Identification of Pyogenic Bacteria in Human and Animals Conventionally and Non-nucleic Acid Molecularly Using MALDI-TOF MS

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A B S T R A C T

Sepsis is considered a race to death between pathogens and host immune system, associated with a high mortality worldwide. Until now, blood cultures (BCs) are still the "gold standard" for identification of pathogens causing sepsis. Its major drawback is long turn around time (TAT) of 24–48 h up to several days, which is not compatible with the need for early sepsis diagnosis. Molecular assays as matrix-assisted laser- desorption ionization time-of-flight mass spectrometry(MALDI-TOF MS) reduce the TAT. Herein, the main aim of the study is to develop rapid and highly sensitive molecular proteomic non-nucleic acid assay for identification (ID) of the most common bacteria causing sepsis and pyogenic infections in human and animals. Eighty clinical samples, including 50 animal pus samples and 30 neonatal whole blood samples were subjected for conventional and molecular identification using VITEK-MS. About 70 % of samples were BC positive, including 33/50(66 %) of animal pus samples and 23/30 (76.7 %) of neonatal blood samples. The identified bacterial species were only three conventionally versus twelve using VITEK-MS, with variable confidence value (C.V) that was 99.9 % for 55/58 (94.8 %) isolates, 50 % for 2/58 (3.4 %) isolates and 26.5 % with 1/58 (1.74 %) isolate. The species-level identification for Streptococci was more challenging than for Staphylococci and Enterococci with only 50 % C.V for *S. dysagalactiae* subsp. *dysagalactiae*. Culture provided concordant species- and genus-level identification with VITEK-MS for 24/58(41.3 %) and 34/58(58.6 %) of isolates, respectively. The overall concordance was 100 % for all isolates except *P. aeruginosa* was 87.5 %.

Keywords: VITEK-MS, sepsis, pyogenic bacteria

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1. INTRODUCTION

Pyogenic bacteria is the usual cause of suppuration, including *S. aureus, S. pyogenes, P. aeruginosa, Proteus* spp, *E. coli, Klebsiella* spp, *C. perfringes* and Bacteroides. They occur in abscesses, burn or wound infections, cellulitis, bites, suppurative lymphadenitis and pyomyositis. An abscess is a localized collection of pus into a cavity formed by tissue liquefaction(Siqueira & Rocas 2013), living, dead and disintegrated neutrophils,

living and dead bacteria, cells debris and inflammatory exudates (Verma 2012).

Sepsis is a severe inflammatory response to infection, that rapidly developed and produced high mortality of 200,000 deaths per year(Chun et al. 2015). Bacteraemia is bacterial circulation in blood, whereas sepsis is bacteraemia with clinical signs as fever, hypotension, tachycardia and tachypnea (Dreyer 2012). Sepsis is caused by microbial invasion from local infection into bloodstream, (Reinhart et al. 2012),evolving as ≥ 2 systemic inflammatory response syndrome (SIRS) criteria (Kaukonen et al. 2015) to severe sepsis, septic shock (Gaieski et al. 2013),multiple organ dysfunction syndrome (MODS) andmultiple organ failure (MOF), in order of increasing severity. Sepsis is caused by Gram-positive (GP) and Gram-negative (GN) bacteria, anaerobes, fungi, viruses or parasites (Dellinger et al. 2013 and Iskander et al. 2013). Bacterial sepsis is a dangerous disease with high mortality rates.*S. aureus* and *Streptococcus* spp are the most frequent GP bacteria causing sepsis (Morel et al. 2015), whereas *E. coli* (Schaub et al. 2014), *K. pneumoniae* and *P. aeruginosa* are the predominant GN bacteria (Ranieri et al. 2012). Sepsis is mono-microbial infection, with minor polymicrobial infections (10–20 %) (Ngo et al. 2013), while pyogenic infections are polymicrobial or monomicrobial, endogenous or exogenous (Verma 2012).

