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Comparison of the conventional technique and 16S rDNA gene sequencing method in identification of clinical and hospital environmental isolates in Morocco

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Early and effective diagnosis of infectious diseases of bacterial origin is a critical key in the management of public health. In hospitals, accurate identification of bacterial isolates is an essential task for the microbiological control. Small subunit ribosomal RNA gene (16S rDNA) sequence analysis is usually used for the identification and classification of bacteria. To evaluate the accuracy of 16S rDNA gene sequencing in the identification of bacteria, 58 clinical and hospital environmental isolates were identified by both conventional and molecular techniques. The comparison between the conventional identification and the 16S rDNA gene sequence identifications showed that the genus identification overlapped for both methodologies in 93.1% of the cases and the species identification in 60.34% of the cases, 16S rDNA gene sequencing had a high percent accuracy as compared to the conventional methods. The obtained results suggest that combination of conventional methods and 16S rDNA gene sequencing provide a more accurate identification of clinical and environmental bacteria to enhance the human health management.

Key words: Clinical bacteria, conventional identification, 16S ribosomal RNA, DNA sequencing.

INTRODUCTION

During the last decades, nosocomial infection was registered with constant increase worldwide. A bleaker picture emerged with the discovery of extreme form of drug resistance, especially for new generation drugs that pose a great threat to the success of patients' treatment (Podschum and Ulmann, 1998). Hospital environments are responsible of the dissemination of micro-organisms for different distances and progressive contamination of various supports, including surfaces (Bonten et al., 1996; Boyce et al., 1997) air and water (Curtis, 2008), and

constitute therefore a possible source of nosocomial infections (Danforth et al., 1987; Maki et al., 1982; Huang et al., 2006; Sexton et al., 2006). Thus, accurate identification of bacterial isolates from the hospital environment is an essential task for the microbiological control.

In Morocco, like other developing countries, bacterial identification is still based on the use of conventional techniques, including Gram staining, colonies morphologies, growth requirements and enzymatic and/or metabolic activities. This phenotypic approach presents some

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inherent problems: they are time consuming, fail to identify some bacteria because of the variability of characteristics generated with stress or evolution, and the test results may be based on an individual and subjective interpretation (Stager and Davis, 1992; Ochman et al., 2005). These techniques cannot distinguish between strains belonging to the same species. Moreover, the corresponding database may not yet include newly described species or unusual microorganisms (Stager and Davis, 1992).

Currently, molecular approach based on the 16S rDNA gene sequencing is used in different clinical laboratories for routine identifications, especially for slow-growing, unusual or fastidious bacteria, but also for bacteria that are poorly differentiated by conventional methods (Drancourt et al., 2000; Kupila et al., 2003; Tsai et al., 2004; Petti et al., 2005). Identification based on the 16S rDNA sequence is of interest because ribosomal small subunit exists universally among bacteria and includes regions with species-specific variability, which makes it possible to identify bacteria to the genus or species levels by comparison with databases in the public domain (Vandamme et al., 1996). A direct comparison of rDNA sequences is probably the most powerful tool for the tracing of phylogenetic relationships between bacteria from various sources, such as environmental or clinical specimens (Drancourt et al., 2000) and their identification (Stackebrandt and Goodfellow, 1991).

16S rDNA sequencing could be performed on DNA from bacterial cultures but also directly from specimens to study the diversity of microorganisms without culturing (Lane et al., 1985; Gray and Herwig, 1996; Gill et al., 2006; Rajendhran and Gunasekaran, 2008). van der Heijden et al. (2000) have clearly demonstrated the interest of using 16S rDNA sequencing in the identification of novel organisms of unknown or poorly defined pathogenicity from patient samples.

The efficacy of bacterial identification by 16S rDNA sequencing and conventional techniques was already evaluated. Reported results clearly demonstrate that the 16S rDNA sequences is more efficient than classical phenotypic methods for the identification of atypical bacteria of clinical origin (Morgan et al., 2009) and from freshly isolated from a natural environment (Boivin-Jahns et al., 1995).

