Serotyping of *Acinetobacter calcoaceticus*

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SUMMARY Serotyping of *Acinetobacter calcoaceticus* by direct immunofluorescence and a capsule swelling reaction is described. One hundred isolates, including 12 from an outbreak in a neonatal department, were studied. Ninety five per cent of the isolates were typable by immunofluorescence and could be divided into 30 separate types, but 42.1% of typable strains, including 11 from the outbreak, were of one type. Typing results by the capsular swelling reaction generally followed those of immunofluorescence methods.

The role of *Acinetobacter calcoaceticus* as an opportunistic pathogen is now well recognised. In a multicentre study¹ by the Centers of Disease Control, Atlanta, Georgia, over a period of four years *A. calcoaceticus* was the causative organism in 1372 of 180 982 cases of infections reported to the centre.

In recent years a number of outbreaks due to this organism have been reported, mostly in association with patients in hospital.²⁻⁴ In the absence of a suitable typing system it has not always been possible to establish the source of infection and route of transmission in such outbreaks. This paper describes the development of a serotyping system by direct immunofluorescence and a capsular swelling reaction.

Material and methods

One hundred isolates of *A. calcoaceticus* were included in the study. Eighty three of these isolates were obtained from clinical hospital laboratories, five from the hospital environment and 12 from an outbreak in a neonatal unit (Stone and Das, unpublished observations) (Table 1).

The isolates were identified as *A. calcoaceticus* on the basis of the following criteria: non-motile; oxidase negative; nitrate, indole, and H₂S negative; non-fermentative Gram negative bacilli or coccus bacilli. The strains were maintained in cooked meat broth at room temperature and subcultured monthly to check the viability and purity.

PREPARATION OF ANTISERA

A pilot study was carried out to identify antigenically distinct strains of acinetobacters. For this purpose,

10 isolates were selected at random. Antigen was prepared from a saline suspension of an overnight culture on nutrient agar at 37°C. The suspension was heated at 100°C for 1 h and was washed in sterile normal saline three times and diluted to give a final suspension of 10⁶-10⁸ organisms per millilitre. The antigen was injected into rabbits at 3-5 day intervals in the following sequence: 0.3 ml subcutaneously, 0.4 ml intramuscularly; 0.4 ml into a foot pad, 0.4 ml into another foot pad; 0.4 ml intravenously, and 0.5 ml subcutaneously.

Serum samples obtained before immunisation were used as controls. Preliminary bleeding was carried out seven days after the last injection and antibody titres in the sera were determined. If satisfactory ($\geq 1/64$) a final bleeding was carried out after the last injection. When the titre was $> 1/64$, the procedure was repeated.

Fluorescein labelled antirabbit immunoglobulin (Wellcome Diagnostics, Dartford, England) was used in indirect immunofluorescence tests. The

Table 1 Sources of acinetobacter strains

No of strains	Source	Clinical information
40	Urine	? urinary tract infection
19	Burns	Significance uncertain
13	Wounds	Probable contaminants except three which were considered clinically significant
3	Conjunctiva	Clinical conjunctivitis
3	Sputum	Pneumonia in immunosuppressed patients
2	Dialysis fluid	Infection in patients with continuous ambulatory peritoneal dialysis
3	Cerebrospinal fluid	Hydrocephalus (2), meningitis (1)
5	Hospital environment	(Air and water)
12	Outbreak isolates	Outbreak of pulmonary infection in a special care baby unit

reagent was reconstituted according to manufacturer's instruction and stored at -20°C in 0.5 ml aliquots. The working dilution was selected as the one which gave clearly positive results with four randomly selected preparations of homologous antigen and antisera and negative results with four heterologous antigen and antisera preparations. The test was carried out with each batch of reagents and the dilution was made with phosphate buffered saline (PBS, pH 7.4) immediately before the test. The optimum dilution varied between 1/20 and 1/25.

Working dilutions of antisera were determined by titration with homologous antigens and selecting for use the titre which was twice the concentration of the dilution yielding 2+ positive results (see below). The titre varied between 1/128 and 1/512.

