Molecular Diagnosis of *Streptococcus Pneumoniae* Acute Meningitis and Profile of Sensitivity of Usual Antibiotics in Bangui, Central African Republic

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Abstract: *A study was conducted from June 2012 to May 2013 at the National Clinical Biology and Public Health Laboratory (NCBPHL) in Bangui, Central Africa Republic (CAR), on cerebrospinal fluid (CSF) coming from town's hospitals. This study aims to assess the prevalence of Streptococcus pneumoniae strains of and their profile of resistance to antibiotics in CSF.*

The CSF was collected from patients admitted to the Bangui town's hospitals and suspected of meningitis according to WHO definition of sentinel surveillance protocol (SMR Guide, 2011). On the Basis of number of white blood cells greater than or equal to 5 mL, the CSF was analyzed in the laboratory of the hospital for preliminary tests (cytology, latex agglutination test (LAT) and Gram). An aliquot was transferred into trans-isolate communities and sent to NCBPHL for confirmation tests by rtPCR, LAT and culture. Susceptibility test was done on strains isolated as recommended by the Antibiogram the Committee of the French Society of Microbiology (CASFM).

Microscopic examination after Gram staining performed on 79 samples allowed us to note the presence of germs in 47 samples (25.54%). The bacterial culture has led to the isolation and identification of bacteria in 40 specimens (21.74%). Latex agglutination test was positive for 60 samples (33.33%). The rtPCR performed on the same samples allowed us to register 79 positive samples with a confirmation rate of 42.93%. The report of the biology technique and conventional bacteriology has achieved a score of 50.63%. The results of the antibiogram carried out on S. pneumoniae strains revealed a sensitivity rate of 92.2% to ceftriaxone, 85% to Amoxicillin+clavunique acid, ciprofloxacin and gentamicin. On the contrary, S. pneumoniae strains showed a resistance against Oxacillin (30%) and penicillin (35%).

S. pneumoniae is an important cause of invasive disease, especially in children and the elderly. However, the emergence of antibiotic resistant genotypes emphasizes the importance of regulated use of these molecules in order to prevent their evolution.

Keywords: *S. pneumoniae*, Antibiotics, meningitis, Central Africa Republic, Sensitivity, Prevalence.

1. INTRODUCTION

Streptococcus pneumoniae continues to be a major etiological agent through the world, causing many diseases including otitis, sinusitis, pneumonia, sepsis and meningitis [1]. *S. pneumoniae* meningitis is an invasive disease striking especially children, elderly and immuno compromised patients. WHO estimates that about 800 000 children die every year of pneumococcal disease, 90% occur in developing countries [2].

 In Sub-Saharan Africa, *S. pneumoniae* meningitis is responsible for 250,000 to 400,000 child deaths per year [3-5]. The borders of the bacterial culture for identification of *S. pneumoniae* and serological tests for the detection of antibodies and antigens lack of sensitivity and specificity. They pose a real problem in diagnosis of bacterial meningitis for adequate care [6, 7].

This germ presents additional opportunities for identification in certain infections, especially in non-sterile samples from the sampling sites. The identification of *S. pneumoniae* was conventionally based on the bile solubility, optochin sensitivity, AccuProbe and Gen Probe pneumococcal identification test. It has also been reported in the literature that isolation of Probe-LVS from clinical samples may give positive or variable reactions in achieving these standards diagnostic tests for the detection of this germ [8, 9]. Therefore, special attention should be taken to monitor and identify the germ in the clinical field.

Previous studies showed that hybridization of the two strands of DNA-DNA by real-time polymerase chain reaction (rtPCR) technique on the basis of autolysin A (lytA), pneumococcal adherence to surface (PAS), and gene sequences can reliably distinguish *S. pneumoniae* and *S. pseudopneumoniae* [10]. The autolysin A sequences of *S. pneumoniae* and *S. pseudopneumoniae* were carefully analyzed and specific pneumococcal alleles were identified according to Llull et al. [11] the work. To confirm *S. pneumoniae* species in the different isolates, they have developed a standard based on these sequences technique, followed by an enzymatic digestion step. Thus, researchers are exploring the use of techniques based on most recent nucleic acid such as rtPCR to improve the diagnosis of pneumococcal disease.

The advantages of rtPCR amongst different techniques of conventional bacteriology were the short duration (one day) of germs identification in the samples, a treatment based on a specific germ, the detection of bacterial DNA of patients under antibiotic treatment. This new technology rtPCR showed high sensitivity in the field of the detection of some viruses of respiratory diseases [12, 13]. Real time PCR assays for *S. pneumoniae* have been reported in the literature [14, 15].

