FINGERPRINTING *ACINETOBACTER* STRAINS FROM CLINICAL SOURCES BY NUMERICAL ANALYSIS OF ELECTROPHORETIC PROTEIN PATTERNS

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SUMMARY. A total of 57 strains of *Acinetobacter calcoaceticus* was fingerprinted by SDS-PAGE of cellular protein. All strains were also examined by conventional, API and N/F-Tek methods, and antibiotic sensitivity patterns were determined. In general, using the API 20E and N/F-Tek methods, it was possible to assign isolates of *A. calcoaceticus* to the two accepted “biotypes” or “varieties”, *A.c. anitratus* and *A.c. lwoffii*, but these methods did not offer useful subdivision of the biotypes. Gel electrophoresis permitted subdivision of the strains into clusters in a manner which suggests that the technique may be valuable in typing strains isolated during outbreaks of infection in hospital.

INTRODUCTION

The genus *Acinetobacter* consists of a single species *calcoaceticus* divided into two morphologically and biochemically dissimilar biotypes, *anitratus* and *lwoffii* (International Committee on Nomenclature of Bacteria, 1971).

Many epithets have been applied to *Acinetobacter* since the first report by Beijerinck (1911), who used the name *Micrococcus calcoaceticus*. Other names include: *Diplococcus mucosus*, *Moraxella glucidolytica*, *Neisseria winogradskyi*, *Lingelsheimia anitrata*, *Alcaligenes haemolysans*, *Mima polymorpha*, *Herellea vaginicola*, *Bacterium anitratum* and *Achromobacter anitratus*. The genus *Acinetobacter* was described by Brisou and Prévot (1954).

That the genus has created problems of identification is evidenced by (i) its present name *Acinetobacter*, which simply means a non-motile rod, (ii) the species name *calcoaceticus* which is derived from the use by Beijerinck of a medium enriched with calcium acetate, and (iii) the biotype *anitratus* which takes its name from the apparent inability of the organism to reduce nitrate to nitrite in usual laboratory media. This mixture of obscure names and negative biochemical characteristics reflects the bacteriologists' inability to satisfactorily identify and name the genus.

Despite the initial difficulty in ascribing an organism to the genus *Acinetobacter*, there is no particular difficulty in differentiating between the two biotypes *lwoffii* and *anitratus* using media common in routine diagnostic work. Biotype *anitratus* is oxidative and can usually be identified by API 20E (API, Basingstoke, Hants) or

UniN/F-Tek (Flow Laboratories, Irvine, Scotland) plus a few ancillary tests, e.g., motility, catalase, oxidase, growth at 42°C, cell morphology by Gram's stain and colony morphology on a medium such as Cysteine Lactose Electrolyte-Deficient medium (CLED). Biotype *lwoffi*, on the other hand, neither oxidises nor ferments glucose in the OF medium of Hugh and Leifson (Cowan, 1974) but does often produce an alkaline reaction at the surface of the unsealed tube. This organism is almost inert and is therefore difficult to place in the genus *Acinetobacter* unless its non-reactivity is relied upon as a differential characteristic. Both biotypes are oxidase-negative and catalase-positive, fail to grow in the absence of oxygen, hydrolyse Tween 80 and assimilate some carbohydrates, e.g., succinate, fumarate, DL-malate and DL-lactate (Martin *et al.*, 1981).

The problem which many workers have in identifying the organisms in the genus *Acinetobacter* is exemplified by the large number sent to the National Collection of Type Cultures (NCTC) each year for identification. Indeed, in our experience organisms in the genus *Acinetobacter* (especially *A.c. lwoffi*) are frequently mis-identified in clinical laboratories.

The integrity of the genus has been confirmed by the work of Juni (1972) who used a strain of *Acinetobacter* which was competent for genetic transformation to prove the genetic relatedness of 265 strains having the characteristics of *Acinetobacter* but with a variety of synonyms.

