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Comparison of Biolog GEN III MicroStation semi-automated bacterial identification system with matrix-assisted laser desorption ionization-time of flight mass spectrometry and 16S ribosomal RNA gene sequencing for the identification of bacteria of veterinary interest

P. Wragg [a,*], L. Randall [b], A.M. Whatmore [b]

[a] Animal Health and Veterinary Laboratories Agency-Penrith Regional Laboratory, Penrith, Cumbria CA11 9RR, United Kingdom
[b] Department of Bacteriology, Animal Health and Veterinary Laboratories Agency-Weybridge, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom

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ABSTRACT

Recent advances in phenotypic and chemotaxonomic methods have improved the ability of systems to resolve bacterial identities at the species level. Key to the effective use of these systems is the ability to draw upon databases which can be augmented with new data gleaned from atypical or novel isolates. In this study we compared the performance of the Biolog GEN III identification system (hereafter, GEN III) with matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) and 16S rRNA gene sequencing in the identification of isolates of veterinary interest. The use of strains that had proven more difficult to identify by routine methods was designed to test the systems' abilities at the extremes of their performance range. Over an 18 month period, 100 strains were analysed by all three methods. To highlight the importance of identification to species level, a weighted scoring system was devised to differentiate the capacity to identify at genus and species levels. The overall relative weighted scores were 0.869:0.781:0.769, achieved by 16S rRNA gene sequencing, GEN III and MALDI-TOF MS respectively, when compared to the 'gold standard'. Performance to the genus level was significantly better using 16S rRNA gene sequencing; however, performance to the species level was similar for all three systems.

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1. Introduction

Following a period of technological refinement in recent decades, automated or semi-automated metabolic methods have predominated in high-throughput microbiology laboratories processing routine clinical specimens. However, intra-species variation, together with the frequent recognition of new species, began to undermine the accuracy of less-sophisticated phenotypic systems (O'Hara, 2005). Such systems were found to be limited either by virtue of a fundamental incompatibility with particular groups or by a lack of flexibility within the reference database to recognise novel profiles and offer 'intuitive' solutions. 16S rRNA gene sequencing offered improved resolution at the genus and, in many cases, species level. Nonetheless, genomic approaches are not without limitations and the freedom to deposit sequences within publically-accessible databases, without peer-review, threatened the integrity of the database and confidence in the result (Mignard and Flandrois, 2006; Woo et al., 2008). Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) has become widely recognised as one of the most adaptable of the chemotaxonomic methods, finding application in both clinical diagnostic and research settings (Bessède et al., 2011; Hijazin et al., 2012; Bizzini and Greub, 2010). MALDI-TOF MS systems are available which provide extensive databases and are equipped with software capable of drawing on both local and manufacturer-derived data. Whilst not as well recognised as in other technologies, development in classical phenotypic approaches continued. Biolog (Biolog Inc., Hayward, USA) adapted the familiar principle of substrate utilisation by coupling metabolic activity to the simultaneous reduction of a redox dye, measured colourimetrically within a 96 well ELISA plate format (Bochner, 1989, 2008). Earlier manifestations utilised specific plates according to Gram reaction but, although successful, remained less effective than 16S rRNA gene sequencing in the identification of 'atypical bacteria' to the species level (Morgan et al., 2009). Accuracy varied according to taxa; specificity was highest with Gram-positive fermenters and lowest with unreactive non-fermenters (Holmes et al., 1994). However, with more biochemically reactive taxa, the system displayed a valuable capacity to identify species which were otherwise 'difficult to identify' and, in some cases, a capability to detect relationships between

* Corresponding author. Tel.: +44 1768 885295; fax: +44 1845 525224.
E-mail address: peter.wragg@ahvla.gsi.gov.uk (P. Wragg).

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epidemiologically-related strains (Jánosi et al., 2009; Lanka et al., 2010). In its most recent manifestation, GEN III, a single plate was designed to cover both Gram-negative and Gram-positive aerobic taxa, reducing costs and improving flexibility. This study was designed to indicate the relative potential of the above methods as 'front-line' identification tools within the Animal Health and Veterinary Laboratories Agency (AHVLA), by assessing their performance against a range of isolates referred to a specialist determinative bacteriology laboratory.

