

Resistance Phenotypes and Molecular Characteristics of *Staphylococcus aureus* Associated with Pleurisy in Patients at the Mali Hospital, Teaching Hospital.

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ABSTRACT

Background: *Staphylococcus aureus* (*S. aureus*) and *S. aureus* resistant to Methicillin (MRSA) are among the pathogens strongly implicated in hospital infections. Data on the resistance and molecular characteristics of this bacteria are rare in Mali.

Objective: This study aimed to evaluate the antibiotic resistance phenotypes and virulence factors of *S. aureus* isolates from pleural fluid infections in hospitalized patients.

Methods: Pleural effusion samples were obtained by thoracentesis for bacteriological examination from October 2021 to December 2022 at the hospital Mali, teaching hospital. Standard microbiological procedures were used for bacterial identification. The disk diffusion method was used to identify Methicillin -resistant *S. aureus*. The PCR amplification method was used to detect the following genes: *lukE /D*, *sek*, *sel* and *sep*.

Results: During the period, 36 isolates of *S.aureus* 26 (72.2% MRSA and 10 (27.8%) MSSA (Methicillin -susceptible *S.aureus*) were isolated from 1526 hospitalized patients. The frequency of bacterial pleurisy was 284 (18.6%). *S. aureus* was isolated in 36 (12.7%) of the cases, of which 26 (72.2%) were MRSA. There was no significant difference between genders ($p = 1.00$). The median age of patients was 37.5 years. All *S. aureus* isolates showed 100% resistance to penicillin-G, 2.8% to fusidic acid, 5.6% to fosfomycin, 8.3% to daptomycin, 11.1% to clindamycin and 13.9% vancomycin. Leukocidin *lukE /D* was detected in 11.1% of patients, *sek*, *sel* and *sep* toxins were not found.

Conclusion: In this study, we found a high frequency of *S. aureus* (and MRSA) in patients with pleurisy at Mali Hospital. Only leukocidin *lukE /D* was found. Our results suggest that the current empiric treatment protocol for pleurisy, which includes ciprofloxacin and gentamicin, should be revised and modified by these molecules in accordance with the WHO AwaRe classification of antibiotics: moxifloxacin, teicoplanin, daptomycin, intravenous or oral vancomycin, fusidic acid, intravenous fosfomycin or linezolid as a last line.

Introduction

Bacterial pleuritis is a common and widespread condition with significant mortality and morbidity [1]. *Staphylococcus aureus* (*S. aureus*) is associated with high mortality and morbidity in most hospitals. It is considered an opportunistic pathogen and a common cause of infections such as pneumonia, sepsis, endocarditis, osteomyelitis, meningitis and toxic shock syndrome, with high levels of antimicrobial resistance [2]. Additionally, *S. aureus*, particularly methicillin-resistant *S. aureus* (MRSA), is a common cause of nosocomial infections and is considered a major public health problem [3]. Sub-Saharan Africa remains a region facing significant health care challenges, and the prevalence and morbidity of pleural fluid infections are of great concern. A study carried out in Chad revealed a prevalence of 13.6%. Only 4% of cases were of bacterial origin, excluding tuberculous pleurisy [4]. In another study conducted in Niger in 2016, the two authors reported that the etiology of pleuritis was dominated by tuberculosis. Conversely, a South African study carried out over a period of 5 years reported pleuritis of bacterial origin in more than half of the cases. The main pathogens isolated were *S. aureus*, *Streptococcus pneumoniae*, tubercle bacillus and *Klebsiella pneumoniae*, in that order. Research on pleural infection in sub-Saharan Africa has highlighted the predominance of *S. aureus* as the most common causative organism [5]. *S. aureus* can evade the defense barriers of the host's immune system by producing various enzymes and toxins. In fact, a strong correlation between toxins and disease has been reported. The prevalence of MRSA is not known in Mali. *S. aureus* produces numerous virulence factors, such as enterotoxins, exoenzymes, immunomodulatory factors, hemolysins, leukocidins, proteases, and exfoliative toxins, which allow it to adhere to a surface, invade, or destroy. Avoid the immune system and, furthermore, cause toxicity to the host [6]. Pleural infections can result from various conditions and factors, such as location,