There have been numerous reports during the last 40 years dealing with the association between *Acinetobacter* and infection. Most prominent are the reports of meningitis, septicaemia and infected wounds (De Bord, 1948; Torregrosa and Ortiz, 1961; Retalliau *et al.*, 1979; Morgan and Hart, 1982; Hoppe, Potel and Malottke, 1983).

Whilst the role of the organism as an opportunistic pathogen is undisputed, its significance as a primary pathogen is doubtful, and its frequent occurrence in soil, water (Baumann, 1968) and human skin (Taplin, Rebell and Zaias, 1963; Somerville and Noble, 1970; Al Khoja and Darrell, 1979; Gaughan, White and Noble, 1979) should serve as a caution when *Acinetobacter* is proposed as the cause of an infection (Hoppe *et al.*, 1983). However, there have been well documented outbreaks of nosocomial infection caused by *Acinetobacter* and these have been of particular concern because of the pattern of multiple resistance so often seen (Buxton *et al.*, 1978; French *et al.*, 1980; Holton, 1982). Biotype *anitratus* is frequently resistant to gentamicin, tobramycin and tetracycline, although nearly always sensitive to minocycline. Biotype *lwoffi*, however, much less frequently displays multiple resistance and resistance to aminoglycosides, although it is often resistant to trimethoprim, clindamycin, cephalexin and penicillin. It is the pattern of resistance and the difficulty of identification that has prompted this study, which is concerned with the "fingerprinting" of *Acinetobacter* strains by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE).

MATERIALS AND METHODS

Selection and sources of strains. Sixty one strains were used: 34 *Acinetobacter calcoaceticus* var. *anitratus*, 23 *A. calcoaceticus* var. *lwoffi*, two *Alcaligenes denitrificans*, and one strain each of *Pseudomonas pseudocaligenes* and *Branhamella catarrhalis*. No particular criteria were used in their selection. Table I shows their sources: NCTC (reference and "acquisition" strains, the

TABLE I
Source and identification of strains

Identification	Strain no.	Laboratory no.	Source
<i>Acinetobacter calcoaceticus anitratus</i>	NCTC7250	54	Cerebrospinal fluid
	NCTC7412	57	Urine
	NCTC7422	58	Leg burn
	NCTC7461	59	Tuberculous sinus
	NCTC7462	60	Tuberculous sinus
	NCTC7363	55	Source unknown
	NCTC7364	56	Source unknown
	NCTC7844	62	Source unknown
	NCTC9427	63	Meningitis
	NCTC10290	64	Skin abscess
	NCTC10291	65	Infants liver
	NCTC10292	66	Source unknown
	R898	43	Clinical isolate (source unknown)
	R1194	45	Clinical isolate (source unknown)
	R37981	49	Catheterised penis
	R40297	52	Eye swab
	A2/82	11	Abdominal abscess
	A30/82	9	Blood culture
	A34/81	8	Mouth ulcer
	A67/81	5	Urine
	A69/82	4	Sputum
	A86/81	1	Lamb
	A123/81	21	Blood culture
	A135/81	20	Wound
	A145/82	19	Sputum
	A181/81	18	Wound
	A231/81	15	Cerebrospinal fluid
	A285/81	14	Ear
	A364/80	23	Wound swab (leg)
	A383/81	24	Urine
	A493/81	25	Blood culture
	A560/81	26	Experimental contaminant
	A695/80	27	Sputum
	A823/80	29	Pus
<i>Acinetobacter calcoaceticus lwoffii</i>	NCTC5867	53	Source unknown
	W235A	35	Axilla
	W235N	34	Nasal
	W240A2	36	Axilla
	W275A1	37	Axilla
	W284(1)	38	Axilla
	RO/E	41	Otitis externa
	RBux	40	Peritoneal dialysis fluid
	R050	42	Clinical isolate (site unknown)
	R991	44	Clinical isolate (site unknown)
	R22334	46	Nail clipping
	R23744	47	Blood culture
	R23938	48	Hickman catheter
	R38168	51	Clinical isolate (site unknown)
	A18/82	10	High vaginal swab
	A34/82	7	Cerebrospinal fluid
	A75/82	3	Wound swab
	A84/81	2	Hydraulic fluid
	A96/81	22	Urine
	A188/82	16	Blood culture
	A295/81	13	Wound swab
	A377/81	12	Eye swab
	A748/80	30	Eye swab
<i>Alcaligenes denitrificans</i>	W187A2	32	
	W187N3	31	
<i>Pseudomonas pseudoalcaligenes</i>	W230A2	33	
<i>Branhamella catarrhalis</i>	NCTC3622	6, 61, 28, 50, 39, 17	