Moreover, the efficacy of genotypic identification using 16S rDNA sequencing was clearly demonstrated in the identification of microorganisms misidentified by conventional methods (Petti et al., 2005; Cherkaoui et al., 2009).

The great potential of the molecular approach has been reported for Gram-positive rods and coryneiform bacterial identification (Bosshard et al., 2003; Tang et al., 2000), for Gram-positive, catalase-negative cocci (Bosshard et al., 2004) and for Gram-negative rods (Tang et al., 1998; Coenye et al., 2002; Ferroni et al., 2002).

Subsequent studies have supported the use of broad range 16S rDNA PCR as a valuable adjunct for increasing

diagnostic sensitivity of some bacterial diseases (Lu et al., 2000; Pandit et al., 2005; Xu et al., 2005), particularly in culture-negative cases (Fenollar et al., 2006).

Moreover, the 16S rDNA identification is the only effective diagnostic method that could provide an etiological diagnosis when the patient is receiving antibiotics, or when the causative agent is a fastidious bacterium (Kupila et al., 2003; Tsai et al., 2004). Thus, poorly described, rarely isolated or phenotypically aberrant strains could be better identified by 16S rDNA gene sequencing.

Moreover, this technique could lead to the discovery and description of novel pathogens and facilitate the identification of non-cultured bacteria.

Currently, 16S rDNA gene sequence analysis is more expensive than most traditional identification methods, for routine identifications. However, for difficult organisms, multiple identification methods often must be used, which increases the cost (Patel et al., 2000; Patel, 2001; Wilck et al., 2001; Cook et al., 2003; Hall et al., 2003; Voldstedlund et al., 2008). Such exhaustive phenotypic testing potentially delays turnaround time without the added benefit of accuracy.

Generally, bacterial identification is based on the full length 16S rDNA gene sequencing (1500 bp), but several studies described the use of the initial 500 bp sequence which provides a sufficient discrimination between strains because this region shows a high genetic diversity (Kattar et al., 2001).

Thus, this study was planned to compare bacterial identification by 16S rDNA sequencing and the conventional techniques, using samples from the hospital environment in order to assess the use of this molecular approach for identifying bacteria in a routine clinical microbiology screening.

MATERIALS AND METHODS

Study design

The comparative study was conducted on 58 bacterial strains, isolated from a hospital survey during 2010. Isolates were collected from patients and the hospital environment including hands of hospital personnel and admitted patients, and various surfaces and locations. Isolates were identified by both conventional technique and molecular approach based on 16S rDNA ID. The accuracy of 16S rDNA ID was evaluated by comparing results of 16S rDNA gene sequencing to the results of the conventional technique considered as gold standard approach.

Conventional identification

Spits were inoculated onto the Chocolat enriched or bacitracin agar in 5% CO₂, and for *Neisseria* sp., the 5% sheep blood agar was used. Blood was injected into two or more "blood bottles" with specific media for aerobic and anaerobic organisms and sub cultured onto 5% sheep blood agar for *Streptococcus pneumoniae*, Chapman agar plates for Gram-positive *Staphylococcus*, Desoxycholate citrate lactose agar (DCL agar) plates for Gram-negative bacteria and Chocolate agar for exigent bacteria.

Cerebrospinal fluid was inoculated onto Chocolat enriched/bacitracin agar in 5% CO₂. The urine specimens were inoculated

onto phosphate buffered saline agar, Cystine Lactose Electrolyte Deficient agar and Mac Conkey agar. The other clinical specimens were inoculated onto DCL agar, 5% sheep blood agar, selenite broth for *Salmonella* species, Chocolat enriched/bacitracin agar in 5% CO₂ for *Neisseria* sp. and *Haemophilus* sp.

Swabs were vortexed and subcultured on Chapman agar plates for Gram-positive bacteria, DCL agar plates for Gram-negative bacteria and Chocolate agar for exigent bacteria. The plates were incubated for 18 to 24 h at 37°C and visible colonies were further subcultured and incubated for 24 h at 37°C.

