Table 2. Differentiation of isolates.

Test	*S. lugdunensis*	*S. warneri*
Site of infection	Wound	Bacteraemia
Catalase	Pos	Pos
Coagulase (slide)	Neg*	Neg
Coagulase (tube)	Neg	Neg
DNase	Neg	Neg
API profile	2716150	6230113
Penicillin	R	R
Erythromycin	R	R
Methicillin	S	S
Tetracycline	S	R
Vancomycin	S	S

*some auto-agglutination

full identification would aid treatment, or when the Staphylase test and DNase agar results are discordant. Susceptibility testing now follows NCCLS guidelines and hopefully misreporting of sensitivity patterns is now avoided.

References

1 Qureshi AH, Hannan A. The prevalence and susceptibility patterns of MRSA. *Pak J Pathol* 1991; **2**: 41–4.
2 Hafeez A. Hospital and community-acquired MRSA. *Inf Dis J Pak* 1996; **3**: 12–3.
3 Siddiquei GM, Karamat KA, Hannan A. Prevalence of MRSA: a study at PAF Hospital, Sargodha. *Pak J Pathol* 1999; **10**: 26–8.
4 Bukhari MH, Iqbal A, Khatoon N *et al*. A laboratory study of susceptibility of MRSA. *Pak J Med Sci* 2004; **20**: 229–33.
5 Tenover CF, Lancaster MV, Hill CB *et al*. Characterisation of staphylococci with reduced susceptibilities to vancomycin and other glycopeptides. *J Clin Microbiol* 1998; **34**: 1020–7.
6 Smith TL, Pearson ML, Wilcox KR *et al*. Emergence of vancomycin resistance in *Staphylococcus aureus*. *N Engl J Med* 1999; **340**: 493–501.
7 Schimpff SC, Young VM, Greene WH *et al*. Origin of infection in acute non-lymphocytic leukaemia: significance of hospital acquisition of potential pathogens. *Arch Intern Med* 1972; **77**: 707–14.
8 Bauer AN, Kirby WMM, Sherris JC, Tuck M. Antibiotic susceptibility testing by a standardised single disc method. *Am J Clin Pathol* 1966; **45**: 493–6.
9 National Committee for Clinical Laboratory Standards. *Performance standards for the antimicrobial disc susceptibilty test* (4th edn). M2-T4, PA: Villanova, 1988.
10 Marples RR. Laboratory characteristics of methicillin-resistant *Staphylococcus aureus* (MRSA) strains. *PHLS Microbiol Digest* 1993; **10**: 218–21.
11 Kloos WE, Bannerman TL. Update and clinical significance of CoNS. *Clin Microbiol Rev* 1994; **7**: 117–40.
12 Mortlock S. Bacteraemia among patients attending a cancer hospital in Lahore, Pakistan. *Br J Biomed Sci* 2000; **57**: 119–25.
13 Vandenesch F, Etienne J, Reverdy ME, Eykyn SJ. Endocarditis due to *Staphylococcus lugdunensis*: report of 11 cases and review. *Clin Infect Dis* 1993; **17**: 871–6.
14 Ling ML, Yeo M. *Staphylococcus lugdunensis*: report of first case of skin and soft tissue infection in Singapore. *Singapore Med J* 2000; **41**: 177–8.
15 Kamath U, Singer C, Isenberg HD. Clinical significance of *Staphylococcus warneri* bacteraemia. *J Clin Microbiol* 1992; **30**: 261–4.
16 Center KJ, Reboli AC, Hubler R, Rodgers GL, Long SS. Decreased vancomycin susceptibility of CoNS in a neonatal ICU: evidence of spread of *Staphylococcus warneri*. *J Clin Microbiol* 2003; **41**: 4660–5.

Antibiotic resistance and identification of uncommon Gram-negative bacteria isolated from sputum of adult patients with cystic fibrosis

C. GILLOW*, A SHAW*, J. E. MOORE*, J. S. ELBORN†‡ and P. G. MURPHY*

Northern Ireland Public Health Laboratory, Department of Bacteriology; †Northern Ireland Regional Adult Cystic Fibrosis Centre, Belfast City Hospital; and ‡Department of Respiratory Medicine, Queen's University, Level 8, Belfast City Hospital, Belfast, Northern Ireland, UK

The organisms *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex are well established and recognised as major causes of lung infection in cystic fibrosis (CF) patients.[1] They are important in CF lung pathology as they initiate a vicious cycle of infection and inflammation, leading to persistent lung damage, which is mediated via the host's own immune system. The clinical management of these organisms is highly problematic in terms of extensive antibiotic multidrug resistance and infection control. Descriptions of other unusual Gram-negative bacterial species colonising the lungs of CF patients are limited and little is known about the negative contributions such unusual organisms make to the pathogenesis of the disease, including the burden of antibiotic resistance.

