A new serotyping method for *Klebsiella* species: development of the technique

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SYNOPSIS A new serotyping method for *Klebsiella* species using indirect immunofluorescence is described. Nonspecific fluorescence has been minimized by carrying out the capsular antigen-antibody reaction at pH 9.0.

Commercial antisera have been tested with the 72 antigenic types of *Klebsiella*, and appropriate dilutions of each pool and specific antisera have been proposed for use in routine typing. Dilutions were chosen to allow strong fluorescence with each type and its specific antiserum and minimal fluorescence with cross reacting antisera.

Where the pool antisera gave a weak reaction for one or more of the component types, it is recommended that the specific antisera for these types be added to the pool dilution.

The few remaining cross reactions, with the pool and specific antisera in test dilution, are listed in a table. The unique cross reacting patterns of particular types have been found to be useful in identification.

Typing *Klebsiella* by the fluorescent antibody technique is easy to perform and interpret; the results are reproducible, and it is less expensive than the existing capsular swelling method as it is more sensitive and requires less concentrated antisera. This new method of typing should facilitate detailed epidemiological studies of the mode of transmission of *Klebsiella* species in hospitals and thus allow more effective infection control measures to be instituted.

During the 1960s, Gram-negative bacilli became increasingly important as a cause of hospital infection, as a result of better control and prevention of infection caused by Gram-positive organisms and a true increase in the incidence of Gram-negative sepsis. *Klebsiella* species have played an especially significant role in this trend. Hospital epidemics have been described, often caused by strains resistant to commonly used antibiotics (Montgomerie *et al*, 1970; Price and Sleight, 1970; Finland, 1971; Martin *et al*, 1971; Selden *et al*, 1971).

It is difficult to investigate the epidemiology of infections without reliable methods for detecting identical strains of the same species. Phage typing has been invaluable in studying the epidemiology of *Staphylococcus aureus* sepsis and has greatly facilitated the introduction of measures to terminate or prevent epidemics of infection (Williams *et al*, 1966), but the application with *Klebsiella* has been limited

(Parker, personal communication). So far other typing methods have had limited success in studying the spread of this organism. Bacteriocine typing has been shown to be insufficiently discriminating for *Klebsiella* strains (Hall, 1971) while the capsular-swelling methods developed by Ørskov (1955a), Kauffmann (1949), and Edwards and Fife (1955) have not become widely adopted, in spite of their apparent simplicity, except by a few specialist reference centres.

Capsular swelling is a very subjective test where the readings can be so ambiguous that only a very trained eye can hope to discern a reaction, and even experience of the method does not always guarantee complete success. Multiple cross reactions have also been described (Edwards and Ewing, 1968; Casewell, 1975).

Fluorescent antibody methods have been used to identify many bacteria either directly with a fluorescein-labelled (FITC) antiserum or indirectly with a specific antiserum and a labelled conjugate. The use of indirect immunofluorescence to detect specific

capsular antigens of *Klebsiella* species was complicated by a large number of cross reactions between the 72 antigenic types and the specific antisera. Because of the convenience and availability of the commercial antisera various techniques were employed to try to overcome this difficulty but these proved unsuccessful or impractical. It was then decided to vary the pH of the incubation of the bacteria with the antisera in view of the possibility that cross reacting antibody may be of low avidity and would be preferentially inhibited by an acidic or alkaline background.

This paper shows how employing pH 9.0 with the fluorescent antibody (FA) method reduces the non-specific cross reactions, thus allowing this technique to be used for *Klebsiella* typing. Once the appropriate dilutions of the antisera had been established and the possible cross reactions defined, the FA technique was considered to be a workable method for typing *Klebsiella*. An evaluation of this method is made in an accompanying paper in this issue.

Material and Methods

CULTURES

Reference strains of 72 recognized *Klebsiella* capsular types were obtained from Dr I. Ørskov, of Statens Seruminstitut, Copenhagen, and were kept at room temperature on nutrient agar slopes. For the experiments, isolates of *Klebsiella* were subcultured onto Worfel-Ferguson Medium (Difco) for 18-24 hours at 37°C to promote capsule production.

