J. Hyg., Camb. (1983), **90**, 461–473
Printed in Great Britain

Comparison of different methods for bacteriocin typing of *Klebsiella* strains

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(*Received* 14 *September* 1982; *accepted* 5 *January* 1983)

SUMMARY

Epidemiological analysis of *Klebsiella* strains from nosocomial infections needs a simple, stable and reproducible technique of typing. We have evaluated three bacteriocin typing methods for *Klebsiella* spp. by means of 15 bacteriocin-producing strains, with special reference to stability and reproducibility.

With the three methods indicator strains and clinical strains were retyped on different days under constant test procedures. Stability of bacteriocins was tested by titration immediately after preparation and after 4 weeks of storage at −70 °C. Thereafter, reproducibility of typing was tested by means of these freshly prepared bacteriocin lysates and of portions of the same lysates stored at −70 °C. A moderate reproducibility was obtained with one method after two typing experiments: 79·2 % and 61·3 % for indicator strains and clinical strains respectively. The other two methods gave a much lower reproducibility of 38·5 % and 32·5 % for indicator strains and of 11·1 % and 25·5 % for clinical strains after two experiments.

The reproducibility decreased after retyping three, four or five times. These methods are simple to perform but their usefulness for epidemiological studies is doubtful. Possible causes of the lack of reproducibility of the methods are discussed.

INTRODUCTION

Hospital-acquired infections with *Klebsiella*, especially with multiply resistant strains, have become increasingly common (Martin, Yu & Washington, 1971; Casewell & Philips, 1978; Curie *et al.* 1978; Cooke *et al.* 1979). The incidence of *Klebsiella* infections in hospital patients with coliform infections is reported to be as high as 16 % (Cooke *et al.* 1979).

Several epidemics and single cases of nosocomial cross-infection with this micro-organism have been described, especially among patients on urological wards and in (neonatal) intensive care units (Price & Sleigh, 1970; Hable *et al.* 1972; Hill, Hunt & Matsen, 1974; Casewell *et al.* 1977). For epidemiological purposes typing of microbial isolates is necessary to establish the sources of infection and the routes of transmission. Many different typing methods for *Klebsiella* strains have been described, including capsular serotyping (Casewell, 1975; Riser, Noone & Bonnet, 1976), biotyping on its own or in combination with serotyping (Rennie

https://doi.org/10.1017/S0022172400029107 Published online by Cambridge University Press

& Duncan, 1974), bacteriophage – although not widely used – (Ślopek *et al.* 1967) and bacteriocin typing.

Typing *Klebsiella* strains by bacteriocin sensitivity has been described by several authors, using different methods and a different number of bacteriocin-producing strains (Ślopek & Maresz-Babcryszyn, 1967; Hall, 1971; Buffenmyer, Rycheck & Yee, 1976; Heddell & Mitchell, 1978; Edmondson & Cooke, 1979; Israïl, 1980*a*, *b*). The percentage of typable strains has varied from 58·5% (Ślopek & Maresz-Babcryszyn, 1967) to 97% (Edmondson & Cooke, 1979). However, little or no attention has been paid to reproducibility and stability in bacteriocin-typing methods of *Klebsiella* strains. Hall (1971) did mention the low discriminating power of the scrape and streak method due to the large number of weakly sensitive reactions with low reproducibility. Buffenmyer *et al.* (1976) also felt the lack of reproducibility to be a major problem in their typing procedure with mitomycin C-induced bacteriocin preparations. Evaluating a bacteriocin-typing method for *Klebsiella* strains by the use of cellulose acetate membranes on solid media, Heddell & Mitchell (1978) did not provide any exact data about reproducibility and stability. Edmondson & Cooke (1979), using the growth in broth method with mitomycin C induction, reported a reproducibility of 67% if patterns differing by one reaction were regarded as distinct and a reproducibility of 89% by the application of a two-difference rule. The aim of this study was to evaluate the published methods of bacteriocin typing of *Klebsiella* strains with special reference to stability and reproducibility.

MATERIALS AND METHODS

Bacterial strains

The following bacterial strains were used in this study:

15 bacteriocin-producing strains of *Klebsiella* (labelled P 1 to P 15) and 16 indicator strains of *Klebsiella* (labelled I 1 to I 16), kindly supplied by Professor E. Mary Cooke, Department of Microbiology, University of Leeds LS2 9NL, U.K.

