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Dynamics of respiratory tract long-term colonization of *Haemophilus influenzae* in 30 patients with cystic fibrosis shows a marked increase in hypermutable strains.

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ABSTRACT

The persistence and variability of 188 *Haemophilus influenzae* isolates in respiratory tract of 30 cystic fibrosis (CF) patients during a long period of time (March 1994 to February 2001) was studied. Serotyping, biotyping, antibiotic susceptibility testing, DNA fingerprinting and analysis of outer membrane protein (OMP) profiles were performed in all isolates. A total of 115 distinct pulsed field gel electrophoresis (PFGE) restriction profiles were identified. Ninety per cent of patients were co-colonized with two or more *H. influenzae* clones over the studied period. A third of the patients were cross-colonized with one or two *H. influenzae* strains; 11% of the clones persisted 3 or more months. Biotype, outer membrane protein profiles and resistance profiles showed variation along the studied period, even in persisting clones. Interestingly, 4 isolates (2.1%) recovered from 3 patients were type f capsulate, three of them belonging to the same clone. ß-lactamase production was detected in 23.9% of isolates while 7% of the ß-lactamase negative isolates presented diminished susceptibility to ampicillin (ß-lactamase negative ampicillin resistance phenotype). Remarkably, 21.3% of the *H. influenzae* isolates presented decreased susceptibility to ciprofloxacin, which were mainly observed in persisting clones. Of the *H. influenzae* isolated from CF patients, 18 (14.5%) exhibited a mutator phenotype in comparison with 1 (1.4%) from non-CF patients ($p < 0.0001$). Ten patients (33.3 %) were colonized by hypermutable strains over the study period. Moreover, multiresistance phenotype and long term clonal persistence were significantly associated, in some cases, for up to seven years. Despite of dynamic situation of *H. influenzae* bronchial colonization in CF patients, these results suggest the persistence of better adapted (more resistant?) *H. influenzae* clones.

INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disorder resulting from mutations in a gene on the long arm of chromosome 7 (11)**.** The gene product is the cystic fibrosis transmembrane conductance regulator (CFTR), which regulates and facilitates transport of electrolytes across epithelial cell and other membranes (10).

Haemophilus influenzae is regularly involved in chronic lung infections and acute exacerbations of CF patients (26). To better study the persistence of this bacteria several typing methods have been applied: serotyping, biotyping, outer membrane protein electrophoresis (OMP) and genotyping methods as random PCR (1, 17, 18, 35)**.**

On the other hand, long-term antibiotic therapy is widely used for the treatment of lung infection in CF patients. The risk of development of antibiotic resistance in these is clearly higher than in other patients, and that also applied for *H. influenzae* (17). The first ciprofloxacin-resistant *H. influenzae* strains in Spain were recovered from cystic fibrotic patients, and studied by our groups (4, 9).

The goals of the present study were to carry out a prospective follow up of CF patients over time, to determine: i) whether these patients maintained the same *H. influenzae* strains or were subsequently colonized by different strains; ii) to study the antimicrobial susceptibility profiles in comparison non-CF patients; and iii) to discern if antimicrobial resistance and persistence were associated. Since other studies showed high proportions of hipermutable strains in CF patients carrying *Pseudomonas aeruginosa* (22) and *Staphylococcus aureus* (25), we decided to study if a similar trend may occur in the case of *Haemophilus influenzae* strains.

MATERIALS AND METHODS

Patients. During the period 1994-2001, thirty CF patients (20 males and 10 females) with a history of *Haemophilus influenzae* infections were identified at three different hospitals of Spain (Ramón y Cajal from Madrid, Lozano Blesa from Zaragoza, and Virgen de las Nieves from Granada). The dynamics of *Haemophilus influenzae* colonization in all these patients were studied for a mean period of median 3,4 years (range 1-6.7 years).

Bacterial isolation. Bacterial isolates were recovered from respiratory secretions, most of them expectorated sputum specimens during scheduled assessment or pulmonary exacerbations.. Samples were homogenized with N-acetyl-cysteine and processed by a modified quantitative technique (38). Columbia 5% blood, MacConkey, mannitol-salt, and a selective *Burkholderia cepacia* agar media were incubated on air for 24 h at 37 ºC plus 24 h at 25 ºC. In addition, bacitracin-chocolate agar was plated and incubated on 5 % CO_2 for 48 h. at 37 ºC. A culture was considered positive for *H. influenzae* when growth of this organism was observed, irrespectively of the bacterial count.

Bacterial identification and Biotyping. The isolates were identified as *Haemophilus influenzae* on the basis of the following biological characteristics: gram-negative small rods requiring X and V factors for growth (Mast Diagnostics, Merseyside, U.K). All the strains were serotyped by a co-agglutination test (Phadebact Haemophilus Test, Boule Diagnostics AB, Huddinge, Sweden) including both specific anti-type b antibodies and a pool of specific antitype a, c, d, e, and f antibodies. Discrimination among these serotypes was done with specific a to f antisera obtained in our laboratory, and eventually confirmed by the PCR technique (7). To evaluate whether the initial *H.*

influenzae population isolated from patients could be composed by a mixture of different strains, we selected 9 individual colonies from 11 samples and carried out a complete bacterial identification for each one of them.

The presence of a mutator phenotype was studied in 124 *H. influenzae* isolates comprising the 111 different PFGE patterns (four more were excluded as their rifampin MICs were ≥ 4.0 μg/ml), and all the 13 OMP variants obtained from the sputum of 30 CF patients. As a control group for the susceptibility and hipermutability studies, a collection of 188 epidemiologically unrelated *H. influenzae* isolates and 71 from non-CF patients matched by time of isolation, geographical source and anatomical origin were also studied. Biotyping was performed according to the method described by Kilian (12).

