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Comparison of clinical acinetobacter strains using a carbon source growth assay

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SUMMARY

A quantitative carbon source growth assay, comprising ten carbon sources, was used to compare acinetobacter strains from three hospitals. The strains had been obtained during episodes of increased prevalence of isolations and were, for each hospital, assumed to be epidemiologically related. This assumption was supported by the electrophoretic protein profiles of the strains. Univariate analysis of growth data showed significant differences between strains from the three hospitals. Moreover, cluster analysis revealed that the major pattern in the data was related to the epidemiological origin of the strains. Exceptions to the epidemic-related pattern were observed. Thus, apart from epidemiological factors, other factors might contribute to carbon source growth profiles of the strains. It is concluded that the carbon growth assay may be useful to distinguish roughly between acinetobacter strains from different sites of origin. Further studies are required to analyse additional factors which influence carbon source growth of strains.

INTRODUCTION

Bacteria of the genus *Acinetobacter* have recently emerged as nosocomial pathogens, which sometimes cause serious infections in hospitalized patients [1–3]. Therefore, an increase of isolations of these bacteria in hospital wards should be notified quickly. In addition, typing methods have to be used in order to characterize isolates. Several typing methods for acinetobacters have been described, e.g. phage typing [4], bacteriocin typing [5], biotyping [6] and protein electrophoretic typing [7, 8]. These methods are not widely applied. For epidemiological purposes, relatively simple typing methods are needed which can be performed in the routine microbiology laboratory.

The present study was undertaken to investigate whether growth of acinetobacters on various carbon sources could be used to assess similarities between strains. Growth characteristics were determined in a micro-assay by measuring optical densities using a photometer. The method was used to investigate

primarily whether acinetobacter isolates from a number of hospitals could be distinguished from their growth patterns. The acinetobacters were obtained from clinical specimens during periods of increased prevalence. Results were interpreted in relation to the epidemiological data of strains and their cell envelope protein profiles obtained by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE).

MATERIALS AND METHODS

Hospitals

The acinetobacter isolates of the study were obtained from three hospitals designated by capitals R, D and V, i.e. the initials of the cities of location, Rotterdam, Dordrecht and Venlo in The Netherlands. Epidemic site R is a 1200 bed teaching hospital. In the period 1981–2, an increased isolation of acinetobacters was observed in several departments of the hospital, in particular in several intensive care units (ICUs). Epidemic sites D and V are district general hospitals with 570 and 465 beds respectively. The increase of isolations in site D was observed in the surgery department in the period May to September 1984. The epidemic spread in site V occurred in the ICU in the period September to December 1986 and has been described in detail elsewhere [9].

Bacteria

Eighty-four acinetobacter clinical isolates from the three epidemic sites R (n = 36), D (n = 28) and V (n = 20) were studied. These strains were obtained from different patients over periods of 9, 4 and 2 months in R, D and V respectively and were selected from those received during the epidemic episodes.

Strains were identified as *A. calcoaceticus in sensu lato* [10] by conventional methods [11] and were of the glucose acidifying phenotype.

SDS–PAGE of cell envelope proteins

The method used has been described previously [8]. Briefly, cell envelope fractions were obtained by ultrasonic disruption of cells and fractional centrifugation. For SDS–PAGE, discontinuous systems consisting of a stacking and a running gel of 3% and 11% acrylamide were used. Profiles, consisting of 30–40 bands of different staining intensity in the apparent molecular weight range (M_r) of 14000–97000 (14–97 K), were compared visually.

Carbon source growth assay

The following substrates (C-sources) were used: lactic acid (C1), DL-aspartic acid (C2), D(−)ribose (C3), D(+)xylose (C4), malonic acid (C5), L(−)leucine (C6), L(−)tryptophan (C7), fumaric acid (C8), DL-histidine (C9) and propionic acid (C10). Chemicals C1 (90% pure), C3, C4, C6 and C7 (for biochemical purposes) and C5 and C10 (analytical grade) were obtained from Merck (Darmstadt, FRG), C2 (Sigma grade) from Sigma (St Louis, MO, USA) and C9 (> 99% pure) from Fluka (Buchs, Switzerland).

