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RESEARCH ARTICLE

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Detection of the major bacterial pathogens among children suffering from empyema in Ahvaz city, Iran

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Introduction: Empyema is one of the important causes of pediatric hospital admissions.

Aim: In this study, we had investigated the frequency rates of *S aureus*, *S pneumoniae*, *H influenzae*, and *P aeruginosa* using PCR and bacterial culture among children suffering from empyema in Ahvaz city, Iran.

Methods: This was a descriptive study conducted on the patients hospitalized in ICUs of two teaching Hospitals of Ahvaz, Iran, between March and September 2018 on 105 pleural fluid (PF) samples of the children less than 16 years of age with the diagnosis of empyema thoracis. These specimens were inoculated on the bacterial culture media and identified using biochemical characteristics. Then, the existence of the four pathogens mentioned above was evaluated using PCR method.

Result: In this study, these bacteria agents were identified in 81 (77.14%) and 30 (28.57%) cases using the PCR assay and bacterial culture, respectively. Moreover, the PCR assay identified the infectious agents in 51 (68%) of PFs where the culture method failed. *S pneumoniae* (63 cases) was recognized as the most common pathogen, followed by *P aeruginosa* (19 cases), *S aureus* (15 cases), and *H influenzae* (9 cases) using the bacterial culture and PCR. Co-infections were detected in 21 samples (20%) using PCR and one sample using the bacterial culture (*P aeruginosa* and *S pneumoniae*).

Conclusion: In this study, we found the higher frequencies of these microorganisms using PCR than culture. In addition, we showed that PCR was a sensitive and accurate method that unaffected by antibiotic therapy and could detect well co-infections.

KEYWORDS
bacterial culture, Empyema, PCR

1 | INTRODUCTION

Empyema thoracis is known as the most common complication of bacterial pneumonia. Moreover, it occurs in 5%-10% of patients suffering from pneumonia. The incidence rates of empyema are increasing in both children and adults, and have occurred approximately 10 cases /10 000.[1] On the other hand, it is an important cause of the hospital admission with a morbidity and mortality rate up to 10%.[2] The different causative organisms are responsible for the pleural infections in various countries. Hence, the analysis of the local epidemiological

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data is necessary. In general, aerobic bacteria are considered as the dominant microorganisms isolated from positive cultures, while anaerobic bacteria account exclusively for up to 15% of cases of pleural infections.[3,4] *Streptococcus pneumoniae* is the most common cause of empyema. Other important bacterial agents, which are becoming increasingly frequent in the pleural infections, are *Staphylococcus aureus*, *Streptococcus pyogenes*, *Haemophilus influenzae*, *Mycoplasma pneumoniae*, *Pseudomonas aeruginosa*, and other *Streptococcus spp*[1]

A fast and correct diagnosis followed by a period of appropriate antibiotic treatment is imperative for the mortality reduction in children.[5] Currently, the bacterial culture is the gold standard method for the diagnosis of the presence of bacterial pathogens in clinical samples. However, the detection rates of bacteria from clinical samples using culture are as low as between 17% and 42%.[6] The low rates can be explained to be due to some growth requirements or the administration of broad-spectrum antibiotics before obtaining pleural fluid (PF) samples for culture.[7] Unlike the bacterial culture, the molecular methods such as PCR have more sensitive for the detection of causative microorganisms. Moreover, PCR has many potential advantages, including positivity of the results in the early course of infection, and the lack of impacts of the prior administration of antibiotics on the results.[8] To our knowledge, in our region, the prevalence of the causative bacterial agents of empyema among pediatrics is not determined yet. Hence, this study was aimed to investigate the frequency rates of the major bacterial pathogens among children suffering from empyema using the bacterial culture and PCR in Ahvaz city, Iran.

2 | MATERIALS AND METHODS

2.1 | Design study

This was a descriptive study conducted on the patients hospitalized in ICUs (intensive care unit) of two Hospitals of Abouzar and Imam Khomeini (Ahvaz, Iran) between March and September 2018.