Outer membrane proteins profiles. An overnight culture of *Haemophilus influenzae* was collected in 10 ml of 10 mM HEPES buffer (pH 7.5), and the suspension was subjected to sonication. Cellular debris was removed by centrifugation at 26,450 x g for 3 min (3000 g for 10 min). The supernatant was then removed and centrifuged at 26,450 x g for 90 min at 4 ºC. The pellet containing the cell membrane material was re-suspended in HEPES buffer and sodium lauryl sarcosylate (2% in HEPES buffer) for 10 min. Each preparation was then centrifuged at 26,450 x g for 60 min at 4ºC. The resulting pellet was diluted in distilled water to a concentration of 1,400 μg of protein per ml. Solutions were then stored at –70 ºC until all samples were processed. Twenty-five microliters of each membrane suspension were added to 50 μl of sample buffer containing 50 % distilled water, 12.6 % 0.5 M Tris-HCl (pH 6.8), 10.5 % glycerol, 21 % sodium dodecyl sulfate (SDS) (10 % solution), and 5% bromophenol blue (0.1% solution). The final solution was boiled for 5 min. Samples were loaded onto SDS-

polyacrylamide (4% stacking gel and 10 % separating gel), prepared, and run according to the method of Laemmli (13).

Pulsed-field gel electrophoresis (PFGE). Total DNA was prepared and PFGE was performed as described previously (14). The restriction endonucleases *Sma* I and Bsp 120 I (Apa I) (MBI, Fermentas, Vilnius, Lithuania) were used at the manufacturer´s suggested temperature. Restriction fragments were separated by PFGE in agarose 1% gel in 0.5 X TBE buffer (45 mM Tris, 45 mM boric acid, 1.0 mM EDTA, pH 8.0) with the BioRad CHEF Mapper apparatus, Hercules, California, U.S.A.). The initial pulse time of 5 s was increased linearly to 50 s over 23.3 h at 6 V/cm at 13 ºC. λ ladders were applied as molecular size markers, size 48.5-1000 kb (PFGE Marker I- Boehringer-Mannheim, Germany). Gels were then stained with ethidium bromide and photographed under ultraviolet light. Fragment patterns were compared visually and interpreted by the software (MVSP Shareware). Similarity analysis and dendrograms were done with this database system on the basis of molecular mass patterns.

Antimicrobial susceptibility testing. For antibiotic susceptibility screening purposes, the disk diffusion susceptibility test for *H. influenzae* was performed on standard Haemophilus test medium to the following antibiotics: ampicillin, amoxicillin-clavulanic acid, cefaclor, cefixime, cefuroxime, cefotaxime, sulphamethoxazole-trimethoprim, chloramphenicol, tetracycline, kanamycin, rifampin, ciprofloxacin and nalidixic acid. The isolates were classified as susceptible, intermediate or resistant according to the criteria of the NCCLS (20). Minimal inhibitory concentration (MIC) was determined by the Epsilon-test (E-test) method including the following antibiotics: ampicillin, amoxicillin-clavulanic, cefotaxime, sulphametoxazole-trimethoprim, chloramphenicol, ciprofloxacin and nalidixic acid. The control strains were *H*

influenzae ATCC numbers: 49247 and 51907. ß-lactamase activity was studied by the chromogenic cephalosporin test with nitrocephin as the substrate.

Determination of mutation frequencies. To determine mutation frequencies, one bacterial colony was resuspended in 20 ml of Brain-Heart Infusion Broth (BHI) and grown at 37ºC with 5% CO_2 overnight. Bacterial cells were then collected at 3,000 rpm for 5 min and re-suspended in 1 ml of BHI. A 100 μl sample from this suspension as well as from successive dilutions was plated onto Haemophilus-Test-Medium (HTM) with and without rifampin (10 μg/ml). After 48 h of incubation, the number of colonies was counted, and the mutation frequencies were determined as its relative proportion to the total count of viable organisms plated. For every strain yielding > 30 colonies on antibiotic containing medium, the experiment was repeated in triplicate, and results were indicated as a mean. A strain was considered to have a mutator phenotype when the mutation frequency was higher than 10^{-7}, i.e., at least ten times higher than the average mutation frequency found for *H. influenzae* strains in non-cystic fibrosis patients (22, 25).

Analysis of the results. Management of data and statistical calculations were carried out using the Whonet Programme (WHO/CSR/DRS/99.1, World Health Organization). Mean mutation frequencies were compared by the Mann-Whitney Test; categorical variables were compared by Fisher's exact test. *p* values were considered significant at $p \leq 0.5$

RESULTS

Frequency and Density of *Haemophilus influenzae* colonization. 794 sputum specimens from 30 CF patients were analysed (median 26,5, range 4-82 per patient). The median follow-up was 40,8 months (range 12 - 80). In total, 188 *H. influenzae* strains were obtained (mean number of isolates per patient 6,3, range 1-27) from the 30 patients studied (Table 1). In the group of eleven clinical patients (see Materials and Methods) in which nine independent *H. influenzae* colonies were studied, only one clonal type was identified per patient, suggesting that co-colonization is a rare event in our series. The quantitative study in the sputa showed a CFU / ml average of 10^8 (range, 10^3 -10^9). The cross-colonising clones persisting for more that 3 months did not reached higher cell densities in the sputa than the total average (7.7×10^7 and 9.1×10^7, respectively).

Serotypes and Biotypes. Four (2.1%) of the 188 isolates were serotype f, three of them belonging to the same clone, despite being isolated from three different patients. The remaining 184 strains, from 27 patients, were nontypable. The most frequent biotype was biotype II (30.3%) followed by biotypes I (27.7%), III (17%), V(10.6%), VI (6.4%), VII (3.7%), VIII (2.7%) and IV (1.6%). Removing duplicate strains (considering only the first isolate of each clone) the most frequent biotype was II (36.5%), followed by the biotypes III (25.2%), I (21.7%), V (6.1%), VI (3.5%), VII (3.5%), IV (1.7%) and VIII (1.7%). Six (5,2%) out of the 115 *H. influenzae* clones obtained as defined by the PFGE profile (see below) showed biotype changes in successive isolations in the same patient.

