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Biochemical and serological investigations on clinical isolates of klebsiella

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SUMMARY

A series of 925 clinical isolates of klebsiella was examined by serological and biochemical typing. To perform serological typing (capsular swelling) 77 capsular antisera were prepared, tested against the type strains and grouped in 13 pools. With this serotyping method 80 % of the cultures were typable and 63 distinct types could be recognized.

All strains were typable biochemically by means of the numerical coding system of the API-20E system supplemented by digits derived from 15 additional conventional biochemical tests. With the API-20E system 24 different biotypes could be distinguished whereas the combination of API-20E and the 15 additional tests produced 93 biotypes. Maximum discrimination of strains was achieved by the combination of serological and biochemical typing (256 bioserotypes). The reproducibility, typability and discriminating power of the biotyping system was not inferior to serotyping. For epidemiological purposes biotyping can replace serotyping of *Klebsiella* species, especially in laboratories less well equipped.

INTRODUCTION

Klebsiella species are isolated with a relatively high frequency in hospital-acquired infections. This has led to the development of typing methods for this organism that would facilitate epidemiological investigations, since identification in routine clinical laboratories to generic or species level is unsufficient for this purpose. Capsular serotyping has been documented as the most reliable typing technique. Different methods have been used for capsular serotyping, such as capsular swelling (Casewell, 1972), indirect immunofluorescence (Riser, Noone & Poulton, 1976; Riser, Noone & Bonnet, 1976), counter-current immunoelectrophoresis (Palfreyman, 1978) and coagglutination and latex agglutination (Onokodi & Wauters, 1981). These techniques, of which capsular swelling is the most traditional, have been successfully used by several workers for epidemiological purposes (Casewell & Philips, 1978; Riser, Noone & Thompson, 1978; Seal *et al.* 1981; Smith, Digori & Eng, 1982).

Bacteriocin typing on its own has not yet proved reliable in distinguishing

strains because of its lack of reproducibility (Simoons-Smit *et al.* 1983). Bacteriophage typing (Slopek *et al.* 1967) is also not widely used as a typing technique in epidemiological analysis.

Extended studies of the biochemical properties of *Klebsiella* strains have been made in the past by Ørskov (1955, 1957). Usually three or six biotypes are distinguished by biochemical typing (Cowan *et al.* 1960; Edwards & Ewing, 1972). To subdivide *Klebsiella* strains into more distinct types for epidemiological purposes several authors have introduced biochemical typing schemes either by conventional test methods (Rennie & Duncan, 1974; Barr & Hogg, 1979; Haverkorn & Michel, 1979), or by commercially available biochemical test systems (de Silva & Rubin, 1977). The presence of multiple biotypes in single specimens and a biotype reproducibility of 64% were reasons for de Silva & Rubin (1977) questioning the value of biochemical typing of *Klebsiella pneumoniae*. A combination of biotyping and serotyping (Rennie & Duncan, 1974), or biotyping, serotyping and phage-typing (Rennie *et al.* 1978) was more discriminating than either method alone, thus promising to be a more useful aid in epidemiological studies.

This study deals with our experience in the production of capsular antisera and the subsequent serological typing (capsular swelling) of clinical isolates of klebsiella and in the biochemical typing of these strains with a combination of the commercial API-20E system and additional conventional tests. A critical evaluation of both typing methods, alone or in conjunction, has been made with special reference to typability, reproducibility and discriminating power. Furthermore, attention has been paid to the question whether biochemical typing could replace the more complicated serotyping methods for epidemiological purposes.

MATERIALS AND METHODS

Bacterial strains

A collection of 925 strains was obtained from routine clinical specimens submitted to the diagnostic microbiology laboratory of the Academic Hospital of the Free University. For clinical purposes they were identified as *Klebsiella pneumoniae* (sensu lato), *K. oxytoca*, *K. ozaenae* or *K. rhinoscleromatis* by the Enterotube II system (Hoffmann–La Roche, Basle, Switzerland) or sometimes by the API-20E system (API-system S.A. Montalieu Vercieu, France). A collection of 72 capsular type strains was in part kindly provided by Prof. E. M. Cooke, University of Leeds, and in part obtained from the National Collection of Type Cultures (NCTC). Strains were maintained on agar slants at room temperature and in a mixture of 1 ml trypticase soy broth and 1 ml of glycerol 60% (v/v) at −20 °C and at −70 °C. For each experiment strains were checked for purity.

