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Prediction of Antibiotic Resistance in *Streptococcus pneumoniae* by Detection of Resistance Genes by Direct Quadruplex PCR in CSF from Patients with Acute Bacterial Meningitis

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Abstract

Introduction: The bacterial etiology of meningitis in Burkina Faso is dominated by *Neisseria meningitidis* (*Nm*), *Streptococcus pneumoniae* (*Spn*) and *Haemophilus influenzae* (*Hi*), which are used epidemically. Low culture yields make it difficult to obtain data on the susceptibility profile of these pathogens. In the absence of such data, this study of resistance genes was initiated. **Method:** The aim of this study was to determine the wild-type gene *pbp2b*, the resistance *genes mef* and *ermB*, *cat* and *tetM* using Quadruplex real-time PCR directly on LCS samples, without prior extraction. **Results:** A total of 188 pneumococcal PCR-positive CSF samples were tested for resistance genes. The *pbp2b gene* was the most represented (166/188), followed by the *tetM gene* (158/188), the *cat gene* (37/188), the *mef gene* (25/188) and the *ermB gene* (10/188). Serotype 1 was dominant in 97/188 cases, with 22/97 carrying the *cat gene*, 9/97 the *mef gene*, 95/97 the *pbp2b gene*, 94/97 the *tet gene* and none the *ermB gene*. **Conclusion:** This study showed that around 88% of pneumococci carried the wild-type *pbp2b gene*, meaning that the majority were wild-type to penicillins. On the other hand, more than 80% of pneumococci carry the *tetM gene*, unlike

ermB, *mef* and *cat*, whose carriage rates are 5, 13 and 19% respectively.

Keywords

Pneumococcus, Gene-*tetM*, Gene-*ermB*, Gene-*pbp2b*, Gene-*cat*, Resistance

1. Introduction

Bacterial meningitis occurs in epidemic form every year in Burkina Faso, which is part of the African meningitis belt [1]. The most implicated pathogens according to surveillance data are *Neisseria meningitidis* (*Nm*), *Streptococcus pneumoniae* (*Spn*), and *Haemophilus influenzae* (*Hi*) [2] [3]. Also, based on epidemiological surveillance data, *Nm* was the most frequent cause of meningitis, and from 2011, Spn began to gain ground, becoming the most detected bacterium in meningitis cases since 2014 [4]-[6]. The identification of these bacteria in the surveillance is done by PCR (real-time and conventional) or by culture. Most pathogens are identified by PCR due to the high negativity and contamination rates of the culture as well as the low number of CSF inoculated in trans-isolate transport (TI) media. Indeed, between 2010 and 2015, out of 15,312 samples sent to the national meningitis reference laboratory (LNRm) as part of the surveillance, only 7474 (48.8%) were inoculated on TI with just 1204 culture-positive samples compared to 5625 positive cases detected by PCR [3]. This situation considerably limits the ability to establish the antimicrobial susceptibility profile of bacteria responsible for meningitis due to the lack of sufficient number of isolates. This does not allow for data on the emergence of resistance within the surveillance of bacterial meningitis in Burkina Faso. To address the lack of phenotypic data on pneumococcal resistance to antibiotics, this study was initiated to screen for resistance genes that could be predictive of potential resistance in the different strains tested.

2. Materials and Methods

2.1. Study Site and Sample Collection

This is a prospective study that involved samples from confirmed cases of pneumococcal meningitis. These samples were collected between 2016 and 2019 in 25 health districts in Burkina Faso. The samples were handled at the National Meningitis Reference Laboratory (LNRm) based at the Charles De Gaulle Pediatric University Hospital Center (CHU-PDG) and at CDC Atlanta.