SLIDE PREPARATION

Slides of 0.8 mm thickness (Microslides, Baird and Tatlock Ltd) were washed in detergent (Pyronex, Diversey), rinsed thoroughly in tap water, distilled water, and then acetone, and air dried. Ten discrete drops of glycerol were applied to a slide which was then coated with Teflon from an aerosol spray (Fluoroplast 82: PTFE spray) and the glycerol was washed off, leaving 10 wells about 2.5 mm diameter for the bacterial suspension (Chessum, 1970). A medium-sized colony or an equivalent amount from a streak of the culture from the Worfel-Ferguson plate was suspended in about 1 ml of phosphate buffered saline (PBS) at pH 7.2 (Oxoid PBS tablets) and diluted to a density of about 0.03-0.04 at a wave length of 500 nm, yielding an average of 100 bacteria per field at $\times 50$ magnification. Distilled water cannot be used for the suspension as it appears to elute the capsular material from the cell, and there is little fluorescence as a result. Drops of the bacterial suspension were added to the well spaces and allowed to dry; the smears were heat fixed and the slides were stored at room temperature until required.

ANTISERA

Individual and pool antisera were obtained in powdered form from Difco and, after reconstitution with sterile, distilled water, were stored at 4°C. Working dilutions were made with Britton Robinson Buffer (BRB), a universal buffer covering a range of pH 2.6-12.0 (McKenzie, 1969) at whatever pH was required using a standard dropper Pasteur pipette. The diluted antisera could be kept at 4°C for many weeks and still retain their activity.

A sheep antirabbit globulin fluorescent conjugate (Wellcome) was reconstituted in sterile distilled water and stored in 0.1 ml amounts at -20°C. Before use an 0.1 ml aliquot was diluted in 0.9 ml normal saline. This saline solution was kept at 4°C and 1/40 working dilutions in PBS were made daily from this (total dilution of 1/400).

ROUTINE FA TECHNIQUE

Bacterial smears were individually covered with the antiserum, and the slides were placed in an incubation chamber (plastic box) at room temperature for 20 minutes to prevent dehydration, rinsed with tap water for 20 minutes, and air dried at room temperature. A drop of the working dilution of the conjugate was added to each well and incubated at room temperature for 20 minutes. The slides were rinsed again for 20 minutes with tap water, air dried at room temperature, mounted in Polarfluor mountant B (Polaron), and kept at -20°C until read. The fluorescence was observed using a Union Inverted Fluorescence Microscope (Heimer and Taylor, 1972) or a Reichert employing fluorescence-free immersion oil (Polarfluor 8) or Reichert glycerin ($n_D = 1.455$) and was graded according to the standard system + to + + + +.

ALTERATION OF pH

Several different antisera which had shown a great deal of cross reaction in preliminary studies were tested against their specific types and several cross reacting strains. The pH of the initial antigen-antibody reaction was varied from pH 5.0 to pH 11.0 by dilution of the antisera in the appropriate buffer at dilutions of 1/20 to 1/160. This was incubated with the specific and cross reacting bacteria for 20 minutes and the usual routine method was followed. One to four tests were run with eight different antisera and their corresponding specific types and two to 11 other strains at each pH. The experiments were done in duplicate, triplicate or quadruplicate, and averages were taken for each test as well as an average of all tests for each antiserum.

PHOTOGRAPHY

The photographs of the bacteria in each set were

taken at the same magnification, camera settings, and time exposure on an automatic Leitz microscope using Kodak film, Ektachrome-B High speed (EHB 135-20) ASA 125.

TEST OF POOL ANTISERA

The pool antisera were analysed at dilutions of 1/20 and 1/40 to find the appropriate dilution for typing, ie, the dilution which gave strong fluorescence for each of the pool components while keeping cross reactions at a minimum. Each of the 18 pools was run against each of the 72 types at each dilution and repeated as necessary.

SUPPLEMENTING POOLS

When a particular type did not react strongly enough with its specific pool, at a dilution favourable to the other pool components, a drop of the specific antiserum of the weak type was added to the pool dilution maintaining a final dilution of 1/20 and 1/40. If necessary, the concentration of the supplemented type was increased until adequate.

