Comparison of peptide nucleic acid fluorescence *in situ* hybridization assays with culture-based matrix-assisted laser desorption/ionization-time of flight mass spectrometry for the identification of bacteria and yeasts from blood cultures and cerebrospinal fluid cultures

A. Calderaro, M. Martinelli, F. Motta, S. Larini, M. C. Arcangeletti, M. C. Medici, C. Chezzi and F. De Conto

Unit of Microbiology and Virology, Department of Clinical and Experimental Medicine, Faculty of Medicine and Surgery, University of Parma, Parma, Italy

Abstract

Peptide nucleic acid fluorescence *in situ* hybridization (PNA FISH) is a molecular diagnostic tool for the rapid detection of pathogens directly from liquid media. The aim of this study was to prospectively evaluate PNA FISH assays in comparison with culture-based matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) identification, as a reference method, for both blood and cerebrospinal fluid (CSF) cultures, during a 1-year investigation. On the basis of the Gram stain microscopy results, four different PNA FISH commercially available assays were used ('*Staphylococcus aureus*/CNS', '*Enterococcus faecalis*/OE', 'GNR Traffic Light' and 'Yeasts Traffic Light' PNA FISH assays, AdvanDx). The four PNA FISH assays were applied to 956 positive blood cultures (921 for bacteria and 35 for yeasts) and 11 CSF cultures. Among the 921 blood samples positive for bacteria, PNA FISH gave concordant results with MALDI-TOF MS in 908/921 (98.64%) samples, showing an agreement of 99.4% in the case of monomicrobial infections. As regards yeasts, the PNA FISH assay showed a 100% agreement with the result obtained by MALDI-TOF MS. When PNA FISH assays were tested on the 11 CSF cultures, the results agreed with the reference method in all cases (100%). PNA FISH assays provided species identification at least one work-day before the MALDI-TOF MS culture-based identification. PNA FISH assays showed an excellent efficacy in the prompt identification of main pathogens, yielding a significant reduction in reporting time and leading to more appropriate patient management and therapy in cases of sepsis and severe infections.

Keywords: Bacteraemia, blood culture, cerebrospinal fluid, fungaemia, matrix-assisted laser desorption/ionization-time of flight, peptide nucleic acid fluorescence *in situ* hybridization

Original Submission: 30 September 2013; **Revised Submission:** 29 November 2013; **Accepted:** 29 November 2013

Editor: F. Allerberger

Article published online: 13 January 2014

Clin Microbiol Infect 2014; **20:** O468–O475

10.1111/1469-0691.12490

Corresponding author: A. Calderaro, Associate Professor of Microbiology and Clinical Microbiology, Faculty of Medicine and Surgery, Unit of Microbiology and Virology, Department of Clinical and Experimental Medicine, University of Parma, Viale A. Gramsci, 14 - 43126 Parma, Italy

E-mail: adriana.calderaro@unipr.it

Introduction

Rapid detection and identification of microorganisms in blood specimens have been advocated in order to shorten the turnaround time (TAT) for appropriate management of patients suffering from bacteraemia and fungaemia [1–3]. While the automated, continuously monitoring blood culture (BC) systems have reduced the delay in detecting the presence of blood-borne bacteria and fungi, identification of such microorganisms still requires subculturing the microorganism onto solid media [4,5]. At present, different techniques for microorganism identification from positive BCs, such as real-time PCR, DNA microarrays, matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) and peptide nucleic acid fluorescence *in situ* hybridization (PNA FISH), are available to shorten the TAT for the detection of Gram-positive cocci (GPC), Gram-negative rods (GNR) and *Candida* spp. [6–10].

Peptide nucleic acid fluorescence *in situ* hybridization is a molecular technology that rapidly identifies directly from liquid cultures the bacteria and yeasts most frequently responsible for bloodstream infections [7,11,12]. In recent years the MALDI-TOF MS has emerged as a powerful tool for microbial species identification after they have grown on solid media [13–17].

To date, studies utilizing PNA FISH assays have been reported for the detection of GPC, *Candida* spp. and GNR, separately [1,18–22] or in comparison with standard culture methods [7]. The aim of the present study was to prospectively compare PNA FISH assays with culture-based MALDI-TOF MS for the identification of bacteria and yeasts in positive BCs in a clinical routine setting. The second aim was to evaluate the identification accuracy of the PNA FISH assays from cerebrospinal fluids (CSF) inoculated into BC media.

