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Electrophoretic Transfer from Polyacrylamide Gel to Nitrocellulose Sheets, a New Method To Characterize Multilocus Enzyme Genotypes of *Klebsiella* Strains

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A new method for multilocus enzyme electrophoresis, based on electrophoretic transfers to nitrocellulose after polyacrylamide-agarose gel electrophoresis was explored. Electrophoretic separation was performed on 1-mm-thick slab gels with 6-μl samples of bacterial extracts and was followed by serial 5-min consecutive transfers. The transferability of 19 metabolic enzymes of *Klebsiella* strains was studied and allowed the simultaneous examination of one enzyme in the separation gel and at least five enzymes on nitrocellulose sheets. The resolution of enzyme bands was increased on nitrocellulose; thus, well-separated bands were recorded for nucleoside phosphorylase, peptidase, and phosphoglucose isomerase whereas their mobility variants could not be clearly distinguished in the separation gel because of stain diffusion. The study of genetic relationships of 42 strains of *Klebsiella pneumoniae* and 24 strains of *Klebsiella oxytoca* demonstrated the reliability of the method, since clustering analysis of electrophoretic types, based on electrophoretic polymorphism of 10 metabolic enzymes, showed two main clusters well correlated with the two species. The 57 electrophoretic types described confirm the usefulness of the method for the study of genetic relationships between closely related strains.

Multilocus enzyme electrophoresis has been developed in recent years in microbiology as an alternative method to biotyping, serotyping, resistotyping, or bacteriophage typing for the characterization of bacterial strains. The method is powerful in bacterial population genetics since electromorph frequencies can be equated with allele frequencies at structural gene loci (12, 13, 21) and has found applications in bacterial epidemiology (4) and systematics (1).

Selander and coworkers (21) used starch gel electrophoresis which allows the detection of several different enzymes in horizontal slices of the gel. Polyacrylamide gel electrophoresis has also been used for the study of enzyme profiles of *Candida* species (10) and of esterase electrophoretic polymorphism (8, 17), but only one enzyme is visualized on each gel. An alternative method using the examination of numerous enzymes from a single polyacrylamide gel by serial electrophoretic transfers to ion-exchange paper (11) has been described for eucaryotic population genetics.

The aim of this work was to explore a new electrophoretic method for the simultaneous study of several bacterial enzymes, based on electrophoretic transfers from a thin polyacrylamide-agarose gel onto nitrocellulose membranes. The method was evaluated for interspecies and intraspecies differentiation of strains of *Klebsiella pneumoniae* and *Klebsiella oxytoca*.

MATERIALS AND METHODS

Bacterial strains. Sixty-six strains of *Klebsiella* spp., isolated from urine, blood cultures, expectorations, and various other clinical sources were tested; after biochemical identification, 42 were strains of *K. pneumoniae* (including *K. pneumoniae* ATCC 10031) and 24 were strains of *K. oxytoca*.

Preparation of bacterial extracts. Strains were grown in 20 ml of Bacto Luria-Bertani Broth, Miller (Difco Laboratories, Detroit, Mich.), for 18 h at 37°C with shaking in a reciprocating water bath. The cells were harvested by centrifugation (20,000 × *g* for 20 min at 4°C) and washed with 100 mM $NaH_2PO_4 \cdot 2H_2O$–100 mM $Na_2HPO_4 \cdot 12H_2O$ buffer (pH 7.2). The pellet was diluted in its own volume of buffer, and the cells were disrupted by sonication (Vibracell T. M.; Bioblock Scientific, Illkirch, France) five times for 20 s at 0°C. Cell debris was removed by centrifugation at 20,000 × *g* for 20 min at +4°C. The supernatant was divided into aliquots and stored at −20°C until used. Protein concentration in bacterial extracts was determined by the Bradford method (2) with Coomassie blue-based reagent and bovine albumin fraction V (Pierce Chemical Co., Rockford, Ill.) as standard. The bacterial extracts contained 15.9 ± 4 μg of protein per μl (mean ± standard deviation).

