Contents

ABSTRACT

The aim of this study was to achieve diagnosis and serotyping of empyema cases using real-time polymerase chain reaction directly on pleural fluid in a large cohort of Italian children hospitalized with CAP complicated by empyema in pre- and post-pneumococcal conjugate vaccine (PCV13) era.

One hundred and thirty five children 0-16 years admitted with a diagnosis of parapneumonic effusion (PPE) to 24 Pediatric Hospitals all over Italy were included in the study.

Realtime PCR was performed in all 135 patients and was positive for at least one of the tested pathogens in 109/135 (80.7%) patients. *Streptococcus pneumoniae* was the most frequent and was found in 84/109 (77.1%) samples. In non-pneumococcal PPE pathogen more frequently found were *Streptococcus pyogenes* (8/135 cases) in younger (mean age 3.89 years) and *Fusobacterium necrophorum* or *nucleatum* (5/135 cases) in adolescents (mean age 11.49 years).

Between 2007 and 2010 (before the inclusion of Prevenar 13 in the Italian schedule) we found 81 cases of PPE. *Streptococcus pneumoniae* was found in 45/81 (55.6%). Betweeen 2011 and January 2014 we found 55 cases of PPE. *Streptococcus pneumoniae* was the most frequent and was found in 39/55 (70.1%).

We have shown that in Italy there was not an increase in the incidence of other pathogens in PPE after the introduction of Prevenar13.

RT-PCR allowed serotyping in 81/84 (96.4%) patients with pneumococcal PPE. Actually, serotype distribution in PPE demonstrated a large preponderance of serotypes 1, 3 and 19A (non-PCV7 serotypes). Serotype 1 appeared significantly associated with complications and older age. Since the mean age for PPE is usually older than 3 years,

at this point of time the direct effect of PCV13 vaccination in reducing PPE incidence is not yet evident . However in the 3 years since PCV13 has been introduced in Italy we did not found pneumococcal PPE in children aged <3,5 years. No cases in children aged <2 years, no cases in vaccinated children.

In 47 patients both cultures of pleural fluid and RT-PCR were performed at the same time. In the subgroup of 34 patients in which both tests were performed and etiologic diagnosis was obtained, the diagnosis was obtained by RT-PCR only in 30/34 (88.2%) patients, by both tests in 4/34 (11.8%) and by culture only in none. Realtime PCR appears 8.5 times more sensitive in individuating an etiologic agent in PPE and is significantly more sensitive than culture in achieving etiologic diagnosis of PPE.

Introduction

Empyema is a severe complication of community-acquired pneumonia (CAP); its incidence has recently shown a marked increase in the pediatric population all over the world (1–5), and a role of aggressive serotypes of *S. pneumoniae*, such as serotype 1 (6,7) has been hypothesized.

Empyema appears to be increasing despite the decrease of pneumonia cases obtained through *S. pneumoniae* vaccination (8–10).

Actually, *Streptococcus pneumoniae* continues to be the principal etiological agent of empyema in the pediatric age, even though an increase in the incidence of other pathogens has been described (11). Microbiological diagnosis of empyema is essential in order to monitor, during the years, the evolution in etiology and the role of different epidemiologic factors or mass vaccination.

Culture techniques fail to identify a causative organism in over 70% of empyema cases, most likely due to previous antibiotic therapy (11–13). However it is absolutely uncommon that pleural drainage could be performed before antibiotic therapy is started. Recently, pneumococcal antigen detection using immunochromatography assay has been shown to be a useful technique in diagnosis of pneumococcal infection on pleural fluid (14) but it gives no information on serotypes.

Molecular techniques such as Realtime PCR (RT-PCR) can be applied directly on biological samples and can be efficiently used for diagnosing and serotyping of bacterial diseases (6,15,16). These methods do not require viable bacteria, need small sample volumes, and appear more sensitive than cultural methods (6,15). Therefore those methods could enable identification of a pathogen in the majority of ‘culture-negative’ cases (6)(17)(11).

The aim of this study was to achieve diagnosis and serotyping of empyema cases using Realtime polymerase chain reaction directly on pleural fluid in a large cohort of Italian children hospitalized with CAP complicated by empyema in pre- and post-pneumococcal conjugate vaccine (PCV13) era.

