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Survey of *Pseudomonas aeruginosa* genotypes in Belgian colonised Cystic Fibrosis patients.

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Genotyping of *P. aeruginosa* from CF patients

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ABSTRACT

To examine whether patients shared genotypes and to compare the genotypes of the isolates from the same patients during two subsequent years, we set up a Belgian databank of P. aeruginosa genotypes of all colonised CF-patients.
Sputum samples from a total of 276 P. aeruginosa colonised patients were sent during 2003 and from a subgroup of 95 patients in 2004. Patients were asked for social contact between each other by questionnaire. All P. aeruginosa isolates exhibiting different colonial morphology on McConkey agar were first genotyped by arbitrarily primed PCR, whereafter single representatives of each RAPD-type were further genotyped by fAFLP-analysis.
For the 213 patients from whom P. aeruginosa could be cultured and 910 isolates, a total of 163 genotypes were found. 75% of patients harboured only one genotype. In most of the limited number of clusters, previous contacts could be suspected. The same P. aeruginosa genotype was recovered from 80% of the patients, studied during both years.

We concluded that most patients harbour only one P. aeruginosa genotype, despite different colonial morphotypes. There is only a limited number of clusters, and most patients seem to have the same genotype during both years.

INTRODUCTION

Pseudomonas aeruginosa, widely spread in soils and water, has been the leading pathogen in cystic fibrosis (CF) lung pathology during the last three decades [1-3]. The means by which this organism is acquired are not yet fully elucidated. After initial infection, chronic infection and colonisation - as defined by the criteria of Döring *et al*. [4] - causes destruction of lung tissue and reduction of lung function, and finally leads to early death. According to the U.S. CF Foundation database of 1996 the median survival of *P. aeruginosa* colonised CF-patients was 28 years, while the median survival for non-colonised patients was 39 years [5]. Kerem *et al.* [6] demonstrated that patients, colonised with *P. aeruginosa* at the age of 7 years, had a mean FEV_1 (Forced Expiratory Volume in one second) that was 10% lower than that of non-colonised patients.

Currently, some CF-centres report the spread of highly transmissible strains that are multi-resistant and, in some patients, are responsible for primary infection [7-12]. Other studies, however, do not find evidence of clonal spread [13-16].

In Belgium the total number of CF patients, registered in the Belgian registry in 2003 [17], was 843, of whom 750 are followed at the 7 CF centres and 280 are considered as colonised by *P. aeruginosa,* according to the criteria of Döring *et al*. [4].

With this study we prospectively set up a Belgian databank of *P. aeruginosa* genotypes, isolated from colonised CF-patients to examine whether patients shared genotypes. In addition we wanted to compare the genotypes of the isolates from the same patients during two subsequent years.

METHODS

Patients

The 7 Belgian CF-centres, Sint Vincentius Hospital/University Hospital Antwerp, HUDERF-Erasme Hospital (ULB) Brussels, University Hospital of the Free University of Brussels (AZ-VUB), University Hospital Ghent, University Hospital Leuven, University Hospital Liège, and Hospital of the Catholic University of Louvain-la Neuve (UCL), participated in this study.

Samples from a total of 276 *Pseudomonas aeruginosa* colonised CF patients, were sent to the microbiology laboratory of the University Hospital of Ghent during 2003.

Sputum samples were mostly collected during an out-patient consultation, at the end of a physiotherapeutic session to ensure that the samples originated from the deeper airways; when patients were too young or unable to expectorate, a nasopharyngeal aspirate or swab was taken (26 nasopharyngeal samples vs. 250 sputum samples). All centres were asked to send another sputum sample one year later. For a subgroup of 95 patients, this sputum sample was obtained.

Patients were aged from 5 to 54 years (with a mean age of 24.2 years ± 8.8 SD).

Patients had to sign an informed consent and approval of the ethics committee of the University Hospital of Ghent was obtained for this national multi-centre study.

Patients filled in a questionnaire (addendum 1 and 2), assessing the frequency and intensity of current and previous social contacts with other CF patients. CF sibling contacts were not taken into account. Scores arbitrarily assigned to the different possible answers in the questionnaire were agreed among the CF specialists involved in the

study, whereby a subjective 'weight' was given to the type of contact (for example an intimate relationship was scored as the highest risk factor for transmission (score 10) and occasional social contact as a minor risk factor (score 4)).

Ninety-three percent of patients, from whom sputum *P. aeruginosa* was cultured, completed the questionnaire.

Segregation policies are installed in all CF centres, except for one centre. In the outpatient clinics, *P. aeruginosa* colonised patients and non-colonised patients are seen on different days. Care givers are strongly advised not to wear jewellery and to wash hands and stethoscopes between each visit. The patients are asked to wash hands before lung function measurement and to produce a sputum sample in a separate room. Filters of the lung function equipment are always changed between patients. Patients with CF are always hospitalised in a single room and contact with other hospitalised CF patients are strongly discouraged.

These recommendations date from the mid nineties and most centres implemented them in the following years.

Microbiology

Sputum samples were inoculated onto McConkey agar (BBL Becton Dickinson, Cockeysville, Md.). After two days of incubation at 37°C, differently looking lactose negative colonies were picked, subcultured on 5% sheep blood agar (BBL) and tested for oxidase. Only oxidase positive colonies were further identified, using tDRNA-PCR [18].