Klebsiella strains (111) were obtained from routine clinical specimens (from neonates in the neonatal intensive care unit). They were identified as *Klebsiella* by the API 20E-system (Analytab Products Inc., Plainview, N.Y.). Pure cultures of each isolate were maintained on nutrient agar slants at room temperature and in a mixture of 1 ml trypticase soy broth and 1 ml of 60% glycerol at −20 °C and at −70 °C.

Each experiment was performed with pure cultures, started from a single colony.

Media and reagents

Proteose peptone no. 3 (PP3) broth contained 20 g of proteose peptone no. 3 (Difco Laboratories, Detroit, Michigan) and 5 g of sodium chloride per litre of distilled water (pH 7·3).

Proteose peptone agar no. 3 was prepared by adding 12 g Oxoid agar no. 3 to the PP3 broth (pH 7·3).

Phosphate-buffered saline (PBS): NaCl 8 g, K_2HPO_4 1·21 g, KH_2PO_4 0·34 g per litre of distilled water (pH 7·3).

Trypticase Soy Broth (TSB) was obtained from BBL (Cockeysville, Md., U.S.A.). Mitomycin C (Sigma Chemical Company) powder was dissolved in sterile distilled

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water to yield a concentration of 40 μg/ml and stored in amounts of 5 ml in the dark at −20 °C. Before usage a 5 ml vial was thawed and diluted in sterile distilled water to yield a concentration of 10 μg/ml.

Cellulose acetate membranes grade 0·45, 85 mm in diameter, were obtained from Oxoid Ltd., Basingstoke, England.

Bacteriocin typing of Klebsiella

Method 1. The growth in broth method, developed by Edmondson & Cooke (1979) was used with the following modification: mitomycin C-induced lysates were spotted onto well-dried PP3-agar plates with the aid of a multiple inoculator (Ridgeway-Watt system, Denley Instruments, Sussex, UK) before test strains were applied.

After diffusion of the spots into the agar, lawns of test strains were poured over the agar plates as described by the authors. This modification resulted in clearer inhibition zones and resolved our problems of weak inhibition zones around the control spots, which were shown to be caused by mechanical disruption of the lawns of the test strains. Unless otherwise stated fresh bacteriocin lysates were used for every experiment.

Method 2. A method with mitomycin C incorporated in PP3 agar plates. Exponential phase cultures of producer strains were prepared as described in method 1 and spotted on to well-dried PP3-agar plates containing 0·1 μg mitomycin C per ml. After incubation (18 h, 37°) plates were sterilized by chloroform vapour for 30 min. Plates were then removed and left inverted and open for 30 min. at room temperature to evaporate. Lawns of test strains were prepared as described in method 1, except that 0·2 ml of the 1:10 dilution in PBS was added to 6 ml agar 0·7% (w/v).

Method 3. Method described by Heddell & Mitchell (1978) in which after diffusion of the bacteriocins through cellulose acetate membranes on solid media, suspensions of test strains were applied. We used PP3-agar plates instead of MacConkey, resulting in greater inhibition zones because of the less mucoid growth of *Klebsiella* on PP3 agar than on MacConkey agar.

In all three methods positive zones of inhibition were recorded and converted to a five digit code by using the simplified notation (Table 1) as described by Farmer (1972). Zones with weak inhibition were regarded as positive in coding.

Titration of bacteriocin lysates

The bacteriocin preparations were serially diluted eightfold in PP3-broth, ranging from 1:8 to 1:512 and then twofold to 1:65536 except for P 6, P 9, P 10 to P 15 which were diluted twofold from 1:2 to 1:256 (Buffenmyer *et al.* 1976).

One μl drops of each dilution were then applied to PP3-agar plates and allowed to diffuse into the agar.

Lawns of corresponding indicator strains to each producer strain were prepared as described in method 1 and poured over the spotted PP3-agar plates. Plates were incubated at 37 °C for 18 h. The reciprocal of the highest dilution of bacteriocin, which gave a clear zone of inhibition, was defined as the titre of the bacteriocin preparation.