PATIENTS AND METHODS

Study Design

This observational study was conducted from April 2007 through January 2014 in children admitted, with a diagnosis of empyema complicating CAP, to pediatric hospitals or pediatric wards of general hospitals in Italy. Hospitals from all Italian regions were invited to participate.

Case definition

CAP was diagnosed as previously described (6) on the basis of clinical signs and radiologic examination. Empyema (or parapneumonic effusion, PPE) was defined as loculated pleural fluid on chest X-ray, chest ultrasound or computed tomography (CT), and/or any pleural fluid parameters consistent with empyema (cloudy, bloody, or purulent appearance; white blood cells count >50,000 x 109/L; pH<7.1; lactic dehydrogenase level >1,000 IU/L; glucose level <40 mg/dL; positive Gram stain, and/or culture, and/or molecular testing) (18).

Patients

All patients aged 0–16 years admitted with a diagnosis of PPE to the participating centers during the study period were included in the study. The exclusion criteria were severe concomitant disease and nosocomial acquired infections. To exclude the latter, children who had been admitted to the hospital or had been evaluated in the day hospital or emergency department in the previous 14 days were excluded from the study. Written informed consent was obtained from all parents or guardians. The study was approved by the local institutional review board.

Pleural fluid sample

Pleural fluid obtained by pleural puncture was evaluated by Realtime PCR; clinicians were allowed to choose when to also request pleural fluid cultures. They were requested

to described why they did or did not request pleural fluid culture. For culture purposes, 4–6 mL of pleural fluid were immediately sent to the local laboratory. Samples for molecular tests were saved at room temperature and sent to the central laboratory within 3 days (Immunology Laboratory, Anna Meyer Children's University Hospital, Florence, Italy) with use of a fast freepost carrier. The samples were delivered by the following day, and the molecular tests were performed within 2 h after delivery; 200 microL of whole pleural fluid was used for both diagnosis and serotyping by RT-PCR.

Realtime PCR

Realtime PCR was performed as previously described (6,15). Primers and probes for 14 pathogens, usually considered as the most frequently in cause in PPE, were used (Table 1). All the samples found positive for *S.pneumoniae* were serotyped by Realtime PCR (6) using 33 primer couples and probes. All the positive results (with the exception of *Streptococcus pneumoniae*) have been confirmed using sequence analysis of 16s gene. Pneumococcal serotyps were divided into PCV7 serotypes (4,6B,9V,14,18C,19F,23F), PCV13 serotypes (7serotypes with the addiction of 1,3,5,6A,7F,19A) and non PCV13 serotypes (all the serotypes not included in the PCV13 vaccine).

Etiological diagnosis of parapneumonic effusion (PPE)

Etiological diagnosis of PPE was performed in the presence of culture positive for any pathogen or in the presence of RT-PCR positive for the pathogens evaluated. If there was no increase in fluorescent signal before the 45th cycle, the sample was assumed to be negative by RT-PCR.

Statistical Analysis

Data were processed with the SPSSX 11.0 statistical package (SPSS); P <.05 was considered to be statistically significant. Results were expressed as mean levels and standard deviations or as median and interquartile range as appropriate. The $\chi 2$ test was

used to assess group differences in categorical variables. Odd ratios and 95% confidence intervals (CIs), when possible, were calculated. For continuous variables, Student's *t* test was used with logarithmic transformation of non-normal distributed variables.

RESULTS

One hundred and thirty five children with PPE (76 males [56.29%]; mean age 5,22 years; interquartile range: 2.98-5.99 years) were included in the study. Patients were recruited from 24 hospitals that are in 9 of 20 Italian regions. The 9 regions (Piemonte, Lombardia, Veneto, Emilia Romagna, Toscana, Lazio, Marche, Campania, Sicilia) are distributed all over the country and are among the most populous regions in Italy, so that they represent 90.1% of the Italian population aged <16 years.

Etiological diagnosis of parapneumonica effusion (PPE)

Realtime PCR was performed in all 135 patients and was positive for at least one of the tested pathogens in 109/135 (80.7%) patients. *Streptococcus pneumoniae* was the most frequent and was found in 84/109 (77.1%) samples; *Streptococcus pyogenes* was the second in frequency with 8/109 (7.4%) cases. The complete distribution of all etiologic agents is shown in Figure 1.