Genotyping

For each patient, all *P. aeruginosa* isolates exhibiting different colonial morphology on McConkey were first genotyped by arbitrarily primed PCR, using alkaline cell lysis for DNA-extraction and Randomly Amplified Polymorphic DNA-fingerprinting analysis (RAPD)

Ready-to-Go beads (Amersham Biosciences AB, Uppsala, Sweden) and primer ERIC2 (AAGTAAGTGACTGGGGTGAGCG) at an annealing temperature of 35°C, as described previously (18). This enabled us to reduce the number of isolates that were subsequently genotyped by the more laborious fluorescent amplified fragment length polymorphism analysis (fAFLP), since only single representatives of each RAPD-type were further genotyped by this procedure. The AFLP-technique is described earlier [19].

Statistical analysis

Values of the 'inter patient contact score' as obtained from the questionnaires did not approach the normal distribution (Kolmogorov-Smirnov Z-statistic $p < 0.001$), therefore all analysis involving questionnaire scores were done under the nonparametric assumption. In order to assess putative differences in number of inter-patient contacts between patients with a unique *P. aeruginosa* genotype compared to patients who share at least one *Pseudomonas* genotype with at least one unrelated patient, median 'inter patient contact scores' were calculated for both groups and compared with the Median test. Dispersion of score values around a median value is presented as interquartile (p25 – p75) ranges. Differences in the distributions of score values between two groups were assessed with the Mann-Whitney U test for two independent samples. Strength of association was expressed as (crude) odds ratios (OR) with 95% confidence intervals (95% CI) to the estimated OR. For any reported measure, statistical significance was accepted, if the two-tailed probability level was <0.05. All analyses were performed with the statistical software package SPSS v. 12.0 (Chicago, Illinois).

RESULTS

P. aeruginosa isolates of a total of 213 out of 276 *P. aeruginosa* colonized patients were genotyped using AFLP-analysis. For 63 patients no *P. aeruginosa* could be isolated, because culture remained negative (n = 10), because technical problems occurred (n = 29) or because another gram negative organism was cultured (n = 24: 6 patients harboured *Achromobacter xylosoxidans* instead of *P. aeruginosa*, 15 patients harboured *Stenotrophomonas maltophilia*, one patient harboured both and 2 patients were colonised with *Burkholderia cepacia*).

A total of 910 *P. aeruginosa* isolates were genotyped using RAPD-analysis.

After excluding isolates from the same patient with an identical RAPD-pattern, 272 isolates from the patients together with an additional 3 reference strains (i.e. epidemic strains from the UK: the Liverpool, Manchester and Midlands strain, kindly provided by Prof. T. Pitt (Public Health Laboratory Services, London, UK) were typed with fAFLP, a technique which is more reproducible than RAPD-analysis and which yields digitized fingerprints allowing large numbers of fingerprints to be compared by computer.

For the 213 patients and 910 isolates, a total of 163 genotypes were found, based on AFLP-analysis. The majority of patients (160) had one genotype, 48 patients had 2 genotypes and 5 patients had 3 genotypes (Fig1).

A limited number of clusters, i.e. 13, with 'cluster' defined as a group of patients carrying *P. aeruginosa* isolates with the same genotype, was observed. There were three additional sibling clusters. The sizes of the clusters and the centre origins are listed in Table 1.

Sixty-six patients (sibling clusters excluded), i.e. 30 %, carried a *P. aeruginosa* isolate with a shared genotype. Five (2.3 %) of them were part of two clusters.

There were six 2-person clusters, one cluster of 4 patients, one of 5 patients, two of 9, two of 10 and one of 12 patients.

Eleven of the 13 non sibling clusters contained patients of multiple centres. In 10 out of 11 multi-centre clusters, former contact between patients could be established, such as stay in a rehabilitation centre (rehabilitation centre A (rehab A) and rehabilitation centre B (rehab B)), attendance to a CF-camp and/or social contact.

When comparing the 'inter patient contact score' between cluster patients and non-cluster patients, there was a significant difference between both groups (100% of the cluster patients filled in their questionnaire versus 90% of the non-cluster patients) (Fig 2). Although mean age of both groups was comparable (24.7 ± 9.1 for the non cluster patients versus 23.2 ± 8.2 years for the cluster group), the cluster patient group (n = 66) reported on average a significantly higher 'inter patient contact score' compared to the non-cluster group (n = 132) (rank-sum $p < 0.001$), i.e. a median inter patient contact score 9.0 (inter-quartile range 5.0 to 14.0) was observed with patients sharing a *P. aeruginosa* genotype with at least one other (unrelated) patient versus a median score of 4.0 (inter-quartile range 0 to 7.0) among patients with a unique *P. aeruginosa* fingerprint ($p < 0.001$).

During 2003, siblings (n = 24) invariably presented with at least one similar *P. aeruginosa* genotype at the level of the sibling pair (n = 12). Siblings represented 4.5% of the non-cluster group of patients (6/132) compared to 27.3% of the cluster group of patients (18/66) ($p < 0.001$), indicating that siblings are actually much more prone to be involved in the spread of *P. aeruginosa* among CF patients (odds ratio = 7.9, 95% CI = 3.0 to 21.0, $p < 0.001$).

The latter observation might be explained by differential inter-patient contact rates, considering that sibling patients (n=24) tended to report on average a higher number of inter-patient contacts compared to unrelated patients (n=174) with median 'inter-patient contact' score values of 5.0 (interquartile range 4.0 to 14.0) and of 4.0 (interquartile range 0.0 to 9.0), respectively, a difference that was marginally significant ($p=0.051$) within the limits of our sample size.