This study was performed on PF samples of the children less than 16 years of age with the diagnosis of empyema thoracis, as the consequence of a late pneumonia. Moreover, we collected these samples from patients with CAP (community-accrued pneumonia) or HAP (hospital-accrued pneumonia). The clinical condition of these patients had necessitated the drainage of PF. Empyema thoracis was diagnosed based on the macroscopic presence of the pus in PF samples and the clinical observation of chest X-ray. Exclusion criteria of the participants were the esophageal rupture, severe coagulopathy that contraindicates thoracentesis, immunodeficiency, abdominal or thoracic surgery, collagen vascular disease, or melioidosis.[8] Finally, we included all patients hospitalized in this period with the diagnosis of empyema who met the inclusion and exclusion criteria mentioned above.

Before enrollment to this study, the parents of patients had completed the informed consent forms. Study protocol and the consent form were reviewed and approved by ethics committees of Islamic Azad University, Branch of Yasouj, Iran.

2.2 | Microbiologic culture

In this study, PF samples were collected from patients with empyema for the detection of infectious agents. All of these samples were transferred to the microbiology laboratory of University of Medical Sciences of Ahvaz, Iran, during one hour. PF specimens were streaked on blood agar and chocolate agar (Merck-Germany) under the microaerophilic (8% CO_2) and aerobic conditions at 37°C and monitored daily to five days. The bacteria isolates were identified using the standard laboratory methods. Briefly, first Gram stain, colony morphology, and growth characteristics on the culture media were examined and then some additional tests were performed on these colonies. For example, optochin test and bile solubility were established for identification of *S pneumoniae*; coagulase test was established for *S aureus* and a requirement to the x and v factors for the identification of *H influenzae*.[9]

2.3 | DNA extraction

PF samples were concentrated by centrifugation (13 000 *g*, 5 minutes), and the pellets were used for DNA extraction. Before DNA extraction, a brief modification was performed on the samples. Moreover, an additional incubation at 37°C for 15 minutes with 15 µL of lysozyme (10 mg/mL) was carried out for proper lysis of the bacterial cell wall. Then, DNA was extracted using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) in accordance with the manufacturer's instructions.

TABLE 1 Primer sequences and annealing temperatures used in this study

Gene	Microorganism	Primer 5' to 3'	Annealing Tm	Amplicon size	Ref.
ecfX	*P aeruginosa*	F-ATGGATGAGCGCTTCCGTG R-TCATCCTTCGCCTCCCTG	52	528 bp	11
lytA	*S pneumoniae*	F-CATGGCGCCTTCTTTAGCGTC R-GAGTTCATGACGGACTACCG	54	546 bp	8
bexA	*H influenzae*	F-TATCACACAAATAGCGGTTG R-GGCCAAGAGATACTCATAGA	55	182 bp	10
femA	*S aureus*	F-CAGTCACTAGCTGGGCCACTT R-GTCAATTAGGTTATGTCGGAG	50	256 bp	8
mecA	MRSA	F-TGGCTATCGTGTCAC AATCG R-CTGGAACTTGTTGAGCAGAG	51	304 bp	8

2.4 | PCR

The molecular identifications of the major bacterial pathogens were performed by the amplifications of species-specific genes. The PCR targets were the*lytA* gene for *S pneumoniae*,[8] the *P1* gene for *H influenzae*,[10] the *femA* gene for *S aureus*,[8] and the *ecfX* gene for *P aeruginosa*.[11] The primer sets used for the identification of *P aeruginosa*, *S pneumoniae*, *H influenzae*, and *S aureus* and their annealing temperatures are listed in Table 1. The Uniplex PCRs for each target organism were prepared in a final volume of 20 μL. In all of these PCRs, the distilled water was used as the negative control. The amplification mixture consisted of 1U of Taq DNA polymerase, 1.5 mmol/L $MgCl_2$, 200 μmol/L dNTP, 0.4 μmol/L of each primer, 10x PCR buffer, 5 μL of template DNA, and distilled water up to a final volume of 20 μL. The amplification process was performed in a Mastercycler Nexus Thermal Cycler Gradient (Eppendorf, Hamburg, Germany) with one cycle of initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing temperatures following Table 1 and extension at 72°C for 60 seconds, with a final extension for 7 minutes at 72°C.[8,10,11] The PCR products were visualized on 1% agarose gel stained with safe stain.

