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ORIGINAL RESEARCH PAPER

Simultaneous detection of *Neisseria meningitidis, Streptococcus pneumoniae* and *Haemophilus influenzae* by quantitative PCR from CSF samples with negative culture in Morocco

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ABSTRACT

Bacteriological cultures from cerebrospinal fluids (CSF) have less sensitivity and specificity compared to quantitative PCR (RT-PCR), and multiple facts still conduct to the increase of negative culture. The aims of this study are to determine the molecular epidemiology and the simultaneous detection of bacterial meningitis in Morocco by using RT-PCR and compared this molecular approach with culture method to improve the etiological diagnosis of meningitis. The CSFs were collected over one-year period in 2018 in different hospitals covering all regions of the Kingdom of Morocco, from patients with suspected meningitis. The results showed the confirmation rate per culture recorded a rate of 33% and the RT-PCR of 70%. Molecular epidemiology is predominant of *Neisseria meningitidis* followed by *Streptococcus pneumoniae* and a dramatic reduction in meningitis due to *Haemophilus influenzae* following the introduction of conjugate vaccine in 2007. Also, the epidemiological profile shows a sex ratio M/F of 1.4 and a median age of 2 years. The national distribution showed a predominant of meningococcal disease followed by pneumococcal disease, especially a dominance of *N. meningitidis* over *S. pneumoniae* in two regions and a slight predominance of *S. pneumoniae* in the other two regions over *N. meningitidis*. Our research shows that culture in our country has less sensitivity and specificity than RT-PCR in diagnosis of bacterial meningitis and that molecular biology technique at bacteriology laboratories is desirable for diagnosis, early management of meningitis cases and in the context of the surveillance of meningitis in Morocco in parallel with culture.

KEYWORDS

bacterial meningitis, molecular epidemiology, negative culture, quantitative PCR, simultaneous detection, vaccine

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INTRODUCTION

A suspicion of bacterial meningitis requires urgent care at hospitals; often on admission a rapid antibiotic therapy is administered to reduce mortality and morbidity. And the frequent

etiology of bacterial meningitis in the most countries are *Neisseria meningitidis, Streptococcus pneumoniae* and *Haemophilus influenzae* [1]. In many developing countries, the surveillance of bacterial meningitis is hampered by limited use of bacterial culture in peripheral laboratories and high frequency of negative cultures [2]. In addition, the recent increase in the practice of early antibiotic therapy prior to performing the lumbar puncture is a factor that decreases the ability to confirm the diagnosis of bacterial meningitis with culture method [3]. In Morocco, meningitis is a clinical, therapeutic and public health emergency, as it is considered one of the contagious diseases that must be declared to those responsible for monitoring and confirming epidemiological diseases, the examination of cerebrospinal fluid (CSF) in the laboratory is frequently used to confirm acute bacterial meningitis [4]. The culture in the laboratory of medical bacteriology of CSF from patients suspected of meningitis is currently considered the gold standard for diagnosing bacterial meningitis and providing information on the evolution of antibiotic resistance used in empirical treatment [5]. However, many lumbar punctures with suspected meningitis are culture negative which calls into question the sensitivity of the culture method [6]. Furthermore, the use of PCR for the detection of bacterial DNA from CSF is now rapidly becoming widespread, and employing this assay can significantly increase the speed and accuracy of identification of the pathogen [7].

The molecular approach using the RT-PCR is fast, reliable, and easily implementable into a laboratory routine for bacterial meningitis confirmation, especially for patients who previously started antimicrobial therapy, and can substantially improve clinical diagnosis and epidemiological measures of meningitis disease burden [8]. Thus, it has added value for the detection of the three pathogens responsible for bacterial meningitis like *N. meningitidis, S. pneumoniae, H. influenzae* and can overcome the disadvantage if antibiotics have been administered prior to lumbar puncture (antibiotic pre-treatment) [9]. In our context, the routine implementation of the RT-PCR laboratories in parallel with the culture will help improve the detection of bacterial strains in the CSF, improve epidemiological surveillance, especially the management of confirmed cases, and contact cases. The aims of this study are to determine the molecular epidemiology and the simultaneous detection of bacterial meningitis in Morocco by using RT-PCR and compared this molecular approach with culture method to improve the etiological diagnosis of meningitis.

