ABSTRACT
A prospective study in 99 hospitalized children <5 years with community-acquired pneumonia was conducted to evaluate the contribution of a nested PCR (nPCR) to detect Streptococcus pneumoniae (Spn) and to analyze the association between nPCR results, chest radiograph findings and white blood cell count (WBC) for differentiating pneumococcal from viral aetiology of pneumonia. Besides, 20 children of similar age, without any signs of infections were studied as control group. In all samples were performed DNA extraction from plasma was performed in all samples, followed by an in vitro amplification by nPCR, using commercial reagents and oligonucleotides specific for the pneumolysin gene. Furthermore, in all patients, the presence of respiratory viruses in nasopharyngeal aspirates was studied by direct immunofluorescence, the presence of respiratory viruses. Five patients had pneumococcal positive culture and 10 patients have viral evidence. Thirty six samples were nPCR positive and 63 were nPCR negative. In the 5 patients with pneumococcal positive culture, Spn was also detected by nPCR. Among the 63 patients with nPCR negative, 70% received prior antibiotic treatment. The proportion of patients with WBC > 15 x 10^9/L was 58% and 43%, in patients with nPCR positive and negative results, respectively (P= 0.14). The proportion of alveolar and unilateral infiltrates was significantly higher in nPCR positive children (72% vs 45%, P= 0.003). There was a significant association between alveolar-unilateral infiltrates and WBC>15 x 10^9/L among patients with positive nPCR results (P= 0,02). Interstitial infiltrates were seen in both negative and positive nPCR (P= 0,32). Finally, we consider that nPCR provides an important contribution to confirm pneumonia pneumococcal and highlight mixed viral/ bacterial infection.

Keywords: Streptococcus pneumoniae; Nested PCR; Diagnosis, Differential; Pneumonia
RESUMEN
Se realizó un estudio prospectivo de 99 niños menores de 5 años, hospitalizados con diagnóstico clínico de neumonía adquirida en la comunidad para evaluar la contribución de una nested-PCR para detectar *Streptococcus pneumoniae* y analizar la asociación entre los resultados de esta técnica molecular, hallazgos radiológicos y recuento de glóbulos blancos para diferenciar neumonías neumocócicas de virales. También se estudiaron 20 niños sin signos de infección alguna (grupo Control). En todas las muestras se realizó una extracción de ADN a partir del plasma, con posterior amplificación por nPCR, utilizando reactivos comerciales y oligonucleótidos específicos para el gen de la neumolisina. Además, en los aspirados de los 99 niños se estudió la presencia de virus respiratorios por Inmunofluorescencia. Solo 5 pacientes desarrollaron Spn en el hemocultivo y en 10 se detectó la presencia de virus sincicial respiratorio (VSR). En los pacientes estudiados, 36 dieron nPCR positivas y 63 negativas. En los 5 pacientes con cultivo positivo, Spn también fue detectado por nPCR. De los 63 pacientes nPCR negativas, 70% recibió tratamiento previo de antibióticos. La proporción de pacientes con WBC> 15 x 10^9/ L fue de 58% y 43%, en los pacientes con resultados nPCR positivos y negativos, respectivamente (P = 0,14). La proporción de infiltrados alveolares y unilateral fue significativamente mayor en los niños nPCR positivo (72% vs 45%, P = 0,003). Se observó una asociación significativa entre la imagen de infiltrados alvéolar-unilateral y leucocitos> 15 x 10^9 / L en los pacientes con resultados positivos nPCR (P = 0,02). Infiltrados intersticiales fueron vistos en nPCR tanto negativas como positivas (P = 0,32). Por último, consideramos que nPCR ofrece una importante contribución para confirmar neumonía neumocócica y resaltar infección mixta viral / bacteriana.