The Northern Ireland Regional Adult Cystic Fibrosis Unit has recently identified several bacterial species as a result of a full biochemical speciation protocol. These species also show considerable antibiotic resistance and clinical persistence/recurrence, and thus this study aims to perform an enhanced investigation of their antibiotic resistance profiles, as well as the ease with which they can be identified.

Correspondence to: Dr. John E. Moore
Northern Ireland Public Health Laboratory, Department of Bacteriology, Belfast City Hospital, Belfast BT9 7AD, Northern Ireland,
Email: jemoore@niphl.dnet.co.uk

Table 1. Phenotypic identification of isolates examined in this study, as well as antibiotic disc susceptibility to 12 antibiotic agents.

			Antibiotic susceptibility											
Reference	Species	API profile	Cip	Co	Caz	Az	Atm	Imi	G	Tob	Mem	Cpo	Taz	Tem
CF/96/01	*Alcaligenes xylosoxidans*	1040477	R	R	R	R	R	R	R	R	R	R	MR	R
CF/96/09	*Pseudomonas stutzeri*	1144455	R	S	R	R	R	R	MR	S	R	R	R	R
CF/96/16	*Aeromonas salmonicida*	5447704	MR	S	S	MR	S	S	S	S	S	S	S	S
CF/96/25	*Burkholderia cepacia* complex Grew on *B. cepacia* agar: R to polymyxin. PCR-confirmed as *B. cepacia* complex	No profile.	R	R	S	S	R	R	R	R	S	R	S	S
CF/96/31	*Stenotrophomonas maltophilia*	1472345	R	R	S	S	R	R	R	R	S	S	S	R
CF/96/32	*Pseudomonas fluorescens*	0157555	S	S	S	MR	R	R	S	S	S	S	S	R
CF/96/40	*Alcaligenes xylosoxidans*	1040457	R	R	S	S	R	S	R	R	S	R	S	
CF/96/50	*Aeromonas* sp.	NA												
CF/96/51	*Acinetobacter lwoffi*	5144555	S	S	S	S	S	S	S	S	S	S	S	S
CF/96/54	*Stenotrophomonas maltophilia*	1472345	R	S	MR	R	R	R	R	R	R	R	MR	R
CF/96/59	*Chromobacterium violaceum*	5144555	S	S	S	S	S	R	S	S	S	S	S	S
CF/96/63	*Aeromonas salmonicida*	100404	R	R	R	R	R	MR	R	R	R	R	MR	R
CF/96/64	CDC gp IV C-2 organism	0200455	R	S	R	R	R	R	R	S	R	MR	S	R
CF/96/67	*Ralstonia pickettii*	1340465	R	S	R	R	R	R	R	S	R	R	S	S
CF/96/68	*Pseudomonas putida*	0040555	R	S	R	R	R	R	R	S	R	R	S	S

Ciprofloxacin (Cip), colistin sulphate (Co), ceftazidime (Caz), azlocillin (Az), aztreonam (Atm), imipenem (Imi), gentamicin (G), tobramycin (Tob), meropenem (Mem), cefpirome (Cpo), tazobactam (Taz) and temocillin (Tem). S=sensitive, MR= moderately resistant, R=resistant.

Fifteen isolates of interest to the study were chosen from a stored frozen (–80^{0}C) batch of a number of collected sputum samples submitted from various adult CF patients (Table 1) over several years. It is worth emphasising that all isolates were collected from patients during active clinical infection. Mixed colonies were separated and other species (eg staphylococci, streptococci, *P. aeruginosa*) were separated following their identification, using routine characterisation tests (eg Gram stain, oxidase and catalase tests, visual characteristics etc.).[2,3] Thus, the final collection consisted of isolates in pure culture selected to satisfy the aims of the investigation.

Pure cultures of each isolate were grown and specific organisms were identified using the API 20 NE (bioMérieux,

Table 2. Minimum inhibitory concentration (μg/mL) of 15 isolates against tazobactam, ceftazidime, imipenem and meropenem.