Polyacrylamide-agarose gel electrophoresis. Horizontal 1-mm-thick slab gels contained 0.8% (wt/vol) agarose, 6% (wt/vol) polyacrylamide, and 1.6% (wt/vol) *N,N'*-methylene-bisacrylamide and were polymerized by 0.03% (vol/vol) TEMED (*N,N,N',N'*-tetramethylethylenediamine) and 0.012% (wt/vol) ammonium persulfate. Agarose (indubiose A37HAA) was obtained from IBF Biotechnics (Villeneuve la Garenne, France); acrylamide and *N,N'*-methylene-bisacrylamide were from Serva (Heidelberg, Germany); TEMED was from E. Merck AG (Darmstadt, Germany); and ammonium persulfate was from Prolabo (Paris, France). Gel buffer was 37.5 mM Tris–30 mM glycine, pH 8.7. Electrophoresis was performed in a 2117 Multiphor System (LKB Products, Bromma, Sweden) with 10 mM Tris–330 mM glycine buffer (pH 8.7) as electrode buffer. Samples of bacterial extracts (6 μl) were laid on the gel with a rubber guide, and electrophoresis was conducted at 4 V/cm for 30 min and 8 V/cm for

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3 h and 30 min, until the dye marker (bromophenol blue) had run 8.5 cm.

Electrophoretic transfers. Five copies of the separation gel were made by a series of electrophoretic transfers onto nitrocellulose sheets (BA 85; Schleicher & Schuell, Inc., Keene, N.H.). These transfers were performed in a Multiphor II Novablot System (LKB Products) by using a horizontal sandwich of the gel and one sheet of nitrocellulose between filter papers soaked in electrode buffer (1 mM Tris–33 mM glycine, pH 8.7) and the graphite cathode and anode plates. Electrophoretic transfer from the gel to each nitrocellulose sheet was run for 5 min at 25 mA or 100 mA, depending on the enzyme tested.

Staining of enzymes. Selective staining for the following enzymes was performed as described by Selander et al. (21): alcohol dehydrogenase (ADH), mannitol-1-phosphate dehydrogenase, malate dehydrogenase (MDH), malic enzyme (ME), 6-phosphogluconate dehydrogenase, NAD-dependent glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, threonine dehydrogenase, NADP-dependent glutamate dehydrogenase (GDH), nucleoside phosphorylase (NSP), glutamic-oxaloacetic transaminase (GOT), adenylate kinase (ADK), phosphoglucomutase (PGM), alkaline phosphatase (ALP), L-phenylalanyl-L-leucine peptidase (PEP), aconitase (ACO), mannose phosphate isomerase, phosphoglucose isomerase (PGI), and α-naphthyl acetate esterase (EST).

Statistical analysis. Statistical analysis of the data was performed by using the computer program designed by T. S. Whittam and R. K. Selander for multilocus enzyme electrophoresis (21). A version adapted for IBM-compatible personal computers was kindly supplied by J.-C. Piffaretti (Lugano, Switzerland). Genetic diversity coefficients (the degree to which the enzymes varied) were calculated for each enzyme locus from allele frequencies, by using the formula $H = 1 - \Sigma x_i^2 [n/(n-1)]$, where x_i is the frequency of the ith allele, n is the number of electrophoretic types (ETs), and $n/(n-1)$ is a correction for bias in small samples. Genetic distances between pairs of strains were expressed as the proportion of loci at which dissimilar alleles occurred. A dendrogram was generated by the average-linkage method of clustering of ETs from a matrix of coefficients of pairwise genetic distances between ETs (21).