**Palabras claves:** *Streptococcus pneumoniae*; Nested PCR; Diagnóstico diferencial; Neumonía

INTRODUCTION
Community-acquired pneumonia (CAP) is a common cause of outpatient visits and hospital admission for children worldwide, presenting a high level of morbidity and mortality [1, 2]. Several bacteria and viruses, and their combinations, can cause the infection. Many published studies have addressed the differentiation of bacterial from viral pneumonia using clinical [3-5], radiological [6-9], and haematological tests [4, 10-13] but these methods have not been found to be sufficiently reliable in differential diagnosis. Suggestive clinical signs, blood or pleural cultures, laboratory indices of inflammation such as white blood cell count as well as neutrophilia and radiographic findings correlate poorly with etiologic diagnosis [3, 11-12, 14-15]. *Streptococcus pneumoniae* (Spn) is the most common bacterial agent of CAP among children under 5 years of age in developed countries [5, 16-18]. For this reason, isolation of Spn from blood or pleural fluid remains as the gold standard test [5, 15]. However, in children with pneumonia, blood culture yields a sensitivity lower than 10% due to most of them presents no bacteremia [17, 19-20], small sample volumes and/or prior antibiotic therapy [10, 21-22]. Moreover, blood culture presents an additional drawback: they may take several days in order to give a positive result. Molecular methods, such as nested-PCR (nPCR), are both rapid and specific tests to detect very small amounts of a specific microorganism and they are being used with increased frequency for the diagnosis of several infectious diseases including pneumococcal pneumonia [23-29].

The goals of the present work were to evaluate the contribution of a nPCR technique for the rapid diagnosis of pneumococcal pneumonia and to analyze the association between nPCR results, chest radiograph findings and white blood cell count (WBC) for differentiating pneumococcal from viral aetiology of pneumonia.

**MATERIAL AND METHODS**
**Subjects**
Between April 2011 to March 2012, 99 consecutive patients (3 to 59 months) admitted to the Hospital de Niños de Santa Fe “Dr. O. Alassia” (Santa Fe, Argentina) with clinical and radiological evidence of pneumonia, were enrolled in a prospective study. Written informed consent was obtained of the child’s parents/guardians before enrolling each child in the study. The project was reviewed and approved by the Research Ethics Committee of the Facultad de Bioquímica y Ciencias Biológicas (Universidad Nacional del Litoral) and the Ethics Committee of the above mentioned hospital.

Children were excluded if there were underlying chronic diseases. Only 2 children had previously received the pneumococcal conjugate vaccine. Chest radiological reading was performed by a paediatric radiologist, blind to clinical information.
The findings were classified as: alveolar and/or interstitial, unilateral or bilateral pneumatic changes and pleural fluid. In addition, 20 age-matched children without any signs of infection, which attending to the Hospital for pre-operative studies, were included as control group.

**Clinical samples**

Blood samples were obtained from all patients within 24 h of admission for blood cultures, white blood cell count (WBC) and nPCR. Virus detection in nasopharyngeal aspirates was performed using immunofluorescence. The test panel included seven common respiratory viruses (respiratory syncytial virus (RSV), influenza A and B virus, parainfluenza 1, 2 and 3 virus and adenoviruses). When the children presented pleural effusion, the fluids were cultured. Also, blood samples for PCR were collected from all control patients.