		Minimum inhibitory concentration			
Reference	Species	Tazobactam	Ceftazidime	Imipenem	Meropenem
CF/96/01	*Alcaligenes xylosoxidans*	64	24	>32	>32
CF/96/09	*Pseudomonas stutzeri*	>256	>256	>32	>32
CF/96/16	*Aeromonas salmonicida*	0.25	0.047	0.64	0.012
CF/96/25	*Burkholderia cepacia* complex	8	2	>32	3
CF/96/31	*Stenotrophomonas maltophilia*	8	2	>32	2
CF/96/32	*Pseudomonas fluorescens*	2	1.5	3	2
CF/96/40	*Alcaligenes xylosoxidans*	0.38	3	3	0.125
CF/96/50	*Aeromonas* sp.	0.19	0.094	0.094	0.023
CF/96/51	*Acinetobacter iwoffi*	<0.016	1.5	0.032	0.064
CF/96/54	*Stenotrophomonas maltophilia*	32	3	>32	>32
CF/96/59	*Chromobacterium violaceum*	0.25	1	>32	0.094
CF/96/63	*Aeromonas salmonicida*	16	16	1.5	32
CF/96/64	CDCgp iv c-2 organism	12	4	12	6
CF/96/67	*Ralstonia pickettii*	0.75	3	>32	6
CF/96/68	*Pseudomonas putida*	0.75	2	>32	4

BRITISH JOURNAL OF BIOMEDICAL SCIENCE 2005 **62 (1)**

France) and their API profile was recorded with sample collection dates. The polymerase chain reaction (PCR) was employed in the investigation and used to confirm that the resistant organisms were not *B. cepacia*, as described by Campbell *et al*.[4]

Modified Stokes' technique[3] was employed for routine disc susceptibility testing using ciprofloxacin (Cip), colistin sulphate (Co), ceftazidime (Caz), azlocillin (Az), aztreonam (Atm), imipenem (Imi), gentamicin (G), tobramycin (Tob), meropenem (Mem), cefpirome (Cpo), tazobactam (Taz) and temocillin (Tem). The findings were recorded as either sensitive (S), of moderate resistance (MR) or resistant (R) to these drugs (Table 1). Minimum inhibitory concentrations (MICs) were determined using the PDM Epsilometer E-test method (AB Biodisk, Solna, Sweden).

A 10^7 dilution of each microorganism in 3 mL saline was prepared and inoculated on sheep blood agar (150 mm plates) and left to dry for 2 h. A concentration gradient strip of each of the four antimicrobial agents used (meropenem, tazobactam, ceftazidime and imipenem) was placed carefully on each plate and the 15 plates were incubated at 37ºC for 24 h. Each plate was assessed for growth and the points at which bacterial growth showed complete inhibition for each antimicrobial agent were recorded as the MICs after incubation.

Phenotypic identification of the 15 isolates is shown in Table 1. All isolates were Gram-negative and belonged to one of nine genera represented. All isolates were identified using phenotypic identification systems, with the exception of isolate CF/96/25, which was positive when examined with the *B. cepacia* complex PCR. All other isolates were PCR-negative when challenged with these specific primers.

Susceptibility of the isolates to the 12 antibiotic agents tested is shown in Table 1. *Alcaligenes xylosoxidans* (API 1040477) and *Aeromonas salmonicida* (API 100404) were the most resistant of the species, being sensitive to none of the 12 antibiotics examined. Another strain of *Alcaligenes xylosoxidans* (API 1040457) was resistant to six antibiotics, but another *Aeromonas salmonicida* (API 5447704) isolate was resistant to only two antibiotic agents. The bacterium *Stenotrophomonas maltophilia* (API 1472345) in a 34-year-old patient was extensively resistant (i.e., resistant to 11 drugs), but interestingly another isolate of the same species from an 11-year-old patient was resistant to only seven antibiotics. An unusual organism, classified as a CDC group IV c-2 bacterium, was shown to be resistant to nine of the agents tested. The most sensitive of the species was confirmed to be the *Chromobacterium violaceum* (API 5244555) isolate, which was sensitive to 11 of the 12 antibiotics tested.

From the disc sensitivity tests, the ability of the antibiotics used to inhibit these relatively resistant organisms varied as follows (from the most potent to the weakest): tazobactam (10 organisms sensitive), colistin sulphate (nine sensitive), meropenem/tobramycin/ceftazidime (seven sensitive), temocillin (six sensitive), azlocillin/cefpirome (five sensitive), gentamicin (four sensitive), ciprofloxacin/aztreonam (three sensitive) and imipenem (two sensitive).