The BCs remain the gold standards for BSI diagnosis(Dellinger et al. 2013). Phenotypic characterization from positive BCs include sub-culture, microscopy and biochemical tests (Laakso 2013).Classic BCs are time-consuming(Chang et al. 2015) and require 48–72 h for bacterial growth, up to 5 days (Loonen et al. 2014) for fastidious bacteria(Mulvey et al. 2012), 4–24 h for identification and an additional 24 h for antimicrobial susceptibility test (Cambau & Bauer 2015) with results are usually false negative (Liesenfeld et al. 2014). Owing to long TAT, a safety strategy utilizes empirical antibiotics in the first hour of sepsis onset (Dellinger et al. 2013). However, this initial treatment is inadequate for 47% of patients (Jernberg et al. 2010).A delay or failure in identification extends the antibiotic courses causing deleterious adverse effects as development of antibiotic resistant bacteria, collateral damage to normal gut flora, opportunistic pathogen colonization as *C. difficile* and/or *Enterobacteriaceae* selection with transferable drug resistance (Farrell et al. 2013).The early detection within the first 6-12 h is essential for favorable outcome in sepsis (Wang et al., 2014). Rapid molecular assays are needed especially in emergency units and acute critical- care settings (Stoneking et al. 2013).They are performed on positive BC(s) or directly on blood (Jordana-Lluch et al. 2013), either amplified nucleic acid- based techniques (NATs),non-amplified NATs or non-NATs(Kothari et al. 2014)such as MALDI-TOF MS which has become an attractive option to rapidly identify many pathogens. It is used in this study as a recent rapid proteomic method identifying bacteria by their conserved ribosomal proteins(Chun et al. 2015). Therefore, the main aim of the study is to develop rapid and highly sensitive molecular proteomic non-nucleic acid assay for identification (ID) of the most common bacteria causing sepsis and pyogenic infections in human and animals.

2. MATERIALS AND METHODS

A total of 80 clinical samples were used in this study, including 50 pus samples (code no 1a: 50a) from animal abscesses and 30 neonatal whole blood samples (code no 1h: 30h) with suspected sepsis admitted to neonatal intensive care unit (NICUs) at El-Ahrar General Hospital, Zagazig, Sharkia, Egypt. All neonates received at least 5 days of antibiotics. The most frequent clinical signs

were respiratory distress and mild perinatal asphyxia, while the most frequent laboratory abnormalities were neutrophilia, leukopenia and thrombocytopenia. Clinical data of cases and patients are not shown.

Bacterial isolation and identification

Pus and whole blood samples were inoculated on blood agar and incubated aerobically or with 5 % CO_2 at 37°C for 24 h up to 5 days(Faria et al. 2015). Blind subcultures were made on solid media, including MacConkey's and eosin methylene blue (EMB) for isolation of Gram-negative (GN) bacteria; mannitol salt agar (MSA)and Edward's agar for isolation of Gram-positive (GP) bacteria with 5 % CO_2 incubation for isolation of streptococci, as well as Brain Heart Infusion (BHI) broth at pH 9.6 and incubated at 10- 45˚C for isolation of enterococci.

Conventional identification of bacterial isolates

Suspected clinical isolates were identified using Gram's stain and biochemical tests (Boutin et al. 2015), including catalase and coagulase for staphylococcal isolates; salt and bile tolerance and esculin hydrolysis tests for enterococcal isolates; oxidase, IMVC, urease and TSI tests for GN isolates. Bacterial isolates were preserved frozen in BHI broth with 30 % glycerol at -20 °C (Quiles et al. 2015).

Molecular identification of bacterial isolates by MALDI-TOF MS

A pure colony of fresh overnight sub-culture on blood agar was spotted directly onto a well of target slide using a 1.0 µl loop, covered with 1 µl matrix solution and air dried for 5 min at room temperature to co-crystallize with sample, before insertionintoVITEK-MS instrument for target interrogation and generation of spectra (Luo et al. 2015). In case of polymicrobial BC(s),colonies had variant morphologies were selected and analyzed separately (Barnini et al. 2015).