Isolation and identification of microorganisms were done according to standard procedures. Bacteria were identified by examination of colonial morphology, haemolytic characteristics on appropriate agar media, Gram staining, rapid tests (catalase, oxidase, coagulase/Dnase, optochin disc, bile solubility, spot indole, latex agglutination), and classic and API galleries (BioMérieux, France) (Baron and Finglod, 1996).

Bacterial DNA extraction

Bacterial DNA was extracted using the Sigma's GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, France), according to manufacturer instructions. Briefly, 1.5 mL of bacterial broth culture was pelleted at 12,000 to 16,000 xg for 2 min; cells were resuspended in 180 µL lysis solution A for Gram-negative bacteria or in 200 µL of lysozyme (200 units/ml) for Gram-positive bacteria. Then, 20 µL of Proteinase K was added to the cell suspension. After incubation at 55°C for 30 min, 200 µL of lysis solution C was added to the suspension. The suspended cells were then incubated at 55°C for 10 min. DNA was purified using GenElute Miniprep Binding Columns (Sigma-Aldrich, France). DNA is then eluted in sterile distilled water and stored at -20°C until use.

16S rDNA gene sequencing

The 16S rDNA gene was amplified using primers fD1 (5-AGA GTT TGA TCC TGG CTC AG-3') and rP2 (5'-AAG GAG GTG ATC CAG CC-3'), as described by Weisburg et al. (1991). PCR was performed using 2.5 µl of 10X buffer, 1.5 mM MgCl₂, 0.2 mM of each dNTPs, 0.4 µM of each primer, 1 U of Platinum Taq Polymerase (Invitrogen) and 5 µl (30 ng/µl) of template DNA in a 25 µl reaction volume under the following conditions : 4 min at 96°C (initial denaturation), 35 cycles of 10 s at 96°C (denaturation), 40 s at 52°C (annealing), 2 min at 72°C (extension), and one final step of 4 min at 72°C (extension cycle) employing the PCR thermocycler "Verity" (Applied Biosystems). The amplified fragments were electrophoresed on 1% agarose gels and detected using ethidium bromide along with molecular weight markers. The PCR products were purified using EXOSAP-IT (USB, USA) and bidirectionally sequenced on an ABI 3130xl automated sequencer (Applied Biosystems) using BigDye Terminator version 3.1 Kits with the same primers as those used for the amplification. Analysis of electrophoregram was done with the sequencing Analysis Software version 5.3.1 (Applied Biosystems). The consensus sequences were edited and compared with published sequences available in GenBank, using Blast tool of the NCBI. The criterion used to identify an isolate to the genus or species level, was suggested by several researches: 97 and 99% identity in 16S rRNA gene sequence to identify an organism to the genus and the species level respectively (Drancourt et al., 2000; Stackebrandt et al., 2002; Bosshard et al., 2003; Harris and Hartley, 2003; Clarridge, 2004; Janda and Abbott, 2002).

Comparative analysis

Results for each sample were compiled in a local database. For each isolate, comparative analysis was made between the conven-

tional method and 16S rDNA gene sequencing, at genus and species levels, to genus level only, or no correlation. The percent accuracy for 16S rDNA gene sequencing method was calculated using the number of identifications out of the total number of samples tested.

RESULTS

16S ribosomal DNA gene sequencing

Bacterial strains collected from the Ibn Sina Hospital fell into one of the two designated categories of this study: Gram-negative rods and Gram-positive cocci. Conventional identification showed that 84.5% of the isolates are Gram-negative bacteria (49/58) and the main isolates were *Klebsiella pneumonia* (18 isolates), *Escherichia coli* (7 isolates) and *Acinetobacter baumannii* (6 isolates). The conventional method allowed also the identification of 10 *Pseudomonas*, including 4 *Pseudomonas aeruginosa*; 8 *Staphylococcus*, including 2 *Staphylococcus warneri*, 1 *Staphylococcus cohnii*, 1 *Staphylococcus sciuri*, 1 *Staphylococcus aureus* and 2 *Staphylococcus haemolyticus*; 6 *Enterobacter*, 2 *Proteus*, including 1 *Proteus mirabilis*; and 1 *Streptococcus*.

