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ORIGINAL ARTICLE

Direct identification of major Gram-negative pathogens in respiratory specimens by respiFISH® HAP Gram (−) Panel, a beacon-based FISH methodology

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Abstract Rapid detection of microorganisms in respiratory specimens is of paramount importance to drive the proper antibiotic regimen to prevent complications and transmission of infections. In the present study, the respiFISH® HAP Gram (−) Panel (miacom diagnostics GmbH, Duesseldorf, Germany) for the etiological diagnosis of hospital-acquired pneumonia was compared with the traditional culture method for the detection of major Gram-negative pathogens in respiratory specimens. respiFISH® combined the classical fluorescence in situ hybridization (FISH) technology with fluorescence-labeled DNA molecular beacons as probes. From September 2011 to January 2012, 165 samples were analyzed: the sensitivity and specificity were 94.39 and 87.93 %, respectively. Only six pathogens (3.6 %) were not identified with respiFISH®, while seven specimens (3 %) provided false-positive results. This beacon-based identification shortens the time to result by at least one work day, providing species-level identification within half an hour. Considering the high sensitivity and specificity and the significant time saving, the introduction of bbFISH® assays could effectively complement traditional systems in microbiology laboratories.

Abbreviations

bbFISH®	Beacon-based fluorescence in situ hybridization
HAP	Hospital-acquired pneumonia
VAP	Ventilation-acquired pneumonia
TAT	Total turn-around time
CNA	Columbia nalidixic acid agar
BAL	Bronchoalveolar lavage
PPV	Positive predictive value
NPV	Negative predictive value
PCR	Polymerase chain reaction
IC	Interval confidence

Introduction

Lower respiratory tract infections represent a very common clinical occurrence, either in community or in nosocomial sets. Microorganisms can easily invade the respiratory tree in case of a temporary failure of mechanical or immunological barriers, as happens in hospital-acquired pneumonia (HAP) and ventilation-acquired pneumonia (VAP).

The relative prevalence of specific pathogens responsible for HAP/VAP vary considerably, depending on the characteristics of the patient population, the duration of hospitalization and mechanical ventilation prior to the onset of pneumonia, the exposure to antibiotic therapy, and the methods and criteria used for diagnosis [1, 2].

In a review published in 2002, Chastre and Fagon compared data from 24 microbiology reports about bronchoscopic diagnostic methods used to confirm 1689 episodes of VAP. Overall, aerobic Gram-negative bacilli represented 58 % of

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isolated pathogens and Gram-positive cocci made up 35 % [3]. These percentages are largely confirmed by a review published in May, 2013 [4].

An important feature of the microbiology of HAP/VAP is that, in many instances, it is a polymicrobial infection. In order to ensure adequate antibiotic therapy, the empiric antibiotic regimen should include multiple agents with a broad spectrum of activity. However, unnecessarily broad antibiotic coverage can promote the further development of resistant bacterial strains, higher rates of antibiotic-related complications, and increased costs, mainly attributable to the long duration of stay.

The use of molecular methods can help in the rapid pathogens identification involved in respiratory infections. This appears quite advantageous, even though the results of molecular assays have to be confirmed by conventional culture methods and antimicrobial susceptibility tests will only be available after culture [5].

The fluorescence in situ hybridization (FISH) technique is based on fluorescently labeled oligonucleotide probes that complementarily bind to specific target sequences in the ribosomal RNA of bacteria, yeasts, or other organisms. Target sequences are naturally present in bacteria at a concentration high enough to enable visual detection of the specific fluorescent signal. FISH applies to the specimens without subculturing and can directly detect those organisms that can be difficult or time consuming to detect with traditional culture methods, especially when more than one species is present in the sample, as in the case of a polymicrobial HAP/VAP [6]. FISH as well as bbFISH® technologies were soon introduced to medical microbiology and have been used ever since in various fields of diagnostics of human infectious diseases, with emphasis on situations when a speedy identification is crucial or the pathogen is difficult to culture: sepsis, meningitis, endocarditis, respiratory tract infections, especially those of cystic fibrosis patients, screening for intrapartum *Streptococcus agalactiae* carriage [7–9].

respiFISH® HAP Gram (−) Panel, made by miacom diagnostics GmbH (Duesseldorf, Germany), combines the classical FISH technology with fluorescently labeled DNA molecular beacons as probes, to develop a simple procedure known as the beacon-based FISH (bbFISH®) technology [10, 11].