A = "acquisition strains" sent by clinical laboratories to NCTC for identification.

R = isolates received from a single hospital.

W = strains from healthy children, isolated during other studies.

latter believed to be epidemiologically independent), patients in a single hospital, and healthy school children. In addition, one ovine strain and one isolate from hydraulic fluid were included.

All strains were subjected to a range of biochemical tests which included the use of API 20E and N/F-Tek kits. Strains were also tested for their sensitivity to 12 antibiotics by the Kirby Bauer test method with disks from Oxoid Laboratories.

Strains were designated as *Acinetobacter calcoaceticus* according to the criteria of API 20E and N/F-Tek kits which gave comparable results. All are gram-negative cocco-bacilli. Using the API 20E system, all are negative for ONPG, oxidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, production of nitrite and nitrogen from nitrate, H₂S production, tryptophane deaminase, indole formation, and utilisation of mannitol, inositol, sorbitol, rhamnose, and amygdalin; all are non-motile and non-fermentative; VP is positive in 11% of each biotype. The biotypes differ in the following tests: citrate utilisation, *anitratus* 71% positive and *lwoffii* 11% positive; urease, 0% and 4%; gelatinase, 14% and 4%; glucose utilisation, 86% and 2%; sucrose utilisation, 3% and 0%; melibiose utilisation, 89% and 2%; arabinose utilisation, 66% and 4%; nitrite from nitrate, 6% and 4%; growth on MacConkey agar, 89% and 83%; oxidative in OF system, 100% and 21% respectively. It is evident that there is scope for considerable variation within the biotypes. *Acinetobacter* strains differ principally from species of *Neisseria* (*Branhamella*), *Pseudomonas*, *Achromobacter*, *Alcaligenes* and *Moraxella* in that the latter are all oxidase positive.

Culture media and growth conditions. Strains were grown on CLED agar (Oxoid CM30) for 48 h at 37°C before biochemical and sensitivity testing and before harvesting for electrophoresis.

Preparation of protein extracts. Approximately 0.3 g wet weight of cells was harvested and washed by centrifugation in 4 ml of distilled water. The pellets were resuspended in equal volumes of sample treatment buffer (0.0625M Tris-HCl pH 6.8, SDS 2%, glycerol 10%, 2-mercaptoethanol 5%), heated at 100°C in an oven for 10 min and then centrifuged at 38 000 *g* for 30 min at 4°C in an HS 18 centrifuge (MSE). The supernates were stored at -20°C.

Electrophoresis. Patterns of whole-cell proteins were prepared in 10% homogeneous polyacrylamide gels in the presence of SDS using a rigorously standardised method based on that of Laemmli (1970), described by Jackman (1983) and Jackman, Feltham and Sneath (1983). The resulting patterns (100 mm in length, dried, and stained with Coomassie Blue) were scanned by a densitometer interfaced with a microcomputer as described by Jackman (1982).

Analysis of patterns. Numerical taxonomy, based on the patterns, was performed using the Correlation coefficient and UPGMA clustering (Sneath and Sokal, 1973) and programs described by Jackman *et al.* (1983).

RESULTS

The use of API 20E and N/F-Tek methods for identification generally resulted in a satisfactory classification into the biotypes *anitratus* and *lwoffii*, but failed to provide discrimination at a sub-biotype level for epidemiological purposes.

