

# Identification and Antimicrobial Susceptibility Testing of Microorganisms From Positive blood Cultures by a Combined Lysis-centrifugation Method with MALDI-TOF MS and VITEK2 System

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## ABSTRACT

**Background:** Rapid identification and the application of antimicrobial susceptibility testing (AST) to microorganisms causing bloodstream infections is pivotal to guide antimicrobial therapy. This study aims to: 1) utilize the Lysis-Centrifugation Method (LCM) not only for identification of microorganisms from positive blood culture bottles, but also for direct AST full panel by Vitek<sup>®</sup>2 system (bioMérieux, Inc. France) and by disc diffusion plate (Kirby Bauer Method) and 2) analyze the accuracy of these combined methods.

**Methods:** 124 mono-microbial positive blood culture bottles were included in this study. An aliquot was subjected to LCM and used for the identification by the MALDI-TOF System. Moreover the microbial pellet was used for direct AST testing full panel by VITEK<sup>®</sup>2 system and by Kirby Bauer Method.

**Results:** 123 isolates were correctly identified to the species level and 1 isolate was identified to the genus level. Comparing the two utilized AST methods, it was observed that Gram-positive isolates showed an agreement rate of 96.6% (58/60). *Enterococcus faecalis* was the only microorganism with a major error rate of 0.6% (2/324) related to erythromycin. Among the Gram-negative, the overall agreement rate was 93.3 (56/60). *Klebsiella pneumoniae*, *Escherichia coli* and *Enterobacter spp.* were the major cause of minor error rates (0.6%, 4/709) and major error rates (1.1%, 8/709). Among the yeasts, results showed an agreement rate of 100% (4/4).

**Conclusions:** Our simple and cost-effective sample preparation method is very useful for rapid identification as well as AST of microorganisms directly from positive blood culture bottles in a clinical setting.

**Keywords:** Bacteremia, LCM, MALDI-TOF MS, VITEK<sup>®</sup>2, AST.

## Introduction

The presence of microorganisms in the blood or bloodstream infections (BSI), confirmed by a positive culture <sup>[1,2]</sup>, is a major cause of morbidity and mortality throughout the world <sup>[3-7]</sup>. During the period from 2000 to 2010, mortality from septicemia grew by 17% <sup>[8]</sup> and recent reports still show mortality to range from 34 to 52% <sup>[9]</sup>.

Microorganisms enter the bloodstream through various portals, including dissemination from a previous or concomitant infection and access via surgical sites, intravenous catheters, and other vascular access devices <sup>[10,11]</sup>. Bloodstream infections can be caused by a wide variety of microorganisms, commonly *Escherichia coli*, *Klebsiella spp.*, *Staphylococcus aureus*, *Enterococcus spp.* and yeast. These infections can lead to increased mortality, long-term disability, excess length of stay in hospitals, large

additional costs for health systems, and high costs as well as loss of quality of life for patients and their families <sup>[7]</sup>.

Rapid identification and the application of antimicrobial susceptibility testing (AST) to microorganisms causing bloodstream infections is pivotal to guide antimicrobial therapy <sup>[12]</sup>, helping to reduce the detection time of the antimicrobial treatment and improving patient response.

The standard protocol to diagnose a bloodstream infection involves liquid medium blood cultures that remains the gold standard to establish its etiology <sup>[13]</sup>.

Currently, steps in routine phenotypic identification involves microscopic observation, sub-culturing and analysis of various biochemical reactions in order to establish an empiric treatment <sup>[14]</sup>. This process could take several days, especially if fastidious and slow-

growing microorganisms are present [15]. An alternative to the phenotypic identification, it is possible to perform a genotypic sequencing technology, such as 16S rRNA, but it is still expensive for a routinely diagnosis [16].

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was recently introduced in the clinical microbiology for microorganisms' identification. Although it identifies bacteria and yeasts within a few minutes, it requires isolated colonies, which takes 18-48 hr of incubation [17]. To reduce the time needed for identification and AST, some investigators tried to inoculate microorganisms present in positive blood culture bottles directly into MALDI-TOF MS system [18-20]. However, direct identification requires sample preparation steps since blood culture bottles contain proteins/debris which could interfere with the spectra of the microorganisms [21]. Therefore, lysis solutions [22], the lysis-filtration method [12] or commercial kits [19] were utilized for sample preparation. However, some of these procedures are laborious and/or expensive [23].