In non-pneumococcal PPE pathogen more frequently found were *Streptococcus pyogenes* (8/135 cases) in younger (mean age 3.89 years) and *Fusobacterium necrophorum* or *nucleatum* (5/135 cases) in adolescents (mean age 11.49 years).

Between 2007 and 2010 (before the inclusion of Prevenar 13 in the Italian schedule) we found 81 cases of PPE. *Streptococcus pneumoniae* was found in 45/81 (55.6%). The complete distribution of all etiologic agents is shown in Figure 2.

Betweeen 2011 and January 2014 we found 55 cases of PPE. *Streptococcus pneumoniae* was the most frequent and was found in 39/55 (70.1%); (in one patient both *S.pyogenes* and *S.pneumoniae* were found at the same time); the other etiological agents are described in Figure 3.

In 47 patients both cultures of pleural fluid and RT-PCR were performed at the same time; RT was positive for *Streptococcus pyogenes* in 4/47 cases, *Staphilococcus aureus*

in 2/47, *Fusobacterium* (1 *necrophorum*, 3 *nucleatum*) in 4/47, *Pseudomonas aeruginosa* in 1/37, *Streptococcus pneumoniae* in 24/47 and was negative for all pathogens tested in 12/47. In those 47 patients pleural fluid culture was positive in 4 cases (2 *Streptococcus pyogenes* and 2 *Streptococcus pneumoniae*) and confirmed RT-PCR results. Cultures was never positive in samples negative by RT-PCR.

Overall, in the subgroup of 34 patients in which both tests were performed and etiologic diagnosis was obtained, the diagnosis was obtained by RT-PCR only in 30/34 (88.2%) patients, by both tests in 4/34 (11.8%) and by culture only in none. Realtime PCR appears 8.5 times more sensitive in individuating an etiologic agent in PPE and is significantly more sensitive than culture in achieving etiologic diagnosis of PPE (odds ratio and confidence limit not calculable, Cohen's k, 0.06; $p<10-6$, by McNemar's test).

Among clinicians who chose to request cultural tests, 6/39 (15.4%) answered that they did that because they followed the local diagnostic procedure for severe infections. Among clinicians who chose not to request cultural tests 9/39 (23.1%) answered they did not have microbiology facilities at the time of the test and 24/39 (61.5%) answered they had stopped using cultural tests on pleural fluid because of frequent negativity of the test.

Antibiotic treatment had been received before pleural drainage by all children studied.

Serotyping of S.pneumoniae

RT-PCR allowed serotyping in 81/84 (96.4%) patients with pneumococcal PPE. The distribution of serotypes before and after the introduction of PCV13 vaccination is summarized in Figures 4. Serotype 1, 3 and 19A were the most frequent before 2010, respectively 17/45 (37.8%) , 11/45 (24.4%) and 5/45 (11.1%) cases. Serotype 1, 3, 7F and 19A are the most frequent after the introduction of PCV13, respectively 18/38 (47.3%) , 10/38 (26.3%), 6/38 (15.8%) and 2/38 (5.3%) cases. We have not found non-

PCV13 serotypes in children with PPE. None of the children affected by pneumococcal PPE due to PCV13 serotypes had been vaccinated with PCV13.

The mean age of children with pneumococcal PPE did not differ in the two study periods (2007-2010 or 2011-2013), before or after the introduction of PCV13: respectively 4.9 years before 2011 and 4.3 years in the period 2011-2013, p=ns.

The mean age of children with pneumococcal PPE due to serotypes 1, 3 and 19A where respectively 5.24, 3.50 and 3.39 years with no difference between pre and post 2011.

Distribution over time of parapneumonic effusion (PPE)

The trend of CAP complicated by empyema was fluctuating over time and is shown in Figure 5a. The trend is similar if we consider only pneumococcal PPE (Figure 5b). There is a clear peak in 2009 when there was H1N1 influenza pandemic and 15/21 (71.4%) cases of pneumococcal PPE occurred between October and March.

The pneumococcal distribution showed the absence of PCV7 serotypes since 2009, after 4 years from the start of PCV7 vaccination. Non-PCV serotypes in PPE have not increased over the time either before or after PCV13 (Figure 6).