The correlation of 16S rDNA gene sequencing to conventional method was evaluated to determine percent accuracy of 16S rDNA gene sequencing for each level of identification (genus and species, genus, and no correlation). In this study, the comparison between the conventional identification and the 16S rDNA gene sequence identifications showed that the genus identification overlapped for both methodologies in 93.1% (54/58) of the cases and the species identification in 60.34% of the cases (35/58).

There was no correlation between conventional method and 16S rDNA gene sequencing for 6.90% (4/58) of the study isolates.

Concordance at the genus and specie levels

The concordance at genus and specie levels was obtained for 35 strains (Table 1). Among them, 34 were Gram-negative bacteria and 1 was Gram-positive bacteria. For Gram-negative bacilli, Gram stain morphology and a manual biochemical profile appeared most consistent with identification as a *K. pneumonia* (17 isolates), *E. coli* (6 isolates), *P. aeruginosa* (4 isolates), *Acinetobacter baumannii* (6 isolates) and *P. mirabilis* (1 isolates).

The only one Gram-positive cocci, giving a concordance at genus and specie levels, belonged to the *S. aureus* specie.

Concordance at the genus level

A total of 19 isolates, including 12 Gram-negative bacilli and 7 Gram-positive cocci, gave a concordance only at genus level (Table 2). Indeed, for Gram-negative bacteria, conventional identification was limited to genus;

Table 1. Comparison of conventional and 16S rRNA sequencing identification methods for bacteria/genus and specie levels.

	Strain	Identification by conventional methods	Using 16S rRNA gene sequencing (EzTaxon)
	2	<i>Klebsiella pneumoniae</i>	<i>Klebsiella pneumoniae</i> subsp. rhinoscleromatis
	4	<i>Klebsiella pneumoniae</i>	<i>Klebsiella pneumoniae</i> subsp. ozaenae
	15U	<i>Klebsiella pneumoniae</i>	<i>Klebsiella pneumoniae</i> subsp. rhinoscleromatis
	36	<i>Klebsiella pneumoniae</i>	<i>Klebsiella pneumoniae</i> subsp. ozaenae
	46	<i>Klebsiella pneumoniae</i>	<i>Klebsiella pneumoniae</i> subsp. rhinoscleromatis
	51	<i>Klebsiella pneumoniae</i>	<i>Klebsiella pneumoniae</i> subsp. rhinoscleromatis
	52	<i>Klebsiella pneumoniae</i>	<i>Klebsiella pneumoniae</i> subsp. rhinoscleromatis
	55	<i>Klebsiella pneumoniae</i>	<i>Klebsiella pneumoniae</i> subsp. rhinoscleromatis
	59	<i>Klebsiella pneumoniae</i>	<i>Klebsiella pneumoniae</i> subsp. rhinoscleromatis
	60	<i>Klebsiella pneumoniae</i>	<i>Klebsiella pneumoniae</i> subsp. ozaenae
	61	<i>Klebsiella pneumoniae</i>	<i>Klebsiella pneumoniae</i> subsp. rhinoscleromatis
	62	<i>Klebsiella pneumoniae</i>	<i>Klebsiella pneumoniae</i> subsp. ozaenae
	64U	<i>Klebsiella pneumoniae</i>	<i>Klebsiella pneumoniae</i> subsp. pneumoniae
	67	<i>Klebsiella pneumoniae</i>	<i>Klebsiella pneumoniae</i> subsp. rhinoscleromatis
	69	<i>Klebsiella pneumoniae</i>	<i>Klebsiella pneumoniae</i> subsp. ozaenae
	73	<i>Klebsiella pneumoniae</i>	<i>Klebsiella pneumoniae</i> subsp. ozaenae
Gram -	83	<i>Klebsiella pneumoniae</i>	<i>Klebsiella pneumoniae</i> subsp. ozaenae
	6	<i>Escherichia coli</i>	<i>Escherichia coli</i> strain U 5/41
	32	<i>Escherichia coli</i>	<i>Escherichia coli</i> strain U 5/41
	44E	<i>Escherichia coli</i>	<i>Escherichia coli</i> KCTC 2441
	72	<i>Escherichia coli</i>	<i>Escherichia coli</i> strain U 5/41
	82	<i>Escherichia coli</i>	<i>Escherichia coli</i> KCTC 2441
	120	<i>Escherichia coli</i>	<i>Escherichia coli</i> KCTC 2441
	48	<i>Pseudomonas aeruginosa</i>	<i>Pseudomonas aeruginosa</i> LMG 1242
	71	<i>Pseudomonas aeruginosa</i>	<i>Pseudomonas aeruginosa</i> LMG 1242
	81	<i>Pseudomonas aeruginosa</i>	<i>Pseudomonas aeruginosa</i> LMG 1242
	109P	<i>Pseudomonas aeruginosa</i>	<i>Pseudomonas aeruginosa</i> LMG 1242
	40E	<i>Acinetobacter baumannii</i>	<i>Acinetobacter baumannii</i>
	49	<i>Acinetobacter baumannii</i>	<i>Acinetobacter baumannii</i>
	50	<i>Acinetobacter baumannii</i>	<i>Acinetobacter baumannii</i>
	53U	<i>Acinetobacter baumannii</i>	<i>Acinetobacter baumannii</i>
	U56	<i>Acinetobacter baumannii</i>	<i>Acinetobacter baumannii</i>
	100U	<i>Acinetobacter baumannii</i>	<i>Acinetobacter baumannii</i>
	113	<i>Proteus mirabilis</i>	<i>Proteus mirabilis</i>
Gram +	43E	<i>Staphylococcus aureus</i>	<i>Staphylococcus aureus</i> subsp. aureus Mu50