Although inter patient contact scores of siblings may actually be correlated data at the sibling pair level, numbers of colonized siblings were too low in our survey to assess and account for such a correlation and therefore score values of all patients were handled as independent observations. To ascertain however, that a potential interaction at the sibling pair level did not bias our primary results in comparing the score values of the cluster and non-cluster groups of patients, the analysis was also repeated by including only a single value for each sibling pair (a single mean score for each sibling pair, the lowest value of each sibling pair, and the highest value of each sibling pair, respectively) but these analyses did not alter our results.

None of the Belgian *P. aeruginosa* genotypes matched with the 3 UK strains.

For a total of 95 patients, sputum samples were collected from two subsequent years (2003 and 2004) and genotyped by AFLP (see fig.3). In total, the same genotype could be recovered from 76 patients (80%) in both years.

We compared the genotypes that were newly acquired in 2004 with the genotypes already present in the patient's centre in 2003. None of the 'new' genotypes accorded with the known centre genotypes, except for 1 patient, whose novel genotype appeared to be identical to a genotype recovered from his sibling in 2003.

DISCUSSION

This study is, to our knowledge, the first to compare the *P. aeruginosa* genotypes of most colonised CF patients within one country. Most studies examine the variety of genotypes within a centre [12, 14, 20, 21, 22, 23] and do not reflect the national situation.

The only comparable study was carried out in the UK [24], where a national survey was set up to identify and characterize transmissible *P. aeruginosa* strains in CF patients in England

and Wales and for which isolates were requested from over 120 hospitals and a sample size of approximately 20% of the CF population in each centre was attempted, but not always reached.

Different PCR fingerprinting techniques have been developed and different names have been used for identical techniques. Terms such as amplification fragment length polymorphism (AFLP), arbitrarily primed PCR (AP-PCR), DNA amplification fingerprinting or random amplification of polymorphic DNA (RAPD) are often used indiscriminately and create a ' Tower of Babel' phenomenon. In a letter to the editor in the Journal of Clinical Microbiology in 2003 several CF physicians and microbiologists therefore emphasized the need for harmonization of techniques and technique designations for genotyping clinical isolates of *P. aeruginosa* from CF patients [25]. Most of the epidemiological research studies [12, 16, 23] were based on Pulsed Field Gel Electrophoresis (PFGE).
Since our laboratory had already built up experience to genotype other species with RAPD and fAFLP [19], we chose to use these techniques. This choice was supported by a publication of Speijer *et al.* [26], which showed that AFLP analysis was comparable with PFGE and RAPD analysis for *P. aeruginosa* isolates. D'Agata *et al*. [27] also confirmed these findings.

Theoretically, it can be imagined that strains with slightly different fingerprints have acquired only recently mutations which make them differ from each other. As such they may be clonally related, whereas the different fingerprints suggest otherwise. The opposite can be true as well: strains with identical fingerprints can in reality differ from each other, because not all genomic differences are revealed by the fingerprint, which is obtained by looking at only some regions of the genome. When obvious differences exist in parts of the genome that are

not addressed by the technique, these will be overlooked and the technique will yield identical fingerprints for genotypically different strains.

Therefore, it can be stated that genotyping studies are an approximation of the true genetic relatedness among the strains studied. But, whether cross infection is underestimated due to the fact that strains with slightly different fingerprints belong to the same genotype anyway is difficult to say, since overestimation on the other hand is possible as well, as a consequence of genotypically different strains with coincidentally identical fingerprints.

However, the study of Speijer et al. [26], as discussed above, showed that the clustering obtained with three different genotyping techniques (RAPD, AFLP and PFGE), which address different regions of the genome, was concordant, and given the fact that we also found concordance between AFLP and RAPD in this and our previous study [19], we assume that the obtained discrimination reflects the true occurrence of genotypes and that there is neither under- nor overestimation of cross infection.

For the 213 patients and 910 isolates tested , a total of 163 genotypes were found, indicating that different morphotypes in one patient often have the same genotype.

This conclusion is supported by Hoogkamp-Korstanje *et al.* [28] and Da Silva Filho *et al* . [20]. Our previous study [19] in a CF rehabilitation centre also showed that for 76 patients only 71 different *P. aeruginosa* genotypes were found among 749 isolates, indicating that in individual patients isolates with different colonial morphology mostly belonged to the same genotype.

In this national study 75% of the colonized patients carried only one genotype, during 2003. This confirms the data by Mahenthiralingam *et al*. [29] and by our previous study [19] where more than half of the patients (49 out of 76 patients) carried only one genotype, 20 carried two genotypes and seven carried three genotypes.

For 24 out of 279 patients, thought to be colonized by *P. aeruginosa*, another gram negative organism was identified.

Some of the isolates, identified genotypically as *A. xylosoxidans* or *S. maltophilia*, seemed to be considered initially as atypical *P. aeruginosa* in the routine laboratory. Due to the diversity of colonial morphologies and biochemical reactivity encountered, misdiagnosis of gram negative non-fermenters cultured from CF sputum may occur. In one study, misidentification of 11% of *A. xylosoxidans* strains was reported [30].

In our previous report about the occurrence of two large clusters of *A. xylosoxidans* in a CF rehabilitation centre population, the routine hospital laboratory initially also misidentified this organism as *P. aeruginosa* [31].

There was only a limited number of clusters (n = 13 + 3 sibling clusters) and a limited number of patients harbouring one of these *P. aeruginosa* cluster genotypes.

These findings were similar to the data of the Vancouver CF-centre [16], and of the Brazilian study of Da Silva Filho *et al.* [20].

