Pseudomonas aeruginosa **transmission is infrequent in New Zealand cystic fibrosis clinics**

Short title: *P. aeruginosa* transmission in CF clinics

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ABSTRACT

Pseudomonas aeruginosa is an important pathogen in cystic fibrosis (CF). Although most patients harbour unique *P. aeruginosa* isolates, some clinics report patients sharing common strains. The overall importance of person-to-person transmission in *P. aeruginosa* acquisition and whether routine patient segregation is necessary remains uncertain. We therefore investigated the extent of *P. aeruginosa* transmission in New Zealand CF clinics.

We assessed New Zealand's seven major CF centres, combining epidemiological data with computer-assisted *Sal*I DNA fingerprinting of 496 isolates from 102 patients.

One cluster of related isolates was significantly more prevalent in the largest clinic than expected by chance. The seven patients with isolates belonging to this cluster had more contact with each other than the remaining patients attending this centre. We found no other convincing evidence of transmission in any of the other smaller clinics. Three *P. aeruginosa* strains believed to be transmissible between patients in Australian and British CF clinics are present in New Zealand, but there was no definite evidence they had spread.

P. aeruginosa transmission is currently infrequent in New Zealand CF clinics. This situation could change rapidly and ongoing surveillance is required. Our results confirm that computer-assisted *Sal*I DNA fingerprinting is ideally suited for such surveillance.

INTRODUCTION

Once *Pseudomonas aeruginosa* is established within the lungs of patients with cystic fibrosis (CF) there is an accelerated decline in pulmonary function, quality of life and life-expectancy [1]. Most CF patients acquire *P. aeruginosa* strains from environmental sources [2]. However, siblings are often infected with indistinguishable clones suggesting person-to-person transmission instead of common environmental exposure [3]. Furthermore, in several European, English and Australian CF clinics, many patients share a common strain [2, 4-7]. Some strains appear in several clinics [8, 9], others superinfect patients already chronically colonised with unrelated *P. aeruginosa* isolates [10], while some are associated with worse outcomes and increased treatment costs [6, 7, 11-13]. In contrast, others have found only small clusters of genetically similar isolates from CF patients lacking geographic or temporal relationships other than attending the same clinic [14 , 15, 16]. Rather than from cross-infection such clusters could be due to common environmental sources or limited discriminatory power of typing methods [17].

Consequently, *P. aeruginosa* transmission between CF patients may occur, but only after prolonged close contact, as in siblings, or from small numbers of strains with heightened potential for transmission. Nonetheless, British and, more recently, Australian CF Trust infection control guidelines and the European consensus report recommend that all CF patients with *P. aeruginosa* be segregated to reduce cross-infection [18-20]. Centres in Australia and Europe that implemented these recommendations have been successful at restricting potentially transmissible strains [21, 22]. However, segregation-based infection control policies are expensive, difficult to implement and adversely affect patient wellbeing without reducing sporadic *P. aeruginosa* acquisition [22]. It is therefore important to determine whether cross-infection is occurring within individual clinics.

Previously, we described a computer-assisted DNA fingerprinting method, which is simple, fast and cost-effective [23]. The DNA fingerprints are stable [23], the pattern score-based genetic distances correlate with DNA sequence-based genetic distances [23], and its discriminatory power matches that of pulse-field gel electrophoresis (PFGE), the current gold standard for molecular typing *P. aeruginosa* isolates [23-25]. A robust pattern scoring system used with the DNA fingerprinting technique largely overcomes problems associated with noncategorical molecular typing methods [17]. Previously, using computer-assisted *Sal*I fingerprinting, we found indications of possible transmission in one New Zealand clinic based upon *P. aeruginosa* isolates from CF patients being genetically less diverse than isolates obtained from patients without CF [23]. However, we did not directly establish this resulted from CF clinic attendance. We therefore conducted a multi-centre study to determine the likelihood of *P. aeruginosa* transmission in New Zealand CF clinics by combining epidemiological data with *Sal*I fingerprinting. The clinics were located in cities between 100 and 1100 kilometres apart, each employed standard hygiene methods, but most had not implemented patient segregation.