Table 2 shows the quantitative results of the E-test performed using meropenem, imipenem, ceftazidime and tazobactam. Minimum inhibitory concentrations (i.e., the smallest amount of antibiotic needed [in μg/mL] to completely inhibit bacterial growth) were found for each of the 15 organisms cultured. Overall, organisms ranging from the most to the least susceptible to these drugs were as follows: *Chromobacterium violaceum, Aeromonas salmonicida, P. fluorescens, Acinetobacter Iwoffii, Alcaligenes xylosoxidans, Burkholderia cepacia* complex organism, CDC gp IV c-2 organism and *Stenotrophomonas maltophilia*. The most resistant organisms were *P. stutzeri, P. putida* and *Ralstonia pickettii*.

The antibiotic gradient strip used for ceftazidime and tazobactam was 0–256 μg/mL. Eight organisms were more strongly inhibited by ceftazidime, two were more strongly inhibited by tazobactam and five were inhibited equally by both. For meropenem and imipenem the gradient strip was 0–32 μg/mL, and these two were compared separately. Six organisms were more strongly inhibited by meropenem, three were more strongly inhibited by imipenem and six were affected equally. Although meropenem was the most active agent when compared with tazobactam, imipenem and ceftazidime, after consideration of the full antibiogram data using disc susceptibility testing, colistin was the most active.

Presently, there are limited published data on the identification, pathogenic role and antibiotic resistance of uncommon organisms, such as those described in this study, in the lungs of CF patients. It is axiomatic in clinical microbiology that the more effort expended in looking for different species the more are found. The traditional and obvious pathogens (i.e., *P. aeruginosa* and *B. cenocepacia*) are routinely identified because they are routinely sought.[5–7]

This study shows that an extended bacteriological protocol will uncover many other related species, some of which may contribute to lung damage directly or possibly act as co-pathogens in CF. With the exception of the *B. cepacia* complex organism, all the organisms, which are relatively uncommon compared to the major bacterial pathogens (e.g., *P. aeruginosa, B. cenocepacia, Staphylococcus aureus* and *Haemophilus influenzae*) associated with CF, were relatively easily to identify by conventional phenotypic methods and thus did not require the use of molecular methods.

The assessment of a culture as either a pathogen or a colonising non-pathogen is often difficult. Existence of these related species, particulary those with broad antibiotic resistance, should not be ignored.[7] Additionally, the resistant nature of these organisms presents not only infection control issues but also a therapeutic dilemma, in terms of finding effective antibiotic treatment, when such organisms are deemed to be clinically significant. □

CG was supported by a Summer Elective Bursary from The Royal College of Pathologists.

References

1 Miller MB, Gilligan PH. Laboratory aspects of management of chronic pulmonary infections in patients with cystic fibrosis. *J Clin Microbiol* 2003; **41**: 4009–15.

2 *Cowan and Steel's identification of medical bacteria* (2nd edn). Cambridge: University Press, 1974.

3 Stokes and Ridgway. *Clinical microbiology* (6th edn). London: Arnold, 1987.

4 Campbell PW, Phillips JA, Heidecker GJ *et al*. Detection of *Pseudomonas (Burkholderia) cepacia* using PCR. *Pediatr Pulmonol* 1995; **20**: 44–9.
5 Lipuma JJ. Update on the *Burkholderia cepacia* complex. *Curr Opin Pulm Med* 2005; **11**: 528–33.
6 Canton R, Cobos N, de Gracia J *et al*., on behalf of the Spanish Consensus Group for Antimicrobial Therapy in the Cystic Fibrosis Patient. Antimicrobial therapy for pulmonary pathogenic colonisation and infection by *Pseudomonas aeruginosa* in cystic fibrosis patients. *Clin Microbiol Infect*. 2005: **11**: 690–703.
7 Conway SP, Brownlee KG, Denton M, Peckham DG. Antibiotic treatment of multidrug-resistant organisms in cystic fibrosis. *Am J Respir Med* 2003; **2**: 321–32.

Analysis of 16S-23S intergenic spacer regions of the rRNA operons in *Tsukamurella pulmonis*

J. E. MOORE*, J XU*† and B. C. MILLAR*

*Northern Ireland Public Health Laboratory, Department of Bacteriology, Belfast City Hospital, Lisburn Road, Belfast, Northern Ireland BT9 7AD, UK; and †Department of Pathogenic Biology, Xian Jiatong University, Xi'an, Shaanxi Province, People's Republic of China

The genus *Tsukamurella* was first described by Collins *et al*.[1] in 1988 following the reclassification and further molecular and phenotypic charactization of *Gordana aurantiaca, Rhodococcus aurantiaca* and other related organisms including *Corynebacterium paurometabola*, which were distinct from the other mycolic acid-containing actinomycetes The genus is phylogenetically related to the genera *Nocardia, Gordonia, Streptomyces, Rhodococcus, Corynebacterium* and *Mycobacterium* and taxonomically comprises of at least eight described species (www.bacterio.cict.fr) including *Tsukamurella inchonensis, T. paurometabola, T. pseudospumae, T. pulmonis, T. spumae, T. strandjordii, T. tyrosinosolvens* and *T. wratislaviensis.*