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RESULTS

Titration of bacteriocin lysates

The bacteriological activity of freshly prepared mitomycin C-induced bacteriocin preparations was determined against the corresponding indicator strains. In repeated titrations we could not find any activity in the bacteriocin lysate of P 14 and we therefore did not use this producer strain in further experiments.

The producer strains P 1 to P 5, P 7 and P 8 showed high activity in their bacteriocin preparation (titre > 2048) whereas strains P 6, P 9 to P 15 failed to produce high concentrations of bacteriocin (Table 2). Titres were reproducible in repeated experiments (with a maximal deviation of one dilution). Although isolation of bacteriophages is possible in the same way as described here for bacteriocins, we do not have the impression that bacteriophages influenced our typing system, because dilution of bacteriophage suspensions should have resulted in separated plaques on sensitive strains. We never observed these plaques.

Bacteriocin typing method 1

The indicator strains I 1 to I 16 were retyped three times on three different days by this method, with freshly prepared bacteriocin lysates and under constant test procedures. Table 3 shows the results of retyping these strains. Of the indicator strains 12 (75 %) gave the same results three times. Discrepancies were observed in four strains (25 %), either from positive or weakly positive to negative or the reverse. From the four deviating strains two gave the same results twice and deviated the third time by one or two reactions. The other two strains showed on the occasions three different patterns (differing by two or more reactions). In the same way 111 patient strains were typed on two different days. Of these strains 105 (94·6 %) with 23 different patterns, and 101 (91 %) with 14 different patterns were typable on the two different days. Only eight patterns were in common. One type predominated with 64 and 72 strains on both typing days. There were 59 strains which showed this predominant pattern on both the first and the second occasion.

In total 68 strains (61·3 %) gave the same results twice, 18 (16·2 %) differed in one reaction, 14 (12·6 %) differed in two reactions and 11 (9·9 %) differed in three or more reactions (Fig. 1). The differences in bacteriocin patterns could not be ascribed to certain producer strains. The reproducibility and the number of different reactions in the patterns after typing the indicator strains twice and thrice and the clinical strains twice with method 1 can be read from Table 4. To determine whether the low reproducibility was due to a varying degree of bacteriocin production by the producer strains, freshly prepared bacteriocin lysates were divided in small amounts and stored at −70 °C.

The effect of storage on the titre of bacteriocin lysates was assessed by titration of freshly prepared lysates and those after 4 weeks of storage. There was no marked decrease in activity (at the most one dilution) after 4 weeks of storage at −70 °C. Overnight storage at +4 °C gave a remarkable decrease in activity of several two-fold dilutions. Sixteen indicator strains and 27 of the 111 clinical strains were typed four times, first with freshly prepared bacteriocin lysates and three times after that with an interval of about 4 days with the rest of the lysates stored at

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Table 1. *Simplified notation for reporting bacteriocin types*

Results of three tests	Notation	Results of two tests	Notation
+ + +	1	+ +	A
+ + −	2	+ −	B
+ − +	3	− +	C
− + +	4	− −	D
+ − −	5		
− + −	6		
− − +	7		
− − −	8		

Table 2. *Titres of bacteriocin preparations of producer strains against their corresponding indicator strains*

Producer strain/ indicator strain	Titre	Producer strain/ indicator strain	Titre
P 1–I 1	65536	P 6–I 6	16
P 2–I 2	4096	P 9–I 9	8
P 3–I 3	32768	P 10–I 10	32
P 4–I 4	65 536	P 11–I 2	32
P 5–I 5	16384	P 12–I 3	2
P 7–I 7	8192	P 13–I 11	256
P 8–I 8	2048	P 14–I 12	–
		P 15–I 13	8

−70 °C. Different batches were used for every typing run, so that every batch was frozen and thawed only once.

Of the 16 indicator strains only eight strains (50 %) gave the same results four times. Discrepancies were seen in eight strains of which five strains showed the same pattern thrice and differed once by one reaction. Three strains demonstrated the same pattern twice but gave one or two different reactions on the other two typing experiments.

Of the 27 clinical isolates only 12 strains (44·4 %) gave the same bacteriocin pattern four times. Of the remaining strains 13 (48·2 %) gave identical patterns thrice. Two strains gave the same results twice but a different pattern twice. In total, 17 different bacteriocin patterns were obtained in the four typing runs but only one pattern was predominant and observed four times.

