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Detection by Culture and by Multiplex Real-Time PCR of *Staphylococcus aureus* and *Staphylococcus* spp. in Vaginal Secretions and Urines in Patients Received at Saint Camille Hospital of Ouagadougou, Burkina Faso

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Staphylococci are bacteria involved in various pathologies and varying degrees of severity. They are one of the first causative agents of nosocomial and community infections with humans and animals as natural habitats. The genus *Staphylococcus* is subdivided into two groups, coagulase-negative staphylococci, the most isolated of which is *Staphylococcus aureus* and coagulase-positive staphylococci and constitutes about fifty species and subspecies. Some of these species are implicated in urinary tract and vaginal tract infections and are a real health problem especially for women. The aim of the study was to detect *S. aureus* and *Staphylococcus* spp. in vaginal secretions and urine using culture and real-time PCR. The microbiological analysis was done using the conventional methods adopted at Saint Camille's hospital laboratory. Molecular analysis was done using the Sacace multiplex PCR kit.
We examined a total of 97 samples including 77 urine samples and 20 vaginal secretion samples. At culture, no *Staphylococcus* was isolated. On the other hand, with the same culture-negative samples, we were able to identify two *S. aureus* and two *Staphylococcus* spp. in the urine and three *Staphylococcus* spp in vaginal secretions. The study then demonstrates the difference in sensitivity between the two identification techniques and confirms that there is a possibility of infection in case of negative culture. It is then necessary to involve rt-PCR in routine analyses.

Keywords: Real-time multiplex PCR; culture; S. aureus; Staphylococcus spp, Burkina Faso.

1. INTRODUCTION

Urinary infection is defined by microbial multiplication within the urinary tract, associated with a local inflammatory reaction. Bacteria and inflammatory cells are found in urine which is normally sterile and therefore indicates an infectious process [1]. Urinary tract infections (UTIs) are almost always bacterial in origin, although viruses, fungi, and parasites can also infect the urinary tract. More than 85% of UTIs are caused by bacteria from the intestine or vagina [2]. They can be located in the lower urinary tract (cystitis, urethritis, prostatitis, epididymitis) or upper urinary tract (pyelonephritis or pyelitis [3]. UTIs are among the most common bacterial infections, affecting 150 million people each year worldwide [4].

The vagina also represents a complex ecosystem in which numerous microbial species are present in varying numbers and proportions. Microorganisms exist in a finely tuned mutualistic relationship with the host and provide the first line of defense against colonization and infection by pathogens. When normal flora is unbalanced or destroyed, many germs can proliferate and cause major health problems for women [5]. Staphylococcal species are among the germs most frequently isolated in cases of urinary infection [6] as well as during vaginal infections [7]. Various parameters give them virulence, the genome, the capsule, protein A, the cell wall, toxins, enzymes [8] as well as the formation of the biofilm [9,10]. Several studies carried out in Burkina Faso as well as in other African countries have demonstrated the extent of vaginal and urinary Staphylococcal infections [11-15].

The genus *Staphylococcus* includes two main groups, coagulase-negative staphylococci (CNS) and coagulase-positive staphylococci (CPS), which have been defined based on their ability to produce the enzyme coagulase. Among staphylococci, *S. aureus*, belonging to the SCP group, and *S. epidermidis*, from the SCN group, are the most frequently isolated from each group [16]. Staphylococci are ubiquitous in nature, with humans and animals as primary reservoirs. Around 50% of healthy individuals carry these bacteria [17]. These bacteria colonize the skin and mucous membranes of humans [18].

Culture is the conventional. method used to identify antimicrobial susceptibilities of staphylococci. However, it has limitations. This method is laborious and time-consuming and

certain microorganisms are not culturable on bacteriological media [19]. However, rapid and accurate identification of germs is important for choosing the most appropriate antibiotic and predicting clinical outcome. Many molecular methods have been developed to detect bacteria. The most common is PCR of the 16S subunit gene of bacterial RNA. It makes it possible to demonstrate a bacterial infection but is insufficient, alone, for the precise identification of the bacterial strain which requires old tests such as the hybridization of specific probes or the sequencing of nucleic acids. Recently, real-time PCR has been shown to be a very sensitive and rapid method for the detection and quantification of microbial species [20].