A total of 34 Gram- and 1 Gram+ bacteria isolated from patients or hospital' environment have shown a concordance of identification at genus and specie levels by both conventional and 16S rDNA gene sequencing methods.

Pseudomonas or *Enterobacter*. Molecular identification based on 16S rDNA sequencing allowed the identification of two distinct species among *Pseudomonas* isolates, *P. aeruginosa* (5 isolates) and *P. Moorei* (1 isolate), and two distinct species among *Enterobacter* isolates; *E. cancerogenus* (4 isolates) and *E. hormaechei* (2 isolates).

Conventional identification showed that all Gram-positive bacteria reported in Table 2 belonged to *S. aureus* specie (7 isolates). However, the molecular approach

revealed that there are 3 *S. haemolyticus*, 2 *S. warneri*, 1 *S. cohnii* subsp. Urealyticus and 1 *S. sciuri* subsp. Sciuri.

Absence of concordance

Table 3 summarises results of 4 isolates (3 Gram-negative and 1 Gram-positive bacteria) giving no concordance between conventional identification and molecular characterisation. The 3 Gram-negative isolates were identified

Table 2. Comparison of conventional and 16S rRNA sequencing identification methods for bacteria/genus level.

	Strain	Identification by conventional methods	Using 16S rRNA gene sequencing
Gram -	65	<i>Pseudomonas</i>	<i>Pseudomonas aeruginosa</i> LMG 1242
	70	<i>Pseudomonas</i>	<i>Pseudomonas aeruginosa</i> LMG 1242
	74	<i>Pseudomonas</i>	<i>Pseudomonas moorei</i> RW10
	77	<i>Pseudomonas</i>	<i>Pseudomonas aeruginosa</i> LMG 1242
	78	<i>Pseudomonas</i>	<i>Pseudomonas aeruginosa</i> LMG 1242
	79	<i>Pseudomonas</i>	<i>Pseudomonas aeruginosa</i> LMG 1242
	39	<i>Enterobacter</i>	<i>Enterobacter cancerogenus</i> LMG 2693
	53	<i>Enterobacter</i>	<i>Enterobacter cancerogenus</i> LMG 2693
	54	<i>Enterobacter</i>	<i>Enterobacter cancerogenus</i> LMG 2693
	64	<i>Enterobacter</i>	<i>Enterobacter cancerogenus</i> LMG 2693
	48E	<i>Enterobacter</i>	<i>Enterobacter hormaechei</i>
	55U	<i>Enterobacter</i>	<i>Enterobacter hormaechei</i>
Gram +	168P	<i>Staphylococcus aureus</i>	<i>Staphylococcus haemolyticus</i>
	5	<i>Staphylococcus aureus</i>	<i>Staphylococcus warneri</i> ATCC 27836
	80	<i>Staphylococcus aureus</i>	<i>Staphylococcus warneri</i> ATCC 27836
	33	<i>Staphylococcus aureus</i>	<i>Staphylococcus cohnii</i> subsp. urealyticus
	58	<i>Staphylococcus aureus</i>	<i>Staphylococcus sciuri</i> subsp. sciuri
	56	<i>Staphylococcus aureus</i>	<i>Staphylococcus haemolyticus</i>
	57	<i>Staphylococcus aureus</i>	<i>Staphylococcus haemolyticus</i>

A total of 12 Gram- and 7 Gram+ bacteria isolated from patients or hospital environment have shown a concordance of identification at genus level only by both conventional and 16S rDNA gene sequencing methods.