2. Materials and methods

2.1. Selection and initial characterisation of isolates

The isolates used in this study ($n = 100$) consisted predominantly of field strains derived from clinical specimens submitted to the AHVLA's Determinative Bacteriology laboratory (AHVLA, Bury St Edmunds, UK), although a number of strains from the National Collection of Type Cultures (NCTC, Colindale, London) were also incorporated. Isolates were cultured onto Columbia agar (Oxoid, Basingstoke, UK) supplemented with 5% sheep blood and incubated according to growth requirements, either aerobically or in a carbon dioxide-enriched environment (7.5% CO_2), at 37 °C for 18–24 h.

2.2. Biolog MicroStation with GEN III microplate system

In the majority of cases, a single colony was selected and emulsified into 'inoculating fluid A' (Biolog) for subsequent inoculation on to the MicroPlate test plate (Biolog). More fastidious organisms, including capnophilic strains, were cultured on alternative media, according to the manufacturer's instructions, and inocula prepared to a specified transmittance using a turbidimeter, as specified in the user guide. For each isolate, 100 μl of the cell suspension was inoculated into each well of the MicroPlate, using a multichannel pipette and incubated at 37 °C for 20 h, either aerobically or in 7.5% CO_2, according to growth characteristics. MicroPlates were read in the MicroStation semi-automated reader after 20 h and results interpreted by the identification system's software (GEN III database, version 5.2.1). The system indicated which isolates could not be identified after 20 h and required further incubation. Such isolates were re-incubated and re-read between 3 and 6 h later. Those which remained unidentified after 26 h were recorded as having no identification.

2.3. Identification by 16S rRNA gene sequencing

Crude bacterial lysates were prepared directly from culture plates by suspending bacteria from a clonal culture in 100 μl of RT-PCR grade water (approximately McFarland Standard 2.0) and placing in a hot block at 100 °C for 10 min. A ~1400 bp fragment of the 16S rRNA gene of the bacterial strains was amplified using the universal primer pair 27 F 5′ AGAGTTTGATCCTGGCTCAG 3′ and 1389R 5′ ACGGGCGGTGTG TACAAG 3′. Resulting PCR amplicons were sequenced in-house with forward and reverse primers 5′ GTTGCGCTCGTTGCGGGACT 3′ and 5′ CTCC TACGGGAGGCAGCAG 3′ using an ABI 3730XL DNA Analyser (Applied Biosystems, Warrington, UK) and standard sequencing methods. Data from both strands was aligned in SeqMan (DNASTAR Lasergene 9 Suite) to generate a contig of up to 700 bp. Sequencing was repeated/excluded from study when available from only one primer or when the contig length was less than 500 bp following editing. The consensus sequences were then used to compare with online databases (NCBI BLAST — http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Ribosomal Database Project (http://rdp.cme.msu.edu/). Identification criteria of >99% sequence identity for identification to species level were applied (Drancourt et al, 2000) where matches had to be to the species type strain. The identities of type strains, as well as accession numbers in NCBI for equivalent 16S rRNA gene sequences, are available at http://www.bacterio.cict.fr/ for all validly published bacterial species although the reader should be aware that there are often multiple sequence depositions for type strains some of which are of better quality than those linked above.

2.4. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)

A Bruker Autoflex II machine (Bruker Daltonik GmbH, Bremen, Germany) was used for MALDI-TOF MS analysis, using standard Bruker protocols available online and as described previously (Eigner et al., 2009). Resulting unknown spectra were compared to the Bruker MSP (Main Spectral Projection) reference spectra (MALDI Biotyper reference library version 3, Bruker Daltonik) using MALDI Biotyper software version 2.0.10.0 to obtain identification based on a score relating to degree of match to reference spectra. To improve the likelihood of obtaining a good score, isolates were spotted on the MALDI TOF MS target plate in quadruplicate and then fired in quadruplicate, resulting in up to sixteen spectra; the best score being accepted as the result (replicate spectra from replicate spots and firings generally yielded the same identification, although scores varied according to the quality of the individual spectrum). The confidence levels for MALDI-TOF MS identifications were those assigned by the Biotyper software; isolates with a score above 2.000 were considered to be identified to the species level with varying levels of confidence, isolates with scores of 1.7 to 1.9999 were identified to the genus level only and isolates with scores of <1.7 were not identified. However, if scores of ≥ 2.000 gave rise to more than one species, the confidence level was taken to be "to genus" only.

