequivocally showed strong decreases in IPD rates caused by the PCV serotypes. Simultaneously, serotype replacement by non-vaccine targeted serotypes occupying nasopharyngeal niches vacated after vaccination was observed. A recent example of this is the emergence of multidrug resistant serotype 35B in IPD isolates from USA and France (**Figure 1**) [5-7].

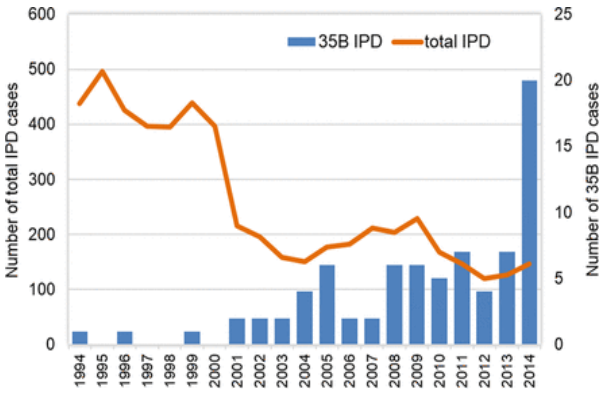

Figure 1. Number of serotype 35B invasive isolates by year from 1994 to 2014 in the USA, contrasting declining IPD incidence after vaccination campaigns. Figure from ref. [5].

Further, an important, and as yet underestimated, role is thought to be played by non-encapsulated pneumococcal strains (NeSP), currently not targeted by vaccination. These strains have either deleted or dysfunctional *cps* genes, or have novel genes in place at this locus [8]. NeSP strains have been isolated from patients with invasive and non-invasive pneumococcal disease, but are typically more frequently associated with non-invasive diseases like conjunctivitis (85%) and OM (8%) [9].

1.2 Changing vaccination recommendations in Belgium

In Belgium, the 23-valent polysaccharide vaccine or PPV23 (currently Pneumovax 23®, MSD) is licensed since 1995. It is currently recommended by the Superior Heath Council (CSS/HGR) for the prevention of pneumococcal infections in persons ≥2 years of age, presenting with an increased risk of mortality and severe morbidity due to pneumococcal infections, and often sequentially combined with PCV13 (Prevenar®, Pfizer) [4]. The national infant vaccination program was initiated in 2007 with PCV7 (Prevenar®, Pfizer), and was updated to PCV13 in 2011. In 2015-2016, the program changed from PCV13 to PCV10 (Synflorix®, GSK). Very recently (HGR 9519, December 2018), a new advice was published by the Superior Heath Council which recommended again the use of the 13-valent vaccine in children ≤2 years of age, alongside an explicit statement stating that this advice could be reconsidered upon the release of new data.

1.3 The underdiagnosed and non-surveilled non-invasive population

Current national and international pneumococcal surveillance is strongly focused on IPD. However, it is now commonly accepted that NIPDs by far account as the major part of all pneumococcal infections worldwide. O'Brien and colleagues reported in 2009 that out of 14.5 million pneumococcal cases, 95.6% were cases of NIPD [1]. In a more recent meta-study by the Johns Hopkins School of Medicine, it was estimated that for every adult bacteraemia case there are at least three non-invasive pneumococcal infections [10], resulting in huge societal and economic costs [11].

Impelled by the lack of surveillance on NIPD, the majority of studies on pneumococcal vaccine effectiveness and serotype replacement are based on IPD data. However, **sheer extrapolation of IPD dynamics to NIPD has been called into question by recent studies**, using serotype-specific urinary antigen technology, which indicated discrepancy between persistent pneumococcal serotypes and the decline of the same serotypes causing IPD [12-14]. To get more insights in this matter, the Belgian Health Care Knowledge Centre (KCE) recently reviewed four observational studies from Denmark, Spain and Portugal (2), and the placebo arm of the CAPITA randomized controlled trial in the Netherlands [15-20]. In the infant populations of the study sites, PCV vaccine choice over time (PCV7, PCV10 and PCV13) and uptake differed. Three studies limited the detection of non-bacteraemic pneumonia to pneumonia with a positive culture of airway respiratory samples such as sputum or broncho-alveolar fluid, while the remaining study used both respiratory and urinary samples. Despite these differences, all studies concluded that the serotype distribution of bacteraemic and non-bacteraemic pneumococcal disease differed substantially in terms of vaccine serotype coverage (**Figure 2**) [15].

