Identification of gram-positive anaerobic cocci by use of systems for detecting pre-formed enzymes

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Summary. Two systems for detecting pre-formed enzymes, RapID ANA and a prototype system from API, were compared in a blind study for their ability to identify 69 gram-positive anaerobic cocci isolated from clinical specimens. Both systems were able to identify *Peptostreptococcus anaerobius*, *Ps. asaccharolyticus* and *Ps. micros* accurately without the need for further tests. The prototype API system identified all isolates of *Ps. magnus* correctly, but the RapID ANA system misidentified several isolates as *Ps. micros*. Numerous different enzyme patterns were found with the indole-negative, butyrate-producing cocci (*Ps. prevotii* and *Ps. tetradius*), suggesting that this group of organisms may be heterogeneous. We conclude that kits for detecting preformed enzymes are of considerable potential for the identification of gram-positive anaerobic cocci in clinical laboratories.

Introduction

Gram-positive anaerobic cocci (GPAC) are major components of the commensal flora of man (Finegold, 1977) and important causes of human disease (Wren *et al.*, 1977; Bourgault *et al.*, 1980; Gorbach, 1985). Their taxonomy was previously based on gas-liquid chromatography (GLC) of fatty-acid metabolic products and carbohydrate-utilisation tests (Holdeman *et al.*, 1977), but most GPAC have very little or no saccharolytic activity (Ezaki and Yabuuchi, 1985). Recent attempts to provide a more satisfactory classification have been based on the homology groups and guanine-plus-cytosine content of the DNA (Ezaki *et al.*, 1983; Huss *et al.*, 1984), GLC analysis of cell fatty acids and metabolic products (Lambert *et al.*, 1979), and analysis of cell-wall structure (Weiss, 1981). However, they have provided conflicting data and it is not surprising that little effort is made to identify isolates of GPAC in most clinical laboratories.

Most GPAC use the breakdown products of proteolysis as their major energy source (Rogosa, 1974; Ezaki and Yabuuchi, 1985). Several commercial systems that detect pre-formed bacterial enzymes, including a range of proteolytic enzymes, have been marketed recently, so their ability to identify GPAC in a clinical laboratory was investigated. Several clinical isolates were characterised according to the scheme of Sutter *et al.* (1986); they were then examined blindly with two pre-formed

enzyme systems—RapID ANA and a new prototype system from API.

Materials and methods

Bacterial strains

A set of 95 isolates of gram-positive anaerobic cocci was originally tested; 82 were clinical isolates from St Bartholomew's Hospital, and 13 were strains from patients undergoing urological surgery at the London Hospital, Whitechapel. None of the organisms grew in an aerobic atmosphere containing CO₂ 5–10% after incubation for one week.

Initial identification

For isolates, gram-stained smears were examined and indole production was tested with Indole Spot disks (Lab M, Bury) after incubation for 48 h on non-selective agar (Brucella Medium Base, Difco, with horse blood 6%). Production of volatile and non-volatile fatty acids was determined by GLC (Holdeman *et al.*, 1977), after incubation for 48 h in Robertson's Cooked Meat broth (Southern Group Laboratories, Hither Green, London). Antibiotic sensitivity testing was performed by the antibiotic disk method, with metronidazole 5-µg and vancomycin 5-µg disks (Mast Laboratories, Merseyside) and sodium polyanethol sulphonate (SPS) 1-mg disks (Oxoid). Organisms that were resistant to metronidazole or vancomycin were not examined further.

Of the original 95 strains, 82 could be identified by the scheme of Sutter *et al.* (1986); 69 of these were tested for their pre-formed enzyme patterns (58 general clinical strains and 11 urological strains).