We previously published a study describing a Lysis-Centrifugation Method (LCM) that can be used for microbial identification by ID Vitek®2 System (bioMérieux, Inc. France) directly from positive blood cultures detected by Oxoid Signal Blood system and by BD BACTEC™FX [24] by MALDI-TOF MS system. Results of this research demonstrated that ID obtained by LCM method correctly identified a 91.4% of microorganisms responsible of the mono-microbial bacteremia with an agreement to the species and the genus level, if compared with the standard method Vitek®2 [24].

The present study aims to: 1) utilize LCM not only for identification of microorganisms from positive blood culture bottles, but also for direct antimicrobial susceptibility testing (AST) full panel by Vitek®2 system (bioMérieux, Inc. France) and by disc diffusion plate (Kirby Bauer Method) and 2) analyze the accuracy of these combined methods.

## Materials and Methods

**Samples:** This research was performed at the Laboratory of Microbiology (DLC01) of “Umberto I” Hospital in Rome. A total of 124 consecutive positive blood cultures, obtained from patients of the Department of “Anesthesiology and Intensive Care” and “Internal Medicine” of the same hospital in the period from April 2015 to March 2016, were included in this study.

Out of 124 blood samples, 70 were collected and inoculated in BD BACTEC™ Plus Aerobic/F culture vials and incubated in the automated system BD BACTEC™FX, whereas 54 samples were collected and inoculated in

Oxoid Signal Blood Culture System Medium (OXOID S.p.A.). Only mono-microbial cultures were selected.

Blood culture bottles signaling positive were removed from BD BACTEC™FX or visualized as CO<sub>2</sub> production in the Oxoid Signal Blood Culture System Medium and an aliquot was taken for Gram staining, subjected to LCM and used for the identification by the MALDI-TOF System (Bruker Detection Corp, Bruker Nano GmbH Germany). Moreover the microbial pellet obtained from LCM was used for direct AST full panel by Vitek®2 system (bioMérieux, Inc. France) and by disc diffusion plate (Kirby Bauer Method).

In parallel, an aliquot taken from positive blood culture bottles was sub-cultured on solid media. Isolates grown from such culture media were used for identification (MALDI-TOF System) and AST Vitek®2 System (bioMérieux, Inc. France).

**LCM for Positive Blood Cultures Detected by Oxoid Signal Blood System:** 5 ml of blood culture was centrifuged to 3000 rpm for 10 minutes in order to obtain a bacterial suspension pellet that was finally resuspended in 4 ml of distilled water in order to lyse red blood cells [24].

**LCM for Positive Blood Cultures Detected by BD BACTEC™FX :** 5 ml of blood culture sample was centrifuged to 3000 rpm for 15 minutes. In order to obtain a bacterial suspension pellet without traces of blood, subsequent washings with 5 ml of 0.45% ammonium chloride solution (NH<sub>4</sub>Cl, Sigma-Aldrich Co. LLC) were carried out. Finally the pellet was resuspended in 5 ml of distilled water to remove traces ammonium chloride and protein residues.

**MALDI-TOF System Identification:** The pellet was smeared on a MALDI-TOF target plate and processed for a rapid microbial identification. According to the microscopic observation the pellet was processed in the following way:

- **For Gram-positive Bacteria:** a small amount of the pellet was spread on two spots of a target plate both processed with 1 µl of 70% formic acid (HCOOH, Sigma-Aldrich Co.LLC) and subsequently with 1 µl of the acid α-cyano-hydroxycinnamic matrix solution (HCCA, Bruker Daltonik, GmbH).
- **For Gram-negative Bacteria:** a small amount of the pellet was spread on two spots of a target plate and processed with 1 µl of HCCA matrix solution.
- **For Yeast:** a small amount of the pellet was dissolved on two spots of a target plate and processed with 1 µl of 70% formic acid (HCOOH, Sigma-Aldrich Co. LLC). Subsequently 1 µl of HCCA matrix solution was added, as above mentioned.

The plate was placed in the mass spectrometer MALDI-TOF System and analyzed for protein mass patterns identification within a few minutes.

**Antimicrobial Susceptibility Testing:** The microbial pellet obtained from LCM was utilized in order to obtain a suspension preparation adjusted to a McFarland standard of 0.5 for Gram-positive and Gram-negative bacteria and used for standard microdilution method AST Vitek®2 System. The same bacterial suspension was used to perform susceptibility disc diffusion test (Kirby Bauer Method).

Yeast suspension was used to carry out antifungal susceptibility tests by Vitek®2 card and Sensititre YeastOne (Thermo-Fisher).