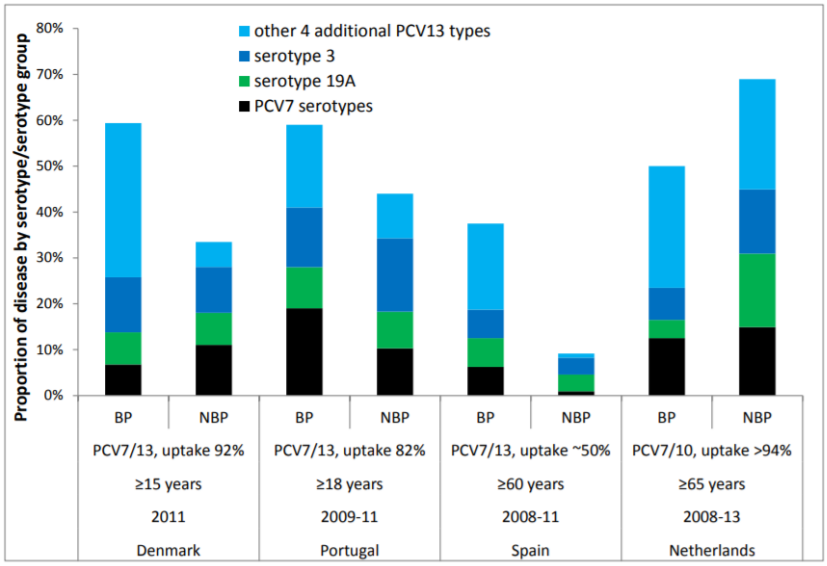

Figure 2. Proportion of serotype categories in bacteraemic/invasive pneumonia (BP) and non-bacteriaemic/non-invasive pneumonia (NBP) in the Netherlands [16], Denmark [17], Spain [18] and Portugal [19,20]. Figure from the Belgian CSS/HGR rapport [15].

These results concurred with the few non-EU studies conducted in USA, Britain and Korea, showing that frequencies of vaccine serotypes among NIPD vary between 28.2 and 57.4%, depending on the specimen type and patient age [21-23].

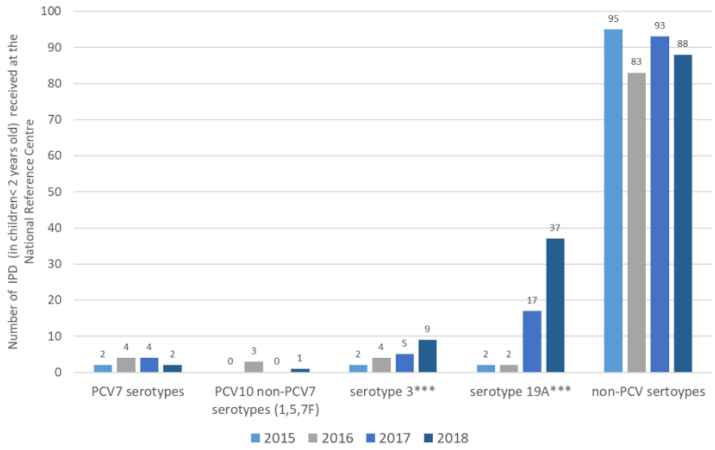

Figure 3. Situation in Belgian infants (<2y). ***serotypes present PCV13 which are absent in PCV10. Data from the Belgian NRC for pneumococcal diseases.

In Belgium, 78.4% of all IPD infections in 2017 were caused by non-vaccine targeted serotypes, which is more than what is found in our neighbouring countries. In infants below 2y, this figure drops to 64.2% in 2018 (**Figure 3**). No information on serotype distributions for NIPD are available.

1.4 Sciensano holds historical collection of non-invasive pneumococcal isolates

Between 1995 and 2015, Raymond Vanhoof (MD, PhD) led a team at Sciensano investigating the evolution of antibiotic resistance in NIPD isolates from CAP patients (as well as from children with acute otitits media). For 12 consecutive winter periods, his team collected between 300 and 500 clinical isolates using a network of 15 peripheral hospital-based laboratories distributed across the country. Consequently, we hold a collection of >5,000 strains for which extracted gDNA, complete phenotypic MIC profiles and associated metadata are already available (**Figure 4** for age distribution). This study, funded by Bayer, focused on antimicrobial resistance and did not routinely included serotyping.