DISCUSSION

The present study, performed on a large cohort of 135 Italian children hospitalized for CAP complicated by empyema in 24 hospitals in Italy, demonstrates that *Streptococcus pneumoniae* was the most frequent pathogen involved.

In USA from 2001 and in Europe some years later, there was a significant reduction of invasive pneumococcal disease (IPD) thanks to the use of the first pneumococcal conjugate vaccine (PCV7).

The incidence of PID was reduced by 77% on the group of younger children (aged <1 years); by 83% on children aged 1 years and by 73% on children aged > 2 years (19). Also pneumonia and non-invasive pneumococcal diseases (8) as acute otitis media were reduced (20). Similar data, obtained by our group in Italy confirmed those results (Azzari et al., personal data, in publication). The increase of CAP complicated by empyema has been demonstrated since the beginning of nineties and could not be stopped with the use of PCV7 vaccine. Actually, serotype distribution in PPE demonstrated a large preponderance of serotypes 1, 3 and 19A (non-PCV7 serotypes). Serotype 1 appeared significantly associated with complications and older age and the important role of serotype 1 in all pneumonia cases and especially in complicated ones had been suggested by many reports (2,3,18,21). Since the mean age for PPE is usually older than 3 years, at this point of time the direct effect of PCV13 vaccination in reducing PPE incidence is not yet evident. However in the 3 years since PCV13 has been introduced in Italy we did not found pneumococcal PPE in children aged <3,5 years. No cases in children aged <2 years, no cases in vaccinated children. We can assume that all pneumococcal PPE by 1,3, 7F and 19A serotypes will decrease in the future.

A shift toward non-vaccine serotypes has been described after mass vaccination (22). This is not the case of PPE in Italy. We have not found non-PCV13 serotypes in children with PPE. Actually, as previously described, PPE pulmonary complications are strongly associated with the most aggressive serotypes 1,3 and 19A (6).

We could not find an increase in PPE due to pathogens different from *S.pneumoniae*.

Actually, *Streptococcus pneumoniae* continues to be the principal etiological agent of empyema in the pediatric age, even though an increase in the incidence of other pathogens has been described in USA and Europe (11,23). We have shown that in Italy there was not an increase in the incidence of other pathogens in PPE. In non-pneumococcal PPE pathogen more frequently found were *Streptococcus pyogenes* (8/123 cases) in younger (mean age 3.89 years) and *Fusobacterium necrophorum* or *nucleatum* (5/123 cases) in adolescents (mean age 11.49 years).

Our data confirm that RT-PCR is significantly more sensitive than culture for diagnosis of CAP complicated by empyema so that a number of clinicians had stopped using cultural tests on pleural fluid because of frequent negativity of the test. It's obvious that a child or adult who is subject to pleural drainage has already received a long antibiotic treatment. The treatment reduces the probability of the cultural test to be positive.

RT-PCR is simple and known to be less expensive (15) than culture-based methods. Molecular methods have demonstrated (24) a sensitivity that is significantly higher that of culture-based methods.

Surveillance of invasive bacterial disease and associated complication is necessary and will give information, in the next years, on the direct effect of PCV13 vaccination in reducing parapneumonic effusion.