Given the limitations of culture which is the routine analysis for the detection of staphylococci in our laboratories, we are considering also adopting multiplex real-time PCR which is also a germ detection technique. We will then compare the results obtained with the two methods in order to highlight the ideal method for routine analyzes in our laboratories. Our work will be divided into four parts: firstly, the bibliographical review, secondly the material and methods used, thirdly the results obtained and fourthly a discussion of the results obtained.

2. METERIALS AND METHODS

2.1 Samples Collection

Our study took place in two laboratories in Burkina Faso, Pietro Annigoni Biomolecular Research Center (CERBA) and Saint Camille Hospital. This is a cross-sectional study over a period of five months, March-July 2023. The sampling and bacteriological analyzes were carried out in the laboratory of Saint Camille hospital while the molecular analyzes were carried out at CERBA. A total of 97 samples were collected, i.e. 77 urine samples and 20 vaginal secretion samples.

2.2 Bacteriological Analysis

The identification of microorganisms was done using conventional methods adopted at the centers. The samples are inoculated on ordinary agar (e.g. BCP or Uriselect) and incubated for 18-24 hours. A Gram stain is subsequently carried out to obtain information on the type of bacteria (Gram+ or-) and the appropriate identification method.

Biochemical examinations were carried out to properly identify the associated bacterial species. This is the catalase test which makes it possible to differentiate staphylococci from streptococci.

Isolation was carried out on Chapman selective medium. Chapman medium is particularly used; it only allows staphylococci, halophilic germs which tolerate high concentrations of NaCl up to 7.5%, to grow after 24 to 48 hours (which for this reason inhibits most other germs). This selective medium is made differential by the addition of 1% Mannitol and an acidity indicator, phenol red. The latter allows both the isolation of Mannitol-fermenting staphylococci from a sample containing a mixture of germs and directs us towards *S. aureus* or another species of Mannitol-fermenting staphylococci. To differentiate *S. aureus* from other staphylococci, the DNase test was performed. This test is used to determine the ability of staphylococci to hydrolyze DNA and use it as a source of carbon and energy for growth. Thus, *S aureus* is DNase positive and the others are DNase negative.

2.3 Molecular Analysis

2.3.1 Sample pretreatment

We proceeded with a pretreatment step which consisted of the elimination of undesirable constituents by prior washing.

2.3.2 Extraction

The urines and vaginal secretions, stored in a refrigerator (2-8°C) were first subjected to a manual extraction process, to release the DNA from the bacteria before initiating the real-time PCR reaction. The extraction kit used is the Sacace DNA-Sorb-B Kit.

The pretreated sample was added to the Eppendorf tube containing the lysis solution. Centrifugation-vortex steps followed and then the sorbent, absorbing DNA, was added to the mixture. Washing steps followed to remove all impurities. The DNA was subsequently suspended in elution buffer. A negative control sample should go through all stages of DNA extraction. Sterile physiological saline solution can be used as a negative control sample with the volume according to DNA extraction kit used. If amplification is not performed in the same day of extraction, the processed samples can be

stored at 2-8°C for at maximum period of 5 days or frozen at –20°/-80°C.

2.3.3 Quantification and verification of the purity of DNA extracts

We quantified and verified the purity of the DNA with the biodrop. This step was important before initiating the amplification, we had to check if there was DNA in our extracts and also detect possible contaminants.