This constitutes an extremely valuable collection that dates from before the introduction of pneumococcal vaccination programs. In the context of the proposed study, this collection would serve to retrospectively assess the impact of successive vaccination campaigns on serotype distribution among NIPD isolates in Belgium.

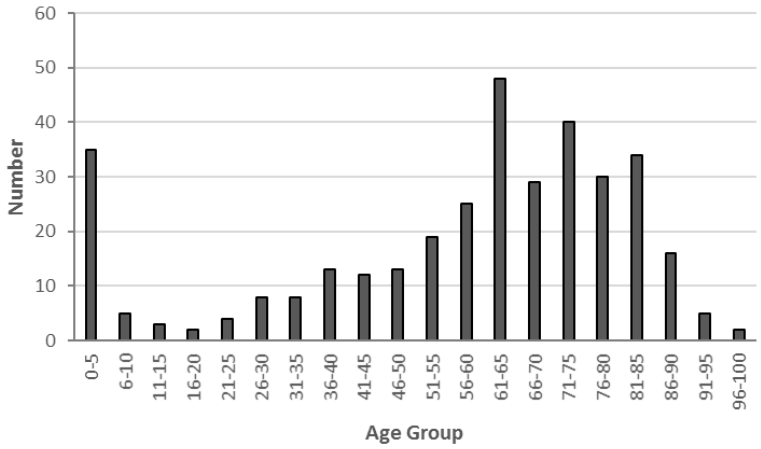

Figure 4. Age distribution of the patients for which cultures were included during the NIPD surveillance study in 2012-2013 using the same network of laboratories [24].

1.5 Implications for empiric treatment guidelines?

Penicillin and macrolides have been the mainstay in the treatment of respiratory diseases for decades [25], but the worldwide spread of drug-resistant clones translated into increased usage of fluoroquinolones [26,27]. Recently, the World Health Organisation (WHO) included penicillin-resistant *S. pneumoniae* in the list of 12 critical drug-resistant pathogens [28]. Nevertheless, current empiric treatment guidelines support the continued use of penicillin and amoxicillin for drug-susceptible isolates. For risk patients, Belgian Antibiotic Policy Coordination Committee (BAPCOC) recommends use of either a respiratory fluoroquinolone, or combination therapy with beta-lactam antibiotic (high-dose amoxicillin, amoxicillin-clavulanate, or, alternatively, a second- or third-generation cephalosporin) plus a macrolide [29].

Reminiscent of the studies on serotype replacement and global surveillance, studies on pneumococcal drug-resistance are strongly biased towards IPD isolates. However, it is conceivable that the non-invasive population functions as a 'laboratory' for the development of drug resistance. During a nation-wide multicentre survey spanning nearly two decades (1995-2014), researchers from Sciensano unequivocally reported higher rates of antibiotic resistance in the non-invasive population, as compared to the invasive strains (**Figure 5**) [30]. Therefore, including antimicrobial resistance screening for non-invasive strains would improve pneumococcal surveillance, improve empiric treatment guidelines and add the possibility to detect circulating drug-resistant clones.

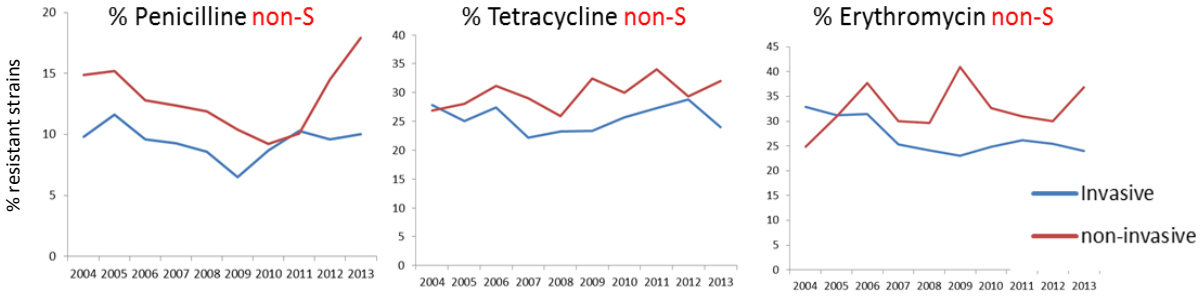

Figure 5. Drug non-susceptibility in invasive versus non-invasive *S. pneumoniae* isolates in Belgium (2004-2013). Data from IPD is annually reported by the National Reference Centre for pneumococcal disease. Data for NIPD was collected in the framework of the multicentre study (1995-2014) on drug susceptibility of non-invasive *S. pneumoniae*.

