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Investigation of a pseudo-outbreak of '*Pseudomonas thomasii*' in a special-care baby unit by numerical analysis of SDS–PAGE protein patterns

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SUMMARY

Forty-two cultures of pseudomonas comprising 28 clinical isolates from a pseudo-outbreak on a Special-Care Baby Unit and 14 reference strains, including 9 type strains, of various *Pseudomonas* species, were characterized by one-dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) of whole-cell proteins. The protein patterns were highly reproducible and were used as the basis for a numerical analysis which divided the strains into 9 phenons. Two of the 28 clinical isolates were identified by biochemical tests as *P. pickettii* and their identification was confirmed by SDS–PAGE as they fell in the same phenon as the type strain of the species. The remaining 26 isolates, which could not be identified on phenotypic tests, fell in the same phenon as three reference strains of '*P. thomasii*'. The protein patterns provided the first clear evidence that *P. pickettii* and '*P. thomasii*' were separate taxa and that the 'outbreak' was polymicrobial in origin, in line with the probable aqueous source of contamination. We conclude that high-resolution SDS–PAGE of proteins provides an effective method of identifying and differentiating pseudomonads, especially where this cannot be done adequately using conventional biochemical tests.

INTRODUCTION

Pseudomonads are ubiquitous and are commonly found in both soil and water. Nine different species of *Pseudomonas* have been identified in bottled drinking water [1]. Included amongst these was *P. pickettii* which has been implicated in a number of infections in the hospital environment [2, 3]. An organism provisionally named '*P. thomasii*' caused bacteraemia, urinary and respiratory-tract infections in 40 patients via contaminated softened, deionized and distilled water manufactured in a hospital pharmacy [4]. '*P. thomasii*' is phenotypically similar to *P. pickettii* and has caused a number of other outbreaks also via aqueous solutions. '*P. thomasii*' [5] and a similar organism [6] were each recovered from the distilled water supplies in other hospitals. The name '*P. thomasii*' appears in quotations as the name has never been validly published because of the difficulty of distinguishing this organism from *P. pickettii*.

Currently, there are problems in classifying, and therefore in identifying, pseudomonads whose biochemical reactions closely resemble those of *P. pickettii*. Various biogroups, including Groups IVd, Va-1, Va-2 and '*P. thomasii*' have been associated with *P. pickettii* in the past. However, a numerical phenotypic analysis of all these organisms showed that whilst heterogeneity was present there were insufficient differential characters to warrant recognition of any of these groups as separate species [7]. Riley and Weaver [8] concluded that Group Va-2 and *P. pickettii* were probably the same organism, by GLC of whole-cell hydrolysates. DNA–DNA hybridization has indicated that Groups Va-1 and Va-2 are also the same organism [9] but revealed a somewhat lower level of relatedness between *P. pickettii* and '*P. thomasii*' [7]. A further similar organism, from the soil of a rice paddy, has been described [10].

High-resolution polyacrylamide gel electrophoresis (PAGE) of bacterial proteins has been used for identification at the species, sub-species and infra sub-specific levels [11, 12]. The technique has been useful in the delineation of species and sub-species of, for example, *Campylobacter* [13, 14], *Providencia* [15] and Group EF-4 [16].

The aim of the present study was to compare the high-resolution 1-D SDS–PAGE whole-cell protein patterns of a number of pseudomonas isolates from a pseudo-outbreak [17] on a Special-Care Baby Unit (SCBU). A computerised analysis of protein patterns was employed to gain an objective evaluation of the technique as a method for identifying and differentiating the organisms involved in a polymicrobial 'outbreak' where biochemical characterization was of only limited success.

MATERIAL AND METHODS

Bacterial cultures

A total of 42 cultures of pseudomonas was examined in this study and they are listed in Table 1. Twenty-eight of these cultures were isolated as part of a hospital 'outbreak' on a SCBU, 16 of these were from 13 infants and 12 from bottles of Amies transport medium. The remaining 14 strains were reference strains (including the type strains of nine species of *Pseudomonas*) and were included as study references. Initial examination of the 'outbreak' strains using biochemical tests showed that two were *P. pickettii* and that the remainder belonged to one or more similar species. Since *P. pickettii* is a member of rRNA homology group II [18] the reference strains selected included other members of this group together with biochemically similar species of rRNA homology groups I, III and IV (Table 1).

