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Difficult-to-identify bacteria: how use of 16S rDNA PCR and gene sequencing can help

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Universal or 'broad range' eubacterial polymerase chain reaction (PCR) was performed on a collection of 70 phenotypically difficult-to-identify bacterial isolates, including 31 atypical mycobacterial isolates, originating from the routine service of an NHS clinical microbiology laboratory in the UK. 16S rDNA PCR was performed using two sets of universal primers to generate a composite amplicon of 1068 bp, which was sequenced to obtain each isolate's identity. In most cases, sequence analysis was able to identify the isolates examined with relative ease, with the respiratory section (atypical mycobacteria and cystic fibrosis) and blood culture work as the main beneficiaries of adoption of molecular identification methods. When the use of molecular identification methods is justified, employment of partial 16S rDNA PCR and sequencing provides a valuable and reliable method for the identification of bacteria that have proved difficult to identify by phenotypic techniques.

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Correct identification of bacterial organisms in clinical microbiology is an important laboratory function. Such organisms may be presented for identification from sporadic clinical cases, from outbreaks or for epidemiological or surveillance purposes. Such identification, to date, has relied largely on phenotypic schema, including initial examination of colonial morphology and Gram stain, which today is usually followed by some form of semi-automated identification scheme, generally based on biochemical differentials such as the API identification schemes, the BBL Crystal Scheme or Vitek 2.

However, one problem with such systems is that their databases are incomplete for some organisms of clinical significance, thereby creating identification anomalies in trying to identify correctly such cultures, even to the genus level. Molecular methodologies offer an alternative laboratory mechanism for the identification of such organisms, particularly the employment of 16S rDNA PCR and sequencing techniques. Over the past decade, particularly with the adoption of PCR in biomedical science, such techniques have been employed increasingly in routine laboratory diagnostics.

Given that 16S rDNA PCR and automated sequencing has become a routine tool in many specialist and reference microbiology laboratories, little more can be added to the description of the method. However, what is of interest is how such techniques can aid the busy service laboratory, and what value such techniques add. Hence, the aim of this study is to discover which organisms are being forwarded for molecular analysis from the phenotypic section, due to difficulties in identification.

Viable culturable bacterial isolates (n=70; 31 atypical mycobacterial isolates and 39 difficult-to-identify isolates from various microbiology laboratory sections) were examined in order to obtain an identification by 16S rDNA PCR and direct automated sequencing, in accordance with a standard protocol.[1] All isolates originated from the routine microbiology service of a large UK hospital trust, which processes approximately 160,000 clinical specimens per annum and has specialist centres for adult cystic fibrosis (CF) and haematological malignancy.

Atypical mycobacterial isolates were examined from the archive of such isolates stored in the Northern Ireland Mycobacterium Reference Laboratory, situated within the same clinical microbiology department. The criteria for forwarding such isolates for molecular analysis included (i) poor (or no) discrimination of identification of the isolate using combinations of phenotypic and conventional identification assays, as performed by experienced biomedical scientists, and (ii) the likelihood of the isolate being of potential clinical significance by the consultant medical microbiologist. All isolates requiring such molecular analysis were processed for 16S rDNA PCR and direct automated sequencing, as described previously.[1]

A total of 70 isolates were obtained for 16S rDNA PCR and sequencing purposes, as detailed in Tables 1 and 2. Polymerase chain reaction amplifications on high-quality genomic DNA preparations of these isolates generated

amplicons of expected size for all the isolates examined. Subsequent sequencing of the amplicons and sequence analysis permitted reliable identification in the majority of cases, and the resulting sequences have been deposited in GenBank (Tables 1 and 2). Of the 39 difficult-to-identify isolates examined, 31 (79%) were identified confidently to the species level, seven (18%) only to the genus level, and one isolate (3%), which was not identified to the genus level, probably represented a novel taxon. Of these, most isolates (25/39, 64%) originated from respiratory cultures, particularly CF isolates.

It was surprising to note that eight isolates of *Pseudomonas aeruginosa* were forwarded for molecular identification from the CF section. Conventionally, it is believed that use of the API 20NE identification scheme (bioMérieux) is able to identify this species reliably; however, it was noted that the API gave anomalous profiles, resembling the presence of an atypical *P. aeruginosa* clone within the adult CF patients attending the regional adult CF centre. Furthermore, experience with such biochemically variant *P. aeruginosa* is not unique. Moissenet *et al*.[2] recently described the phenotypic problems associated with the correct identification of non-fermenting Gram-negative rods, including *P. aeruginosa*, from CF sputum.

Table 1. Description of the identification of 39 bacterial isolates by 16S rDNA PCR and sequencing, examined in this study, including organism identification, source and submitted GenBank Accession number.