a. Generation of mass spectra

Spectra were recorded in positive linear mode within a mass range from 2000 to 20,000 Da (laser frequency of 20 Hz, acceleration voltage 20 kV, IS2 voltage maintained at 18.6 kV, extraction delay time 200 ns). For each spectrum, 500 laser shots (50 laser shots from different positions of the target spot, 10 times) were collected by MS. The generated mass fingerprints were processed by computer software and the advanced spectrum classifier algorithm which was associated with VITEK-MS and identified an organism by comparing the obtained spectrum (presence and absence of specific peaks) with those of the typical spectrum of each species in a reference spectra database (Verroken et al. 2015).

b. Interpretation of MALDI-TOF MS results

Confidence value (C.V)

It represented similarity of specific peaks between generated and database spectra. Samples had single identification with any C.V highly confident at species level, whereas only genus-level identification result was obtained when C.V of a 'low discrimination' (LD), including species of the same genus. A 'No ID' result was denoted if (i) no identification provided or (ii) identification including species of different genera. Samples with 'No ID' were retested and repeated result was considered as final. A C.V of 99.9 % meant a perfect match and that of 60- 99.8% indicated spectra, close to that of a reference spectrum (Luo et al. 2015).

The VITEK-MS and conventional results were compared and correlated as follows; total concordance, when MALDI-TOF MS and phenotypic identification agreed at species level, MS was considered correct species identification; partial concordance, both methods matched at genus level; no reliable result, when VITEK- MS provided

No ID; no peaks, when no protein spectrum was obtained; and discordant results, when identification by both methods did not match.

3. RESULTS

After BC incubation at 37 ºC for 24- 48 h aerobically with/without 5 % CO_2, 56/80 (70 %) samples were BC positive, including 33/50 (66 %) animal pus samples and 23/30 (76.7 %) neonatal blood samples. Bacterial isolates were identified by standard conventional phenotypic methods (Fig. 1),whichrevealed that among pus samples, 31 were monomicrobial and only 2 samples (code no 4a and 23a) were polymicrobial at Gram stain.Whereas, all blood samples appeared monomicrobial at Gram stain.

Distribution of bacterial isolates from pus and blood culture

All 58 bacterial isolates of animal and human origin were identified as 30 (51.7 %) GP, including Staphylococcus (n= 23), Streptococcus (n= 5), Bacillus (n= 1) and non-Bacillus spp (n= 1) and 28 (48.3 %) GN isolates, including *Enterobacteriaceae*(n= 19) and non-*Enterobacteriaceae* (n= 9) (Table 1).