AST-P632, AST-P592, AST-N202 (bioMérieux, Inc. France) were used for AST of staphylococci, enterococci and Enterobacteriaceae, respectively according to the identification results.

The resulting minimum inhibitory concentration (MIC) values were classified into clinical categories of susceptible, intermediate or resistant following the European Committee on Antimicrobial Susceptibility Testing – EUCAST recommendations, 2014.

For susceptibility, disc diffusion test (Kirby Bauer Method) antimicrobial discs were chosen among those recommended by EUCAST guidelines and common with the standard method AST Vitek®2.

The comparison between AST Vitek®2 system and Kirby Bauer Method was expressed in terms of agreement, very major error (false susceptibility), major error (false resistance), or minor error (susceptible/resistance versus intermediate susceptibility).

**Statistical Analysis:** We compared LCM to current standard practice in an effort to gain insight into how the process would improve clinical work flow. Fig. 1 shows a flow chart for identification and AST of microorganisms obtained directly from positive blood culture bottles.

Chi-square test or Fisher's exact test was used for statistical comparisons. A P value < 0.05 was considered statistically significant.

## Results

Out of 124 positive blood samples included in this study, 70 were collected and inoculated in BD BACTEC™ Plus Aerobic/F culture vials and incubated in the automated system BD BACTEC™ FX, whereas 54 samples were collected and inoculated in Oxoid Signal Blood Culture System Medium (OXOID S.p.A.).

Within these selected positive blood cultures, 60 Gram-positive, 60 Gram-negative and 4 yeasts were correctly

identified with MALDI-TOF system by LCM: 123 isolates were identified to the species level and 1 isolate was identified to the genus level. The list of isolates are reported in Table 1 and Table 2.

The identified microorganisms were then analyzed for direct antimicrobial susceptibility testing by disc diffusion plate (Kirby Bauer Method) and by microdilution method AST by Vitek®2 (bioMérieux, Inc. France).

The comparison of the two antimicrobial susceptibility testing utilized methods was showed in Table 1. It was observed that Gram-positive isolates showed an agreement rate of 96.6% (58/60) (Table 1). *Enterococcus faecalis* (N = 2) was the only microorganism responsible of the disagreement with a major error rate of 0.6% (2/324) (Table 1). Among the Gram-negative, the overall agreement rate was 93.3 (56/60) (Table 1). *Klebsiella pneumoniae* (N = 2), *Escherichia coli* (N = 1) and *Enterobacter spp.* (N = 1) were the major cause of this disagreement with a minor error rate of 0.6% (4/709) and a major error rate of 1.1% (8/709) (Table 1).

In Table 3 the discordant antibiotics obtained from comparison by AST Vitek®2 and Kirby Bauer Systems are showed. Regarding Gram-positive, erythromycin (N = 2) was the only antibiotic with a major error rate related to *Enterococcus faecalis* (N = 2) (Table 3).

Concerning Gram-negative [*Klebsiella pneumoniae* (N = 2), *Escherichia coli* (N = 1) and *Enterobacter spp.* (N = 1)], Gentamicin (N = 2), Amikacin (N = 1) and Ceftazidime (N = 1) were the discordant antibiotics obtained from comparison by AST Vitek®2 System and Kirby-Bauer System. This discrepancy was classified as a minor error (Table 3). Moreover, amoxicillin/clavulanic acid (N = 3), Cefotaxime (N = 1), Ciprofloxacin (N = 2), Piperacillin/tazobactam (N = 1) and Cefepime (N = 1) were an attribute of major error rates (Table 3).

Among the yeasts, the comparison of antimicrobial susceptibility testing results between Sensititre YeastOne® System (Thermo Fisher Scientific comparison) and AST-yeast Vitek®2 System (bioMérieux, Inc. France) showed an agreement rate of 100% (4/4) with the absence of very major errors, major errors and minor errors (Table 2).

Finally, the Table 4 reported the AST by Vitek®2 and Kirby Bauer Method percentage comparison results according to the type of blood culture bottles included in the study. Out of 124 positive blood cultures no significative discordance was observed within the Oxoid Signal Blood and BD Bactec bottles: in fact there was an agreement of 98.1% and of 93% (p= 0.830) in the Oxoid Signal Blood and BD Bactec, respectively (Table 4).