1.6 Key points

From the information above, it is clear that:

- Current recommendations for vaccination and empiric treatment are strongly influenced by the current surveillance focus on IPD. Information on serotype distribution and drug resistance dynamics in NIPD is scarce. However, a discrepancy in serotype distribution between IPD and NIPD isolates is present in neighbouring countries.
- In Belgium, there was a recent (2015-2016) switch in the infant vaccination program to PCV10 and a recommended reversal to PCV13 in December 2018. The influence on serotype distributions in NIPD is unclear, although this information is essential in vaccine cost-effectiveness analyses [31].

2 Study aims

The main objective of this study is to generate insight in the serotype dynamics for NIPD in Belgium, in association with changing vaccination programs. We aim to serotype non-invasive *S. pneumoniae* isolates prospectively collected in Belgian routine laboratories, during a period of 20 months. Simultaneously, a similar characterisation study will be performed retrospectively on a selection of more than 5,000 NIPD strains collected between 1995 and 2014 in Belgium.

Additionally, this projects aims to do the surveillance of emerging serotypes, clones and drug resistances. Using Whole Genome Sequencing (WGS), the genetic divergences between IPD and NIPD isolates will also be studied, in collaboration with the National Reference Centre (NRC) for pneumococcal diseases (UZ Leuven, Belgium).

3 Type of study

This will be an observational study collecting data on *S. pneumoniae* serotypes causing NIPD in Belgium, in relation with changing vaccination programs.

4 Conducting the study

The study will be conducted by Sciensano in collaboration with the NRC for pneumococcal diseases and 12 peripheral clinical laboratories, which all agreed in writing to prospectively collect non-invasive *S. pneumoniae* samples in the framework of this study. These labs are geographically spread over Belgium to avoid clonal bias in sampling (**Table 1**).

Table 1. Peripheral network of participating clinical laboratories.

Hospital	Contact	City	
CHR Citadelle	Dr. M. Carpentier	Liège	
CHU Mont-Godinne	Dr. D. Huang	Yvoir	
ZNA	Dr. K. Camps	Antwerpen	
Virga-Jessaziekenhuis	Dr. K. Magerman	Hasselt	
Hôpital de Jolimont	Dr. N. Ciupilan	Haine-St.-Paul	
AZ Delta	Dr. S. Vervaeke	Roeselare	
AZ St. Jan	Dr. E. Nulens	Brugge	
AZ. Jan Palfijn	Dr. L. Ide	Gent	
Imeldaziekenhuis	Dr. J. Frans	Bonheiden	
Hôpital Princesse Paola	Dr. Ph. Lefèvre	Marche-en-Famenne	
Clinique St. Joseph	Dr. J.S. Goffinet	Arlon	
CHU de Charleroi	Dr. S. Lali	Charleroi	

The proposed project workflow is schematically depicted in **Figure 6**. All stages in this work will be performed according ISO15189 standards.