Separation of whole-cell proteins by SDS-PAGE produced patterns containing up to 40 visually detectable bands (fig. 1). Densitometric scanning resolved more than 25 distinct peaks. The reproducibility of the technique was measured by running a replicate protein sample on each gel used in the experiment. The mean similarity between replicates was 91%, equivalent to a test error of about 5% which is within the limits suggested by Sneath and Johnson (1972).

Differentiation between biotypes *lwoffii* and *anitratus* was clear cut in several of the groups resolved by the similarity matrix at above the 85% level (fig. 2). This supports the concept of two biochemically dissimilar groups which have a close genetic relationship and consequently fall within the same species *A. calcoaceticus*. Three groups contained only strains identified as *A. c. anitratus* and a further three contained only strains of *A. c. lwoffii*. Group 2B contained one strain of *A. c. lwoffii* amongst eight

FIG. 1.—Gel of protein extracts of one strain of *Brachnamella catarrhalis* (centre) and ten NCTC strains of *A. calcoaceticus* var. *anitratus*, stained with Coomassie Blue.

of *A.c. anitratus* whilst group 4B contained two strains of *A.c. lwoffii* and four of *A.c. anitratus* (table II).

Numerical taxonomy revealed 11 groups clustering above 85% similarity as shown in the dendrogram (fig. 2).

Group 1 consisted of four NCTC strains of *A.c. anitratus* that were biochemically similar. They shared antibiotic resistance to sulphonamide, trimethoprim, cephalixin, clindamycin, ampicillin and penicillin. Three of the four shared intermediate resistance to erythromycin. The ovine strain (A86/81, no. 1) showed 78% similarity to this group, but was biochemically different and lacked resistance to a number of antibiotics.

Group 2A consisted of eight strains of *A.c. anitratus* from a variety of clinical

FIG. 2.—Dendrogram showing relationship between strains based on PAGE. Strains within each group (1, 2A, 2B, 2C, 3, 4A, 4B, 5, 6, 7, 8) have at least 85% similarity. (See table I for identifications of strains.)

sources; and these again were biochemically similar, apart from three strains which lacked urease. Five of these strains were resistant to gentamicin and tetracycline, and all but one were resistant to erythromycin. In other respects their sensitivity patterns resembled those of group-1 strains.

Group 2B had an 84% similarity to group 2A but showed much greater biochemical diversity and included one strain of *A.c. lwoffii*. Additionally one strain showed greater antibiotic resistance including gentamicin and tetracycline. Other strains had resistance patterns similar to those of group-1 strains.

Group 2C consisted of three strains of *A.c. lwoffii* which were almost inert biochemically. They were generally more sensitive to antibiotics and in particular lacked resistance to sulphonamides. Strain 84/81 (no. 2), which was isolated from hydraulic fluid and had a resistance pattern of the group-1 type, had 81% similarity to groups 2A, 2B and 2C.

Group 3 consisted of one strain of *A.c. anitratus* and one of *A.c. lwoffii*.

Group 4A contained four biochemically inert strains which were all identified as *A.c. lwoffii*. They lacked resistance to sulphonamide but were resistant to trimethoprim and clindamycin. One strain was sensitive to penicillin.

Group 4B contained strains of both *A.c. anitratus* and *A.c. lwoffii*. One strain was from the axilla of a healthy child, and one from an abdominal abscess. The other four isolates were from a single hospital; all four were resistant to tobramycin, and one showed intermediate resistance to amikacin.

Group 5 consisted of very sensitive strains of *A.c. lwoffii*, all of which were biochemically inert. Four of these strains were obtained from the axillae or noses of healthy school children and the other was a clinical isolate from a patient with a Hickman catheter.

Group 6 strains were all *A.c. anitratus*, and biochemically similar. Two were resistant to tetracycline, gentamicin and tobramycin. One other strain showed intermediate sensitivity to tetracycline but was sensitive to gentamicin and tobramycin.