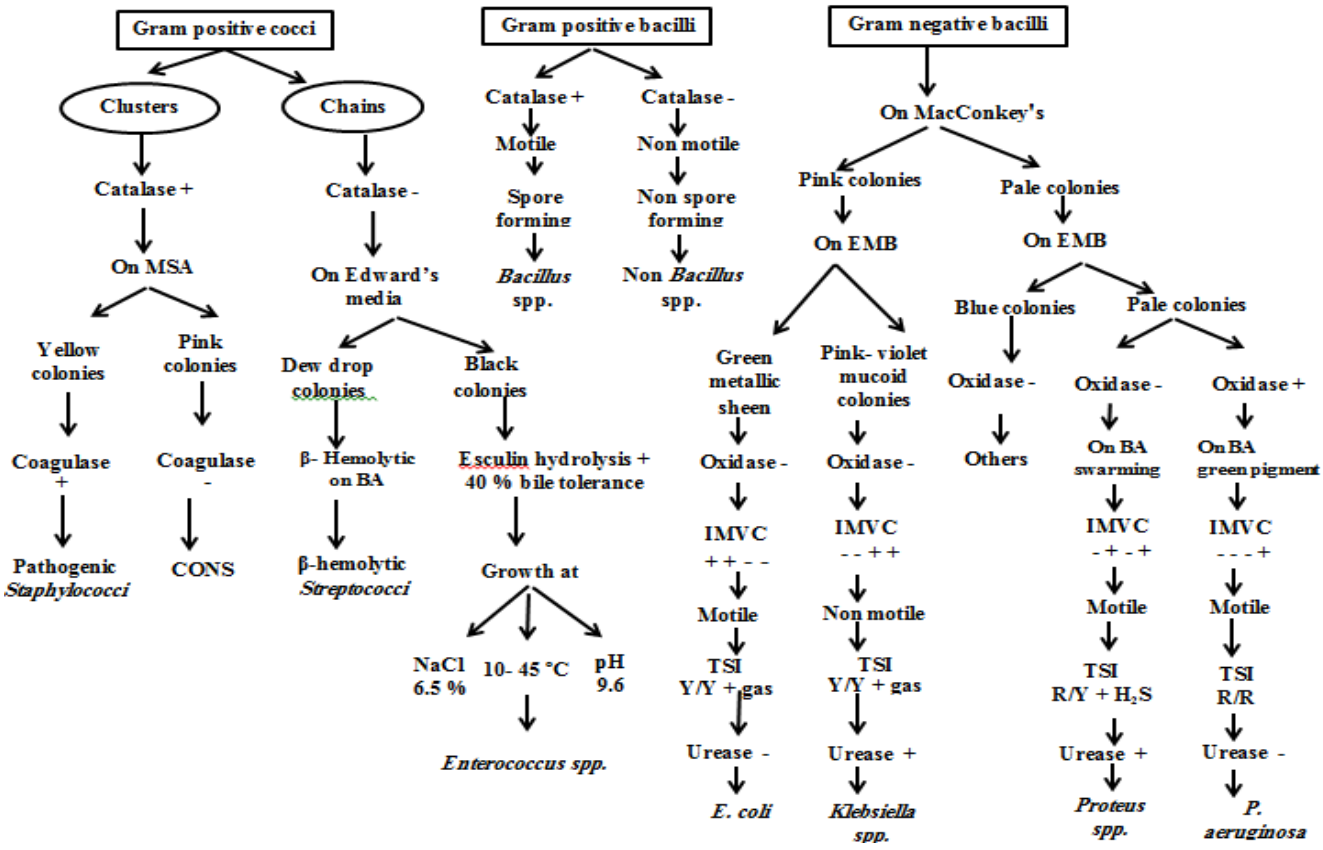

Fig.1. Flowchart of culture and phenotypic identification of bacterial isolates recovered from animal pus and neonatal blood with suspected sepsis. BA, blood agar; Y, yellow; R, red; MSA, mannitol salt agar; EMB, eosin methylene blue agar; TSI, triple sugar iron agar test; IMVC, indole, methyl red, Vogusproskour and citrate utilization tests; CONS, coagulase negative Staphylococci.

Table 1. Distribution of bacterial isolates from pus and blood samples

Bacterial species	Animal pus (n= 35)		Neonatal blood (n= 23)		Correct ID at	
	No.	(%)	No.	(%)	Species level	Genus level
Gram positive cocci						
Staphylococcus aureus	6	(17.14)	4	(17.39)	10	0
CONS	7	(20)	6	(26.08)	0	13
Enterococcus spp.	2	(5.71)	1	(4.34)	0	3
β hemolytic Streptococci	2	(5.71)	0	0	0	2
Gram positive bacilli						
Bacillus spp.	1	(2.85)	0	0	0	2
Non*Bacillus* spp.	1	(2.85)				
Total GPbacteria	19	(54.28)	11	(47.82)	10	20
Gram negative bacilli						
Escherichia coli	4	(11.42)	2	(8.69)	6	0
Klebsiella spp.	1	(2.85)	7	(30.43)	0	8
Proteus spp.	5	(14.28)	1	(4.34)	0	5
Pseudomonas aeruginosa	6	(17.14)	2	(8.69)	9	0
Total GN bacteria	16	(45.71)	12	(52.17)	15	13
Total isolates (n=58)	35		23		25	33