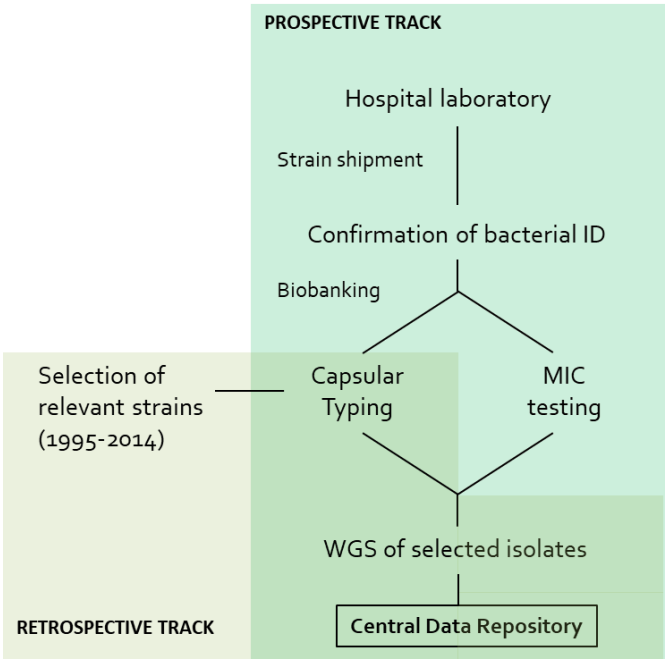

Figure 6. Study design.

4.1 Prospective track

Regarding the prospective track (**Figure 6**), each month from September 2020 to May 2022 (20 months), twelve laboratories listed in **Table 1** committed to send the first 15 unduplicated isolates of *S. pneumoniae* collected from patients meeting the following criteria:

Inclusion criteria:

- Patients living in Belgium at the time of the study,
- from whom unduplicated *S. pneumoniae* isolates were collected in routine practices,
- from non-invasive upper (e.g.: pus/fluid from nasal sinus or from otitis media fluid) or lower respiratory tract (e.g. sputum bronchial or endotracheal aspirate, bronchoalveolar fluid (BAL),…) clinical samples.
- from patients diagnosed with pneumonia, sinusitis and otitis.

Exclusion criteria:

- Patients for whom *S. pneumoniae* was simultaneously isolated from blood or another usually sterile specimen.

Consequently, a theoretical maximal amount of 3,600 non-invasive *S. pneumoniae* isolates will be prospectively collected, although the actual number will probably be lower given the lower prevalence of NIPD during the summer period. For every shipped strain (with associated metadata) during the study period, the laboratory will be compensated by payment of €20.

The transfer of the isolates (under dry ice) from hospital laboratories to the “Sciensano Unit ‘Antibiotics & Resistance’ (Site Engeland - Engelandstraat 642, 1180 Uccle)” will be organised in the last week of every month, using the Sciensano internal sample transport service. Isolates will be accompanied by an electronic record sheet, listing the following metadata **upon availability** :

- isolate number
- Hospitalised (HOS), residing in a long-term care facility (LCF), ambulatory (AMB), or other (to be specified).
- admission date (hospitalised patients only)
- date of specimen collection
- patient age
- patient sex
- sample source including collection method
- clinical diagnosis
- vaccination status of the patient for *Streptococcus pneumoniae*
- For bacterial cultures from sputum, the amount of epithelial cells and white blood samples
- Other potential respiratory pathogens isolated (e.g. *H. influenzae*, *M. pneumoniae*, …)

For all isolates sent, following analyses will be performed at Sciensano:

- Identification and confirmation of isolates by reference methods prior to use.
- Capsular typing using an FT-IR based method that will be developed in the scope of the project (section 5.4).
- Characterization of NeSP strains by gene sequencing of the *cps* locus.
- Antibiotic susceptibility tests by microdilution (MIC).
- Whole-Genome Sequencing (WGS) for multi-drug resistance and non-typeable strains or if clonality of the same serotypes originating from different, but geographically linked, patients needs to be assessed.

4.2 Retrospective track

As described in Section 1.4, Sciensano holds a collection of >5,000 NIPD strains (1995-2015) for which extracted gDNA, complete MIC profile and associated metadata are already available. From every uneven year, we will randomly select 150 isolates, without bias towards age and gender. On these strains, we will perform FT-IR capsular typing, which will have been developed during the first months of this project (section 5.4). Typing of the isolates of the retrospective study will be performed in the summer period of 2021, when few isolates of the ongoing prospective study (2020-2022) are expected.

4.3 Gannt Chart

* Intermediate reports will not be published publicly, and can only be consulted by participating hospitals and funding partners.