PFGE-typing profiles. On the basis of the PFGE patterns, using *Sma* I as restriction enzyme, we obtained 112 different profiles from 188 isolates; three strains were non-

digested with *Sma* I but they were digested with *Bsp* 120 I . The same number was assigned to identical restriction patterns obtained from different strains. PFGE patterns with coefficients of similarity greater that 85% were considered to define to a particular clone. For instance, PFGE patterns 1A and 1 B (Table 1) were considered the same clone 1 according to the Tenover criteria (31). We also tested the isolates with other restriction enzyme (*Bsp* 120 I), obtaining the same clonal distribution (Fig. 1, 2, 3). In some cases, strains with the same PFGE profile had a variation in their OMP profile; hence, we designed them with lower case letters next to the numbers of the PFGE pattern (Table 1 and Fig. 5).

Persistence

The 115 *H. influenzae* PFGE patterns persisted in average 2.5 months (range 1-80). Thirteen PFGE patterns (11.3%) persisted for 3 or more months in their respective patients. In several patients, *H. influenzae* strains with indistinguishable PFGE patterns persisted during a long period of time (Table 1). Figures 1,2 and 3 show the colonization course in three selected patients: 1, 3 and 28. In 24 patients (80%), the first strain of *H. influenzae* was isolated only once, being replaced by other strains in subsequent cultures. In 27 patients (90%), two or more different strains were isolated over the period of study.

Thirteen (11.4%) of the 114 clones (115 PFGE patterns) suffered OMP variations (Table 1 and Fig. 4); the greatest number of OMP variants was found in patient 3. In patients 14, 15 and 16, with long follow-up periods (33 to 50 months), only one *H. influenzae* strain was isolated (Table 1).

Cross-colonizing strains.

Five clones were isolated from more than one single patient: Clone 19 was isolated from patients 6 and 7 the same month (Table 1), persisting one month in the last patient. Clone 39 colonized patients 8 and 14. Clone 41 was found in patients 8, 9 and 12. Clone 52 was obtained from patients 13, 16 and 20. Clone 70 (a type f isolate) colonized patient 20 during one month and 2.5 years later appeared in patient 21 (table 1). In total 10 patients were cross-colonized with one or two strains from other patients. However, strains from patients sharing identical clones did not always present the same biotype: the biotype of clone 19 was IV in patient 6 but was II in patient 7; the biotype of clone 52 was II in patient 12 but was VIII in patient 16.

Antibiotic susceptibility. Among the 188 *H. influenzae* strains, diminished ampicillin susceptibility was found in 29.2 %; 23, 9% produced ß-lactamase and 5.3 % presented diminished susceptibility (MIC ≥2 μg/ml) to ampicillin (β-lactamase negative ampicillin resistance (BLNAR)-phenotype). Considering only the first isolate of each one of the different 115 clones, the corresponding figures are 29.6 % for ampicillin reduced susceptibility, 26.1 % for beta-lactamase production and 3.5 for BLNAR (Table 2). All isolates were susceptible to cefotaxime, cefixime, amoxycillin-clavulanate. Only one strain was beta-lactamase-positive amoxicillin/clavulanate-resistant (BLPACR)

Regarding the ciprofloxacin susceptibility, 40 (21.3%) of the 188 *H. influenzae* isolates had MICs >1 μg/ml in comparison with 9 (7.8%) from 115 first isolates of each PFGE pattern ($p = 0.002$).

According to NCCLS breakpoints (20), chloramphenicol and amoxicillin-clavulanate exhibited susceptibility rates equal to or higher than 85%. The antibiotics with the lowest intrinsic activity (as measured by MIC_{90}) were trimethoprim-sulfamethoxazole and ampicillin, both displaying an $MIC_{90} \geq 32$ μg/ml.

Eleven (36.7 %) patients were colonized by multiple resistant *H. influenzae* clones (defined as resistance or diminished susceptibility to three or more antimicrobials of different antibiotic classes). The most common combinations of multidrug resistance were: ampicillin, cefaclor, chloramphenicol, ciprofloxacin and cotrimoxazol. Only three patients (5,9 and 10, Table 1) were colonized by totally antibiotic susceptible strains.

Antibiotic resistance and *H. influenzae* persistence. Of the 13 PFGE patterns (11.3 %, 12 clones) that persisted for at least three months, three did not change its antimicrobial susceptibility over time; three acquired additional antibiotic resistance markers, three lost at least one resistance, and four suffered apparent changes in sensitivity. The most frequently acquired resistance determinants were tetracycline in six PFGE patterns, and kanamycin in three PFGE patterns. Of the 12 clones that persisted for more than three months, 4 had diminished susceptibility to two or more antibiotics and persisted for 1,545 days (51.5 months; range 15-80) on average, compared with a period of 291 days (9.7 months; 3-26) for the 8 more susceptible persistent clones ($p = 0.0001$, Fisher´s exact test).

Twenty-three patients (76.7 %) yielded at least three consecutive *H. influenzae* strains, irrespectively of their clonality. In four (13.3 %) patients no changes were observed in the antibiotic susceptibility patterns; in six patients (20 %) the isolates increased their antibiotic resistance markers; in five (16.7 %) the isolates diminished its antibiotic resistance. In three (10 %) the strains initially increased its antibiotic resistance but it decreased further on; in two (6.7 %) the strains initially lost resistance determinants but they increased afterwards; and in three (10 %) there was a continuous fluctuation in their antibiotic susceptibility. Older patients (patient 1, 37 years; patient 2, 18 years and patient 28, 20 years at the beginning of the study) were colonized with

more strains resistant to different antibiotics such as ciprofloxacin (nalidixic acid), cotrimoxazol, chloramphenicol, ampicillin and cefaclor.

Antibiotic susceptibility of CF patients strains versus non-CF patients control strains. We observed a significantly higher frequency of resistance to ampicillin (55/188, 29.2%) and decreased susceptibility to ciprofloxacin (40/188, 21.2%) among cystic fibrosis patients than among the patients of the control group (39/188, 20.7% and 0% respectively) (Table 3). Similarly, a significant higher proportion of BLNAR (10/188, 5.3%) and resistance to two or more antibiotics (62/188, 32.9%) were found in the strains from the CF group in comparison with the non-CF control strains (20/188, 10.6%) (Table 3).