Other centres however reported large clusters with the same genotype. A paediatric CF centre in Victoria, Australia [12] showed that 55% of the 118 *P. aeruginosa* colonized CF children carried the same genotype and the Manchester CF centre [9] had to deal with a multi-resistant strain carried by 14% of its 154 *P. aeruginosa* colonised patients. In the Liverpool CF centre [8] 60% of 92 *P. aeruginosa* colonised children harboured the same strain.

A Norwegian study [11] showed that only 7/60 patients had a distinct genotype, one large main cluster of 27 patients (45%) and remaining clusters of 2 to 4 patients. Patients were known to have contact during holiday camps and training courses.

In the nationwide survey of Scott and Pitt [24], 72% of patients harboured strains with unique genotypes, which matches with our results. In their study small clusters of related strains were evident in some centres, presumably indicating limited transmission of local strains. The most prevalent strain ('Liverpool' genotype) accounted for 11% of patient isolates from 15 of the 31 examined centres. The second most prevalent strain ('Midlands1') was recovered from 86 patients in nine centres and clone C (originally described in Germany) was found in 15 patients from 8 centres. A fourth genotype, identical to the 'Manchester' strain, was found in three centres.

The Liverpool, Manchester and Midlands strain were not detected in the Belgian CF population. Our data did not point to a Belgian 'problem' genotype, carried by many patients, since the largest cluster containing 12 patients (5.5% of the studied population).

We are not aware whether these cluster genotypes are multi-resistant, since susceptibility testing was not performed during this study.

In our study most clusters, i.e. 11 out of 13, contained patients from different CF centres. The vast majority of these patients had spent time in one of the two Belgian rehabilitation centres (rehab A and rehab B), or had participated in a CF camp and at least 2 patients had even shared a hospital room with another non sibling CF patient in the past.

For instance, the largest cluster of 12 patients (cluster 4) contained 6 patients who had stayed several years ago in rehabilitation centre B and 4 patients who had stayed in rehabilitation centre A, whereby the remaining two patients were siblings that had stayed in both centres (for prolonged periods). One could speculate that this sibling pair caused the spread of this cluster genotype. In cluster 6, eight out of the 10 patients previously stayed in rehabilitation centre A. The clustering of the isolates from the remaining 2 patients remains unexplained, since they never stayed there, and since they mentioned close contacts only with

each other, but not with others from cluster 6.

In cluster 8, three of the 5 patients went on a CF holiday camp (the majority of them however could not specify which camp and how long, since these camps took place more than 10 years ago).

One 2-person cluster (cluster 13) contained 2 young school children, followed at the same CF centre: these girls were close friends, and, though they were discouraged to do so, they always came together to the centre, with the same car. They went to the same physiotherapist, shared the same classroom and even wanted to be hospitalised at the same moments. In these two children obviously patient-to-patient transmission had occurred (within the setting of an in- ánd outpatient CF clinic).

Since segregation between *P. aeruginosa* colonised and non colonised patients has been installed in almost all Belgian CF centres (except for centre B) and rehabilitation centres since the mid-nineties, patient-to-patient transmission is suspected to have occurred before that period.

In our previous study in one of the two Belgian rehabilitation centres [19], during 2002 and 2003, 38 of the 45 patients with a cluster strain already carried this strain upon arrival at the CF-centre. Therefore, we could not exclude that acquisition of this strain from a common source, or from another patient, occurred during one of the previous stays in the CF-centre, before more stringent infection control measures were introduced.

That study could establish that the risk of patient-to-patient-transmission during the study period was relatively low (10%), and that the risk of persisting colonisation with a newly acquired strain during the study period was still lower (4%).

In this study siblings carried the same genotype. We did not take into account the sibling clusters, nor did we ask for sibling contacts in the questionnaire, since it could be considered as 'obvious' for siblings to share genotypes [13, 32, 33].

In this Belgian cohort study siblings (total n = 24) represented 4.5% of the non-cluster group of patients (6/132) compared to 27.3% of the cluster group of patients (18/66) ($p < 0.001$) indicating that siblings are actually much more prone to be involved in the spread of *P. aeruginosa* among CF patients (odds ratio = 7.9, 95% CI = 3.0 to 21.0, $p < 0.001$).

We could speculate that siblings stay in a rehabilitation centre more often than non siblings, because the burden of having 2 children with CF (and having to spent a lot of time to their treatment) 'forces' the parents to send their children to these centres from time to time.

It is also possible that siblings are more willing to stay in a rehabilitation centre or to attend a holiday camp, since they don't have to go alone (in contrast with the non sibling patients).

For a subgroup of 95 patients, genotyping was performed for two subsequent years. The vast majority continues to carry its own predominant strain (80%). Of those who had a 'new' genotype in 2004, only one patient had a genotype that matched with a genotype from his own centre in 2003. The strain with the matching genotype however had been isolated from his sister in 2003. Therefore, we could speculate that this strain was already present in the patient during 2003, but had been overlooked, or that it had been newly acquired from his sister in the period between both samplings. Since no other 'new' genotypes in 2004 seemed to match with the 'known centre genotypes' of 2003, we could state that patient-to-patient transmission probably did not occur within the Belgian centres for this subgroup of patients.

A limitation of the study is the lack of validation of the questionnaire. As mentioned in Methods the scores were arbitrarily assigned, since no other scoring system, evaluating the

amount and intensity of social contacts has been used in CF studies.

Although this scoring system remains subjective this questionnaire enabled us to assign to some degree an 'inter patient contact score' to each patient.