Table I. Biochemical reactions of RapID ANA system

Test code	Substrate	Test
PO4	<i>p</i> -nitrophenylphosphate	hydrolysis of phosphate ester
ONPG	<i>o</i> -nitrophenyl- β -D-galactoside	hydrolysis of glycoside ester
aGLU	<i>p</i> -nitrophenyl- α -D-glucoside	
bGLU	<i>p</i> -nitrophenyl- β -D-glucoside	
aGAL	<i>p</i> -nitrophenyl- α -D-galactoside	
aFUC	<i>p</i> -nitrophenyl- α -D-fucoside	reduction to a red formazan
NAG	<i>p</i> -nitrophenyl-N-acetylglucosamine	
TTZ	triphenyltetrazolium	
ADH	arginine	
TRE	trehalose	production of basic compound
LGY	leucylglycyl- β -naphthylamide	fermentation
GLY	glycyl- β -naphthylamide	hydrolysis of β -naphthylamide ester
PRO	prolyl- β -naphthylamide	
PHE	phenylalanyl- β -naphthylamide	
ARG	arginyl- β -naphthylamide	
SER	seryl- β -naphthylamide	indole production
PYR	pyrrolidonyl- β -naphthylamide	
IND	tryptophane	

Inoculation of pre-formed enzyme kits

All strains were coded and plated out to check for viability and purity. A single colony was sub-cultured on to freshly prepared Columbia blood agar (Oxoid) with horse blood 6% and Vitamin K1 0.1%, and the plates were incubated for 48 h. A separate observer who did not know the identity of the organisms inoculated the kits according to the instructions of the manufacturer with a bacterial suspension of density McFarland standard 4 (bacterial count *c.* 10^9 cfu/ml). Kits were then incubated aerobically at 37°C for 4 h.

The RapID ANA system

The RapID ANA system (Mercia Diagnostics, Guildford, Surrey) is based on 18 biochemical reactions (table I) (Karachewski *et al.*, 1985). Eight of the cupules are bifunctional; reactions were read before and after the addition of reagents, and were described as either positive or negative depending on the colour change. Tests were assigned numerical values, and the resulting six digit code was interpreted with the RapID ANA Code Compendium for identification.

The prototype API system

This new system (from API Laboratory Products, Basingstoke, Hants) consists of 32 biochemical reactions

Table II. Biochemical reactions of API prototype system

Test no.	Test code	Test
1	ADH	Arginine dihydrolase
2	GDC	Glutamate decarboxylase
3	aGAL	Hydrolysis of glycoside ester
4	bGAL	
5	aGLU	
6	aFUC	
7	aARA	
8	bGAL-6PH	
9	N-Ac-GLU-NH2	
10	bGLU	
11	bGUR	
12	Glucose	Utilisation of monosaccharide
13	Mannose	
14	Raffinose	
15	Trehalose	
16	Fructose	
17	Urea	Urease
18	Indole	Indole production
19	Nitrate	Nitrate reduction
20	PAL	Alkaline phosphatase
21	ARG	Hydrolysis of aminopeptide
22	PRO	
23	HIS	
24	LEU-GLY	
25	PHE	
26	GLU-GLU	
27	LEU	
28	PYRA	
29	TYR	Catalase production
30	LANA	
31	GLY	
32	SER	
33	CAT	

Reactions 3–11 test for the hydrolysis of a glycoside ester to release a coloured *p*-nitrophenol derivative.

Reactions 21–32 test for the hydrolysis of an aminopeptide linkage to release an orange 2-naphthylamine derivative. In reaction 20, an *o*-phosphate linkage is hydrolysed.

(table II). Tests 1–17 are spontaneous colour reactions. Indole production (test 18) requires the addition of James's reagent (James *et al.*, 1986) and nitrate production (test 19) the addition of sulphanic acid and dimethyl- α -naphthylamine. Tests nos. 20–32, for alkaline phosphatase and aminopeptidase production, require the addition of lauryl sulphate and Fast Blue BB. Catalase production (test 33) is detected by the addition of H₂O₂ 2% to cupule 7 (aARA). Reactions were graded as strongly positive, weakly positive, very weakly positive or negative according to a supplied colour chart, and a pre-formed enzyme profile generated for each isolate. Organisms were grouped according to their identification by the methods of Sutter *et al.* (1986), and the pre-formed enzyme pattern characteristic of the species deduced.