3. Results

3.1. Scoring of result according to resolution to genus or species

A summary of the results is shown in Table 1. In order to reflect the value of species-level identification to the diagnostic laboratory, a weighted scoring system was devised based on comparison to the 'gold standard' represented by 16S rRNA gene sequencing and referred to as 'Presumptive identification' in Table 1. Where the presumptive identification was indeterminate by 16S rRNA gene sequencing alone, reference to any unequivocal phenotypic characteristics, or the use of type strains, was made to further establish the species of the majority of strains ($n = 76$). Where the presumptive identification could not be substantiated to species level, strains could only be compared at the genus level ($n = 24$). Identifications to the genus and species level were allocated scores of 1 and 3, respectively, with the exception of the two *Salmonella* isolates, where the maximum achievable score was 1. Failure of any technique to provide identification resulted in a score of 0. The theoretical maximum achievable score for any technique, based on comparison with 76 strains confirmed to species level and 24 to genus level, was 252. Overall, the relative weighted scores for the three methods were 0.869:0.781:0.769, achieved by 16S rRNA gene sequencing, Biolog and MALDI-TOF MS respectively, when compared to the 'gold standard'.

3.2. Biolog GEN III identification system

The GEN III software provided clear indications of the degree of uncertainty for genus and species level identifications. Although all the gold standard strains were represented on the manufacturer's database, some could not be identified at either genus or species level ($n = 15$). This was particularly notable in the less metabolically-active isolates, for example *Riemerella* spp., where discrimination relies on activity in very low numbers of wells. 56 isolates were correctly identified at the species level; a further 29 were identified as far as genus, with no misidentifications, giving an overall capability of 85 strains identifiable to at least genus level. The overall weighted score was 197/252.

Table 1
Identification of isolates.