2.2. Laboratory Analysis

Species identification was conducted by culture and single-plex RT-PCR and serotyping by RT-PCR and conventional PCR. All PCRs were performed directly on the CSFs without prior extraction [7] [8]. The PCR for species identification targeted *lytA gene* for *S. pneumoniae*, sodC gene for *N. meningitidis* and hpd gene for *H. influenzae* [9]. Pneumococci serotyping was performed by a series of 12

RT-PCR quadriplex reactions and 8 conventional PCR triplex reactions which allow the determination of 48 and 24 serotypes respectively [10] [11]. The screening for antibiotics resistance genes was done by the direct PCR Quadruplex [10] [12]. Five antibiotic resistance genes were searched for. These are the wild-type *Penicillin banding protein* 2*b* (*pbp2b*) *gene* carried by penicillin-sensitive pneumococci, the *macrolide efflux* (*mef*) and *erythromycin methylase* (*ermB*) *genes*, both responsible for macrolide resistance, the *cat gene* responsible for phenobarbital resistance, and the *tetM gene* responsible for tetracycline resistance (Table 1). The choice of these antibiotics is justified by the fact that they are used in various treatment protocols for pneumococcal infections such as meningitis. The study of pneumococcal sensitivity to antibiotics consisted of testing the antibiotics discs using the disk diffusion method. The results were interpreted in accordance with the recommendations of antibiogram committee of French society of antibiogram and the European Committee on Antimicrobial Susceptibility Testing (CASFM-EUCAST).

Table 1. Probe primers used to detect resistance genes [12].

Oligonucleotide	Sequence (5'-3')	Nucleotide position	GenBank accession no.
lytA-F	ACGCAATCTAGCAGATGAAGCA	1841014	AE005672
lytA-R	TCGTGCGTTTTAATTCCAGCT	1840961	
lytA-Probe	5'-FAM-TGCCGAAAACGCTTGATACAGGGAG-3'-BHQ1	1840985	
ermB-F	CTTGGATATTCACCGAACAC	766	AB111455
ermB-R	TTGGTTTAGGATGAAAGCAT	844	
ermB-Probe	5'-ROX-AAGTCTCGATTCAGCAATTGCTTAAG-3'-BHO2	807	
mef-F	TATGGAGCTACCTGTCTGGA	291	AF227520, U83667
mef-R	GGTACTAAAAGTGGCGTAACC	375	
mef-Probe	5'-HEX-CCGTAGCATTGGAACAGCTTTTC-3'-BHQ1	333	
pbp2b-F	CTGTTTGGACCATATAGGTATTT	1494906	AE007317
pbp2b-R	CAATTCTTGGTATACTCAGGCT	1494976	
pbp2b-Probe	5'-Cy5-TCCAGAGCTTGGACCGCTGTGATA-3'-BHQ3	1494938	

3. Results

3.1. Sociodemographic Characteristics

A total of 188 pneumococci detected by real-time in the CSF of patients with meningitis were used to screen for resistance genes. Among these patients, 100 were men and 88 were women, with a male/female ratio of 1.14. The distribution by age group shows that 54.25% (n = 102) were children aged 3 to 15 years, 25% (n = 47) were adults aged 16 to 59 years, 18.1% (n = 34) were infants aged 1 to 30 months, 2.12% (n = 4) were elderly people over 60 years, and finally 0.53% (n = 1) were a 5-day-old newborn (Figure 1).