The C-sources were added to the basal medium of Gilardi [12] to a final concentration of 0·03 M. After pH-adjustment at 6·5, the C-source solutions were

Table 1. *Coefficients of variation of the assay*

C-source	Intra-assay (%)	Inter-assay (%)
C1	4	4
C2	9	9
C3	10	11
C4	19	19
C5	4	5
C6	15	17
C7	18	18
C8	8	9
C9	8	11
C10	46	46

stored at −20 °C and filter sterilized (Sterivex-GV, Millipore, Bedford, MA, USA) directly before use. Effort was taken to standardize manipulations, growth conditions and incubation times in order to minimize experimental error [13, 14]. Aliquots of 250 μl sterilized saline were applied to each well of the first column of flat bottom microtitre plates (Greiner, Nürtingen, West Germany). One of the ten C-sources was added to each of the columns 2 to 11, in aliquots of 250 μl for each well. Fresh bacterial suspensions in saline were prepared from cultures on blood agar, grown at 30 °C for 24 h. The suspensions were adjusted to an optical density of 0·06 ($\pm$0·01), at 623 nm (UC 200 photometer, Vitatron, Meyvis, Bergen op Zoom, The Netherlands), light path 10 mm. Aliquots of 20 μl of the bacterial suspensions were added to the wells of rows A to G. No bacteria were added to the wells of row H in order to check sterility of C-sources. The trays, covered by lids, were incubated at 30 °C in a ventilated incubator containing a water reservoir for humidity. After incubation for 48 h, trays were shaken for 10 seconds (Microshaker, Dynatech, Billingshurst, UK). Optical densities (O.D.) at 620 nm were measured by using a Titertek Multiscan MCC 340 (Flow Laboratories, Irvine, Scotland). The first column, supplied with saline, served as a blank. For all strains, inoculation of C-sources was performed on one day. All manipulations were carried out by the same two individuals.

In preliminary experiments the reproducibility of the assay was assessed. The intra- and inter-assay variation for the various C-sources is given in Table 1.

Biometrical analyses

The growth data as determined by the optical densities of the various C-sources were investigated by both univariate and multivariate methods. Univariate comparison between the epidemic sites R, D and V was carried out using the Kruskal–Wallis test (K–W), supplemented by pairwise comparisons using Mann–Whitney's test (M–W) when the former test indicated statistically significant differences ($P < 0{\cdot}05$).

To evaluate the growth data multivariately, cluster analysis was used. By this method the most striking pattern of variation between the strains was revealed. Strains were grouped on the basis of similarities in their growth characteristics. The grouping thus obtained, depicted in a dendrogram, was compared with

groupings based on hospital, hospital department, moment of sampling, body sites and other features.

The similarities between the strains, characterized by their growth on C-sources (O.D. × 1000), were calculated using the mean city block distance [15], i.e.

$$d(a, b) = 1/n \sum_{i=1}^{n} |p_a(i) - p_b(i)|,$$

where, $d(a, b)$ is the dissimilarity between strain a and b, n is the total number of C-sources ($n = 10$), $|p_a(i) - p_b(i)|$ is the absolute difference in C-assimilation with the ith C-source between strain a and b.

Clusters were generated by agglomerative cluster analysis. Ward's method [16] was used because of its strong pattern-generating capacities [17, 18].

RESULTS

Protein profiles

Protein profiles of strains were inspected for similarity. Strains, the patterns of which were indistinguishable when run in adjoining lanes, were allocated to the same provisional pattern group. Seven pattern groups were established, for the purpose of this study arbitrarily coded with arabic numbers 1–7. The patterns of these groups (Fig. 1) were characterized by one heavily stained band and by 30–40 less densely stained bands. Differences between the patterns were seen in the less densely stained bands, for example, between pattern 1, 2 and 3 in M_r range 45–66 K, and between patterns 2, 4 and 7 in M_r range 12–36 K. The differences were relatively small, in particular between patterns 3 and 5, but reproducible. For each epidemic site, the majority of strains could be assigned to one specific pattern group, pattern 1 for site R, pattern 3 for site D and pattern 5 for site V (Table 2). For each site, a few strains deviated from the main pattern, e.g. three strains from site R were allocated to pattern 3.

Carbon source growth profiles

Table 3 gives the median values of sources C1–C10 according to the site of sampling (i.e. epidemic sites R, D and V). Except for sources C5, C8, C9 and C10, there were statistically significant differences in median levels between epidemic sites. In the case of C3 and C7 significant differences were present between all sites. Fig. 2 shows both measurements (C3 and C7), isolated as well as in combination, in relation to the epidemic site, Apart from a few outlying observations, most measurements from site V were well separated from both other sites, while sites D and R were less separated.