Strain reference	Identification				Weighted score		
	Presumptive	Biolog Gen III	MALDI-TOF MS	16S rRNA gene sequencing	Gen III	MALDI	16S
KL176	*Acinetobacter* sp.	No identification	*Acinetobacter* sp.	*Acinetobacter* sp.	0	1	1
17-B147-07-07	*Actinobacillus* Bisgaard taxon 26	No identification	*Actinobacillus* sp.	*Actinobacillus* Bisgaard taxon 26	0	1	3
15/S245/1/12	*Actinobacillus lignieresii*	*A. lignieresii*	*A. lignieresii*	*A. lignieresii*	3	3	3
KL135	*A. lignieresii*	*Actinobacillus* sp.	*A. lignieresii*	*A. lignieresii*	1	3	3
NCTC 12370	*Actinobacillus pleuropneumoniae*	*A. pleuropneumoniae*	*A. pleuropneumoniae*	*A. pleuropneumoniae*	3	3	3
14-P414-12-08	*Actinobacillus porcinus*	*A. porcinus*	No identification	*A. porcinus*	3	0	3
521 (SC0217)/KL:B102	*Actinobacillus porcitonsillarum*	*A. porcitonsillarum*	No identification	*A. porcitonsillarum*	3	0	3
KL46	*Actinobacillus* sp.	*Actinobacillus lignieresii*	*Actinobacillus lignieresii*	*Actinobacillus* sp.	1	1	1
6 (SC0217) NCTC10840	*Actinobacillus suis*	*A. suis*	*A. equuli*	*Actinobacillus* sp.	3	1	1
21/P91/5/11-2	*Actinomyces hyovaginalis*	*A. hyovaginalis*	*Actinomyces* sp.	*Actinomyces* sp.	3	1	1
23/P127/2/12	*Actinomyces hyovaginalis*	*A. hyovaginalis*	*Actinomyces* sp.	*Actinomyces* sp.	3	1	1
29/S78/2/12	*Arcanobacterium pluranimalium*	*A. pluranimalium*	*A. pluranimalium*	*A. pluranimalium*	3	3	3
29/B68/7/11	*Avibacterium gallinarum*	*A. gallinarum*	*Avibacterium endocarditis*	*Avibacterium* sp.	3	1	1
84 (SC0217) 29/B48/6/08	*Avibacterium gallinarum*	*A. gallinarum*	*A. endocarditis*	*A. gallinarum*	3	1	3
22/B144/4/11	*Avibacterium* sp.	No identification	*Avibacterium* sp.	*Avibacterium* sp.	0	1	1
16/B99/8/11	*Avibacterium* sp.	*Avibacterium volantium*	*A. gallinarum*	*Avibacterium* sp.	1	1	1
21/B187/11/11	*Avibacterium* sp.	*A. gallinarum*	*Avibacterium* sp.	*Avibacterium* sp.	1	1	1
21-B407-07-07	*Avibacterium* sp.	*Avibacterium avium*	No identification	*Avibacterium* sp.	1	0	1
10 (SC0217) NCTC7464	*Bacillus cereus*	*Bacillus* sp.	*Bacillus cereus*	*Bacillus* sp.	1	3	1
23/P338/11	*Bacillus licheniformis*	*B. licheniformis*	*Bacillus* sp.	*B. licheniformis*	3	1	3
21/C1/9/11	*B. licheniformis*	*B. licheniformis*	No identification	*B. licheniformis*	3	0	3
23/C425/2/12	*B. licheniformis*	*B. licheniformis*	*Bacillus* sp.	*B. licheniformis*	3	1	3
16/S195/2/12	*Bacillus* sp.	*Bacillus* sp.	No identification	*Bacillus* sp.	1	0	1
16/C254/2/12	*Bacillus* sp.	*Bacillus* sp.	*Bacillus* sp.	*Bacillus* sp.	1	1	1
12 (SC0217) G4382	*Bibersteinia trehalosi*	*B. trehalosi*	No identification	*B. trehalosi*	3	0	3
17-C364-08-07	*B. trehalosi*	*B. trehalosi*	No identification	*B. trehalosi*	3	0	3
21/P91/5/11-1	*Bordetella bronchiseptica*	*B. bronchiseptica*	No identification	*Bordetella* sp.	3	0	1
KL172	*Bordetella* sp.	*B. bronchiseptica*	*Bordetella* sp.	*Bordetella* sp.	1	1	1
13/M6/2/12	*Corynebacterium hansenii*	*Corynebacterium* sp.	*C. hansenii*	*C. hansenii*	1	3	3
21/M95/2/12	*C. hansenii*	*Corynebacterium* sp.	*C. hansenii*	*C. hansenii*	1	3	3