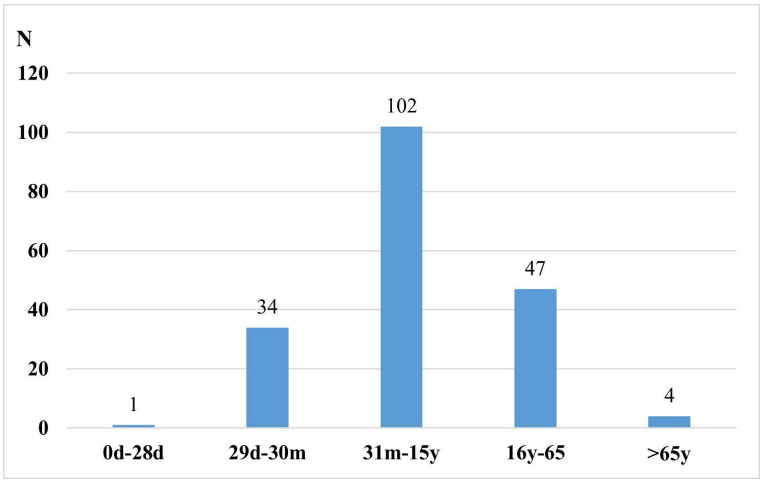
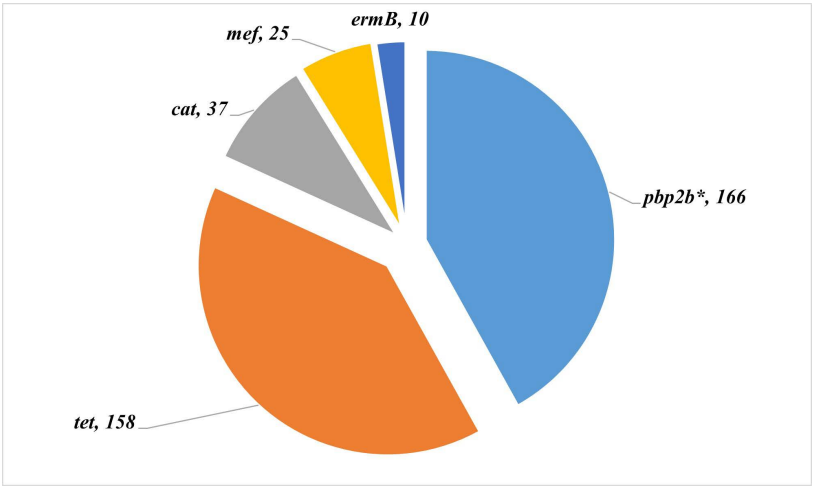


Figure 1. Age distribution of patients with isolated pneumococcal CSF.

3.2. Overall Prevalence of Resistance Genes

Overall prevalence of resistance genes of the 188 samples processed, the wild-type *pbp2b gene* was the most prevalent, accounting for 88.3% (n = 166) of cases, followed by the *tetM gene* in 84.04% (n = 158), the *cat gene* in 19.68% (n = 37), the *mef gene* in 13.3% (n = 25), and the *ermB gene* in 5.32% (n = 10) (Figure 2).



*Wild-type *pbp2b* gene.

Figure 2. Number of strains carrying the wild-type *pbp2b gene*, *ermB gene*, *mef gene*, *cat gene* and *tetM* gene out of the 188 *Streptococcus pneumoniae* strains included in the study.

3.3. Detection of Resistance Genes by Serotype

3.3.1. Wild Type *pbp2b* Gene

Approximately 88% (166/188) of pneumococci carry the wild type *pbp2b* gene. The distribution of this resistance gene by serotype yields a carriage rate of 97% (95/97) for serotype 1, 66% (7/21) for serotype 12F/44, 57% (8/14) for the 8 serotype 35B, 100% for the 6 serotype 2s, and 90% for the 11 non-typeable serotypes (Table 2).

Table 2. Recapitulative of genes prevalence according to *Spn* serotypes.