2.5 | Statistical method

The descriptive statistics and chi-square test with a level of significance of $P < 0.05$ were performed in SPSS version 16.00. In addition, the sensitivity and negative predictive value (NPV) were calculated for both PCR and bacterial culture techniques on PF samples, while the culture was considered as the gold standard.

3 | RESULT

In total, 105 PF samples were analyzed by both PCR and bacterial culture. Their mean age was 9.87 ± 0.58 years (age range 0.3 to 15 years old) and the boy-to-girl ratio was 1.7:1. Demographic data were available from these 105 patients; of these, 8 cases (7.61%)were fully vaccinated against *H influenzae* type b (median age 1.81 years, range 1-2 years); 4 cases (3.8%) were partially vaccinated (median age 0.47 years, range 0.4-0.5 years); and 93 cases (88.57%) were not vaccinated (median age 6.46 years, range 0.3-15 years). In this study, the pneumonia in 76 patients was diagnosed as CAP and in the 29 remaining patients was HAP.

The PCR assay detected the bacteria agents in 77.14% (81 cases) of PF samples while the bacterial culture identified the pathogens in 30 cases (28.57%). Moreover, the PCR assay identified the infectious agents in 51 (68%) PF samples where the culture method failed.

Table 2 shows the frequencies of these microorganisms in PF samples using the bacterial culture and PCR. Also, the amounts of NPV and sensitivity of the PCR assay compared to the bacterial culture of PF samples are shown in Table 3. According to these results, the amounts of NPV and sensitivity were high for all of these pathogens.

TABLE 2 Frequencies of bacterial pathogens using bacterial culture and PCR in pleural fluids

Organism	Culture n (%)	PCR n (%)
S aureus	5 (4.8)	15 (14.3)
H influenzae	3 (2.9)	9 (8.6)
S pneumoniae	13 (12.4)	63 (60)
P aeruginosa	9 (8.6)	19 (18.1)
MRSA	Unknown	9 (8.5)

TABLE 3 Determination of NPV and sensitivity of the PCR assay compared to the bacterial culture in pleural fluids

Organism	Sensitivity (%)	NPV (%)
S aureus	100	100
H influenzae	100	100
S pneumoniae	100	100
P aeruginosa	77.8	97.7
MRSA	-	-

The co-infections were detected in 21 samples (20%) by PCR and one sample using the bacterial culture (*P aeruginosa* and *S pneumoniae*). The details of the co-infection results are described in Table 4. According to the data, the co-infection of *P aeruginosa* and *S pneumoniae* was the most common co-infection in children with empyema.

In this work, seventy-nine (75.23%) patients had received antibiotic before sampling from pleural fluid and their bacterial culture was negative in 68 (86.1%) of these 79 patients. However, the antibiotic treatment before sampling had no effect on PCR results ($P > 0.05$). Table 5 shows the effects of antibiotic treatment on the results of the bacterial culture and PCR.

4 | DISCUSSION

Bacterial pneumonia is the most common cause of thoracic empyema, especially in the pediatric age-group. Pleural effusion during the course of nonspecific bacterial pneumonia can progress to empyema for several reasons, including malnutrition, immunodeficiency, irregular antibiotic treatment, the delay in diagnosis of pneumonia, and disappearance of the signs and symptoms of pneumonia.[12]

In our study, the most common bacterial agents of pleural effusion were identified by the bacterial culture and conventional PCR of PF samples. We indicated that the yield of the positive results was much low using the microbiologic culture compared to PCR (28.57% vs 77.14%). This low sensitivity can be explained to be due to the antibiotic treatment before sampling, the low bacterial load in sample, or the presence of bacterial agents with the stringent requirements.[7] As expected, a higher proportion of PF