Materials and Methods

Patients and clinical samples

From January to December 2012, 12 051 BCs collected from 4713 patients (2104 female and 2609 male; median age 68 years, range 1 day–101 years) with clinical suspicion of sepsis or other severe infections, such as endocarditis, pneumonia or meningitis, were routinely sent to the Bacteriology Section of the Unit of Clinical Microbiology of the University Hospital of Parma for the diagnosis of bacterial infections. Among the 1907 positive BCs (belonging to 1114 patients), 921 samples (belonging to 886 patients) were selected for the PNA FISH assays. The PNA FISH assays were generally performed on the first sample of each patient; they were eventually repeated in samples of the same patient following the first one if the Gram stain result was different from that of the first sample or in samples collected at least 15 days from the first one. This was in order to check whether the same or a new microorganism was involved in a second episode of sepsis.

If a set of BCs from a patient (aerobic plus anaerobic bottles) was positive, a single bottle was evaluated (the first that tested positive).

In the same period, 35 (belonging to 34 patients) of the 138 positive samples (belonging to 55 patients) were selected for *Candida* spp. PNA FISH assay, following the same criteria as for bacteria. The 138 positive samples were obtained from 3740 blood samples collected from 1194 patients (544 female and 650 male; median age 68 years, range 1 day–99 years) with clinical suspicion of fungaemia and routinely sent to the Mycology Section of the above-mentioned Unit of Clinical Microbiology.

Among the 410 samples of CSF (belonging to 197 patients; 88 female and 109 male; median age 49 years, range 1 day–91 years) routinely examined during 2012, a panel of 11 positive BC bottles inoculated with CSF (belonging to 11 patients) was selected, as described above for bacterial BCs, for the PNA FISH assays from 45 positive samples (belonging to 27 patients). These patients presented with hydrocephalus, clinical suspicion of central nervous system infection (meningitis and encephalitis) or polytrauma involving the central nervous system.

The samples analysed in this study were sent to the University Hospital of Parma for routine diagnostic purposes, and the laboratory diagnosis results were reported in the medical records of the patients as a diagnostic answer to a clinical suspicion of sepsis; ethical approval at the University Hospital of Parma is required only in cases in which the clinical samples are to be used for applications other than diagnosis.

Conventional blood/cerebrospinal fluid culture processing and reference method for identification from solid culture

Blood and CSF cultures were screened for microbial growth with the Bactec FX system (Becton Dickinson, Sparks, MD, USA), using BACTEC Plus Aerobic F, Plus Anaerobic F, Plus Ped and Mycosis IC/F bottles (Becton Dickinson) according to standard methods and to the manufacturer's recommendations [23]. As part of a routine culture set for bacterial detection, 8–10 mL of blood were inoculated into each aerobic and anaerobic bottle, and incubated at 37°C; for yeast detection Mycosis IC/F bottles were used. In the case of CSF samples, in parallel with conventional culture on solid media, an aliquot (1–3 mL) was inoculated into a Bactec Plus Ped bottle. When a bottle signalled positive by the Bactec FX instrument a Gram stain was performed and an aliquot was subcultured onto blood agar, MacConkey agar, Schaedler agar and/or Sabouraud dextrose agar with chloramphenicol (KIMA, Piove di Sacco-PD, Italy) and incubated at 37°C in the appropriate conditions according to bottle type and Gram staining result. After sufficient (at least two to three colonies) pure microbial growth was achieved (24–72 h), identification tests were performed by a Microflex LT- MALDI Biotyper mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany, supplied by Becton Dickinson, Italy) in the case of Gram-negative bacteria and by a VITEK MS mass spectrometer (bioMérieux, Marcy L'Etoile, France) in the case of Gram-positive bacteria and yeasts, according to the laboratory workflow.

Matrix-assisted laser desorption/ionization-time of flight mass spectrometry

Matrix-assisted laser desorption/ionization-time of flight mass spectrometry was performed according to the manufacturer's instructions. A single bacterial colony of fresh overnight

culture was smeared directly in duplicate onto a 'MSP-96 polished steel' target plate (Bruker Daltonics) or onto a polymeric target slide (VITEK MS-DS; bioMérieux) and covered with 1 μL of matrix, a saturated solution of α-cyano-4--hydroxy cinnamic acid (Bruker Daltonics) in 50% acetonitrile with 2.5% trifluoracetic acid (Sigma-Aldrich, Milan, Italy) for the MALDI Biotyper method or α-cyano-4-hydroxy cinnamic acid in acetonitrile/ethanol/water 1:1:1–3% trifluoracetic acid (bioMérieux) for the VITEK MS method. The plate was then left to dry at room temperature for 5 min. For yeasts, before applying the matrix solution, 1 μL of formic acid (VITEK MS-FA; bioMérieux) was added to the plate and air dried for 5 min.

