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Evaluation and application of an improved bacteriocin typing method for *Klebsiella aerogenes*

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SUMMARY A bacteriocin typing method was evaluated using 200 strains of *Klebsiella aerogenes*, 93% of which fell into 11 distinct types. The typing technique was successfully applied to the monitoring and control of hospital cross-infection.

Nosocomial infection with Gram-negative bacteria has become increasingly common (Finland, 1973). *Klebsiella* species in particular have been responsible, and the apparent ease with which these organisms can spread, especially to debilitated patients, is a matter of concern (Price and Sleigh, 1970; Hill *et al.*, 1974).

Ascertainment of routes of cross-infection depends on accurate identification of bacterial species and of strains within species so that identical strains may be recognised (*Lancet*, 1971). The *Klebsiella* species commonly responsible for nosocomial infections is *K. aerogenes*. Several methods for typing these organisms have been developed. The most widely used is serological typing. This has been used successfully in epidemiological studies on its own (Hill *et al.*, 1974) and in conjunction with biochemical tests (Rennie and Duncan, 1974). However, the procedure is time consuming, even when sera are obtained from commercial sources and are used by experienced workers (Casewell, 1975; Riser *et al.*, 1976). Bacteriophage typing of *K. aerogenes* has been of limited value because many strains are non-typable (Asheshov, 1974, personal communication).

The use of bacteriocins produced by Klebsiella for typing was described by Slopek and Maresz-Babczyszyn (1967). Using eight bacteriocins only 58·5% of strains were typable. Using 10 bacteriocins Hall (1971) was able to type 77% of 800 strains tested. Nevertheless, the method was not discriminating enough. The present study was undertaken to develop a typing method which was both practicable and discriminating and could therefore be used in the detection and subsequent control of *K. aerogenes* cross-infection. The technique was applied to the monitoring of outbreaks of *K. aerogenes* infections in a general hospital over a two-year period. The typing method used bacteriocins produced by *Klebsiella* which inhibit the growth of other strains of *Klebsiella*. They are referred to in this paper as klebcins. Results reported by Hall (1971), using 10 of the same klebcin-producer strains that we used, differed considerably but different strains were typed by a different typing procedure.

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Material and methods

Strains were obtained from routine clinical specimens, environmental samples, and macerated food examined in the bacteriology department of Law Hospital. Those responding as indicated to the following tests were classified as *K. aerogenes;* indole−, glucose+, gas from glucose+, lactose+, inositol+, urease+, motility−, citrate ultilisation+. The methods used were those described by Cowan (1974) with the exception of methods for determining motility and citrate utilisation. These are described below.

This classification would therefore include bacteria classified by Cowan (1974) as *K. aerogenes*, *K. pneumoniae*, and *K. atlantae* and by Edwards and Ewing (1972) as *K. pneumoniae*. Indole-positive strains designated as *K. oxytoca* by Cowan (1974) were not included in the study.

Defined synthetic medium as described by Cruickshank *et al.* (1969), modified for detecting motility and citrate utilisation, was prepared in 100-ml quantities. Two agar tablets (Oxoid CM49) and a 'knife tip' of triphenyltetrazolium chloride (TTC) were added to each 100 ml. The medium was autoclaved at 121°C for 15 min, cooled to 50°C, and dispensed in 3-ml amounts in sterile bijou bottles.

For use a stab inoculum of the test organism was made and incubated at 37°C for 48 hours.

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Since sodium citrate was the only carbon source in the defined synthetic medium the reduction of TTC to red triphenylformazan indicated the ability of the test organism to utilise citrate as a sole carbon source. A single red streak was produced by non-motile 'citrate utilisers', whereas motile citrate-utilising organisms produced a diffuse pink colour in the medium.

A selective medium was devised that allowed *K. aerogenes* to multiply while inhibiting many other enterobacteria. 0·15% bile salts (Oxoid No. 3) were added to basal defined medium without glucose (Cruickshank *et al*., 1969) in which citrate was the sole carbon source.

Clinical specimens, environmental swabs, and macerated food were directly cultured onto MacConkey agar plates (Oxoid No. 2) and were also placed into 25-ml universal containers containing 10 ml of the selective medium. This medium was then subcultured on to MacConkey agar after three and seven days' incubation at 37°C.