The panel is able to detect the most common Gram-negative pathogens and it appears to be a useful and promising innovation among new diagnostic tools. A panel for Gram-positive pathogens has very recently been approved for in vitro diagnostics (IVD) and European certification (CE).

Objectives

The aim of this study was to assess the possible benefits of this molecular technology for routine practice.

The study was conducted by comparing the total turnaround time (TAT) of both the bbFISH® test and a conventional identification assay.

Materials and methods

Specimens

A total of 165 respiratory samples were included in this study; 85 were sputa, 79 bronchial secretions (75 bronchial aspirates and four bronchial lavages), and one tracheal aspirate. From September 2011 to January 2012, samples were delivered to the Microbiology Laboratory of Foundation Polyclinic of Tor Vergata in Rome, Italy, and to the Microbiology Laboratory of University Polyclinic G.B. Rossi, Verona, Italy.

A minority of samples (6.1 %) came from outpatients, monitored as previously hospitalized; the great majority (93.9 %) came from inpatients: 60 from the intensive care unit and emergency (36.4 %), 30 from internal medicine (18.2 %), 22 from the respiratory disease unit (13.3 %), 16 from the infectious disease unit (9.7 %), ten from the transplantation, hematology, and oncology units (6.1 %), seven from rheumatology (4.2 %), six from surgeries (3.6 %), and four from the pediatric unit (2.4 %). All the patients were selected as hospitalized for a minimum of 3 days.

Conventional microbiological identification

In order to fluidize mucous specimens, Sputasol buffer solution was used (Oxoid; Basingstoke, UK). The Sputasol was prepared according to the manufacturer's instructions. Portions of sputum or bronchial secretions were diluted 1:1 (v/v) in this buffer, mixed by vortex, and shaken for 15′ at room temperature.

20 μl of the so-treated samples were seeded on five different agar media plates: CNA agar plus optochin disk, MacConkey agar, mannitol salt agar, chocolate agar plus bacitracin disk, and Sabouraud dextrose agar.

Bronchial lavages (BAL) were centrifuged at 3750 g for 20′ and an aliquot of the pellet was streaked on the surface of the same sets of the above-reported agar plate media. Tracheal tube lavage, as well as tracheal secretion, were treated as described for BAL. Cultures were incubated at 37 °C for 24 h in aerobic or micro-aerobic conditions (CNA and chocolate agar plates). At the end of incubation, they were examined for the presence of significant pathogens, and, in case of negativity, reincubated for up to 5 days. All samples underwent microscopic examinations. After the fluidization and/or the centrifugation, 30 μl of each specimen were spread onto a slide surface and air dried. The slides were colored using a Gram stainer PREVI Color (bioMérieux; Marcy l'Etoile, France), according to the manufacturer's instructions. The

specimens were microscopically evaluated according to Bartlett's score [12].

When significant pathogens were grown, fresh isolates were identified using GN/GP/NH cards from the VITEK 2® system (bioMérieux, Marcy l'Etoile, France).

bbFISH® assay

In this study, the Gram-negative panel (respiFISH® HAP Gram (−) Panel) was used. Tests were run directly on the specimens.

An aliquot from sputum or bronchial secretions was diluted in Clinical Sample Buffer (included in the kit), applied, dried, and fixed onto an eight-field glass slide placed on a hotplate pre-warmed to 52±1 °C. Samples were treated with enzymatic lysis solution at 52±1 °C until they were completely dried: this step takes about 10 min. After rinsing in absolute ethanol, hybridization with DNA molecular beacon probes was carried out in a hotplate hybridization chamber at 52±1 °C, followed by incubation in a Stop Solution bath for 1 min. Slides were dried, covered with mounting medium, and evaluated under a fluorescence microscope. Two filter sets were required; one detected the probes labeled with ATTO550 (red channel, absorption maximum of 554 nm/emission maximum of 576 nm), and the other filter set detected the probes labeled with FAM (green channel, absorption maximum of 494 nm/emission maximum of 520 nm). The first field of the slide serves as an intrinsic control for the procedure: it contains a probe that detects most eubacteria, giving a positive signal only in the red channel. When turning to the green channel, no fluorescence should be visible. In the remaining fields, there may be pairs of probes, labeled with either FAM or ATTO 550, giving either a red or green fluorescent signal when the specific target is encountered. If a specific target is not encountered, the unbound probes are returned into the initial closed conformation and no fluorescent signal is generated. The hands-on time of the bbFISH® assay was approximately 30 min.