The outcome of the cluster analysis of C-growth data of strains using Ward's criterion is shown in the dendrogram (Fig. 3). The major pattern in the data was related to the epidemic sites R, D and V. At a dissimilarity level indicated by the arrow, the following three clusters were distinguished.

Cluster I. This cluster, which showed up as a clearly aberrant group of strains, comprised five strains which grew poorly on one or more C sources. Four strains

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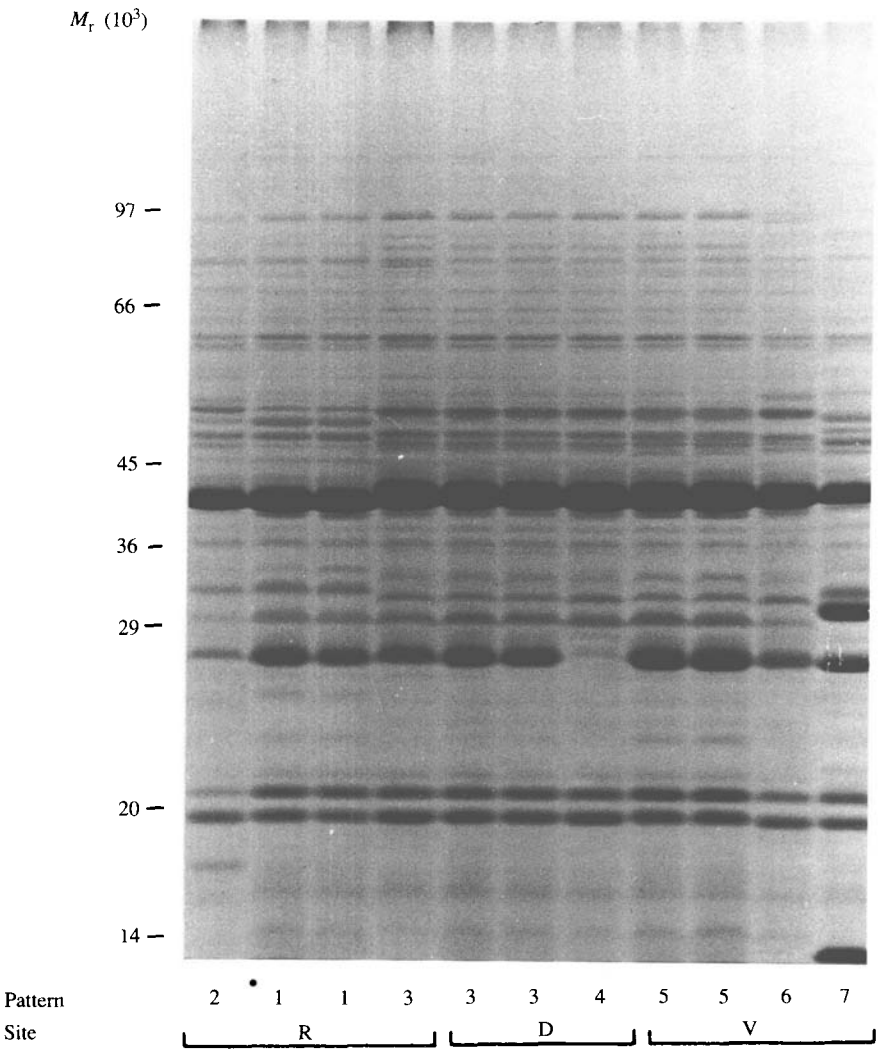

Fig. 1. Different cell envelope patterns observed in acinetobacter strains from sites of origin (hospitals) R, D and V. M_r, apparent molecular weight.

originated from epidemic site R. One of these strains was aberrant in its quantitative antibiotic resistance pattern (data not shown). One strain from epidemic site V deviated from other strains from the same site with respect to its protein profile (profile 7, Fig. 1, Table 1).

Cluster II. This cluster, which was well separated from other clusters, contained 12 of a total of 20 strains from site V. The strains were of uniform protein profile (pattern 5) and were associated with a hospital outbreak which was described previously [9].