Frequency of hypermutation. Of the *H. influenzae* isolated from CF patients, 18 (14.5%) exhibited a mutator phenotype, compared with one (1.4%) from non-CF patients ($p = 0.002$). 10 patients (33.3 %) were colonized by hypermutable strains over the study period. The mutation frequency distributions are shown in Fig. 5. The mean mutation frequency in the two groups (3.4×10^{-7} for 124 strains in the CF group and $1,22 \times 10^{-8}$ in 71 strains in the non-CF group) significantly differed ($p<0.0001$, Mann-Whitney U test). Two groups of isolates from CF patients were distinguished: a group of non-mutators, with a mean mutation frequency of $2.4 \pm 2.5 \times 10^{-8}$, and a group of mutators, with a mean mutation frequency of $2.2 \pm 4.9 \times 10^{-6}$ (Fig. 5). The mutation frequency of the only mutator strain in non-CF patients was $1,9 \times 10^{-7}$; the remaining strains had a low mutation frequency (mean: $9.7 \times 10^{-9} \pm 1.4 \times 10^{-8}$) (Fig. 5).

Of the 18 hypermutable strains, 16 presented resistance to one of more antibiotics (seven were multiple-antibiotic resistant, one was resistant to ampicillin and co-

trimoxazol, five were resistant to co-trimoxazol, two to tetracycline, one to ampicillin) and two were fully antibiotic-susceptible.

DISCUSSION

Several studies have previously investigated the epidemiology of long term *Pseudomonas aeruginosa* colonization in the respiratory tract of patients with CF, but there are a limited number of studies dealing with *H. influenzae* (17, 18, 26). We describe here the dynamics of *H. influenzae* colonization in 30 CF patients over a long period of time (median 40.8 months, range 12-80 months), a longer time than in the only previous study (18). Sequential isolates were obtained in 27 patients. Only in 20 % of the patients the same clone was repeatedly recovered during the time of observation. This proportion was identical using PFGE (this study) or RAPD-typing (18).

As other authors (17, 37), we observed that all biotypes are recognized among *H. influenzae* isolates from CF patients. In our series, the order of biotype isolation by frequency (considering first isolate of each PFGE clones) was II (36%) III (25%), I (21%) followed by V, VI, VII and VIII (in total 17 %). These proportions are almost identical to those found in the Netherlands, with the exception of biotype VIII, hyper-represented in the Möller series (17). As these authors, we have also observed biotype fluctuations among persistent and cross-colonizing clones. No apparent association between biotypes and outer membrane protein profiles was found, in accordance with

previous observations (2). Nevertheless, as biotypes, variations over time were observed in persisting clones (Table 1), corroborating the low power as typing marker of this technique (34).

In our study, only three patients kept all their strains totally susceptible to the antimicrobials studied. We have observed greater antibiotic susceptibility figures among the 115 first isolates of each PFGE pattern (114 clones) than in the total 188 *H. influenzae* isolates, suggesting that persistence increases the levels of resistance. The usual ciprofloxacin MIC of *H. influenzae* is ≤ 0,03 μg/ml (23). We have shown that *H. influenzae* strains with MIC ≥ 0.12 μg/ml present target mutations (9). It is noteworthy that the number of strains with a ciprofloxacin MIC > 1.0 μg/ml in our study increased from 7.8% in the 115 first isolates to 21.3% in all 188 isolates. The two most persistent clones (clone 1 from patient 1, which persisted 80 months and clone 101 from patient 28, which persisted 72 months) presented a high MIC for ciprofloxacin (4).

The large proportion of strains with diminished susceptibility (MIC > 1,0 μg/ml) to ciprofloxacin (21.3 %) in our study is noteworthy, and the highest reported to date in the literature for *H. influenzae* from any geographical or clinical location. Note that the overall frequency of ciprofloxacin resistance in *H. influenzae* in Spain do not exceed 0.1 % (16).

Interestingly, the rate of ampicillin-resistance in our CF *H. influenzae* isolates, although higher than in the Netherlands (Möller 1998), was almost identical (29.2 %) to the average frequency of ampicillin-resistance in Spain (30.1 %) (16). BLNAR isolates from CF were more abundant among persistent strains (5.3%) than among the first isolated strains of each clone (3.5%).

In general, this study suggests that CF *H. influenzae* strains are much more antibiotic-resistant than strains from non-CF patients. Resistance to two or more

antimicrobials occurred in 62 (32.9%) of all *H. influenzae* isolates (Table 3) and 11 (36.7%) of patients; analogous observations have made for other bacterial pathogens, such as *P. Aeruginosa, S. Maltophilia,* and *S. Aureus* (22, 25, 32). The increased resistance rates in these patients have been associated with high and persistent consumption of antimicrobial agents (15). However, the effect of high rates of consumption on the frequency of antibiotic resistance is expected to be significantly amplified in the presence of hypermutable (mutator) bacteria.

Our measurements of mutation frequency corroborated in *H. influenzae* the tendency of a significant proportion (14.5%) of hypermutable strains previously found in CF patients with *Pseudomonas* (19.5%) (22) and *Staphylococcus aureus* (14.6%). It has been shown that hypermutation strongly correlates with antibiotic resistance (22), and this probably also occurs with *H. influenzae*. It is to note that the mechanisms of resistance derived from mutational events in *H. influenzae* (such as ciprofloxacin-resistance and co-trimoxazol) are more influenced by hypermutation than those mediated by horizontal gene transfer (such as beta-lactamases). We cannot rule out the possibility that hypermutation may originate other selective advantages related to the adaptation and survival in the local conditions of the CF lung.

Mutation frequency (Fig. 5) was less polarized in *H. influenzae* than in *Pseudomonas* and similar to the *Staphylococcus*, with a wide range of distribution.