Biochemical tests

For biotyping we used (1) the API-20E system and (2) 15 conventional test media (complementary to API-20E) described in Table 1.

Commercial media were prepared according to the manufacturers' instructions. The inoculum was prepared by suspending single colonies from 18 h cultures on nutrient agar in sterile water (API-20E) or phosphate-buffered saline, pH 7·4 (PBS) (conventional test media).

Table 1. *Biochemical tests for biotyping of klebsiella (complementary to API-20E)*

Test	Medium constituents/reference	Maximum incubation time/incubation temperature	
Acetate	Acetate agar (Merck) Bromothymolblue 0·0008 %	7 days	37 °C
Starch	Potato starch 10 % (Cowan & Steel, 1974 (method 1))	5 days	30 °C
KCN	KCN-broth (Cowan & Steel, 1974)	2 days	37 °C
Motility	Motility medium (Difco) Bacto TTC 10 %	18 h	37 °C
Gluconate	Gluconate broth (Cowan & Steel, 1974)	2 days	37 °C
Malonate	Malonate broth (Difco) Bromothymolblue 0·0025 %	2 days	37 °C
Glucose (gas)	Phenol red peptone water (Oxoid) glucose 1 %	5 days	37 °C
Lactose	Phenol red peptone water (Oxoid) lactose 1 %	5 days	37 °C
Dulcitol	Bacto Pepton 1 % dulcitol 1 % Bromothymolblue 0·02 %	5 days	37 °C
Methyl red (MR)	MR–VP medium (Oxoid)	2 days	37 °C
D-Tartrate Mucate	Organic acid medium Phenol red 0·0008 % (Cowan & Steel, 1974)	14 days	37 °C
Adonitol	Phenol red peptone water (Oxoid) Adonitol 1 %	5 days	37 °C
Sorbose	Phenol red peptone water (Oxoid) Sorbose 1 %	5 days	37 °C
Gelatinase	Nutrient gelatin (Cowan & Steel, 1974)	90 days	22 °C

Inoculation, incubation, reading and transcribing the results to a seven-digit code for the API-20E system were done according to the instructions given by the manufacturer.

The total incubation time of the complementary tests is shown in Table 1. The tests were read daily with the exception of gluconate oxidation and methyl red (MR) which were read only after 2 days, starch hydrolysis which was read after 5 days and the gelatinase test which was read weekly.

The results of the biochemical tests were recorded in accordance with the instructions of Cowan & Steel (1974), or by changes in the colour of the indicators incorporated in the media. Gluconate oxidation was read after the addition of one Clinitest (Ames and Co.,). Gas production from glucose was detected in a Durham tube. The results of the 15 conventional tests were converted to a 5-digit code, in

Table 2. *Cross-reactions of antisera at titre*

Antiserum	Cross-reacting antigen
6	46
33	35
34	9
41	43
43	41
47	80
61	63
63	10, 61
69	74

Table 3. *Pool composition*

Pool no.	Constituent antisera					
I	1	2	3	4	5	7
II	6	46	8	9	34	11
III	12	13	14	15	16	17
IV	18	19	20	21	22	23
V	24	25	26	27	28	29
VI	30	31	32	33	35	36
VII	37	38	39	40	41	43
VIII	42	44	45	48	49	50
IX	51	52	53	54	55	56
X	57	58	59	60	62	64
XI	10	61	63	65	66	67
XII	68	69	74	80	47	70
XIII	71	72	79	81	82	

the same way as in the API-20E system by dividing the biochemical tests in groups of three tests in the sequence of Table 1.