**Polymerase chain reaction**

Blood samples collected in EDTA were centrifuged and remaining plasma was used for DNA extraction as follows: plasma (100 ul) was diluted with an equal volume of 0.1 M HCl-Tris buffer pH 8.0. The mixture was incubated at 100°C for 15 min, centrifuged at 10,000 X g for 5 min and the supernatant was stored at - 20°C until PCR assay. The nPCR was performed using primers described by Salo et al. (28) first-round (outer) PCR primers Ia (5-ATTCTCTGAACAGCTACCAACGA-3) and Ib (5-GATTCCTCTGCTTTTCAAGTC-3) amplify a 348-bp region. The second-round (inner) PCR primers Ila (5-CCCCCTTCTTCTGCGGTGTA-3) and IIb (5-TGAGCCGATTATTTTTTACTG-3) amplify a 208-bp region of the pneumolysin gene. In the first round PCR, 2.5 ul of DNA were added to the PCR mixture to give a final volume of 25 ul. Reaction mixture contained 1X Green Go-Taq PCR buffer (Promega Corp.), 20 pmol of each primer. Nested PCR was carried out using the same PCR mixture conditions with primers Ila and IIb, with 1ul of the first PCR transferred to a new tube containing 24 ul of PCR mixture. DNA amplification (first and nested-PCR) was performed on a programmable thermal cycler (Multigene Gradient TC 9600-G, Labnet) with the following parameters: 94°C for 10 min followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, with a last cycle at 72°C for 7 min. PCR products were analyzed by electrophoresis on agarose gels in the presence of ethidium bromide, observed under UV illumination and photographed using orange filter. Three different controls were included in all PCR reactions. DNA extracted from S. pneumoniae ATCC 49619 was used as positive control. Serum samples from healthy adult volunteers were used as negative controls. Sterile distilled water instead of DNA was used in the reagents controls.

**Statistical analyses**

The standard X2 test was used to compare the proportions between the groups. Odds ratio (and its confidence interval) were also computed.

**RESULTS**

The mean age of the patients was 20.4 months (range, 3 to 59 months). Thirty four children were >2 and 65 were <2 years old. Sex distribution was similar. Five patients (5%) with pneumonia had pneumococcal positive culture, four of them from blood, one from pleural effusion and viral evidence was found in 10% of the cases.

The distribution of the radiographic patterns was as follows: alveolar (61%, 59/96), interstitial (23% 22/96), alveolar-interstitial (16% 15/96), unilateral (58%, 56/96), bilateral (42%, 40/96) infiltrate and pleural effusion (11%).

The different combinations of the above mentioned patterns are shown in Tables 1 and 2. There were no associations between radiographic patterns and children’s age: 57% (37/65) of patients <2 years old presented both alveolar and unilateral infiltrates compared to 50% (17/34) of patients >2 years (P= 0.46; OR= 1.3, 95%CI: 0.6 - 3.0).

Thirty six (36%) samples were nPCR positive and 63 (64%) were nPCR negative. No association among PCR results and children’s ages (P= 0.54; OR= 0.8; 95% CI: 0.3-1.8) was found. In the 5 patients with pneumococcal positive culture Spn was also detected by nPCR.

Among the 36 patients with nPCR positive, 8 presented pleural fluid. In these nPCR positive cases there were 5 mixed viral/bacterial infections. Seven patients (19%) did not received prior oral antimicrobial therapy and 29 patients (81%) were treated with oral antibiotics before admission during different preceding periods (2 days, n= 23 and 3 days, n=6).

In the nPCR negative group, 70% (44/63) received prior antibiotic treatment (11/44 presented radiological pattern of bacterial pneumonia and WBC >15x10⁹/L) and 3 children under antibiotic treatment for 72 to 120 h before admission presented pleural effusion. Viral infection was detected in 5 of these nPCR negative patients.

In relation to WBC, they were grouped as count > or <15 x 10⁹/L. The proportion of patients...
with WBC > 15 x 10^9/L was 58% (21/36) and 43% (27/63), in patients with nPCR positive and negative results, respectively (P = 0.14, OR=1.9; 95% CI: 0.8-4.3).

The combination of nPCR results, radiographic findings and WBC counts are showed in Tables 1 and 2. It was found that the proportion of alveolar and unilateral infiltrates was significantly higher in children nPCR positive than in those children with negative nPCR (72% vs 45%, respectively; P = 0.003; OR= 3.5; 95% CI: 1.4-8.4). Furthermore, there was a significant association between alveolar-unilateral infiltrates and WBC > 15 x 10^9/L within patients with positive nPCR results (P= 0.02; OR=5.3; 95% CI: 1.1-25.7). On the contrary, interstitial infiltrates were seen in both negative and positive nPCR (P = 0.32; OR= 2.3; 95% CI: 0.8-6.9). Two (10%) of 20 blood samples nPCR assay from the control group were positive. Among the samples from studied patients, one case nPCR and virus detection positive, with no radiological evidence of bacterial etiology, and satisfactory discharge without antibiotic treatment during the hospitalization, makes us suspect that it is a false positive.