In summary, our findings confirm that, in the Belgian CF population, different colonial morphotypes of *P. aeruginosa* from the same CF patient usually belong to the same genotype. We could also state that genotypic diversity among *P. aeruginosa* strains is large in Belgian CF patients. We could describe only a limited number of clusters. The situation is different from one country to another and depends probably on multiple factors such as number of patients per centre, presence of highly transmissible strains, segregation measures. Most clusters in our study could probably be explained by previous social contacts (mostly during previous stays in rehabilitation centres and during holiday camps). Eighty percent of a subgroup of patients continued to carry its own predominant strain during 2 subsequent years, suggesting a small genotype variability in the same patient despite the large genotype diversity in this survey.

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References

1. Mearns MB, Hunt GH, Rushworth R. Bacterial flora of respiratory tract in patients with cystic fibrosis. *Arch Dis Child* 1972; 47: 902–907.

2. Hoiby N. Epidemiological investigations of the respiratory tract in patients with cystic fibrosis. *Acta Pathol Microbiol Scand* 1974; 85: 541.

3. Fitzsimmons S. The cystic fibrosis patient registry report. *Pediatr Pulmonol* 1996; 21: 267–275.

4. Döring G, Conway SP, Heijerman HGM, Hodson ME, Hoiby N, Smyth A, Touw DJ. Antibiotic therapy against *Pseudomonas aeruginosa* in cystic fibrosis: a European consensus. *Eur Respir J* 2000; 16: 749–767.

5. Cystic Fibrosis Foundation USA, Patient Registry 1995. Annual data report. Bethesda, MD, Cystic Fibrosis Foundation, USA, 1996.

6. Kerem E, Corey N, Gold R, Levison H. Pulmonary function and clinical course in patients with cystic fibrosis after pulmonary colonisation with *Pseudomonas aeruginosa*. *J Pediatr* 1990; 116: 714–719.

7. Pedersen SS, Koch C, Hoiby N, Rosendal K. An epidemic spread of multiresistent *Pseudomonas aeruginosa* in a cystic fibrosis centre. *Antimicrob Chemother* 1986; 17: 505–516.

8. Cheng K, Smyth RL, Govan JR. Spread of β-lactamresistant *Pseudomonas aeruginosa* in a cystic fibrosis clinic. *Lancet* 1996; 348: 639–642.

9. Jones AH, Doberty C, Govan JR, Dodd NE, Wels AK. *Pseudomonas aeruginosa* and cross-infection in an adult fibrosis clinic. *Lancet* 2001; 358: 557–558.

10. McCallum S, Corkill J, Gallagher M, Ledson MJ, Host CA, Walshaw MJ. Cross-infection with a transmissible *Pseudomonas aeruginosa* strain in chronically colonized adult cystic fibrosis patients. *Lancet* 2001; 358: 358–360.

11. Fluge G, Oyeniyi B, Hoiby N, et al. Typing of *Pseudomonas aeruginosa* strains in Norwegian cystic fibrosis patients. *Clin Microbiol Infect* 2001; 7: 238–243.

12. Armstrong D, Nixon C, Carzino R, et al. Detection of a widespread clone of *Pseudomonas aeruginosa* in a paediatric cystic fibrosis clinic. *Am J Respir Crit Care Med* 2002; 166: 983–987.

13. Speert DP, Lawton D, Damm S. Communicability of *Pseudomonas aeruginosa* in a cystic fibrosis summer camp. *J Pediatr* 1982; 101: 227–229.

14. Speert DP, Campbell ME. Hospital epidemiology of *Pseudomonas aeruginosa* from patients with cystic fibrosis. *J Hosp Infect* 1987; 9: 11–21.

15. Tubbs D, Lenney W, Alcock P, Campbell CA, Gray J, Pantin C. *Pseudomonas aeruginosa* in cystic fibrosis: cross-infection and the need for segregation. *Respir Med* 2001; 95: 147–152.

16. Speert DP, Campbell ME, Henry DA, Milner R, Taha F, Gravelle A,. Davidson AGF, Wong LTK, Mahenthiralingam E. Epidemiology of *Pseudomonas aeruginosa* in cystic fibrosis in British Columbia, Canada. *Am J Respir Crit Care Med* 2002; 166: 982–993.

17. Sevens C, Jansen H. The Belgian CF Registry. *Percentile*, 2005;10, nr 4: 98-103.

18. Baele M, Baele P, Vaneechoutte M, Storms V, Butaye P, Devriese LA, Verschraegen G, Gillis M, Haesebrouck F. Application of tRNA Intergenic Spacer PCR for Identification of Enterococcus Species. *J Clin Microbiol* 2000; 38: 4201–4207.

19. Van daele S, Franckx H, Verhelst R, Schelstraete P, Haerynck F, Van Simaey L, Claeys G, Vaneechoutte M, De Baets F. Epidemiology of *Pseudomonas aeruginosa* in a cystic fibrosis rehabilitation centre. *Eur Resp J* 2005; 25: 474-481.

20. Da Silva Filho L, Levi J, Bento C, Rodrigues J, Da Silva Ramos S. Molecular epidemiology of *Pseudomonas aeruginosa* in a cystic fibrosis outpatient clinic. *J Med Microbiol* 2001; 50: 261–267.

21. Jones AM, Dodd ME, Govan JRW, Doherty CJ, Smith CM, Isalska BJ, Webb K. Prospective surveillance for *Pseudomonas aeruginosa* cross-infection at a cystic fibrosis center. *Am J Resp Crit Care Med* 2005; 171: 257-260.