2.3.4 Amplification

The amplification kit used was the Bac Multi-Screen Real-TM. It is a real-time multiplex PCR Kit for the detection of the DNA of opportunistic bacteria of the Bacilli, Beta-proteobacteria and Gamma-proteobacteria classes causing nosocomial and community infections. The kit included a reaction mixture consisting of probes, specific primers for detecting bacterial DNA as well as the internal control (IC), which is an indicator of the quality of the reaction in each individual tube. For each clinical sample and controls are required 1 strip of PCR-Reaction mix-1 and 1 strip of PCR-Reaction mix-2. For each PCR, a negative extraction control and another included in the PCR kit were included. The PCR is carried out in a reaction volume of 35 µL.

2.4 Interpretation of Multiplex Real-Time PCR Results

The reaction results were analyzed automatically using the software supplied with the SaCycler-96TM Real-Time PCR device. Once the PCR is completed, the program displays in the table in the "Result" column: "+" or "-". Based on the PCR results (threshold cycles), the software automatically calculates the concentration logarithms, which are indicated in the line with the name of the microorganism and the corresponding total bacterial mass. The amplified product is detected using fluorophores (FAM, HEX, and Cy5). These dyes are linked to Oligo nucleotide probes which bind specifically to the amplified product. To control the location of the bands in the thermal block of the PCR device, an oligonucleotide with a Rox fluorescent marker was added to the mixture for the amplification of tube #1 and tube #10. It is used by the device as a marker to determine the position of the strips in the thermal block.

The results were interpreted based on the Ct values indicated by each fluorophore. The Ct values for the studied samples were compared with those of the positive controls.

2.5 Data Analysis

The results obtained in PCR and culture were entered and analyzed with Excel 2016 software. We then calculated the sensitivity and specificity of multiplex real-time PCR using bacterial culture as the reference (gold standard) in diagnosing *S. aureus* and *Staphylococcus* spp.

Sensitivity (Se): Capacity of a test to be able to detect as sick subjects who have the disease in a given population; thus measures the ability of a test to eliminate false negatives.

Specificity (Sp): Ability to correctly identify the non-diseased among those who do not have the disease in a given population; thus measures the ability of a test to eliminate false positives.

$$Se = Tp \times 100 / Tp + Fn$$

$$Sp = Tn \times 100 / Tn + Fp$$

Tp (true positives) represents the number of sick individuals with a positive test,
Fp (false positives) represents the number of non-sick individuals with a positive test,
Fn (false negatives) represents the number of sick individuals with a negative test,
Tn (true negatives) represents the number of non-ill individuals with a negative test

3. RESULTS AND DISCUSSION

3.1 Bacterial Culture Results

On urine culture, we detected the presence of *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* germs. All samples of vaginal secretions were culture negative.

The results obtained in culture allowed us to make the distribution of the bacterial strains isolated.