Source of bacterium	16S rDNA-based identification	16S rDNA primers XB1-XB4		Submitted GenBank Accession No.
		Size (bp)	Identity(%)	
RESPIRATORY				
Environmental isolate	*Saccharomonospora viridis*	1001	100	AY114168
Environmental isolate	*Thermoactinomuces vulgaris/candidis*	1015	100	AY114167
Environmental isolate	*Thermoactinomuces sacchari/thalpophlus*	987	100	AY114169
Environmental isolate	*Thermoactinomuces sacchari/thalpophlus*	985	100	
Environmental isolate	*Tsukamurella pulmonis*	1471	100	AY741505
Cystic fibrosis (sputum)	*Burkholderia cepacia*	1016	100	AY360346
Sputum	*Burkholderia gladioli*	1360	100	AY665976
Sputum	*Burkholderia multivorans*	1011	99	
Sputum	*Cellulosimicrobium cellulans*	1514	99	AY665978
Sputum	*Chryseobacterium meningosepticum*	1015	99	AY360341
Sputum	*Haemophilus influenzae*	1018	99	AY360336
Sputum	*Inquilinus limosus*	1006	99	AY360342
Sputum	*Mycobacterium abscessus*	1010	100	AY360327
Sputum	*Ochrobactrum intermedium*	909	100	AY093589
Sputum	*Pandoraea apista*	1021	99	AY360339
Sputum	*Pandoraea pulmonicola*	1020	99	AY360334
Sputum	*Pseudomonas aeruginosa* (x8)	1365	100	AY665977
Sputum	*Stenotrophomonas maltophilia*	985	100	AY360340
BLOOD CULTURE	*Ralstonia paucula*	1015	100	AF495865
Haematology	*Ralstonia paucula*	1021	99	
	Roseomonas mucosa	1017	100	AY360348
	Tsukamurella tyrosinosolvens	1009	100	AY259830
General blood culture	*Acinetobacter* sp.	1026	99	AY380830
	Actinobacillus actinomycetemcomitans	1014	99	AY360355
	Corynebacterium striatum	570	100	DQ018338
	Exiguobacterium sp.	1024	99	AY360351
	Haemophilus paraphrophilus	1024	98	AY360333
	Klebsiella pneumoniae	999	99	
	Neisseria meningitidis	1024	99	AY360345
	Peptostreptococcaceae bacterium	973	100	AF521147
	Streptomyces sp.	961	99	AY360338
	Unidentified bacterium	997	92	

Table 2. Comparison of the molecular and phenotypic identification of 31 isolates of atypical *Mycobacterium* isolates.

Identification	16s rDNA sequencing result XB1-XB4		Submitted GenBank Accession No.	Culture results
	Size	Identity(%		
M. malmoense	1017	100		*M. malmoense*
M. malmoense	1021	100		*M. scrofulacium*
M. malmoense	1012	99		*M. malmoense*
M. asiaticum	1010	99		*Mycobacterium* sp.
M. xenopi	1015	99	AY082373	*M. xenopi*
M. malmoense	993	100		*M. malmoense*
M. malmoense	987	100		*M. malmoense*
M. malmoense	1015	100		*M. malmoense*
M. gordonae	1014	99		Environmental scotochromogen
Mycobacterium sp.	1015	99	AY360325	*Mycobacterium* sp.
M. asiaticum	1021	99	AY360326	Environmental scotochromogen
M. xenopi	1021	99		*M. xenopi*
M. malmoense	1011	99		*M. malmoense*
M. garstri/M. kansasii	981	100	AY360332	*M. garstri/M. kansasii*
M. malmoense	1017	100		*M. malmoense*
M. malmoense	948	100		*M. malmoense*
M. malmoense	970	100		*M. malmoense*
M. abscessus	1010	100	AY360327	*M. abscessus*
M. malmoense	1019	100		*M. malmoense*
M. abscessus	1007	100		*M. abscessus*
M. avium	1014	100	AY360329	*M. avium*
M. malmoense	999	100		*M. malmoense*
M. gordonae	1018	99		*M. gordonae*
M. malmoense	1023	100		*M. malmoense*
M. malmoense	1001	100		*M. malmoense*
M. malmoense	1020	100		*M. malmoense*
M. gordonae	1021	99	AY360328	Environmental scotochromogen
M. garstri/M. kansasii	1012	100		*Mycobacterium* sp.
M. malmoense	1021	100	AY360330	*M. malmoense*
M. malmoense	1018	100		*M. malmoense*
M. bovis/M. africanum	1021	100	AY360331	*M. bovis*

The remaining 14 isolates originated from blood cultures, with four out of 14 isolates from patients with a haematological malignancy. In relation to the atypical mycobacterial isolates examined, there was concordance in the majority of isolates examined (25/31; 81%). 16S rDNA techniques were able to identify 26/31 isolates to the species level, with four isolates being identified to one of two possible species.

