Video Article

Preparation of a Blood Culture Pellet for Rapid Bacterial Identification and Antibiotic Susceptibility Testing

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Abstract

Bloodstream infections and sepsis are a major cause of morbidity and mortality. The successful outcome of patients suffering from bacteremia depends on a rapid identification of the infectious agent to guide optimal antibiotic treatment. The analysis of Gram stains from positive blood culture can be rapidly conducted and already significantly impact the antibiotic regimen. However, the accurate identification of the infectious agent is still required to establish the optimal targeted treatment. We present here a simple and fast bacterial pellet preparation from a positive blood culture that can be used as a sample for several essential downstream applications such as identification by MALDI-TOF MS, antibiotic susceptibility testing (AST) by disc diffusion assay or automated AST systems and by automated PCR-based diagnostic testing. The performance of these different identification and AST systems applied directly on the blood culture bacterial pellets is very similar to the performance normally obtained from isolated colonies grown on agar plates. Compared to conventional approaches, the rapid acquisition of a bacterial pellet significantly reduces the time to report both identification and AST. Thus, following blood culture positivity, identification by MALDI-TOF can be reported within less than 1 hr whereas results of AST by automated AST systems or disc diffusion assays within 8 to 18 hr, respectively. Similarly, the results of a rapid PCR-based assay can be communicated to the clinicians less than 2 hr following the report of a bacteremia. Together, these results demonstrate that the rapid preparation of a blood culture bacterial pellet has a significant impact on the identification and AST turnaround time and thus on the successful outcome of patients suffering from bloodstream infections.

Video Link

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Introduction

Bloodstream infections and sepsis in hospitalized patients are a major cause of morbidity and mortality. Thus, mortality related to bloodstream infections is observed in about 14% to 37% of hospitalized patient and may increase to 35% in intensive care units patients [1-3]. The rapid identification of the infectious agent is pivotal to guide optimal antimicrobial treatment and to increase the successful outcome of antimicrobial therapy [4,5]. The rapid analysis of Gram stains from positive blood culture has already a significant impact on the adaptation of antimicrobial therapy [6,7] but accurate identification of the infectious agent is required to provide the best adapted antibiotic treatment to the patients. For instance, different antibiotic treatment regimens have to be implemented following bacteremia with enterococci and streptococci that are difficult to distinguish by Gram staining. Similarly, identification at the species level is required to detect Gram negative enterobacteria encoding a chromosomal *ampC* gene which confer an increased resistance to β-lactams [8].

With a positive blood culture, the conventional diagnostic approach is to subculture the infectious agent on different agar plates, which requires several hours of additional incubation prior identification with various approaches including biochemical tests, growth on different selective media and automated microbial identification systems. The time to results of a conventional diagnostic approach is of about 1 to 3 days.

The emergence of the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) technology for rapid identification of microorganisms has provided a new tool to quickly identify microorganisms from colonies grown on agar plates but also directly from positive blood cultures (**Figure 1**) [9-12]. The use of MALDI-TOF to identify an infectious agent from blood cultures has significantly reduced the time to results to a few minutes instead of the hours and days required by traditional methods. As discussed by Croxatto *et al.* [13], the efficiency of MALDI-TOF identification relies on different parameters including the microorganism's purity and quantity. These two criteria are easily obtained from discrete colonies grown on agar plates but required a pre-analytical treatment for bacterial enrichment and purification from complex samples such as blood culture, which contain multiple cellular and protein components that may interfere with MALDI-TOF identification.

Various microorganisms' isolation methods from blood culture have been used in a number of studies including saponin or other mild detergents method for bacterial extraction [9,14], serum separator method [10], lysis centrifugation methods [12] and commercially solutions such as the sepsityper kit. Our bacteriology diagnostic laboratory has developed a simple blood-culture bacterial pellet preparation based on ammonium chloride erythrocyte-lysis which allow fast identification of bacteria and yeast by MALDI-TOF and automated identification systems (**Figure 2**) [15]. This blood-culture pellet preparation also provide a sample for other direct downstream applications such as Gram staining, automated PCR-based

diagnostic tests such as POCT-PCRs for the rapid detection of methicillin-resistant *Staphyloccocus aureus* (MRSA), and antibiotic susceptibility testing with automated AST systems and/or by disk diffusion assays on agar plates (**Figure 3**).