MATERIALS AND METHODS

Statement of ethics

Samples from this study were collected as part of the national meningitis surveillance system in Morocco. The laboratory of medical bacteriology at the National Institute of Hygiene is the national laboratory of meningitis. All patient identity information was treated confidentially.

Data

The data were entered using Excel software and the statistical calculations were performed using Epi Info version 7 from the CDC (Centers for Disease Control and Prevention, Atlanta, Georgia, USA). The qualitative variables were expressed in terms of number and percentage. The comparison of the qualitative variables was made by the x^2 test and the exact Fisher test. A P-value < 0.05 was considered as statistically significant.

Design of the study

It is an epidemiologic descriptive study of Cerebrospinal fluid (CSF) samples analyzed in the Meningitis Reference Laboratory at the National Institute of Hygiene in Morocco. A total of 90 CSFs were collected over a one-year period in 2018 in different hospitals covering all regions of the Kingdom of Morocco, from patients with suspected meningitis. 60 CSFs are negative culture and 30 CSFs are positive culture.

Molecular study of CSF

Biological samples of CSF were submitted to investigate by PCR based assay to the Meningitis Reference Laboratory at the National Institute of Hygiene in Morocco. The three main bacteria that are *N. meningitidis, S. pneumoniae* and *H. influenzae* were analyzed by molecular biology using RT-PCR in three separate tubes. For each performed test a negative and positive control were made.

Primers and probe design

We used specific primers and probes of the target genes of the three bacterial species, ***ctrA*** (F753:TGTGTTCCGCTATACGCCATT, R846:GCCATATTCACACGATATACC,Pb820i(FAM):AACCTTGAGCAA"T"CCATTTATCCTGACGTTCT), ***hpd*** (hpd F822: ACGCAATCTAGCAGATGAAGCA, hpd R952: TGCATCTTTACGCACGGTGTA,Pb896i(FAM):TTGTGTACACTCCGT"T"GGTAAAAGAACTTGCAC) and ***lytA*** (F373:ACGCAATCTAGCAGATGAAGCA,R424: TCGTGCGTTTTAATTCCAGCT,Pb400i(FAM):TGCCGAAAACGC"T"TGATACAGGGAG) were respectively the target gene of *N. meningitidis, H. influenzae* and *S. pneumoniae* [10].

Extraction of bacterial DNA

Bacterial DNA was extracted from the CSF upon receipt with the use of QIAamp DNA Mini Kit (QIAGEN).

Amplification

To identify *N. meningitidis, S. pneumoniae* and *H. influenzae*, RT-PCR screen amplification was performed of *ctrA, lytA* and *hpd* target genes, respectively, with specific oligonucleotide primers previously described. The DNA was included in each assay from standard strains (*N. meningitidis* ATCC 13090, *S. pneumoniae* ATCC 49619, and *H.*

influenzae ATCC 10211) as positive controls and the reaction mixture without DNA target as negative control.

The DNA extracted from each CSF sample was amplified in three separate tubes. The three tubes were introduced into the same amplification run, each targeting one of the three bacteria studied using primers and probes specific to each organism. The protocol was validated according to the instructions of the reference laboratory for meningitis based on the CDC.

A volume of 2 µL of the extracted DNA was added to 12.5 µL TaqMan PCR Master Mix, 2 µL forward primer, 2 µL reverse primer, 2 µL probe and 4.5 µL sterile PCR grade water in the PCR tube to reach a final amplification volume of 25 µL.

The DNA was amplified in Real-Time BIORAD thermocycler (CFX 96™ Real time BIORAD), with the TaqMan system using the following PCR conditions for the three targeted microorganisms are used: 1 cycle of 50 °C for 2 min, 1 cycle of 95 °C for 10 min, 50 cycles of 95 °C for 15 s +60 °C for 1 min.