Genes / Serotypes	*pbp2b*					*tetM*					*cat*					*mef*					*ermB*				
	Negative		Positive		T	Negative		Positive		T	Negative		Positive		T	Negative		Positive		T	Negative		Positive		T
	N	%	N	%		N	%	N	%		N	%	N	%		N	%	N	%		N	%	N	%	
1	2	2.06	95	97.94	97	3	3.09	94	96.91	97	75	77.32	22	22.68	97	88	90.7	9	9.28	97	97	100	0	0	97
12F/44	7	33.33	14	66.67	21	4	19.05	17	80.95	21	17	80	4	19.05	21	18	85.7	3	14.3	21	20	95.2	1	4.8	21
35B	6	42.86	8	57.14	14	0	0	14	100	14	12	85.71	2	14.29	14	10	71.4	4	28.6	14	7	50	7	50	14
2	0	0	6	100	6	2	33.33	4	66.67	6	5	83.33	1	16.67	6	6	100	0	0	6	6	100	0	0	6
8	0	0	5	100	5	3	60	2	40	5	4	80	1	20	5	3	60	2	40	5	5	100	0	0	5
10A	1	20	4	80	5	4	80	1	20	5	4	80	1	20	5	3	60	2	40	5	4	80	1	20	5
14	0	0	3	100	3	0	0	3	100	3	3	100	0	0	3	3	100	0	0	3	3	100	0	0	3
23F	0	0	2	100	2	0	0	2	100	2	2	100	0	0	2	1	50	1	50	2	2	100	0	0	2
7F/7A	0	0	3	100	3	2	66.67	1	33.33	3	2	66.67	1	33.33	3	3	100	0	0	3	3	100	0	0	3
3	0	0	1	100	1	1	100	0	0	1	1	100	0	0	1	0	0	1	100	1	1	100	0	0	1
5	0	0	1	100	1	0	0	1	100	1	0	0	1	100	1	0	0	1	100	1	1	100	0	0	1
18C	0	0	1	100	1	0	0	1	100	1	0	0	1	100	1	1	100	0	0	1	1	100	0	0	1
25F/25A38	0	0	2	100	2	0	0	2	100	2	2	100	0	0	2	2	100	0	0	2	2	100	0	0	2
34	1	50	1	50	2	0	0	2	100	2	2	100	0	0	2	2	100	0	0	2	2	100	0	0	2
24FAB	0	0	2	100	2	1	50	1	50	2	2	100	0	0	2	2	100	0	0	2	2	100	0	0	2
33F	1	50	1	50	2	1	50	1	50	2	2	100	0	0	2	1	50	1	50	2	2	100	0	0	2
7CB1/7CB2	0	0	2	100	2	1	50	1	50	2	2	100	0	0	2	2	100	0	0	2	2	100	0	0	2
6A	0	0	1	100	1	0	0	1	100	1	1	100	0	0	1	0	0	1	100	1	1	100	0	0	1
5B/15C	0	0	1	100	1	0	0	1	100	1	1	100	0	0	1	1	100	0	0	1	1	100	0	0	1
11A	1	100	0	0	1	1	100	0	0	1	0	0	1	100	1	1	100	0	0	1	1	100	0	0	1
17F	0	0	1	100	1	0	0	1	100	1	0	0	1	100	1	1	100	0	0	1	1	100	0	0	1
19A	1	100	0	0	1	0	0	1	100	1	1	100	0	0	1	1	100	0	0	1	0	0	1	100	1
23B	0	0	1	100	1	0	0	1	100	1	1	100	0	0	1	1	100	0	0	1	1	100	0	0	1
NT	3	9.09	11	90.91	11	7	54.55	6	45.45	13	12	90.91	1	9.09	13	13	100	0	0	11	13	100	0	0	11
TOTAL	22	11.70	166	88.30	188	30	15.96	158	84.04	188	151	80.32	37	19.68	188	163	86.7	25	13.3	188	178	94.7	10	5.3	188

N: Frequency. %: percentage. T: Total. NT: Not Typable.

3.3.2. *ermB* and *mef* Gene

The *ermB* gene was detected in 5.3% (10/188) of pneumococcal cases, but no serotype 1 was found with *ermB* gene. Serotype 35B carried this gene predominantly, with approximately 50% (7/14), followed by serotype 10A with 20% (1/5), and serotype 12F/44 with approximately 4% (1/20) (Table 2). For the *mef* gene, 13.3% (2/188) of the pneumococcal strains were carriers. Serotype 1 carried this gene in approximately 9% (9/88), serotype 12 in approximately 14% (3/18), and serotype 35B in approximately 28% (4/10) (Table 2).