KLEBCIN TYPING

Klebcin-producer strains (that is, Fkt 3, 4, 5, 7, 23, 24, 27, 30, 77, and 9137) were supplied by Dr G. A. J. Ayliffe, Summerfield Hospital, Birmingham and were those originally used by Hall (1971). Four other producer strains (AM 1, 4, 7, and 21) were isolated in our laboratory in the following way. Twenty-five strains of *K. aerogenes* from different patients were tested for their ability to produce klebcins. Each strain was grown in 10 ml of nutrient broth at 37°C for 24 hours. Then 3 ml of the broth culture was poured on to a dry MacConkey agar (Oxoid No. 2) plate and the excess fluid was removed. Inoculated plates were then left at room temperature until the agar surface was dry.

The remainder of each broth culture was centrifuged and the supernatants were spotted on to the inoculated MacConkey plates. The plates were incubated at 37°C for six to eight hours. Inhibition of growth by a culture supernatant, which when serially diluted failed to form plaques, indicated the production of klebcin rather than bacteriophage.

The typing method was a modification of that described by Noy *et al.* (1974), who reported the inhibition of unknown organisms by klebcins. Cellulose acetate membranes 6 cm in diameter (Grade 0·45, Oxoid) were placed in the centre of well-dried MacConkey agar plates. Four plates were used for each test organism. Separate areas on the membranes were inoculated with culture from agar plates of each of the 14 producer strains. The inoculum site was about 0·5 cm in diameter. Seven producer strains were inoculated on to one membrane and the other seven on to another membrane. This was done in duplicate. After incubation the membranes were removed from the agar plates. Strains to be tested were spread on to MacConkey agar plates, so as to obtain single colonies, and incubated overnight at 37°C. A colony of each test strain was suspended in 3 ml of 0·15-M NaCl. The four MacConkey agar plates, from which the membranes had been removed, were then flooded with the suspension of the test strain and the excess fluid was removed. When dry the plates were inverted and incubated at 37°C for about six hours. Any inhibition of growth caused by klebcin which had diffused through the membrane into the agar was recorded. Only zones which exhibited complete inhibition of growth were used in the typing scheme. Strains of known klebcin sensitivity were included as controls.

ANTIBIOTIC SENSITIVITY TESTS

All strains of *K. aerogenes* were tested for their sensitivity to carbenicillin (100 mg/l), oxytetracycline (10 mg/l), cephaloridine (25 mg/l), and kanamycin (33 mg/l). Sensitest agar plates (Oxoid CM409) containing these antibiotics were inoculated with three-hour broth cultures using a multi-inoculator. Resistance was assessed by comparison with inoculated antibiotic-free Sensitest agar plates after incubation for 18 hours at 37°C.

In addition, sensitivity to gentamicin (10 μg), colistin (10 μg), and ampicillin (10 μg) was determined by a disc diffusion technique. Sensitest agar plates were flooded with an inoculum (about 10^6 organisms/ml) of the test organism in 0·15-M NaCl. When dry, Oxoid Multidisks were applied and the sensitivity pattern was recorded after 18 hours' incubation at 37°C.

Results

KLEBCIN TYPING

Two hundred and fifty-three strains of *K. aerogenes* were used to evaluate the technique. Fifty-three were repeat cultures of a klebcin type identical with previous cultures from the same sites in individual patients and were therefore excluded from the analysis. In 33 cases these strains with identical klebcin types were isolated two or more times over periods of two to three weeks. This demonstrates both the reproducibility of the typing method and the stability of klebcin types.

The klebcin typing of 200 strains of *K. aerogenes* fell into 11 distinct groups (Table 1 shows the inhibition patterns). The sources of the 200 strains are shown in Table 2. Particular klebcin types did not seem to be isolated significantly more from one clinical source than another.

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Table 1 *Klebcin typing scheme*

Klebcin type	*Sensitivity to klebcins from producer strains:*													
	Fkt										*AM*			
	3	*4*	*5*	*7*	*23*	*24*	*27*	*30*	*77*	*9137*	*1*	*4*	*7*	*21*
1		+		+		+					+	+	+	+
2		+		+		+	+				+	+	+	+
3	+	+	+	+		+	+	+	+		+	+	+	
4		+		+		+				+	+	+	+	+
5		+		+		+	+				+	+	+	
6														+
7	+	+	+	+		+	+	+	+		+	+	+	+
8		+		+		+					+	+	+	
9							+							
10							+							+
11	+	+	+	+		+		+	+		+	+	+	+
12	+		+					+	+					+
13		+		+		+				+	+	+	+	
14					+		+							
15	+	+	+	+		+		+	+		+	+	+	

+ zone of complete inhibition.