Table 5 shows the reproducibility after typing the indicator strains and the clinical strains two, three or four times with the stored bacteriocins. The number of different reactions in the deviating patterns after four typing runs is also given. From these results the conclusion can be drawn that the lack of reproducibility cannot be explained by a varying production of bacteriocins. After more than two typing runs the differences in results were not restricted to certain producer or indicator strains, but showed a random distribution. This phenomenon results in a rapid decrease in the percentage of strains showing identical patterns after three or more runs.

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Table 3. *Results of retyping* 16 *indicator strains* (*method* 1)*

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P15	Notation I	Notation after retyping† II	III
I 1	+	+	+	+	+	–	+	+	–	–	–	–	–	–	1228 D	1228 D	1228 D
I 2	+	+	+	+	+	–	–	+	+	+	+	+	–	–	1241 D	1241 D	1241 D
I 3	+	+	+	+	+	–	+	+	+	+	+	+	–	–	1211 D	1211 D	1211 D
I 4	+	+	+	+	+	–	+	+	+	+	+	+	–	–	1211 D	1211 D	1211 D
I 5	+	+	+	+	+	–	+	+	+/–	+/–	+/–	+/–	–	–	1211 D	1211 D	1211 D
I 6	–	–	–	+/–	+/–	+/–	–	–	–	–	–	–	–	–	8188 D	8188 D	8$\underline{7}$88 D
I 7	+	+	+	+	+	–	+	+	–	–	–	–	–	–	1228 D	1228 D	1228 D
I 8	+	+	+	+	+	–	+	+	–	–	–	–	–	–	1228 D	1228 D	1228 D
I 9	+	+	+	+	+	+/–	+	+/–	+	+	+	+	–	–	1111 D	11$\underline{3}$1 D	11$\underline{7}$1 D
I 10	+	+	+	+	+	–	–	+	+	+	+	+	–	–	1241 D	1241 D	1241 D
I 11	+	+	+	+	+	+	–	–	–	–	–	–	+	–	1188 B	1188 B	1188 B
I 12	+	+	+	+	+	–	–	+	–	–	–	–	–	–	1268 D	1268 D	1268 D
I 13	–	–	+/–	+/–	+/–	+	+/–	–	–	–	–	–	+	+/–	7158 A	71$\underline{32}$ A	$\underline{8788}$ A
I 14	+/–	+/–	+/–	+/–	+/–	+	–	–	+/–	+/–	+/–	+/–	+	+/–	1171 A	11$\underline{31}$ A	1171 A
I15	+	+	+	+	+	–	+	+	+/–	+/–	+/–	+	+/–	–	1211 B	1211 B	1211 B
I16	–	–	+	+	+	–	+	+	+	+	+	+	+	–	7288 B	7288 B	7288 B

* Symbols: + = clear zone of inhibition of indicator strain by bacteriocin; – = no inhibition; +/– = weak inhibition (recorded as + in notation).

† Deviating code numbers (compared to first typing results) are underlined.

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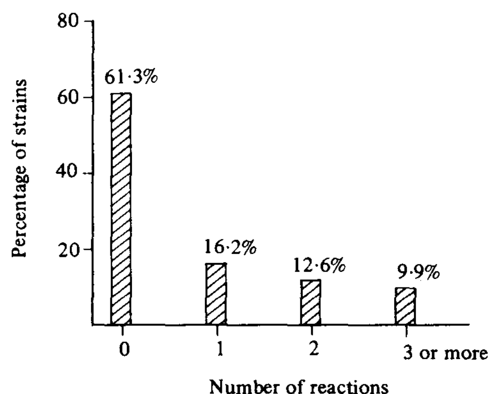

Fig. 1. Reproducibility of bacteriocin typing of 111 clinical strains twice with method 1. Percentage of strains versus the number of reaction differences between the patterns.

Bacteriocin typing method 2

With this method we tested 16 indicator strains on four different days and 27 clinical strains on two different days. Of the 16 indicator strains only two (12·5 %) gave the same bacteriocin pattern four times, six strains (37·5 %) gave the same results thrice, but in the deviating patterns only one reaction difference was observed. Of the remaining strains six (37·5 %) gave the same pattern twice and two strains (12·5 %) differed in bacteriocin pattern four times.