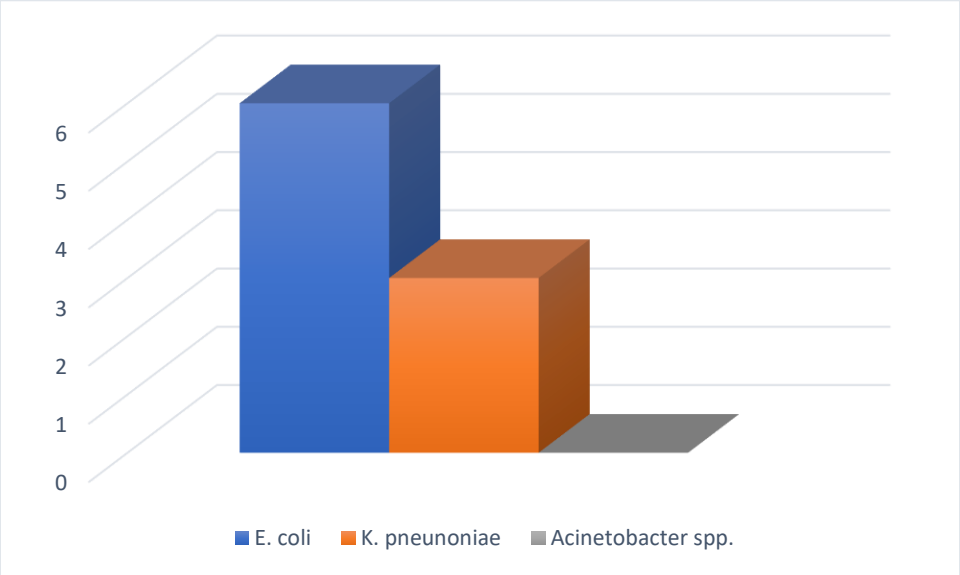

Fig. 1. Distribution of germs isolated in culture

3.2 Multiplex Real-time PCR Results

3.2.1 Quantification of extracts

We obtained values ranging from 33 to 200 µg/mL. The R ratio gave a purity ranging from 0.8-1.871. Our results confirmed the presence of DNA in our extracts in sufficient quantity. The purity values obtained confirm that our extractions were well carried out.

3.2.2 Amplification

No staphylococci were isolated in urines or in vaginal secretions on culture. Furthermore, the analysis of negative culture samples by multiplex real-time PCR allowed us to detect the presence of two *Staphylococcus* spp. and two *S. aureus* in the urine thus 22% of the detected germs and three *Staphylococcus* spp. in the vaginal swabs thus 15% of the samples. Among the samples analyzed, a co-infection was observed in one of the urine samples (*E. coli* and *S. aureus*).

The comparison of the results obtained with the two techniques is summarized in the following Table 1.

3.2.3 Sensitivity – Specificity

Based on the results obtained, the specificity and sensitivity of PCR were calculated with the culture technique as a reference (gold standard). The results obtained are shown in the following Table 2.

Fig. 2 corresponds to the results automatically interpreted using the software supplied with the SaCycler-96TM real-time PCR instrument. It represents an example of a sample (No. 4) positive for *Staphylococcus* spp. with a Ct value of 25.8. The cycle threshold (Ct) value is the number of cycles required for the PCR to detect the target microorganism [21].

Table 1. Comparison of target germs isolated in the two techniques

Types of exams	Urines (target germs identified)	Vaginal swabs (target germs identified)
Bacterial culture	None	None
Real-time multiplex PCR	22% (2 *S. aureus* and 2 *Staphylococcus* spp.)	15% (*Staphylococcus* spp.)

Table 2. Performance of PCR tests in the detection of *S. aureus* and *Staphylococcus* spp.

Test	Sensitivity	Specificity
Real-time multiplex PCR	100%	93,26%

Results

Multiplex detection

N	Identificator	Fam Cp	Hex Cp	Rox Cp	Cy5 Cp	Cy5.5 Cp
A1	4	25,7	24,9			
B1	4		25,7			
C1	4	35,5	25,6			
D1	4	36,5	25,6			
E1	4		25,7		34,3	
F1	4		25,6		25,8	
G1	4		25,6			
H1	4	33,9	25,6			
A2	4'		27,3			
B2	4'		25,2			
C2	4'		25,7			
D2	4'		25,5		30,2	
E2	4'		25,2			
F2	4'	33,3	25,5			
G2	4'		25,8			
H2	4'		26,2			
A3	5	26,7	25,2			
B3	5		25,5			
C3	5		27,5			
D3	5	37,3	27,5			
E3	5		27,2		28,6	

Fig. 2. Example of a positive sample (Sample Number 4) for *Staphylococcus* spp. with a Ct value of 25.8

Target seeds are detected by a fluorescent signal in the FAM, HEX, ROX and Cy5 fluorophore channels.
HEX detects internal control
Fam detects several germs but in position 6 it is S. aureus
CY5 detects several germs but in position 6 it is Staphylococcus spp.
The fluorescent marker Rox was added to the mixture for the amplification of tube #1 and tube #10. It is used by the device as a marker to determine the position of the strips in the thermal block