In group 7 were two isolates of *Alc. denitrificans*, from the nose and axillae of a healthy school child, showing 98% similarity. They had sensitivity patterns similar to those of the *A.c. lwoffii* strains in Group 4A, i.e., resistance to trimethoprim, cephalixin, clindamycin and penicillin. These strains were included in the study to test the ability of the system to segregate other gram-negative genera. Similarity between these strains and the groups of *Acinetobacter* already described was 68%. Similarly an axillary strain from a healthy school child, identified as *Ps. pseudoalcaligenes*, showed 69% similarity to the *Acinetobacter* species used in this study.

Group 8 consisted of six replicate samples of *B. catarrhalis*—one on each gel—used to measure reproducibility.

DISCUSSION

Commercial biochemical identification kits that could be used for *Acinetobacter* have been disappointing. The limited biochemical profiles obtained were not useful in differentiating the organisms in this study, other than into the two biotypes, *lwoffii* and *anitratus*. The profiles were chosen to coincide with tests that would be readily available in routine diagnostic work (Cowan, 1974). Antibigrams appeared to relate significantly to some of the electrophoretic groups, in particular resistance to tetracycline, gentamicin and tobramycin. Sensitive strains also clustered together. However, acquisition or loss of resistance plasmids would confuse an epidemiological study based on antibigrams alone.

Pagel and Seyfried (1976) have split the genus into "phenons" using biochemical and morphological data, but their organisms were isolated from two distinctly different environments, i.e., aquatic and clinical.

Considerable work has been done to produce a serological typing scheme for *A. calcoaceticus* but so far a satisfactory scheme has not been found (French *et al.*, 1980;

TABLE II
Antibiotic-resistance patterns of strains

Group	Laboratory no.	Identification	Resistance to										
			Cn	Tob	S	W	Cl	E	Da	Mn	Te	P	Pn
...	1	<i>A.c. anitratus</i>	S	S	S	R	S	S	R	S	S	S	S
1	55	<i>A.c. anitratus</i>	S	S	R	R	R	I	R	S	S	R	R
		"	S	S	R	R	R	I	R	S	S	R	R
		"	S	S	R	R	R	I	R	S	S	R	R
		"	S	S	R	R	R	S	R	S	S	R	R
...	2	<i>A.c. lwoffii</i>	S	S	S	R	R	S	R	S	S	R	S
2A	5	<i>A.c. anitratus</i>	R	S	R	R	R	R	R	S	R	R	R
		"	R	S	S	R	R	R	R	R	R	R	R
		"	R	S	R	R	R	R	R	S	R	R	R
		"	S	S	S	R	R	R	R	S	S	R	R
		"	R	S	R	R	R	R	R	S	R	R	R
		"	R	S	S	R	R	R	R	S	S	R	R
		"	R	S	R	R	R	R	R	I	S	R	R
		"	S	S	S	R	R	I	R	S	R	R	R
2B	8	<i>A.c. anitratus</i>	R	S	R	R	R	S	R	S	R	R	R
		"	S	S	S	R	R	I	R	S	S	R	R
		"	S	S	S	R	R	S	R	S	S	I	S
		"	S	S	R	R	R	I	R	S	S	R	R
		"	S	S	S	R	R	R	R	S	S	R	R
		"	S	S	R	R	R	I	R	S	S	R	R
		"	S	S	R	R	R	I	R	S	S	R	R
		"	S	S	R	R	R	R	R	S	S	R	R
2C	10	<i>A.c. lwoffii</i>	S	S	S	R	S	S	R	S	S	S	S
		"	S	S	S	R	R	S	R	S	S	R	R
		"	S	S	S	S	R	S	R	S	S	R	R
3	14	<i>A.c. anitratus</i>	S	S	S	R	R	S	R	S	S	R	R
		<i>A.c. lwoffii</i>	S	S	S	R	S	S	R	S	S	S	S
...	20	<i>A.c. anitratus</i>	R	S	R	R	R	R	R	S	I	R	R
...	30	<i>A.c. lwoffii</i>	S	S	S	S	S	S	S	S	S	S	S
4A	7	<i>A.c. lwoffii</i>	S	S	S	R	R	S	R	S	S	R	S
		"	S	S	S	R	S	S	R	S	S	R	S
		"	S	S	S	R	R	S	R	S	S	R	S
		"	S	S	S	R	S	S	R	S	S	S	S
...	63	<i>A.c. anitratus</i>	S	S	S	R	R	I	R	S	S	R	S
4B	37	<i>A.c. lwoffii</i>	S	S	S	R	R	S	R	S	S	R	S
		"	S	S	S	R	R	I	R	S	S	R	S
		"	S	R	S	R	R	S	R	S	S	R	S
		"	S	R	S	R	R	S	R	S	S	R	S
		"	S	R	S	R	R	S	R	S	S	R	S
		"	S	R	S	R	R	S	R	S	S	R	S
5	48	<i>A.c. lwoffii</i>	S	S	S	S	S	S	R	S	S	S	S
		"	S	S	S	S	S	S	S	S	S	S	S
		"	S	S	S	S	S	S	S	S	S	S	S
		"	S	S	S	S	S	S	S	S	S	S	S
		"	S	S	S	R	S	S	R	S	S	S	S
...	53	<i>A.c. lwoffii</i>	S	S	S	S	S	S	S	S	S	S	S
...	40	<i>A.c. lwoffii</i>	S	S	S	R	R	S	R	S	S	R	S
6	9	<i>A.c. anitratus</i>	S	S	S	R	R	S	R	S	S	R	R
		"	R	R	R	R	R	I	R	S	R	R	R
		"	R	R	R	R	R	I	R	S	R	R	R
7	31	<i>Alc. denitrificans</i>	S	S	S	R	R	S	R	S	S	R	S
		"	S	S	S	R	R	S	R	S	S	R	S
...	33	<i>Ps. pseudoalcaligenes</i>	S	S	S	R	R	S	R	S	S	R	S
8	6	<i>B. catarrhalis</i> (Reproducibility control)											
		"											
		"											
		"											
		"											
...	26	<i>A.c. anitratus</i>	S	S	S	R	R	S	R	S	S	R	R
...	47	<i>A.c. lwoffii</i>	S	S	S	I	S	S	R	S	S	S	S
...	12	"	S	R	S	R	R	S	R	S	S	R	S
...	49	<i>A.c. anitratus</i>	S	S	S	R	R	S	R	S	S	R	S
...	16	<i>A.c. lwoffii</i>	S	S	S	R	S	S	R	S	S	S	S