22. Griffith AM, Jamsen K, Carlin**,** Grimwood K, Carzino R, Robinson PJ, Massie J, Armstrong DS. Effects of segregation on an epidemic strain in a cystic fibrosis clinic. *Am J Resp Crit Care Med* 2005; 171: 1020-1025.

23. Edenborough FP, Stone HR, Kelly SJ, Zadik P, Doherty CJ, Govan JRW. Genotyping of *Pseudomonas aeruginosa* in cystic fibrosis suggests need for segregation. *J Cyst Fibr* 2004; 3: 37-44.

24. Scott FW, Pitt TL. Identification and characterization of transmissible *Pseudomonas aeruginosa* strains in cystic fibrosis patients in England and Wales. *J Med Microbiol* 2004; 53: 609-615.

25. Moore E, Goldsmith CE, Elborn SJ, Murphy PG, Gilligan PH, Fanning S, Hogg G Towards "Molecular Esperanto" or the Tower of Babel? (The need for harmonization of techniques for genotyping clinical isolates of *P. aeruginosa* isolated from patients with Cystic Fibrosis) *J Clin Microbiol* 2003, 41;11: 5347-5348

26. Speijer H, Savelkoul PHM, Bonten MJ, Stobberingh EE, Tjhie JH. Application of different genotyping techniques for *P. aeruginosa* in a setting of endemicity in an intensive care unit. *J Clin Microbiol* 1999;37:3654-3661

27. D'Agata EM, Gerrits MM, Tang YW, Samore M. and Kusters JG. Comparison of pulsed-field gel electrophoresis and amplified fragment-length polymorphism for epidemiological investigations of common nosocomial pathogens. *Infect Control Hosp Epidemiol* 2001;22(9):550-554

28. Hoogkamp-Korstanje J, Meis J, Kissing J, Van Der Laag J, Melchers J. Risk of cross-colonization and infection by *Pseudomonas aeruginosa* in a holiday camp for cystic fibrosis

patients. *J Clin Microbiol* 95; 33: 572–575.

29. Mahenthiralingam E, Campbell M, Foster J, Law J, Speert D. Random amplified polymorphic DNA typing of *Pseudomonas aeruginosa* isolates recovered from patients with cystic fibrosis. *J Clin Microbiol* 1996; 34: 1129–1135.

30. Saiman L, Chen Y, Tabibi S, San Gabriel P, Zhou J, Liu Z, Lai L, Whittier S. Identification and antimicrobial susceptibility of *Alcaligenes xylosoxidans* isolated from patients with cystic fibrosis. *J Clin Microbiol* 2001; 39: 3942-3945.

31. Van daele S, Verhelst R, Claeys G, Verschraegen G, Franckx H, Van Simaey L, De Ganck C, De Baets F, Vaneechoutte M. Shared genotypes of *Achromobacter xylosoxidans* strains isolated from patients at a cystic fibrosis rehabilitation center. *J Clin Microbiol* 2005; 43: 2998-3000.

32. Grothues D, Koopmann U, Van der Hardt H, Tümmler B. Genome fingerprinting of *Pseudomonas aeruginosa* indicates colonization of cystic fibrosis siblings with closely related strains. *J Clin Microbiol* 1988; 26: 1973–1977.

33. Renders NHM, Sijmons MAF, van Belkum A, Overbeek SE, Mouton JW, Verbrugh HA . Exchange of *Pseudomonas aeruginosa* strains in cystic fibrosis siblings. *Res Microbiol* 1997; 148: 447-454.

Table 1. *P. aeruginosa* genotypes shared in Belgian CF patients

	Number of patients	Centre							
		A	B	C	D	E	F	G	H
Cluster 1	2		1		1				
Cluster 2	2				1[b]				1
Cluster 3	4			1	1	1		1	
Cluster 4	12				2x2S[c]	6[b]			2
Cluster 5	2					2			
Cluster 6	10	2[b]			2S+1[c]	3			2
Cluster 7	2				1[b]	1			
Cluster 8	5		1		1	3			
Cluster 9	10		2			2x2S+2[c]		2S[c]	
Cluster 10	9		3[b]			2	1	1	2S
Cluster 11	2				1				1
Cluster 12	9	2	1		2S+1[c]	1		2S[c]	
Cluster 13	2				2				

Legend:

[a] Centres A and H form one CF centre on different locations in the same city.

[b] The *P. aeruginosa* strains of one or more of these patients are part of two clusters.

[c] 2S: two siblings or one sibling pair, 2x2S: two sibling pairs, 2S+1: one sibling pair and one unrelated patient.