No., number; GP, Gram positive; GN, Gram negative; ID, identification

Proteomic molecular identification of bacterial isolates by MALDI-TOF-VITEK-MS

The 58 bacterial isolates of animal and human origin were identified by VITEK-MS assay and 12 bacterial species were detected, comprising the most common pathogens causing pyogenic infection and sepsis in animals and neonates.Correct species level identification was consistently obtained for all isolates with a variable C.V, including *S. aureus* (n= 10), *S. epidermidis* (n= 12), *Staphylococcusxylosus* (n= 1), *E. faecalis* (n= 3), *S. dysagalactiae subspp. dyasgalactiae* (n= 2), *Bacillus licheniformis* (n= 1) and *Lactobacillus rhamnosus* (n= 1) among 30 GP isolates; in addition to *E. coli* (n= 6), *K. pneumonia* (n= 8), *P. mirabilis* (n= 6), *P. aeruginosa* (n= 7) and *Delftiaacidovorans* (n= 1) among 28 GN isolates. Overall, VITEK-MS correctly identified to a species level 55/58 (94.8 %) isolates with 99.9 % C.V; 2/58 (3.4 %) isolates with 50 % C.V and 1/58 (1.7 %) isolate with 26.5 % C.V identified as *L. rhamnosus* in one animal isolate (Table 2).

Distribution of bacterial species among pus and blood isolates using VITEK-MS

In the 35 animal isolates, GP were more common than GN bacteria with a frequency of 19/35 (54.2 %) and 16/35 (45.7 %) respectively. The two polymicrobial pus samples were confirmed by VITEK-MS as *B. licheniformis* and*E. faecalis* as well as*E. coli* and *P. mirabilis* with a C.V of 99.9 %.The 23 human isolates included 11/23 (47.82 %) Gp and 12/23 (52.17 %) GN bacteria (Table 2).

Table 2. Distribution of bacterial isolates identified by VITEK-MS from pus and blood samples

Bacterial species	Animal pus(n= 35)		Neonatal blood(n= 23)		Total	C.V %
	No.	(%)	No.	(%)		
Gram positive cocci						
Staphylococcus aureus	6	(17.14)	4	(17.3)	10	99.9
Staphylococcus epidermidis	6	(17.14)	6	(26)	12	99.9
Staphylococcus xylosus	1	(2.85)	0	-	1	99.9
Enterococcus faecalis	2	(5.71)	1	(4.34)	3	99.9
S. dysagalactiae subspp dysagalactiae	2	(5.71)	0	0	2	50
Gram positive bacilli						99.9
Bacillus licheniformis	1	(2.85)	00		1	26.5
Lactobacillus rhamnosus	1	(2.85)	00		1	
Total GP bacteria	19	(54.28)	11	(47.82)	30	
Gram negative bacilli						
Escherichia coli	4	(11.42)	2	(8.69)	6	99.9
Klebsiellapneumonia	1	(2.85)	7	(30.43)	8	99.9
Proteus mirabilis	5	(14.28)	1	(4.34)	6	99.9
Pseudomonas aeruginosa	5	(14.28)	2	(8.69)	7	99.9
Delftiaacidovorans	1	(2.85)	0	0	1	99.9
Total GNbacteria	16	(45.7)	12(52.17)		28	
Total	35		23		58	

Culture versus VITEK-MS

The results obtained by VITEK-MS and routine culture methods were compared (Table 3).Twelvebacterial species were identified using VITEK-MS versus three only by conventional methods.*P. aeruginosa*was detected in 8 isolates by culture and 7 isolates by VITEK-MS. This misidentification was due *Delftiaacidovorans* were oxidase positive, pale colonies on MacConkey's and EMB agar.

Table 3. Discrepancy between culture and VITEK-MS results

VITEK-MS result	No.	Culture result	No.	Concordance VITEK-MS × culture				
				Concordant at Species level		Concordant at Genus level		NC
Gram positive cocci								
Staphylococcus aureus	10	*Staphylococcus aureus*	10	10/ 10	(100 %)			1
Staphylococcus epidermidis	12	CONS	13	12/12	(100 %)			
Staphylococcus xylosus	1		-	-		-		
S. dysagalactiaesppdysagalactiae	2	β hemolytic Streptococci	2			2/2	(100 %)	
Enterococcus faecalis	3	*Enterococcus spp.*	3			3/3	(100 %)	
Gram positive bacilli								
Bacillus licheniformis	1	*Bacillus spp*	1			1/1	(100 %)	
Lactobacillus rhamnosus	1	Non spore forming bacilli	1			1/1	(100 %)	
Gram negative bacilli								
Escherichia coli	6	*Escherichia coli*	6	6/6	(100 %)			
Klebsiella pneumonia	8	*Klebsiella spp.*	8			8/8	(100 %)	
Proteus mirabilis	6	*Proteus spp.*	6			6/6	(100 %)	1
Pseudomonas aeruginosa	7	*Pseudomonas aeruginosa*	8	7/8	(87.5 %)			2
Delftiaacidovorans	1		-					
Total	58		58	35		21		4