**Table 1: Comparison of antimicrobial susceptibility testing results between the AST-Vitek®2 System and Kirby Bauer System from blood cultures Oxoid Signal Blood and BD BACTEC.**

Microorganism	N° of Isolates	Agreement Susceptibility Test (Vitek®2/Kirby)	No Agreement (Vitek®2/Kirby)	N° of Antimicrobial Tested	Antibiotics In Agreement	Minor Error	Major Error	Very Major Error
Gram-positive	60	58	2	324	322 (99.4%)	0	2 (0.6%)	0
Staphylococcus aureus	18	18	-	90	90	-	-	-
Staphylococcus epidermidis	19	19	-	95	95	-	-	-
Staphylococcus haemolyticus	1	1	-	5	5	-	-	-
Staphylococcus lentus	1	1	-	5	5	-	-	-
Staphylococcus hominis	4	4	-	20	20	-	-	-
Staphylococcus capitis	5	5	-	25	25	-	-	-
Enterococcus faecalis	9	7	2	63	61	-	2	-
Enterococcus faecium	3	3	-	21	21	-	-	-
Gram-negative	60	56	4	709	697(98.3%)	4 (0.6%)	8 (1.1%)	0
Klebsiella pneumoniae	25	23	2	300	298	1	1	-
Klebsiella oxytoca	1	1	-	12	12	-	-	-
Escherichia coli	19	18	1	228	224	1	3	-
Enterobacter spp.	1	-	1	12	6	2	4	-
Acinetobacter baumannii	2	2	-	24	24	-	-	-
Enterobacter cloacae complex	1	1	-	12	12	-	-	-
Pseudomonas aeruginosa	6	6	-	72	72	-	-	-
Pseudomonas oryzae	1	1	-	12	12	-	-	-
Proteus mirabilis	2	2	-	24	24	-	-	-
Serratia marcescens	1	1	-	12	12	-	-	-
Stenotrophomonas maltophilia	1	1	-	1	1	-	-	-
<b>TOTAL</b>	<b>120</b>	<b>114</b>	<b>6</b>	<b>1033</b>	<b>1019</b>	<b>4</b>	<b>10</b>	<b>0</b>

**Table 2: Comparison of antimicrobial susceptibility testing results between Sensititre YeastOne® System (Thermo Fisher Scientific comparison) and AST-yeast Vitek®2 System (bioMérieux, Inc. France) from positive blood culture (OXOID SIGNAL BLOOD/ BACTEC ) after by using LCM.**

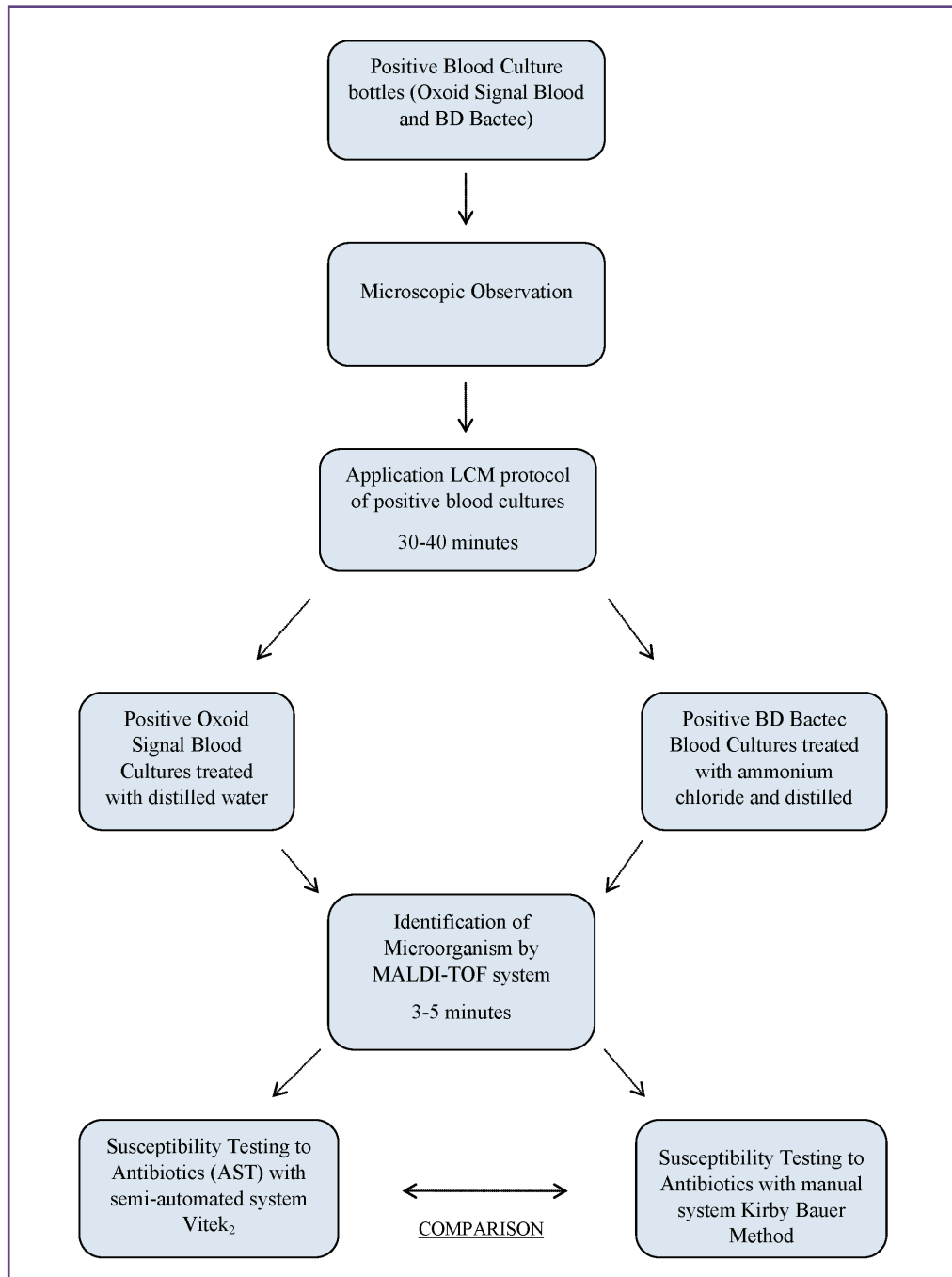
Microorganism	N° of Isolate	Agreement (Sensititre YeastOne®/Ast-Yeast)	No Agreement	N° of Antifungals Tested	Minor Error	Major Error	Very Major Error
Yeasts	4	4	0	24	0	0	0
Candida albicans	2	2	-	12	-	-	-
Candida tropicalis	2	2	-	12	-	-	-