TABLE 4 Co-infection results of pathogens using bacterial culture and PCR

Microorganisms	PCR (n)	Bacterial culture (n)
S aureus and *S pneumoniae*	4	0
S aureus and *S pneumoniae* & *P aeruginosa*	1	0
S aureus and *P aeruginosa*	1	0
S aureus and *H influenzae*	2	0
P aeruginosa and *S pneumoniae*	13	1

TABLE 5 Effects of antibiotic treatment on the bacterial culture and PCR

	Antibiotic before PF sampling		
	Yes (n = 79)	No (n = 26)	P value
Positive PCR result	23 (88.5%)	58 (73.41%)	0.177
Negative PCR result	3 (11.53%)	21 (26.58%)	
Positive culture result	19 (73.07%)	11 (13.92%)	>0.001
Negative culture result	7 (26.92%)	68 (86.07%)	

samples were positive when the bacterial culture was used in combination with PCR analysis. In our study, 75.23% of patients had initiated antibiotic treatment before sampling. As expected, the use of antibiotics before PF sampling did not affect the bacteria identification by PCR ($P = 0.177$). However, there was a clear difference between the treated and untreated groups when the culture method was performed for the identification of the bacteria ($P < 0.001$). Moreover, the chance of obtaining a positive result with antibiotic consumption had increased notably to 7-fold (from 10.47% to 75.23%), when the PCR assay was used instead of the bacterial culture. In our study, the PCR identified these infectious agents in 68% of cases (51 out of 75) with the negative PF culture results. In agreement with our research, Menezes-Martins *et al*[8] and Lochindarat *et al*[13] confirmed the advantage of PCR compared to the culture method for identification of these bacterial agents in the negative cultures.

In our study, the values of NPV and sensitivity of PCR compared to culture on PF samples for these four pathogens were calculated separately. As shown in Table 2, the PCR technique had the high NPV and sensitivity values for these pathogens, suggesting the characteristics of a desirable screening test.

In our study, *S pneumoniae* was recognized as the most frequent bacterial agent identified by both the PCR and bacterial culture. This finding was similar to the study conducted on Canadian children,[14] where the frequency rate of *S pneumoniae* was as same as our study (60% vs 62%). Also, *S pneumoniae* was the most common pathogen in a Spanish population[15] (25.6%), but it was found with the less frequency than our study. The differences in the frequency rates reported in these studies might be due to the diversity in the age ranges of enrolled patients. In our study using PCR, *S pneumoniae* was recognized in 79.4% of PFs with a negative culture. In consistent with our study, in research of Menezes *et al*,[8] the identification of *S pneumoniae* with PCR was unaffected by antibiotic consumption, Although this effect was seen well on the bacterial cultures. Hence, the use of PCR for finding pneumococci as one of the main pathogens in empyema cases is recommended to all laboratories. Since in our study, *S pneumoniae* was the main cause of empyema among children, the introduction of the pneumococcal vaccine into childhood immunization programs can reduce the incidence and morbidity of invasive pneumococcal disease in this age-group. However, unfortunately, pneumococcal conjugate vaccine had not been introduced in Iran yet.

In our study, *P aeruginosa* was recognized as the second cause of empyema (nine cases by the microbial culture and 19 cases by PCR). The frequency rates of *P aeruginosa* reported in other studies were lower than ours.[3,7,9,16] The low frequency may be due to the exclusive use of microbiologic method for the detection of this organism or the low prevalence of risk factors associated with the pseudomonas infections in their studies. For example, the low rate of detection of *P aeruginosa* in the research of Strachan *et al*[4] was due to the lack of availability to the PCR strategy for the identification of this organism. *Pseudomonas aeruginosa* is an opportunistic pathogen that often colonized in immunocompromised patients.[17] In our study, most patients with the positive results for *P aeruginosa* had at least one of the risk factors predisposing to the pseudomonas infections (data not shown).