Culture media and conventional biochemical tests

Strains were maintained on nutrient agar containing (g/l): Nutrient Broth No. 2 (Oxoid CM 67), 25; New Zealand agar, 12; and grown in nutrient broth (as above) with shaking for 3 h at 37 °C.

Attempts were made to determine the identity of the 28 'outbreak' isolates by a range of up to 72 conventional biochemical tests and processing the results through the probability matrix of Holmes and colleagues [19]. This matrix includes detailed biochemical test data on 20 species of *Pseudomonas* isolated from clinical material (the probability entries of the matrix are based on an examination of 1901 strains, in up to 83 tests each).

Table 1. *Strains analysed by whole cell SDS–PAGE protein patterns*

Ref. no. in dendrogram	Strain no.	Patient: Source	Protein type
'Pseudomonas thomasii'			
1	NCTC 10893	Blood	1a
2	NCTC 10894	Blood	1a
3	NCTC 10895	Blood	1c
4	CL154/88	A: Surface swab, index case	1a
5	CL155/88	B: Surface swab	1a
6	CL35/89	C: Umbilical swab	1a
7	CL36/89	D: Ear swab	1b
8	CL37/89	E: Umbilical swab	1b
9	CL39/89	F: Ear swab	1b
10	CL41/89	G: Ear swab	1b
11	CL43/89	H: Ear swab	1a
12	CL44/89	I: Ear swab	1b
13	CL45/89	J: Ear swab	1b
14	CL46/89	K: Umbilical swab	1a
15	CL48/89	E: Ear swab	1a
16	CL49/89	L: Rectal swab	1b
17	CL50/89	J: Umbilical swab	1a
18	CL51/89	M: Nasal swab	1a
19	CL53/89	Amies bottle, SCBU	1b
20	CL54/89	Amies bottle, SCBU	1a
21	CL55/89	Amies bottle, SCBU	1b
22	CL56/89	Amies bottle, SCBU	1a
23	CL58/89	Amies bottle, SCBU	1a
24	CL59/89	Amies bottle, SCBU	1a
25	CL61/89	Amies bottle, SCBU	1a
26	CL63/89	Amies bottle, SCBU	1d
27	CL65/89	Amies bottle, SCBU	1a
28	CL66/89	Amies bottle, SCBU	1a
29	CL67/89	Amies bottle, SCBU	1d
P. pickettii			
30	NCTC 11149*	Tracheotomy patient	3
31	CL47/89	A: Groin swab, index case	3
32	CL57/89	Amies bottle, SCBU	3
P. cepacia			
33	NCTC 10743*	Onion	8
P. gladioli			
34	CL105/89; L1	Respiratory tract, cystic fibrosis	7
P. acidovorans			
35	NCTC 10859†	Soil	9
36	NCTC 10683*	Soil	9
P. testosteroni			
37	NCTC 10698*	Soil	2
P. alcaligenes			
38	NCTC 10367*	Swimming pool	6
P. mendocina			
39	NCTC 10897*	Soil	6
P. pseudoalcaligenes			
40	NCTC 10860*	Sinus drainage	6
P. stutzeri			
41	NCIB 11358*	Spinal fluid	5
P. diminuta			
42	NCTC 8545*	Water	4

* Type strain.
† Strain now deleted from NCTC catalogue, duplicated in error.

Preparation of protein samples and electrophoresis

Approximately 0·02–0·04 g wet weight of the bacteria were harvested after centrifugation (2500 g) from nutrient broth, and suspended in about 60 μl of double-strength lysis buffer (20% v/v glycerol, 2% v/v 2-mercaptoethanol, 4% w/v SDS and 70% v/v stacking gel buffer) in a micro-centrifuge tube. The protein samples were then extracted as described previously [20].

Samples were run on discontinuous SDS–polyacrylamide gels which were cast to allow for a 10 mm stacking gel. The final polyacrylamide concentrations were 10% w/v for the separation gel and 5% w/v for the stacking gel. Full details of the methods used in gel preparation and electrophoresis were described previously [21].

Scanning of gels and computations

The stained protein patterns in the dried gels were scanned using a LKB Ultroscan XL laser densitometer (Pharmacia-LKB Biotechnology, Sweden). Absorbance was recorded at 160 μm intervals along the gel yielding 625 values per 10 cm gel. The absorbance range was set from 0·1–0·8 absorbance units (full scale). A rectangular line beam (800 × 50 μm) was used to scan each track three times (with no overlap in scan positions) resulting in a multiple track scan of 2·4 mm width. Multiple scanning was carried out in order to reduce the effect of inconsistencies which may be encountered across a track. The mean absorbance of the area scanned was recorded, via an RS232C interface, as raw data on the magnetic disk of a computer.