Van Schilfgaarde et al (36) suggested that penetration of *H. influenzae* between epithelial cells *in vivo* might contribute to the persistence of this microorganism in CF patients in the presence of antibiotics and antibodies, despite the fact that the isolated bacteria are fully susceptible to these antibacterial activities *in vitro*. Besides of this, it does appear that the prolonged microbial colonization / infection that is characteristic of the CF lung results from defects in the innate or non-specific immune system (10).

H. influenzae cross-infection with five clones among ten different patients was observed. This could be due to close contact among these patients, and/or that these clones have an special ability to invade the respiratory tract. One of these five clones was serotype F. Non-encapsulated *H. infuenzae* isolates are almost always from CF patients, although encapsulate serotype b may be occasionally isolated, most often from CF children less than 5 years of age. In our study, with the exception of four type f isolates all *H. influenzae* were nontypeable. To the best of our knowledge, this is one of the first CF studies to isolate capsulated type f strains. In other research of our laboratory (3), with different type of patients, we found 18 strains of serotype f in a total of 38 respiratory samples from 1996 to 2000, suggesting that serotype f strains often colonize the upper respiratory tract.

In summary, using PFGE typing we characterized 115 genotypes among 188 isolates of *H. influenzae,* in 30 CF patients over a long follow up ranging from one to seven years. Although persistence of the same clone was only observed in nearly 12% of patients, chronic colonization with multiple *H. influenzae* clones was observed in the majority of patients. Interestingly, antimicrobial resistance was associated with strains persistence over long periods of time.

REFERENCES

1. **Barenkamp, S. J., R. S. Jr. Munson, and D. M. Granoff.** 1981. Subtyping isolates of *Haemophilus influenzae* type b by outer membrane protein profiles. J Infect Dis **143**:668-76.
2. **Bilton, D., A. Pye, M. M. Johnson, J. L. Mitchell, M. Dodd, A. K. Webb, and Stockley R. A., and L. Hill**. 1995. The isolation and characterization of non-typeable *Haemophilus influenzae* from the sputum of adult cystic fibrosis patients. Eur Respir J **8(6)**:948-53.
3. **Campos J, F. Román, M. Pérez-Vázquez, , B. Aracil, J. Oteo, and J. E. Cercenado**. 2003. Antibiotic resistance and clinical significance of *H. influenzae* type F. J. Antimicrob. Chemother. (In press).
4. **Campos, J., F. Román, M. Georgiou, C García, R. Gómez-Lus, R. Cantón, and H. Escobar, F. Baquero**. 1996. Long-term persistent of ciprofloxacin-resistant *Haemophilus influenzae* in patients with cystic fibrosis. J. Infect. Dis. **174:**1345-7.
5. **Cerquetti M., M. L. Ciofi degli Atti, G. Renna, A. E. Tozzi, M. L. Garlaschi, and P. Mastrantonio**. 2000. Characterization of non-type B *Haemophilus influenzae* strains isolated from patients with invasive disease. J Clin Microbiol. **38** (12):4649-52.
6. **Doern G., J. H. Gerlach, P. R. Jorgensen, C. Murray, Thornsberry, and J. A. Jr.Washington.** 1991. Quality control limits for disk diffusion and broth

microdilution susceptibility tests with Haemophilus test medium. Diagn. Microbiol. Infect. Dis. **14**:485-493.

7. **Falla, T. J., D. W. Crook, L. N. Brophy, D. Maskell, J. S. Kroll, and E. R. Moxon**. 1994. PCR for capsular typing of *Haemophilus influenzae*. J. Clin. Microbiol. **32(10)**:2382-6.

8. **Gazagne L., C. Delmas, E. Bingen, and H. Dabernat**. 1998. Molecular epidemiology of ampicillin-resistant non-beta-lactamase-producing *H. influenzae*. J Clin Microbiol. 36(12):3629-3635.

9. **Georgiou, M., R. Muñoz, F. Román, R. Cantón, R. Gómez-Lus, J. Campos, and A. G. de la Campa**. 1996. Ciprofloxacin-resistant *Haemophilus influenzae* strains possess mutations in analogous positions of Gyr A y Par C. Antimicrob. Agents Chemother. **40**:1741-4.

10. **Hart C. A., and C. Winstanley. 2002**. Persistent and aggressive bacteria in the lungs of cystic fibrosis children. Br Med Bull. **61**:81-96.

11. **Kerem B., J. Rommens, J. Buchanan, D. Markewicz, T. Cox, A. Chakranarti, M. Buchwald and L. Tsui**. 1989. Identification of the cystic fibrosis gene: genetics analysis. Science. **245**:1873-80.

12. **Kilian M.** 1976. A taxonomic study of the genus *Haemophilus*, with the proposal of a new species. J Gen Microbiol . **93**:9-62.

13. **Laemmli U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**:680-685.

14. **Lee J. J., and H. O. Smith** . 1998. Sizing of the *Haemophilus influenzae* Rd genome by pulsed-field agarose gel electrophoresis. J. Bacteriol. **170**:4402-4405.

15. **Lyczak J.B., C. L. Cannon, and G. B. Pier**. 2002. Lung infections associated with cystic fibrosis. Clin Microbiol Rev. **15**:194-222.

16. **Marco, F., J. García de Lomas, C. García-Rey, E. Bouza, L. Aguilar, C. Fernández-Mazarrasa, and The Spanish Surveillance Group for Respiratory Pathogens**. 2001. Antimicrobial susceptibilities of 1,730 *Haemophilus influenzae* respiratory tract isolates in Spain in 1998-1999. Antimicrob Agents Chemother. **45(11)**:3226-28.

17. **Möller, L. V., H. Grasselier, J. Dankert, and L. van Alphen**. 1996. Variation in metabolic enzyme activity of persistent *Haemophilus influenzae* in respiratory tracts of patients with cystic fibrosis. J. Clin. Microbiol. 1926-29.

18. **Moller, L. V., A. G. Regelink, H. Grasselier, J. E. Dankert-Roelse, J. Dankert, and L. van Alphen** . 1995. Multiple *Haemophilus influenzae* strains and strain variants coexist in the respiratory tract of patients with cystic fibrosis. J. Infect. Dis. **172(5):**1388-92.