The results of retyping the 27 clinical isolates on two different days are graphically presented in Fig. 2. Only three strains (11·1 %) gave the same pattern on both days. With the 27 strains 14 bacteriocin patterns were observed on both typing days, but only two patterns were in common. Table 6 shows the results of these experiments. The bacteriocin patterns obtained by this method were completely different from patterns observed in the preceding method.

Bacteriocin sensitivity patterns of different colonies of the same strain

Of every indicator strain five colonies were tested for bacteriocin sensitivity on the same day with method 2. In 11 of the 16 indicator strains two or more morphologically different colony types were observed.

Only seven strains (43·8 %) showed no differences in bacteriocin patterns of the five colonies of each strain. Three of the five strains with morphologically identical colony types belonged to this group.

In six strains (two strains with identical colony types) one or two differences between the patterns of the five colonies were observed and in three strains there were very major differences between the observed patterns. No correlation was observed between variations in colony type and bacteriocin pattern.

Bacteriocin typing method 3

The 16 indicator strains and 27 clinical strains were retyped on five different days. The results are given in Table 7.

Of the 16 indicator strains only one (6·25 %) gave the same bacteriocin pattern five times. With three strains (18·75 %) the same pattern was observed in four of

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Table 4. *Typing of* Klebsiella *strains with method* 1

(Reproducibility is given as the percentage of the strains showing identical patterns after two or three typing runs.)

	Indicator strains (16)		Clinical strains (111):
	2 runs*	3 runs†	2 runs†
Same pattern 3 ×		75	
Same pattern 2 ×	79·2	12·5 (1–2)	61·3
No pattern identical	20·8	12·5 (2–4)	38·7 (1–6)

* Percentages are the mean of all possible combinations of 2 after three typing runs.
† Number of different reactions in the deviating patterns after various typing runs is given in parentheses.

Table 5. *Effect of storage of bacteriocin suspensions at* −70 °*C on the reproducibility of klebcin typing using method* 1

(Percentage of strains showing identical patterns are given.)

	2 runs*	3 runs*	4 runs†
	Indicator strains (16)		
Same pattern 4 ×	—	—	50
Same pattern 3 ×	—	57·8	31·25 (1)
Same pattern 2 ×	70·8	32·8	18·75 (1–2)
No pattern identical	29·2	9·4	0
	Clinical strains (27)		
Same pattern 4 ×	—	—	44·4
Same pattern 3 ×	—	56·5	48·2 (1–5)
Same pattern 2 ×	70·4	41·7	7·4 (2–3)
No pattern identical	29·6	1·8	0

* Percentages are the mean of all possible combinations of 2 or 3 after four typing runs.
† See footnote, Table 4.

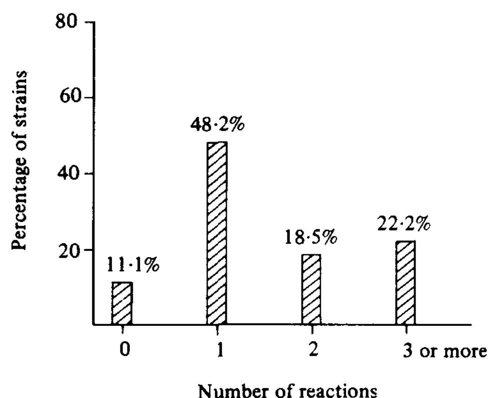

Fig. 2. Reproducibility of bacteriocin typing of 27 clinical strains twice with method 2. Percentage of strains versus the number of reaction differences between the patterns.

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Table 6. *Typing of* Klebsiella *strains with method* 2

(Reproducibility is given as the percentage of strains showing identical patterns after various typing runs.)

	Indicator strains (16)			Clinical strains (27):
	2 runs*	3 runs*	4 runs†	2 runs†
Same pattern 4 ×	—	—	12·5	—
Same pattern 2 ×	—	21·9	37·5 (1)	—
Same pattern 2 ×	38·5	50	37·5 (1–3)	11·1
No pattern identical	61·5	28·1	12·5 (4–5)	88·9 (1–5)

* See footnote, Table 5.
† See footnote, Table 4.

