Clinical Presentation of Invasive Pneumococcal Disease in Spain in the Era of Heptavalent Conjugate Vaccine

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Background: The aim of this study was to analyze the rate of incidence, clinical presentation, serotype, and clonal distribution of invasive pneumococcal disease (IPD) in the era of heptavalent pneumococcal conjugate vaccine (PCV7) in Barcelona, Spain.

Methods: This was a prospective study comprising all children <5 years with IPD who were managed in 2 tertiary-care, pediatric hospitals between January 2007 and December 2009. IPD was defined as the presence of clinical findings of infection together with isolation or detection of DNA of *Streptococcus pneumoniae* in a sterile fluid sample.

Results: In this study, 319 patients (53.3% male), mean age 29.6 months, were included. Comparing rates in 2007 and 2009 (76.2 and 109.9 episodes/100,000 population, respectively), an increase of 44% (95% confidence interval, 10%–89%) was observed. The main clinical presentation was pneumonia (254 episodes, 79.6%), followed by meningitis (29, 9.1%), and bacteremia (25, 7.8%).The diagnosis was made by positive culture in 123 (38.6%) patients and in 196 (61.4%) by real-time polymerase chain reaction. Serotype study was performed in 300 episodes, and 273 (91%) were non-PCV7 serotypes. The most frequent serotypes were 1 (20.7%), 19A (15.7%), and 3 (12.3%). A minimal inhibitory concentration ≥0.12 μg/mL to penicillin was detected in 34.4% of isolates. Sequence type 306 expressing serotype 1 was the most frequent clonal type detected (20.3% of studied strains).

Conclusions: IPD continues to increase in Barcelona, and the rate is higher than previously reported as a result of low sensitivity of bacterial culture. Non-PCV7 serotypes were responsible for 91% of episodes and pneumonia was the main clinical presentation.

Key Words: *Streptococcus pneumoniae*, pneumococcal conjugate vaccine, pneumonia, serotype, MLST

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Streptococcus pneumoniae is a major cause of morbidity and mortality worldwide, especially among young children, despite the availability of antibiotic treatment and vaccines. The World Health Organization estimates that every year more than one million children younger than 5 years die of invasive pneumococcal disease (IPD), mainly in developing countries.[1]

The imbalance between host factors and virulence of the pathogen is partly responsible for the production of IPD. The main virulence factor of pneumococcus is the polysaccharide capsule, with 93 serotypes with differing pathogenicity.[2]

Following introduction of pneumococcal conjugate vaccine (PCV7) in the United States, there was a dramatic decline in IPD rates and drug-resistant pneumococci.[3,4] However, in Spain and other countries, we observed a significant increase in the rate of IPD caused by non-PCV7 serotypes and a slight reduction in the rate of IPD caused by PCV7 serotypes.[5] There was a change in the main serotypes associated with IPD, and this change was associated with changes in clinical types of IPD,[6] a reduction in the rate of antibiotic-resistant strains causing IPD, and the emergence of previously established virulent clones of non-PCV7 serotypes.[5]

The introduction of real-time polymerase chain reaction (PCR)-based methods that specifically identify capsular DNA in direct sample offer a sensitive, rapid, and simple approach for the surveillance of IPD.[7] Different authors have reported that molecular methods can be used directly on sterile biologic samples, improving the ability to diagnose IPD.[8–12] At present, little is known about the epidemiologic characteristics, clinical presentation, and outcome of IPD including episodes with negative bacterial culture. The purpose of this study was to determine the epidemiologic variables, clinical presentation, current trends, and serotypes and clones of *S. pneumoniae* among children in Barcelona, Spain, after the implementation of PCV7, in 2001, including patients with negative culture who were diagnosed by real-time PCR.

MATERIALS AND METHODS

Patients and Definitions

We performed a prospective study comprising all children <5 years with IPD managed in 2 tertiary-care pediatric hospitals in Barcelona (Spain) during a 3-year period (January 2007-December 2009). These 2 centers serve a pediatric referral population of 134,662 children <5 years (around 27% of the Catalan pediatric population <5 years).[13]

An episode of IPD was defined as the presence of clinical findings of infection together with isolation and/or DNA detection of pneumolysin *(ply)* gene and an additional capsular gene of *S. pneumoniae* by real-time PCR in any sterile body fluid such as blood, cerebrospinal fluid, pleural fluid, or articular fluid.