3.3.3. *tetM* Gene

Approximately 84% (158/188) of pneumococci carried the *tetM* resistance gene. And among the carrier serotypes, 97% (94/97) of serotype 1 were found, 81%

(4/17) of serotype 12F/44, 100% of the 14 serotype 35B, and 45% (6/13) of non-typeable (Table 2).

3.3.4. *cat* Gene

Approximately 19% of serotypes carried the chloramphenicol acetyltransferase (*cat*) gene, including approximately 22% (22/97) of serotype 1, followed by serotype 12F/44 with approximately 19% (4/21) (Table 2).

3.3.5. Modified *pbp2b* Associated with the *ermB*, *mef*, and *cat* Genes

Among the pneumococci carrying the modified *pbp2b* gene (*pbp2b* negative), 31.81% carried the *ermB* gene, 22.72% the *mef* gene, and 9.09% the *cat* gene (Table 3).

Table 3. Prevalence of macrolide resistance genes among penicillin-resistant pneumococcal strains (*pbp2b* gene negative).

Associated resistance genes	negative *pbp2b gene*: n = 22	
	n	%
ermB gene	7	31.81
mef gene	5	22.72
cat gene	2	9.09

3.4. Surface Protein Genes *pili1 and pili2*

Of the 188 pneumococci, we identified 7 strains (3.72%) that carried pili1, and 4 strains (2.12%) that carried pili2. Among the 7 pili1-positive pneumococci, 1 carried the *cat* gene, 6 the *teMt* gene, 2 the *mef* gene, 2 the *ermB* gene, and 1 the modified *pbp2b* gene. Among the four pili2-positive pneumococci, 2 carried the *cat* gene, 2 the tet*M* gene, and 1 the *mef* gene; however, none carried the modified *ermB* and *pbp2b* genes (Table 4).

Table 4. Prevalence of resistance genes according to the presence of pili1 and pili2.

Genes	*cat*	*ermB*	*mef*	*pbp2b*	*tetM*
pili1					
Positive n (%)	1 (2.7)	2 (20)	2 (8)	6 (3.61)	6 (3.8)
Negative n (%)	36 (97.3)	8 (80)	23 (92)	160 (96.39)	152 (96.2)
Total	37	10	25	166	158
pili2					
Positive n (%)	2 (5.41)	0 (0)	1 (4)	4 (2.41)	2 (1.27)
Negative n (%)	35 (94.59)	10 (100)	24 (96)	162 (97.59)	156 (98.73)
Total	37	10	25	166	158

3.5. Susceptibility Profile of Culture-Isolated Strains

Among the 188 pneumococci, only seven strains were culture-isolated and tested

for susceptibility, with 25% resistance to penicillin G, 20% resistance to oxacillin, and 100% resistance to chloramphenicol.

4. Discussion

Of the 188 pneumococci included in the study, serotype 1 remains the most prevalent strain with approximately 52%, followed by serotype 12F/44 with approximately 9.9%. Since the establishment of surveillance, serotype 1 has remained the predominant serotype in pneumococcal meningitis cases in Burkina Faso. This predominance persists despite the introduction of the PCV13 vaccine in 2013, which includes serotype 1, unlike serotype F12/44, which is not included in the vaccine [13] [14]. This high frequency of serotype 1 is common to other countries in the subregion, such as Niger, where the prevalence between 2003 and 2011 was 54.3% [15].