Table 1 – Primer and probe used in RT-PCR

target	Forward primer	Reverse primer	Probe
S. pneumoniae	acgcaatctagcagatgaagc	tgtttggttggttattcgtgc	FAM_tttgccgaaacgcttgatacaggg
P. aeruginosa	ccggagaccttcagcaacat	gacgccggagttgaggaa	FAM_atcctggccaagcgcatccg
E.coli	cggaagcaacgcgtaaactc	gcgtcgcagaacattacattg a	FAM_acccgacgcgtccgatcacct
S. aureus	gtttctatatcaactgtagcttctttatcca	cattaaaggtgtgcaaaagatggt	FAM_ acgttgaataattgtacgattctgacg
K.pneumoniae	ggc sca rta tca gtt cga ctt	ccc ttc rat atc ctt ccc ttt c	FAM_tct gcg tcc gtc cct cgg
F.necrophorum	tggatgccaatggagtta	gagaggtctttccgacc	FAM_tggatcggaagtggagc
HI B/C	ggc gaa atg gtg ctg gta a	ggc caa gag ata ctc ata gaa cgt t	FAM_cac cac tca aac gaa tga gcg tgg
S. pyogenes	Gca ctc gct act att tct tac ctc aa	gtc aca atg tct tgg aaa cca gta at	FAM_cga caa ctc atc aag gat ttc tgt tac ca
Salmonella	gggcaataagctggtttartgc	ggaatgtggcggattgatg	FAM_cctgtaaacgcgcagccgcct
Hi	ggt gca ttc gca gct tca g	gat tgc gta atg cac cgt gtt	FAM_ttg ttt ata aca acg aag gga cta acg t
M. pneumoniae	ggcagtcaacaaaccacgtatg	cgtatcggcgtaacacaaagg	FAM_tgtttccaaaatcgttcccgaa
PIV1	tga ttt aaa ccc ggt aat ttc tca t	cct tgt tcc tgc agc tat tac aga	FAM_acg aca aca gga aat c
PIV2	agg act atg aaa acc att tac cta agt ga	aag caa gtc tca gtt cag cta gat ca	FAM_atc aat cgc aaa agc tgt tca gtc act gct ata c
PIV3	tga tga aag atc aga tta tgc ata tc	ccg gga cac cca gtt gtg	FAM_tgg acc agg gat ata cta caa agg caa aat aat att tct c

Figure 1 - The complete distribution of all etiologic agents in children with PPE (2007-2013)

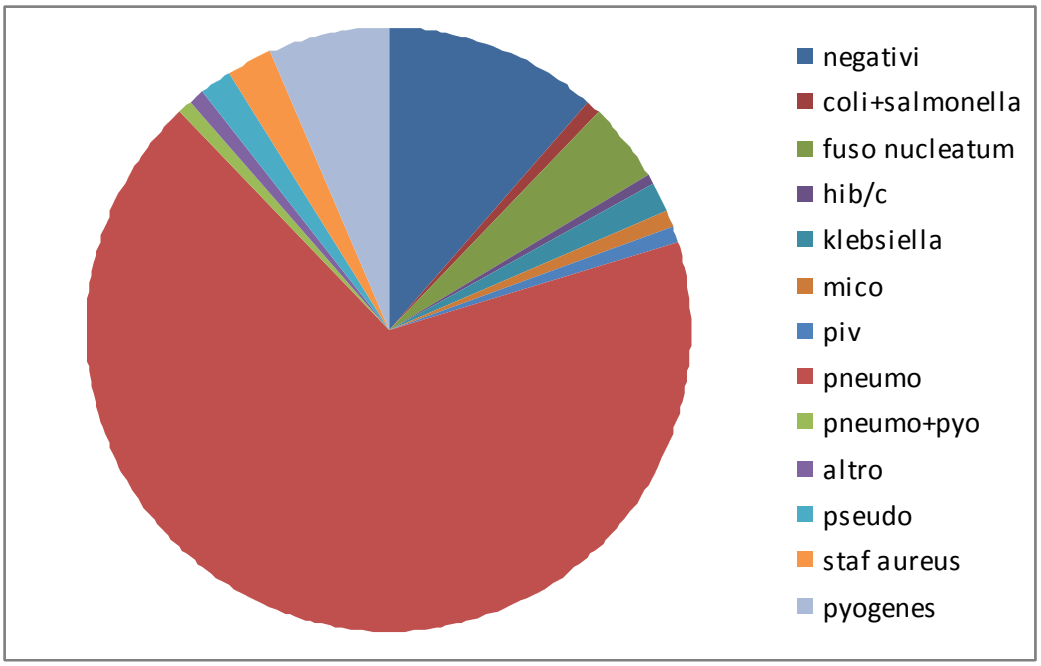


Figure 2 - The complete distribution of all etiologic agents in children with PPE before the introduction of 13-valent conjugate anti-pneumococcal vaccine (PCV13) (2007-2010)