Data Collected and Analyzed

Epidemiologic characteristics included age, gender, immunization status against *S. pneumoniae* (when written records were available), underlying medical condition, group child care attendance, antibiotic treatment and/or respiratory infection before the diagnosis of IPD, history of breast-feeding, household size, and exposure to tobacco smoke.

Clinical characteristics including clinical presentation, intensive care unit (ICU) admission, complications, antibiotic treatment and duration, days of hospitalization, and clinical outcome were also recorded.

Microbiologic Bacterial Culture and Antimicrobial Susceptibility Studies

All pneumococcal isolates were identified by standard microbiologic methods that remained constant during the study period. Agar dilution technique was used to determine the minimal inhibitory concentration (MIC) of several antibiotics, including penicillin and cefotaxime. American Type Culture Collection 49619 (serotype 19F) was used as a control. Susceptibility to penicillin and other antibiotics was defined according to the 2008 meningeal break points by the Clinical Laboratory Standards Institute.[14] Isolates with intermediate- or high-level resistance were defined as nonsusceptible.

DNA Detection of *S. pneumoniae* by Real-time PCR

Detection of *ply* gene of *S. pneumoniae* was performed by real-time PCR according to a published assay.[7]

Serotype Identification

Serotyping of strains isolated by culture was carried out by the Quellung reaction, using antisera provided by the Statens Serum Institut (Copenhagen, Denmark), or by dot-blot serotyping.[15] MICs and serotyping of the strains were performed at the National Center for Microbiology (Majadahonda, Spain). Detection of pneumococcal serotypes in negative culture clinical samples but *ply* pneumococcal gene positive was performed according to a published multiplex real-time PCR methodology.[16] This procedure includes the DNA detection of conserved *wzg* capsular gene of *S. pneumoniae* and other different genes selected to distinguish 24 serotypes (1, 3, 4, 5, 6A/C, 6B/D, 7F/A, 8, 9V/A/N/L, 14, 15B /C, 18C/B, 19A, 19F/B/C, 23A, and 23F).

Serotypes were classified into the following groups: PCV7 serotypes (4, 6B, 9V, 14, 18C, 19F, and 23F), PCV10 serotypes (PCV7 serotypes plus 1, 5, 7F), and PCV13 serotypes (PCV10 serotypes plus 19A, 6A, 3).

Clonal Study

Clonal composition of strains was analyzed using multilocus sequence typing (MLST) as reported elsewhere.[17] The assignment of alleles and sequence types (ST) was carried out using the software at the pneumococcal web page www.mlst.net. Analysis of ST and assignment to clonal complex (CC) were performed with the eBURST program.[18] STs that shared 6 of 7 alleles (single locus variants) were considered a CC.

Statistical Analysis

Rates of IPD, defined as the number of episodes per 100,000 population, were calculated using the annual estimates of pediatric population obtained from the Department of Statistics in Catalonia[13] and the percentage of capture of both hospitals among total hospitalization in children <5 years. In Catalonia county, these hospitals captured, during the study period, 25.4% of all pediatric hospitalizations <2 years and 32.2% of pediatric hospitalizations between 2 to 5 years.

We used the χ^2 test or Fisher exact test to compare proportions and Student *t* test to compare means. Statistical analyses were performed using SPSS for Windows, version 17.0 (SPSS), and Epi Info, version 6.0 (Centers for Disease Control and Prevention). We calculated 95% confidence intervals (CIs), and 2-sided *P* values ≤0.05 were considered to be statistically significant.

RESULTS

During the study period, 319 episodes of IPD were identified in 319 patients, including 170 male patients (53.3%) and 149 female patients (46.7%), with a mean age of 29.6 months (standard deviation [SD]: 15.7). One hundred ninety-two episodes (60.2%) were in children aged 24 to 59 months, 99 (31.0%) in children aged 7 to 23 months, and 28 (8.8%) in children <6 months of age.

There was clearly seasonal variation. 73% of episodes were detected during cool months (October to March) versus 27.2% during warm months (April-September), $P < 0.001$.

Two hundred forty-four (76.5%) patients reported group child care attendance, 144 (45.1%) patients had a viral respiratory infection by history during the month before IPD, and 44 (13.8%) had received antibiotic treatment the month before IPD.