4.1. Resistance Genes

4.1.1. Wild-Type *pbp2b* Gene

Of the 188 samples, the wild-type *pbp2b* gene was detected in 166 cases (88.3%). This gene is one of the six wild-type penicillin-binding protein (*pbp*) genes identified in pneumococcus. Its qualitative and quantitative modification leads to an increase in the MIC in the pneumococcal strain as observed with the phenotype of pneumococci with decreased sensitivity to penicillin (PDSP). This resistance to penicillin can be crossed with all beta-lactams whose phenotypic expression is a function of the degree of increase in the MIC of each molecule [16]. The results found in this study mean that there is a strong prediction of penicillin sensitivity of the bacteria detected in 88.3% of cases. The 11.7% negative PCR for the *pbp2b gene* corresponding to the absence of the wild-type target could therefore correspond to an alteration of wild *pbp2b*, thus corresponding to a strong prediction of resistance of these pneumococci in accordance with the validation study of the method that we used for the search for this gene. Indeed, it appears from this study that the wild *pbp2b* gene is associated in 99.44% (179/180 strains) of cases with sensitivity to penicillin among the strains tested [12]. The few rare strains that we isolated from this study showed a low phenotypic resistance rate to peni G and oxacillin at 25% and 20% respectively, thus corroborating this weak resistance observed according to the search for the wild *pbp2b* gene [17]. Other studies prior to the period of ours have also reported low rates of resistance to penicillin. Strains isolated from 2010 to 2012 in several health districts of Burkina Faso had a resistance rate of approximately 10% to oxacillin [18]. Unlike the *pbp2b* gene, the *tetM*, *cat*, *ermB* and *mef* genes are genes of resistance acquired by bacteria through mobile genetic elements such as transposons. This means that their detection is associated with a high probability of resistance in the detected pneumococci.

4.1.2. The *ermB* and *mef* Resistance Gene

Approximately 5% of serotypes carried the *ermB* gene, 13% the *mef* gene, and 1% both genes. No serotype 1 carried the *ermB* gene, and most of this serotype was

negative for the *mef* gene (approximately 91%). Serotype 35B constitutes the majority of the predominant serotypes carrying these genes, with approximately 50% (7/14) for the *ermB* gene and 29% (4/14) for the *mef* gene. It is followed by serotype 12F/44 with approximately 4% (1/21) for the *ermB* gene and 14% (3/21) for the *mef* gene. These are macrolide resistance genes and one of the major mechanisms of this resistance is the expression of efflux proteins encoded by the *mef* gene [19] [20]. The *mef* gene confers a low to moderate level of resistance to macrolides with 14 and 15 carbon atoms and not to lincomycin and streptogramin B [21]. On the other hand, the *ermB* gene confers resistance to all macrolides in general including lincomycin and streptogramin [21]. The presence of these genes allows a prediction of resistance of our strains because studies have reported significant rates of individual or associated carriage of these genes in strains resistant to erythromycin [22]. The results found in this study reflect a low level of resistance of our strains to 14- and 15-carbon macrolides, as well as lincomycin and streptogramin. These low carriage rates of these resistance genes in our strains corroborate the prevalence of pneumococcal resistance to macrolides before the introduction of the PCV13 vaccine in 2013 in the country [18].

4.1.3. The *tetM* Resistance Gene

The *tetM* gene was detected in 84.04% of cases in our study. Among the major serotypes, all serotypes 35B carried this gene, followed by serotype 1 (approximately 97%) and serotype 12F/44 (approximately 81%). Tetracycline resistance in pneumococci results from the acquisition of one of two resistance determinants, *tetM* or *tetO*. These genes confer resistance by ribosome protection following the acquisition of Tet protection protein which can lead to cross-resistance to all cyclins [16]. According to some studies, this gene is in most cases associated with resistance to tetracycline in resistant pneumococcal strains which allows a strong prediction of resistance when it is present [23]. Therefore, the 84% detection of this gene in this study could thus correspond to 84% resistance of pneumococci to tetracyclines. This high resistance rate could be explained by the long-term use of cyclins in the treatment of acute bacterial meningitis in Burkina Faso. Indeed, before 2013 the rate of resistance of pneumococci to tetracycline in Burkina Faso was already estimated at around 90% [18] and the carriage rate of the *tetM* gene in our study corroborates this rate of phenotypic resistance. Although tetracyclines are not the antibiotics of first choice in the treatment of meningitis, this strong prediction of resistance could compromise future therapeutic strategies aimed at combining them with cephalosporins to reduce mortality in meningitis [24].