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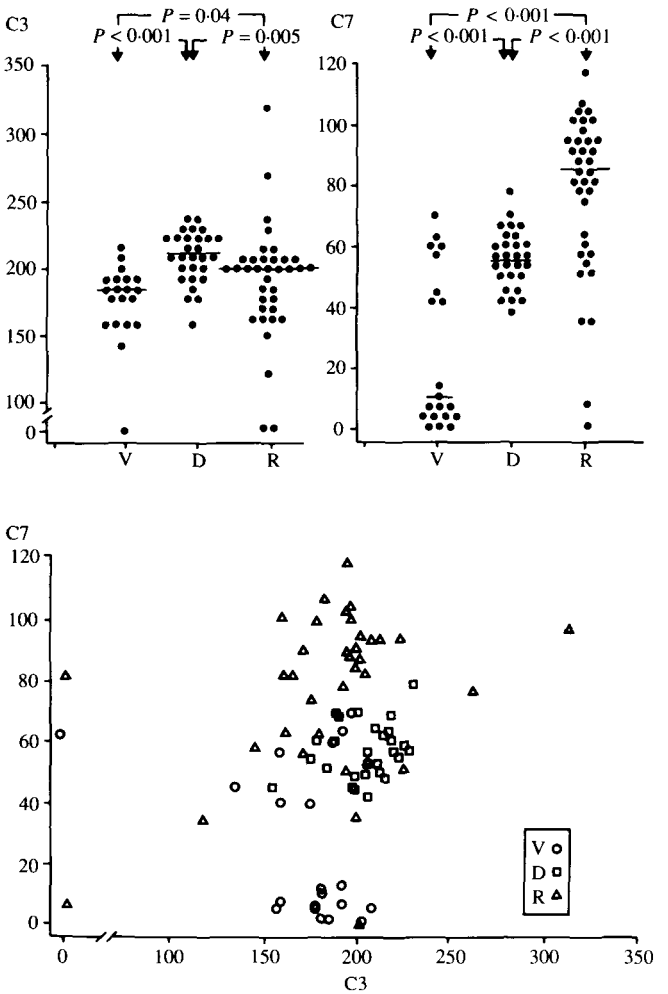

Fig. 2. Measurements of growth on carbon sources C3 and C7 according to sites of origin R, D and V, isolated (upper panel) and in combination (lower panel) expressed in optical densities (×1000). Bars denote median values.

Table 2. *Distribution of protein patterns in acinetobacter strains from the three epidemic sites.* Patterns are shown in Fig. 1*

Epidemic site	Pattern						
	1	2	3	4	5	6	7
R	32	1	3	—	—	—	—
D	—	—	27	1	—	—	—
V	—	—	—	—	18	1	1

* Hospitals in different cities in The Netherlands.

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Table 3. *Median values and ranges (between brackets) of measurements of growth on C-sources C1-C10 (optical densities ×1000 at 620 nm) according to epidemic site*

	Epidemic site			Significance
	V	D	R	(K–W)
C1	290 (182–324)	263 (219–285)	262 (3–300)	< 0·001
C2	327 (177–342)	328 (289–385)	314 (1–372)	0·04
C3	180 (0–208)	206 (154–230)	194 (1–313)	< 0·001
C4	101 (0–130)	123 (87–246)	107 (0–163)	< 0·001
C5	351 (0–374)	352 (326–382)	346 (0–388)	0·4
C6	75 (28–89)	96 (72–124)	101 (38–136)	< 0·001
C7	11 (1–70)	56 (42–79)	84 (1–119)	< 0·001
C8	176 (137–196)	172 (136–200)	170 (4–205)	0·7
C9	239 (208–268	247 (213–296)	236 (1–268)	0·09
C10	24 (0–57)	26 (8–52)	23 (0–43)	0·7

Statistically significant (M–W: $P < 0{\cdot}05$) differences: C1 (V *v.* D, V *v.* R), C2 (V *v.* R, D *v.* R), C3 (V *v.* D, V *v.* R, D *v.* R) C4 (V *v.* D, D *v.* R), C6 (V *v.* D, V *v.* R), C7 (V *v.* D, V *v.* R, D *v.* R).