METHODS

Patients, data collection and isolates

Participants attended one of seven (Table 1) clinics caring for 269 CF patients (102 adults aged $\geq$ 15-years; clinic size 21-57), representing 71% of children, 84% of adults and 75% of New Zealand CF patients. Treatment and infection control protocols were similar between hospitals [26]. Inpatients were nursed either in single rooms with their own bathrooms or in wards with non-CF patients. Contact between CF patients was discouraged, respiratory equipment was not shared, in-line filters, mouthpieces and connectors for pulmonary function equipment were discarded or sterilised after use, and physiotherapy conducted separately. Segregation of all CF

individuals in outpatient clinics was practised by Starship, Christchurch (paediatric clinic only) and Dunedin hospitals. The other hospitals segregated only those harbouring *Burkholderia cepacia complex* or methicillin-resistant *Staphylococcus aureus*. The Ministry of Health's Multi-Region Ethics Committee approved the study as a clinical audit.

Following informed consent, single sputum or oropharyngeal specimens were collected from CF patients attending routine clinic appointments during July 2004-August 2005. Their physician entered centre, age, gender, sputum microbiology and, for the previous 12 months, dates of hospitalisation and clinic attendances, best recorded body-mass index z-scores and percent predicted forced expiratory volume in 1-second (FEV_1) values into a secure database.

Respiratory samples were inoculated onto selective media to detect CF respiratory pathogens by standard techniques [27]. When *P. aeruginosa* was identified, five randomly selected colonies were each inoculated into glycerol broth and stored at -70^0C (see Supplementary material).

Representative isolates of clonal strains from CF patients in Liverpool [4], Manchester [5], Melbourne (2 pulsotype1 isolates) [6] and Brisbane (pulsotype2) [7] were kindly provided by John Govan, University of Edinburgh, Andrew Jones, Manchester Adult CF Centre, David Armstrong, Monash University, Melbourne and Scott Bell, the Prince Charles Hospital, Brisbane respectively.

P. aeruginosa genotyping

DNA fingerprinting was performed as described previously [23]. Briefly, following electrophoresis and ethidium bromide staining of *Sal*I digests of genomic DNA, the number of

restriction fragments in molecular weight brackets (defined by an XV molecular weight standard [Roche Diagnostics, Auckland] loaded in alternate lanes) was counted for each strain, and used to calculate genetic distances (see Supplementary material). Distances were displayed as unweighted pair group method with arithmetic mean (UPGMA) trees using Paup*4.0 (Sinauer Associates, Sunderland, Mass). Five colonies per patient were typed. PFGE of *SpeI*-genomic DNA digests of *P. aeruginosa* isolates was also performed [28].

Statistical analysis

Simple descriptive statistics, binomial, *t* and Fisher's exact tests were performed using Stata software (Stata Corporation, College Station, Version 10). Statistical significance was determined by $P < 0.05$ and Bonferroni correction applied when multiple comparisons were made.

RESULTS

Frequency of *P. aeruginosa* in the study population

Of 269 patients attending seven clinics, 102 (38%) had *P. aeruginosa* in sputum (n=97) or oropharyngeal (n=5) cultures. The characteristics of these 102 patients are shown in Table 2.

Evidence for transmission at one centre

Table 1 shows that 496 *P. aeruginosa* isolates from 102 CF patients underwent DNA fingerprinting. This included two patients who moved during the study and provided samples in two clinics. The table also displays the degree of contact between patients. Figure 1 shows the relationships between isolates as a UPGMA tree. If a patient had several isolates with identical patterns, only one of these is included in the tree. Four strains highly prevalent in CF clinics overseas are also included.

Transmission results in clusters of identical or very similar DNA types in different patients. To arrive at a meaningful threshold for identifying clusters, we determined in 50 instances the genetic distance between two independent scores of the same pattern, in two different DNA preparations. The average distance was 0.094, the upper 95% confidence limit was 0.118. We therefore used 0.118 to define clusters and above this distance we were confident of distinguishing different patterns.