In Central African Republic, the identification of germs in the diagnosis of bacterial meningitis is very little documented. It is in this point of view that this technique was introduced in the country, as part of a WHO technical support in Central Africa in the domain of surveillance of invasive bacterial meningitis. The techniques are based on the amplification of the lyt A gene located in DNA of *S. pneumoniae* with a high sensitivity [16, 17]. It is in this context that this study was conducted in NCBPHL of Bangui (CAR) to assess the prevalence of *S. pneumoniae* strains and their antibiotic resistance profiles in CSF.

2. MATERIALS AND METHODS

2.1. Collection Samples of Cerebrospinal Fluid (CSF)

From June 2012 to May 2013, NCBPHL received CSF samples, coming from three universities hospitals (Amitié, Communautaire, and Complexe pédiatrique de Bangui) of Bangui in CAR. Patients exhibiting fevers, vomiting, headache, stiff neck or any other symptoms were diagnosed clinically and defined as suspected cases of meningitis [18]. The CSF has systematically collected and analyzed by the hospital laboratory. On Basis of the number of white cells/mL greater or equal to 5, an aliquot was transferred to the Trans-Isolate medium and sent to NCBPHL of Bangui. Thus, 184 CSF samples were received for confirmation tests.

2.2. Diagnosis of Cerebrospinal Fluid

Cytology, Gram stain and the LAT (tests using Biorad Pastorex kit) were carried out in the laboratories of different sampling sites. The culture and rtPCR confirmation tests were carried out in NCBPHL of Bangui. The preferred confirmatory diagnosis is based on the cultivation of isolating germs on Polyvitex chocolate agar media in fresh blood and blood cooked for the identification of the etiologic agent. Each strain of *S. pneumoniae* isolated in NCBPHL, susceptibility testing was performed according to the recommendations of the French Society of Microbiology [19]. Indeed, seven antibiotics (BioRad, Marnes la coquette, France) belonging to four families were tested. These antibiotics were Amoxicillin+clavulanic acid (AMC: 20 µg), Ceftrioxone (CRO: 30µg), penicillin (P: 10 µg), Chloramphenicol (C: 30 µg), ciprofloxacin (CIP: 5 µg), oxacillin (OX: 5 µg) and Gentamicin (CN: 15 µg). *Escherichia coli* ATCC 25922 strains were used as the quality control strain [19].

2.3. Molecular Tools

The rtPCR was also used to identify *S. pneumoniae* species. The QIAamp DNA Mini Kit from Qiagen was used for the extraction of DNA from CSF. An additional step to aid lysis of the bacterial cell wall was added by reporting to the manufacturer's instructions. LCRs were treated with a mixture of

lysozyme (0.04g/ml final) and mutanolysin (2,500 units/ml final concentrations) freshly prepared in TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0). The sequences of primers and probes and the amplification protocol used were those previously described by Mothershed *et al.* [20] and Dolan *et al*. [21]. The ABI 7500 Life Technologies was used for amplification of the bacterial DNA and the amplification cycle numbers was generated based on the amount of DNA extracted in amplicans. The results were determined by a value displayed on the computer screen connected to the ABI 7500 [21, 22].

2.4. Data Analysis

To assess the performance of rtPCR method in the detection and identification of this organism, the rate of confirmation of the sensitivity and specificity were calculated (Sensibility = Positive true/(Positive true + Negative false) and the Specificity = Negative true/(Negative true + Positive false). The sample was considered negative true after a negative reaction to the pathogen sought by all tests.

A sample was considered as false negative if the pathogen was detected by CSF culture, but has not been highlighted by the rtPCR or false positive if the pathogen was detected sought by latex and not by rtPCR. These explanations have been applied to all techniques. The performance was calculated taking as reference the results of the rtPCR.

The data collected were entered and analyzed using Excel and Epi Info 2000™ 3.3 software. The ANOVA test was used to compare mean age patients. Fisher and Yates chi square tests were used to compare proportions and a p-value of 0.05 was assumed to be statistically significant.

3. RESULTS

In this study, 184 CSF samples coming from suspected cases of bacterial meningitis were analyzed. The sex ratio was 1.07. The results of this study showed that the mean age was 14 years with a minimum of 0 and a maximum of 70 years. The age lower than or equal to 10 years was most represented (45.57%) of the patients suspected (Table 1).

Table 1. *Distribution of Streptococcus pneumoniae strains according to age groups.*

Age groups	Number of *Streptococcus pneumoniae* cases	Percentages
≤10 years	36	45.57%
11 - 20 years	22	27.85%
21 - 30 years	6	7.59%
31 - 40 years	8	10.13%
> 40 years	7	8.86%
TOTAL	79	100%

Gram stain test performed on 79 samples revealed 47 positive cases (25.54%). The bacterial culture has allowed the isolation and identification of bacteria in 40 samples or 21.74% confirmation rate. The latex agglutination test was positive on 60 samples (32.61%). The rtPCR performed on the same samples revealed 79 positive cases or 42.93% confirmation rate (Table 2).