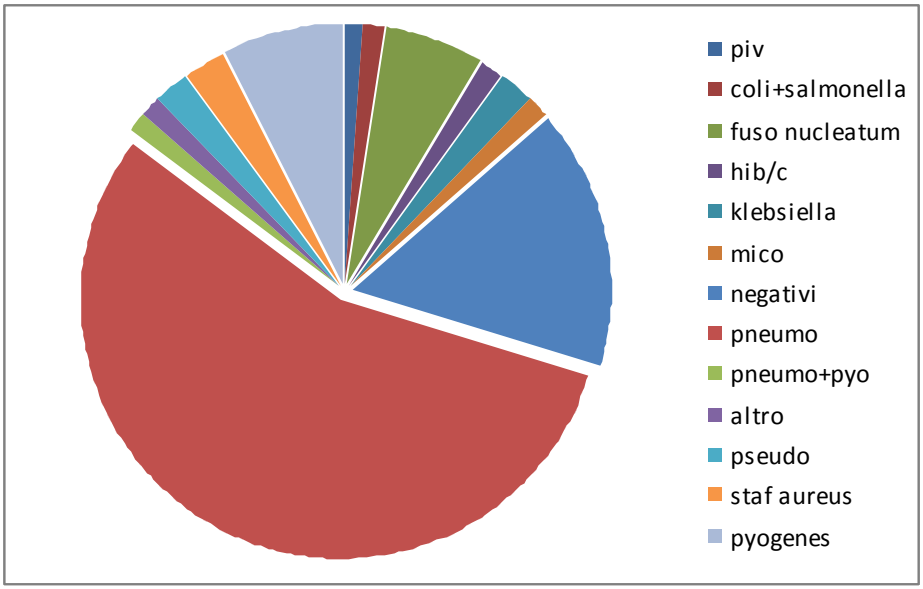
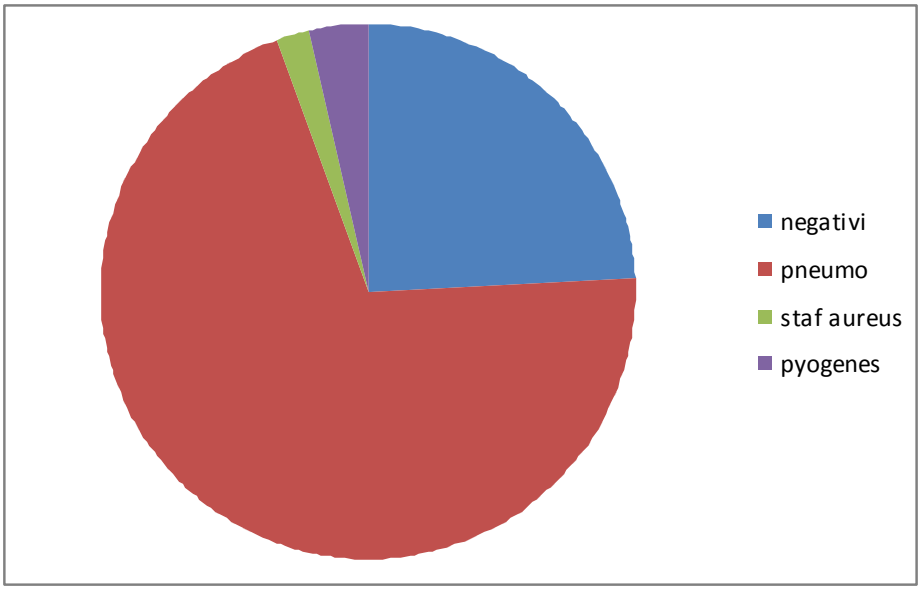
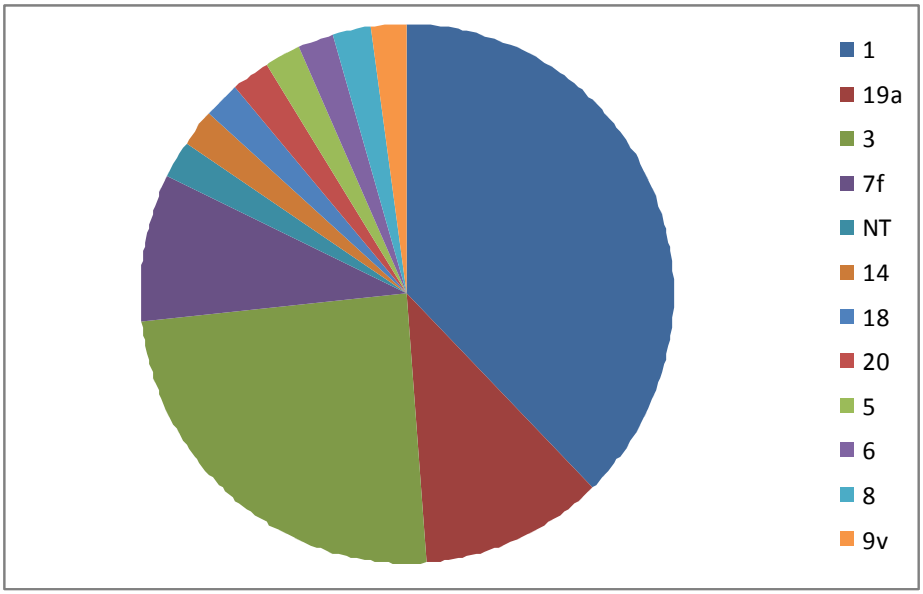