For the Microflex LT mass spectrometer (Bruker Daltonics) system, in each plate *Escherichia coli* (Bruker Bacterial Test Standard, Bruker Daltonics) was used as a positive control. For MALDI-TOF VITEK MS, each plate was calibrated and validated with a control strain of *E. coli* (ATCC 8739) tested at the beginning and at the end of the use of each plate. For both Microflex LT and the VITEK MS system, the spectra were automatically obtained in the linear positive mode within a mass range from 2000 to 20 000 Da and analysed by the FlexControl software (version 3.3.63; Bruker Daltonics) and the Myla software (version 3.1.0–15; bioMérieux), respectively.

Peptide nucleic acid fluorescence *in situ* hybridization assays

On the basis of the Gram stain microscopy result (from the positive blood/CSF culture bottles), four different probe sets for PNA FISH (AdvanDx, Woburn, MA, USA) were used according to manufacturer's instructions: '*Staphylococcus aureus*/CNS PNA FISH' (afterwards named as SA) in the case of samples containing GPC in pairs or clusters for the identification and differentiation of *S. aureus* (appearing with a green fluorescence) and coagulase-negative staphylococci (CNS) (red), '*Enterococcus faecalis*/OE PNA FISH' (EF) in the case of GPC in chains identifying *E. faecalis* (green) and other enterococci (red), 'GNR Traffic Light PNA FISH' (GNTL) for GNR distinguishing *E. coli* (green), *Klebsiella pneumoniae* (yellow) and *Pseudomonas aeruginosa* (red) and 'Yeasts Traffic Light PNA FISH' (YTL) for yeasts distinguishing *Candida albicans*/*Candida parapsilosis* (green), *Candida tropicalis* (yellow) and *Candida glabrata*/*Candida krusei* (red).

Briefly, a drop of liquid culture placed on a microscope slide was fixed for 20 min at 55°C, hybridized with a drop of the specific PNA probe for 30 min at 55°C and washed for 30 min. Slides were examined at 100× on a fluorescent microscope equipped with a dual band filter. Probe-specific PNA FISH positive and negative control slides were obtained from the manufacturer and utilized for quality control each time the PNA FISH assay was performed.

A PNA FISH result was considered concordant or discordant with that obtained by culture-based MALDI-TOF MS, taking into account the species detectable by PNA probes. A negative result by PNA FISH was considered concordant with MALDI-TOF MS when the species identified was not included in the spectrum of those recognized by PNA probes.

Reference bacterial and yeast strains

To assess the detection limit of each PNA FISH assay, the reference microbial strains listed in Table 1 were prepared in physiological saline solution (bioMérieux) from fresh agar culture and adjusted to defined organism concentrations by plate colony counting. Each PNA FISH probe set was tested in duplicate with the corresponding microorganism suspensions containing from 10^7 to 10^4 CFU/mL, which included the detection limit declared by the manufacturer (10^5 CFU/mL). A panel of 16 microbial strains served as negative controls in the specificity testing.

Results

Analytical sensitivity and specificity of peptide nucleic acid fluorescence *in situ* hybridization

The detection limits for SA, EF, GNTL and YTL assays were determined to be 10^6 CFU/mL by serial dilutions of positive cultures.

The specificity of the probes used in the PNA FISH assays was assessed by demonstrating the absence of fluorescent signal from all strains different to those recognized by the molecular assay, except from *Shigella sonnei*, which cross-reacted to create a green signal in the GNTL PNA FISH assay. Conversely, a fluorescent signal was obtained as expected when the specific probes were used on the corresponding reference strains.

Evaluation of peptide nucleic acid fluorescence *in situ* hybridization assays on blood cultures

Among the 921 positive blood samples submitted to PNA FISH assays for bacteria detection, 860 samples (93.4%) contained only one microorganism and 61 (6.6%) had mixed infections as detected by MALDI-TOF MS applied to conventional cultures. Out of the 860 samples with monomicrobial infections, 315 samples (36.6%) contained GNR and 545 (63.4%) GPC (Table 2). The identification obtained by the PNA FISH assays was concordant, with regard to the species recognized by PNA probes, with that obtained by MALDI-TOF MS from conventional cultures in all samples, except in five cases (99.4%): three samples containing GNR and negative to GNTL and two samples containing GPC and positive for CNS by SA.

TABLE 1. Reference bacterial and yeast strains used for determination of the PNA FISH detection limit and specificity testing