Table 2 *Sources of 200 strains of* Klebsiella aerogenes *with klebcin type*

Klebcin type	*Urine*	*Sputum*	*Pus*	*Necropsy*	*Throat, mouth swabs*	*Sink swabs, floor waters*	*Ear swabs*	*Eye swabs*	*Faeces*	*Other*	*Total*
1	16	8	2	1	—	—	1	—	—	—	28
2	15	4	—	2	—	—	—	1	2	—	24
3	—	2	—	—	—	—	—	—	—	—	2
5	6	6	1	1	—	1	1	—	—	—	16
6	27	14	8	—	1	2	—	—	1	1 skin swab	54
7	5	2	1	—	—	—	—	—	—	1 blood culture	9
8	11	8	3	2	—	2	1	—	—	1 umbilical swab	28
9	3	1	—	—	1	—	—	—	—	—	5
10	2	—	1	1	—	—	—	—	—	—	4
11	5	1	2	—	—	—	—	—	—	—	8
15	2	3	1	—	—	2	—	—	—	—	8
Non-typable	7	3	2	1	1	—	—	—	—	—	14
Total	99	52	21	8	3	7	3	1	3	3	200

Strains of *K. aerogenes* with klebcin types 4, 12, 13, and 14 were not found in this study.

EPIDEMIOLOGY

Between January and June 1973 six patients in the intensive care unit (ICU) at Law Hospital were found to be infected with *K. aerogenes*. In each case the organisms had similar antibiotic sensitivity patterns and were of klebcin type 6. During the same period *K. aerogenes* was isolated from nine patients in two adjacent surgical wards (A, male, and B, female). The organisms isolated from five of these patients were also of klebcin type 6 and again had similar antibiotic sensitivity patterns. Patients were sometimes transferred between the wards and the ICU.

To determine the vehicles of cross-infection swabs were taken from sink overflows, drains, and floors in the ICU. This environmental sampling yielded no *K. aerogenes* until June 1973 when type 6 was isolated from a whitlow on an ICU nurse's finger.

In August and September 1973 type 6 strains were isolated from three more patients in these wards. Details of the initial isolations of klebcin type 6 *K. aerogenes* from individual patients in the ICU and wards A and B between January and September 1973 are summarised in Table 3. Thereafter for over three months there were no isolations of this strain from these wards or the ICU and there was no evidence of *K. aerogenes* cross-infection in the hospital. Then in January 1974 a type 6 strain was isolated from the urine of an ICU patient (case 1, Table 4).

During the following six weeks three more ICU patients were infected. In March 1974 the sister of the ICU acquired a urinary tract infection with a type 6

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Table 3 *Initial isolations of klebcin type 6* K. aerogenes *from ICU and wards A and B January-September 1973*

Case no.	*Ward*	*Date*	*Source*	*Antibiotic sensitivity pattern*						*Other sites of subsequent isolation*
				A	*Carb*	*T*	*G*	*CS*	*K*	
1	A	25/1	Sputum	R	R	S	S	S	S	
2	ICU	12/2	Urine	R	R	S	S	S	R	
3	ICU	22/2	Sputum	R	R	S	S	S	R	
4	A	27/2	Urine	R	R	S	S	S	R	
5	A	28/2	Sputum	R	R	S	S	S	R	
6	ICU	7/3	Sputum	R	R	R	S	S	R	Faeces, urine, pus (abdominal wound)
7	ICU	20/3	Urine	R	R	R	S	S	R	
8	A	11/5	Urine	R	R	R	S	S	R	
9	ICU	19/5	Pus (peritoneum)	R	R	R	S	S	R	
10	ICU	19/5	Urine	R	R	R	S	S	R	Sputum
11	B	28/5	Urine	R	R	R	S	S	R	
12	ICU	28/5	Pus (whitlow on finger)	R	R	R	S	S	R	
13	ICU	4/6	Drains and overflows	R	R	R	S	S	R	
14	A	3/8	Wound (mid thigh)	R	R	S	S	S	S	
15	ICU	8/8	Urine	R	R	S	S	S	R	
16	A→ICU	6/9	Abdominal wound	R	R	S	S	S	R	Sputum

R = resistant; A = ampicillin; G = gentamicin; S = sensitive; Carb = carbenicillin; CS = colistin; T = oxytetracycline; K = kanamycin.