19. **Moller, L. V., A. G. Regelink, H. Grasselier, L. van Alphen, and J. Dankert.** 1998. Antimicrobial susceptibility of *Haemophilus influenzae* in the respiratory tracts of patients with cystic fibrosis. Antimicrob Agents Chemother. **42**:319-24.

20. **National Committee for Clinical Laboratory Standards**. 2002. Performance Standards for Antimicrobial Susceptibility Testing; Twelfth Informational Supplement. Vol 22 No 1. M2-A7 and M7-A5. Wayne, Pennsylvania.

21. **O´Callaghan C. H., A. Morris, S. M. Kirby, and Shingler. 1972**. Novel method for detection of B-lactamases by using a chromogenic cephalosporin substrate. Antimicrob Agents Chemother. **1**:283-288.

22. **Oliver, A, R. Cantón, P. Campo, F. Baquero, and J. Blázquez**. 2000. High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. Science **288**:1251-4.

23. **Pérez-Vázquez M., F. Román, M.C. Varela, R. Cantón and J. Campos**. 2003. Activities of 13 quinolones by three susceptibility testing methods against a collection of *Haemophilus influenzae* isolates with different levels of susceptibility to ciprofloxacin: evidence for cross-resistance. J. Antimicrob. Chemother. **51(1)**:147-51.

24. **Pfaller, M. A., C. Wendt, R. J. Hollis, R. P. Wenzel, S. J. Fristschel, J. J. Neubauer, and L. A. Herwaldt**. 1996. Comparative evaluation of an automated ribotyping system versus pulsed-field gel electrophoresis for epidemiological typing of clinical isolates of *Escherichia coli* and *Pseudomonas aeruginosa* from patients with gram-negative bacteremia. Diagn. Microbiol. Infect. Dis. **25**:1-8.(17).

25. **Prunier A. L., B. Malbruny, M. Laurans, J. Brouard, J. F. Duhamel, and R. Leclerq**. 2003. High rate of macrolide resistance in *Staphylococcus aureus* strains from patients with cystic fibrosis reveals high proportions of hypermutable strains. 2003. J. Infect. Dis. **187**:1709-16.

26. **Rajan S. and L. Saiman**. 2002. Pulmonary infections in patients with cystic fibrosis. Semin. Respir. Infect. 17 (1):47-56.

27. **Regelink, A. G., D. Dahan , L. V. Moller , J. W. Coulton , P. Eijk , P. Van Ulsen , J. Dankert , and L. Van Alphen.** 1999. Variation in the composition and pore function of major outer membrane pore protein P2 of *Haemophilus influenzae* from cystic fibrosis patients. Antimicrob. Agents Chemother. **43(2)**:226-32.

28. **Renders, N., L. Licciardello, C. Ijsseldijk, M. Sijmons, L. Van Alphen, H. Verbrugh and A. van Belkum.** 1999. Variable numbers of tandem repeat loci in genetically homogeneous *Haemophilus influenzae* strains alter during persistent colonization of cystic fibrosis patients. FEMS Microbiol Lett **173 (1)**:95-102.

29. **Schmid, J., E. Voss, and D. R. Soll.** 1990. Computer-assisted methods for assessing strain relatedness in *Candida albicans* by fingerprinting with the moderately repetitive sequence Ca3. J Clin Microbiol. **28(6):**1236-1243.

30. **Sorensen B., E. S. Falk, E. Wisloff-Nilsen, B. E. Bjorvatn, and B. E. Kristiansen**. 1985. Multivariate analysis of *Neisseria* DNA restriction endonuclease patterns. J. Gen. Microbiol. **131**:3099-3104.

31. **Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan**. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis criteria for bacterial strain typing. J. Clin. Microbiol. **33**:2233-2239.

32. **Valdezate, S., A. Vindel, A. Echeita, F. Baquero and R. Cantón**. 2002. Topoisomerase II and IV quinolone resístanse-determining regions in *Stenotrophomonas maltophilia* clinical isolates with different levels of quinolone susceptibility. Antimicrob Agents Chemother. **46(3):**665-71.

33. **Valdezate, S., A. Vindel, L. Maiz, F. Baquero, H. Escobar, and R. Cantón**. 2001. Persistence and variability of Stenotrophomonas maltophilia in cystic fibrosis patients, Madrid, 1991-1998. Emerging Infectious Diseases. **7**:113-122.

34. **Van Alphen L. 1992**. Epidemiology and prevention of respiratory tract infections due to nonencapsulated *Haemphilus influenzae*. J Infect Dis. **165 Suppl 1**:S177-80.

35. **Van Belkum A., B. Duim, A. Regelink, L. Moller, W. Quint, and L. Van Alphen**. 1994. Genomic DNA fingerprinting of clinical *Haemophilus influenzae* isolates by polymerase chain reaction amplification: comparison with major outer-membrane protein and restriction fragment length polymorphism analysis. J. Med. Microbiol. **41**(1):63-8.

36. **Van Schilfgaarde M., P. Eijk, A. Regelink, P. van Ulsen, V. Everts, J. Dankert, and L. van Alphen**. 1999. Haemophilus influenzae localized in epithelial cell layers is shielded from antibiotics and antibodies and antibody-mediated bactericidal activity. Microbial Pathogenesis **26**:249-262.

37. **Watson K. C, E. J. C. Kerr, and M. Baillie.** 1988. Temporal changes in biotypes of *Haemophilus influenzae* isolated from patients with cystic fibrosis. J. Med. Microbiol. **26**: 129-32.

38. **Wong K., M. C. Roberts, L. Owens, M. Fife, and A. L. Smith**. 1984. Selective media for the quantification of bacteria in cystic fibrosis sputum. J. Med. Microbiol.**17**:113-119.