Table 7. *Typing of* Klebsiella *strains with method* 3

(Reproducibility is given as the percentage of strains showing identical patterns after various typing runs.)

	2 runs*	3 runs*	4 runs*	5 runs†
	Indicator strains (16)			
Same pattern 5 ×	—	—	—	6·25
Same pattern 4 ×	—	—	10	18·75 (1)
Same pattern 3 ×	—	16·9	28·75	31·25 (1–2)
Same pattern 2 ×	32·5	48·1	42·5	31·25 (1–3)
No pattern identical	67·5	35·0	18·75	12·5 (2–6)
	Clinical strains (27)			
Same pattern 5 ×	—	—	—	7·4
Same pattern 4 ×	—	—	10·4	14·8 (1–2)
Same pattern 3 ×	—	14·1	14·8	7·4 (2)
Same pattern 2 ×	25·5	33·7	42·3	55·6 (2–7)
No pattern identical	74·5	52·2	32·5	14·8 (4–6)

* Percentages are the mean of all possible combinations of 2, 3 or 4 after five typing runs.
† See footnote, Table 4.

the five typing experiments. The deviating patterns in this strain differed by only one reaction.

As for the 27 clinical isolates, two strains (7·2 %) did not vary in bacteriocin pattern five times and four strains (14·8 %) showed the same results four times. The majority of the strains (55·6 %) gave the same pattern only twice and four strains showed on every typing attempt a different result. There was only one pattern which was common in the five typing runs of the 27 strains, whereas 13 to 16 patterns could be observed on the different days.

Reproducibility

The overall reproducibility of the three different methods after various typing runs is given in Table 8. The reproducibility of bacteriocin typing of method 1 is superior to the methods 2 and 3. In all methods a decrease in reproducibility is observed after more than two typing experiments.

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Table 8. *Comparison of reproducibility of bacteriocin typing using different methods*

(Percentages of strains showing identical patterns after various typing runs are given.)

	2 runs	3 runs	4 runs	5 runs
	Indicator strains (16)			
Method 1	79·2	75	n.d.*	n.d.*
Method 1 (storage of bacteriocins at −70 °C)	70·8	57·8	50	n.d.*
Method 2	38·5	21·9	12·5	n.d.*
Method 3	32·5	16·9	10·0	6·25
	Clinical strains (27)			
Method 1	61·3	n.d*	n.d*	n.d*
Method 1 (storage of bacteriocins at −70 °C)	70·4	56·5	44·4	n.d.*
Method 2	11·1	n.d*	n.d*	n.d*
Method 3	25·5	14·1	10·4	7·4

* n.d. = not done.

DISCUSSION

Of the typing methods for *Klebsiella* species for epidemiological purposes serological typing seems to be the most reliable. However, this method has the disadvantage of needing 77 capsular antisera (not commercially available) and of tedious microscopic work. From a practical standpoint bacteriocin typing has the advantage that it can be performed in the routine laboratory since this method does not require any special materials or instruments.

Bacteriocin typing of *Klebsiella* species is nearly always based on the sensitivity of *Klebsiella* for bacteriocins because of the low frequency of bacteriocin production in *Klebsiella* strains. Edmondson & Cooke (1979) found 35·8 % of the *Klebsiella* strains to be bacteriocin producers, Hall (1971) 40 %, but Israïl (1980*a*) using the Abbott Shannon set of standard strains found 63 % of *Klebsiella* strains to be bacteriocinogenic.

Several methods have been described for bacteriocin typing of *Klebsiella* strains. The cross-streaking method of Hall (1971) gave, in our hands, certain problems because of the difficulty of removing bacterial growth of mocoïd strains by scraping with a glass microscopic slide. The author herself found the method to be of limited value because of lack of reproducibility in the weakly sensitive reactions.

In the literature, little attention is paid to the reproducibility of the different methods for bacteriocin typing of *Klebsiella* strains. In our opinion reproducibility is a very important condition for the usefulness of a bacteriocin-typing method in the routine laboratory. We therefore studied the reproducibility of different bacteriocin-typing methods.