Antigen for the test was prepared by suspending three to five colonies from an overnight culture in 1 ml of PBS, pH 7.4. Strains producing M or mucoid colonies were selected, as opposed to S forms with flat, bluish or greyish white colonies. Some strains produced a mixture of M and S forms while others predominantly or exclusively produced M or S forms. On repeated subcultures variation from the M to S form and vice versa was also noted. The suspension was mixed thoroughly before use.

A thin film of antigen in a well of a Teflon coated slide was allowed to dry in air and then fixed by gently passing over a flame. The slides were degreased in acetone before use. The mixture of antigen and antiserum was incubated in a moist chamber at 37°C for $1\frac{1}{2}$ to 2 h, washed three times with PBS, dried with blotting paper, incubated with fluorescein labelled antirabbit serum at 37°C for $1\frac{1}{2}$ to 2 h, washed three times with PBS, dried with blotting paper, and examined for fluorescence with a Reichert's/Leitz immunofluorescence microscope.

Results were considered positive when cellular outlines were seen as complete and distinct. Intensity of fluorescence was graded from $\pm$ to 2+. Absence of fluorescence or $\pm$ fluorescence or

incomplete peripheral staining, irrespective of the intensity of fluorescence, was considered negative.

The following controls were used in the tests and results were recorded only when the control (a) was positive and (b) and (c) were negative: (a) homologous antigen and antiserum; (b) heterologous antigen and antiserum; (c) preimmunisation rabbit serum and antigen.

Antigens of other organisms—namely, NCTC strains of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*—were also used as negative controls.

The capsular swelling test was carried out with 30 type specific strains (see results) and the corresponding antisera. One loopful of neat antiserum was mixed with an equal volume of a 6 h broth culture of the organism. The mixture was allowed to react for 2 min, after which a drop of eosin stain was added and a film was made. A mixture of heterogenous antigen and antiserum was used as a negative control. Clear delineation of capsule was recorded as positive. With strains where the capsular swelling was not clear cut, the results were recorded as doubtful.

Thirty type specific strains were tested by the immunofluorescence method, and 10 of those strains were tested by the capsular swelling test on four occasions over a period of six months to determine reproducibility.

Results

In the pilot study (Table 2), six antigenically distinct strains could be identified. Strains 1, 2, 3, and 9 were of one type; strains 6 and 10 were of another type; strains 4, 5, 7, and 8 were of four different types.

Six specific antisera obtained from the above experiment were used to screen the rest of the acinetobacter strains. A second batch of 10 strains was selected from those which were negative for further antiserum preparation.

Using the techniques described, 30 antigenically

Table 2 Immunofluorescence test: pilot study

Antigen	Antisera									
	1	2	3	4	5	6	7	8	9	10
1	+	+	+	—	—	—	—	—	+	—
2	+	+	+	—	—	—	—	—	+	—
3	+	+	+	—	—	—	—	—	+	—
4	—	—	—	+	—	—	—	—	—	—
5	—	—	—	—	+	—	—	—	—	—
6	—	—	—	—	—	+	—	—	—	+
7	—	—	—	—	—	—	+	—	—	—
8	—	—	—	—	—	—	—	+	—	—
9	+	+	+	—	—	—	—	—	+	—
10	—	—	—	—	—	+	—	—	—	+

Table 3 Typing of 100 isolates by immunofluorescence

No of strains tested	Types				
	1	2	3-5	6-30	Untypable
100	40*	12	11	32	5

*Includes 11 isolates from outbreak.

distinct strains and their antisera were obtained. Table 3 shows the results of serotyping 100 strains with these antisera. Ninety five per cent of the strains were typable and could be divided into 30 separate recognisable types. Of the typable strains 42.1% were of the same type, designated type 1 in this study, and included 11 of the outbreak strains.

On repeat testing, the same results were obtained with both immunofluorescence and bacteriocin methods on each occasion. Some isolates, however, had changed from M phase to predominantly S phase during the six month period between the experiments. M phase colonies were selected and subcultured for the tests.

Discussion

A. calcoaceticus, as defined in *Bergey's Manual* (8th edition), has been known by a variety of synonyms. *Mima polymorpha*, *Herellea vaginicola*, *Bacterium anitratum*, *Neisseria winogradsky* and B5W are only a few of them.