In this work, we describe the different steps for the preparation of the blood-culture bacterial pellet as explained by Prod'hom *et al.*[15] (**Figure 4**). We will also describe the protocols for three of the main applications that can be performed on the blood culture pellet: Identification by MALDI-TOF [15], identification (ID) and antibiotic susceptibility testing (AST) with the automated systems [16] for *Enterobacteriaceae* and staphylococci and automated PCR-based diagnostic test for the detection of MRSA [17].

Protocol

This protocol has been developed and validated following the research and development processes and ethical rules of our institution before being implemented as a routine tool.

1. Preparation of a Blood Culture Bacterial Pellet by Ammonium Chloride Erythrocyte-lysing Procedure

1. Preparation of a positive blood culture for subsequent centrifugation.
    1. Sterilize the blood culture cap. Add 70% ethanol on the cap of the bottle and burn it.
    NOTE: Do not perform this step under a laminar flow hood.
    2. Move the bottle to a laminar flow hood. Collect 5 ml of the blood culture with a 20 G syringe.
    3. Add the 5 ml of blood culture in a 50 ml centrifuge tube containing 45 ml of sterile water (H_2O) and mix the sample.

2. Preparation of a blood culture bacterial pellet by lysis centrifugation.
    1. Centrifuge the sample at 1,000 x g for 10 min at RT.
    2. Remove the supernatant (which contains mainly H_2O and blood cells) with a laboratory vacuum pump.
    3. Resuspend the pellet in 1 ml of home-made ammonium chloride lysing solution (0.15 M NH_4Cl, 1 mM $KHCO_3$). Break down the pellet by scraping and by vortexing the tube and centrifuge the sample at 140 x g for 10 min at RT.
    4. Discard the supernatant which mainly contains red blood cells debris.
        1. If the pellet remained hemorrhagic, resuspend the pellet in 2 ml of sterile and distilled H_20 to further lyse residual red blood cells and to wash the pellet. Centrifuge the sample at 140 x g for 10 min at RT and discard the supernatant.
    5. Resuspend the pellet in 200 µl of H_2O

3. Subculture of blood culture on various selective and non-selective agar media.
    1. Collect 1 ml of the blood culture with a 20 G syringe.
    2. Inoculate 1 drop of blood culture into four quadrants on blood agar, chocolate agar, McConkey agar and Schaedler agar.
    3. Incubate at 37 °C the blood agar and chocolate agar plates in a 5% CO_2 atmosphere, the McConkey agar in a normal atmosphere and the Schaedler agar plate in anaerobic conditions.
    4. Follow bacterial growth on the different media.
    5. Check for bacterial purity.

2. Identification by MALDI-TOF MS

1. Direct identification from the blood pellet.
    1. Transfer 1 µl of the blood pellet on a MALDI target plate and let it dry on a 42 °C heating platform.
    2. Overlay the sample with 1 µl 70% formic acid and let it dry on a 42 °C heating platform.
    3. Overlay the sample with 1 µl of MALDI matrix (saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile-2.5% trifluoroacetic acid) and let it dry on a 42 °C heating platform.
    4. Proceed to MALDI-TOF MS analysis with a MALDI-TOF MS system as described by the manufacturers.
    5. If the identification score is invalid at the species level (for instance with a score < 2), perform a protein extraction of the blood pellet sample.

2. Identification after protein extraction from the blood culture pellet.
    1. Mix 20 µl of the pellet with 1 ml of 70% ethanol and centrifuge at 13,000 x g for 2 min at RT.
    2. Discard the supernatant and add 25 µl of 70% formic acid and 25 µl of 100% acetonitrile to the pellet. Vortex vigorously to resuspend the pellets. Resuspend tough pellets by breaking them down with pipette tips before vortexing.
    3. Centrifuge at 13,000 x g for 2 min at RT. Transfer 1 µl of the supernatant (which contain extracted proteins) on a MALDI target plate and let it dry on a 42 °C heating platform. Proceed as described in 2.1.2.

3. Bacterial Identification and Antibiotic Susceptibility Testing with an Automated Microbial System

1. Preparation of a bacterial suspension for bacterial identification and AST with an automated microbial system.
    1. Prepare a plastic tube containing 3 ml of sterile 0.45% NaCl solution compatible with the automated microbial system.

2. Add a sufficient volume of the blood culture pellet in the 0.45 % NaCl solution to obtain a bacterial suspension corresponding to a McFarland turbidity of 0.6 to 0.8. Measure the turbidity using a densitometer machine.
   NOTE: The volume of the blood culture bacterial pellet varies from 50 to 200 µl to achieve a McFarland turbidity of 0.6 to 0.8.