TAT evaluation

The laboratory analytical TAT is a reliable performance indicator, which measures the efficiency of a laboratory in producing results [13]. TAT is commonly defined as the time elapsed between ordering a laboratory test and reporting of the result. In this study, TAT was considered as the time elapsed between sample arrival into the laboratory and the availability of results by either the bbFISH® assay or the conventional identification assay (VITEK 2®) [14–17].

In this study, the TAT was evaluated as the total time required to achieve pathogens identification for the culturing method (the time elapsed from the delivery of the specimens to the laboratory until the identification of microorganisms) or the time to obtain a final result by the bbFISH® assay (the time elapsed from the delivery of the specimens to the laboratory until the time to conclude the bbFISH® assay).

All statistical analyses were performed using MedCalc® software.

Results

A total of 165 samples were tested by traditional culture and bbFISH® methods to identify pathogens. By culturing assay, nine specimens resulted negative and 156 were positive. Among the positive specimens, 42 showed mixed cultures, including Gram-positive pathogens, saprophytes, and resident *Streptococcus* spp. The agreement between bbFISH® and conventional culture results was 92.13 %, with 152 samples concordant for both methods, while 13 (7.87 %) were not concordant. In this study, the conventional routine methods (VITEK 2®) have been considered as the gold standard identification assay, thereby the deviations between results have not been rechecked by polymerase chain reaction (PCR) or sequencing assay.

The results are summarized in Table 1: among the 13 incorrect identifications, six were false-negative and seven were false-positive results; thus, the respiFISH® HAP Gram (−) Panel test showed a sensitivity of 94.39 % and a specificity of 87.93 %, with interval confidence (IC) of 88.19–97.91 and 76.70–95.01, respectively.

The positive predictive value (PPV) was 93.51 % and the negative predictive value (NPV) was 89.47 % with ICs of 87.10–97.35 and 78.48–96.04, respectively.

These parameters were also calculated for each of the 12 different Gram-negative bacterial pathogens included in one entire test (see Table 1): for *Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Moraxella catarrhalis*, and *Proteus mirabilis*, no false-positive or false-negative results occurred, with sensitivity, specificity, PPV, and NPV of 100 %.

Of the total of 165 samples, 57 were identified at the family level as Enterobacteriaceae: one false-negative result occurred in a sputum sample, where *Enterobacter aerogenes* was identified by the traditional method but missed by the respiFISH® kit probe; in two cases, false-positive results were obtained: *Stenotrophomonas maltophilia* and *Sphingomonas paucimobilis* grown on plates and were incorrectly identified as Enterobacteriaceae by the respiFISH® kit.

Regarding *Pseudomonas aeruginosa*, 36 true-positives were counted, with no false-positive results but three false-negatives: the respiFISH® kit probe did not detect *P. aeruginosa* isolates that were found on culture; in one case, it was a mixed culture of both *E. aerogenes* and *P. aeruginosa*.

Fourteen true-positive and one false-negative results were counted for *Acinetobacter* ssp. Ten *S. maltophilia* were

Table 1 Results of the identification of Gram-negative bacteria from respiratory specimens using the bbFISH® technique [respiFISH® HAP Gram (−) I Panel]. Culture and identification by the VITEK 2® system was considered the gold standard method. Positive predictive values (PPVs), negative predictive values (NPVs), sensitivity, specificity, and positive and negative likelihood ratios are expressed as percentages. Interval confidence (IC) is in shown in parentheses