Figure 3 - The complete distribution of all etiologic agents in children with PPE after the introduction of 13-valent conjugate anti-pneumococcal vaccine (PCV13) (2010-2013)



Figures 4 - The distribution of *Streptococcus pneumoniae* serotypes before and after the introduction of PCV13.



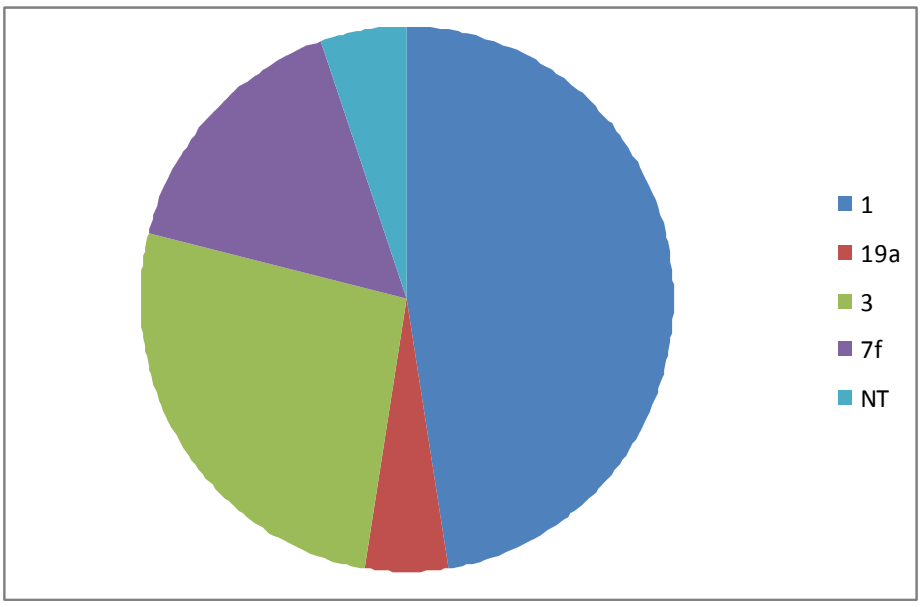


Figure 5a - Trend of PEE over the time (2006-2013)

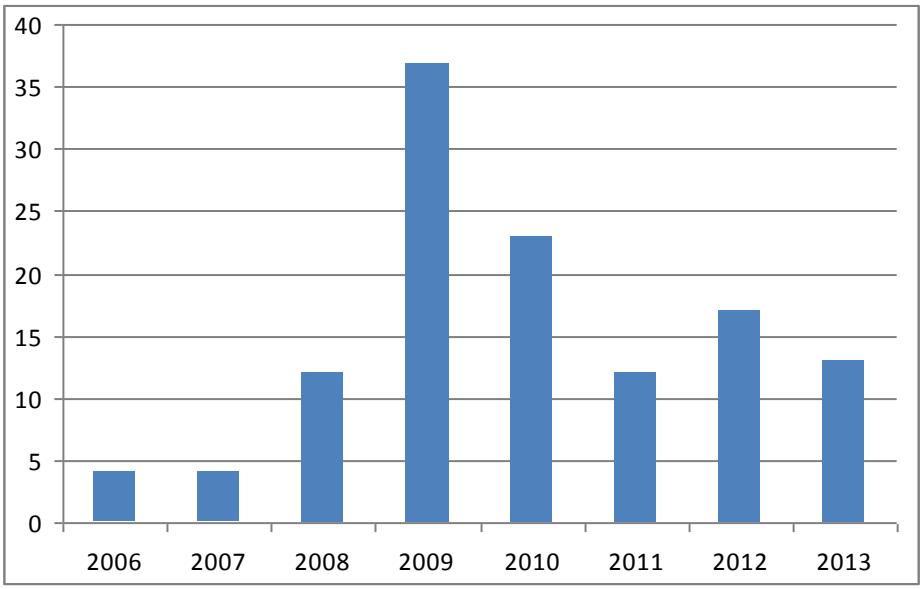


Figure 5b - Trend of pneumococcal PEE over the time (2006-2013)