Antibiotics tested: Cn = gentamicin, Tob = tobramycin, S = sulphonamide, W = trimethoprim, Cl = cephalixin, E = erythromycin, Da = clindamycin, Mn = minocycline, Te = tetracycline, P = penicillin, Pn = ampicillin. (With amikacin, all strains were sensitive except no. 45 which showed intermediate resistance.) S = sensitive, R = resistant, I = intermediate.

Vivian, Hinchcliffe and Fewson, 1981; Holton, 1982). Even if one were to be produced, it would be very expensive to use and would remain the province of specialised laboratories.

The object of this preliminary study was to discover whether SDS-PAGE was an appropriate tool for identification and typing of *Acinetobacter*. A collection of clinical isolates, isolates from healthy volunteers, a veterinary isolate and one isolate from hydraulic fluid were examined to discover if they would resolve into groups, and whether such groups were associated with any particular characteristics, e.g., source of isolation, antibiogram or biochemical characteristics.

The study has shown that subdivisions can be made which appear meaningful in epidemiological terms. Four of five strains from healthy children appeared in one cluster (5) and a close relationship was demonstrated for four of the 12 strains from a single hospital (cluster 4B). The use of SDS-PAGE would thus seem potentially to have a very important role for epidemiological investigations and may also be useful for confirming identifications. In the former case, visual comparisons of gel tracks might be adequate, removing the necessity for time consuming densitometric analysis. Further studies will concentrate on the investigation of hospital outbreaks of infection to determine the value of SDS-PAGE fingerprinting as an epidemiological tool.

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