Two hundred twenty-five (70.5%) patients reported a history of breast-feeding, and 122 (38.2%) patients had been exposed to tobacco smoke. The mean household size was 4 cohabitants (SD: 1.2, range: 2–10).

According to the criteria of the American Academy of Pediatrics,[19] only 5 of 319 (1.5%) children were at high risk of IPD, including 2 children with malignant disease who were receiving immunosuppressive therapy, 1 with diabetes mellitus, 1 with congenital cyanotic cardiopathy, and 1 with pulmonary emphysema.

Concerning immunization status for *S. pneumoniae*, 168 (52.8%) cases had received at least 1 dose of PCV7, although only 141 (44.3%) were considered fully vaccinated by age.

Incidence

Rates of IPD increased between 2007 and 2009; the incidence of IPD in <5 years in 2007 was 76.2 cases/100,000 population; in 2008, it was 82.2 cases/100,000 population; and in 2009, it was 109.9 cases/100,000 population. Comparing rates in 2007 and 2009, there was an increase of 44% (95% CI: 10%–89%; $P = 0.008$). There was a significant increase in the rate of pneumonia during the study period: an increase of 81% (95% CI: 33%–148%; $P = 0.001$) comparing 2007 versus 2009. There were no significant changes in the rates of meningitis and bacteremia during the study (Table, Supplemental Digital Content 1, http://links.lww.com/INF/B14 shows rate of IPD in children according to age group during 2007–2009).

Clinical Presentation

Overall, the clinical diagnosis of patients included in this study was pneumonia in 254 (79.6%) patients, meningitis in 29 (9.1%), bacteremia in 25 (7.8%), arthritis or osteomyelitis in 6 (1.9%), sepsis in 3 (0.9%), and cellulitis in 2 (0.6%). Among pneumonia cases, 51 (20.1%) were noncomplicated pneumonia, 171 (67.3%) were empyema, and 32 (12.6%) parapneumonic pleural effusion.

Table 1 shows the distribution of positive samples detected by culture and by real-time PCR according to main clinical presentations.

Children were admitted to the hospital for 310 (97.2%) of the 319 episodes. The mean length of stay was 10.8 days (SD: 7.5). The longest mean stay by clinical presentation was 18.25 days (SD: 13.19) for meningitis. Of note, patients with noncomplicated pneumonia have no statically differences in the median age,

TABLE 1. Distribution of Positive Samples Detected by Culture and by Real-time PCR According to Main Clinical Presentations

	Positive Blood Culture	Positive Plasma Real–time PCR	Positive Pleural Effusion Culture	Positive Pleural Effusion Real–time PCR
Noncomplicated Pneumonia (n = 51)	18	39		
Parapneumonic pleural effusion (n = 32)	5	18	0	12
Empyema (n = 171)	18	75	32	151
			CSF Culture	CSF Real–Time PCR
Meningitis (n = 29)	15	18	20	19
Bacteremia (n = 25)	25	2		

gender, days of hospitalization, and total days of antibiotic in the groups "positive blood culture" and "only plasma real-time PCR positive."

The mean of days of antibiotic therapy (including extrahospital treatment) was 17.8 days (SD: 6.8). Arthritis and osteomyelitis were the diagnosis with the longest duration of antibiotic therapy (28.17 days, SD: 11.78).

Forty-four children (13.8%) were admitted to the pediatric intensive care unit. Overall, 27 of 29 episodes of meningitis (93.1%), 14 of 254 (5.5%) episodes of pneumonia, and 3 of 3 (100%) episodes of sepsis were admitted to ICU.

Among children admitted to ICU, 22 (51.2%) had received at least 1 dose of PCV7, but only 19 (44.2%) were fully vaccinated for age.

Of the 319 patients, there were 4 (1.3%) deaths, 3 patients with meningitis and 1 with sepsis. Thirty-four patients (10.7%) had sequelae associated with *Streptococcus pneumoniae*: neurologic sequelae in 15 of 29 (51.7%) meningitis episodes and pulmonary sequelae in 17 of 254 (6.7%) children with pneumonia.

Serotypes, Molecular Study, and Antibiotic Susceptibility

Diagnosis was established in 123 (38.6%) episodes by culture and in 196 (61.4%) by real-time PCR.