DISCUSSION

In the present study we tried have a rapid diagnosis of pneumococcal pneumonia by using a nPCR. We have previously reported the sensitivity and specificity of the assay: in our hands as little as 10 CFU/ml Spn can be detected and the specificity was shown by its negative results for other organisms including closely related streptococci. In this paper, we analyzed plasma from 99 children (<5 years old) with suspected of community acquired pneumonia. Nested PCR demonstrated to be 8 times more sensitive than blood and pleural fluid culture for Spn detection: 36 samples (36%) yielded positive results in the nPCR whereas only 5 patients had positive blood and pleural fluid culture. Similar to Michelow, there were no associations between nPCR results and children’s age.

On the other hand, several studies have suggested that bacterial pneumonia cannot be differentiated from non-bacterial pneumonia on the basis of the chest radiograph. In clinical practice, alveolar infiltrates on chest radiograph are often considered to indicate bacterial etiology of pneumonia but clinical studies have failed to confirm this concept.

Virkki et al. recently found that most children with an alveolar infiltrates and, in particular lobar infiltrates, have laboratory evidence of a bacterial pneumonia, whereas interstitial infiltrates are seen in both viral and bacterial cases although there were significantly more pneumonias with interstitial infiltrates in viral infection. Turner et al. studied the chest radiographs of 37 paedriatric outpatients with pneumonia and documented viral or bacterial infection: alveolar infiltrates were found in 38% of the cases with bacterial pneumonia and in 67% of those of viral pneumonia. Toikka et al. reported 84% and 9% of alveolar and interstitial infiltrates, respectively in children with blood culture positive pneumococcal pneumonia. Korpri et al. found that alveolar pneumonia was associated with bacterial infection in 74% and with sole viral...
infection in 26% of the cases (6). In agreement with Toikka (15), Virkki (7) and Korppi (6) we found that the proportion of alveolar and unilateral infiltrates was significantly higher in children with evidence of pneumococcal pneumonia (positive nPCR) than in those children showing negative nPCR results. In addition, there was a strong association among nPCR positive results and alveolar infiltrates with a WBC > 15 x 10^9/L count. We also found that 100% of children culture-positive for Spn were correctly identified by nPCR.

In the group of patients with negative blood cultures and positive nPCR, radiological findings, WBC count and quick and favorable clinical evolution after empirical antibiotic treatment were all suggestive of bacterial etiology. Hence we conclude that nPCR becomes a fast and useful tool for clinicians, confirming the suspected pneumococcal pneumonia. Only one case was considered “false-positive”.

Several factors interfere the sensitivity of nPCR tests, causing false-negative results, such as: the presence of inhibitors in blood or incomplete removal of them by dilution, types of blood components for testing, antibiotic treatment before admission, quick phagocytosis of bacteria by macrophages (23, 29). Any of these reasons could explain the negative nPCR results in 11 patients who presented radiological pattern of bacterial pneumonia and WBC >15x10^9/L in the group of 44 children that had received prior antibiotic treatment. These results generate an important question: are true or false negatives? Currently, we cannot know it due to the absence of a sensitive reference standard.

On other hand, nPCR identified Spn in 10% of control subjects (specificity 90%). A similar proportion was reported in previous studies, by Falguera et al. (4%) (22), Salo et al. (6%) (28), Dagan et al. (17%) (23). The last one reported that specificity was strongly affected by nasopharyngeal colonization by Spn, causing false-positive results among both healthy children and those with viral respiratory tract infections. Despite these limitations -which must be taken into account- as well as the absence of a sensitive “gold standard” test, we consider that the nPCR (due to both a sensitivity and speedness greater than cultures) provides an important contribution to confirm pneumococcal pneumonia and highlight mixed viral/ bacterial infection.

REFERENCES


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