Cluster III. This contained nearly all strains from site R and all strains from D. Within this cluster, several subgroups were distinguishable. One of these subgroups (subcluster a, Fig. 3) contained exclusively strains from site R; these strains belonged to protein profile group 1 (Fig. 1). Another subcluster (subcluster b, Fig. 3) comprised almost exclusively strains from site D; these belonged to protein profile group 3. Several other subclusters within cluster III were mixtures of strains from D, R and V. Some of the strains in these mixtures deviated from the majority of strains from the same site with respect to their protein profile. For example, one strain in a mixed cluster (subcluster c), originating from site D, was of the deviating pattern 4 (top of the dendrogram); another strain, originating from site V was of pattern 6 (centre of dendrogram), whereas other strains from V were of pattern 5. Also, several strains from site R, which clustered together with strains from D, belonged to pattern 3, i.e. the main pattern for strains from D (subcluster c, upper part of dendrogram).

The strains from site R were obtained from 36 patients in 12 different wards. Twenty-six of the patients had been nursed in more than one ward during the period of hospitalization. No clear relationship was observed between the grouping of these strains within cluster III and department and moment of isolation or other features, e.g. antibiotic sensitivity spectrum.

In addition to the cluster analysis based on Ward's criterion, an analysis was performed using the UPGMA clustering criterion [19]. Results of this second analysis (not shown) largely agreed with the dendrogram shown in Fig. 3.

DISCUSSION

The ability of micro-organisms to grow on specific carbon sources is determined by several factors, e.g. the metabolic constitution and cell membrane character-

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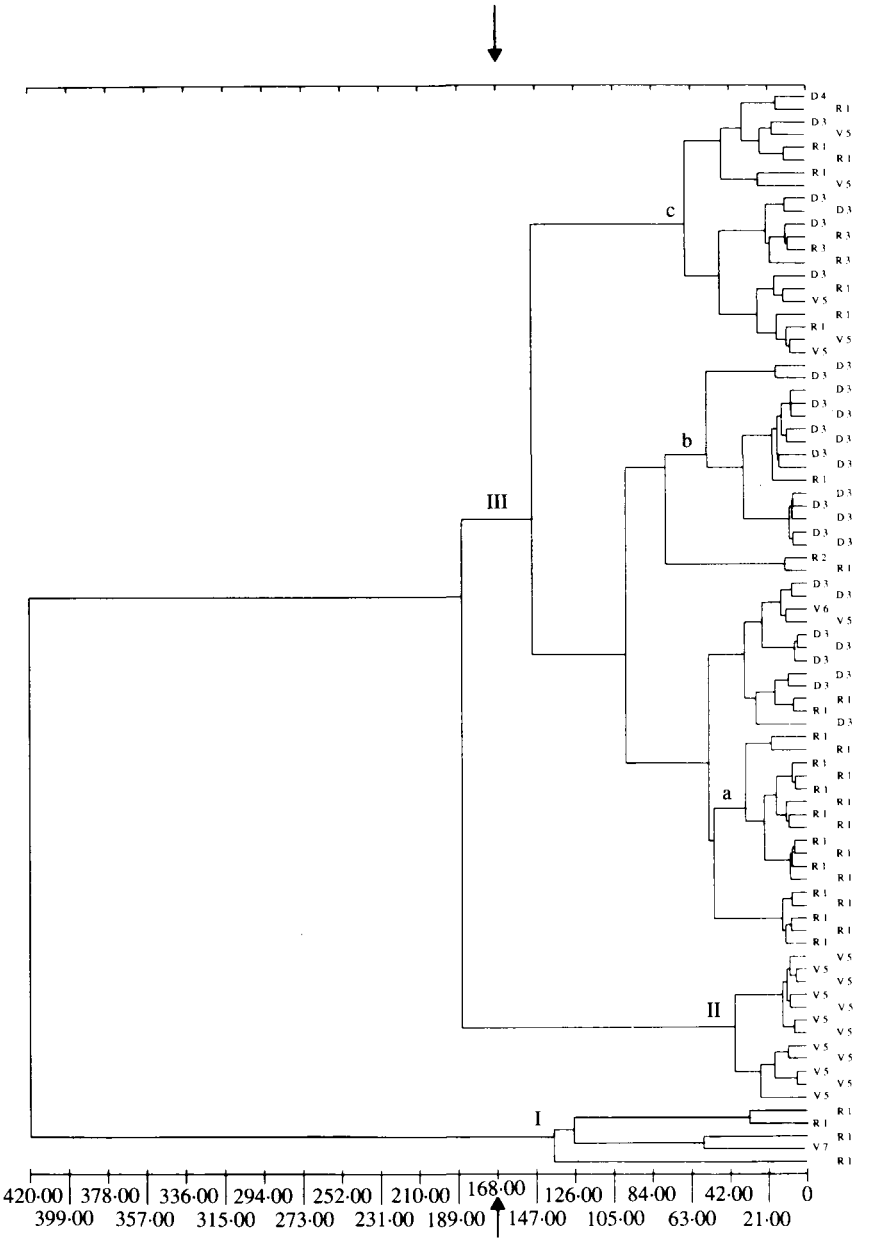