demographics, and comorbidities (Park et al. 2023). Although a positive pleural fluid culture defines an infection, the microbiological profile can be very diverse (Foley and Parrish 2023). The emergence of resistance in *S. aureus* is attributed to various mechanisms, including production of enzymes that inactivate antimicrobial agents, activation of antimicrobial efflux pumps, reduction of bacterial cell permeability to antibiotics, and modification of the target site of [7]. The emergence of virulent and drug-resistant strains of *S. aureus*, particularly MRSA, poses a significant challenge for the treatment and management of staphylococcal infections. These methicillin-resistant strains cause infections that are difficult to treat because they are resistant to several antibiotics, including betalactams, aminoglycosides, and macrolides. The main mechanism of resistance to penicillin is the production of betalactamase, which inactivates penicillin by breaking down its beta-lactam ring. Another mechanism associated with the presence of penicillin-binding protein 2a (PBP2a) is encoded by the *mecA* gene, which is carried on the staphylococcal cassette mec chromosome (SCCmec). Furthermore, the *blaZ* gene is involved in penicillin resistance in staphylococci and encodes the production of β -lactamase [8].

Our study is the first to our knowledge in Mali to prospectively search for the presence of several virulence factors on clinical isolates of *S. aureus* and MRSA. Additionally, it determines antibiotic resistance phenotypes in *S. aureus* isolates and in pleurisy patients.

The main contribution of the present work was to describe the clinical characteristics, antibiotic susceptibility analysis and virulence profile of *S. aureus* isolates isolated from patients with pleurisy who were hospitalized. The results of this investigation could help researchers better understand the nature of these deadly bacteria that endanger the lives of people suffering from pleurisy.

Methods

Study design and population

The present study was carried out as a cross-sectional investigation from October 2021 to December 2022 at the Mali Hospital in the thoracic surgery department with hospitalized patients presenting with pleural effusions.

Bacterial identification

Previously obtained pleural effusion samples were routinely inoculated with a brain-heart infusion (BHI) and anaerobic blood culture bottle and incubated at $35^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 18 to 24 hours. Subsequently, fresh blood agar, enriched chocolate agar and Sabouraud agar were inoculated using the aforementioned broths. The identification of the isolated bacteria was carried out using conventional methods, including Gram staining, morphological and biochemical tests, the colonies were then subjected to agglutination using the Pastorex® Staph plus kit (BioMérieux, Marcy Etoile-Lyon).

An automated Phoenix M50 system (BD, Stockholm, Sweden) with the identification panel (PMIC/ID-600) specifically designed for Gram-positive bacteria was used to confirm this identification.

Antimicrobial susceptibility testing

The antibiotic susceptibility of the isolates was assessed by the Kirby bauer disk diffusion method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines [9]. The antibiotics tested were penicillin-G (10 µg), cefoxitin (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), moxifloxacin (5 µg), gentamicin (10 µg), amikacin (30 µg), tobramycin (10 µg), trimethoprim + sulfamethoxazole (1.25-23.75 µg), linezolid (10 µg), tetracycline (10 µg), fosfomycin (200 µg), clindamycin (2 µg), erythromycin (15 µg) and fusidic acid (10 µg). MRSA isolates were identified using a cefoxitin disk (30 µg) (all from Bio-Rad, USA). The method for determining the minimum inhibitory concentration (by microdilution according to ISO 20776-1) using the Phoenix M50 CLSI 2019 automated system (10) was

used to test the sensitivity of these four antibiotics: [10] daptomycin, oxacillin, teicoplanin and vancomycin. *S. aureus* isolates resistant to erythromycin but susceptible to clindamycin were classified into the MLS b phenotype inducible. *S. aureus* isolates resistant to erythromycin and clindamycin (factor B) were classified into the constitutive MLS b phenotype. *S. aureus* isolates that had isolated resistance to amikacin were classified into the K phenotype (Kana^R). *S. aureus* isolates that were resistant to amikacin and tobramycin were classified as KT phenotype. *S. aureus* isolates that were resistant to amikacin, tobramycin and gentamicin were classified into the KTG phenotype which is associated with partial resistance to amikacin and netilmicin [11].