Fig 1. Flow chart of the study design and of the genotyping results

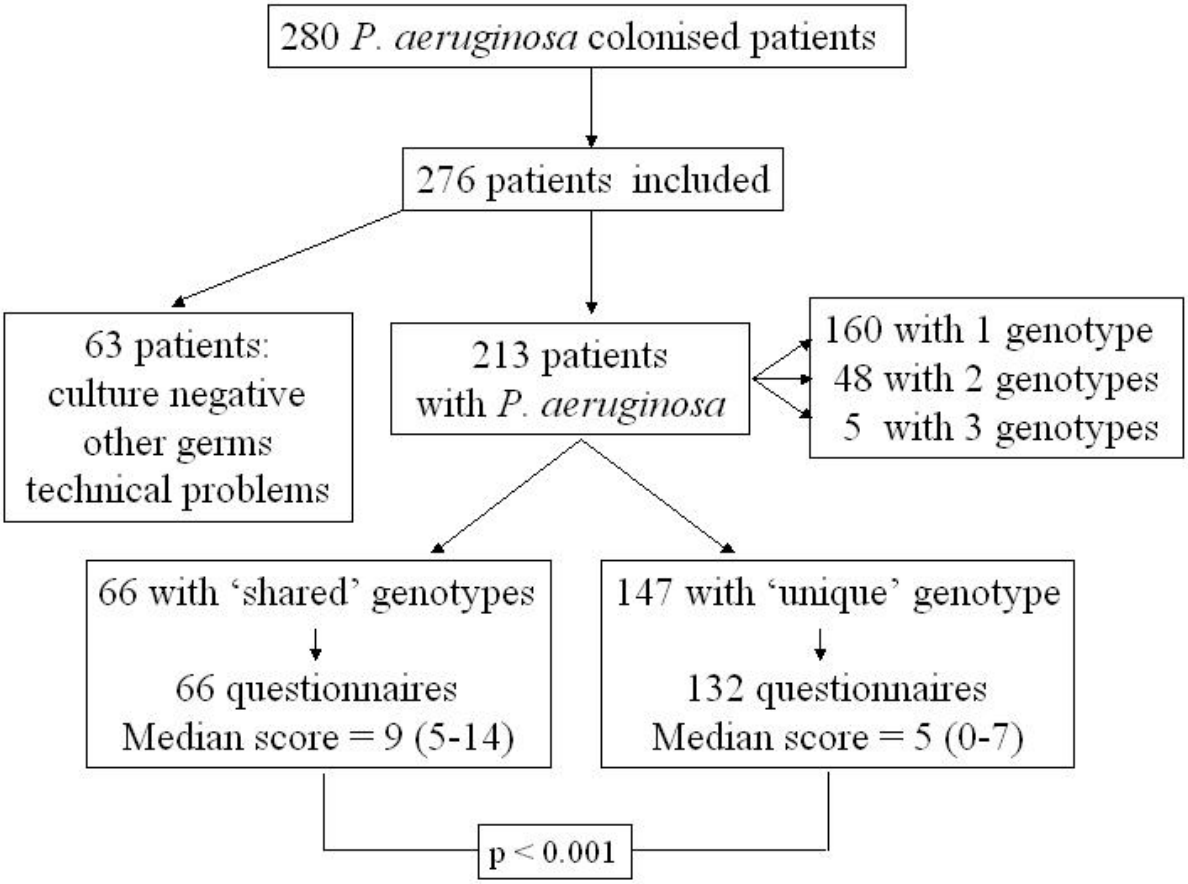


Fig 2. Score of inter patient contacts among patients with a unique *P. aeruginosa* genotype (non-cluster group) compared to that of patients sharing a *P. aeruginosa* genotype with at least one other unrelated patient (cluster group)

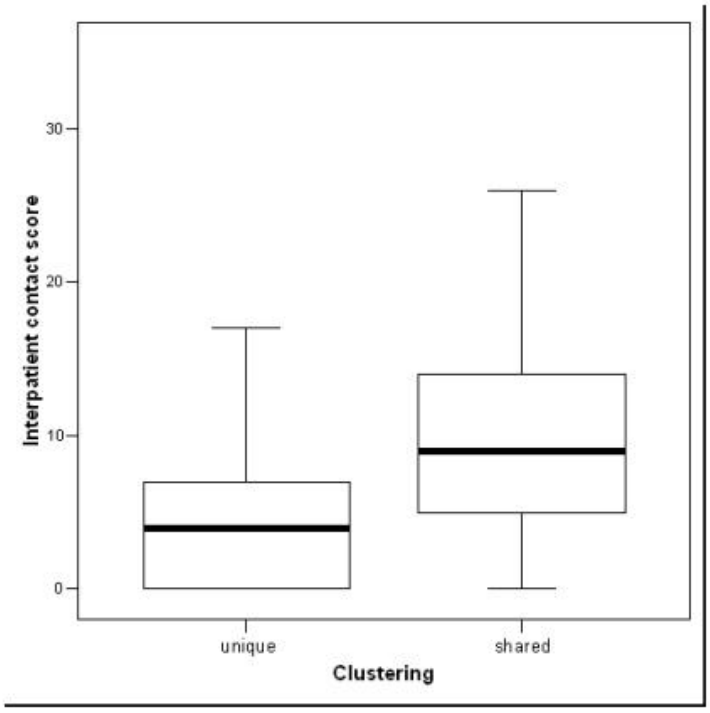
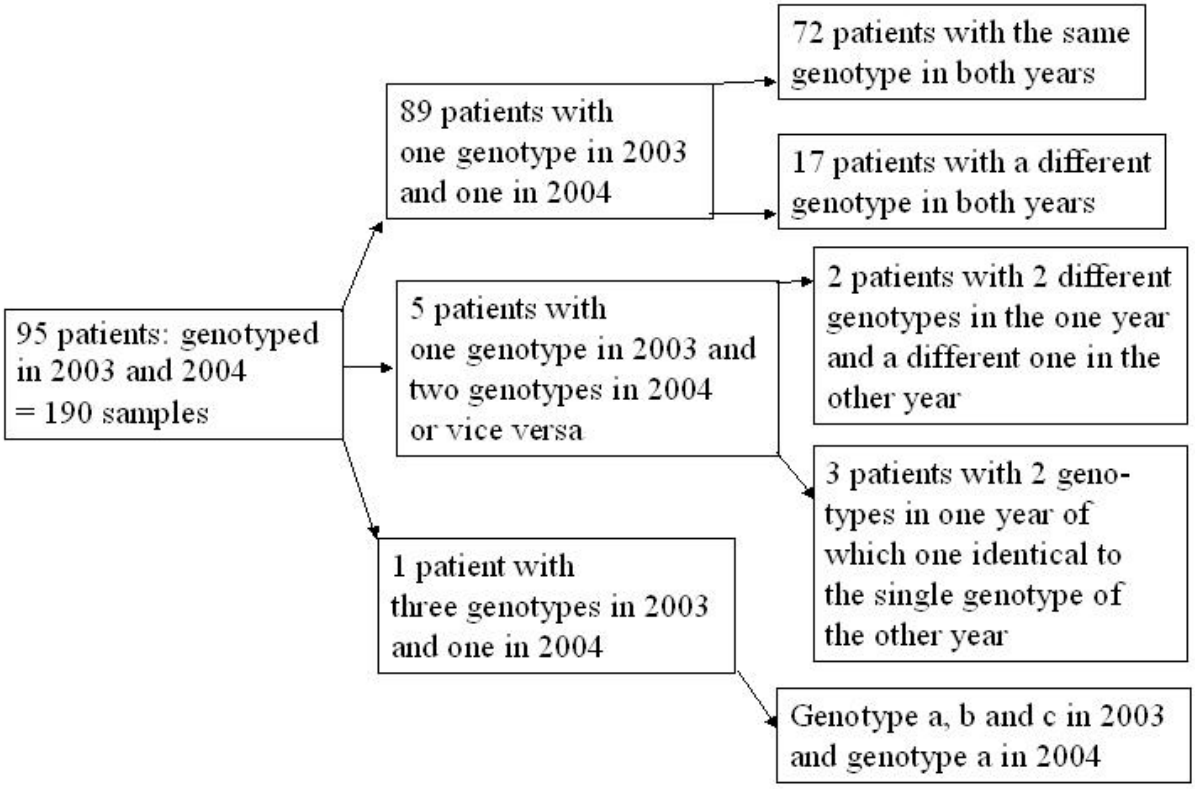