The initial (stacking gel/separation gel interface) and final (bromophenol blue marker) bands were deleted and protein patterns corrected for gel-to-gel variation using a reference bacterial standard (CL43/89: Table 1, no. 11). A replicate of the reference bacterial standard on the three subsequent gels used in the study was used to calibrate patterns against the reference on the first gel. Segmented linear correction was performed using a total of 17 discernible marker positions (usually peaks) on the reference pattern and by marking the same positions on the calibration pattern replicate. Linear correction (expansion or compression) to the reference distances was carried out within each of the 16 defined segments for each track by three-point quadratic interpolation [22]. The length-corrected traces on the reference gel were composed of 565 absorbance values after removal of the initial and final bands. A general background trend (0·4: fraction of absorbance) in each trace was removed to increase discrimination between patterns. Similarity between all possible pairs of traces was expressed as the Pearson product-moment correlation coefficient, which was converted for convenience to a percent value. The best fit between each pair of traces was obtained by laterally shifting one corrected trace with respect to the other in single-point steps of 160 μm up to three points on either side of the initial alignment. Strains were then clustered by the method of unweighted pair group average linkage (UPGMA). Computations were carried out on a Compaq 386 microcomputer using a program package written in Turbo Pascal.

RESULTS

Biochemical characterization of the outbreak organisms

On the probability matrix of Holmes and colleagues [19] only two of the 'outbreak' isolates could be identified, as *P. pickettii*, with identification scores > 0·999. The remaining 26 'outbreak' isolates failed to reach identification level and although no significance can be attached to the identification scores, the most likely taxa suggested were *Pseudomonas* species, principally *P. acidovorans*, *P. pickettii*, *P. stutzeri* and *P. testosteroni*. There were, however, characters which excluded the 26 isolates from each of these species. Although different species were suggested as possible identifications for different isolates, given the low identification scores it requires little heterogeneity in biochemical characters to change the species achieving the highest identification score. Thus the variety of *Pseudomonas* species suggested did not necessarily indicate that the remaining isolates belonged to different species. Indeed, examination of the biochemical data revealed relatively low heterogeneity, principally in the following characters: Hugh and Leifson O-F test, KCN tolerance, malonate utilization and production of acid from ethanol. The identity of the reference strains was confirmed on the same probability matrix (with identification scores > 0·999 for the respective taxa), except for *P. gladioli* which was not included in the matrix and the '*P. thomasii*' strains which identified as *P. pickettii*.

General features of PAGE protein patterns

One-dimensional SDS–PAGE of whole-cell protein extracts of the 42 cultures included in this study produced patterns containing approximately 40 discrete bands with molecular weights of 18–100 kDa. Proteins of < 18 kDa were not resolved under the electrophoretic conditions used in this study. PAGE protein patterns are illustrated in Fig. 1.

Reproducibility

The protein patterns of the isolates examined were highly reproducible both within and between gels. Replicate (four) protein samples of CL43/89 (Table 1, no. 11) run on different gels, and separate gel runs, gave similarity values of $96·5 \pm 1·6$ %. Molecular weight protein standards were also included on each gel and in this case estimates of their similarity were $97·3 \pm 0·9$ % although they provided a less objective measure of reproducibility as they were based on only four bands. The level of reproducibility achieved in this study was greater than that quoted by Jackman [11] and was well above the minimum acceptable value of 80% [23]. Previous studies using similar methods have reported levels of at least 93% similarity between duplicate samples in separate electrophoretic runs [15, 16, 21]. The dendrogram and protein types recognized in the analysis proved to be extremely robust when the computations were repeated using different levels of trace alignment and background subtraction.