In 67 patients all their isolates were identical or could be assigned to one cluster specific to this patient (grey boxes in Figure 1) indicating that their isolates were derived from a single infecting strain. Isolates from the remaining 35 patients were too different to be placed into single patient-specific clusters (although often they were still more similar to each other than to isolates from any other patient). For two patients who moved during the study and were sampled twice, GRE142/DUN242 and GRE144/WAI249 (lower case isolate labels in Fig.1), isolates obtained at both centres fell into the same clusters, with one exception (gre144 3).

Some clusters contained isolates from two to nine different patients (black boxes in Fig.1). Such clustering can result from transmission, clonal expansions predating and unrelated to transmission in the patient population under study, or simply because no typing method produces an infinite number of types [17]. To estimate how much clustering is expected without transmission in this patient population, we calculated the frequency of distance values < 0.118 when only isolates obtained at different hospitals were compared (i.e. isolates lacking direct epidemiological connection; 1.9%; 336/17,078 comparisons). Therefore without transmission

1.9% of distance values resulting from all comparisons between isolates from different patients in Fig. 1 should be below 0.118. However, 2.2% (486/21,366) comparisons had distance values <0.118; 65 more than expected without transmission.

We next assessed if any of the clusters in Fig.1 involved a significantly higher proportion of patients from one centre, as expected if a cluster were resulting from transmission. This was the case only for the largest cluster (involving nine patients), isolated almost exclusively from patients attending the Greenlane CF Centre (7/38 Greenlane patients had isolates that were part of this cluster, compared to 2/66 patients from other clinics; Fisher's exact test $P = 0.012$). Also, patients with isolates belonging to the cluster had more centre-related contact with each other than the remaining Greenlane patients (Fig. 2; $P = 0.015$; the sum of 21 pairwise physical distance category values for the seven patients was lower than the sum of 986/1000 sets of 21 values randomly chosen from a matrix of all remaining Greenlane patient pairwise physical distance category values), consistent with the hypothesis that transmission was health care-related.

We found no significant (t test) differences in several health status indicators (body-mass index z-scores, FEV_1 values, frequency of clinic attendance or days in hospital) between the nine patients carrying strains belonging to this shared cluster and all other study patients or between the seven Greenlane patients carrying these strains and all other Greenlane patients. Neither did the sex or age distribution of patients infected with cluster isolates differ significantly (Fisher's exact and t tests) from that of the remaining patients (data not shown).

Prevalence of potentially transmissible overseas strains in New Zealand

Introduction and spread of highly transmissible overseas strains is a potential threat to New Zealand patients. We therefore included representatives of four such strains (see Methods) in our analysis to see if such strains might be spreading in New Zealand. We found New Zealand isolates clustering with three of the strains, and confirmed that isolates with *Sal*I types highly similar to the overseas strains also had PFGE types indistinguishable or closely related to those of the overseas strains (see supplementary Fig. S1). The Manchester and pulsotype1 strains each clustered with isolates from a single New Zealand patient. The adult (GRE126) with an isolate indistinguishable from the Manchester strain had lived in London and it was there his initial *P. aeruginosa* infection was first detected. Another patient (CHR119) possessed isolates similar to Melbourne pulsotype1 isolates and had previously holidayed in Queensland, although without making contact with Australian CF clinics or patients. Brisbane pulsotype2 isolates formed a cluster with isolates from four New Zealand patients. Two (WEL101 and GRE144/WAI249) had lived previously in Brisbane where one first acquired *P. aeruginosa* before immigrating to New Zealand. Both had mixed freely with Australian CF patients while receiving treatment. The remaining two patients (GRE114, GRE185) had isolates resembling pulsotype2 and were treated in the same centre as, but were never in direct contact with, a patient previously exposed to pulsotype2 overseas (GRE144/WAI249). Thus at most three patients are likely to have acquired overseas strains in New Zealand.

No general correlation between centre–related patient contact and *Sal*I pattern-based distance between their isolates

Many of the centres in this study had very small patient numbers, and in these transmission may only lead to very small clusters, and thus be difficult to detect by assessing clustering. We

therefore also used an alternative approach of searching for transmission, by determining if the genetic distance between patients' isolates tends to decrease with increased patient contact.