The serotyping study was done in 300 (94%) of the total IPD episodes. In 120 (40%), the serotyping study was carried out with strain isolates from culture, whereas 180 (60%), were done with direct samples by multiplex, real-time PCR. Overall, 23 different serotypes were identified. Nevertheless, there was a large number (76, 25.3%) of samples with *ply* and *wzg* gene positive but no specific gene of 24 serotypes tested, so we considered these as "other serotypes." The most frequent among identified serotypes were serotype 1 (62; 20.7%), 19A (47; 15.7%), and 3 (37; 12.3%). Of the 300 episodes, 27 (9%) were caused by PCV7 serotypes and 273 (91%) were caused by non-PCV7 serotypes. One-hundred nineteen (39.7%) were caused by PCV10 serotypes and 209 (69.7%) by PCV13 serotypes. Of 27 patients who had IPD attributed to PCV7 serotypes, 5 were well vaccinated. The characteristics of vaccinated children with IPD caused by PCV7 serotypes are shown in Table 2.

There were significant differences in the clinical presentation among the most prevalent serotypes detected in the study: serotype 1 and serotype 3 were significantly associated with pneumonia, whereas the clinical presentation of episodes caused by serotype 19A was more diverse (Table 3). Among episodes resulting in death, 3 were caused by non-PCV7 serotypes (serotypes 7F, 27, and 6A) and 1 by vaccine serotype 23F in an unvaccinated child.

PCV7 serotypes were significantly present in younger children (mean age, 21.2 vs. 30.4 months in IPD caused by non-PCV7 serotypes; $P = 0.004$). In addition, IPD by PCV7 serotypes was associated with a higher rate of sequelae than non-PCV7 serotypes (25.9% vs. 9.9%; $P = 0.02$). In contrast, non-PCV7 serotypes were associated mainly with pneumonia: 81.3% of total episodes caused by non-PCV7 versus 48.1% of episodes caused by PCV7 serotypes; $P < 0.001$ (Table 3).

TABLE 2. Characteristics of Vaccinated Children With Invasive Pneumococcal Disease Caused by PCV7 Serotypes

Sex	Age (mo)	Clinical Presentation	Previous Disease	Serotype
Female	5	Bacteremia	Methylmalonic acidosis	19F
Female	13	Bacteremia	Retinoblastoma (neutropenia)	19F
Female	45	Pneumonia	No	19F
Male	29	Pneumonia	No	14
Female	50	Pneumonia	No	14

Molecular analysis by MLST was performed for 108 of 123 (87.8%) strains isolated by culture. Overall, when comparing our data with isolates listed in the MLST database, there were 46 different STs, including 8 new ST profiles (ST3437, serotype 23F; ST3436, serotype 38; ST4827, ST2948, and ST4826, serotype 19F; ST4676, serotype 27; ST5195, serotype 19A; ST4834 serotype 7F). Of these, 50% new ST expressed PCV7 serotypes. eBURST analysis using the stringent 6/7 identical loci definition grouped the 46 ST into 6 CCs and 34 singletons (Fig. Supplemental Digital Content 2, http://links.lww.com/INF/B15, shows clonal distribution of 108 invasive isolates from pediatric patients obtained by use of the output of eBURST, version 3. Each circle represents single MLST, with the area proportional to the number of isolates of that ST. Black lines represent single-locus variants).

Six CCs or ST accounted for 55.9% of total collection: ST306 (n = 22 isolates serotype 1), ST320 (n = 9 isolates serotype 19A), CC289 (n = 8 isolates serotype 5), ST191 (n = 8 isolates serotype 7F), ST1201 (n = 7 isolates serotype 19A), and CC276 (n = 5 isolates serotype 19A and 1 serotype 24B).

Comparative analysis of our serotype and ST results with those published in the MLST database showed that 5 of our STs expressed serotypes different than those previously reported (capsular switching): ST101 (serotype 15C), ST109 (serotype 23F), ST230 (serotype 24B), ST433 (serotype 28), and ST2372 (serotype 23F). Antibiotic susceptibility was available for 120 of 123 (97.5%) strains.