Numerical analysis

Numerical analysis of PAGE total protein profiles based on the determination of the Pearson product-moment correlation coefficient and UPGMA clustering

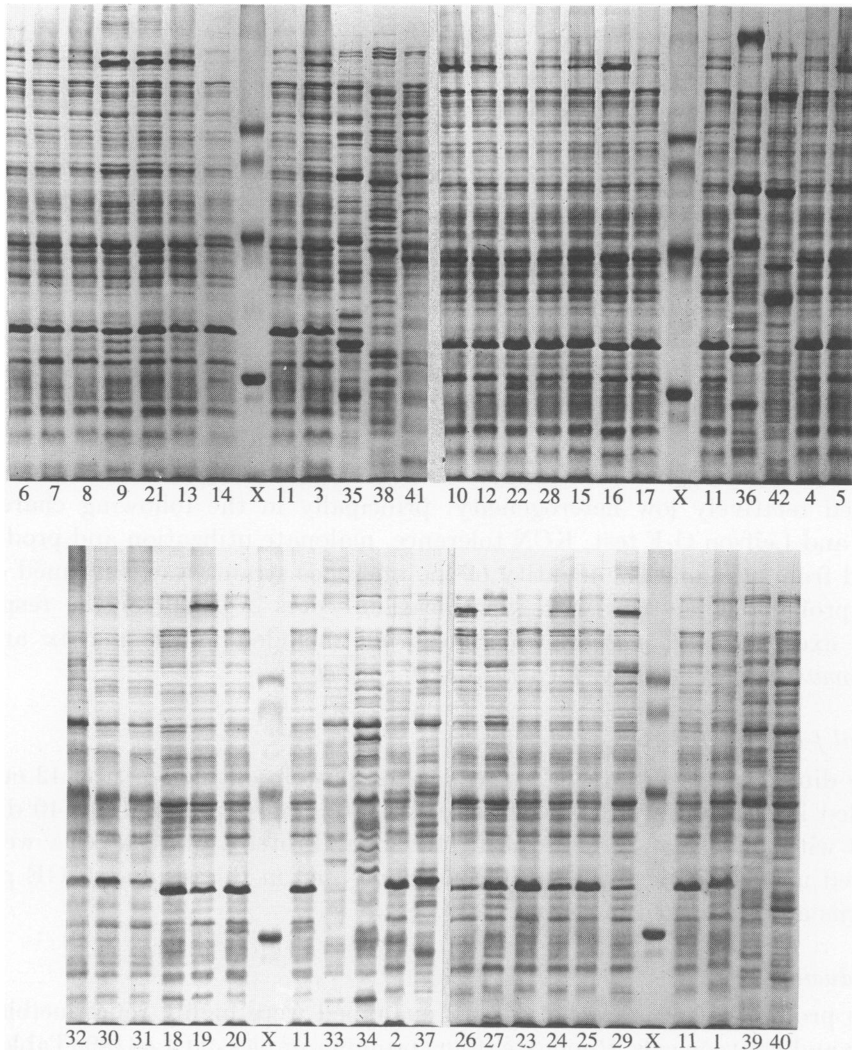

Fig. 1. Electrophoretic protein patterns of '*Pseudomonas thomasii*' pseudo-outbreak isolates and reference strains of other *Pseudomonas* species. The numbers refer to those used in Table 1 and Fig. 2. Molecular weight markers (track labelled X) are (from top to bottom): ovotransferrin, 76–78 kDa, albumin 66·25 kDa; ovalbumin, 42·7 kDa, carbonic anhydrase 30 kDa; myoglobin, 17·2 kDa.

revealed that, at the 63% (S) similarity level, the 42 pseudomonas isolates formed a total of nine distinct phenons (phenons 1–9), as shown in the dendrogram (Fig. 2). Each phenon represented a different species except phenon 6 which comprised three species, *P. alcaligenes*, *P. pseudoalcaligenes* and *P. mendocina*, which are closely related to each other within the same rRNA homology group. The two 'outbreak' isolates identified as *P. pickettii* on biochemical tests were included in phenon 3 together with the type strain of this species, thereby confirming their identity. Since hitherto '*P. thomasii*' had been considered a biotype within *P. pickettii*, it was expected that the three reference strains of '*P. thomasii*' would have been included in the same phenon as the type strain of *P. pickettii*. However,