Table 4 *New isolations of klebcin type 6* K. aerogenes *from ICU*

Date (1974)	*Case no.*	*Source*	*Other sites of isolation*
Jan. 4	1	Urine	
20	2	Tracheal secretions	Postoperative wound
Feb. 6	3	Tracheal secretions	
19	4 (transferred to ward A)	Wound (bowel) fistula	
Mar. 23	5	Urine	
May 27	6	Urine	
June 3	7	Faeces	

strain and at the end of May another patient was infected. A type 6 strain was isolated from the faeces of an ICU patient at the beginning of June. During this period the environment and the food entering the ICU were regularly sampled for the presence of *K. aerogenes*. Type 6 strains were isolated from sliced boiled ham which was supplied to the ICU from the main hospital kitchen at the end of April. Investigations revealed that the slicing machine used was contaminated with type 6 *K. aerogenes*. The source of this contamination was not established.

On 19 February an ICU patient was transferred to ward A. Twelve days later a type 6 strain was isolated from the sputum of another patient who developed a chest infection in ward A. Five more patients became infected during the next two months, as shown in Table 5. These infections included urinary tract infections, postoperative wound infections, and one case of septicaemia.

For two months there were no new isolations until the end of July 1974, when a toe amputation wound became infected. It was the beginning of the most severe outbreak of *K. aerogenes* infections in this ward. From August until December 1974 10 more patients were infected (Table 5). At the end of August seven of these patients were in the ward at the same time. Type 6 *K. aerogenes* was isolated from the faeces of nearly all these patients. During this time these organisms were isolated from sinks, toilets, and a bath but not from the air or food.

Table 5 *New isolations of klebcin type 6* K. aerogenes *from ward A*

Date (1974)	*Case no.*	*Source*	*Other sites of isolation*
Mar. 3	1	Sputum	
19	2	Urine	
Apr. 25	3	Urine	Postoperative wound and faeces
29	4	Urine	
May 7	5	Urine	Faeces
15	6	Blood culture	
July 26	7	Toe amputation	Urine, faeces, below knee amputation wound
Aug. 8	8	Bile (CBD)	Postoperative wound, faeces, sputum
20	9	Urine	
Sept. 4	10	Colectomy wound	
6	11	Appendectomy wound	Faeces
11	12	Prostatectomy	Bed sores, faeces
19	13	Colostomy wound	Faeces
25	14	CSU	Faeces
Oct. 29	15	CSU	(Rectal swabs not taken)
Nov. 11	16	Faeces (diarrhoea)	
23	17	Sputum	

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The problem subsided only when the highly infected patients were discharged or died in the closing months of 1974. Interestingly, one patient was removed to a medical ward. Although this ward contained several small six-bedded compartments five more patients became infected over a three-month period.

Discussion

KLEBCIN TYPING

Sensitivity to klebcins from Fkt 4, Fkt 7, Fkt 24, AM 1, AM 4, and AM 7 always occurred together, as shown in Table 1. These klebcin-producer strains were all klebcin type 6. It was therefore decided to omit Fkt 7, Fkt 24, AM 1, AM4, and AM 7 from the typing scheme and to use only Fkt 4.

Sensitivity to klebcins from Fkt 3, Fkt 5, Fkt 30, and Fkt 77 also always occurred together. These klebcin-producing strains were all klebcin type 6 except Fkt 3, which was non-typable. It was therefore decided to omit Fkt 5, Fkt 30, and Fkt 77 as they did not contribute to the overall typing scheme. These three strains evidently produce klebcins with the same spectrum of activity as Fkt 3 since unknown organisms exhibit the same sensitivity patterns.

There was no evidence of sufficient klebcin production by Fkt 23 and Fkt 9137; their use was therefore discontinued.

These omissions left a typing scheme (Table 6) in which only Fkt 3, Fkt 4, Fkt 27, and AM 21 were used as producer strains. With these four producer strains 93% of the 200 strains of *K. aerogenes* tested were divided into 11 types and only 7% were non-typable. The search for different klebcin-producer organisms will probably reduce the percentage of non-typable strains still further.