Micro organisms	Origin	Used for detection limit	Used for specificity testing
Acinetobacter haemolyticus	NEQAS 8539	–	GNTL
Candida albicans	ATCC 24433	YTL	–
Candida dubliniensis	NEQAS 9463	–	YTL
Candida glabrata	NEQAS 0142	YTL	–
Candida kefyr	ATCC Y050	YTL	YTL
Candida krusei	ATCC 62589	YTL	–
Candida lusitaniae	NEQAS 8863	–	YTL
Candida parapsilosis	ATCC 22019	YTL	–
Candida tropicalis	ATCC 750	YTL	–
Enterococcus casseliflavus	ATCC 700327	EF	SA
Enterococcus faecalis	ATCC 51299	EF	SA
Escherichia coli	ATCC 25922	GNTL	–
Haemophilus influenzae	NEQAS 9531	–	GNTL
Klebsiella pneumoniae	ATCC 700603	GNTL	–
Listeria monocytogenes	NEQAS 9483	–	GNTL, SA, EF
Neisseria gonorrhoeae	NEQAS 9928	–	GNTL
Pseudomonas aeruginosa	ATCC 27853	GNTL	–
Saccharomyces cerevisiae	NEQAS 9005	–	YTL
Serratia marcescens	NEQAS 8859	–	GNTL
Shigella sonnei	ATCC 25931	–	GNTL
Staphylococcus aureus	ATCC 29213	SA	EF
Staphylococcus epidermidis	Clinical isolate[a]	SA	–
Streptococcus agalactiae	ATCC 12386	–	SA, EF
Streptococcus pneumoniae	NEQAS 8886	–	SA, EF
Trichosporon mucoides	ATCC 204096	–	YTL

ATCC, American Type Culture Collection; NEQAS, National External Quality Assessment Service, United Kingdom; GNTL, 'GNR Traffic Light PNA FISH'; PNA FISH, peptide nucleic acid fluorescence *in situ* hybridization; YTL, 'Yeasts Traffic Light PNA FISH'; EF, '*E. faecalis*/OE PNA FISH'; SA, '*S. aureus*/CNS PNA FISH'.
[a]Identified by conventional biochemical profile using the Vitek 2 System (bioMérieux).

As regards the three GNTL-negative cases, two were identified as *P. aeruginosa* and one as *K. pneumoniae*. In the other two cases a red fluorescence, referring to CNS, was produced with isolates of *Micrococcus luteus*.

In 53 out of the 61 samples (87%) with mixed infections (Table 3), PNA FISH assays gave concordant results with those obtained by MALDI-TOF MS identification for the species recognized by PNA probes (10 GNR, 25 GPC and 18 GNR + GPC). In the remaining eight samples (13%), PNA FISH failed to reveal one of the species involved (two *K. pneumoniae*, one *E. coli* and five *Staphylococcus epidermidis*).

The molecular identification obtained by YTL for the 35 positive blood samples totally agreed with the culture-based MALDI-TOF MS identification (Table 4) with regard to the species recognized by the PNA probes. In particular, the 26 samples positive for *C. albicans*/*C. parapsilosis* by PNA FISH were identified as *C. albicans* in 22 cases (in one case in association with *C. glabrata*) and as *C. parapsilosis* in four cases, the eight samples positive for *C. glabrata*/*C. krusei* were *C. glabrata* in six cases (in one case in association with *C. albicans*) and *C. krusei* in two cases, and the sample positive for *C. tropicalis* was confirmed by MALDI-TOF MS. In the last case, which was YTL-negative, MALDI-TOF MS identified *Candida lusitaniae*, a species not included in the panel of PNA probes.

Evaluation of peptide nucleic acid fluorescence *in situ* hybridization assay on cerebrospinal fluid cultures

Nine out of the 11 samples of CSF submitted to PNA FISH gave positive results: six CNS, one *S. aureus*, one *K. pneumoniae* and one *E. coli*. All PNA FISH results were in agreement with those obtained by MALDI-TOF MS identification. In the remaining two samples, the PNA FISH assays gave negative results as expected because the conventional culture method identified one isolate of *Streptococcus agalactiae* and one of *Streptococcus parasanguinis*, respectively.

Discussion

The PNA FISH technology, commercially available as a diagnostic assay, represents a rapid (TAT 90 min) and reliable technique for the identification of the most frequent bacteria and yeasts responsible for sepsis and provides faster identification than methods based on conventional culture.

In the present study, for the first time to our knowledge, the results of the PNA FISH assays are prospectively compared with those obtained by the MALDI-TOF MS identification applied to conventional culture during a 1-year study in a large tertiary-care setting. In our laboratory the workflow, the load and the type of activity requires two MALDI-TOF MS instruments, which complement each other in terms of analytical efficiency for the different groups of microrganisms [24,25].

Regarding the performance of PNA FISH assays, the overall identification accuracy was 98.64% (ranging from 98.58% for bacteria to 100% for yeasts), increasing to 99.4% when considering monomicrobial infections.