Fig 3. Comparison of genotypes of 95 patients, sampled during both 2003 and 2004


95 patients: genotyped in 2003 and 2004 = 190 samples
89 patients with one genotype in 2003 and one in 2004
72 patients with the same genotype in both years
17 patients with a different genotype in both years
5 patients with one genotype in 2003 and two genotypes in 2004 or vice versa
2 patients with 2 different genotypes in the one year and a different one in the other year
3 patients with 2 genotypes in one year of which one identical to the single genotype of the other year
1 patient with three genotypes in 2003 and one in 2004
Genotype a, b and c in 2003 and genotype a in 2004

Addendum 1

Questionnaire for parents of CF patients younger than 14 years

1. Does your child have contact with other CF patients in the family, other than brothers or sisters (now or in the past)? YES/ NO

   If yes, with whom?……(initials only) YES = score 8

2. Does your child have contact with other CF patients in the classroom (now or in the past)? YES/ NO

   If yes, with whom?……(initials only) YES = score 7

3. Does your child have contact with other CF patients at school (now or in the past)? YES/ NO

   If yes, with whom?……(initials only) YES = score 4

4. Did your child ever stay in a rehabilitation centre? YES/ NO

   If yes, in which rehabilitation centre and for how long?…… YES = score 5

5. Did your child ever attend a CF camp? YES/ NO

   If yes, which camp and for how long?…… YES = score 5

6. Has your child ever shared a hospital room with another CF patient (brothers and sisters excluded)? YES/ NO

   If yes, with whom?……(initials only) YES = score 5

7. Does your child have social contacts with other CF patients (excluding contacts already asked for in previous questions)? YES/ NO

   If yes, with whom?……(initials only) YES = score 4

TOTAL SCORE=

Remark 1: ‘social contacts’ are ‘physical’ contacts (playing, talking etc.), not written or internet contacts!

Remark 2: you are not obliged to answer these questions, if you don’t want to.

Remark 3: this questionnaire is sent to the investigating lab, under closed envelop.

The members of your CF-centre will not be able to look at the answers.

Addendum 2.

Questionnaire for adolescents and adults with CF

1. Do you have contact with other CF patients in the family, other than brothers or sisters (now or in the past)? YES/ NO

   If yes, with whom?……(initials only) YES = score 8

2. Do you have contact with other CF patients in the classroom (now or in the past)? YES/ NO

   If yes, with whom?……(initials only) YES = score 7

3. Do you have contact with other CF patients at school or at work (now or in the past)? YES/ NO

   If yes, with whom?……(initials only) YES = score 4

4. Did you ever had a sexual relationship with another CF patient? YES/NO

   If yes, with whom?……(initials only) YES = score 10

5. Did you ever stay in a rehabilitation centre? YES/ NO

   If yes, in which rehabilitation centre and for how long?…… YES = score 5

6. Did you ever attend a CF camp? YES/ NO

   If yes, which camp and for how long?……..YES = score 5

7. Have you ever shared a hospital room with another CF patient (brothers and sisters excluded)? If yes, with whom?……(initials only) YES/ NO YES = score 5

8. Do you have social contacts with other CF patients (excluding contacts already asked for in previous questions)? YES/ NO

   If yes, with whom?……(initials only) YES = score 4

TOTAL SCORE =

Remark 1: ‘social contacts’ are ‘physical’ contacts (talking, going out together etc.), not written or internet contacts!

Remark 2: you are not obliged to answer these questions, if you don’t want to.

Remark 3: this questionnaire is sent to the investigating lab, under closed envelop.

The members of your CF-centre will not be able to look at the answers.