Table 2. *Results comparison of rtPCR, to culture, to LAT and to Gram stain tests.*

Tests	Tests Results		
	Positive cases (percentages)	Negative cases (percentage)	Total
rtPCR	79 (42,93)	105 (57,07)	184
Culture	40 (21,74)	144 (72,26)	184
Latex	60 (32,61)	124 (67,39)	184
Gram stain	47 (25,54)	137 (74,46)	184

P-value = 0, 0001

The sensitivity and specificity of rtPCR compared to culture, Gram stain test and latex agglutination test was assessed. It appears that the sensitivity and specificity of rtPCR and culture were respectively

98,57% and 73.92%. The sensitivity of rtPCR and latex agglutination test was 96.67% and the specificity was 83.33%. For rtPCR and the Gram stain, the sensitivity of 97.87% and specificity of 75.91% were registered. All P-values were of 0.0001 with Odd ratio superior 1 (table 3).

Table 3. *Sensitivity and specificity results of the rtPCR, culture, LAT and Gram stain test*

Variables	Results of rtPCR		OR	P-value
	Positive	Negative	(IC95%)	
Gram stain results	Sensibility = 97,87%	Specificity =75,91%		
Positive	46	1	146,36	0,0001
Negative	33	104	(19,44-101,93)	
Latex results	Sensibility = 96,67%	Specificity =83,33%		
Positive	58	5	59,59	0,0001
Negative	20	101	(21,25-167,12)	
Culture results	Sensibility = 98,57%	Specificity =73,92%		
Positive	40	1	136,26	0,0001
Negative	39	104	(12,24-1001,93)	

OR= Odd ratio, CI = Confidence Interval

The report of the technique of conventional biology and bacteriology has achieved a score of 50.63% (Table 4).

Table 4. *Contribution of rtPCR in the confirmation of suspected cases.*

Total number of LCR samples	Total number of suspected cases	Number of positive cases with culture	rtPCR confirmation rate
184	79	40	50.63 %

The results of susceptibility test carried out on *S. pneumoniae* strains showed an excellent sensitivity rate of 92.2% to ceftriaxone, 85% to Amoxicillin + clavunique acid, ciprofloxacin and gentamicin. On the contrary, these strains revealed a resistance against Oxacillin (30%) and penicillin (35%) (Figure 1).
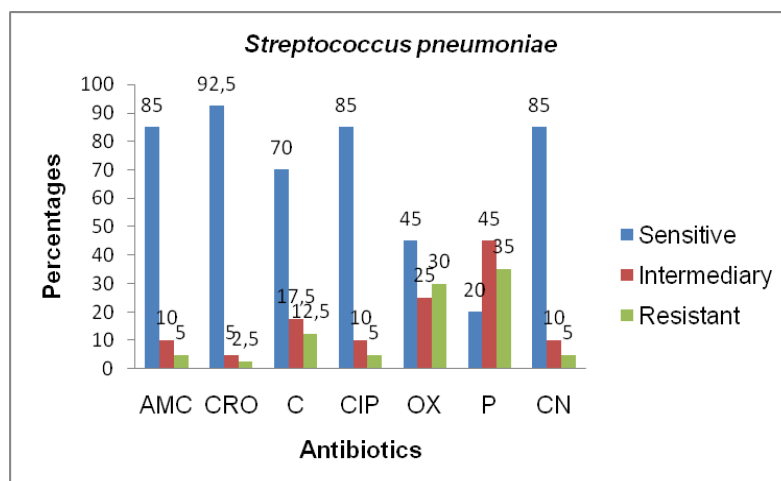

Figure 1. *Sensitivity percentages of S. pneumoniae strains isolated in Bangui (AMC: Amoxicillin + clavulanic acid; CRO: Ceftrioxone; P: Penicillin; C: Chloramphenicol; CIP: ciprofloxacin; OX: oxacillin; CN: Gentamycin).*

4. DISCUSSION

The rapid and precise identification of pathogenic bacteria is very important for the timely implementation of management case, control and monitoring of disease. Approaches based on the rtPCR have been widely used for the detection of human pathogens because of their high sensitivity and fast response time to disease [23, 24, 25].

This prospective study conducted in NCBPHL of Bangui emphasizes the frequency and severity of bacterial meningitis in the Central African population. The results of this study showed that the mean

age was 14 years with a minimum of 0 and a maximum of 70 years. The age lower than or equal to 10 years was most represented (45.57%) among patients suspected. Similar studies carried out in Burkina Faso and Egypt revealed a rate of 50% and 40% in children with age lower or equal to 5 years received in hospitals for meningitis [26, 27]. Data of study conducted on meningitis in North region of Cameroon showed the predominance of patients with age lower than 10 years and sensitivity rate of 41% [28].