2. Inoculate the automated Gram-positive (GP) or Gram-negative (GN) cards for identification and the automated AST cards for antimicrobial susceptibility testing of Gram-positive cocci and Gram-negative bacteria as described by the manufacturer.
   NOTE: Identification with GP or GN cards is not applied if MALDI-TOF MS identification score value is >2. Check the purity of the bacterial suspension by subculturing the bacterial suspension on blood agar (GP and GN) and MacConkey agar (GN) plates.

4. Antibiotic Susceptibility Testing by Disk Diffusion Assay

The disk diffusion assay is described by the European committee on antimicrobial susceptibility testing (EUCAST, Version 3.0, April 2013, www.eucast.org).

1. Preparation of a bacterial suspension to perform antimicrobial susceptibility testing by disk diffusion assay.
   1. Prepare a glass tube containing 3 ml of sterile 0.9% NaCl solution.
   2. Add a sufficient volume of the blood culture pellet in the 0.9% NaCl solution to obtain a bacterial suspension corresponding to a McFarland turbidity of 0.5. Measure the turbidity using a densitometer machine.
      NOTE: The volume of the blood culture bacterial pellet varies from 50 to 200 µl to achieve a McFarland turbidity of 0.6 to 0.8.
   3. Vortex the bacterial suspension.

2. Inoculation of bacterial suspension onto Mueller-Hinton agar (MH) or Mueller-Hinton agar-fastidious organisms agar (MHF) (MH supplemented with 5% defibrinated horse blood and 20 mg/L of β-Nicotinamide adenine dinucleotide).
   1. Dip a sterile cotton swab in the bacterial suspension and gently remove excess fluid by turning the swab on the inside wall of the glass tube.
   2. Spread the bacterial suspension evenly onto the entire surface of the MH/MHF agar plate by swabbing in three directions or by using an automated plate rotator.

3. Application of antimicrobial disks on inoculated agar plates.
   1. Apply closely the disks on the surface of the dried inoculated agar plates manually or by using a susceptibility disk dispenser.
   2. Incubate the plate at the appropriate temperature and atmosphere as described in the antimicrobial susceptibility testing EUCAST disk diffusion method (Version 3.0, April 2013, www.eucast.org).

5. Automated PCR-based Diagnostic Test for the Detection of MRSA

1. Using a micropipette, transfer 50 µl of the blood culture pellet into the sample reagent vial provided with the MRSA automated PCR-based diagnostic test kit and vortex during 20 sec.
2. Using a 1 ml micropipette, dispense the sample into the specimen port of the cartridge. Insert the cartridge into the automated PCR system and start the assay as described by the manufacturer.

Representative Results

In the study performed by Prod'hom *et al.*[15], bacterial pellets obtained by ammonium chloride lysis centrifugation of 122 positive blood culture from 78 patients were analyzed by MALDI-TOF MS. Out of 122 positive blood culture, 95 (77.9%) were correctly identified at the species level and one (0.8%) at the genus level. The remaining 26 (21.3%) blood culture pellets gave no reliable identification by MALDI-TOF. Among those, 21 were gram positive bacteria including 13 streptococci and 5 coagulase-negative staphylococci. Importantly, 10 out of the 13 unidentified streptococci were *Streptococcus pneumoniae* which often possess a difficult-to-lyse capsule and are hardly distinguished from species of the *S. mitis* group using MALDI-TOF MS. Five Gram-negative bacteria were not identified by MALDI-TOF, among which four difficult-to-lyse encapsulated bacterial species including two *Klebsiella pneumoniae* and two *Haemophilus influenzae*. Thus, correct identification by MALDI-TOF was obtained in 78.7% of blood culture pellets, which represents an efficient performance compared to the 84.1% of identification obtained from conventional identification of bacterial colonies grown on agar plates [11].