We first determined if the closest-related counterparts of isolates from patients in a given clinic were isolates from other patients at the same clinic more often than expected by chance. This was not the case (see supplementary table 1). We next checked if, within each clinic, the average genetic distance between patients' isolates decreased as the degree of physical contact (and opportunity of transmission) increased (Fig. 3). For comparison we also included in the analysis the distribution of genetic distances between isolates from the same patient and the distribution of genetic distances between isolates from a given centre and isolates from all other centres. Only isolates from different siblings were generally much more similar to each other than isolates from pairs of patients treated in different centres, indicating that the multiple *P. aeruginosa* isolates in most sibling pairs were derivatives of a single strain. In contrast, the distribution of genetic distances between isolates from pairs of patients with various degrees of contact through treatment at the same centre was not significantly different from that between isolates from patient pairs treated at different clinics, although at Greenlane the median genetic distances in all but one of these categories were lower than those in category VII. We also failed to detect a dependence on the frequency of low genetic distance values on centre–related patient contact (see Supplementary material).

Lastly, we determined if four patients who first acquired *P. aeruginosa* during the course of the study (WEL110, CHR235, WELL243 and PAL257) harboured isolates closely related to those from other CF patients at the same clinic. In each case the isolates in our sample closest related to theirs were not from the same centre.

DISCUSSION

Transmission of *P. aeruginosa* between New Zealand CF patients appears infrequent. Isolates from patient pairs with frequent treatment-related contact are generally not more closely related than those from patients attending different centres. This also indicates that, with the possible exception of siblings who often share similar strains, social contact generally did not lead to significant transmission: otherwise isolates from patients treated in the same centre should be more closely related than isolates from patients treated in different centres hundreds of kilometres apart.

Our only evidence for healthcare-related transmission is a cluster of isolates in seven of 38 patients at Greenlane. The cluster is overrepresented in Greenlane where patients with isolates belonging to the cluster were in closer contact with one another than other Greenlane patients. Also the cluster's size (97 distance values < 0.118 among cluster isolates from Greenlane) roughly matches the excess of distance values < 0.118 in our patient population indicative of transmission.

Evidence for transmission is also consistent with our earlier findings [23] of reduced genetic diversity of isolates from CF patients compared with isolates from other Greenlane patients, but together the two studies also indicate that transmission is uncommon at Greenlane. The cluster's prevalence has not significantly changed in the eight years since the earlier study, when cluster isolates were present in three of 20 CF patients [23], and had not increased among five patients participating in both studies where one of these patients (GRE 107) had cluster isolates on both occasions. Furthermore, three of the remaining four patients (GRE102, 105, 123 and 145) also retained their original strains. Likewise pulsotype2 strain, capable of spread overseas, had not

done so in Greenlane to a readily demonstrable degree. Two patients may have acquired the strain while at Greenlane. However, no direct epidemiological connection was identified, and the main Greenlane cluster can account for all transmission–related clustering in our sample, arguing against transmission as a cause of the pulsotype2 cluster. Thus, because typing methods do not give an unlimited number of patterns and sample only a small part of the genome [17, 23], the pulsotype2 isolates from the two patients may only be similar to, but not derived from, the overseas pulsotype2 strain.

Current infection control measures thus appear to protect patients from transmission in New Zealand clinics, except for the cohort presently attending Greenlane where a recent report also suggested cross-infection by *B. multivorans* may have taken place [29]. When interpreting these data, the small size of New Zealand CF clinics must be considered. Patients in smaller centres usually have less contact with one another [30] and thus less opportunity of transmitting strains. Certainly, the clinic where we found evidence for transmission was by far the largest in our study, with substantially more patient contact compared to other centres. Our results raise the possibility that the existing body of research, based largely on findings in large CF clinics, may overestimate the risk of cross-infection among the many CF patients that are still treated in smaller centres. Furthermore, smaller sized centres also make it harder to detect transmission by visual inspection of dendrograms. However, the analysis in Fig.3 and our analysis based on the expected frequency of distance values <0.118 in the entire sample without epidemiological contact should have revealed if transmission occurred in other centres. It could be argued that the latter analysis may have underestimated transmission amongst our patients, since its calculations are based on the assumption that patients treated in different centres lack any epidemiological connection. This is not entirely accurate, as patients do move between clinics. Two such