RESULTS

Gel electrophoresis and electrophoretic transfer. The 1-mm-thick polyacrylamide-agarose gels allowed small sample volumes (6 μl) of protein extracts and improved the resolution of mobility variants compared with thicker gels. These gels could be easily moved from the electrophoretic separation plate to the transfer cathodal plate without any damage. Nineteen metabolic enzymes were found in all strains and were studied subsequently. To evaluate the efficacy of transfer, we tested, for each of the 19 enzymes, the staining on nitrocellulose sheets after five electrophoretic transfers from a single gel (Table 1). Fourteen enzymes could be electrophoretically transferred without loss of clarity or resolution. Furthermore, the resolution of bands of NSP, PEP, and PGI was increased on nitrocellulose because protein or stain diffusion was reduced compared with the gel. After five electrophoretic transfers, the separation gel could still be stained for the corresponding enzyme. Five enzymes (ME, threonine dehydrogenase, GOT, ADK, and PGM) could not be detected on nitrocellulose in the present transfer conditions, but could still be stained on the separation gel after five transfers for the detection of other enzymes. Results were recorded in the form of photographs since fading occurs after a few days (Fig. 1).

TABLE 1. Electrophoretic transferability of 19 enzymes in *Klebsiella* strains

Enzyme[a]	Transfer on nitrocellulose membranes[b]	Transfer conditions
ADH	+	25 mA, 5 min
M_1P	±	25 mA, 5 min
MDH	+	25 mA, 5 min
ME	−	25 mA, 5 min
6PG	±	25 mA, 5 min
GP_1	+	25 mA, 5 min
LDH	+	25 mA, 5 min
THD	−	25 mA, 5 min
GDH	+	100 mA, 5 min
NSP	+	25 mA, 5 min
GOT	−	25 mA, 5 min
ADK	−	25 mA, 5 min
PGM	−	25 mA, 5 min
ALP	+	25 mA, 5 min
PEP	+	25 mA, 5 min
ACO	+	25 mA, 5 min
MPI	+	25 mA, 5 min
PGI	+	25 mA, 5 min
EST	+	25 mA, 5 min

[a] M_1P, mannitol-1-phosphate dehydrogenase; 6PG, 6-phosphogluconate dehydrogenase; GP_1, NAD-dependent glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate dehydrogenase; TDH, threonine dehydrogenase; MPI, mannose phosphate isomerase.

[b] +, enzyme activity on nitrocellulose membranes after transfers; −, no enzyme activity on nitrocellulose after transfer; ±, decreased enzyme activity on nitrocellulose membranes after transfer.

Evaluation of the method by the analysis of enzyme polymorphism in *Klebsiella* strains. In order to examine whether the method was readily applicable to the determination of multilocus chromosomal genotypes, we used it to analyze the genetic diversity of 66 strains of *K. pneumoniae* and *K. oxytoca*. The mobility variants of the 11 following enzymes were analyzed: ADH, MDH, ME, GDH, NSP, GOT, ALP, PEP, ACO, PGI, and EST. ME and GOT were stained on the separation gel, and the other enzymes were stained on nitrocellulose membranes. PGI was found to be monomorphic and was not used in subsequent genetic analysis. The 10 other enzyme loci were polymorphic, with a mean number of alleles per locus of 5.7. The genetic diversity of the loci in all strains tested ranged from 0.410 (GOT) to 0.772 (MDH) and were also calculated for *K. pneumoniae* (mean genetic diversity = 0.559) and *K. oxytoca* (mean genetic diversity = 0.673) (Table 2).

Fifty-seven ETs were described among the 66 strains. ETs 9, 11, 33, and 43 were common to four, five, two, and two strains, respectively. The genetic relatedness of the ETs is shown in the dendrogram of Fig. 2. Two main clusters are separated by a genetic distance of 0.85, containing 40 of the 42 strains of *K. pneumoniae* (cluster I) and 22 of the 24 strains of *K. oxytoca* (cluster II), respectively. The four overlapping strains were further studied by gas production from lactose at 44°C (fecal coliform test): strains of ETs 49 and 52, which appear in cluster I, failed to produce gas and were therefore assigned to *K. oxytoca*, whereas strains of ETs 17 and 32 produced gas and were confirmed as *K. pneumoniae*.