P aeruginosa is the second cause of HAP, especially in the patients hospitalized in ICU.[18] In our study, empyema among 17 out of 19 cases with the positive results for the pseudomonas infections was recognized as the outcome of HAP. On the other hand, according to the data obtained from the hospital laboratories, the pseudomonas infections reported from these 17 patients had occurred during >72 hours or more after hospital admission. *P aeruginosa* is also reported as one of the main causes of ventilator-acquired pneumonia (VAP).[19] VAP is considered as the second most common of nosocomial infections among mechanically ventilated patients in the ICUs.[20] In our study, 8 out of 17 patients had VAP.

Also, we showed that the most common co-infection of *P aeruginosa* with other pathogens was associated with the colonization of *P aeruginosa* and *S pneumoniae* (13 cases), followed by *P aeruginosa*, *S aureus* and *S pneumoniae* (1case) and *P aeruginosa* and *S aureus* (1case). Our data showed that these 14 cases with the co-infections of *S pneumoniae* and *P aeruginosa* had been initially infected with *S pneumoniae* in community, *for example*, they had acquired CAP and later acquired HAP due to *P aeruginosa*. Moreover, ventilator had been used for all of these 14 cases that among them only 8 cases had been infected with *P aeruginosa* due to the contamination water used in some ventilators and then acquired VAP. Also, HAP due to *P aeruginosa* in the remaining cases was due to other risk factors predisposing to the pseudomonas infections such as underlying

diseases, immunocompromise condition, or malignancy(data not shown).

In the present study, *S aureus* was introduced as the third cause of empyema (five cases by microbial culture and 15 cases by PCR). However, some researchers were reported this bacterium as the main cause of empyema.[8,13,21,22] *S aureus* is associated with high rates of morbidity and mortality in hospitals worldwide and is responsible for more than 20% of all nosocomial pneumonia cases.[23] In our study, empyema among eight cases with the positive result for *S aureus* was due to HAP. Among them, ventilator was only used for the two cases with COPD symptoms. The methicillin-resistant *S aureus* strains now have accounted for 20 to 50% of cases of HAP and VAP.[24] In our study, the *mecA* gene was found in nine *S aureus* strains (60%). In similar to our study, the high incidence of the *mecA* gene in PF samples was reported by other researchers.[4,13,25]

In this present study, *H influenzae* was recognized as the fourth cause of empyema (3 cases by microbial culture and nine cases by PCR, in total 8.75%). The frequency rates of this organism in other regions using the bacterial culture or PCR were lower than our study, ranged from 0.4% to 3.2%.[4,7,21,26] Since *H influenzae* is an organism with complex requirements, the use of the molecular methods or other techniques independent of the microbiologic growth is suggested to all laboratories. In our study, for the identification of *H influenzae*, we amplified the *P6* gene of this organism. This gene is encoded by both typable strains and non-typable strains of *H influenzae*,[27] and hence, we could not differentiate between these strains. *H influenzae* type b is the most aggressive type of this organism. In Iran, the immunization against *H influenzae* type b has been initiated since November 2014. In our study, only 7.6% of patients were fully vaccinated against this organism. This low rate can be explained due to a long delay in the introduction of this vaccine into the national immunization program.

The main limitation of our study was the lack of availability to the real-time PCR strategy for the diagnosis of accurate counts of each pathogen. In addition, we could not determinate the serotype types of *S pneumoniae* and *H influenzae* using serology or molecular techniques.

5 | CONCLUSION

In this study, we found the higher frequency of these microorganisms by PCR than culture. In addition, we showed that PCR was a sensitive and accurate method that unaffected by antibiotic therapy and could detect well co-infections.

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AUTHOR CONTRIBUTIONS

Samaneh Youfpour and Tahereh Navidifar carried out the laboratory tests. Mansour Amin participated in designing the study. Tahereh Navidifar participated in drafting the article. Also all authors approved the final article.

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