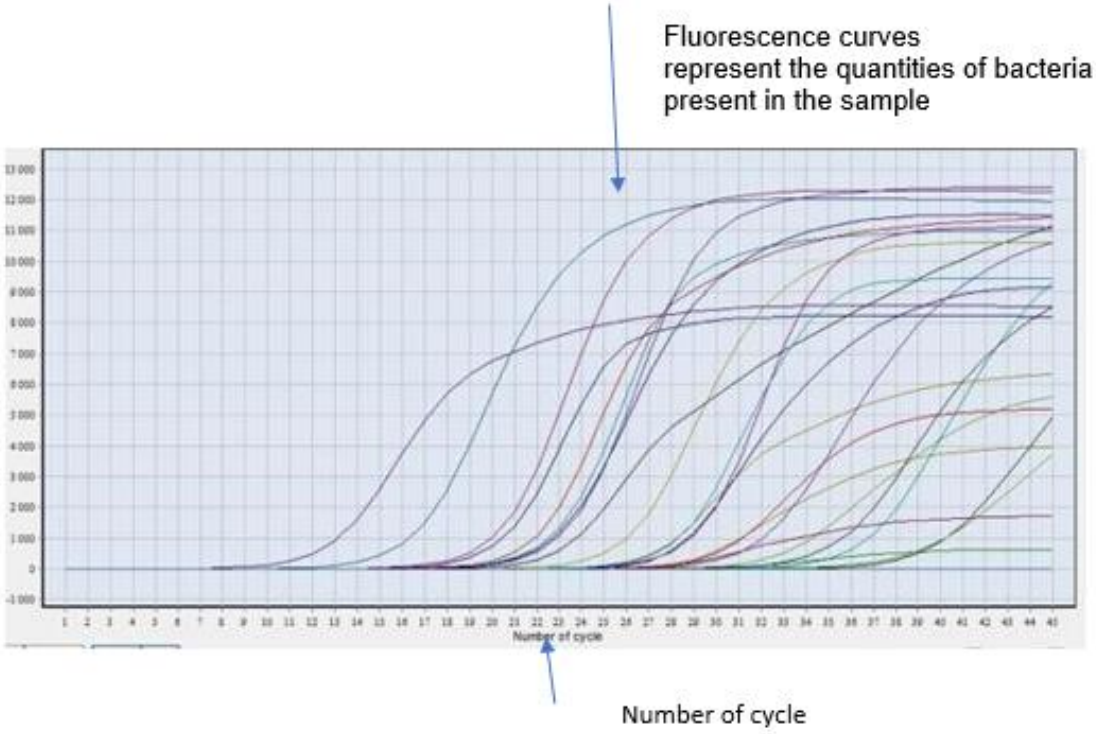

Fig. 3. Descriptive curve automatically interpreted using the software supplied with the SaCycler-96TM real-time PCR instrument

4. DISCUSSION

The objective of our study was to detect *S. aureus* and *Staphylococcus* spp. by culture and by Multiplex real-time PCR in vaginal secretions and urine and then to carry out a comparative study of these two techniques. The microbiological analysis was carried out using conventional methods adopted at Saint Camille hospital and the molecular analysis was carried out with the Sacace, Bac Multi-Screen Real TM kit. The basic principle of culture is to promote the growth of bacteria by placing them in favorable conditions, evaluate bacterial abundance by determining, if possible, the extent of bacterial colonies, and identify organisms by evaluating their phenotypic characteristics. In multiplex real-time PCR, the principle is to extract all the DNA from the sample, amplify the targeted sequences of the bacterial genomes present and evaluate the number of bacteria according to the detection limits (Ct values) or copy number of the genome.

The results demonstrated the potential utility of real-time PCR for the detection of staphylococci. It has good sensitivity (100%) and good precision (93.6%) for the detection of staphylococci. This technique was able to detect seven staphylococci, including four staphylococci in urine (22%) and three in vaginal secretions (15%) with negative cultures. In addition, the PCR performance time was reduced (maximum 4 hours) compared to culture which can last up to 72 hours.