CROSS REACTION WITH POOLS

When the 18 pools were run against the 72 types, the pools that reacted strongly were recorded, and any pool other than the specific pool reacting with a particular type was considered to constitute a cross reaction.

TEST OF SPECIFIC ANTISERA

Each of the 72 types was tested against its specific antiserum and the type antisera of any pool reacting strongly with it and repeated when necessary. A dilution of 1/32 was used unless the specific antisera were weak, in which case the concentration was increased until adequate for identification.

DILUTION OF SPECIFIC ANTISERA

When a particular type reacted strongly with other type antisera, these were recorded as cross reactions. The specific and cross reacting antisera were diluted 1/64, 1/128, and 1/256 until the specific antisera gave a stronger fluorescence than the cross reacting antisera and they could be easily distinguished. This was run in quadruplicate and any necessary repeats were run in duplicate. The remaining antisera which could not be separated by this method were considered to be inherent multiple reactions.

Results

EFFECT OF pH ON FLUORESCENCE

Figures 1-5 illustrate the effect of pH on the initial antigen-antibody reaction as expressed in the change in fluorescence. The cross reacting types often gave

Fig 1 Effect of pH of antigen-antibody reaction on fluorescence of K7 antiserum + specific type K7 and several other strains. Average of two tests, one with three and one with two replicates, dil 1/20.

Fig 2 Effect of pH of antigen-antibody reaction on fluorescence of K20 antiserum + specific type K20 and several other strains. Average of two tests, one with four and one with two replicates, dil 1/40.

Fig 3 Effect of pH of antigen-antibody reaction on fluorescence of K1 antiserum + specific type K1 and several other strains. Average of two tests, one with three and one with four replicates, dil 1/20.

Fig 4 Effect of pH of antigen-antibody reaction on fluorescence of K8 antiserum + specific type K8 and several other strains. One test, average of three replicates dil 1/20.

Fig 5 Effect of pH of antigen-antibody reaction on fluorescence of K7 antiserum + specific type K7 and several other strains. One test, average of three replicates, dil 1/20.

fluorescence equal to or greater than that of the specific types from pH 5.0 to 7.0. Non-specific fluorescence decreased with rising pH (also fig. 6), while the fluorescence of the specific types was enhanced, reaching an optimum at pH 9.0 (also fig. 7). Over the range of pH 5.0 to pH 11.0 the greatest difference between the fluorescence of the specific and non-specific types was observed at pH 9.0.

Occasionally, cross reacting strains were still found to give a definite fluorescence at pH 9.0, but this could usually be distinguished from specifically reacting strains by the degree of fluorescence. Specific types which gave good (++++) fluorescence at pH 7.0 often displayed a characteristic fluorescence of an even more intense quality at pH 9.0.

TEST OF POOL ANTISERA

The majority of the pool components were found to

Fig 6a

Fig 6b

Fig 6 Reduction of fluorescence of cross reacting type from pH 7.0 to pH 9.0, dil 1/20: (a) antiserum K19 v antigen K10 at pH 7.0, ++++ fluorescence; (b) antiserum K19 v antigen K10 at pH 9.0, ++ fluorescence.

give optimum fluorescence and a minimum of cross reaction at a dilution of 1/40; therefore, this was chosen as the working dilution for the pool antisera.

SUPPLEMENTING POOLS

Some individual components of the pool antisera did

Fig 7a

Fig 7b

Fig 7 Enhancement of fluorescence of a specific type from pH 7.0 to pH 9.0, dil 1/20; (a) antiserum K20 v antigen K20 at pH 7.0, ++ fluorescence; (b) antiserum K20 v antigen K20 at pH 9.0, ++++ fluorescence.

not give strong fluorescence at the chosen 1/40 dilution of the pools. The specific antisera of these types were added to this dilution of the pools until appropriate fluorescence was attained. Table I indicates the pools and the specific type antisera with