Fig. 3. Dendrogram of cluster-analysis of 84 acinetobacter strains characterized by their growth on carbon sources. Horizontal axis: dissimilarity level at which the strains are grouped. This level is determined by Ward's criterion and based on the mean city block distance. Arrow denotes the cutting level for separation main clusters. Vertical axis: the strains grouped according to the similarity of their growth on the carbon source. R, D and V denote epidemic sites of origin (hospital) of the strains. Protein profiles are represented by numericals 1–7; main clusters by I–III and subclusters by a–c.

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istics. Diversity with respect to the use of different C-sources by bacteria has appeared useful in classification [14, 20, 21]. Baumann and co-workers [22] distinguished nutritional groups within the genus *Acinetobacter*, although the groups were not sharply distinguishable. Recently, Bouvet and Grimont [6, 23] developed schemes for identification and typing of acinetobacter strains, which are largely based on C-growth tests. In contrast to these investigations, the present study has no taxonomic pretention. The purpose of this study was to investigate whether C-growth may be useful in distinguishing between strains, primarily in relation to their epidemiological origin. The number of C-sources used was relatively low. Instead of using qualitative (binary) data, as was done in other studies [22, 23], we used quantitative data. Strains were, for each hospital, assumed to be epidemiologically related. This assumption was supported by the protein profiles of the strains. Univariate statistical analysis showed significant differences in median levels between the strains when these were *a priori* separated according to their epidemiological origin. The overlap of the measurements when considered for each carbon source separately, however, was generally large. For some C-sources, differences could only be demonstrated between two of the three hospitals, while other C-sources did not show significant differences. For two C-sources, C3 (D(−)ribose) and C7 (L(−)tryptophan), statistically significant differences existed between all of the three epidemic sites. For C4 (D(+)xylose) and C6 (L(−)leucine), significant differences between some sites were observed, in spite of the moderate reproducibility of results of these C-sources (Table 1). The lack of reproducibility of C10 (propionic acid), possibly caused by the poor growth of the strains on the C-source, might account for the lack of statistically significant differences. In future experiments, this C-source might be replaced by a more discriminating C-source.

By cluster analysis, similarities between individual strains and grouping of strains are studied without any *a priori* separation. Thus, the main pattern in the data is studied. This pattern was evaluated for its correlation with several characteristics of the individual strains. The major pattern of the cluster analysis of C-growth data was related to epidemic sites (e.g. cluster II). Exceptions to the pattern were evaluated. Some of these might be explained on the basis of characteristics of the strains. Several strains deviated from strains from the same site in carbon source growth capacities (strains of cluster I) or in protein profile (e.g. strains of pattern 3 from site R). These strains may have represented occasional clinical isolates, unrelated to the epidemic spread. Strains from site R, a large teaching hospital, were in comparison to those from site V more scattered over the dendrogram. As indicated in the methods section, the period of increased numbers of isolations of acinetobacter in site R was longer as compared to sites V and D and many departments were involved. Therefore, strains from site R may have been of endemic rather than of epidemic origin. The passage of strains in many patients in a prolonged period may have contributed to the heterogeneity observed in the growth tests. In addition, experimental intra-strain variation [14, 24] may have contributed to the variation, although rigid standardization was applied in the present assay in order to minimize experimental error.

The present study was initiated to develop an easy-to-perform assay for the screening of strains in epidemiological surveys. The cluster analysis revealed a

pattern related to epidemic sites. From this, it is concluded that the assay is a useful method in order to distinguish roughly between epidemic sites. However, not all strains assumed to be epidemiologically related were grouped together. Additional factors, largely unknown, contributed to the variation. As this variation may complicate application of C-growth tests in epidemiology, further studies are required to assess these sources of variation.

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