Table 3. Comparison of conventional and 16S rRNA sequencing identification methods for bacteria/absence of concordance.

	Strain	Identification by conventional methods	Using 16S rDNA gene sequencing
Gram -	55E	<i>K. Pneumoniae</i>	<i>Escherichia coli</i> O157
	63	<i>Proteus</i>	<i>Delftia tsuruhatensis</i> T7
Gram +	63U	<i>E. coli</i>	<i>Klebsiella pneumoniae</i> subsp. ozaenae
	28	<i>Streptococcus</i>	<i>Aerococcus urinaeequi</i>

A total of 3 Gram- and 1 Gram+ bacteria isolated from patients or hospital environment have shown no concordance of identification by conventional and 16S rDNA gene sequencing methods.

by the conventional technique as *K. pneumoniae*, *E. coli* and *Proteus*, whereas the molecular approach revealed a misidentification of these isolates at genus level identifying them respectively as *E. coli*, *K. pneumoniae* and *Delftia tsuruhatensis*.

Moreover, a strong discordance was obtained with the Gram-positive bacteria which was identified as *Streptococcus* by conventional technique but identified as *Aerococcus urinaeequi* by 16S rDNA sequencing approach.

These results have demonstrated that the sequencing method allowed in several cases the possible identification of subspecies and strains of bacteria while the conventional methods failed to do so.

DISCUSSION

Worldwide, molecular approaches have emerged in clinical microbiology practices. Their high sensitivity, specificity and the short time required to perform the procedure explain the great interest given to these techniques in diagnostic laboratories. Rapid and accurate identification of bacterial isolates is a fundamental task in clinical microbiology, and provides insights into etiologies of infectious disease (Clarridge, 2004; Woo et al., 2008) and appropriate antibiotic treatment (Harris et al., 2002). Although conventional phenotypic methods are relatively inexpensive, easy to perform without the need for specialized instrumentation and allow identification of most com-

monly encountered bacteria, they fail to identify some special groups of bacteria, rare bacteria or bacteria with ambiguous profiles. Indeed, mistakes in identifying rarely encountered or phenotypically aberrant isolates are probably quite common in clinical microbiology laboratories. Sometimes, it is even difficult to know whether a bacterium has been incorrectly identified. Moreover, phenotypic methods rely on the availability of pure culture and are dependent on subsequent growth characteristics and biochemical profiling.

Boivin-Jahns et al. (1995) have reported that the occurrences of misidentification of bacteria are very much decreased by using the 16S rDNA sequencing method.