Pool	Supplemented Specific Antisera
1	3
2	—
3	—
4	13, 14
5	17, 19
6	11, 22
7	26, 30
8	29, 40
9	—
10	33, 36
11	37, 39, 42 ¹
12	45
13	49, 50
14	55
15	—
16	62, 63
17	—
18	—

Table I The 18 pool antisera and the specific type antisera with which they are supplemented at a concentration of 1/40 in a 1/40 dilution of the pool

¹Supplemented at a concentration of 1/20 in a 1/40 dilution of the pool

which they are supplemented. The type antisera are added at a concentration of 1/40 in a 1/40 dilution of the pool. One exception is pool 11, which is supplemented with types 37 and 39 at a concentration of 1/40, and type 42 at 1/20.

Casewell (1972, 1975) also found that some pools needed to be supplemented in order to obtain a reaction with capsular swelling. Nine of the specific antisera added to the pools for use with fluorescence were also supplemented for use with capsular swelling.

CROSS REACTION WITH POOLS

At a 1/40 dilution of the supplemented pools, most types fluoresced more strongly with their specific pools than with any other; however, some types gave similar fluorescence with two or more pools, often forming a characteristic pattern for that type. The strongest cross reactions between various pools and a particular type are listed in table II.

TEST OF SPECIFIC ANTISERA

Some cross reaction was found among the 72 types and the various specific antisera of the pools that had reacted strongly with them. Table III lists the specific antisera which react with each type at a 1/32 dilution of the antisera.

Type 27 was the only type which did not react with its specific antiserum but reacted instead with antiserum 46.

DILUTION OF SPECIFIC ANTISERA

Dilutions of 1/64 to 1/256 were found to be adequate to distinguish most of the specific from the cross

Type	Specific Pool	Pool Antisera giving Positive Reaction at 1/40 Dilution
1	1	1, 2
3	1	1 (17)
7	2	2 (3)
9	3	3 (4, 10)
10	3	3 (16)
12	8	8 (3, 5, 10, 11, 16, 17, 18)
13	4	8 (1, 2, 4, 5, 9, 11, 12)
20	5	5 (3, 10, 11, 12, 16)
22	6	6, 4, 11 (5, 8, 11, 16)
26	7	7 (2, 9, 10, 11, 12, 15)
37	11	11, 6, 12
40	8	8, 4, 5
41	8	8 (12)
42	11	11, 10
43	9	9 (15)
44	9	9, 5
45	12	12 ¹ , 5, 18 (3, 4, 8, 9, 10, 11, 14, 16, 17)
46	12	12 (18)
47	12	12 (14)
49	13	13 (10)
50	13	13 (10)
68	17	17 (1)
69	17	17 (1)

Table II Cross reactions of pools with certain specific types: weaker reactions in parentheses

¹Background fluoresces with specific antiserum

reacting antisera. Table III indicates the dilutions to be used with multiple antisera reacting with a particular isolate to determine which is the stronger specific antiserum. Those antisera which could not be differentiated are listed in the final column. Weaker reactions are in parentheses.

Discussion

Many cross reactions have been observed among the capsular antigens of *Klebsiella*. The cross reactions which occurred with the fluorescent antibody method had previously limited the use of this technique for typing these organisms. The present studies have indicated a relationship between the degree of cross reaction and pH.

The capsular polysaccharides of the *Klebsiella* serological types have been found to contain uronic acids (Choy and Dutton, 1972) which are often a principal antigenic determinant (Heidelberger and Nimmich, 1972). These influence the specificity of the antigens in which they are present, and when their steric arrangement in different antigens is

Table III Each antigenic type, the specific antisera giving a positive reaction with each type at a dilution of 1/32, the dilution necessary to differentiate cross reacting antisera, and the remaining antisera still giving a strong fluorescence: weaker reaction in parentheses