McFarland standard 0.5 (1.5×10^8 colony forming units/ mL) was prepared. Antibiotic disks were placed on the inoculated Mueller-Hinton agar plates (Marnes-la-Coquette, France) with the appropriate distances, and then the plates were incubated at 37°C for 16–18 h. Inhibition zones were measured and interpreted according to EUCAST guidelines, 2019. *S. aureus* ATCC 25923 was used as a quality control strain.

Of note, multidrug resistance was defined as lack of susceptibility to at least one agent from three or more antibiotic classes, as described previously [12].

DNA extraction and PCR

DNA was extracted according to the heat shock method described, with modifications [13]. Briefly, pure colonies were suspended in 200 µl of Tris-EDTA solution, heated at 100°C for 10 minutes, and then immediately placed at -20°C for 5–10 minutes. After centrifugation at 12,000 rpm for 10 min, the resulting supernatant was used as a DNA template. The quantity of each DNA extract was determined by measuring absorbance at 260 nm to estimate DNA concentration and calculating the A260/A280 ratio to determine purity using the NanoDrop One/One^c (ThermoFisher Scientific, Waltham, MA USA). DNA was considered pure when the A260/A280 ratio was between 1.6 and 2.0 [8].

S. aureus virulence factor genes

We searched for five virulence genes including a leukocidin (lukE /D), the enterotoxin genes k, l and p (sek, sel and sep) using the list of primers presented in the Table 1. The four virulence genes were detected by conventional PCR using the appropriate specific primers and amplicons electrophoresed on a 1.5% agarose gel with 0.5

µg/ mL ethidium bromide dye. Primer genes were selected based on previous studies from the primer stock of the National Institute of Public Health (INSP) using primers (Eurofins Genomics Louisville, KY) [14] on a thermal cycler GeneAmp PCR System 9700 (ThermoFisher Scientific, Waltham, MA USA).

Table 1: Primers used in this study

| Primer name | Primer sequence (5' → 3') | Product size (bp) | References |
|-------------------------|---------------------------|-------------------|------------|
| <i>lukE / D_Forward</i> | GAAATGGGGCGTACTCAAA | 269 | [8] |
| <i>lukE / D_Reverse</i> | GAATGGCCAAATCATTCGTT | | |
| <i>sek_Forward</i> | GGAGAAAAGGCAATGAA | 516 | [34] |
| <i>sek_Reverse</i> | TCGTTAGTAGCTTGTGACTCC | | |
| <i>Sel_Forwardt</i> | CGATGTAGGTCCAGGA | 369 | |
| <i>Sel_Reverse</i> | TTCCTGTGCGGTAACCA | | |
| <i>Sep_Forward</i> | TCAAAAGACACCGCCAA | 396 | |
| <i>Sep_Reverse</i> | ATTGTCCTTGAGCACCA | | |

The analysis of the PCR products obtained was carried out by electrophoresis on agarose gel (1.5%). 12 µl of the PCR products are mixed with 4 µl of loading buffer, xylene cyanol, then loaded onto the agarose gel. 5 µl of a size marker (269bp 369 bp 396 bp 516 bp) are also deposited on the gel. The migration is carried out on a Power Pro 330 V 700 mA 150 W voltmeter at 120 Volt 79 mA for one hour.

Cycling conditions were as follows: an initial denaturation of one cycle at 98 °C for 3 min; 35 cycles of denaturation at 98°C for 15 seconds, annealing at 61°C and primer extension at 72°C for 15 seconds; and a final extension at 72 °C for 1 min, Table 2. After electrophoresis on agarose gel, the PCR products were read using a UV Transluminator, Vilber Lourmat 230 V 50 HZ, France.