RESULTS

In this study, 90 CSFs were studied. The characteristics of the collected CSF samples show that 52% belongs to the age class 0–2 years and *N. meningitidis* is dominant as *S. pneumoniae* for ages under 15 years. The male gender is 58% versus 42% female. The results also show that the NNE region has 59% of meningitis cases and that the geographical distribution shows a dominance of *N. meningitidis* compared to the *S. pneumoniae* in the NNE region and RSK region while *S. pneumoniae* is slightly dominant in the CN region and EOO regions (Table 1).

The germ frequency shows that *N. meningitidis* was present in 49%, *S. pneumoniae* in 19% and *H. influenzae* in 2% were found positive by RT-PCR in the CSFs samples studied and 30% was negative, whereas by culture *N. meningitidis*, *S. pneumoniae* and *H. influenzae* and other bacteria (*Escherichia coli, Klebsiella pneumoniae* and *Staphylococcus aureus*) had a frequency of 17, 11, 2 and 3% respectively and 67% were negative (Table 2).

Of the 60 negative CSF samples per culture, *N. meningitidis* and *S. pneumoniae* represented 29 and 7 isolates respectively, and 24 CSFs were still negative. Also, among the other 30 positive CSFs per culture: we founded 15 strains of *N. meningitidis*, 10 *S. pneumoniae* and 2 *H. influenzae* confirmed positive by RT-PCR while 3 were negative because these last were *E. coli, K. pneumoniae* and *S. aureus*. This difference in results between the two techniques is statistically significant ($P < 0.05$) (Table 3).

By comparison, other results have been reported: the sensitivity and specificity for culture were 42.86 % and 88.89 % respectively; also the Positive Predictive Value (PPV) and Negative Predictive Value (NPV) were 90 % and 40 % respectively (Table 4).

DISCUSSION

The samples were collected from patients clinically suspected of bacterial meningitis in different hospitals covering the entire regions of the Kingdom of Morocco. The specimens were sent to referral laboratory of meningitis situated

Table 2. Bacteriological profile of cerebrospinal fluids studied in culture and quantitative PCR

Characteristics	Culture % (n)	PCR % (n)
Neisseria meningitidis	17 (15)	49 (44)
Streptococcus pneumoniae	11 (10)	19 (17)
Haemophilus influenzae	2 (2)	2 (2)
Escherichia coli	1 (1)	0 (0)
Klebsiella pneumoniae	1 (1)	0 (0)
Staphylococcus aureus	1 (1)	0 (0)
Negative	67 (60)	30 (27)
Total	100 (90)	100 (90)

Table 1. Characteristics of the CSFs studied

		Neisseria meningitidis	*Streptococcus pneumoniae*	*Haemophilus influenzae*	Negative	Total *
Age* (years)	[0–2]	29 (26)	9 (8)	1 (1)	13 (12)	**52 (47)**
	[2–5]	6 (5)	2 (2)	0 (0)	1 (1)	**9 (8)**
	[5–15]	11 (10)	3 (3)	1 (1)	7 (6)	**22 (20)**
	[15–50]	3 (3)	4 (4)	0 (0)	9 (8)	**17 (15)**
Total*						**100 (90)**
Gender*	F	17 (15)	7 (6)	0 (0)	18 (16)	**42 (37)**
	M	32 (29)	12 (11)	2 (2)	12 (11)	**58 (53)**
Total*						**100 (90)**
Region*	CNR(a)	6 (5)	7 (6)	0 (0)	0 (0)	**13 (11)**
	NNER(b)	29 (26)	7 (6)	1 (1)	22 (20)	**59 (53)**
	RSKR(c)	13 (12)	3 (3)	1 (1)	6 (5)	**23 (21)**
	EOOR(d)	1 (1)	2 (2)	0 (0)	2 (2)	**5 (5)**
Total*						**100 (90)**

*Percentage (Effective); (a) CNR: Casablanca and Neighbors Region. This region includes the Cities of Casablanca and nearby cities/(b): NNER: North and Northeast Region. This region includes the cities of Tanger, Tetouane, Hoceima, Ksar el Kebir, Chefchaouen, Ouazzane and Nador/(c): RSKR: Rabat-Sale-Kenitra Region. This region includes the cities of Rabat, Sale, and Kenitra and nearby cities/(d): EOOR: Er-Rachidia, Ouarzazat Region and Oriental Region. These regions include the cities of Er-Rachidia, Ouarzazat, Oujda and Bouarfa.