M1. Ethical Approval granted

M2. FT-IR based capsular typing test validated for 24 pneumococcal serotypes

M3. Protocol approved by all partners

M4. Preparatory phase finished, with Sensititre™ plates received, database and lab shipments completely prepared

M5. Capsular typing completed for selected non-invasive *S. pneumoniae* isolates (1995-2014)

M6. Capsular typing and MIC testing completed for all NIPD isolates collected during 20 months (2021-2022)

M7. Final statistical analyses on complete dataset finished

Publication 1 (provisional title): An FT-IR based test for fast *cps* typing in *Streptococcus pneumoniae*.
Publication 2 (provisional title): The genetic diversity of Belgian invasive and non-invasive *Streptococcus pneumoniae* strains in an international context.
Publication 3 (provisional title): Distribution of serotypes and drug resistance profiles of Belgian non-invasive *Streptococcus pneumoniae* strains in light of national vaccination campaigns

5 Microbiology

5.1 Sampling

Collecting laboratories will:

- prepare stock cultures from overnight pure cultures on blood agar plates.
- confirm *S. pneumoniae* identification by a slide agglutination test with specific antisera on the colonies, which will subsequently be transferred to a storage tube. A sufficient number of pre-filled ready-to-use tubes will be provided.
- prepare as dense suspensions as possible (preferably the culture from one blood agar plate) in 1-ml-aliquots of sterile BHI broth containing glycerol (10% v/v) in suitable screw-cap tubes, and vortexed thoroughly.
- quick freeze (*e.g.* in liquid nitrogen) immediately after vortexing (if part of the general lab routine), and store tubes preferably at ≤ -70°C.

5.2 Storage

Isolates will be kept at ≤ -70°C. Cultures are stored in Brain Heart Infusion (BHI) broth containing glycerol (10% v/v). For retrieval of frozen stock cultures, the inoculum will be subcultured on sheep blood agar in 5% CO_2.

5.3 Confirmation of correct identification

All isolates will be tested for susceptibility to optochin and bile solubility. In case of doubt or negative reaction, a slide agglutination test (e.g. Slidex pneumo-Kit™, BioMérieux) as well as their MALDI-TOF (Biotyper, Bruker) profile will be examined.

5.4 Typing of cps genes

Capsular typing is performed according to the traditional Quellung reaction. However, in this project we will investigate the use of Fourier transform infrared (FT-IR) spectroscopy to predict the serotype of the most prevalent serotypes [32,33]. It has been reported that discriminatory biochemical fingerprints, observed on FT-IR spectra, consistently correlate with sugar-based coating structures that reflect strain variation. This will allow to perform capsular typing on a medium-throughput scale (96-well plate format), at less than €5 consumable cost per sample. The test will be validated, and published, in collaboration with the NRC for pneumococcal diseases. A downside of this test is that it relies on relative (comparative) measurements, with a large number of controls (n=24) being needed in every run. This implies collaboration with the NRC to traditionally type all strains that were nontypable using FT-IR.

To type nonencapsulated strains, the *cps* locus of the strain will be sequenced to classify the strain in group I (mutation or deletion in *cps* genes) or group II (replacement of the *cps* genes) NeSP. For this last group, 2 subgroups can be distinguished in function of genes that replace the *cps* locus. In the NCC1 subgroup (null capsule clade 1), the *psp*K gene is present, while in the NCC2 subgroup the *cps* genes are replaced by *ali*C and *ali*D.

5.5 *Drug susceptibility testing*

The antibiotic susceptibility test will be performed by broth microdilution in cation-adjusted Mueller-Hinton broth with 2-5% (v/v) lysed horse blood, using custom-made Sensititre™ plates (Thermo Scientific, USA) containing lyophilized antibiotics. The final volume of broth in each of the wells of the microdilution plates is 50 µl, and the inoculum 5 x 10^4 CFU. The inoculum will be prepared by direct suspension of colonies grown for 18-20 hours in 5% CO_2 on sheep blood agar in saline. Inoculum adjustment will be done with a photometric device. Incubations will be carried out at 35°C in an ambient air incubator, and MICs will be read after 20-24 hours using Vizion™ technology. The following strains will be used as first-line control: *S. pneumoniae* ATCC 49619, and *S. pneumoniae* TPN.881 (strain F9 from study 25000/BE-96-01; M phenotype).

In this surveillance study, we will test the antibiotics listed in the table below.