Table 2: PCR program for detection of S.aureus virulence factors

| Cycle stage | Cycle | Time | Temperature |
|------------------------|-----------|----------|-------------|
| - Initial denaturation | 1 cycle | 3 mins | 98°C |
| - Denaturation | 35 cycles | 15 secs | 98°C |
| - Hybridization | | 15 secs | 61°C |
| - Extension | | 10 secs | 72°C |
| - Final extension | 1 cycle | 1 minute | 72°C |

Definitions and statistical analysis

Data were collected via a questionnaire in the Excel sheet and analyzes were carried out using Open Epi software version 3.01. Comparison of frequencies was carried out using Fisher's exact test. A significance level of 0.05 or less for

the value was considered statistically significant. Multidrug resistance was defined as an isolate resistant to at least one antimicrobial agent belonging to at least three different antimicrobial classes [12] and all intermediate

susceptibility profiles were considered resistant for the purposes of this study.

Results

Population characteristics

Of 1526 patients during the study period, the overall prevalence of bacterial pleuritis in the thoracic surgery department was 284 (18.6%). The frequency of sample positivity was 194 (68.3%). *S. aureus* was isolated in 36 (12.7%) cases. Among these 36 patients, 19 (52.8%) were men and 17 (47.2%) were women, which corresponds to a male/female ratio of 1.1. The median age of patients was 37.5.

Antibiotic susceptibility results

S. aureus isolates showed 100% resistance to penicillin-G. In terms of resistance to oxacillin, we observed a resistance frequency of 63.9%.

The resistance profiles of *S. aureus* isolate to various antibiotics are shown in Table 3. MRSA accounted for 26 (72.2%) cases. The aminoglycoside K and KT phenotypes were 17 (46.2%). Among macrolides, the constitutive MLSB phenotype was 2 (7.7%) in MRSA and 1 (10%) in MSSA. In contrast, the inducible MLSB phenotype was 7 (29.9%) in MRSA and 1 (10%) in MSSA. The resistance of MRSA to aminoglycosides was 12 (46.2%), for MSSA it was 3 (30%). Of the 36 *S. aureus* isolates, 4 (11.1%) were multi-resistant; no isolate was resistant to all antibiotics tested.

The frequency of resistance of the most effective antibiotics were fusidic acid 1 (2.8%), fosfomycin 2 (5.6%), daptomycin 3 (8.3%), clindamycin 4 (11.1%) and vancomycin 5 (13.9%).

Table 3: Resistance of *S. aureus* isolates to antibiotics

| Antibiotics | Resistant n (%) |
|---------------------------------|-----------------|
| Penicillin -G | 36 (100) |
| Oxacillin | 29 (80.6) |
| Cefoxitin | 26 (72.2) |
| Amikacin | 17 (47.2) |
| Gentamicin | 15 (41.7) |
| Tobramycin | 19 (52.8) |
| Levofloxacin | 12 (33.3) |
| Ciprofloxacin | 12 (33.3) |
| Moxifloxacin | 9 (25.0) |
| Tetracycline | 35 (97.2) |
| Erythromycin | 28 (77.8) |
| Clindamycin | 4 (11.1) |
| Linolezide | 9 (25.0) |
| Teicoplanin | 5 (13.9) |
| Daptomycin | 3 (8.3) |
| Trimethoprim + sulfamethoxazole | 26 (72.2) |
| Fosfomycin | 2 (5.6) |
| Vancomycin | 5 (13.9) |
| Fusidic acid | 1 (2.8) |

Molecular detection of toxin genes

The toxin known as leukocidin *lukE /D* was detected in 11.1% of patients (n = 4). The

toxins known as *sek*, *sel* and *sep* were not detected in any of the patients.

Discussion

S. aureus is an opportunistic pathogenic bacteria responsible for numerous infections both in hospitals and in the community. This bacteria is the cause of potentially fatal illnesses in humans. MRSA isolates are common causes of emerging nosocomial infections and are considered an important public health problem. *S. aureus* is the leading cause of bloodstream infection in the majority of developed countries [15]. Virulence genes, such as enzymes, toxins, adhesin proteins, and cell surface proteins produced by *S. aureus*, play an important role in the pathogenicity of *S. aureus* strains. Virulence factors play different roles in various diseases [16]. Patients with pleurisy are known to be at increased risk of infections [17].