4.1.4. The *cat* Resistance Gene

The *cat* gene was detected in approximately 19% of serotypes, including approximately 22% (22/97) of serotype 1, followed by serotype 12F/44 with approximately 19% (4/21). It is an inducible resistance through the action of an acetyltransferase and constitutes the main mechanism of resistance to chloramphenicol [25] [26].

Chloramphenicol has been used for a long time in the treatment of meningitis in Burkina Faso but was withdrawn from the market not because of resistance but because of its irreversible hemotoxicity. In surveillance data from Burkina Faso, this antibiotic has always shown good activity, and the resistance rates found in various previous studies corroborate the low rate of *cat* resistance gene detected in this study [17] [18]. However, phenotypic results show 100% sensitivity, but the small number of strains tested means that it is not possible to objectively conclude that there is a discrepancy between phenotype and genotype.

4.1.5. Modified *pbp2b* Gene Associated with the *ermB* and *mef* Genes or the *cat* Gene

Pneumococcal strains with decreased sensitivity to Penicillin often carry resistance mechanisms associated with other antibiotics such as macrolides and phenicols.

In our case, among the strains with modified *pbp2b* genes (*pbp2b* gene negative), 31.81% carried the *ermB* gene, and 22.72% carried the *mef* gene, thus corroborating the prevalence of resistance associated with macrolides of PDSP described in the literature. Indeed, among the resistances associated with PDSP strains, macrolide resistance has a high prevalence, as it is estimated that nearly 80% of penicillin-resistant strains are also resistant to erythromycin [27]. Regarding resistance associated with phenolicols, the incidence of resistance increases particularly in PDSP according to some studies [26] [28] [29], but in ours, only 9% of pneumococci with modified *pbp2* gene carried the *cat* gene. This rate of carriage of the *cat* resistance gene differs from the rate of resistance to chloramphenicol in PDSP strains that has been reported in some countries. Indeed, studies conducted in France reported that 31% to 36% of PDSP strains were resistant to chloramphenicol compared to 13% of pneumococci susceptible to penicillin [28] [29].

4.1.6. Resistance Associated with *pili*

The search for pili1 and pili2 based on resistance genes was negative in more than 80% of cases for pili1 and more than 90% of cases for pili2. Pili are involved in gene transfer through the conjugation process, and the results obtained in this study show that the acquisition of resistance genes in the majority of cases was not associated with pili, *i.e.*, through the conjugation process. The major resistance mechanisms observed in pneumococcus are target modification and the efflux system, through the acquisition of resistance genes. The acquisition of these genes is mainly done by the transformation process because the pneumococcus is recognized by its natural competence [30]. Conjugation is also a gene transfer mechanism encountered in the pneumococcus, for example, the conjugative transposon which is responsible for the wide diffusion of the tetracycline/minocycline *tetM* gene [29]. These conjugative transposons can also carry resistance genes as in the case of TN1545 which carries the *ermB* gene. In this study, we found 3.8% of pili1 and 1.27% of pili2 associated with *tetM* genes out of 158 pneumococcal strains carrying this gene. Two strains out of 10 carrying pilus1 were *ermB*-positive. No *ermB*-positive strain was associated with pilus2 [31].

5. Conclusion

The aim of this study was to screen for pneumococcal resistance genes, which should provide data on the resistance of this pathogen in the context of difficulties in obtaining strains by culture. The results show that around 88% of pneumococci are wild-type to penicillins, as they carry the wild-type *pbp2b* gene. On the other hand, over 80% of pneumococci carry *tetM* genes, while *ermB*, *mef* and *cat genes* carry 5%, 13% and 19% respectively. These data predict a high level of tetracycline resistance. Serotype 1, the most dominant of the serotypes, carries most of the genes tested. As pneumococcus is one of the pathogens most implicated in bacterial meningitis, extending the search for resistance genes to other bacteria, such as Neisseria meningitidis and *Haemophilus influenzae*, will strengthen AMR data for meningitis surveillance in Burkina Faso.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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