502 (SC0217) NCTC10006	*Enterobacter aerogenes*	No identification	*E. aerogenes*	*E. aerogenes*	0	3	3
23/P518/7/11	*Erysipelothrix rhusiopathiae*	*E. rhusiopathiae*	*E. rhusiopathiae*	*E. rhusiopathiae*	3	3	3
16 (SC0217) NCTC 10418	*Escherichia coli*	*E. coli*	*E. coli*	No identification	3	3	0
16/B162/04/08 KL:B103	*Gallibacterium anatis*	*Gallibacterium* sp.	*G. anatis*	*G. anatis*	1	3	3
52 (SC0217) 29/B82/10/07	*G. anatis*	*G. anatis*	*G. anatis*	*G. anatis*	3	3	3
53 (SC0217) 21/B175/11/07	*G. anatis*	*G. anatis*	*G. anatis*	*G. anatis*	3	3	3
57 (SC0217) 17/B264/7/07	*G. anatis*	*G. anatis*	*G. anatis*	*G. anatis*	3	3	3
66 (SC0217) 14/B246/9/07	*G. anatis*	*G. anatis*	*G. anatis*	*G. anatis*	3	3	3
22-B54-09-07	*G. anatis*	*G. anatis*	*Gallibacterium* sp.	*G. anatis*	3	1	3
16/B214/10/11	*Gallibacterium* sp.	*Gallibacterium* genomospecies 1	*Gallibacterium* sp.	*Gallibacterium* sp.	1	1	1
KL144	*Gordonia rubripertincta*	No identification	*G. rubripertincta*	*G. rubripertincta*	0	3	3
24/C107/8/11	*Histophilus somni*	No identification	*H. somni*	*H. somni*	0	3	3
16/C241/1/12-A	*H. somni*	*H. somni*	*H. somni*	*H. somni*	3	3	3
16/C241/1/12-B	*H. somni*	*H. somni*	*H. somni*	*H. somni*	3	3	3
15-B252-7-12	*Klebsiella pneumoniae*	*K. pneumoniae*	*K. pneumoniae*	*Klebsiella* sp.	3	3	1
KL177	*Lactococcus lactis*	No identification	*L. lactis*	*L. lactis*	0	3	3
16/S159/2/12	*Listeria ivanovii*	*Listeria* sp.	*L. ivanovii*	*Listeria* sp.	1	3	1
KL116	*Listeria monocytogenes*	*Listeria innocua*	*L. monocytogenes*	*Listeria* sp.	1	3	1
KL149	*L. monocytogenes*	*Listeria* sp.	*L. monocytogenes*	*L. monocytogenes*	1	3	3
14/S653/7/11	*Listeria* sp.	*Listeria* sp.	*L. monocytogenes*	*Listeria* sp.	1	1	1
16/S27/2/12	*Listeria* sp.	*Listeria* sp.	*L. monocytogenes*	*Listeria* sp.	1	1	1
16/S160/02/12	*Listeria* sp.	*Listeria* sp.	*L. monocytogenes*	*Listeria* sp.	1	1	1
16/S253/2/12	*Listeria* sp.	*Listeria innocua*	*Listeria* sp.	*Listeria* sp.	1	1	1
KL124	*Listeria* sp.	No identification	*Listeria* sp.	*Listeria* sp.	0	1	1
23-S208-01-08	*L. innocua*	*L. innocua*	*L. innocua*	*Listeria* sp.	3	3	1
KL185	*Mannheimia glucosida*	*M. glucosida*	*Mannheimia haemolytica*	*Mannheimia* sp.	3	1	1
9 (SC0217) NCTC10208	*M. glucosida*	*M. glucosida*	*M. haemolytica*	*Mannheimia* sp.	3	1	1
16/M59/5/11	*M. haemolytica*	*M. haemolytica*	*M. haemolytica*	*M. haemolytica*	3	3	3
29/S46/5/11	*M. haemolytica*	*M. haemolytica*	*M. haemolytica*	*M. haemolytica*	3	3	3
14/C286/5/11	*M. haemolytica*	*M. haemolytica*	*M. haemolytica*	*M. haemolytica*	3	3	3
29/S81/5/11	*M. haemolytica*	*M. haemolytica*	*M. haemolytica*	*M. haemolytica*	3	3	3
16/S94/5/11	*M. haemolytica*	*M. haemolytica*	*M. haemolytica*	*M. haemolytica*	3	3	3
23/S311/5/11	*M. haemolytica*	*M. haemolytica*	*M. haemolytica*	*M. haemolytica*	3	3	3
16/S123/5/11	*M. haemolytica*	*M. haemolytica*	*M. haemolytica*	*M. haemolytica*	3	3	3
14/S524/5/11	*M. haemolytica*	*M. haemolytica*	*M. haemolytica*	*M. haemolytica*	3	3	3
16/C85/6/11	*M. haemolytica*	*M. haemolytica*	*M. haemolytica*	*M. haemolytica*	3	3	3