**Table 3: Discordant antibiotics obtained from comparison by AST Vitek®2 and Kirby Bauer Systems.**

MICROORGANISM	Antibiotics showing discrepancy		
	Very major error (0)	Major error (10)	Minor error (4)
Enterococcus faecalis (2)	-	Erythromycin (2)	-
Klebsiella pneumoniae (2)	-	Amoxicillin/clavulanic acid (1)	Gentamicin (1)
Escherichia coli (1)	-	Cefotaxime (1) Amoxicillin/clavulanic acid (1) Ciprofloxacin (1)	Gentamicin (1)
Enterobacter spp. (1)	-	Piperacillin/tazobactam (1) Cefepime (1) Ciprofloxacin (1) Amoxicillin/clavulanic acid (1)	Amikacin (1) Ceftazidime (1)
<b>TOTAL</b>	<b>0</b>	<b>10</b>	<b>4</b>

**Table 4: Percentage comparison results according to the type of blood culture bottles (Oxoid Signal Blood and BD Bactec) between the direct antimicrobial susceptibility testing by Vitek® 2 and Kirby Bauer Method.**

Comparison results AST Vitek2 / Kirby-Bauer			
	Oxoid Signal Blood	BD Bactec	P-value
AGREEMENT	53/54 (98.1%)	65/70 (93%)	0.830
NO AGREEMENT	1/54 (2%)	5/70 (7%)	0.193



**Fig. 1: Flow chart for identification and AST of microorganisms obtained directly from Positive Blood Culture bottles.**

## Discussion

The rapid identification and the application of AST to microorganisms causing bloodstream infections is pivotal to guide antimicrobial therapy, helping to reduce the detection time of the antimicrobial therapy and improving the patient response.

In this study we evaluated the Lysis-Centrifugation Method for identification as well as antimicrobial susceptibility testing of microorganisms directly from positive blood cultures (Oxoid Signal Blood and BD Bactec bottles) by disc diffusion plate (Kirby Bauer Method) and by microdilution method (AST Vitek®2 System bioMérieux, Inc. France) analyzing the accuracy of these combined methods.

During the study period, within the 124 selected positive blood cultures (60 Gram-positive, 60 Gram-negative and 4 yeasts), 123 microorganisms were correctly identified with MALDI-TOF system by LCM to the species level. Only 1 isolate was identified to the genus level. In this case, MALDI-TOF MS has not been able to precisely identify the species responsible for bacteremia (*Enterobacter* spp. instead of *Enterobacter kobei*). Our result is in agreement with what reported by other previous studies, in which MALDI-TOF MST accurately detects *Enterobacter cloacae* complex although it may not discriminate some species within this complex [25-27].