Patients referred to the thoracic surgery department of the Hôpital du Mali teaching hospital for pleurisy presented with *S. aureus* infections. The hospital frequency of *S. aureus* pleurisy was 12.7%. Our results are lower than those reported by NGakoutou R et al, 2022, who cite in a 2006 study reporting a frequency of 15.9% in a pneumological context. This could be explained by changing case management practices. This result is lower than those reported by certain African authors (13.8% in Chad and 42.2% in South Africa) [5, 18]. In developed countries, the hospital prevalence of pleurisy was 7.8 cases in 2017 in France and 9.9 cases per 100,000 inhabitants in Finland in 2017 [19, 20]. An increasing trend was noted in the United States in Missouri. The weighted prevalence of empyema was 95.5 per 100,000 hospitalizations per year. It varied by race and was 68.10 per 100,000 population for white and black patients, respectively [21].

Among the rare studies carried out on the subject in our country, in 2006, Diallo S et al found that the tuberculosis bacillus was the most frequent etiology of pleurisy, representing 37% of cases [22] while B Kéita et al in 1993 found the causative organism in 18 cases (16.7%), of which 14 were Gram positive. The etiology of tuberculosis was proven in 16 cases (27.6%) [23]. In 2023, Kalambry AC et al, in a

study which did not look for the tuberculosis germ, found that *Escherichia coli* represented 44.5% of the bacteria isolated in pleural fluid [24]. This increase would be linked to the emergence of less sensitive bacterial strains or those expressing increased virulence. The male predominance in our study, although there was no significant difference, has been reported by other authors. The median age in our study was 37.5 years, which could be explained by the greater activity at this age of the life cycle in our country.

In our institution, the most prescribed antibiotics to treat staphylococcal pleurisy are gentamicin (GEN) and ciprofloxacin (CIP). Our isolates showed 41.7% resistance to GEN and 33.3% resistance to CIP. Our results are lower than those reported by some authors who found 72.6% resistance to GEN and 71.7% resistance to CIP [25]. For aminoglycosides, there was no significant difference between the presence of the sensitive phenotype in MRSA and MSSA ($p = 0.05$). According to the American Association of Thoracic guidelines Surgery (AATS) and the British Thoracic Society (BTS), aminoglycosides should be avoided in the management of pleurisy, while other authors advise [13] the use of these molecules in the treatment of infections with MRSA [20].

S. aureus penicillinase is species specific, it is transmitted by plasmids, it confers resistance to penicillins G, V and A, carboxypenicillins and ureidopenicillins. More than 90% of *S. aureus* isolated from pathological products in hospitals are penicillinase producers [26]. In our study, penicillinase G was observed in 100% of our isolates. This observation confirms the strong trend towards resistance of staphylococci to penicillin. In addition to their intrinsic resistance to beta-lactams, hospital-associated MRSA strains often exhibit a variable but alarming level of multidrug resistance, which limits treatment options to the few remaining effective drugs. Our results are comparable to those found in Burkina Faso in 2024 by Traoré et al who had resistances varying from 83 to 94% [27]. Regarding resistance to

aminoglycosides, we found 46.2% K and KT phenotypes. Our results are far superior to those found by Traoré et al, 2024 in Burina Faso who found 6% and 1% respectively. In Cameroon, Kengne et al. 2020 found 4.5% and 12.5% respectively [28]. The presence of these phenotypes would be due to acquired resistance which is ensured by the production of inactivating enzymes in the strains [29].

MRSA poses a significant threat to public health, particularly in developing countries, due to its ability to cause life-threatening infections [28]. Data on MRSA prevalence in Africa vary in coverage and quality. They are available for South Africa, Nigeria and the countries of the Mediterranean basin but are fragmentary for other countries [30]. Some come from single-center studies, and others from larger but few surveillance systems. Many studies have relied on phenotypic methods to identify MRSA.

In our study, 72.2% of MRSA isolates were encountered. Some authors reported a MRSA prevalence of 80.9% in Cyprus in 2022 [13]. In 2018, a study bringing together data from a large number of countries estimated the prevalence of MRSA between 25 and 50% for Algeria, Morocco and Cameroon compared to 10 to 25% for the Republic of Côte d'Ivoire and the Senegal; Mali had not provided data [30]. The variation between countries could be partly explained by differences in the availability and use of antimicrobials. Due to differences in the extent of collection and testing methods, caution should be used when comparing data between countries.