Table 1 *(continued)*

Strain reference	Identification				Weighted score		
	Presumptive	Biolog Gen III	MALDI-TOF MS	16S rRNA gene sequencing	Gen III	MALDI	16S
29/S75/5/11	*Mannheimia ruminalis*	*M. ruminalis*	*Mannheimia* sp.	*M. ruminalis*	3	1	3
12/C245/11/11	*Mannheimia* sp.	*M. haemolytica*	*Mannheimia* sp.	*Mannheimia* sp.	1	1	1
17/C348/6/11	*Mannheimia varigena*	*M. varigena*	*M. varigena*	*M. varigena*	3	3	3
17/C579/6/11	*M. varigena*	*M. varigena*	*Mannheimia* sp.	*M. varigena*	3	1	3
12-C177-05-12	*M. varigena*	*M. varigena*	*Mannheimia* sp.	*M. varigena*	3	1	3
12-C203-05-12	*M. varigena*	No identification	*M. varigena*	*M. varigena*	0	3	3
17/C348/4/11	*Nocardia* sp.	*Nocardia seriolae*	No identification	*Nocardia* sp.	1	0	1
B1547	*Ornithobacterium rhinotracheale*	*O. rhinotracheale*	*O. rhinotracheale*	*O. rhinotracheale*	3	3	3
13/C5/2/12	*Pasteurella multocida*	*P. multocida*	*P. multocida*	*P. multocida*	3	3	3
21/B66/2/12	*P. multocida*	*P. multocida*	*P. multocida*	*P. multocida*	3	3	3
15/S71/2/12	*P. multocida*	No identification	*P. multocida*	*P. multocida*	0	3	3
11 (SC0217) NCTC 12178	*P. multocida*	*P. multocida*	*P. multocida*	*P. multocida*	3	3	3
14-B6-8-12	*P. multocida*	No identification	*P. multocida*	*P. multocida*	0	3	3
22/C20/8/11	*Psychrobacter sanguinis*	No identification	No identification	*P. sanguinis*	0	0	3
KL186	*Riemerella anatipestifer*	No identification	*Riemerella* sp.	*R. anatipestifer*	0	1	3
8 (SCV0217) B1705	*R. anatipestifer*	No identification	*R. anatipestifer*	*R. anatipestifer*	0	3	3
518 (SC0217) NCTC7832	*Salmonella* Nottingham	*Salmonella* sp.	*Salmonella* sp.	*Salmonella* sp.	1	1	1
5 (SC0217) NCTC 4840	*Salmonella* Poona	*Salmonella* sp.	*Salmonella* sp.	*Salmonella* sp.	1	1	1
KL57	*Serratia marcescens*	*S. marcescens*	*S. marcescens*	*Serratia* sp.	3	3	1
1 (SC0217) stock 177	*Staphylococcus chromogenes*	*S. chromogenes*	*S. chromogenes*	*S. chromogenes*	3	3	3
2 (SC0217) NCTC 11048	*Staphylococcus intermedius*	*S. intermedius*	*S. intermedius*	*Staphylococcus* sp.	3	3	1
21/C189/8/11	*Staphylococcus* sp.	*Staphylococcus* sp.	*Staphylococcus* sp.	*Staphylococcus* sp.	1	1	1
KL92	*Staphylococcus* sp.	*Staphylococcus capitis* subsp. *capitis*	*S. capitis* subsp. *capitis*	*Staphylococcus* sp.	1	1	1
KL111	*Staphylococcus* sp.	No identification	*Staphylococcus* sp.	*Staphylococcus* sp.	0	1	1
12/S209/1/12	*Streptococcus dysgalactiae*	*Streptococcus equi* subsp. *zooepidemicus*	*S. dysgalactiae*	*S. dysgalactiae*	1	3	3
15-C289-7-12	*S. dysgalactiae*	*S. dysgalactiae*	*S. dysgalactiae*	*S. dysgalactiae*	3	3	3
15/S233/6/11	*Streptococcus* sp.	*Streptococcus* sp.	No identification	*Streptococcus* sp.	1	0	1
16/C130/7/11	*Streptococcus* sp.	*Streptococcus* sp.	No identification	*Streptococcus* sp.	1	0	1
3 (SC0217) Y2737	*Streptococcus suis*	*S. suis*	*S. suis*	*S. suis*	3	3	3
Y123	*S. suis*	*S. suis*	*S. suis*	*S. suis*	3	3	3
21-C447-04-08	*S. suis*	*S. suis*	*S. suis*	*S. suis*	3	3	3
23/P434/5/11	*Trueperella pyogenes*	*T. pyogenes*	*Arcanobacterium* sp.	*T. pyogenes*	3	1	3
FD2294	*T. pyogenes*	*T. pyogenes*	*T. pyogenes*	*T. pyogenes*	3	3	3
15-C243-7-12	*Yersinia pseudotuberculosis*	*Y. pseudotuberculosis*	*Y. pseudotuberculosis*	*Yersinia* sp.	3	3	1

3.3. MALDI-TOF MS

The number of strains which could not be identified, at least to genus level, was lower than with GEN III ($n = 12$) whilst the number of strains identified to the species level was marginally lower than with GEN III ($n = 53$). A further 35 strains were correctly identified as far as genus, with no misidentifications at the genus level since, at the time of the study, the change from *Arcanobacterium pyogene*s to *Trueperella pyogenes* had not taken place (Yassin et al., 2011). The total number of strains identifiable at least to genus was therefore 88, with an overall weighted score of 194/252.