	MIC testing	
Antibiotic	**Range (µg/ml)**	**No. Dilutions**
Penicillin G	0.015-8	10
Amoxicillin	0.12-4	6
Moxifloxacin	0.004-8	11
Amoxicillin/Clavulanic acid 2:1	2-16	4
Cefuroxime	0.5-8	5
Cefotaxime	0.016-4	9
Clindamycin	0.016-4	9
Erythromycin	0.25-4	5
Meropenem	0.016-4	10
Levofloxacin	0.008-4	10
Tetracycline	0.25-8	6
Imipenem	0.5-4	4
Trimethoprim/Sulfmethoxazole	0.06-2	6

5.6 *Whole-Genome Sequencing of selected isolates*

Whole-Genome Sequencing (WGS) will be performed for multi-drug resistant and non-typeable strains or if clonality of the same serotypes originating from different, but geographically linked, patients needs to be assessed.

In this project, we budget the sequencing of 300 strains, the selection of which will be made in collaboration with the NRC for pneumococcal diseases to maximize genetic insights in the diversity of circulating IPD and NIPD serotypes.

For every selected strain, gDNA will be extracted using the automated MagCore™ platform (Atrida, NL) using standard procedures for gram-positive bacteria. Illumina MiSeq™ sequencing will be performed in-house, according to internally validated procedures. In brief, high-quality sequences are mapped onto a single reference genome, *S. pneumoniae* ATCC700669. We will use the bioinformatic pipeline designed by the NRC, to maximize the comparability with IPD isolates.

5.7 Data processing and transmission of the results

For every NIPD isolate, all typing data will be collected along with its metadata in a central database using a standardized software format (*e.g.* Microsoft Access), which will be password protected, with aggregated and anonymized data accessible to the participating laboratories.

6 Statistics

6.1 Calculation of the sample size

To estimate the number of samples to be sent by each hospital, we calculated a total sample size based on the main objective; estimating the proportions of circulating serotypes. Based on an estimated serotype distribution of: PCV7= 7.5%, serotype 19A= 7.5%, serotype 3 =5%, 4 additional serotypes included in vaccine PCV13 =30%, serotypes not included in the vaccine non-PCV13 = 50%, we would need a total of 2250 samples to be able to obtain these estimates within 95% confidence intervals with a width of ± 20 during the whole study period (2020-2022). This translates into approximately 2 samples per week from each hospital for the 20 months period (ie total sample size/(number of hospital*weeks of study): 2250/(12*80) ≅ 2).

For the first secondary objective (establishing a statistically significant shift in serotypes from serotypes included in the PCV-13 vaccine to serotypes not included in the PCV-13 vaccine between retrospective and prospective samples), we estimated the necessary sample size to detect a 20% decrease in PCV-13 serotypes (PCV7-serotypes, 19A, 3, 4 additional PCV13) (or 20% increase in non-PCV-13 serotypes) at n=1500 for each time point (1995-2015 and 2020-2022).

6.2 Evaluation

Our final goal is to investigate Belgian trends in serotype distribution in the non-invasive pneumococcal population. The statistical data analysis will be performed with Stat10 software (Stata Statistical Software: Release 13. College Station, TX), in collaboration with Sciensano's Epidemiology Service.

Using data from the Prospective/Retrospective surveillance track:

- Chi-square trend analysis of circulating serotypes within the non-invasive *S. pneumoniae* population, integrating retrospective data (1995-2014) with prospectively collected data (2020-2022). Data will be compared with the serotype dynamics of IPD, annually published by the NRC for pneumococcal diseases.

- Measurement of association strength within each stratum of age, gender and infection type by the prevalence ratios with exact Fisher tests for statistical significance. Particular attention will be given to NeSP isolates, their association with patient type and their influence on drug resistance.
- Chi-square trend analysis of antimicrobial resistance for all tested drug classes, in relation to historic data (1995-2013) and in association with serotype.
- Integrating metadata with WGS, we will perform phylogenetic analysis of sequenced isolates in their national and international context [13,14], and identification of genetic determinants of antibiotic resistance.

7 Access to source data and data in general

Neither of the funding partners can have a role in data collection and interpretation. However, funding partners can request at any time intermediate results or reports. The final report will be put under a six months embargo before open access publishing in Summer 2023.

8 Monitoring and quality assurance

All laboratory analyses will be performed according to ISO15089 standards. An internal project board consisting Alexandra Vodolazkaia (MD, PhD), Ioannis Passaris (Ir, PhD), Pieter-Jan Ceyssens (Ir., PhD) and Raymond Vanhoof (MD, PhD) will monthly follow up experimental results.