Capsular serotyping

Antisera production. Antisera were produced by two methods both involving intravenous inoculation of formalinized cultures into rabbits. Type strains to be used for antiserum production were tested for capsule production after cultivation on Worfel–Ferguson agar (Difco) by the indian-ink wet-film method. Suitable discrete capsule-producing colonies were used for antiserum production. The first method of antisera production was similar to that used by Edwards & Ewing (1972). If a sufficient titre (16 or greater) had not been reached on test bleeding after the last inoculation, two extra inoculations were given. In the case of an insufficient titre after these additional injections, a new antigen was prepared and the method of Edmondson & Cooke (1979) (this method was published during the time we were preparing our antisera) was used with freshly prepared vaccines for each inoculation. Additional injections were also given in this method when the titre was too low.

Testing of antisera. Each capsular antiserum was titrated against its homologous strain and tested in a working dilution (i.e. a little less than the titre dilution) against suspensions of all 77 capsular type strains by capsular swelling (Quellung

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Table 4. *Frequency of API-20E biotypes* (925 Klebsiella *strains*)

Biotype	No. of strains	%
5215773	618	67
5255773	235	25
5205773	20	2
5015773	10	1
5245773	7	1
Others (19)	35	4
	925	100

reaction) (Casewell, 1972). Removal of cross-reactions which could not be eliminated by further dilution was attempted by absorption (Kauffmann, 1954). Persistent cross-reactions after absorption are listed in Table 2.

Composition of pools. The contents of pools of antisera were based on the strong cross-reactions found in testing the antisera against the 77 capsular antigens. The constituent antisera of each pool are shown in Table 3. The final dilution of the constituent antisera of the pools were the same as the working dilution chosen for each of the specific antisera. The pools were tested against the corresponding capsular antigens and subsequently for cross-reactions with the other capsular serotypes. Cross-reactions were removed by absorbing the pools with one or more capsular type strains (Kauffmann, 1954). Mostly absorption with one capsular antigen was sufficient to remove several cross-reactions. No cross-reactions remained in our pools after absorption. Since cross-reactions will vary with each set of pools prepared no cross-reactions of the pools are listed here.

Typing unknown strains. Bacterial strains were cultured on Worfel Ferguson agar for 48 h at room temperature. Bacterial suspensions of these strains in 10% formol-PBS (about 1–5 micro-organisms per high-power field) were first tested for capsular swelling against the 13 pools. Where a positive result occurred with a pool the bacterial suspension was tested against the specific sera included in the pool at working dilution.

RESULTS

Biotyping

In our collection of 925 clinical isolates of klebsiella 24 numerical biotypes were distinguished by the API-20E system. Table 4 shows the most prevalent API-20E biotypes among the 925 strains. When the numerical results of the conventional tests were added to the API-20E biotypes the total number of biotypes could be enlarged to 93. The subdivision by the additional biochemical tests of the two most frequently occurring API-20E biotypes can be read from Table 5. The API-20E biotype 5215773 could be subdivided into 27 biotypes and the API-20E biotype 5255773 into 17 biotypes on the base of the added conventional biochemical tests. Tables 6 and 7 give the percentage of positive reactions in each of the biochemical tests in the API-20E and in the additional conventional tests for the species *K. pneumoniae* (*sensu lato*), *K. oxytoca*, *K. ozaenae*, *K. rhinoscleromatis* and for the total of all *Klebsiella* species. It can be seen that the most discriminating reactions for all *Klebsiella* strains are: indole, dulcitol, tartrate and sorbose. Whereas

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Table 5. *Frequency of subdividing biotypes of API-20E biotypes* 5215773 *and* 5255773 *by additional tests*

Biotype	No. of strains	%
5215773–56363	137	22
–56341	88	14
–56741	79	13
–56361	50	8
–56761	50	8
–52343	43	7
–56343	39	6
–56763	29	5
–56762	21	3
–52743	20	3
–56362	18	3
–56743	18	3
Others (15)	26	5
	618	100
5255773–56767	100	43
–56747	65	28
–56363	14	6
–56343	11	} 13
–56743	11	
–56763	11	
Others (11)	23	10
	235	100

K. oxytoca only differs from the other *Klebsiella* species by indole and mostly by gelatinase production (conventional test) it is clear that *K. ozaenae* and *K. rhinoscleromatis* differ biochemically in many reactions from the other *Klebsiella* species, although our number of these strains is small.