The typability of the 111 clinical strains was as high as 91–95 % using a modification of the method of Edmondson & Cooke (1979) and is in agreement with their results. The reproducibility of 79·2 % for indicator strains and of 61·3 % for

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clinical strains after two typing runs is also comparable with their reproducibility percentages (67 %). However, use of the one- or two-reaction difference rule with a reproducibility of 67 % or 89 % as shown by these authors gives in our opinion difficulties in comparing bacteriocin patterns of strains with each other. Above all, the reproducibility of the weakly sensitive reactions is low. These weakly sensitive reactions appeared mostly with the low-titred bacteriocin preparations (P 6, P 9, P 10–P 15), whereas the bacteriocin lysates with a high titre (P 1–P 5, P 7, P 8) gave more stable results.

This observation is also reported by Buffenmyer *et al.* (1976). Omission of the low producers from the system, or regarding the weakly sensitive reactions as negative, resulted in a great decrease in the discriminating power of the method. Increasing the bacteriocin production by incubating strains in fresh PP_3 broth after induction with mitomycin C resulted in the appearance of new weakly sensitive reactions (unpublished results).

The reproducibility of the bacteriocin typing is relatively high for method 1 after two typing experiments. After three or four experiments the reproducibility results decreased with all three methods, because the variations occurred every time in different strains. This phenomenon makes bacteriocin typing of *Klebsiella* strains as an epidemiological tool unacceptable. Storage of the bacteriocin suspensions at −70 °C was necessary and satisfactory to prevent a decrease in activity. This is in agreement with the observations of Buffenmyer *et al.* (1976). But even storage of the bacteriocin suspensions at −70 °C with a negligible decrease in activity resulted in a reproducibility of only 50 % for indicator strains and of 44·4 % for clinical strains after four experiments

Our results indicate that substantial intra-strain variations in bacteriocin sensitivity may be responsible for these observations. Abbott & Shannon (1958) already mentioned the different sensitivity of epidemiological related *Shigella* strains to colicines. Farmer (1972) also showed in his epidemiological typing of *Serratia* that sensitivity to bacteriocins is a less stable characteristic than production of bacteriocins. Genes for bacteriocin production are often carried on a plasmid. These plasmids are generally very stable and although they can be eliminated by chemicals like acridine orange, they are only occasionally spontaneously lost (Reeves, 1972). Bacteriocin sensitivity is dependent on the adsorption of bacteriocins to receptor sites on the bacterial cell. Alteration in bacteriocin pattern may be due to changes in cell-wall structure (e.g. loss of capsule) and therefore to changes such as the covering of receptor sites or their removal from the cell envelope. As for *Klebsiella* species it is very easy to select colonial variants after serial transfer. Maybe these colonial variants with possibly altered or lost receptor sites are the cause of the low reproducibility. Our results in testing five different colonies of each of the 16 indicator strains yield some support for this supposition. In our hands reproducibility is higher in duplicate experiments of the same colony of a strain on one day (not published). Israil (1980*b*) also mentioned that it is necessary for consistent and comparable results to test strains from the same source simultaneously and under identical experimental conditions.

Recently Bauernfeind, Petermüller & Schneider (1981) reported the use of a streak and point bacteriocin typing method with a typability of 91 % and a

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reproducibility of 83 %. The authors explained the relatively high reproducibility by high stability of their bacteriocins and slower inactivation rates by avoiding the use of suspensions.

Our results, however, clearly show that method 1 (growth in broth) is more reproducible than methods 2 and 3 (solid media). Despite the higher reproducibility, method 1 is still subject to such great variations that its use in epidemiology seems of doubtful value. The lower reproducibility of the bacteriocin typing methods 2 and 3 may be due to the fact that induction of bacteriocin production on solid medium is more variable than in broth (method 2) or to the fact that no induction is used (method 3). Although we did not evaluate the methods with the producer set of Bauernfeind, Petermüller & Schneider (1981), we believe that variation in bacteriocin sensitivity of strains, despite high stability of bacteriocin, will always be a major problem in bacteriocin typing with every set of producer strains. These variations will not only affect the reproducibility of the results in one laboratory but also from one laboratory to another. The lack of reproducibility in typing *Klebsiella* strains by bacteriocins on consecutive days is in our opinion a serious shortcoming in these typing methods for epidemiological purposes. Nevertheless, it can be used for a limited number of strains to confirm other typing methods.

This research was supported by a grant from the Praeventiefonds, 's-Gravenhage, number 28-712.

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