¹Background fluoresces with specific antiserum

Antigenic Type	Specific Antisera giving Positive Reaction at Dilution 1/32	Dilution to resolve Cross Reactions	Remaining Antisera giving Positive Reaction
1	1	—	1
2	2 (69)	1/128	2
3	3, 68	—	3, 68
4	4	—	4
5	5	—	5
6	6	—	6
7	7, 10	1/64	7
8	8	—	8
9	9	—	9
10	10, 61, 7	1/128	10
11	11, 21 (33)	1/64	11
12	12, 29, 42 (41)	1/256	12 (29)
13	13, 12 (29, 21, 30)	—	13, 12 (29)
14	14 (64, 66)	1/64	14
15	15	—	15
16	16	—	16
17	17	—	17
18	18 (44)	1/64	18
19	19	—	19
20	20, 19, 37	—	20, 19, 37
21	21, 11	1/128	21
22	22 (29, 41)	1/128	22
23	23, 22 (37)	1/128	23
24	24	—	24
25	25	—	25
26	26, 22	—	26 ¹ , 22
27	46 (28)	1/64	46
28	28 ¹ , 27, 46	1/256	28, 27
29	29, 42 (12)	1/128	29
30	30 (13)	1/64	30
31	31	—	31
32	32	—	32
33	33	—	33
34	34	—	34
35	35, 33	1/64	35 (33)
36	36	—	36
37	37, 22	1/256	37
38	38	—	38
39	39	—	39
40	40 (17)	1/64	40
41	41 (12, 23, 38)	1/128	41 (12)
42	42 (29)	1/64	42
43	43	—	43
44	44, 18	1/128	44
45	45 (46)	—	45 (46)
46	46 ¹ , 27, 28	1/256	46
47	47	—	47
48	48, 49	1/64	48
49	48, 49	1/128	49
50	50	—	50
51	51	—	51
52	52	—	52
53	53	—	53
54	54	—	54
55	55	—	55
56	56	—	56
57	57	—	57
58	58	—	58
59	59	—	59
60	60	—	60
61	61	—	61
62	62 (63)	—	62 (63)
63	63	—	63
64	64, 66	1/256	64
65	65, 64	—	65, 64
66	66	—	66
67	67	—	67
68	68	—	68
69	69	—	69
70	70, 72	1/64	70
71	71	—	71
72	72	—	72

sufficiently similar, cross reactions take place (Eriksen, 1965).

The uronic acids contain weakly acidic carboxyl groups which are undissociated at a low pH but almost totally dissociated at a higher pH (White *et al.*, 1968). The results of the present studies indicate an increase in specificity with a rise in pH to 9.0, and it is possible that this may be due to the greater ionization of the carboxyl groups of the uronic acids. This may be affecting the binding of antibody by changing the structure and charge characteristics of the antigenic site.

A similar use of the effect of pH is employed by Schmidt and Lennette (1970), who pretreat serum at pH 9.0 for rubella haemagglutination tests to eliminate nonspecific, while fully preserving specific, activity.

Both the pool and specific antisera were improved by conducting the incubation at pH 9. Once the nonspecific fluorescence had been removed it was then possible to investigate the characteristics and limitations of the various antisera.

The pool antisera were found to be inadequate for some of the component types and could be rendered usable only by supplementing them with these particular specific antisera. Casewell (1972, 1975) also found that some of the same types had to be added to pool antisera in order to observe capsular swelling. A dilution of 1/40 was chosen for the pool antisera as this gave the best fluorescence of most of the specific types and the least cross reaction. There still remained some cross reactions between the pools at this dilution, although this was generally much weaker and was usually easy to distinguish from the specific pool. Some degree of identification can be made at this stage by the pattern of cross reacting pools for a particular type.

The majority of the type specific antisera gave optimum fluorescence and minimum cross reaction at a dilution of 1/32. Of the cross reactions observed at this dilution 70% were resolved by further diluting the positive antisera until the specific antiserum gave the greatest fluorescence. Only 13 cross reactions remained among the 72 different types.

One type, type 27, did not react with its specific antiserum. It has also been observed by Casewell to react with antiserum 46 rather than with its own. There was general interaction among antisera 27, 28, and 46, but they could be differentiated by use of the two recommended dilutions. There were also mixed cross reactions among antisera 12, 13, 22, 29, 41, and 42, but each type gave a different cross reacting pattern.