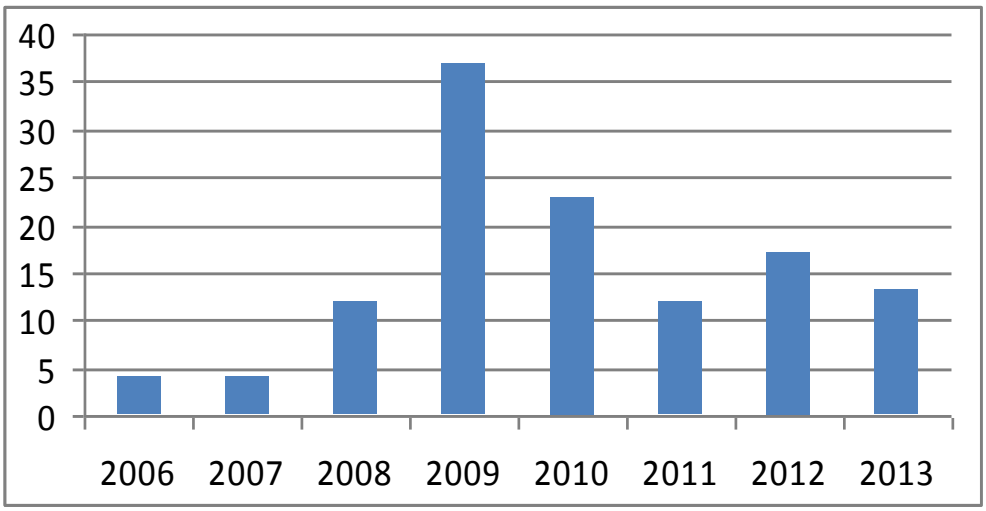
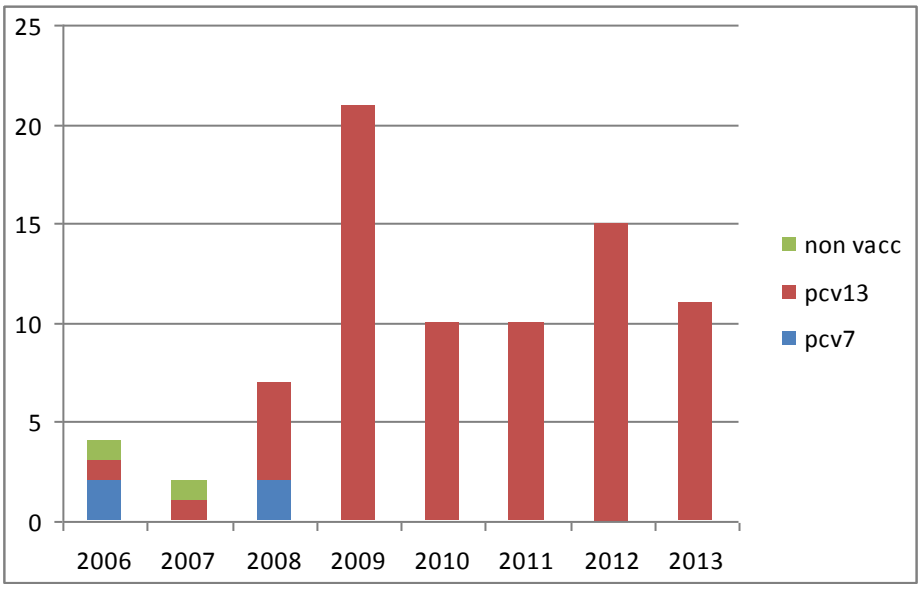


Figure 6 - Distribution of *Streptococcus pneumoniae* serotypes over time



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