Excluding the only identification to the genus level, the concordance rate of identification with MALDI-TOF system by LCM was very reliable (99,2%, 123/124). Moreover, the application of 70% formic acid to Gram-positive spots of a target plate improved the identification of gram-positive bacteria with a perfect overlapping with classical ID using VITEK®2 system (bioMérieux, Inc. France).

The evaluation of LCM for AST of microorganisms directly from the 124 positive blood cultures by disc diffusion plate (Kirby Bauer Method) and by microdilution method (AST Vitek®2 System bioMérieux, Inc. France) showed different results within the isolates.

Comparing the two antimicrobial susceptibility testing utilized methods, it was observed that *Enterococcus faecalis*, within Gram-positive groups, was the only microorganism with a major error rate related to erythromycin.

Among the Gram-negative, *Klebsiella pneumoniae*, *Escherichia coli* and *Enterobacter* spp. were the major cause of minor error rates and major error rates.

*Klebsiella pneumoniae* and *Escherichia coli* showed a minor error rates against Gentamicin. Regarding

*Enterobacter* spp, the discordant agents, as minor error rates, obtained from comparison by AST Vitek®2 System and Kirby-Bauer System were amikacin and ceftazidime. On the other hand, amoxicillin/clavulanic acid, cefotaxime, ciprofloxacin, piperacillin/tazobactam and ceftazidime were an attribute of major error rates in *Klebsiella pneumoniae*, *Escherichia coli* and *Enterobacter* spp.

The percentage of minor and major errors was low among the 1033 microorganism-antimicrobial combinations tested. Although in literature, the coagulase negative staphylococci (CNS) that are common blood culture contaminants, exhibited the most errors among the Gram positive isolates [12, 28], our CNS (N= 30) isolates showed a perfect agreement susceptibility between the Kirby Bauer Method and AST Vitek®2 System.

Among the Gram-negative isolates, previous studies reported the most errors for *Escherichia coli*, *Pseudomonas aeruginosa* and *Proteus mirabilis* isolates [29]. Our data are in agreement with these reports only for *Escherichia coli*. For *Pseudomonas aeruginosa* and *Proteus mirabilis* strains, the comparison of the two antimicrobial susceptibility testing utilized methods revealed no discrepancies. Among our Gram-negative isolates we observed the most errors in *Klebsiella pneumoniae* (N=25) and *Enterobacter* spp. (N=2) as just indicated by Wimmer *et al.* [29].

In the present study, we can conclude that the accuracy of AST was outstanding with both Gram-positive and Gram-negative isolates, since the antibiotics in agreement were 1019/1033 with only 4 minor errors and 10 major errors. This result was comparable or higher to that of other studies [12, 28].

During the study period, the number of positive blood cultures (Oxoid Signal Blood and BD Bactec bottles) for yeast isolates was very low, although the comparison of antimicrobial susceptibility testing results between Sensititre YeastOne® System and AST-yeast Vitek®2 System from positive blood culture after by using LCM was perfectly overlapping. Therefore, the limited number of yeasts strains was not utilized for statistical analysis, but it was included in the total results.

The final goal for using MALDI-TOF MS is to get early ID and AST for better use of antibiotics for treatment of infection and for patient management and hospital costs [30]. In fact, the average time to identification and antimicrobial susceptibility testing by using our LCM protocol for both Vitek®2 Method and Kirby Bauer method was 18-24 hours, directly from positive blood culture.

The results obtained in this study support that our strategy reduced the times for species identification and

susceptibility testing. In fact, particularly in Gram-negative sepsis, it is crucial that an appropriate antibiotic therapy must be administered as soon as possible and this could contribute to have a maximum benefit to patient care.

Further, this new technology may provide better definition of the epidemiology, pathogenesis, and antimicrobial susceptibility of unrecognized or misidentified microorganisms.

An important limitation is that polymicrobial infections were not included during the study period, since as just reported in literature, there is a difficulty in identifying polymicrobial infections.

## Conclusion

Larger future studies should include more isolates, particularly more yeast to confirm our results, as well as polymicrobial infections, to check whether, within polymicrobial infections, a predominant microorganism could be evidenced by LCM without misidentifications.

In summary, our simple and cost-effective sample preparation method is very useful for rapid identification as well as AST of microorganisms directly from positive blood culture bottles in a clinical setting.

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