Table III. Comparison of efficacy of pre-formed enzyme systems

Organism	Number of strains tested	Number of isolates identified by				
		Rapid ANA			API prototype	
		Correct	Inadequate ID*	Incorrect	Correct	Incorrect
<i>Ps. anaerobius</i>	8	6	2	0	8	0
<i>Ps. asaccharolyticus</i>	19	19	0	0	19	0
<i>Ps. indolicus</i> †	1	1	0	0	1	0
<i>Ps. magnus</i>	23	14	3	6	23	0
<i>Ps. micros</i>	5	5	0	0	5	0
Indole-negative butyrate-producing cocci	13	Could not be confidently speciated				
Total	69	45	5	6	56	0

* When the Rapid ANA profile could not differentiate between species, identification was described as 'inadequate ID'.

† The single isolate of *Ps. indolicus* could not be differentiated satisfactorily from *Ps. asaccharolyticus* by standard identification techniques.

Results

The results from both pre-formed enzyme systems agreed well with identification based on standard identification techniques (table III), for all species groups except the indole-negative butyrate-producing cocci—*Ps. prevotii* and *Ps. tetradius* by Ezaki's classification (Ezaki *et al.*, 1983). One indole-positive coccus could not be identified fully by standard methods, but was identified as *Ps. indolicus* by both enzyme systems.

The *Rapid ANA* system was easy to use and could be inoculated rapidly; it required only two additional reagents. The minimum inoculum (1 ml of a McFarland standard 3 suspension) could be harvested from one plate after incubation for 48 h with even slow-growing GPAC. However, some reactions which were critical for correct identification were difficult to interpret.

Ps. asaccharolyticus and *Ps. micros* were easily identified and the profile numbers within each species were very similar. *Ps. magnus* often produced a borderline prolyl- β -naphthylamide reaction (PRO, cupule 3); if the reaction was read as positive, the organism was incorrectly identified as *Ps. micros* (table IV). Two reactions were responsible for producing an unsatisfactory level of identification (described as "inadequate ID" in the Rapid ANA Code Compendium): the glycyl- β -naphthylamide reaction (GLY, cupule 2) with two strains of *Ps. anaerobius*, and the arginine dihydrolyase reaction (ADH, cupule 9) with two strains of *Ps. magnus*.

The indole-negative butyrate-producing cocci produced a large number of different patterns with

both Rapid ANA and the prototype API system. This group of organisms is probably heterogeneous (Ezaki *et al.*, 1983).

The *prototype API* system was slower to inoculate and required the addition of six different reagents. It was frequently difficult to harvest an adequate inoculum (5 ml of a McFarland standard 4 suspension) from one plate after incubation for 48 h, especially with *Ps. micros*. The aminopeptidase reactions (cupules 20–32) were sometimes difficult to interpret, but the critical reactions were almost always unequivocal. As this system is still at the prototype stage, a profile index has not yet been generated. Nevertheless, identification was remarkably easy—strains within each species produced very similar patterns, easily separable from strains of other species (table V). In particular,

Table IV. Organisms misidentified by the Rapid ANA system

Organism	Number of isolates	Misleading test	Rapid ANA results
<i>Ps. anaerobius</i>	2	GLY	90% probability of <i>Ps. tetradius</i> 8% probability of <i>Ps. anaerobius</i>
<i>Ps. magnus</i>	2	ADH	82% probability of <i>Ps. tetradius</i> 18% probability of <i>Ps. magnus</i>
	5	PRO	<i>Ps. micros</i>
	1	ADH, PRO	<i>Ps. tetradius</i>

Table V. Enzyme patterns with prototype API system

Organism	Number of isolates	Characteristic reaction in cupule no.																			
		1	5	12	13	16	18	20	21	22	23	24	25	26	27	28	29	30	31	32	33
<i>Ps. anaerobius</i>	8	—	+	±/+	v	±/+	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—
<i>Ps. asaccharolyticus</i>	17	—	—	—	—	—	+	—	+	—	±/+	—/±	—	—	±/+	—	±/+	—	v	—	—
<i>Ps. indolicus</i>	2	v	—	—	—	—	+	+	+	—	+	v	—	—	±	—	±	±	+	—	—
<i>Ps. magnus</i>	23	v	—	—	—	v	—	v	+	—	±/±	v	—	—	+	+	v	v	±/+	—	v
<i>Ps. micros</i>	5	—	—	—	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+	+	—
Indole-producing saccharolytic organisms	2	—	—	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Indole-negative butyrate- producing organisms*																					
Pattern 1	4	—	—	—	—	—	—	—	+	—	+	—	—	—	±/+	—	±	—	+	—	—
Pattern 2	3	+	—	v	v	v	—	—	+	—	+	v	—	—	+	±	v	v	±	±	v
Pattern 3	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+

Several reactions were always negative and have not been included. Reactions are graded when >90% of the organisms produced the same reaction.