TABLE 1 Results of typing of 188 *Haemophilus influenzae* strains isolated from 30 patients with cystic fibrosis at three different hospitals of Spain

Patient number	Sex	Patient age when isolates (years/months)	Sampling period (mo)	*H. influenzae* number of strains (*)	PFGE pattern	Strain distribution according to PFGE pattern	Persistence of PFGE pattern (mo)	OMP variants	Biotypes
1	M	37yr / 10mo	80 mo	15	1A/1B/2	10/4/1	80/15/0	1Aa,1Ab,1Ac	VI(6), VIII(2), VII(2)-VI-VI
2	F	18yr / 11mo	39 mo	3	3/4	2/1	39/0		VII-V
3	F	12yr / 4mo	41 mo	18	5/6/7/8	14/2/1/1	26/10/0/0	5a(5), 5b, 5c(4)/6a	V-III-II-III
4	M	4yr / 3mo	58 mo	7	9/10/11/12/13	1/2/1/1/2	0/3/0/0/2		II-I-I-II-I
5	M	15yr / 9mo	60 mo	3	14/15	2/1	1/0		II-III
6	F	3yr / 10mo	61 mo	15	16/NT/17/18/**19**/20/21/22/23/24/25/26	2/1/1/1/1/1/1/1/1/1/3	3/0/0/0/0/0/0/0/0/0/8		II-VII-II-I-IV-III-II-III-I-III-III-I
7	F	4 yr / 11mo	77 mo	15	27/28/**19**/29/30/31/32/33/34/35/36/37/38	2/1/2/1/1/1/1/1/1/1/1	<1/0/1/0/0/0/0/0/0/0/0/0/0/0	**19**a	I-I-II-II-V-III-III-II-II-III-II-II-II
8	M	11yr / 2mo	41 mo	3	**39**/40/**41**	1/1/1			II-III-II
9	M	21yr / 5mo	12 mo	3	**41**/42/43	1/1/1			III-III-II
10	F	16yr / 8mo	49 mo	2	44/45	1/1			I-II
11	M	11yr / 9mo	48 mo	3	46/47/48	1/1/1			II-II-I
12	M	1yr / 6mo	37 mo	6	49/**41**a/50/51/**52**/53	1/1/1/1/1/1		**41** a	VIII-II-VII-III-II-II
13	M	5yr / 9mo	51 mo	5	**52** a/54/55/56	1/2/1/1	0/1/0/0	**52** a	II-III-II-II
14	F	8yr / 11mo	33 mo	1	**39**	1			II
15	M	18yr / 1mo	50 mo	1	57	1			I
16	F	6yr / 7mo	41 mo	1	**52**	1			VIII
17	F	5yr / 10mo	48 mo	7	58/59/60/61/62	2/1/1/1/2	9/0/0/0/6		II-V-I-III-II
18	M	4yr / 10mo	40 mo	4	63/64/NT/65	1/1/1/1			I-II-III-I
19	M	22yr / 6mo	28 mo	2	66/67	1/1			IV-III
20	F	3yr / 8mo	38 mo	9	68/69/**70**/71/72/73	1/2/2/1/2/1	0/11/1/0/2/0	72a	III-II-I-V-III-II
21	M	1yr / 2mo	47 mo	7	74/75/76/77/78/79/**70**	1/1/1/1/1/1/1			I-III-V-III-II-II-I
22	F	5yr / 8mo	16 mo	5	80/81/82/83	1/1/1/2	0/0/0/2	83a	II-II-II-VII, II
23	M	2yr / 3mo	40 mo	7	84/85/86/87/88/89	1/2/1/1/1/1	0/2/0/0/0/0		III-II-III-II-II-I
24	M	20yr / 2mo	19 mo	2	90/91	1/1			III-III
25	M	3mo	17 mo	2	92/93	1/1			II-III
26	M	9yr / 7mo	23 mo	3	94/95/96	1/1/1/1			II-II-II
27	M	5yr / 6mo	14 mo	5	NT/97/98/99/100	1/1/1/1/1			III-V-II-II-III
28	M	20yr / 9mo	72 mo	27	101/102/103/104/105	23/1/1/1/1	72/0/0/0/0	101a	I(20), II(2), IV(1)-I-I-I-II
29	M	2yr / 5mo	25 mo	3	106/107/108	1/1/1			VII-VI-I
30	M	6yr / 5mo	20 mo	4	109/110/111	2/1/1	11/0/0		I-I-I

1A, 1B. Clone 1 subtypes.

Lower case letters. OMPs variants.

Bold type. Cross-colonizing strains.

(*) Refers to total number of isolates

TABLE 2. Minimal inhibitory concentrations (MIC) of *Haemophilus influenzae* isolated from cystic fibrosis patients as determined by the Epsilon test.

Antibiotic	Nº isolates[1]	MIC range[2]	MIC_{50} [2]	MIC_{90} [2]	Geometric mean
Ampicillin	188	0.064-256	0.50	64	0.886
	115	0.064-256	0.25	64	0.82
Amoxicillin/Clavulanic-acid	188	0.064-8	0.5	2	0.555
	115	0.064-4	0.5	2	0.472
Cefotaxime	188	0.003-2	0.23	0.125	0.029
	115	0.003-1	0.016	0.06	0.022
Chloram-phenicol	188	0.125-16	0.5	8	0.692
	115	0.125-16	0.5	1.0.	0.534
Co-trimoxazol	188	0.012-64	4	64	1.807
	115	0.012-64	4	32	0.903
Ciprofloxacin	188	0.003-64	0.012	4	0.042
	115	0.003-32	0.012	0.025	0.019

[1] Two figures are specified; first (188), is the total number of isolates; second (115), is the number of PFGE patterns.

[2] Expressed in µg/ml.

TABLE 3. Antimicrobial susceptibility comparison between *H. influenzae* strains isolated from cystic fibrosis patients and controls without cystic fibrosis.

Property	Control isolates (188 strains)	Cystic fibrosis isolates (188 strains)	p[1]
Nº fully antibiotic-susceptible	88	50	**0.006**
Nº chloramphenicol-resistant	23	36	0.12
Nº with ciprofloxacin MIC > 1µg/ml[2]	0	40	**<0.0001**
Nº amoxicillin/clavulanic acid-resistant	1	4	0.37
Nº trimethoprim-sulfamethoxazole-resistant	68	123	**0.001**
Nº ß-lactamase +	38	45	0.54
Nº BLNAR[3]	1	10	**0.01**
Resistance to 2 or more antimicrobials	20	62	**<0.0001**

[1] Fisher's exact test.