bbFISH® Panel: list of pathogens (for each spot)	No. of true-negative results	No. of true-positive results	No. of false-negative results	No. of false-positive results	PPV (IC) %	NPV (IC) %	Sensitivity (IC) %	Specificity (IC) %	Positive likelihood ratio (IC)	Negative likelihood ratio (IC)
Total of specimens (165)	51	101	6	7	93.52 (87.10–97.35)	89.47 (78.48–96.04)	94.39 (88.19–97.91)	87.93 (76.70–95.01)	7.82 (3.90–15.69)	0.06 (0.03–0.14)
Enterobacteriaceae	105	57	1	2	96.61 (88.29–99.59)	99.06 (94.86–99.98)	98.28 (90.76–99.96)	98.13 (93.41–99.77)	52.58 (13.32–207.60)	0.02 (0.00–0.12)
P. aeruginosa	125	36	3	0	100.00 (90.26–100.00)	97.66 (93.30–99.51)	92.31 (79.13–98.38)	100.00 (97.09–100.00)	–	0.08 (0.03–0.23)
E. coli	158	7	0	0	100.00 (59.04–100.00)	100.00 (97.69–100.00)	100.00 (59.04–100.00)	100.00 (97.69–100.00)	–	0.00
Acinetobacter ssp.	150	14	1	0	100.00 (76.84–100.00)	99.34 (96.37–99.98)	93.33 (68.05–99.83)	100.00 (97.57–100.00)	–	0.07 (0.01–0.44)
K. pneumoniae	147	17	0	0	100.00 (80.49–100.00)	100.00 (97.52–100.00)	100.00 (80.49–100.00)	100.00 (97.52–100.00)	–	0.00
S. maltophilia	155	8	0	2	80.00 (44.39–97.48)	100.00 (97.65–100.00)	100.00 (63.06–100.00)	98.73 (95.47–99.85)	78.50 (19.81–311.12)	0.00
K. oxytoca	160	4	1	0	100.00 (39.76–100.00)	99.38 (96.59–99.98)	80.00 (28.36–99.49)	100.00 (97.72–100.00)	–	0.20 (0.03–1.15)
H. influenzae	161	3	0	1	75.00 (19.41–99.37)	100.00 (97.73–100.00)	100.00 (29.24–100.00)	99.38 (96.61–99.98)	162.00 (22.96–1143.14)	0.00
S. marcescens	154	11	0	0	100.00 (71.51–100.00)	100.00 (97.63–100.00)	100.00 (71.51–100.00)	100.00 (97.63–100.00)	–	0.00
M. catarrhalis	164	1	0	0	100.00 (2.50–100.00)	100.00 (97.78–100.00)	100.00 (2.50–100.00)	100.00 (97.78–100.00)	–	0.00
P. mirabilis	162	3	0	0	100.00 (29.24–100.00)	100.00 (97.75–100.00)	100.00 (29.24–100.00)	100.00 (97.75–100.00)	–	0.00
P. vulgaris	161	2	0	2	50.00 (6.76–93.24)	100.00 (97.73–100.00)	100.00 (15.81–100.00)	98.77 (95.64–99.85)	81.50 (20.56–323.11)	0.00

detected by the respiFISH® kit; however, two of them were not confirmed by the traditional culture method, providing a total of eight true-positive and two false-positive results.

Regarding *Klebsiella oxytoca*, four samples were true-positives and one was a false-negative: *E. aerogenes* and *K. oxytoca* were both present on the plate, but no *K. oxytoca* was detected by the respiFISH® kit probe.

Haemophilus influenzae showed three true-positive and one false-positive results, since, in one case, the bacteria on the plate was *Haemophilus parainfluenzae*.

In four samples, both *Proteus mirabilis* and *Proteus vulgaris* were detected by the respiFISH® kit, but two *P. vulgaris* resulted as false-positives, as the culture was consistently negative.

In Table 2, the results of direct Gram stain are compared to the respiFISH® test: no wrong identifications by respiFISH® Panel occurred in case of Gram-positive and Gram-negative cultures, providing a 100 % concordance; in two cases of negative Gram stain results, identifications of *Acinetobacter* and *P. aeruginosa* were missed by the respiFISH® test, as well as in a Gram-variable sample, where *P. aeruginosa* was not detected.

The mean TAT from the bbFISH® system was 11.28 h in Verona and 10.20 h in Rome, while the mean TAT recorded using traditional culture methods was 74.95 h in Verona and 50.85 h in Rome, so the difference between the timing of the two systems (Δt) was 63.67 h in Verona and 40.65 h in Rome, respectively.

Discussion

HAP and VAP are life-threatening conditions requiring rapid diagnosis and identification of the pathogens in order to improve patient outcomes, as well as to reduce the spreading of infection. Bacterial culture of clinical specimens is currently considered the gold standard microbiological method, particularly when followed by an antibiotic susceptibility testing on the isolates, which is helpful in orienting the antibiotic regimen [18].

However, culture assays require a long time to achieve a final result. The bbFISH® method is a novel, qualitative nucleic acid hybridization assay indicated for use as an aid in the diagnosis of HAP/VAP. A rapid distinction between oxidase-positive and -negative pathogens, represented by Enterobacteriaceae and non-fermenting Gram-negative bacteria in the respiFISH® kit, can be very helpful to a prompt therapy and prevention of hospital outbreaks.