movements were recorded during the study and additional movement will have occurred before the investigation. However, the error in our estimated frequency of distance values <0.118 between unconnected isolates is probably small. We had determined earlier [23] that the discriminatory power of our method (probability that two unrelated isolates can be discriminated), was 0.99, using isolates from unrelated non-CF patients where unexpected epidemiological relationships are very unlikely. In the current study we found that 1.9% of pair-wise comparisons between representative isolates from patients treated in different centres were not reliably distinguishable from one another (genetic distance <0.118), equivalent to a discriminatory power of 0.98. This means that isolates from different centres in the present study were approximately as epidemiologically unconnected as isolates from epidemiologically unrelated non-CF patients.

This new estimate of discriminatory power, the finding that *Sal*I patterns are stable over seven to eight years and observing isolates that formed clusters with our method were also indistinguishable by PFGE all confirm *Sal*I typing as an attractive alternative to PFGE, in particular if cost is considered as well. The current study indicates that surveillance of the total New Zealand CF population would cost around $US 8,000 per year.

ACKNOWLEDGMENTS

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FIGURE LEGENDS

Fig. 1. UPGMA tree based on *Sal*I pattern score-based genetic distances between representative isolates (where multiple isolates from the same patients are scored as identical, only one representative is included). Isolate labels reflect the clinic at which a patient was treated: Greenlane (GRE), Starship (STA), Waikato (WAI), Palmerston North (PAL), Wellington (WEL), Christchurch (CHR) and Dunedin (DUN)- followed by a three digit patient number; the fourth digit indicates which of the five isolates from the patient is shown. Lower case isolate names highlight two cases in which patients were sampled twice at two centres (GRE142/DUN242 and GRE144/WAI249). Isolates belonging to overseas strains are from Liverpool (LES), Manchester (MAN), Melbourne (Pulsotype1.1 and Pulsotype1.2) and Brisbane (Pulsotype2). Five different symbols (empty and filled circles, triangles and rhomboids) identify isolates obtained from five sibling pairs. A dashed line indicates the threshold of a genetic distance of 0.118 used for defining clusters of isolates with highly similar patterns. Grey bars show clusters present only on one New Zealand patient. Black bars show clusters present on several New Zealand patients. In cases where a cluster was present on more than two New Zealand patients, the number of patients is shown to the right of the bar.