9 Adverse events

Not applicable.

10 Ethics

Upon approval of the project by at least two contact partners, a general and specific ethical application will be filed, in collaboration with Sciensano's legal department and GDPR officer. Approval of local and ethical committees will be sought.

11 Budget

PERSONNEL

Description	% Effort dedicated to this study	Institutional salary (3 years)	TOTAL
Coordinating Scientist	0.6 FTE	€ 276,372	€ 165,823.2
Statistician	0.2 FTE	-*	€ 0
Lab Technician	0.8 FTE	€ 135,312	€ 108,249.6
		SUBTOTAL	**€ 274,072.8**

STUDY/LAB SUPPLIES

Description	Unit Cost	# of Units	TOTAL
Strain handling (collection, transport)	€ 21.50	3,600	€ 77,400
Strain investigation (ID, MIC testing, geno-serotyping)	€ 24.03	3,600	€ 86,508
Retrospective study	€9.60	1,500	€ 14,400
WGS of 300 strains	€ 94.50	300	€ 28,350
		SUBTOTAL	**€ 206,658**

ADDITIONAL COSTS and FEES

Description	Unit Cost	# of Units	TOTAL
FT-IR development	€ 6,500	1	€ 6,500
Publication fees	€ 4,000	3	€ 12,000
Administration	€ 5,000	1	€ 5,000
		SUBTOTAL	**€ 23,500**

DIRECT COSTS - SUBTOTAL	**€ 504,230,8**
OVERHEADS (20%)	**€ 100,846.2**
TOTAL STUDY BUDGET 2020-2022 (will be equally split among participating companies)	**€ 605,076.9**
MAXIMAL TOTAL BUDGET REQUESTED PER FUNDING PARTNER**	**€ 302,538.5**
MAXIMAL REQUESTED ANNUAL REQUESTED BUDGET**	**€ 100,846.2**

To avoid any conflict of interest, this proposal is send at the same time to the three companies producing pneumococcal vaccines, being Pfizer, GSK and MSD. This project will only be launched if at least 2 out of 3 companies are willing to participate and share the associated costs.

12 References

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13 Changes in Protocol to previous version:

- Figure 6 : Changed the phrase ‘Genetic Serotyping’ to ‘Capsular Typing’
- Section 4.1 :
    - Deleted from exclusion criteria “Patients for whom other bacterial pathogens were detected in sample besides *S. pneumoniae*. This is to limit the inclusion of asymptomatic carriers in the study.”
    - Added to inclusion criteria: “from patients diagnosed with pneumonia, sinusitis and otitis.”
    - Moved to metadata requirements: “For bacterial cultures from sputum and endotracheal aspirate samples, the amount of epithelial cells and white blood samples”
    - Added: “For every shipped strain (with associated metadata) during the study period, the laboratory will be compensated by payment of €20”
- Section 4.3. Changed M2 to “FT-IR based capsular typing test validated for 24 pneumococcal serotypes”
- Section 5.3. *S. pneumoniae* confirmation was changed to match with methods in the NRC, towards “All isolates will be tested for susceptibility to optochin and bile solubility. In case of doubt or negative reaction, a slide agglutination test (e.g. Slidex pneumo-Kit□, BioMérieux) as well as their MALDI-TOF (Biotyper, Bruker) profile will be examined.”
- Section 5.4; Genetic capsular typing is replaced by FT-IR based typing.” Capsular typing is performed according to the traditional Quellung reaction. However, it this project we will investigate the use of Fourier transform infrared spectroscopy to predict the serotype of the most prevalent serotypes [32,33]. It has been reported that discriminatory biochemical fingerprints observed on FT-IR spectra have been consistently correlated with sugar-based coating structures that reflect strain variation. This will allow to perform capsular typing on a medium-throughput scale (96-well plate format), at less than €5 consumable cost per sample. The test will be validated, and published, in collaboration with the National Reference Center for Pneumococcal diseases (UZ Leuven, Belgium).”
- Section 5.5. The table with antibiotics to be tested was adapted, to match the drugs tested at the NRC.
- Section 5.6; WGS data analyses will be done using the pipeline designed by the NRC at UZLeuven