No., number of isolates; NC, not concordant

4. DISCUSSION

The gold standard for diagnosing sepsis is BC. The obtained result revealed a positive BC(s) in 66 % of animal pyogenic infections and 76.7 % of neonatal sepsis. It was higher than those reported by Lukacs & Schrag (2012),Afshari et al. (2012),Loonen et al. (2014)and Roland & Frank (2014)who estimated a positive BC(s) in neonatal sepsis as 40%, 60 %, 40% and 20%, respectively. Failure in bacterial isolation and negative culture may be due to incorrect sampling, inadequate specimen conservation, low microbial concentration in sample, non-culturable bacteria (Rampini et al., 2011), small sample volume, VBNC (Jiang et al., 2013), fastidious, slow growing bacteria, antibiotic pre-treatment (Loonen et al. 2014), intracellular pathogens (Cambau & Bauer 2015), toxin producing pathogens as *S. pyogenes*or *S. aureus* (Liang et al., 2013).

Only2 animal pus samples were polymicrobial, which is explained as in case of co-infections, culture may be biased towards the more readily cultured organism, yielding an incomplete clinical picture (Hasman et al., 2014). The polymicrobial nature of pyogenic infections may be

underestimated by routine culture, due to the fastidious nature of many organisms and the loss of viability during transport or from prior antibiotics (Sibley et al. 2012).As such, DNA from skin microbiota may have been introduced during the venipuncture of the skin. This could account for the abundance of *Staphylococcus* spp. (Rosenthal et al. 2011).*B. licheniformis* is reported to cause neonatal sepsis (Idelevich et al. 2013).It was found in one sample in a mixed culture, forming pleomorphic colonies with its satellite growth around colonies, with no hemolysis. Thesame result was reported by Idelevich et al.(2013).In recent years, MALDI-TOF MS has emerged as a novel method to identify pathogens causing sepsis via extracting acid-soluble microbial proteins in a size range of 2–20 kDa, which are then analyzed by MS to generate a "mass fingerprint." (Chun et al. 2015).It is suitable for species identification since the generated mass profile comes from ribosomal proteins, which aligns with taxonomic classification (Shitikov et al. 2012). It can be used for early bacterial identification in BCs. It surpassed the conventional methods in speed and accuracy (Foster, 2013). With respect to analyte ionization, MALDI is more sensitive than other ionization techniques, as the laser beam is focused on a small portion of the matrix, allowing efficient energy transfer and preventing destruction of the clinical sample. Moreover, the analytes are widely separated within the matrix, preventing ions clustering that can hamper analysis (Clark et al. 2013).Currently, MALDI-TOF applied directly to positive samples enables microbial identification in less than 1 hour, with a maximum identification rate of 97.3% of GN bacteria and 98.4% of GP bacteria (March-Rosselló et al. 2013).Numerous studies reported that it is a rapid, reliable and cost-effective method for bacterial identification. However, it has some lacunae: (i) proper identification is only when spectral database has information about specific genes, (ii) databases should be prepared locally for certain taxa as Streptococcus or Staphylococcus in which geographical variations lead to genotypic and phenotypicvariations (Benagli et al., 2011). The main weakness was its inability to identify all organisms in polymicrobial infections or in mixed cultures, as none or, at best, one single isolate could be identified(Leli et al. 2013).

5. CONCLUSION

Molecular assays particularly, MALDI-TOF MS significantly reduce the TAT, permitting rapid pathogen ID. Speeding up the TAT of positive BC(s) is crucial for accurate targeted antibiotic therapy that decreases the mortality rates and antibiotic resistant strains as well with the option to select more potent and less toxic antibiotics, leading to favorable clinical outcome.

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