Table 3. Combination results of quantitative PCR with culture

Test performed	Characteristics	Effective % (n)	PCR % (n)				P value
			Positive			Negative	
			Neisseria meningitidis	*Streptococcus pneumoniae*	*Haemophilus influenzae*		
Culture	Negative	67 (60)	**32 (29)**	**8 (7)**	**0 (0)**	**27 (24)**	**<0.05**
	Positive	33 (30)	**17 (15)**	**11 (10)**	**2 (2)**	**3 (3)**	
	Total	100 (90)	49 (44)	19 (17)	2 (2)	30 (27)	

From 60 CSFs with negative culture performed by RT-PCR: we found 29 *N. meningitidis*, 7 *S. pneumoniae* were positive and 24 were remained negative. From 30 CSFs with positive culture, we confirmed that *N. meningitidis, S. pneumoniae* and *H. influenzae* represented 15, 10 and 2 respectively. The three bacterial with positive culture and negative RT-PCR were *E. coli, K. pneumoniae* and *S. aureus* (Used as an intern control for PCR). These results showed a difference between RT-PCR and culture. This difference in results was statistically significant ($P < 0.05$).

Table 4. Compare of culture test against quantitative PCR test

Test performed	PCR +	PCR −	Total
Culture +	27	3	30
Culture −	36	24	60

for additional testing with RT-PCR. In this work, molecular epidemiology and simultaneous detection of three major pathogens responsible for bacterial meningitis were performed using a non-commercial RT-PCR for the diagnosis of *N. meningitidis*, *S. pneumoniae* and *H. influenzae*. We also compared this RT-PCR home-made to the bacterial culture method of the three germs implicated in this disease. Additionally, we cited here the technical difficulties encountered and associated with the high rates of negative culture and the demanding transport conditions of these samples as some limits of this study.

The results here showed a median age of 2 years old and the sex ratio M/F was 1.4. Furthermore, the epidemiologic profile of meningitis based in RT-PCR in this study shows a high frequency of *N. meningitidis* followed by *S. pneumoniae* and a remarkable decrease of meningitis due to *H. influenzae*.

In a recent research based on culture confirmation and published in this journal, the phenotypic epidemiology showed that in Moroccan children and adults, meningococcal disease causes more meningitis followed by pneumococcal disease, and *H. influenzae type b* come last with very low rates and mainly reaches children ≤5 years old and unvaccinated adults [11].

In the meningitis belt, cases of meningitis are due on the first to *N. meningitidis* then to *S. pneumoniae* and finally to *H. influenzae* which continues to be reported [12]. During a study on Finland, *S. pneumoniae and N. meningitidis* were the most common etiologies accounting for 78% of cases and *H. influenzae* accounted for 4% of cases [13].

The compared frequency of *S. pneumoniae* reported in this study and other published data would be the result of the introduction of the pneumococcal vaccine PCVs into the national immunization program in 2010, which explain that the pneumococcal diseases caused by vaccine serotypes declined significantly [11, 14]. There is a dramatic regression in cases of meningitis due to *H. influenzae* in children ≤5 years in Morocco and another countries, this decrease in cases of *H. influenzae* was the result of the introduction in 2007 into the national immunization program of the *H. influenzae type b* conjugate vaccine for children [11, 15, 16]. Furthermore, the prophylaxis was based on taking rifampin as dose of 200 mg twice in two days for the eradication of *N. meningitidis* oropharyngeal carried for contacts cases, and prevention vaccination with tetravalent vaccine ACYW135 in the cases of closed communities [11, 17].

At the national level, the PCR based results in this research showed as described above that *N. meningitidis* is dominant followed by *S. pneumoniae*, but the slight difference between the regions of the kingdom may be due to socio-economic factors, the different climates between the regions, also to the strategic location of Morocco which is close to the sub-Saharan and Maghreb countries and also to Europe, which induces a migratory fluidity of the imported strains. Similar results in other studies have already been widely reported and explained [18, 19].