For the vast majority of microorganisms, this test is able to provide species-/genus-/family-specific identification within 30 min following direct Gram straining of sputa and bronchial secretions, greatly reducing the TAT. The identification of Enterobacteriaceae species achieved by bbFISH® was shown

Table 2 Number of samples and percentage of concordance between Gram stain results and respiFISH® identifications. Different results observed by Gram staining are listed in the first column: Gram-positives included positive rods and cocci, Gram-negative included negative rods and cocci, Gram-variable included mixed culture of Gram-positive rods and cocci, Gram-negative rods and cocci, and yeasts, and negative culture indicates no growth on plate

Type of Gram stain	Result of Gram stain (number of samples)	Number of samples identified by respiFISH® HAP Gram (−) Panel	Concordance (%)
Gram-positive	35	0	100 %
Gram-negative	84	82 (one *P. aeruginosa*, one *Acinetobacter* ssp. failed)	97.6 %
Gram-variable	40	39 (one *P. aeruginosa* failed)	97.5 %
Negative culture	9	0	100 %

to be very accurate: the sensitivity and specificity for *E. coli*, *K .pneumoniae*, *S. marcescens*, *M. catarrhalis*, and *P. mirabilis* were 100 %, and this information can guide clinicians to a proper therapy avoiding the increment of multidrug-resistant strains, especially in case of *K. pneumoniae*, which represents an emerging carbapenemases producer (KPC enzymes) [18].

P. vulgaris was recognized twice by the respiFISH® kit in samples where only *P. mirabilis* was present, which is of limited importance from a clinical point of view. But the mistake in the identification of *H. influenzae*, a well-known pathogen, which the conventional method demonstrated to be *H. parainfluenzae* in culture, could lead to an unnecessary or even harmful pharmacological treatment. *H. parainfluenzae* is rarely considered as a pathogen but is frequently an upper airways colonizer [19]. Other rare pathogens not usually involved in HAP/VAP, such as *Burkholderia cepacia* and *S. paucimobilis*, were not identified by respiFISH®, since they are not included in the test.

High sensitivity and specificity were obtained with *P. aeruginosa*, which is one of the most frequent pathogens causing HAP/VAP, but in two cases, the co-presence of *S. maltophilia* was pointed out by the FISH probe. HAP/VAP are often polymicrobial infections, and this information could be very useful for clinicians because *Stenotrophomonas* is more difficult to treat and it could have been underestimated by a slower growth on culture, where *P. aeruginosa* prevailed. It is more likely that the accuracy of the probe would have to be increased for these species. In this study, the culture methods were used as the gold standard, and a limitation was that discrepant results were not confirmed by a third technique, such as PCR or sequencing. No data on previous treatment were recorded, so false-positive results of respiFISH® could be due to antibiotic use.

The results were available sooner by using the bbFISH® assay than by the conventional assay. However, there are multiple interrelated variables that can affect the TAT [13]. The delay in the TAT is primarily due to the pre- and post-analytical phases. The most common reasons for this delay were the order processing time, excessive laboratory queues, and instrument operation times. Moreover, overall delay in the reporting of test results was linked to workflow interruption on weekends and holidays, but the bbFISH® technique avoids this problem, providing same-day identification of the majority of microorganisms. The data were obtained by calculating the workflow of a microbiologist from 8 a.m. to 3 p.m. from Monday to Friday and the TAT is considerably lower than that of microbiological cultural methods. By considering a full-time working day of 8 h, the bbFISH® time saving consists of 5 days in Rome and more than 1 week in Verona, when compared with conventional laboratory identification.

Conclusions

bbFISH® is a novel molecular assay that effectively supplements traditional approaches, speeds up the diagnosis of respiratory infections, and identifies the majority of the most important respiratory pathogens. However, this technique significantly increases cost and technical complexity, both of which are likely to hamper their adoption for routine use in the clinical laboratory setting. This assay has the potential to provide timely and cost-effective information on infection status. Thus, clinicians are able to make more informed decisions regarding appropriate antibiotic therapy at an earlier stage than is possible with culture-based approaches. Additionally, complications can be avoided and hospitalization time may be reduced.

Conflict of interest All co-authors have no specific conflict of interests.

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