Discordant results were observed in five samples with monomicrobial infections (three GNR and two GPC) and in eight samples with mixed infections. The production of a

TABLE 2. Peptide nucleic acid fluorescence *in situ* hybridization (PNA FISH) results for 860 positive blood samples with monomicrobial infections

Gram stain microscopy	Conventional identification by matrix-assisted laser desorption/ionization-time of flight	PNA FISH assays results				
		Escherichia coli	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	Negative	Total
Gram-negative rods (n = 315)		177	44	18	76	315
	E. coli (ESBL n = 10)	177				177
	K. pneumoniae (ESBL n = 3)		44		1	45
	P. aeruginosa			18	2	20
	Acinetobacter baumannii, A. junii, A. calcoaceticus, A. lwofii				11	11
	Aeromonas caviae				1	1
	Bacteroides distasonis, B. fragilis, B. ovatus				8	8
	Campylobacter coli, C. jejuni				2	2
	Citrobacter freundii, C. koseri				3	3
	Clostridium symbiosum[a]				1	1
	Enterobacter aerogenes, E. agglomerans, E. cloacae				16	16
	Fusobacterium necrophorum				1	1
	Klebsiella oxytoca				6	6
	Proteus mirabilis, P. vulgaris				7	7
	Pseudomonas luteola, P. oryzihabitans, P. putida, P. putrefaciens, Pseudomonas sp.				5	5
	Salmonella enterica group B, *S.* Panama, *S.* Paratyphi A				6	6
	Serratia marcescens				4	4
	Sphingobacterium spiritivorum				1	1
	Sphingomonas paucimobilis				1	1
		Staphylococcus aureus	**CNS**	***Enterococcus faecalis***	***Enterococcus* sp.**	**Negative**	**Total**
Gram-positive cocci (n = 545)		108	351	27	15	44	545
	S. aureus (MRSA n = 9)	108					108
	E. faecalis			27			27
	Enterococcus faecium				15		15
	Leuconostoc pseudomesenteroides					1	1
	Micrococcus luteus		2			5	7
	Peptostreptococcus parvulus					1	1
	Staphylococcus auricularis		2				2
	Staphylococcus capitis		32				32
	Staphylococcus epidermidis		181				181
	Staphylococcus haemolyticus		28				28
	Staphylococcus hominis		93				93
	Staphylococcus saccharolyticus		3				3
	Staphylococcus saprophyticus		2				2
	Staphylococcus schleiferi		1				1
	Staphylococcus warneri		4				4
	Staphylococcus xylosus		3				3
	Streptococcus agalactiae					7	7
	Streptococcus anginosus					2	2
	Streptococcus constellatus					2	2
	Streptococcus gallolyticus					3	3
	Streptococcus gordonii					2	2
	Streptococcus mitis, S. infantarius, S. salivarius					9	9
	Streptococcus pneumoniae					11	11
	Streptococcus pyogenes					1	1

ESBL (extended spectrum beta-lactamase) and MRSA (methicillin-resistant *Staphylococcus aureus*) were identified by antimicrobial susceptibility testing.
CNS, coagulase-negative staphylococci.
[a]Misidentified by Gram stain.

bacterial capsule in two strains (1 *K. pneumoniae* and 1 *P. aeruginosa*) and point mutations in the organism rRNA sequence could explain the negative results by GNTL [1,22]. The two positive samples for CNS by SA contained *M. luteus*, which was even misidentified as *S. aureus* by other authors [26]; an explanation could be the strict correlation between *M. luteus* and *Staphylococcus* spp. For these discordant results MALDI-TOF MS identification was confirmed by conventional biochemical methods (data not shown).

The failure to correctly identify the species contained in the eight samples with mixed infections may be due to the lower detection limit of the culture-based method compared with that of the PNA FISH. In our hands, PNA FISH demonstrated a detection limit of 10^6 CFU/mL, which is consistent with the analytical sensitivity of the slide-based staining techniques. The PNA FISH assays allowed us to identify bacteria and yeasts 1.3 days before the MALDI-TOF MS identification and 2 days earlier in the case of polymicrobial infections.

In the light of the good results obtained using the PNA FISH assays on positive BCs, we extended this assay to CSF cultures. The results of the PNA FISH assays totally agreed with those obtained by MALDI-TOF MS from conventional cultures, providing a useful identification with a TAT reduction of 1.1 days. The use of PNA FISH with clinical samples other than blood seems a promising approach, especially for those

TABLE 3. Peptide nucleic acid fluorescence *in situ* hybridization (PNA FISH) results for 61 positive blood samples with polymicrobial infections