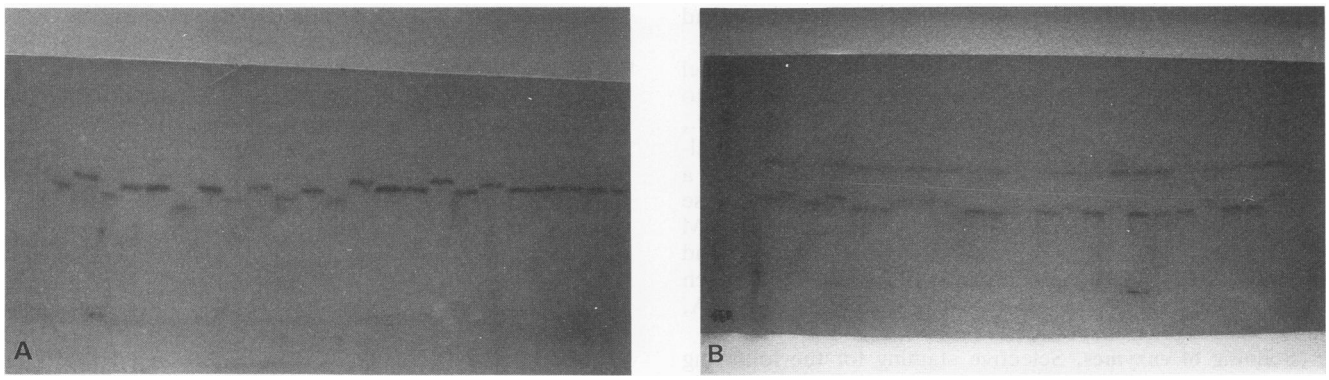

FIG. 1. Electrophoretic variants of two enzymes of *K. pneumoniae* as revealed on nitrocellulose membranes after transfer from the separation gel. (A) Alkaline phosphatase. (B) PEP.

DISCUSSION

Multilocus enzyme electrophoresis has proved to be an efficient method for bacterial population genetics, systematics, and epidemiology (21). However, it is generally considered that a well-documented study requires the analysis of a large number of loci (1, 12, 13), although a limited set of enzymes may be sufficient for the epidemiological characterization of bacterial strains (6). Therefore, starch gel electrophoresis or polyacrylamide gel electrophoresis may be considered as rather time-consuming since numerous electrophoretic separations are necessary. The method developed in this work allows the analysis of at least six enzyme loci in about 25 strains after one separating migration and five copies of the separating gel, and requires only 6-μl samples whereas we previously needed 200 μl for the analysis of five enzymes on multiple polyacrylamide-agarose gels (17). The serial electrophoretic transfers may be achieved in about 45 min, each transfer needing 5 min.

Considering that the separating gel may be still stained after five transfers, it is expected that an increased number of transfers are achievable. However, it would be necessary to determine the optimal order for staining of enzymes in that case, whereas it did not matter when the technique was limited to five transfers, and it seemed to us advisable to propose common transfer conditions for all enzymes. The protein concentration in bacterial extracts may also be a critical problem since ADH, NAD-dependent glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, mannose phosphate isomerase, and EST, which were not detected in preliminary experiments, could be revealed on nitrocellulose membranes by increasing the protein concentration from 10 to 15 μg/μl. Furthermore, 6-phosphogluconate dehydrogenase, ADK, and PGM have a low activity in gels, which becomes undetectable on nitrocellulose after transfer. Whatever these limitations, the combination of staining one enzyme in the gel and five enzymes on nitrocellulose sheets is a significant improvement for the simultaneous analysis of several enzyme loci.

TABLE 2. Number of alleles and genetic diversity of 10 enzyme loci for 66 isolates of *K. pneumoniae* and *K. oxytoca*

Locus	No. of alleles	H^a		
		Klebsiella spp.	*K. pneumoniae*	*K. oxytoca*
ADH	6	0.627	0.556	0.731
MDH	7	0.772	0.784	0.755
GDH	4	0.635	0.629	0.644
NSP	4	0.475	0.540	0.379
GOT	4	0.410	0.447	0.356
ALP	7	0.678	0.619	0.767
PEP	7	0.764	0.736	0.806
ACO	8	0.654	0.512	0.866
EST	6	0.576	0.449	0.763
ME	4	0.455	0.316	0.660
Mean	5.7	0.605	0.559	0.673

[a] *H*, genetic diversity per locus.