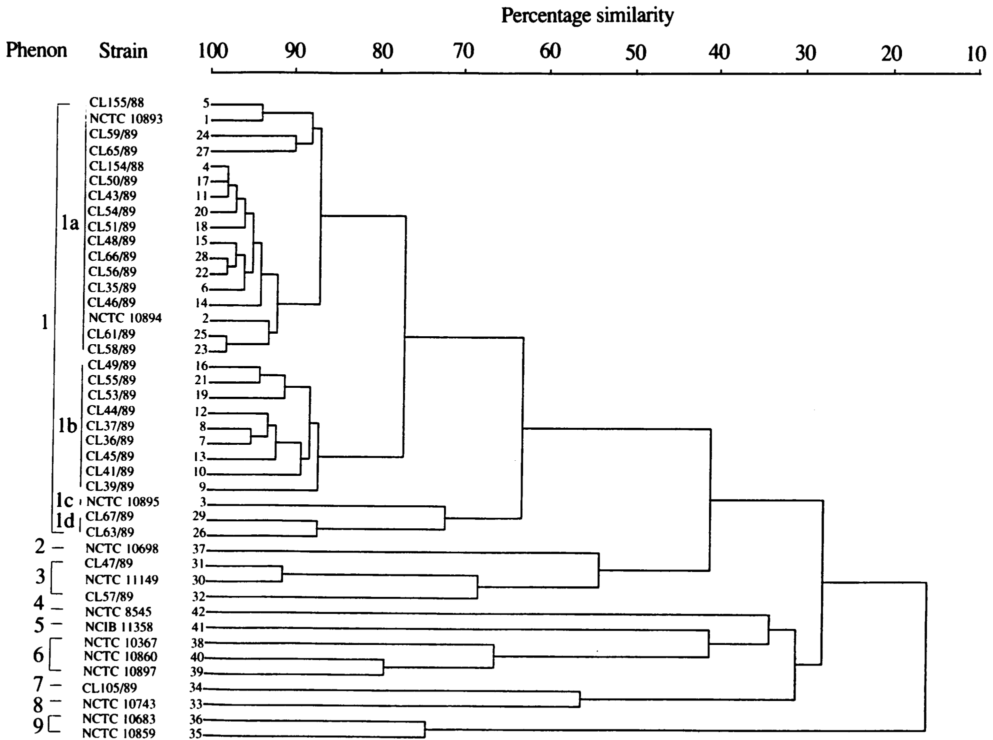

Fig. 2. Dendrogram of the cluster analysis based on total protein content of strains (vertical axis) listed in Table 1. The numbers on the horizontal axis indicate the percentage similarities as determined by the Pearson product-moment correlation coefficient and UPGMA clustering. Phenons were formed at the 63% similarity level.

the reference strains of '*P. thomasii*' were included in phenon 1 together with all of the remaining 26 'outbreak' isolates, which could not be identified using biochemical tests. Despite their close phenotypic similarity, *P. pickettii* and '*P. thomasii*' are clearly distinguishable by SDS–PAGE of their protein patterns (average similarity 34·8%, Table 2). Phenon 1 could be further divided at the 86% S level into four clearly separate sub-phenons, 1a to 1d. The majority of strains fell into sub-phenons 1a and 1b and of the three reference strains of '*P. thomasii*', two fell into sub-phenon 1a and one comprised sub-phenon 1c. Most of the heterogeneity seen in phenon 1 was a reflection of differences in the mobility and density of only two protein bands, one at between 33·0 and 33·4 kDa and the other > 78 kDa. The mean intra- and inter-phenon percentage similarities are shown in Table 2.

'*P. thomasii*' sub-phenons 1a and 1b (comprising 24/26 of the 'outbreak' isolates) and *P. pickettii* (phenon 3) each contained isolates from both patients and Amies bottles. Two swabs were examined from each of three infants (patients A, E and J; Table 1). Strains of the two different species were grown from the swabs of patient A and although only a single species, '*P. thomasii*', was grown from infants E and J, each pair of swabs yielded isolates with patterns representing a different sub-phenon. There did not appear to be any correlation between the phenotypic differences amongst the strains and the different sub-phenons recognized by SDS–PAGE.

Table 2. *Mean intra- and inter-phenon percentage similarity as determined by the Pearson product-moment correlation coefficient (r) and unweighted pair group average linkage clustering. All phenons are formed at 63% level*