Gram stain microscopy	Conventional identification by matrix-assisted laser desorption/ionization-time of flight	PNA FISH assay results	
		Expected	Not expected
Gram-negative rods (n = 13)		10	3
	Enterobacter cloacae+ *Klebsiella oxytoca*+ *Proteus mirabilis*	1	
	Escherichia coli+ *Aeromonas caviae*	1	
	E. coli+*E. cloacae*	2	
	E. coli+*Klebsiella pneumoniae*	2	2[a]
	E. coli+*Pseudomonas aeruginosa*+*Bacteroides fragilis*	1	
	K. pneumoniae+*Aeromonas hydrophila*		1[b]
	K. pneumoniae+*B. fragilis*	1	
	K. pneumoniae+*Citrobacter koseri*	1	
	K. pneumoniae+*K. oxytoca*+*E. cloacae*	1	
Gram-positive cocci (n = 30)		25	5
	Streptococcus parasanguinis+*Actinomyces viscosus*	1	
	Streptococcus mitis+ *Lactobacillus rhamnosus*	1	
	Staphylococcus hominis+ *Streptococcus pneumoniae*	1	
	Staphylococcus epidermidis+ *Staphylococcus xylosus*	1	
	S. epidermidis+*Staphylococcus schleiferi*	1	
	S. epidermidis+*S. hominis*	3	
	S. epidermidis+*Staphylococcus cohnii* ssp. *urealyticus*	1	
	S. epidermidis+*Staphylococcus capitis*	2	
	S. epidermidis+*Streptococcus anginosus*		1[c]
	S. epidermidis+*Pediococcus acidilactici*	1	
	Staphylococcus aureus+*Staphylococcus haemolyticus*	1	
	S. aureus+*Streptococcus agalactiae*	1	
	S. aureus (MRSA)+*S. capitis*	1	
	Enterococcus faecium+*S. haemolyticus*	1	
	E. faecium+*S. epidermidis*	2	
	Enterococcus faecalis+*S. hominis*	1	
	E. faecalis+*S. haemolyticus*	1	
	E. faecalis+*S. epidermidis*	1	4[c]
	E. faecalis+*S. aureus*	2	
	E. faecalis+*E. faecium*+*Staphylococcus caprae*	1	
	E. faecalis+*E. faecium*	1	
Gram-negative rods and Gram-positive cocci (n = 18)		18	0
	E. coli+*E. faecalis*+*C. koseri*	1	
	E. faecalis+*K. pneumoniae*	1	
	E. faecium+*E. coli*	1	
	E. coli+*E. faecium*+*P. mirabilis*+*K. oxytoca*	1	
	S. aureus+*E. coli*	1	
	K. pneumoniae+ *S. epidermidis*	2	
	S. epidermidis+*Salmonella enterica* group B	1	
	S. epidermidis+*Salmonella* Typhimurium	1	
	S. epidermidis+*P. mirabilis*	1	
	S. epidermidis+ *Stenotrophomonas maltophilia*	1	
	E. coli+ *S. epidermidis*	1	
	E. faecalis+*E. cloacae*	1	
	E. faecium+*E. coli* (ESBL)	1	
	E. coli+ *S. mitis*	1	
	E. coli+*K. pneumoniae*+ *Streptococcus gallolyticus*	1	
	S. epidermidis+ *Acinetobacter baumannii*	1	
	K. pneumoniae+ *S. aureus*	1	
Total		53	8

[a]One *K. pneumoniae* and one *E. coli* were not identified by PNA FISH.
[b]*K. pneumoniae* was not identified by PNA FISH.
[c]*S. epidermidis* was not identified by PNA FISH; ESBL (extended spectrum beta-lactamase) and MRSA (methicillin-resistant *S. aureus*) were identified by antimicrobial susceptibility testing.

TABLE 4. Peptide nucleic acid fluorescence *in situ* hybridization (PNA FISH) results for 35 blood samples positive for yeasts

Gram stain microscopy	Conventional identification by matrix-assisted laser desorption/ionization-time of flight mass spectrometry	PNA FISH assay results				
		Candida albicans/ *Candida parapsilosis*	*Candida glabrata*/ *Candida krusei*	*Candida tropicalis*	Negative	Total
Yeasts (n = 35)		26	8	1	1	36
	C. albicans	21				21
	C. parapsilosis	4				4
	C. glabrata		5			5
	C. krusei		2			2
	C. tropicalis			1		1
	Candida lusitaniae				1	1
	C. albicans + *C. glabrata*	1[a]	1[a]			2[a]

[a]One was a mixed infection, *C. albicans* and *C. glabrata.*

samples, submitted in parallel with conventional solid culture and automated liquid culture [27], for which only the latter method gives a positive result.

These data demonstrate that the use of PNA FISH assays in tertiary-care hospitals may improve the laboratory diagnosis of sepsis by identifying or by ruling out certain infections in a shorter period of time as compared with culture-based MALDI-TOF MS identification.

With a further reduction in the TAT of the new-generation PNA FISH technology (QuickFISH assays, based on a 15-min hybridization without washing/mounting steps) and extension of the panel of probes, including other species or resistance markers, such as those for MRSA and ESBL *Enterobacteriaceae*, PNA FISH will become a very useful tool in the diagnosis of bloodstream infections [8,26]. Although innovative applications of MALDI-TOF MS have been proposed to identify nearly all the bacteria and yeasts involved in human infections [6,9], most of the recovery protocols for the direct identification by MALDI-TOF are cumbersome and suffer from variable identification rates and concordance with conventional identification methods [6,9,28–30].