The reliability of this new method was illustrated by the analysis of genetic relationships of 66 *Klebsiella* strains from various clinical sources, which showed that strains of *K. pneumoniae* and *K. oxytoca* formed two well-separated clusters. The taxonomic problem of indole-positive strains has been studied for years. On the basis of DNA-DNA hybridization, these strains have been considered either as different from members of the genus *Klebsiella* (9) or as distinct species within this genus, named *K. oxytoca* (3, 14). In the present work, the differentiation was achieved by analyzing only 10 polymorphic loci, whereas taxonomic studies based on multilocus enzyme electrophoresis often require the analysis of 10 to 20 loci. The electrophoretic polymorphism of bacterial esterases, based on both their substrate specificity and their mobility variants, also provides a good differentiation of these two species (7). Our results, which are in agreement with the known relationships between *K. pneumoniae* and *K. oxytoca*, confirm the usefulness of multilocus enzyme electrophoresis in taxonomy, and emphasize the value of the combined polyacrylamide electrophoresis-electrophoretic transfer method.

Multilocus enzyme electrophoresis is also widely used for the estimation of genetic relationships between closely related strains (12, 16) and is thus a reliable typing method. Various epidemiological markers have been proposed for *K. pneumoniae* and *K. oxytoca*. Serological typing of carbohydrate capsular antigens may be performed by capsular swelling reaction (15) or by indirect fluorescent typing (20) and may be combined with biochemical typing (18). However, capsular typing requires a large battery of high-quality antisera which are not commonly available for many laboratories. For similar reasons, bacteriocin typing (5) and bacteriophage typing (19) have not been widely used. The characterization of electrophoretic mobility variants of metabolic