None of the 120 strains was fully resistant (MIC >8 μg/mL) and 3 (2.5%) were intermediately penicillin-resistant according to nonmeningeal breakpoints. Two of these strains be-

TABLE 3. Epidemiologic Data and Clinical Characteristic of 300 Episodes of IPD Caused by PCV7 Serotypes, Non-PCV7 Serotypes, and the 5 Main Serotypes Detected in the Study

Serotype	No. Episodes	Age (Mean SD)	Sex (Males) N (%)	Clinical Presentation*				PICU Admission N (%)	Outcome	
				Pneumonia N (%)	Bacteremia N (%)	Meningitis N (%)	Others N (%)		Sequelae	Death
PCV7	27	21.2 (13.68)	16 (59.3%)	13 (48.1%)	7 (25.9%)	6 (22.2%)	1 (3.7%)	6 (22.2%)	7 (25.9%)	1 (3.7%)
Non–PCV7	273	30.4 (15.84)	145 (53.1%)	222 (81.3%)	18 (6.6%)	23 (8.4%)	10 (3.6%)	38 (13.9%)	27 (9.9%)	3 (1.1%)
Serotype 1	62	41 (10.48)	34 (54.8%)	62 (100%)	0	0	0	2 (3.2%)	3 (4.8%)	0
Serotype 19A	47	19.21 (10.54)	28 (59.6%)	32 (68.1%)	6 (12.8%)	6 (12.8%)	3 (6.3%)	9 (19.1%)	4 (8.5%)	3 (2.4%)
Serotype 3	37	29.03 (14.11)	16 (43.2%)	36 (97.3%)	0 (0%)	1 (2.7%)	0	6 (16.2%)	5 (13.5%)	0
Serotype 7FA	21	24.71 (16.2)	16 (76.2%)	12 (57.1%)	4 (19%)	4 (19%)	1 (4.9%)	5 (23.8%)	4 (19%)	1 (4.8%)
Serotype 14	12	24.71 (13.3)	9 (75%)	11 (91.7%)	1 (8.3%)	0	0	0	3 (25%)	0

*Other clinical presentations were arthritis or osteomyelitis, sepsis, and cellulitis.

Statistically significant differences (χ^2 test for categorical variables and Student t test for continuous variables) were found for the following.

Mean age: PCV7 versus non-PCV7 serotypes ($P = 0.004$); serotype 1 versus other serotypes ($P < 0.001$); serotype 19A versus other serotypes ($P < 0.001$).

Gender: serotype 7F/A versus other serotypes ($P = 0.03$).

Clinical presentation: serotype 1 versus other serotypes ($P = 0.001$).

PICU admission: serotype 1 versus other serotypes ($P = 0.002$).

Outcomes: PCV7 versus non-PCV7 serotypes ($P = 0.02$).

longed to ST320 expressing serotype 19A and the other belonged to ST2948 expressing serotype 19F. Forty-one isolates (34, 4%) had an MIC $\geq$0.12 μg/mL, and 18 of these isolates (43.9%) were serotype 19A. Regarding cefotaxime, only 2 isolates (1.7%) showed an MIC $\geq$4, and both belonged to ST320-expressing serotype 19A. Regarding meningeal breakpoints, 21 isolates (17.5%) showed a diminished susceptibility to cefotaxime, and serotype 19A account 66.7% of these episodes.

DISCUSSION

This is a prospective study that updates the information about IPD in children in a geographical area without systematic vaccination. The inclusion of episodes with negative culture and only detected by real-time PCR has allowed us to gain greater insight into the burden of the disease and the main serotypes causing IPD in Barcelona. We think that molecular methods can be used directly not only on samples as cerebrospinal fluid, pleural effusion, or arthritis fluid but also in plasma improving the ability to diagnose IPD. The usefulness of real-time PCR in blood has been discussed because some authors found a high rate of detection of pneumococcal DNA in healthy controls associated with nasopharyngeal carriage.[20] However, we consider plasma PCR–positive patients with noncomplicated pneumonia and negative culture as patients with pneumococcus pneumonia and not false positive from pneumococcus colonization. All these patients are clinically compatible with pneumococcus pneumonia (all of them have high fever, cough, crackling, or hypophonesis in the auscultation and radiologic image of alveolar condensation). Moreover, our patients with noncomplicated pneumonia have no statically differences in clinical variables in the groups "positive blood culture" and "only plasma real-time PCR positive." In the same way, other authors have described previously the validity of plasma PCR in diagnosing IPD.[8,11] A low bacterial load could explain the negativity of the culture in these patients. However, more studies in this area will be required to confirm the validity of plasma PCR in determining deep-seated pneumococcal infection.