Capsular serotyping

All of the pooled and monospecific sera gave positive Quellung reactions with the specific type strains. The remaining cross-reactions after absorption are listed in Table 2. Titres of antisera varied between 16 and 512. With this set of antisera it was possible to differentiate serologically 735 (79·5 %) of the 925 clinical isolates. By this typing method 63 distinct types could be recognized (Table 8).

Combination of biotyping and serotyping

As serological and biochemical typing are independent of each other the combination of both methods could be used to increase the number of *Klebsiella* types. Strains which seemed to be the same with one typing method could be further subdivided by the other. Combining the serological (63 types) and the biochemical types (93 types) led in our series of 925 strains to a further subdivision to 256 different bioserotypes. The predominant serotype 18 (108 strains) could be subdivided into 3 biotypes (API-20E) or into 8 biotypes (combination of API-20E and conventional tests), the next common serotype 55 (72 strains) in the same way into respectively 2 and 10 biotypes. The same phenomenon was observed with the most common biotypes, 5215773–56363 (137 strains) and 5255773–56767 (100

Table 6. *Percentage of positive biochemical reactions (API-20E) among* 925 Klebsiella *strains*

Test	*K. pneumoniae* (667 strains)	*K. oxytoca* (250 str.)	*K. ozaenae* (6 str.)	*K. rhino-scleromatis* (2 str.)	All *Klebsiella* spp. (925 str.)
ONPG	100	100	100	0	99·8
ADH (arginine)	0·6	0	16·7	0	0·5
LDC (lysine)	99·1	98·8	50	0	98·5
ODC (ornithine)	0·6	0·4	0	0	99·5
CIT (citrate)	97·9	99·6	0	0	97·5
H_2S	0	0	0	0	0
URE (urease)	96·4	96·4	0	0	95·6
TDA (tryptophan)	0	0	0	0	0
IND (indole)	0	100	0	0	27·0
VP (Voges–Proskauer)	99·5	99·6	0	0	98·7
GEL (gelatinase)	0	0	0	0	0
GLU (glucose)	100	100	100	100	100
MAN (mannitol)	100	100	100	100	100
INO (inositol)	98·4	99·6	100	100	98·7
SOR (sorbitol)	99·7	100	33·3	100	99·3
RHA (rhamnose)	100	100	66·7	100	99·8
SAC (saccharose)	100	100	0	100	99·3
MEL (melibiose)	100	100	100	0	99·8
AMY (amygdaline)	100	100	100	100	100
ARA (arabinose)	99·7	99·6	100	0	99·5

strains), among which respectively 18 and 10 different serotypes could be recognized. Among these 18 and 10 different serotypes, K18 (91 strains) and K69 (59 strains) were respectively the most prevalent.

Reproducibility

As colony variation is very common in klebsiella and may give rise to inconsistency in serotyping by loss of K-antigens we selected morphologically different colonies from a number of strains for testing reproducibility of serotyping. From 111 strains two more or less morphologically different colonies and from 12 strains three morphologically different colony types were serotyped. Eighty-five strains (76·6 %) of the 111 strains kept the same serotype (including 33 non-typable strains) for both colonies, 23 strains (20·7 %) gave a serologically typable and a non-typable result of the 2 colonies and 3 strains showed a different serotype for both colonies. From the 12 strains of which 3 colony types were tested, 3 strains gave the same serotype for the 3 colonies, 6 strains gave the same serotype for 2 colonies and a non-typable result for the third colony and 3 strains showed a typable result for 1 colony and a non-typable result for the other 2 colonies.