One atypical isolate could not be identified to the species level (GenBank Accession No. AY360325), either by molecular or conventional means. Recently, however, the sequence of a new *Mycobacterium* species, *M. arupense*, has been released to GenBank (DQ157760) by a US group. This shows complete homology to the clinical isolate in question, suggesting similarity to the as yet unnamed *Mycobacterium* species. This illustrates that employment of 16S rDNA gene sequencing techniques are evolving and being supplemented and updated on a daily basis by a global laboratory, adding value to this technique as a means of bacterial identification.

The traditional basis for the identification of bacterial organisms has been their isolation or propagation in the laboratory, where biochemical, morphological and serological tests are used to aid in the process. 16S rRNA genes are found in all bacteria and they accumulate mutations at a slow, constant rate over time, hence they may be used as 'molecular clocks'.[3] Highly variable portions of the 16S rRNA sequence provide unique signatures to any bacterium, and also useful information about relationships between them. Alternatively, as 16S rRNA molecules have crucial structural constraints, certain conserved regions of sequence are found in all known bacteria (i.e., the eubacteria) and in all known organisms.

Broad-range PCR primers may then be designed to recognise these conserved bacterial 16S rRNA gene sequences and used to amplify intervening, variable or

diagnostic regions. In bacteria, three genes make up the rRNA functionality (i.e., 5S, 16S and 23S rRNA). Historically, the 16S rRNA gene has been employed most commonly and hence has the most comprehensive database for comparison during searches of unknown sequences. Use of such rRNA-based techniques has gained increasing popularity as a means of identifying organisms that are troublesome and phenotypically difficult to identify. Even with improvements to conventional phenotypic identification techniques, certain genera and species continue to cause diagnostic dilemmas for the routine clinical microbiology laboratory. Given that most clinically significant organisms are relatively easy to identify by routine phenotypic laboratory methods (e.g., API identification scheme), an identification dilemma may still exist with the correct identification of certain genera and species of unusual but clinically significant bacteria.

What does the adoption of such techniques mean for the routine clinical microbiology laboratory? The authors have found these techniques to be particularly useful in respiratory specimens (atypical TB and CF) and in a small proportion of blood cultures, including those from haematology patients. As such, molecular techniques have become commonplace in many specialist and reference laboratories, with the value of associated reports dependent on how these methods perform in the routine setting. Continued use of such techniques will permit the description of emerging bacterial pathogens, which to date have not been described, as illustrated by the publication of several case reports using this technology.[3,4]

For the medical microbiologist, such techniques should be regarded as simply another technology to obtain quality data on the identification of isolates, where interpretive criteria on the clinical significance of the organism is no different to those isolates identified conventionally.[5]

Judging by the authors' experience, specimens for molecular analysis originate from a relatively narrow source (respiratory; CF and atypical mycobacteria) and blood culture, even in a large teaching hospital, indicating that adoption of such technology in all clinical microbiology laboratories is unwarranted and not practical. Hence, such techniques should be restricted to specialist/reference laboratories.

The inability to report accurately the identification of an organism to clinical users of the service may compromise the clinical management of the patient (e.g., if the unidentified organism is important in terms of infection control). When such molecular techniques are adopted, the laboratory is able to provide reliable identification of organisms, which is a fundamental objective of any microbiology service.

In conclusion, when use of molecular identification methods is justified, employment of partial 16S rDNA PCR and sequencing provides a valuable and reliable method of identification for bacteria that prove difficult to identify by conventional phenotypic techniques.

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An unbalanced translocation, der(17)t(1;17)(p13;p11.2), leads to heterozygous loss of *TP53* and is associated with clinical evolution in myelodysplastic syndrome

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Cytogenetic abnormalities are found in half of all myelodysplastic syndrome (MDS) cases.[1,2] Gross karyotypic changes are frequent, including chromosomal gain (most commonly trisomy 8) and chromosomal deletion and loss (most notably monosomy 5 and 7, loss of Y, deletion of 5q and 7q).[3] These changes are usually found in the setting of a complex karyotype.

In comparison to the situation in acute myeloid leukaemia (AML), isolated reciprocal translocations are uncommon in MDS. This has hampered the identification of specific genetic defects, although the prognostic importance of chromosomal abnormalities is well recognised in MDS.[4]

This study reports a der(17)t(1;17) (p13;p11.2) unbalanced translocation as the sole abnormality in a case of MDS showing disease progression.

A 71-year-old woman presented with the symptoms of anaemia. Peripheral blood count showed the following values: haemoglobin: 7.8 g/dL, platelets: 372 x 10^9/L and leucocytes: 3.4 x 10^9/L (66% neutrophils, 20% lymphocytes, 10% monocytes, 2% basophils and 2% myelocytes). Bone marrow examination revealed hypercellular particles with 3% blasts and dysplastic granulopoiesis and megakaryopoiesis. Marked erythroid hypoplasia was noted. Iron staining revealed no ringed sideroblasts.

A diagnosis of refractory cytopenia with multilineage dysplasia (RCMD) was made, in accordance with the World Health Organization (WHO) classification.[5] Cytogenetic analysis performed by overnight fluorodeoxyuridine-

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