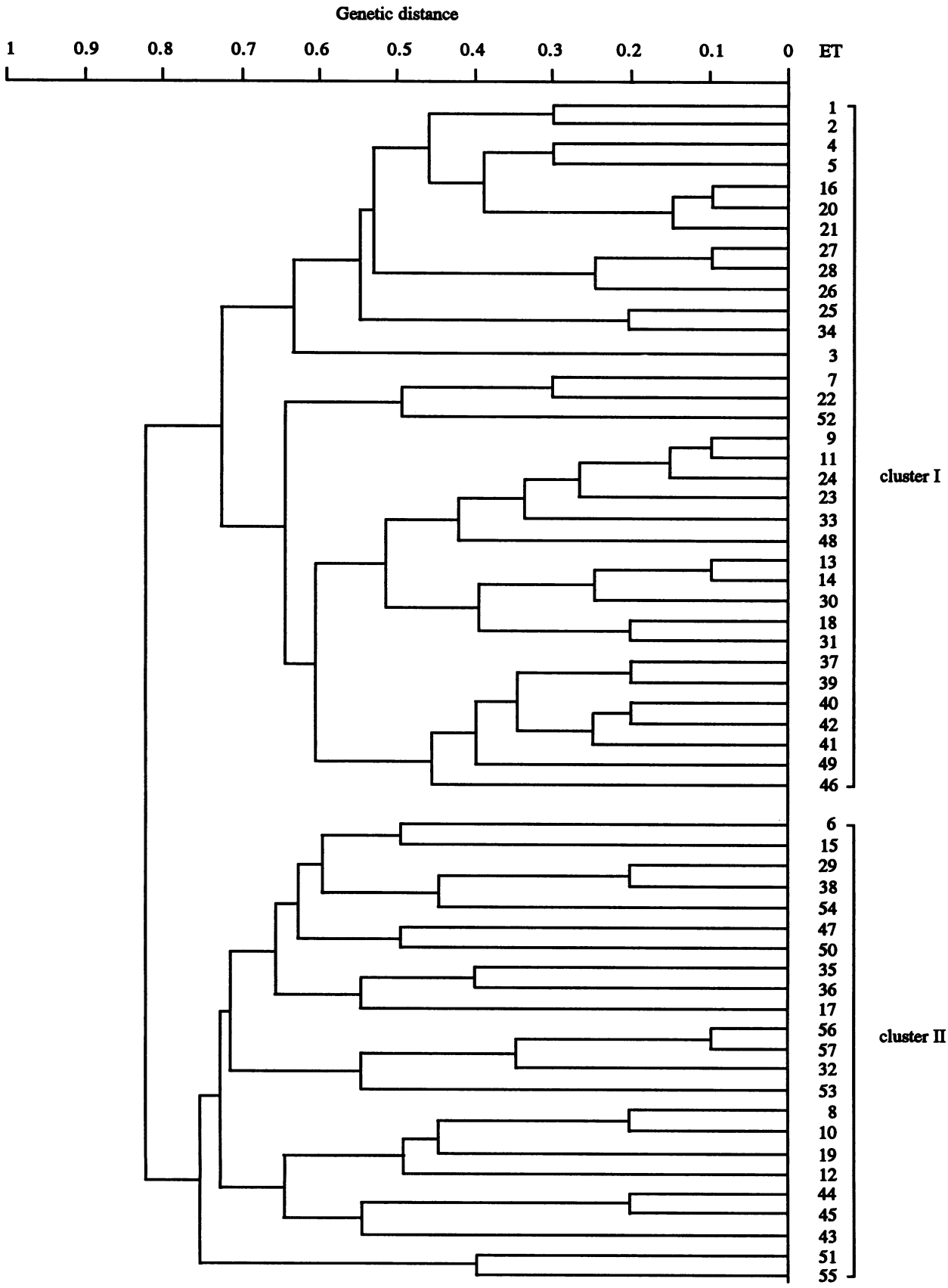

FIG. 2. Relationships among ETs of *Klebsiella* strains. The dendrogram was generated by using the average-linkage method of clustering from a matrix of pairwise coefficients of genetic distances between ETs. Cluster I, *K. pneumoniae* strains (except ETs 49 and 52); cluster II, *K. oxytoca* strains (except ETs 17 and 32).

enzymes needs reagents which are not specific for given bacterial species, and the method is thus a powerful means of epidemiological typing for bacteria exhibiting a significant enzyme genetic diversity. In the present study, the description of 57 ETs for the 66 strains tested gives evidence of the reliability of this typing method for *Klebsiella* strains. ET 9 was common to four strains isolated in the same hospital over a 1-month period. ETs 37, 39, 40, 41, and 42, which are closely related on the dendrogram, were described for five strains isolated in another hospital also for a short time period. These results confirm that strains from a given environment may be derivatives of a limited number of clonal genotypes.

The combined polyacrylamide electrophoresis-electrophoretic transfer method described in this work requires small volumes of bacterial extracts, allows for the rapid examination of several metabolic enzymes, and provides a good definition of enzyme mobility variants. Therefore, it may be proposed as a new method to characterize multilocus enzyme genotypes.