The reproducibility of the biochemical typing method was tested by typing 10 colonies of 10 strains selected from the 925 strains exhibiting variable API-20E and conventional test profiles. These 100 colonies were tested with API-20E and five conventional tests which had shown a tendency to vary in the past in retesting strains. These tests were: D-tartrate, malonate, dulcitol, adonitol and sorbose. The

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Table 7. *Percentage of positive biochemical reactions among* 925 Klebsiella *strains (additional tests to API-20E)*

Test	*K. pneumoniae* (667 strains)	*K. oxytoca* (250 str.)	*K. ozaenae* (6 str.)	*K. rhino-scleromatis* (2 str.)	All *Klebsiella* spp. (925 str.)
Acetate	99·4	99·6	16·7	0	98·7
Starch	0·3	3·2	0	0	1·1
KCN	99·6	100	100	100	99·7
Motility	0	0	0	0	0
Gluconate	99·9	98·4	0	0	98·6
Malonate	88·6	99·6	0	100	91·0
Glucose (gas)	99·4	100	83·3	0	99·2
Lactose	99·9	100	100	0	99·7
Dulcitol	35·2	80·8	0	0	47·2
MR	0·6	0·8	100	100	1·6
D-Tartrate	51·1	60·4	0	0	53·2
Mucate	98·2	100	66·7	0	98·3
Adonitol	91·6	99·6	100	100	93·8
Sorbose	53·8	99·2	33·3	50	65·9
Gelatinase	0·9	77·2	16·7	0	21·6

Table 8. *Capsular types of* 925 *isolates of klebsiella*

Type	Strains No.	Strains %
18	108	12
55	72	8
69	67	7
8	51	5
33	49	5
21	44	5
63	38	4
24	35	4
2, 3 (16–20 each)	34	4
6, 7, 43, 54, 41/43,* 61/63* (11–15 each)	79	9
4, 22, 27, 38, 44, 45, 47, 62 (6–10 each)	58	6
Others (24 types) (2–5 each)	86	9
Others (14 types) (1 each)	14	1
Non-typable	190	21
	925	100

* Cross-reactions not separated.

biochemical tests in the API-20E system showed a reproducibility of 95 % for lysine decarboxylase (LDC), of 99 % for arginine dehydrolase (ADH), of 99 % for urease (URE) and of 100 % for the other tests. As for the conventional tests the reproducibility was 99 % for malonate and D-tartrate and 100 % for adonitol, sorbose and dulcitol.

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DISCUSSION

We investigated the biochemical and serological properties of 925 clinical isolates of *Klebsiella* strains. To specify biotypes which would facilitate any epidemiological analysis of our strains we tested the commercial API-20E system alone and in combination with 15 additional conventional biochemical reactions. The use of a combination of a commercial system with additional conventional tests for biotyping has not, as far as we know, been earlier described.

A total of 24 biotypes could be distinguished among our 925 clinical strains by using the API-20E alone. However, 92% of our strains were classified in two biotypes, in which the indole reaction was the only discriminatory test. This relatively low discriminatory power of the API-20E system is not unexpected, as tests chosen for differentiating genera of Enterobacteriaceae are not necessarily the most discriminating tests for differentiating strains of *Klebsiella*. The low frequency of urea or citrate-negative strains as the only other differences for 96% of our strains increased the need for additional biochemical tests in discriminating *Klebsiella* strains. The combination with our 15 additional tests raised the number of biotypes to 93. We found the most discriminatory reactions for all *Klebsiella* strains in the indole, dulcitol, D-tartrate and sorbose tests. Ørskov (1957) has already reported 36 biotypes of klebsiella on the basis of 11 biochemical tests, in which the four above-mentioned tests and the citrate test had the highest discriminating power. In our series of 925 clinical isolates 250 strains (27·0%) produced indole. This is a relatively high percentage of indole-positive strains compared with the findings of other authors (Eickhoff, Steinhauer & Finland, 1966; Edwards & Ewing, 1972; Rennie & Duncan, 1974) who found 5–16% of their strains indole-positive. In a more recent study, however, Rennie *et al.* (1978) also found a high percentage (35%) of their strains to be indole-positive, but they could not find any relationship between indole production and serotype or phagetype. Amongst the indole-producing strains 77·2% produced gelatinase, all very slowly in the conventional test. Because of the slow gelatin liquefaction in *Klebsiella* species the commercial API-20E system is not suitable for testing this biochemical property. Although gelatinase production can be shown in the conventional test, the long incubation time is a disadvantage for routine purposes or rapid epidemiological analysis. The biochemical differences of *K. ozaenae* and *K. rhinoscleromatis* are clear.