Variations in antisera patterns occasionally occur and should be considered as probably being unique types that are the results of mutation or that have not

yet been investigated.

It is not always necessary to dilute the antisera further to separate cross reactions for an identification. The cross reacting patterns themselves are usually different, and a tentative identification can be made at that stage by comparison with the table of established cross reactions. The antisera can be diluted further for confirmation.

Cross reactions observed with fluorescence are not believed to be caused by the presence of somatic antibodies in the sera. When antisera of types belonging to O group 1 were absorbed with an acapsular organism of that group, the same cross reactions between O group 1 types were still observed. It has also been noted that an organism of a particular group does not cross react even weakly with the antisera of all the other types in that group, and the cross reactions are randomly scattered among the 12 O groups. A bacterium must produce sufficient capsular material for a reaction to occur, otherwise it may not even react with its specific antiserum, as has been observed when using cultures grown on other media.

A few specific antisera give better fluorescence at a higher dilution and should not be missed by an apparently weak reaction at a dilution of 1/32. The list of cross reactions should be checked to find which antisera to include when the cross reacting antisera may appear slightly stronger at the low dilution. A granular fluorescence of the background can be another aid in identification. This is caused by slime material in the suspension which is produced by some of the types. The slime is of the same constitution as the capsule and gives the same specific reaction with the capsular antisera. It can be so concentrated as to mask the fluorescence of the bacteria but will be diluted out with a rise in titre, which allows the capsules to appear more brilliant. Although a few cross reacting antisera have been observed to produce this effect, it is generally specific and will usually indicate a specific reaction.

The titre of antiserum recommended to distinguish each type is based on several replications of the same batch of serum. Titres obtained for capsular reactions are sensitive to antigen excess (Kauffmann, 1949; Ørskov, 1955b), but careful control of the density of suspension will give reproducible titres (Casewell, 1975). Casewell also found that serum with different batch numbers gave the same titres on several occasions. Therefore, the titres proposed for use with fluorescence should be fairly consistent with any batch of antiserum provided the recommended concentration of bacteria is observed.

There are two ways of investigating the reactions of the 72 types and the specific antisera. The first is to discover all the types that react with a particular antiserum, and the second is to find all the antisera

that react with a certain type. Each leads to a different set of information which is not necessarily interchangeable. The latter has been chosen to incorporate into the typing method.

It is interesting from a research point of view to know what types cross react with each antiserum, but it is important for typing to know which antisera react with a certain type. When an unknown is being identified, it will react with one or more antisera, and it is more appropriate to check the results quickly against a table arranged for this purpose which will indicate the specific type.

An important characteristic of this typing method is that it gives a consistent typing of identical organisms regardless of pattern, and that it is possible to know exactly which of the patterns is the specific type. This is necessary for a positive identification of isolated strains in any epidemiological study.

The method that has now been developed can be summarized briefly. The bacteria are suspended in PBS and smears are made. To these are added the pool antisera at a dilution of 1/40 to which weaker types have been added at a recommended dilution. The pools are diluted in a buffer at pH 9.0. After the slides have been rinsed and dried, the conjugate is added at a dilution of 1/400 in PBS. The slides are read by grading the degree of fluorescence.

The pools that have given the strongest fluorescence are broken down into their specific antisera which are tested at a dilution of 1/32 at pH 9.0 following the same procedure. When cross reactions exist after this stage, the type may be identified by the pattern of cross reactions of the antisera diluted as recommended to separate them.

Summary of Method

- 1 Suspended bacteria in PBS to OD 0.03-0.04 at 500 nm.
- 2 Make smears and heat fix.
- 3 Add pool or specific antisera and incubate 20 minutes.
- 4 Rinse under running tap water 20 minutes.
- 5 Air dry.
- 6 Add FITC conjugate and incubate 20 minutes.
- 7 Rinse under running tap water 20 minutes.
- 8 Air dry.
- 9 Mount and store in deep freeze until read.
- 10 Examine microscopically.
 - ++++ brilliant fluorescence
 - +++ bright fluorescence
 - ++ definite fluorescence
 - + faint fluorescence
 - O no fluorescence

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