Even if MALDI-TOF MS directly applied to positive blood bottles is cheaper than PNA FISH (about €3 vs. €40, VAT excluded), it requires expensive instrumentation, a better evaluation in clinical settings and is not useful in the case of mixed infections. For these reasons, although PNA FISH has a limited spectrum of recognized species, it remains at present a quick and easy-to-perform method, which requires only basic laboratory equipment, and the results of this study suggest its usefulness in the case of mixed infections.

The reduction in time to result represents one of the main advantages of the PNA FISH technology, which could affect antibiotic and antifungal utilization, allowing for more targeted and shorter therapy [7], increased benefits for patients and overall reduction of healthcare costs.

Acknowledgements

We thank Dr Emanuela Catellani and Dr Laura Gaita for technical assistance.

Author Contributions

AC, MM and FM: designed the experiments. MM and FM: performed the experiments. AC, MM, FM, SL, MCA, MCM, FDC and CC: analysed the data. MM and FM: wrote the manuscript. AC and CC: conceived and supervised the project.

Transparency Declaration

This study was supported by the Ministry of University and Scientific Research grant FIL (Parma, Italy). All authors declare no conflicts of interest.

References

1. Shepard JR, Addison RM, Alexander BD *et al.* Multicenter evaluation of the *Candida albicans*/*Candida glabrata* peptide nucleic acid fluorescent *in situ* hybridization method for simultaneous dual color identification of *C. albicans* and *C. glabrata* directly from blood culture bottles. *J Clin Microbiol* 2008; 46: 50–55.
2. Trenholme GM, Kaplan RL, Karakusis PH *et al.* Clinical impact of rapid identification and susceptibility testing of bacterial blood culture isolates. *J Clin Microbiol* 1989; 27: 1342–1345.
3. Weinstein MP, Towns ML, Quartey SM *et al.* The clinical significance of positive blood cultures in the 1990s: a prospective comprehensive evaluation of the microbiology, epidemiology, and outcome of bacteraemia and fungaemia in adults. *Clin Infect Dis* 1997; 24: 584–602.
4. Mylotte JM, Tayara A. Blood culture: clinical aspects and controversies. *Eur J Clin Microbiol Infect Dis* 2000; 19: 157–163.
5. Wilson ML, Mitchell M, Morris AJ *et al.*, eds. *Principles and procedures for blood cultures; approved guideline*. CLSI document M47-A. Wayne, PA: Clinical and Laboratory Standards Institute, 2007.
6. Christner M, Rohde H, Wolters M, Sobottka I, Wegscheider K, Aepfelbacher M. Rapid identification of bacteria from positive blood culture bottles by use of matrix-assisted laser desorption – ionization time of flight mass spectrometry fingerprinting. *J Clin Microbiol* 2010; 48: 1584–1591.
7. Harris DM, Hata DJ. Rapid identification of bacteria and *Candida*, using PNA FISH from blood and peritoneal fluid cultures: a retrospective clinical study. *Ann Clin Microbiol Antimicrob* 2013; 12: 1–9.
8. Peters RPH, Savelkoul PHM, Simoons-Smit AM, Danner SA, Vandenbroucke-Grauls CMJE, van Agtmael MA. Faster identification of pathogens in positive blood cultures by fluorescence *in situ* hybridization in routine practice. *J Clin Microbiol* 2006; 44: 119–123.
9. Spanu T, Posteraro B, Fiori B *et al.* Direct MALDI-TOF mass spectrometry assay of blood culture broths for rapid identification of *Candida* species causing bloodstream infection: an observational study in two large microbiology laboratories. *J Clin Microbiol* 2012; 50: 176–179.
10. Wellinghausen N, Wirths B, Franz AR, Karolyi L, Marre R, Reischl U. Algorithm for the identification of bacterial pathogens in positive blood cultures by real-time LightCycler polymerase chain reaction (PCR) with sequence-specific probes. *Diagn Microbiol Infect Dis* 2004; 48: 229–241.
11. Forrest GN, Roghmann MC, Toombs LS *et al.* Peptide nucleic acid fluorescent *in situ* hybridization for hospital-acquired enterococcal bacteraemia: delivering earlier effective antimicrobial therapy. *Antimicrob Agents Chemother* 2008; 52: 3558–3563.
12. Sogaard M, Hansen DS, Fiandaca MJ, Stender H, Schonheyder HC. Peptide nucleic acid fluorescence *in situ* hybridization for rapid detection of *Klebsiella pneumoniae* from positive blood cultures. *J Med Microbiol* 2007; 56: 914–917.
13. Calderaro A, Piccolo G, Montecchini S *et al.* MALDI-TOF MS analysis of human and animal *Brachyspira* species and benefits of database extension. *J Proteomics* 2013; 78: 273–280.
14. Cherkaoui A, Hibbs J, Emonet S *et al.* Comparison of two matrix-assisted laser desorption ionization-time of flight mass