Serological typing seems, from a study of the literature, to be the most stable and reliable technique for typing *Klebsiella* species, but needs the production of 77 capsular antisera since these sera are no longer commercially available. The testing of the antisera produced and the typing of the unknown strains demand a lot of microscopic work. Although this method is tedious, the typability and reproducibility are high. The 80% typability percentage of our strains does not differ much from that of other authors (Rennie & Duncan, 1974; Casewell & Philips, 1978; Cooke *et al.* 1979; Rennie *et al.* 1978). Serotype 18 was the most common serotype among our strains, a serotype which has, as far as we know, never been reported in the literature as the most prevalent serotype among clinical isolates.

Another shortcoming of this typing method is the occurrence of cross-reactions.

Considerable differences between the cross-reactions of capsular antisera produced in different laboratories on different occasions (Edwards & Ewing, 1972; Palfreyman, 1978; Edmondson & Cooke, 1979) as well as in comparison with commercially produced antisera (Casewell, 1975) have been reported. Elimination of cross-reactions can be achieved by dilution, as the titre of such reactions is often lower than the homologous reaction, or by absorption. The only cross-reactions in our antisera which could not be removed at all were from those strains which reacted with antiserum against type strains 41 and 43, and against 61 and 63. In practice this proved to be only a minor disadvantage.

The recognition of many more types by combining serotyping and biotyping (> 200 serobiotypes in our series) is in agreement with the work of Rennie & Duncan (1974). The reproducibility of serotyping was tested with morphologically different colony types of a strain. As for serotyping the reproducibility can be disturbed by the loss of K-antigen of a strain which makes it insufficiently capsulated for capsular swelling. We saw this phenomenon in 23 (21 %) of the 111 strains tested with two morphologically different colonies and in 9 of the 12 strains tested with three colonies. Spontaneous loss of capsular antigen synthesis can occur *in vivo* and *in vitro*, leading to a mixed growth on culture media because of different colony types. It is therefore necessary to select a capsulated colony of a strain from a capsule-enhancing medium for serotyping by capsular swelling. The reproducibility of each of the biochemical tests in the API-20E and five of the conventional tests as tested by ten colonies of a strain was in our hands very high. Standardization of the inoculum, an incubation time of not less than 24 h for API-20E and reading of the tests by the same technician may be responsible for this high reproducibility of our biotyping system. In general, the biochemical characters of micro-organisms are chromosomally determined and highly stable. However, the possibility of transferable plasmid-mediated biochemical properties cannot fully be excluded. As a consequence, elimination from or acquisition of the ability of a particular biochemical reaction might lead to different biotypes in epidemiologically related strains. For complete characterization of outbreak strains, serotyping and/or the analysis of strains by the use of restriction endonuclease digestion might be necessary.

Serological and biochemical typing are both up to the present useful techniques to identify strains of *Klebsiella*. In those laboratories in which serotyping is not available biochemical typing with API-20E in combination with conventional tests can replace serotyping since the discriminating power, reproducibility and typability are in no way inferior to serotyping. However, with the combination of both methods a greater number of *Klebsiella* types can be distinguished which will give more information in epidemiological analysis of klebsiella infections and colonization of the patient. The potential value of these combined serotyping and biotyping methods in epidemiology will be the subject of our continuing study on the 925 clinical isolates.

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