The phenotype Inducible MLSB was more common in MRSA than in MSSA ($p = 0.000$). In contrast, there was no significant difference between the constitutive MLSB phenotype in MRSA and MSSA ($p = 0.405$). We noted a predominance of 56.2% of the constitutive MLSB phenotype versus 22.9% of the inducible MLS b phenotype in Iran [11]. Compared to vancomycin, resistance is 9.6%. No vancomycin resistance was detected in Zambia by the authors in hospital settings [31]. Vancomycin is the drug of choice used in hospital settings to treat MRSA infections. Our results could

perhaps be explained by the possible acquisition of resistance to vancomycin. The choice of this antibiotic to treat MRSA may also cause the emergence of vancomycin resistance [31]. Leukotoxins are a family of two-component, pore-forming toxins with membrane-damaging functions. These bacterial exotoxins share sequence and structural homology and target multiple host cell types. Leukotoxin ED (*LukED*) is one such toxin that *Staphylococcus aureus* produces in order to suppress the host's ability to contain the infection. The recent definition of the important role that *LukED* plays in the pathogenesis of *S. aureus* and the identification of its protein receptors, combined with its presence in epidemic methicillin-resistant *S. aureus* strains, make this leukocidin a potential target for the development of new therapies [8].

We found 7.7% positivity for *lukE /D*. This rate is lower than those obtained by certain authors, 44.3% in 2022 in China and 68.3% in 2017 in the Democratic Republic of Congo [32]. The absence of virulence factors *sek*, *sep* and *sel* during our study could indicate that the isolates obtained do not possess enterotoxin genes.

Clindamycin, linezolid, teicoplanin, daptomycin, fosfomycin, vancomycin, moxifloxacin, and fusidic acid were the most active antibiotics against our isolates during this study. On the other hand, at Point G University Hospital, Maiga A et al. 2017, found that the most active antibiotics were fusidic acid, amoxicillin + clavulanic acid and pristinamycin [33]. In this group of antibiotics, resistance to fusidic acid was 2.8%, implying that this antibiotic still remains active against *S. aureus* isolates.

Limitations

Our study was limited to a single hospital and is therefore not representative of the entire country; however, the profile of the affected population and their behavior are typical of the country. We did not carry out molecular typing (clonal complex, sequence typing, *SCCmec*

typing), nor molecular detection of the *mecA* gene encoding methicillin resistance.

Conclusion

The results of our study indicate that *S. aureus* is common among patients with pleurisy admitted to Mali University Hospital, and the frequency of MRSA is alarming at 72.2%, particularly in the absence of national prevalence assessment. This study can serve as a reference to monitor the evolution of MRSA and set up a surveillance system in the hospital. Our results suggest that the current empirical treatment protocol for pleurisy, which includes ciprofloxacin and gentamicin, could be revised and modified by these molecules in accordance with the WHO AwaRe classification of antibiotics: moxifloxacin, teicoplanin, daptomycin, intravenous or oral vancomycin, fusidic acid, intravenous fosfomycin or linezolid as a last line. Despite the limited exploration of the frequency and severity of MRSA pleurisy in our country, our study highlights the importance of implementing infection prevention measures, such as establishing a team of infection control, MRSA screening on admission and isolation of patients screened positive.

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Ethics and consent

We obtained written consent from each participant. Review and approval of the study protocol was obtained from the ethics committee of the University of Sciences, Techniques and Technologies of Bamako, identifiable under the reference number 2021/228/USTTB of 06/09 /2021.

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Competing interests

The authors have no competing interests to declare.

Author contributions

Aimé Césaire Kalambry, Tchamou Malraux Fleury Potindji Conceptualization, Data curation, Aimé Césaire Kalambry, Ambara Kassogu é, Moussa Karambiry Formal Analysis, Investigation, Methodology, Resources, Dinanibè Kambiré, Writing- Original draft Aimé Césaire Kalambry Conceptualization, Project administration, Supervision, Validation, Writing- review and editing Boubacar Sidiki Ibrahim Dramé, Ibrehima Guindo, Sadio Yéna, Seydou Doumbia and Mahamadou Diakité.

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