The performance of the automated microbial system for bacterial identification (ID) and for AST was tested on 278 bacterial pellets of positive blood culture for Gram-positive cocci in cluster and Gram-negative bacteria. These results were compared to conventional results obtained with the automated microbial system cards from colonies grown on agar plates following subculture of positive blood culture on appropriate media [16]. Direct identification using microbial identification system ID cards can be used when identification of the bacterial pellet by MALDI-TOF MS has failed. Compared to a final identification by MALDI-TOF MS, a correct identification with microbial identification system ID cards was observed in 99% of *Enterobacteriaceae,* 74% of staphylococci, 71% of non-fermentative Gram-negative bacteria and 33% of other Gram-positive cocci. A misidentification was observed in 11% of total cases whereas 8% of bacterial pellets gave no identification. A total of 220 bacterial pellets including 87 *Enterobacteriaceae* and 133 staphylococci were analyzed for AST with automated microbial system AST GN26 and AST 580 cards [16]. The results were analyzed according to definitions given by the US Food and Drug Administration (FDA) for interpretive agreement results, which defined a false susceptible as a very major error (VME) and a false resistant as a major error (ME). AST obtained from direct inoculation of blood culture pellet compared to inoculation from colonies grown on agar plates showed 0.1% VME and 0.3% ME for *Enterobacteriaceae*, and 0.7% VME and 0.1% ME for staphylococci. Thus the performance of AST cards inoculated directly with blood culture pellets are in agreement with the performance criteria accepted by the FDA.

The performance of the PCR-based nucleic acid amplification technology MRSA test targeting the *spa*, *mecA* and *SCC* genes was directly applied on *Staphylococcus aureus* blood culture bacterial pellets identified by MALDI-TOF MS [17]. Based on 106 cases, the detection of

methicillin resistance exhibited a 99% sensitivity and a 100% specificity. Interestingly, the median time to report the nucleic acid amplification technology results from blood culture positivity was equal to 201 min (range 100-430) whereas the median time from MALDI-TOF identification to report similar results was equal to 97 min (range 25-250).

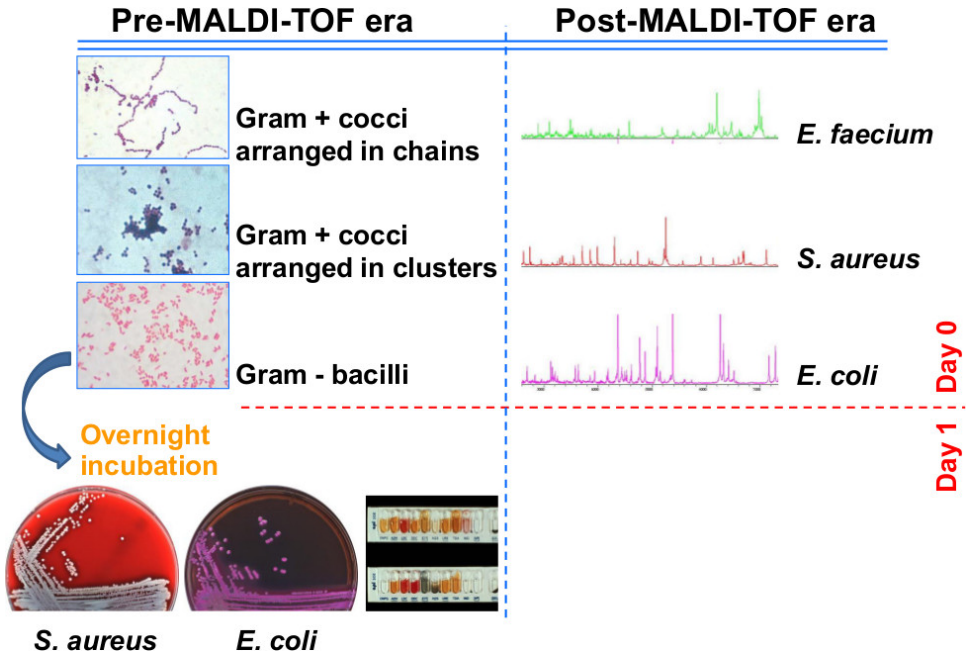

Figure 1. Pre- and post-MALDI-TOF era. Compared to conventional diagnostic approaches which include an O/N subculture of the sample, MALDI-TOF MS identification can be performed within minutes following samples delivery to the diagnostic laboratory. Please click here to view a larger version of this figure.