	Phenon 1 '*P. thomasii*' ($n = 29$)	Phenon 2 *P. testosteroni* ($n = 1$)	Phenon 3 *P. pickettii* ($n = 3$)	Phenon 4 *P. diminuta* ($n = 1$)	Phenon 5 *P. stutzeri* ($n = 1$)	Phenon 6 *P. alcaligenes/ P. pseudoalcaligenes/ P. mendocina* ($n = 3$)	Phenon 7 *P. gladioli* ($n = 1$)	Phenon 8 *P. cepacia* ($n = 1$)	Phenon 9 *P. acidovorans* ($n = 2$)
Phenon 1	79·3 ± 13·0*								
Phenon 2	52·2 ± 15·6	(100)							
Phenon 3	34·8 ± 12·2	53·3 ± 9·2	75·7 ± 11·0						
Phenon 4	21·3 ± 3·9	18	8·7 ± 4·9	(100)					
Phenon 5	28·3 ± 6·7	27	29·0 ± 4·2	32	(100)				
Phenon 6	28·3 ± 7·4	23·7 ± 5·7	24·1 ± 11·7	34·7 ± 3·3	41·0 ± 6·5	70·3 ± 6·9			
Phenon 7	23·8 ± 3·7	27	30·7 ± 6·0	25	32	26·7 ± 9·7	(100)		
Phenon 8	31·1 ± 2·9	37	42·3 ± 6·5	38	37	29·7 ± 6·2	56	(100)	
Phenon 9	12·2 ± 6·9	29·0 ± 11·0	17·0 ± 6·4	28·0 ± 3·0	16·5 ± 8·5	23·8 ± 10·4	24·5 ± 2·5	32·5 ± 4·5	74

* Mean and standard deviation of similarity estimates; n, number of strains in each phenon; number in parentheses indicates only one strain included in phenon.

DISCUSSION

The analysis of bacterial SDS–PAGE protein patterns is an effective means of differentiating medically important bacteria at the species and infra sub-specific levels, especially where other methods are of limited use. The 'outbreak' isolates examined in this study, apart from two which proved to be *P. pickettii*, could not be identified on a probability matrix which included 20 clinically relevant *Pseudomonas* species. This was despite performing up to 72 biochemical characterization tests in a reference laboratory specializing in the identification of various Gram-negative bacteria, including the non-fermenters [19]. This may have been due, in part, to the inability of phenotypic tests to differentiate adequately between *P. pickettii*, '*P. thomasii*' and several similar organisms [7]. Without additional data from other techniques to indicate that all these organisms did not represent a single biochemically heterogeneous species they were all included as a single entry (*P. pickettii*) in the probability matrix [19].

The 'outbreak' isolates were biochemically heterogeneous and whilst the protein patterns reflected this heterogeneity the phenon containing the majority of these strains and the reference strains of '*P. thomasii*' nonetheless formed a single cluster well separated from its nearest neighbour (22% difference between phenon 1 and phenon 2/3). Since the phenons containing the references strains of *P. pickettii* and '*P. thomasii*' gave distinct protein patterns this is the first clear evidence to suggest that these organisms may represent separate species. The polymicrobial nature of the 'outbreak' was further emphasized in that from the index case (patient A), both species were isolated via different swabs. Although SDS–PAGE has here been applied primarily to '*P. thomasii*' the results indicate that the technique can be equally applied to the other *Pseudomonas* species since each appears to give a species-specific pattern (except phenon 6, see Results).

The original 'outbreak' was thought due to a single organism as all isolates shared a common pattern of antimicrobial susceptibility, including aminoglycoside-resistance. Over-reliance on the susceptibility patterns for provisional identification was erroneous in this case as proved by both biochemical tests and the protein patterns. Previously, Phillips and co-workers [4] had also relied heavily on antimicrobial susceptibility patterns for the provisional identification of a similar organism(s) causing an outbreak of infection. Twenty-five of these organisms were, in addition, biochemically identical [7], yet in the present study three of the strains from this original '*P. thomasii*' outbreak fell into two different sub-clusters. Thus in both the latter and the present studies, contrary to the original belief, neither was due to a single strain. These findings correlate well with the suspected aqueous source of contamination in both cases; the implication is that the water-borne route provided a continuous and varied population of microorganisms for contamination.

There have been several reports of contamination of water supplies by antibiotic-resistant pseudomonads [1, 4, 17]. In this 'outbreak' no infections arose but such organisms pose an ever-present threat as in some circumstances serious infections have been caused [4]. This emphasizes the need for constant vigilance in the preparation and storage of aqueous-based hospital products.

Where there is a requirement merely to determine whether strains are identical

or not, which is often the case in outbreaks of infection, a simple visual interpretation of patterns on gels can be successfully used to differentiate isolates. However, for definitive identification of species or electrophoretic types, reference material including type strains should be used together with a high-resolution scanner and numerical analysis by computer.

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