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REFERENCES

1. **Boerlin, P., J. Rocourt, and J.-C. Piffaretti.** 1991. Taxonomy of the genus *Listeria* by using multilocus enzyme electrophoresis. Int. J. Syst. Bacteriol. **41**:59–64.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
3. **Brenner, D. J., J. J. Farmer III, F. W. Hickman, M. A. Asbury, and A. G. Steigerwalt.** 1977. Taxonomic and nomenclature changes in *Enterobacteriaceae*. HEW Publication No. (CDC) 79-8356. Center for Disease Control, Atlanta.
4. **Caugant, D. A., K. Bøvre, P. Gaustad, K. Bryn, E. Holten, E. A. Høiby, and L. O. Frøholm.** 1986. Multilocus genotypes determined by enzyme electrophoresis of *Neisseria meningitidis* isolated from patients with systemic disease and from healthy carriers. J. Gen. Microbiol. **132**:641–652.
5. **Edmonson, A. S., and E. M. Cooke.** 1979. The development and assessment of a bacteriocin typing method for *Klebsiella*. J. Hyg. **82**:207–223.
6. **Gaston, M. A., and M. Warner.** 1989. Electrophoretic typing of *Enterobacter cloacae* with a limited set of enzyme stains. Epidemiol. Infect. **103**:255–264.
7. **Goullet, P.** 1980. Distinctive electrophoretic patterns of esterases from *Klebsiella pneumoniae*, *K. oxytoca*, *Enterobacter aerogenes* and *E. gergoviae*. J. Gen. Microbiol. **117**:483–491.
8. **Goullet, P., and B. Picard.** 1990. Characterization of enterobacteria by esterase specific-activity profiles. J. Gen. Microbiol. **136**:431–440.
9. **Jain, K., K. Radsak, and W. Mannheim.** 1974. Differentiation of the *oxytocum* group from *Klebsiella* by deoxyribonucleic acid-deoxyribonucleic acid hybridization. Int. J. Syst. Bacteriol. **24**:402–407.
10. **Lehmann, P. F., C.-B. Hsiao, and I. F. Salkin.** 1989. Protein and enzyme electrophoresis of selected *Candida* species. J. Clin. Microbiol. **27**:400–404.
11. **McLellan, T., and J. A. M. Ramshaw.** 1981. Serial electrophoretic transfers: a technique for the identification of numerous enzymes from single polyacrylamide gels. Biochem. Genet. **19**:647–654.
12. **Musser, J. M., D. M. Granoff, P. E. Pattison, and R. K. Selander.** 1985. A population genetic framework for the study of invasive diseases caused by serotype b strains of *Haemophilus influenzae*. Proc. Natl. Acad. Sci. USA **82**:5078–5082.
13. **Ochman, H., and R. K. Selander.** 1984. Evidence for clonal population structure in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **81**:198–201.
14. **Ørskov, I.** 1984. Genus *Klebsiella* Trevisan 1885, 105^{AL}, p. 461–465. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. Williams & Wilkins, Baltimore.
15. **Ørskov, I., and F. Ørskov.** 1984. Serotyping of *Klebsiella*. Methods Microbiol. **14**:143–164.
16. **Piffaretti, J.-C., H. Kressebuch, M. Aeschbacher, J. Bille, E. Bannerman, J. M. Musser, R. K. Selander, and J. Rocourt.** 1989. Genetic characterization of clones of the bacterium *Listeria monocytogenes* causing epidemic disease. Proc. Natl. Acad. Sci. USA **86**:3818–3822.
17. **Pons, J.-L., B. Picard, P. Niel, G. Leluan, and P. Goullet.** 1993. Esterase electrophoretic polymorphism of human and animal strains of *Clostridium perfringens*. Appl. Environ. Microbiol. **59**:496–501.
18. **Rennie, R. P., and I. B. R. Duncan.** 1974. Combined biochemical and serological typing of clinical isolates of *Klebsiella*. Appl. Microbiol. **28**:534–539.
19. **Rennie, R. P., C. E. Nord, L. Sjoberg, and I. B. R. Duncan.** 1978. Comparison of bacteriophage typing, serotyping and biotyping as aids in epidemiological surveillance of *Klebsiella* infections. J. Clin. Microbiol. **8**:638–642.
20. **Riser, E., P. Noone, and M.-L. Bonnet.** 1976. A new serotyping method for *Klebsiella* species: evaluation of the technique. J. Clin. Pathol. **26**:305–308.
21. **Selander, R. K., D. A. Caugant, H. Ochman, J. M. Musser, M. N. Gilmour, and T. S. Whittam.** 1986. Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. Appl. Environ. Microbiol. **51**:873–884.