Fig. 1
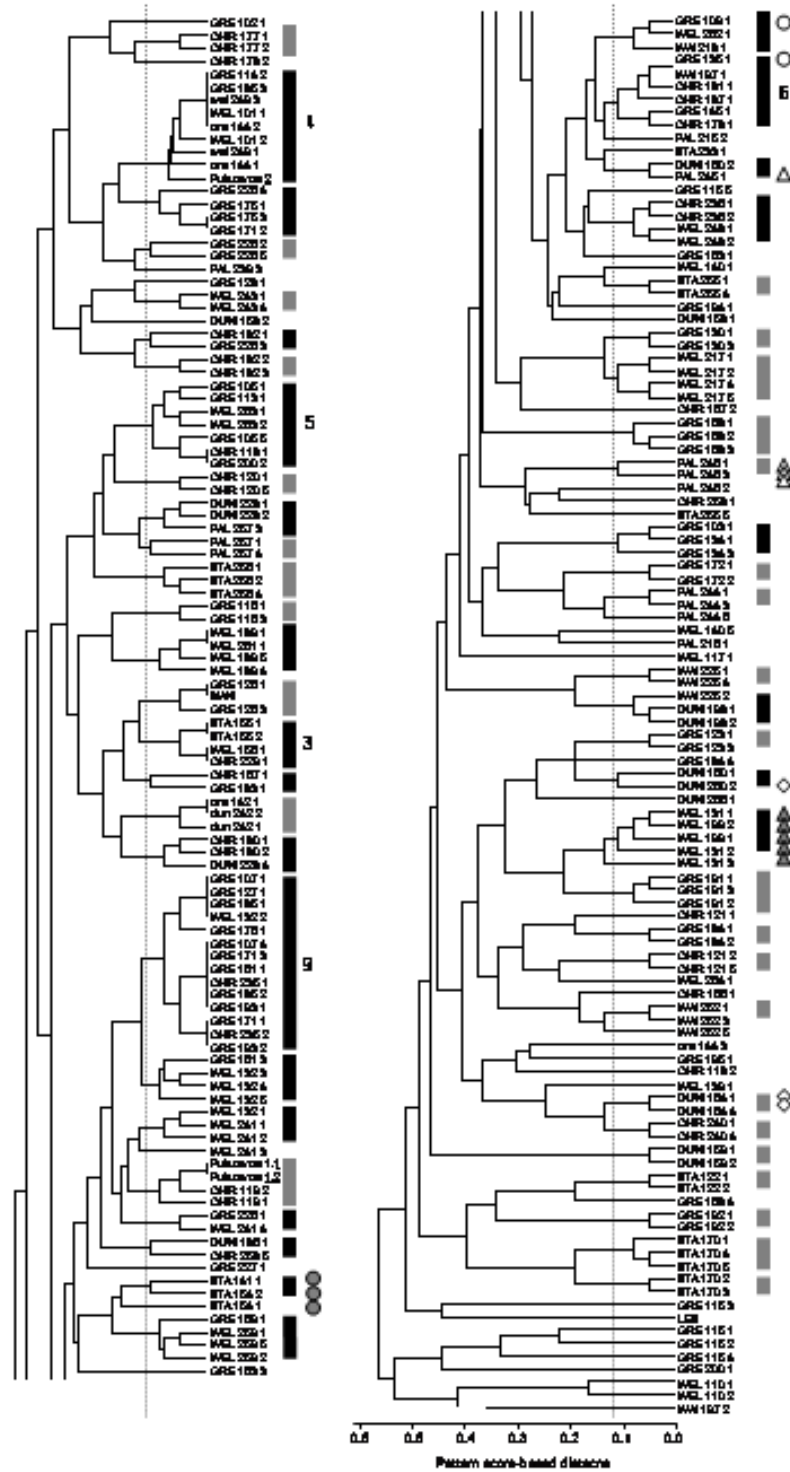
Fig. 2. Distribution of physical distance categories among Greenlane patients contributing to the largest cluster (black bars) and the remaining Greenlane patients (empty bars). See Table 1 for definition of physical distance categories.
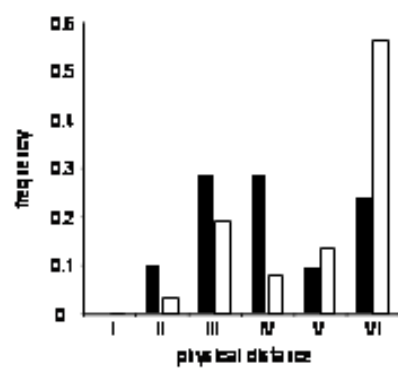
Fig. 2

Fig. 3. Box and whisker plots of the distribution of genetic distances between isolate pairs from patients with different degrees of physical contact in Starship (A), Greenlane (B), Waikato (C), Palmerston North (D) Wellington (E), Christchurch (F), and Dunedin (G) CF clinics. Physical distance categories are defined as in Table 1 except for two additional categories. Category S represents pairs of isolates from the same patient. Category VII represents pairs of isolates from pairs of patients treated in different hospitals, i.e. the genetic distances in category VII are those between isolates obtained in the hospital the figure refers to and isolates in all other hospitals. A grey line represents the median of genetic distances in category VII.