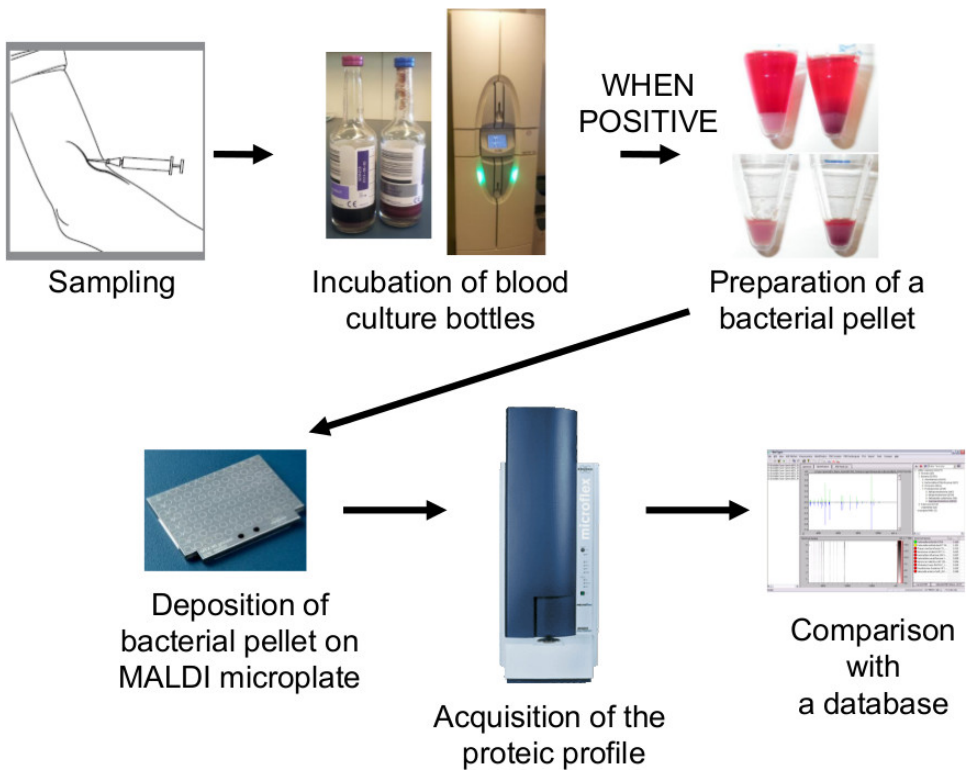

Figure 2. Workflow of blood culture processing from sampling to MALDI-TOF MS identification. When blood cultures are positive, a bacterial pellet is prepared by ammonium chloride lysis centrifugation. This bacterial pellet is then used for direct identification by MALDI-TOF MS. MALDI-TOF MS identification can be obtained within 30 to 60 min following blood culture positivity. Please click here to view a larger version of this figure.