Previous studies of serology of these organisms have not clarified this confusion. The absence of precise and consistent methods of identification has made it difficult to interpret past work. An additional problem is that some undergo phase variation and a change in antigenic character on prolonged storage.^{5,6}

Agglutination tests, capsular swelling reactions, precipitation tests, and immunofluorescence methods have been used to investigate the antigenic relation of these organisms among themselves and with other bacteria.

Tube and slide agglutination tests with live, heated, or formalin killed suspensions have been disappointing. A wide range of cross reactions between homologous and heterologous strains and difficulty of reading end points in the presence of both capsular and somatic agglutination⁵ appear to be the main problems. Autoagglutinability of some of the strains presents additional difficulty.

Capsular swelling reactions using either neat or diluted antisera have been found to be specific⁶ but less sensitive than immunofluorescence and are sometimes difficult to interpret.⁷

Cary *et al.*⁸ studied precipitation tests with Maxted extracts of bacteria as an antigen. Strains of *Morax-*

ella sp, *N. Winogradskyi*, *D. mucosus* and *B. anitratum* were investigated. The exact identities of some of these strains are now uncertain, which makes the result confusing and difficult to interpret.

The direct immunofluorescence method has been used by Marcus *et al.*⁵ to identify strains of *H. vaginicola* (acid producing strains of *A. calcoaceticus*) and to differentiate it from *M. polymorpha* (non-acid producing strains of *A. calcoaceticus*). The names *H. vaginicola* and *M. polymorpha* are now considered obsolete and only one species *A. calcoaceticus* is generally recognised. Marcus *et al.*⁷ identified 28 serotypes of *H. vaginicola* and when strains isolated from one institution were typed, two types occurred more frequently than others. Cunha *et al.*⁹ used this serotyping system to investigate a nosocomial outbreak of pneumonia and identified a single serotype from 10 patients and a contaminated respirometer. In another study,¹⁰ however, only seven of 15 blood culture isolates could be typed by this method.

In the present study, an indirect immunofluorescence method and a capsular swelling reaction have been used to type 100 isolates of *A. calcoaceticus*. "Specific" anticapsular serum was prepared using a bacterial suspension heated at 100°C for 1 h and washed several times. Billing,¹¹ working with soap tolerant variants of *B. anitratum*, noted a "specific," probably capsular, and a "common" surface antigen. Both antigens were stable at 100°C for 1 h. In the process of heating, however, "common" antigen was completely or almost completely detached whereas "specific" antigen was only partly removed.

Antisera obtained in the present study were of high titre and reactions in the immunofluorescence test were distinct. Cross reactions were encountered infrequently and could be easily eliminated by appropriate dilution of antiserum. The time of antigen and antiserum incubation was critical; reproducible results were obtained only when the incubation period was at least 1 h at 37°C. Although sensitivity of the test (95%) was encouraging, the result did not necessarily reflect overall typability of acinetobacter strains because the 100 isolates of this study included the type strains. The lack of discrimination was an obvious disadvantage. The test was reproducible, however, and when the test was used to type twelve outbreak isolates, the result was consistent with other epidemiological evidence. A wider

experience of this method in other investigations is required to confirm its value.

The capsular swelling test was quick and easy to perform, but in the absence of a clear cut result with some strains, subjective error remained a possibility and some experience is necessary to obtain reproducible results.

It is possible that investigation of a large number of isolates might reveal more types, but to attempt a serological classification as with the *enterobacteriaceae*, would entail a considerable amount of work. As Henriksen¹² observed, whether this would be justified, in view of the limited role this organism plays in clinical infection, is arguable. But the limited study reported was of use in investigating an epidemic situation in a neonatal unit.

To be of value for epidemiological investigations a typing method or combination of methods should be sensitive, discriminating and reproducible. Bacteriocin susceptibility testing and polyacrylamide gel electrophoresis are worth exploring for this purpose.

We are grateful to Dr M Casewell, London Hospital Medical College; Dr P Mortimer, PHLS, Coventry; Dr PP Brown, Queen Elizabeth Hospital, Birmingham; Dr CJ Mitchell, PHLS, Leicester; Dr M Osman, PHLS, Nottingham; and Central Public Health laboratory for providing the isolates included in this study. We thank Mrs L Blakeney for typing the manuscript.

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