Various other studies have compared bacteriological culture with real-time PCR. They conclude with identical results to ours in most cases. That's to say rt-PCR has high sensitivity, bacteria that are not detected in conventional bacteriology are identified with real-time PCR. The sensitivity of detecting *S. aureus* has been reported as 97% compared to bacterial culture for milk samples [22] and 89% for clinical mastitis cases [23]. Compared with culture, sensitivity of PCR has been reported to 96.4%, for detecting *S. aureus* in evaluating commercial PCR against bacterial culture for diagnostic *S. aureus* throughout lactation [24].The diagnostic accuracy of PCR-based methods has shown high sensitivity (93-100%) in detection of *S. aureus* compared to culture from clinical mastitis milk [25].

PCR assay had a relative sensitivity of 94.1% in identification of *S. aureus* from metered milk samples [26]. For total *S. aureus*, PCR assay

had a sensitivity of 93.3% [27]. Another study on culture-negative subclinical mastitis milk samples was able to detect *E. coli, S. uberis* or *S. aureus* in 18 out of 26 cases or 69% of samples with real-time PCR [28].

However, although the PCR a good technique for detecting germs, the results must be interpreted with caution because of multiple pre-analytical factors (quality of the sample, sampling media, transport time to the laboratory, etc.) and analytical (preparation of the analysis, quantity of material used, etc.) can influence the performance of molecular tests. There may be false positives in rt-PCR which can be explained by the mechanism of death of the bacteria. Indeed, the destruction of the bacteria leads to the exteriorization of its genetic material, and potentially of the rt-PCR target sequences. Thus this sequence can be amplified and give a positive result where the culture will not find any bacterial colony because no cell multiplication will have taken place [29-32]. Since PCR is based on DNA amplification, false-positive or -negative outcomes may easily occur. In particular, a single PCR cycle results in very large numbers of amplifiable molecules that can potentially contaminate subsequent amplifications of the same target sequence [33,34]. To control such carryover contamination, one must prevent physical transfer of DNA between amplified samples, and between positive and negative experimental controls. For this purpose, preparation of samples for PCR assay must be in a room or biosafety hood separate from that in which the reactions are performed. Using a pipette tip with an aerosol barrier is essential for avoiding cross contamination as well as carryover contamination [33]. In addition, performing PCR is expensive compared to standard culture, probably due to generally expensive reagent prices, which limits its use, particularly in Burkina Faso.

However, a positive PCR result with negative culture does not exclude the presence of live pathogenic bacteria in the patient. When confronted with false positive real- time PCR results, one explanation is that patients may have been receiving an antimicrobial regimen that could inhibit growth of targeted bacteria when cultured, resulting in a false negative culture result [27]. If the cultures come back negative while the patients have symptoms of an infection, the PCR results may reveal germs. In addition, following therapeutic failures, it is necessary to carry out PCR to search for possible co-infection.

In conclusion, real-time PCR is more sensitive for the detection of germs but conventional culture methods are still important, especially for determining antibiotic susceptibility. An appropriate combination of molecular method and conventional culture allows a definitive diagnosis to be made.

5. CONCLUSION

The results of our study showed that the PCR technique is more sensitive compared to culture for the detection of *S. aureus* and *Staphylococcus* spp*.* This makes possible to not exclude the possibility of infection in the event of a negative culture. However, real-time PCR results should be interpreted with caution. To interpret the result of a PCR, it is essential that clinicians and microbiologists share experiences, so that the analytical and clinical levels of interpretation can be combined. It is extremely useful for clinical data to accompany test requests. This will make it possible to exclude false positives in PCR. Every healthcare establishment is faced with staphylococcal infections. Therefore, it would be interesting to have molecular biology techniques allowing local monitoring and accessible to a majority of laboratories. Conventional culture methods are still important, especially for determining antibiotic susceptibility. An appropriate combination of molecular method and conventional culture allows a definitive diagnosis to be made.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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