Tsukamurella infections have emerged over the last decade as a rare but significant cause of serious infection in immunocompromised individuals. For a comprehensive review of these cases see Schwartz *et al*.[2] Further to this review, an additional case of line-related sepsis has been shown,[3] as well as three cases of *Tsukamurella* conjunctivitis, which were treated successfully after 10 days with polymyxin B–neomycin or chloramphenicol eyedrops.[4] Concensus opinion from published reports indicates that underlying serious disease, including haematological malignancy where the patient is immunocompromised, combined with the use of indwelling catheters are important risk factors for infection with this genus.[5] Therefore, such patients are susceptible populations for *Tsukamurella* infection.

Correspondence to Dr. John E. Moore
Email: jemoore@niphl.dnet.co.uk

Little information is available for the 16S-23S intergenic spacer regions of the ribosomal RNA (rRNA) operons of members of *Tsukamurella* species. Therefore, this study aims to examine this region in a wild type isolated from a patient.

An isolate of *T. pulmonis* was isolated from the sputum of a female patient with suspected tuberculosis. The isolate was purified on Colombia agar base (CM331, Oxoid, Basingstoke, UK) supplemented with defibrinated horse blood 5% (v/v; Oxoid) and incubated at 37^{0}C for 48 h. Genomic DNA was extracted from a single colony with the Roche High Purity PCR Template kit (Roche Diagnostics, UK), in accordance with the manufacturer's instructions.

All reaction mixes were set up in a PCR hood in a separate room from that used to extract DNA and the amplification and post-PCR room, in order to minimise contamination in accordance with the laboratory guidelines of Millar *et al*.[6] Reaction mixes (50 μL) contained10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 200 μmol/L (each) dATP, dCTP, dGTP and dTTP, 1.25 units *Thermus aquaticus* (*Taq*) DNA polymerase (Amplitaq, Perkin Elmer), 0.2 μmol/L (each) 16S-23S rRNA region primers (forward primer P11P[7] [5′-GAG GAA GGT GGG GAT GAC GT-3']; reverse primer 23S [5′- CCA AGG GCA TCC ACC CGT –3′]), together with 4 μL DNA template containing approximately 50 ng DNA/mL extract.

Following a 'hot start', the reaction mixtures were subjected to the following thermal cycling parameters in a Perkin Elmer 2400 thermocycler: 96^{0}C for 3 min followed by 40 cycles of 96^{0}C for 1 min, 55^{0}C for 1min, 72^{0}C for 1 min, and a final extension at 72^{0}C for 10 min. During each run, molecular grade water (Biowhittaker, Maryland, USA) instead of DNA was included randomly as a negative control, and *Staphylococcus aureus* DNA was included as a positive control.

Following amplification, samples (15 μL) were removed, electrophoresed (80V, 45 min) in 2% agarose gel (w/v; Gibco, Paisley, UK) in TAE buffer (40 mmol/L Tris, 20 mmol/L acetic acid, 1 mmol/L EDTA [pH 8.3]) and stained with ethidium bromide (5 μg/100 mL). Gels were visualised under ultraviolet (UV) illumination with a gel image analysis system (UVP Products, England) and all images were archived as digital (*.bmp) graphic files.

Amplicons for sequencing were purified with a QIAquick PCR purification kit (Qiagen, UK) and eluted in Tris-HCl (10 mmol/L, pH 8.5) prior to sequencing. Cy-5′-labelled primers (P11P and 23S) were prepared and used for sequencing in the forward and reverse direction. Amplicons were sequenced on the ALF Express II (Amersham-Pharmacia, Buckinghamshire, England) using the Thermo Sequenase fluorescence-labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, UK; RPN 2438): 96^{0}C for 1 min, followed by 25 cycles of 96^{0}C for 10 sec, 50^{0}C for 5 sec, 60^{0}C for 5 sec, followed by a 40C hold. The resulting sequences from both reactions were aligned and compared with those stored in GenBank using the BLASTn alignment software (www.blast.genome.ad.jp/). Sequence homology identity was determined in accordance with criteria described previously.[8]

Polymerase chain reaction amplification of the isolate with the primer pair P11P/23S gave an amplicon of approximately 2000 bp, encompassing the complete 16S rRNA gene (1499