In our study, among the bacterial strains in CSFs detected by culture and used as control for our RT-PCR method, *E. coli, K. pneumoniae* and *S. aureus*. The latter are isolated in newborns less than 7 days old. They were among the bacteria of neonatal meningitis frequently isolated in the newborn [20]. However, these pathogens and others that cause bacterial meningitis in infants like group B *Streptococcus, Listeria monocytogenes, Enterobacter cloacae, Cronobacter sakazakii, Pseudomonas stutzeri, E. coli, K. pneumoniae* and *S. aureus* [21–23].

Moreover, the *ctrA* gene (The capsule transport to cell surface gene) of *N. meningitidis*, the *lytA* gene (autolysin gene) of *S. pneumoniae* and the *hpd* gene (encodes protein D is present in all encapsulated and non-encapsulated) of *H. influenzae* were highly conserved and frequently used in both real-time and conventional PCR [24–27]. Using the PCR method for the diagnosis of meningitis has shown to be superior to the culture for determining the pathogens that most frequently cause bacterial meningitis in CSF, considering that a part of the patients received antibiotics before the diagnostic tests [28], and that PCR is a

complement to classical bacteriology techniques [29]. RT-PCR showed in this study a significantly higher detection rate of bacterial meningitis when compared to culture, the confirmation rate was 33% by culture and 70% by RT-PCR. In this research and by comparison, the sensitivity and specificity for culture were 42.86 % and 88.89 % respectively, but RT-PCR was able to detect several bacterial that had not been identified by culture. Comparison was made on the assumption that the samples tested by RT-PCR were positive or negative. Compared to culture method, RT-PCR sensitivity and specificity for detection of *N. meningitidis, S. pneumoniae* and *H. influenzae* were always higher in many conducted studies, and the RT-PCR sensitivity doesn't change significantly when there was antibiotic activity in the CSF [30]. In addition, RT-PCR has high positive and negative predictive values, with better confirmation of cases [31].

Cerebrospinal fluid cultures have been used traditionally, but over time, the sensitivity and specificity were being decreased by empiric treatment prior to culture. More rapid techniques such as the cryptococcal lateral flow assay (IMMY), GeneXpert MTB/Rif Ultra (Cepheid), HG Pneumo/Meningo Combo (HiberGene Diagnostics), Eazyplex CSF direct System (Amplex Diagnostics GmbH) and FilmArray (Biofire) were the examples of commercial systems that have drastically changed routine meningitis diagnostics [32–35].

The RT-PCR home-made failed to detect bacterial DNA from 27 CSFs samples excluded the three bacterial were confirmed *E. coli, K. pneumoniae* and *S. aureus*, possible reasons could be low bacterial density in some samples or perhaps it is viral meningitis or other disease with atypical fever or true negative results. Also failure of PCR could be due to the presence of an inhibitory substance for the test or the contamination of samples and PCR reagents with irrelevant DNA from various sources remains a problem and considered limitations in this technic [36].

The impact of the implementation of meningitis RT-PCR diagnosis for the simultaneous detection of *N. meningitidis, S. pneumoniae* and *H. influenzae* in the routine of peripheral laboratories in Morocco will improve the early management of meningitis cases, the health information system and strengthen the national meningitis surveillance system. The importance of molecular tests used in the laboratory is crucial for the detection and confirmation of meningitis cases in addition to providing response data, improving surveillance, responding to emergency response questions and establishing a molecular epidemiology network based on molecular confirmation in parallel with culture.

CONCLUSION

In this study, the molecular epidemiology showed a predominance of *N. meningitidis* followed by *S. pneumoniae* and a decrease of cases of *H. influenzae* at national level, but a slightly difference remarked for *N. meningitidis* and *S. pneumoniae* between regions of country. Our research shows that culture has less sensitivity and specificity than RT-PCR home-made for simultaneous detection of three pathogens and in the diagnosis of bacterial meningitis, and that molecular biology technique at bacteriology laboratories is desirable for diagnosis, early management of meningitis cases and in the context of the surveillance of meningitis in Morocco in parallel with culture.

Conflicts of interest: The authors declare no conflict of interest.

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