spectrometry methods with conventional phenotypic identification for routine identification of bacteria to species level. *J Clin Microbiol* 2010; 48: 1169–1175.
15. Drancourt M. Detection of microorganisms in blood specimens using matrix-assisted laser desorption/ionization – time of flight mass spectrometry: a review. *Clin Microbiol Infect* 2010; 16: 1620–1625.
16. Seng P, Drancourt M, Gourlet F *et al.* Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption/ionization – time of flight mass spectrometry. *Clin Infect Dis* 2009; 49: 543–551.
17. van Veen SQ, Claas EC, Kuijper EJ. High throughput identification of bacteria and yeasts by matrix-assisted laser desorption/ionization – time of flight mass spectrometry in conventional medical microbiology laboratories. *J Clin Microbiol* 2010; 48: 900–907.
18. Forrest GN, Mehta S, Weekes E, Lincalis DP, Johnson JK, Venezia RA. Impact of rapid *in situ* hybridization testing on coagulase-negative staphylococci positive blood cultures. *J Antimicrob Chemother* 2006; 58: 154–158.
19. Ibrahim EH, Sherman G, Ward S, Fraser VJ, Kollef MH. The influence of inadeguate antimicrobial treatment of blood stream infections on patient outcomes in the ICU setting. *Chest* 2000; 118: 146–155.
20. Morgan M, Marlowe E, Della-Latta P *et al.* Multicenter evaluation of a new shortened peptide nucleic acid fluorescence *in situ* hybridization procedure for species identification of select Gram-negative bacilli from blood cultures. *J Clin Microbiol* 2010; 48: 2268–2270.
21. Reller ME, Mallonee AB, Kwiatkowski NP, Merz WG. Use of peptide nucleic acid-fluorescence *in situ* hybridization for definitive, rapid identification of five common *Candida* species. *J Clin Microbiol* 2007; 45: 3802–3803.
22. Rigby S, Procop GW, Haase G *et al.* Fluorescence *in situ* hybridization with peptide nucleic acid probes for rapid identification of *Candida albicans* directly from blood culture bottles. *J Clin Microbiol* 2002; 40: 2182–2186.
23. Baron EJ, Weinstein MP, Dunne WM, Yagupsky P, Welch DF, Wilson DM. *Cumitech 1C: blood Cultures IV*. Coordinating editor Baron EJ. Washington D.C.: ASM Press, 2005.
24. Martiny D, Busson L, Wybo I, El Haj RA, Dediste A, Vandenberg O. Comparison of the Microflex LT and Vitek MS systems for routine identification of bacteria by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J Clin Microbiol* 2012; 50: 1313–1325.
25. Kärpänoja P, Harju I, Rantakokko-Jalava K, Haanperä M, Sarkkinen H. Evaluation of two matrix-assisted laser desorption ionization-time of flight mass spectrometry systems for identification of viridians group streptococci. *Eur J Clin Microbiol Infect Dis* 2013, doi: 10.1007/s10096-013-2012-8. [Epub ahead of print].
26. Deck MK, Anderson ES, Buckner RJ *et al.* Multicenter evaluation of the *Staphylococcus Quick*FISH method for simultaneous identification of *Staphylococcus aureus* and coagulase-negative staphylococci directly from blood culture bottles in less than 30 minutes. *J Clin Microbiol* 2012; 50: 1994–1998.
27. Akcam FZ, Yayli G, Uskun E, Kaya O, Demir C. Evaluation of the Bactec microbial detection system for culturing miscellaneous sterile body fluids. *Res Microbiol* 2006; 57: 433–436.
28. Lagacé-Wiens PRS, Adam HJ, Karlowsky JA *et al.* Identification of blood culture isolates directly from positive blood cultures by use of matrix-assisted laser desorption ionization – time of flight mass spectrometry and a commercial extraction system: analysis of performance, cost, and turnaround time. *J Clin Microbiol* 2012; 50: 3324–3328.
29. Leli C, Cenci E, Cardaccia A *et al.* Rapid identification of bacterial and fungal pathogens from positive blood cultures by MALDI-TOF MS. *Int J Med Microbiol* 2013; 303: 205–209.
30. Schubert S, Weinert K, Wagner C *et al.* Novel, improved sample preparation for rapid, direct identification from positive blood cultures using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. *J Mol Diagn* 2011; 13: 701–706.