Microscopic examination after Gram stain test performed on 79 samples allowed to note 25.54% of germs. The bacterial culture revealed isolation and identification of bacteria with a confirmation rate of 21.74%. The reaction was positive with latex agglutination test in 33.33% of samples. The rtPCR performed on the same samples made it possible to detect a confirmation rate of 42.93%. The rtPCR improved the confirmation rate of suspected cases of meningitis over 50.63% compared to culture. Similar results were observed in other countries and after the introduction of molecular biology, the number of cases of meningitis confirmed in laboratory doubled in USA [17, 20]. It has also doubled in England and Wales. This confirmation rate with rtPCR tripled in Greece [29]. The rtPCR has significantly improved the confirmation rate of bacterial meningitis in Burkina Faso (58.5%) and Niger (60.4%) [30,31].

The high sensitivity of 98,57% obtained in the rtPCR with respect to the culture and those of 97.87% and 96.67% respectively compared to the Gram stain test, and the latex agglutination test, demonstrate that the probability of false negative is low. Real time PCR significantly improves the confirmation rate of bacterial meningitis [16, 17]. The inability of the rtPCR to detect a positive sample compare to the culture, Gram stain test may be related to the presence of inhibitors of rtPCR. Indeed, samples of CSF analyzed have not been previously purified.

The specificity rate of rtPCR compared to culture, Gram stain and latex agglutination tests were respectively 72.92%, 83.33%, and 75.91%. These rates are quite low. The high number of LCR samples negative with other techniques and positive with rtPCR explain the low specificity rate of different techniques used in this study. This information revealed the limit of the use of other techniques such as standard methods in the study. Other studies have demonstrated the highest sensitivity of the rtPCR with respect to the latex agglutination test [20, 21].

The sensitivity of *S. pneumoniae* to various antibiotics was determined using the diffusion method on agar medium. Molecules of interest according to their use in human medicine, route of administration, and commercial availability have been used [18].

For this study, *S. pneumoniae* strains showed a sensitivity of 92.5% to ceftriaxone. In a study in American medical centers on patients suffering from meningitis, we observed ceftriaxone susceptibility rate (2 mg/L MIC) on the decrease of 14.4%. In other medical centers in the same country we also noted that susceptibility rate also fell under 5.9% [32, 33]. These results were similar to those obtained in this study, despite the difference between the number of *S. pneumoniae* strains tested with antibiotic in different works. Other authors have noted that more than 95% of *S. pneumoniae* strains isolated in world are currently sensitive to cephalosporins of third generation [34, 35]. Cirpofloxacine, amoxicillin + clavulanic acid and gentamicin showed an efficiency of 85%. On the contrary, these species were resistant to oxacillin and penicillin amoxicillin with respective rates of 30 and 35%. Each one of these strains also has intermediate resistance of more than 21% with these two antibiotics. The mechanism of resistance to penicillin by *S. pneumoniae* involves structural variations in the modes of action of penicillin (binding proteins 1A penicillin, 2X, 2B). These variations result in a reduction of affinity for penicillin [36].

Amoxicillin + clavulanic acid was effective against *S. pneumoniae* strains with a sensitivity rate of 89.74%. This antibiotic is used to replace cases of resistance of the strains to amoxicillin. It is a broad-spectrum antibiotic that inhibits the action of lactamases (enzymes) by clavulanic acid and restore the activity of amoxicillin [34]. Many studies revealed different rates of antimicrobial resistance in patients in different geographic areas of the world. The resistance rate was lower in the northern countries than in developing countries [37]. Antibiotic resistance in the United States was also compared with that of Europe. The countries of southern Europe such as Greece, France, Italy and Spain have a similar antibiotic resistance rates with United States. On the contrary, resistance rate in the United States was higher than that of northern Europe countries [38].

5. CONCLUSION

Bacterial meningitis remains a serious public health problem in the world globally and especially in Africa and Central Africa Republic (CAR). This study conducted in National Clinical Biology and Public Health Laboratory has generated data on the situation of bacterial meningitis in CAR. The age groups lower or equal to 10 years are exposed to this disease. *S. pneumoniae* strains were sensitive to antibiotics, such as ceftriaxone, ciprofloxacin and amoxicillin + clavulanic acid. On the contrary, a resistance has been observed with oxacillin and penicillin. *S. pneumoniae* species continues to be an important cause of invasive disease, especially in children and the elderly. However, the emergence of drug-resistant genotypes emphasizes the importance of a regulated use of antibiotics in order to prevent their spread.

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