The incidence of IPD continues to increase in our geographic area. The incidence is higher than previously reported, presumably as a result of low sensitivity of the bacterial culture, which was the only microbiologic criterion for definition of IPD in previous studies.[5] The hospitals included in the study are the most important ones in pediatrics in Catalonia. Non-PCV7 serotypes cause most IPD episodes, whereas PCV7 serotypes cause only a minority of cases. The change in pneumococcal serotypes causing IPD is associated with a change in clinical presentation and in some epidemiologic characteristics.

Concerning clinical manifestations, the proportion of pneumococcal bacteremia and meningitis is relatively stable, but a significant increase in pneumonia was observed. These changes were observed by others in the United States.[21]The increase in pneumonia has also been observed in other regions of Spain[22] and in other countries such as Denmark and United States.[23,24] A significant proportion of these pneumonias is complicated by empyema, and some of the children (5.5%) developed pulmonary sequelae and required intensive care management. This high proportion of empyema and parapneumonic pleural effusion could also be explained because the study was performed in 2 tertiary-care pediatric hospitals. Of concern is the increase in complicated pneumonias caused by non-PCV7 serotypes. It is important that vaccines against *S. pneumoniae* include serotypes associated with pneumonia, such as serotypes 1 and 3.

The mean age of children with IPD is higher than previously reported in the prevaccine era,[25] and the majority of children with IPD is healthy without any recognized risk factors. This high proportion of healthy children is different from what was recently reported by Kaplan et al.[21] These differences are caused in part by the introduction of a virulent clone of serotype 1, with proven capacity to produce outbreaks, just before the implementation of PCV7 in our country.[5] Serotype 1, which is associated with pneumonia in older healthy children,[22] was the main serotype detected in our series, whereas in Kaplan et al's study, serotype 1 was infrequent.

It is remarkable how many serotypes are involved, which demonstrates the great diversity of pneumococcus. The detection of only 24 serotypes by multiplex real-time PCR methodology is a limitation of the study and raises concern about the high diversity of pneumococcus and the need for accurate surveillance of this disease in the coming years, including new molecular methods to detect a wider range of serotypes.

Despite the low vaccination coverage (approximately 50%), a low rate of infections due to vaccine serotypes were found. These data confirm, as have many other studies, that PCV7 is highly effective against IPD caused by vaccine serotypes, because this vaccine also prevents IPD in adult contacts and nonvaccinated siblings through indirect effect (herd immunity) on pneumococcal transmission.[26]

Regarding the 5 cases of IPD in vaccinated children, it is important to note that 2 of them had a previous disease and had not completed the vaccination schedule, which might explain this failure.

As to the clonal study, it also showed great genetic diversity in the strains that produce IPD in our pediatric population, including the appearance of new ST and capsular switches.

As reported before,[5] ST306 is the most important ST in our population. This ST relates to an increase of empyema.[22] Recently, our group showed that this increase of empyema associated with ST306 may be because of the presence of PsrP,[27] a pneumococcal virulence factor not present in all STs of *S. pneumoniae*. PsrP is an adhesine related to the invasion of pulmonary cells by pneumococcus.

As for the study of antibiotic susceptibility, we previously reported a decrease in the global rate of penicillin resistance if we compare the present rate with that of the prevaccine period.[5] Nevertheless, the presence of strains of serotype 19A, especially those with ST320 having multiple antibiotic resistances, is grounds for concern. PCV13 includes serotype 19A, which we hope will be controlled once the new vaccine is implemented.

One of the limitations of the study is that not all episodes of IPD are detected, as blood cultures and/or PCR *S. pneumoniae* are not performed in all children with fever or suspected of pneumonia. Therefore, some bacteremias and pneumonias have presumably not been diagnosed. However, our guidelines for evaluating children with fever did not substantially change during the study period.

In conclusion, IPD continues to increase in Barcelona, and the rate is much higher than previously reported due to low sensitivity of bacterial culture. Non-PCV7 serotypes were responsible for 91% of episodes, and pneumonia was the main clinical presentation.

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