3.4. 16S rRNA gene sequencing

Only one strain was not identifiable by 16S rRNA gene sequencing (NCTC strain 10418, *Escherichia coli*). Bearing in mind the strict criteria used for calling, 60 strains were identified to species level and 39 to genus. The overall weighted score was 219/252.

3.5. Statistical analysis

The overall performance for the three systems was compared in terms of two binary measures.

The first assumed the diagnosis was correct if the score was at least 1 (identification to genus level) and the second regarded the diagnosis as correct only if the rating score was 3 (identification to species level). McNemar's test was used to compare the proportions of correct outcomes for each pair of systems. For identification to genus level, the proportions of samples with a score of ≥1 for the GEN III and MALDI-TOF MS systems were compared and no significant difference was found between the two systems ($p = 0.69$), indicating similar performance at this level of identification. Further, 16S rRNA gene sequencing performed significantly better than both GEN III and MALDI-TOF MS ($p = 0.0005$ and 0.0034 respectively) in this respect. However, for identification to species level (scores of 3) there was no evidence of a significant difference between the GEN III, MALDI-TOF MS or 16S rRNA gene sequencing systems ($p = 0.7428$, 0.5847 and 0.2295 respectively) indicating that all three techniques displayed broadly similar performance at this level.

4. Discussion

Comparative studies on the performance of identification systems should consider the importance of identification to species level and the significance of misidentification. At a clinical or epidemiological level, erroneous or limited data may result in inappropriate treatment regimes or result in misleading statistical analysis of prevalence. Although the weighted scoring system used in this study emphasised the importance of identification to species level, no attempt was made to quantify the negative effects of misidentification, for example by negative scoring, although examples were relatively few in number. Further, evaluation of the repeatability of the methods was not the prime objective of this study.

Interestingly, rather than misidentifying at the species or, more significantly, at the genus level, GEN III tended to yield a result of "No identification", indicating that the user should seek alternative methods or re-analyse. Misidentifications at the species level ($n = 2$) were confined to *Listeria monocytogenes*, strain KL116, misidentified as *L. innocua*, and *Streptococcus dysgalactiae*, strain 12/S209/01/12, misidentified as *S. equi*. There were some differences in the system's capacity to identify all the

strains within a given species, for example in the case of *Pasteurella multocida*. This may be attributable to intra-species (strain) variation although it is likely that some between-test variation exists. Identification of isolates within the family *Pasteurellaceae* can be particularly challenging and 48 strains were represented in this study. GEN III performed well in this area attracting a total score of 111, with no known misidentifications.