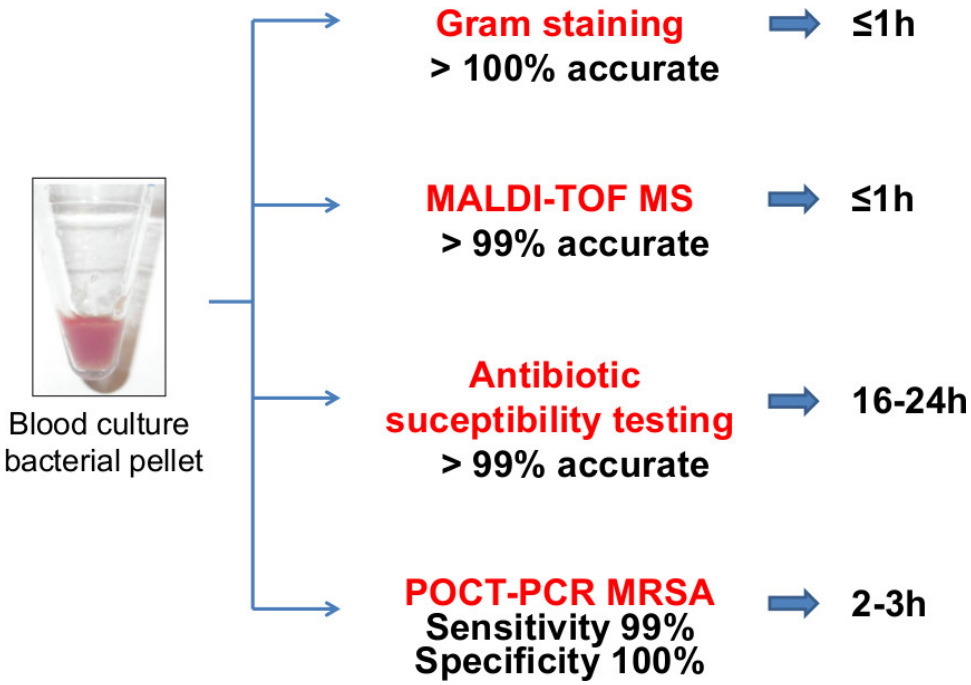

Figure 3. Many downstream applications including Gram staining, MALDI-TOF MS identification, AST using automated systems and/or disk diffusion assays and rapid PCR-based testing such as point of care testing PCRs (POCT-PCR) for the detection of MRSA can be performed directly on the blood culture bacterial pellet. Direct testing from the bacterial blood culture pellets significantly decrease the turn-around time to report ID and AST results which is pivotal to improve the outcome of patients suffering from bloodstream infections. Please click here to view a larger version of this figure.

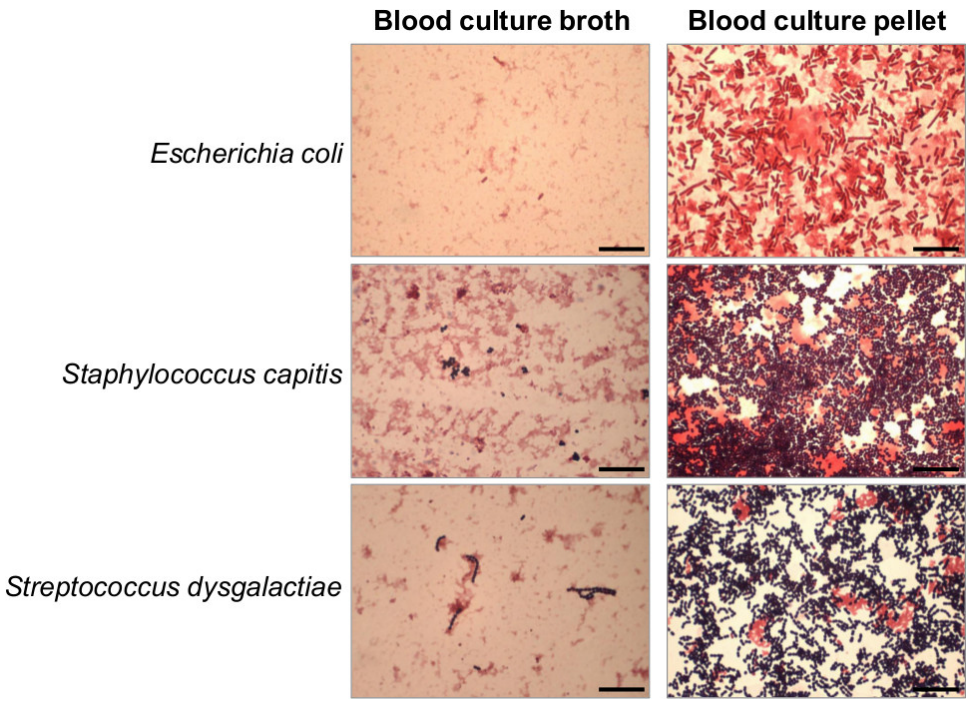

Figure 4. The ammonium chloride lysis centrifugation protocol allows the preparation of an enriched and purified bacterial pellet from positive blood culture broths. Gram staining of positive blood culture of *Escherichia coli*, coagulase-negative *Staphylococcus capitis* and *Streptococcus dysgalactiae* performed before and after the preparation of the blood culture bacterial pellet. The classical morphologies of staphylococci in clusters and streptococci in chains are more easily observed in the native blood culture broth than in the blood culture bacterial pellet. Scale bar = 25 µm. Please click here to view a larger version of this figure.

Discussion

Compared to conventional positive blood culture diagnostic approaches, the rapid acquisition of a bacterial pellet by using the ammonium chloride lysis centrifugation approach reduce the time to report identification by 16 to 24 hr and the time to report AST by 24 to 48 hr (**Figures 1 and 3**).