Fig. 3
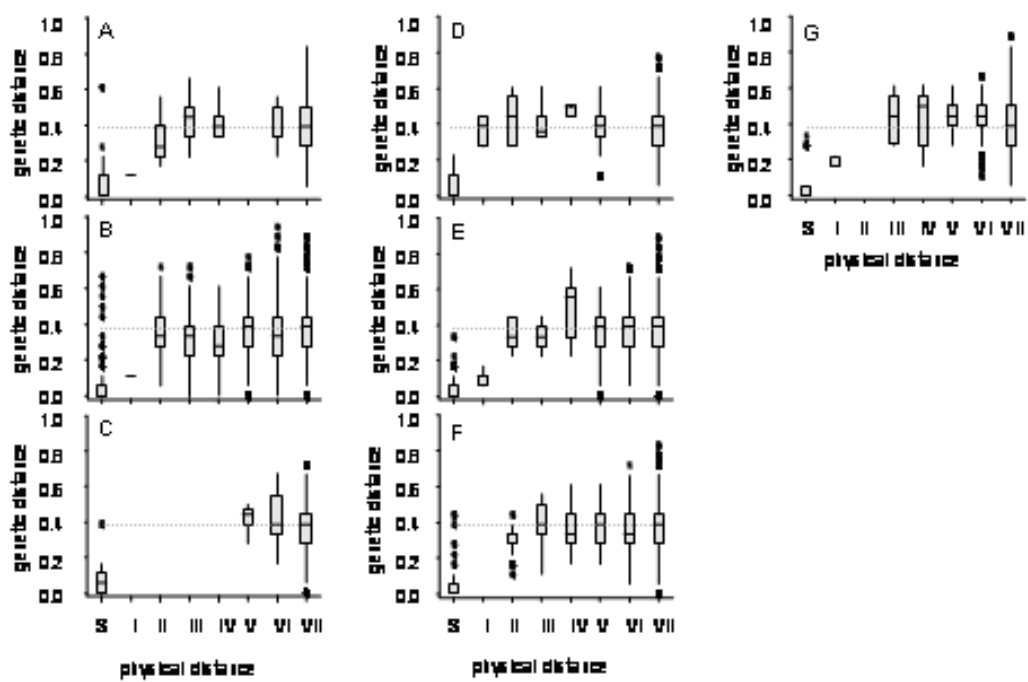
TABLE 1. Patient population and isolates

Clinic[a]	No. of patients sampled[b]	No. of isolates finger-printed	Timeframe of collection[c]	No. of patient pairs in contact category (%)[d]					
				I	II	III	IV	V	VI
Starship (Auckland)	8	39	11/04 – 05/05	1 (4)	4 (14)	15 (54)	5 (18)	0 (0)	3 (11)
Greenlane (Auckland)	38	182	11/04 – 03/05	1 (0)	25 (4)	136 (19)	59 (8)	95 (14)	387 (55)
Waikato	5	24	02/05 – 06/05	0 (0)	0 (0)	0 (0)	0 (0)	3 (30)	7 (70)
Palmerston North	7	31	01/05 – 03/05	1 (5)	2 (10)	4 (19)	2 (10)	12 (57)	0 (0)
Wellington	19	95	07/04 – 08/05	1 (1)	3 (2)	7 (4)	3 (2)	10 (6)	147 (86)
Christchurch	17	81	11/04 – 05/05	0 (0)	10 (7)	6 (4)	12 (9)	16 (12)	92 (68)
Dunedin	10	44	12/04 – 08/05	1 (2)	0 (0)	7 (16)	6 (13)	3 (7)	28 (62)

Table 2: Characteristics of patients with *P. aeruginosa*

	Number of patients
Proportions of patients with *P. aeruginosa*	
Adults ≥ 15 yrs	83/102 (81%)
Children < 15 yrs	19/167 (11%)
Total	102/269 (38%)
Patients with *P. aeruginosa*; n = 102	
Median age (IQR) [a]	20.5 (17,29) yrs
Age range	6 – 65 yrs
Gender (males)	67
Sibling pairs	5
Patients with first acquisition	4
Duration of infection	$0 - 10^{+}$ yrs
Colonial morphology; n = 91	
Mucoid	27
Non-mucoid	26
Mucoid and non-mucoid	38
Co-pathogens [b]	75
Staphylococcus aureus [c]	64
Haemophilus influenzae	13
Stenotrophomonas maltophilia	9
Burkholderia cepacia complex	3
Others [d]	30

[a] interquartile range; [b] 75 patients were infected with between 1 and 3 respiratory pathogens;

[c] all isolates were methicillin-susceptible; [d] 21 Aspergillus species, 5 other gram –ve bacilli and 4

non-tuberculous mycobacteria.