For MALDI-TOF MS, misidentifications at the species level ($n = 5$) occurred for two database-related reasons; either a genuine failure to obtain a correct result (as in the case of two *Avibacterium gallinarum* strains misidentified as *A. endocarditis* and a strain of *Actinobacillus suis* misidentified as *A. equuli*) or due to changes in nomenclature of long-standing (as in the case of the two strains of *Mannheimia glucosida*, formerly *M. haemolytica* serotype A11 and reported as *M. haemolytica*). Identification within the *Pasteurellaceae* resulted in an overall score of 103, including the aforementioned misidentifications. Identification of species within certain genera of the *Pasteurellaceae* has been highlighted as a potential area of weakness for MALDI-TOF MS by other workers (Kuhnert et al., 2012). Improvements in databases will undoubtedly improve performance in the identification of so-called 'difficult-to-identify' bacteria with some workers (Bizzini et al., 2011) reporting identification rates to species level as low as 45.9%. Veloo et al. (2011) achieved species-level identification rates among anaerobes of between 51 and 61%, according to the instrument used. . The necessity to perform specific additional testing where multiple species matches occurred (with log scores ≥ 2.0) and an adjustment to the acceptance criteria where differences in log score of <0.200 occurred in multiple matches led some workers to recommend a capable bacteriologist with knowledge of taxonomy is required to interpret MALDI-TOF MS output (De Bel et al., 2011). Although 16S rRNA gene sequencing achieved the highest overall score (219/250), analysis indicated that, whilst more results were achievable at the genus level, using the strict calling criteria implemented here it was no more capable at the species level than either of the other systems, with recognised areas of weakness in certain genera (for example, *Listeria* sp.). Within the *Pasteurellaceae*, 16S rRNA gene sequencing achieved the highest score (123), with no known misidentifications. The superiority of 16S rRNA gene sequencing over conventional automated phenotypic systems (Fontana et al., 2005; Bosshard et al., 2006) is perhaps not surprising yet studies suggest that, with respect to certain genera, 16S rRNA gene sequencing may be fallible at the species level (Janda and Abbott, 2007; Mignard and Flandrois, 2006). Clearly, the input of a skilled bacteriologist is invaluable regardless of the identification system used.

Database improvement initiatives are ongoing for all three technologies, both manufacturer-led and by research facilities and interest groups around the world. Most importantly, all three methods permit the development of user-defined databases to incorporate strain diversity, an essential feature of any modern identification system. Any system lacking this facility will be quickly hampered by poor quality data and an inability to keep pace with rapid changes in existing nomenclature and the discovery of 'new' species. For 16S rRNA gene sequencing, commercial systems, such as MicroSEQ (Applied Biosystems, Foster City, USA), enable automatic searching of an extensive validated database with the facility to search publicly available databases such as GenBank. When considering use of a public database, comparison with sequence data that has not been peer-reviewed requires skilled interpretation and clearly established criteria for the acceptability of similarity thresholds (Boudewijns et al., 2006; Janda and Abbott, 2007; Woo et al., 2009). Bruker offers a similarly extensive, flexible database, although reference to spectra outside the manufacturer's database requires user-supplementation with validated strains. The possibility of sharing of spectra between laboratories offers great promise, although care will be required to maintain consistency in methods of production. GEN III is equipped with an extensive manufacturer's database which can be supplemented by reference to user-defined data. The extent to which data can be shared between laboratories is not known.

In addition to database requirements, including ease of augmentation, the choice of identification system will involve factors such as speed of turnaround, throughput, training requirements, linkage to existing information management systems and an overall cost analysis. A threshold of 5–7000 identifications per annum has been proposed to justify investment in MALDI-TOF MS (Prod'hom et al., 2012). 16S rRNA gene sequencing remains limited in its application beyond reference and research laboratories due to cost and the requirement for expertise in interpretation (Claridge, 2004). In this context, more affordable options, based on classical phenotypic analysis, may yet retain a role in smaller laboratories not able to justify or resource the initial purchase and routine maintenance costs of MALDI-TOF MS or 16S rRNA gene sequencing systems. A significant advantage of MALDI-TOF MS is the availability of results in a shorter timescale than with traditional phenotypic methods (Cherkaoui et al., 2010) or 16S rRNA gene sequencing, although this assumes that a sufficiently high 'score value', indicating the required level of confidence in the result, is obtained for a single species without the need to resort to repeat runs or alteration of the sample matrix.

5. Conclusion

The relative strengths and weaknesses demonstrated in all three systems highlight the contribution made by the trained bacteriologist in two key areas: Firstly, their ability to select the most appropriate technique for the unknown isolate, using prior knowledge of the fundamental characteristics of the organism (for example, case-history, site of isolation, and primary features such as gross and microscopic morphology) and secondly, their ability to cross-refer the output from the system with these fundamental characteristics. The first skill could be considered 'triaging' and could save time, money and, potentially, prevent the reporting of an erroneous result. The second skill could be referred to as 'sense-checking'. Does the output from the machine correspond with all available data, including phenotype and case history? Thus, although recent advances in technology may superficially indicate a gradual decline in the role of the bacteriologist, it could be argued that their fundamental knowledge has never been more important in assuring that results from the laboratory remain of the highest quality.

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