EPIDEMIOLOGY

The outbreaks of nosocomial infections with type 6 *K. aerogenes* were not part of an ongoing episode before 1973 as this strain had only once been isolated from the ICU and never from wards A and B during 1972.

For three periods of two to three months each (March to May 1973, June to August 1973, and February to May 1974) there were no newly infected patients in the ICU. There did not seem to be any patients acting as reservoirs for type 6 *K. aerogenes* during these periods. Therefore possibly the organisms were able to remain in the environment or were being carried by staff. Environmental contamination with type 6 *K. aerogenes* was shown by the isolation from the ICU sinks in June 1973 and May 1974. The possibility of carriage of type 6 *K. aerogenes* by staff was highlighted by the nurse's infected finger at the end of May 1973 and the ICU sister who contracted a urinary tract infection in March 1974.

Hand washing in ward A, which was a 30-bedded 'Nightingale type' ward, was rendered difficult by only one sink being available in a dressing room at one end. To find *K. aerogenes* in sinks, baths, and toilets used by infected patients is perhaps not surprising. However, every effort must be made to avoid infecting other patients with such organisms. There was only one bath for all patients on ward A and it was cleaned between use with an abrasive, non-disinfectant paste which was introduced for the sake of economy. The reintroduction of a bleach-containing cleaner resulted in no further isolations of *K. aerogenes* from baths sampled after cleaning.

Transmission of pathogens by food has been described (Shooter *et al.*, 1971; Walton and Lewis, 1971). The isolation of type 6 *K. aerogenes* from boiled ham supplied to the ICU and the machine on which it was sliced emphasises the need for strict hygiene to be exercised by kitchen staff and the need for thorough cleansing of machinery used to slice cooked meats. Although the machine was modern in design several parts of it could not be removed for cleaning. It was both impracticable and dangerous to remove the blade. No receptacle was used to collect the food when sliced. Thus assorted particles of meat accumulated on the fixed collecting tray.

This study showed that debilitated patients—which, of course, includes all patients under intensive care—are at far greater risk than others. Surgical patients, particularly those with lower abdominal wounds and those who are catheterised, are also at high risk of infection—often from their own gut which has become colonised with *K. aerogenes* after admission to hospital (Selden *et al.*, 1971). Such colonisation must be guarded against.

Hospital acquired *K. aerogenes* infection does not seem to affect short-stay patients. Seven days was the

Table 6 *New klebcin typing scheme*

Klebcin type	*Sensitivity to klebcins from producer strains:*			
	Fkt 3	*Fkt 4*	*Fkt 27*	*AM 21*
1 (as 4)		+		+
2		+	+	+
3	+	+	+	
5		+	+	
6				+
7	+	+	+	+
8 (as 13)		+		
9 (as 14)			+	
10			+	+
11	+	+		+
12	+			+
15	+	+		

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shortest time taken for infection to develop and in most cases it took considerably longer.

Conclusions

Our scheme would enable hospital laboratories to type cheaply, easily, and rapidly a high proportion of strains of *K. aerogenes*, thus facilitating ascertainment and control of cross-infection with these organisms. The use of klebcin typing in conjunction with biochemical classification and antibiotic sensitivity testing has enabled repeated outbreaks of nosocomial infections with a particular strain of *K. aerogenes* to be recognised and monitored. Possible transmission of pathogens by staff and food has been discovered and several reservoirs of *K. aerogenes*—namely, sinks, baths and toilets, staff, a meat-slicing machine, and the patients' intestinal flora.

This study shows that the control of hospital infection requires an awareness of the problems by all the relevant staff. Doctors and nurses who come into direct contact with patients must recognise the need for careful patient-care procedures, particularly hand washing (Schaberg *et al.*, 1976). Nursing and domestic staff should minimise the risk of cross-infection by cleaning baths between each use with a bleach-containing abrasive powder (Alder *et al.*, 1966). While it is neither practicable nor usually necessary to prepare sterile food, kitchen staff should take the greatest care, particularly when handling cooked meat which is ready for eating (Shooter *et al.*, 1971). Such co-operation should reduce the exposure of patients to hospital strains of *K. aerogenes* and thereby minimise the opportunity for cross-infection.

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