Rapid introduction of appropriate antibiotic therapy is pivotal to improve the outcome of patients suffering from bloodstream infections. Thus, early identification of the infectious agents within 1 hr followed by a rapid AST obtained in about 8 to 16 hr represents a major impact in the clinical management of septic patients. The rapid reporting of Gram staining has already been associated with a great impact on antimicrobial therapy and thus with a decrease in patient morbidity and mortality [6,7]. The Gram staining of the blood culture bacterial pellet may be done to increase sensitivity when the Gram staining of the blood culture broth is negative or weakly positive (**Figure 4**). However, Gram staining performed on the native blood culture broth is recommended to observe typical morphologies such as staphylococci in clusters or streptococci in chains. In a recent prospective study performed on 202 cases of bloodstream infection, MALDI-TOF MS identification from blood culture pellets showed an impact in antibiotic therapy in 35.1% of cases whereas Gram stain had only an additional impact in 20% of cases [8]. Interestingly, the maximum difference of impact between Gram staining and MALDI-TOF MS identification reporting on clinical management was observed when bacteria associated with increased antibiotic resistance such as AmpC-producing *Enterobacteriaceae* were identified by MALDI-TOF MS. Similarly, Martiny *et al.* have shown that MALDI-TOF MS rapid identification may fasten the adaptation of the antibiotic therapy in 13.4% of cases whereas a retrospective study performed by Stoneking *et al.* suggested that a rapid identification of microorganisms causing bacteremia may help adjusting the empirical therapy in 77% of cases either by administrating an additional antibiotic not covered by the initial regimen or by reducing the spectrum of the antibiotics [18,19]. Moreover, the use of a rapid PCR-based assay such as the nucleic acid amplification technology MRSA following *S. aureus* identification by MALDI-TOF MS from blood culture pellets allowed to provide the most appropriate antibiotic therapy in less than 4 hr to patients suffering from *S. aureus* bacteremia [17]. When excluding patients with penicillin allergy, the use of the nucleic acid amplification technology MRSA allowed a significant reduction of anti-MRSA antibiotics misuse such as glycopeptides from 26.1% to 8.1%. Together, these data suggest that rapid sequential reporting of Gram staining, MALDI-TOF MS identification and rapid PCR-based assay from blood culture pellets are significantly improving the antibiotic therapy and thus the clinical management as well as the outcome of patients suffering from bloodstream infections.

MALDI-TOF MS allowed a 78.7% correct identification of blood culture pellets, which is a very good performance compared to a rate of 84.1% identification obtained by conventional MALDI-TOF MS performed on pure colonies grown on agar plates. More that 50% of bloodstream infections are caused by bacterial species such as *Enterobacteriaceae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and enterococci. These bacteria are efficiently identified by MALDI-TOF MS which likely positively influence the good performance of MALDI-TOF MS identification performed directly on blood culture bacterial pellets. The low performance for the identification of some bacterial species by MALDI-TOF MS performed on blood culture pellets is mainly due to similar factors than those observed for the conventional identification from pure colonies grown on agar plates. For instance, several microbial species such as Streptococci belonging to the *mitis* group and coagulase-negative staphylococci are closely related and identified with low accuracy by MALDI-TOF MS. Moreover, a particular composition of the bacterial cell wall of Gram-positive bacteria or the presence of a dense capsule observed in bacterial species such as *Klebsiella pneumoniae* significantly decrease the lysis efficiency and thus the performance of MALDI-TOF MS identification. Finally, the low performance of MALDI-TOF MS for the identification of anaerobic bacteria is mainly due to an incomplete and poor representation of these bacteria in the MALDI-TOF database.

The small reduced performance of MALDI-TOF MS identification from blood culture pellets compared to conventional identification from pure colonies may be due to residual blood protein components or to an insufficient amount of bacteria obtained after lysis-centrifugation. Similarly, the differences observed with the automated microbial system cards inoculated directly with blood culture pellets compared to those inoculated with isolated colonies may be caused by residual blood culture components such as proteins, blood cells and blood medium compounds that may interfere with bacterial growth in the automated microbial system cards and may have a significant impact on both identification and AST. Moreover, the AST results obtained by automated microbial systems need to be checked by disk diffusion assays also performed directly on blood culture bacterial pellets to validate the results of some antibiotics known to present significant discrepancies compared to conventional approaches. However, direct inoculation of the automated microbial system cards with blood culture pellets presents an excellent ID and AST performance for both *Enterobacteriaceae* and staphylococci.

Thus, the bacterial pellet obtained by ammonium chloride lysis centrifugation of positive blood cultures has been validated to directly perform several downstream applications allowing a rapid reporting of essential results such as ID and AST in a significantly reduced TAT compared to conventional approaches. Moreover, additional applications such as extended-spectrum β-lactamases (ESBLs) testing have been validated on similar lysis centrifugation of positive blood culture methodologies [20] suggesting that they could also be applied on the bacterial pellet obtained with the protocol described in this work.

Disclosures

The authors declare that they have no competing financial interests.

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