+ Strongly positive result; ± weakly positive; ± very weakly positive; — negative.

* Four organisms in this group did not fit into any pattern.

there was a clear distinction between *Ps. magnus* and *Ps. micros*—organisms which previously have been separated by the size of the coccus (Holdeman *et al.*, 1977; Sutter *et al.*, 1986). As with the RapID ANA system, the indole-negative butyrate-producing cocci produced various patterns. These patterns were distinct from those produced by the well defined species.

The reproducibility of the prototype API system was investigated by re-testing several organisms blind. The results were very similar (table VI), showing that in optimum conditions the system is highly reproducible. The RapID ANA system has

already been shown to be reproducible (Murray *et al.*, 1985).

The importance of the correct inoculum for the prototype API system was tested by comparing a McFarland standard 2 suspension with a McFarland standard 4 suspension (table VII). Most organisms were identified correctly when a lighter inoculum was used (table V), though some individual reactions were weaker and two organisms could not be definitely identified. The possibility of using a light inoculum would be especially useful for *Ps. micros*, a species which produced very strong reactions with even a weak inoculum.

Table VI. Reproducibility of prototype API system

Organism	Number of strains tested	Number of positive reactions	Number of repeat reactions that were		
			correct	nearly* correct	incorrect
<i>Ps. asaccharolyticus</i>	5	38	26	11	1
<i>Ps. indolicus</i>	1	9	6	3	0
<i>Ps. magnus</i>	3	35	27	8	0
Indole-negative butyrate-producing cocci	5	32	20	11	1

* Repeat reactions were classed as 'nearly correct' when they were one grade out (for instance, negative/very weakly positive), and 'incorrect' when they were two or more grades out.

Table VII. Comparison of inoculum strength for prototype API system

Organism	Number of strains tested	Number of strains correctly identified with	
		McFarland 2	McFarland 4
<i>Ps. anaerobius</i>	3	2	3
<i>Ps. asaccharolyticus</i>	1	0	1
<i>Ps. indolicus</i>	1	1	1
<i>Ps. magnus</i>	5	5	5
<i>Ps. micros</i>	2	2	2
Indole-negative butyrate-producing cocci	1	1	1

Discussion

Identification of GPAC in clinical laboratories is based primarily on the detection of fatty-acid end-products by GLC, with the detection of indole production and the estimation of the size of the coccus as secondary tests (Sutter *et al.*, 1986). However, their taxonomy is still debated (Rogosa, 1974) and their clinical importance not fully appreciated (Gorbach, 1985). GPAC are often not identified as far as the species level in most clinical laboratories.

In this study, both systems based on the detection of pre-formed enzymes were highly effective at identifying *Ps. anaerobius*, *Ps. asaccharolyticus* and

Ps. micros. The RapID ANA system (but not the API prototype) frequently misidentified *Ps. magnus* as *Ps. micros*, but this error can be prevented by looking at the gram-stained film to check the size of the coccus. Neither kit requires specialised equipment, and each can be inoculated and read on the same day. We conclude that these kits make the identification of the well characterised species of GPAC considerably simpler for the clinical laboratory.

A significant proportion of clinical isolates of GPAC do not fit into described species; 13 (15%) of the original 95 in our set could not be confidently identified. Four of these strains produced large quantities of isovaleric acid as their main fatty-acid end-product, and did not correspond with any of the well characterised species. The enzyme patterns of two indole-producing organisms showed saccharolytic but not proteolytic activity—but by the current taxonomy, they are identified as *Ps. asaccharolyticus*. It is likely that further species of GPAC remain to be described. Until the taxonomy of GPAC is more comprehensive, pre-formed enzyme systems will be able to characterise many clinical isolates, but not to identify them.

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