16S rDNA sequencing represents a universal technology that, theoretically, provides solutions to these problems, yielding unambiguous data, even for unusual and slow-growing isolates, often within 48 h (Relman et al., 1990; Patel et al., 2000; Drancourt et al., 2004; Woo et al., 2008; Morgan et al., 2009), which are reproducible among laboratories (Kolbert and Persing, 1999; Drancourt et al., 2004). 16S rDNA sequencing is considered as a reference method for bacterial taxonomy and identification. It has been utilized by several researchers to identify environmental and clinical isolates (Stackebrandt et al., 1993; Drancourt et al., 2000; Clarridge et al., 2001; Clarridge and Zhang, 2002; Bosshard et al., 2003; Song et al., 2003; Drancourt et al., 2004; Drancourt and Raoult, 2005)

On the other hand, 16S rDNA identification is of great interest in determining the appropriate treatment of some isolates that are considered environmental contaminants (Miller and Rhoden, 1991; Drancourt et al., 2004) but can cause opportunistic infections in immunocompromised patients (Morgan et al., 2009, Sontakke et al., 2009), because they are rarely associated with human infection and considered clinically insignificant (Clarridge, 2004). Unlike phenotypic identification, which can be affected by the presence or absence of non-housekeeping genes or by variability in expression of characters, 16S rDNA sequencing provides precise identification of isolates with atypical phenotypic characteristics. In fact, the major advantage of 16S rDNA sequencing is the presence of the 16S rDNA gene in all bacteria; it provides high accuracy for identification of any bacterial organism, reliability and reproducibility (Kolbert and Persing, 1999; Drancourt et al., 2004).

In this study, results clearly demonstrate the feasibility of routinely used method in a microbiological laboratory, allowing the rapid and accurate identification of these pathogens, which is important in clinical and public health interventions. Most of the isolates we analyzed were correctly identified, and the etiological agent was identified even with the use of the single sequencing direction. In almost all cases, the species identified was predictable, but in some cases we identified unusual bacteria such as *D. tsuruhatensis* and *A. urinaeequi*.

For health management strategy, 16S rDNA has been useful in subtyping virulent bacterial strains associated

with outbreaks and can provide some additional information on the prevalence of endemic strains (Sacchi et al., 2002a, b). Moreover, 16S rDNA sequencing has been crucial in the identification of novel and rare bacteria associated with infectious diseases (Clarridge, 2004; Woo et al., 2008).

However, this molecular method has some limitations. There are 'blind spots' within some major genera, in which 16S rDNA sequences are not sufficiently discriminative for the identification of certain species. In these circumstances, alternative targets have to be investigated. For example, *groEL* is a commonly used essential gene other than 16S rDNA which is useful for classification and identification of many groups of bacteria, e.g. staphylococci species (Viale et al., 1994)

Moreover, there is no universal agreement about the percentage similarity required to assign a sequence to a particular species or genus. Globally, the similarity levels, that have been proposed, range from 97% for the genus level to 99% for the species level (Drancourt et al., 2000; Janda and Abbott, 2002; Stackebrandt et al., 2002; Bosshard et al., 2003; Harris and Hartley, 2003; Clarridge, 2004). Assignment to a species can be difficult and relatedness is often more easily shown by alignment and drawing a phylogenetic tree (Clarridge, 2004)

Finally, the interpretation of sequences depends on the database, which constitutes a critical factor to bear in mind when considering the possibility of error. This may be linked to the large number of sequences deposited and to the errors of databases by misidentified strains (Kawamura et al., 1995; Facklam, 2002; Harris and Hartley, 2003).

Conclusion

Our results demonstrate clearly the interest and feasibility to introduce the 16S rDNA gene sequencing method in the clinical specimen protocol in Morocco. Moreover, combination of conventional techniques and molecular approach will improve bacteriological diagnosis and allow specific and efficient identification of pathogenic bacteria, limit nosocomial infections and save human lives.

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