[2] Usual ciprofloxacin MIC in *H. influenzae* is ≤ 0.03 µg/ml (23).

[3]BLNAR, beta-lactamase-negative-ampicillin-resistance.

FIG. 1. PFGE fingerprints of 15 strains from patient 1 in chronological order. The patient harbors 3 patterns: pattern 1A (lanes 1, 3, 5, 6, 10, 11, 12, 13, 14 and 15), pattern 1B (lanes 2, 4, 7 and 8) and pattern 2 (lane 9). Pattern 1B strains showed the same biotype (VI) as six strains (lanes 1, 3, 5, 6, 11 and 15) of the pattern 1A and the pattern

2 (lane 9). In panel A are shown the Sma I-PFGE fingerprints. In panel B the Bsp 120 I (Apa I)-PFGE fingerprints. M is the molecular size marker (PFGE marker).

FIG. 2. PFGE fingerprints of 18 strains from patient 3 in chronological order. The patient harbors 4 clones: clone 4 (lanes 1,2,3,4,5,6,7,8,9,10,11,12, 13 and 16) , clone 6 (lanes 14 and 18), clone 7 (lane 15) and clone 8 (lane 17). Clones 6 and 8 showed the same biotype (III). In panel A are shown the Sma-PFGE fingerprints. In panel B the Bsp 120I (Apa I)-PFGE fingerprints. M is the molecular size marker (PFGE marker). PFGE patterns of the same strains with Bsp 120 I (Apa I), in the second photo.

FIG. 3. PFGE fingerprints of 27 strains from patient 28 in chronological order. The patient harbors 5 clones: clone 101 (lanes 1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 19, 20, 21, 24, 25, 26 and 27), clone 102 (lane 5), clone 103 (lane 18), clone 104 (lane 22) and clone 105 (lane 23). Three strains of the clones 102 (lane 5), 103 (lane 18) and 104 (lane 22), and twenty strains (lanes 1, 2, 3, 4, 6, 7, 8, 9, 11, 12, 13, 14, 16, 17, 19, 20, 21, 24, 25 and 26) of the clone 101 showed the same biotype (I). Clone 105 and two isolates of the clone 101 (lanes 10 and 27) showed the same biotype (II). In panel A are shown the Sma-PFGE fingerprints. In panel B the Bsp 120I (Apa I)-PFGE fingerprints. M is the molecular size marker (PFGE marker).

FIG 4. Variants of Outer membrane protein profiles of seven *H. influenzae* strains belonging to two PFGE patterns (1A and 1B) from patient 1 and isolated in different periods of time. Letters a and b indicate variations in the OMPs.

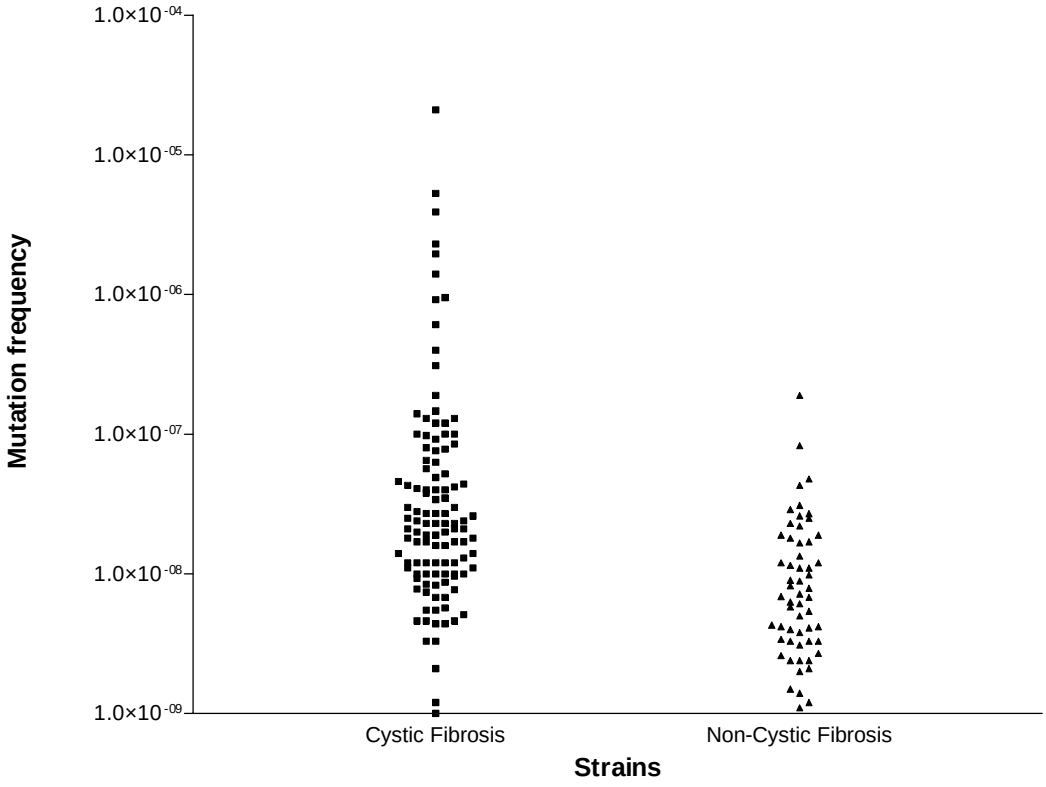

Figure 5. Mutation frequency of the CF *H. influenzae* and non-CF *H. influenzae* strains on rifampin. Each dot represents the mean mutation frequency calculated from 3 experiments for 1 strain. Most strains in both groups yielded mutation frequencies to rifampin resistance $<10^{-7}$. Strains with mutation rates $>10^{-7}$ were considered to be displaying an hypermutable phenotype.

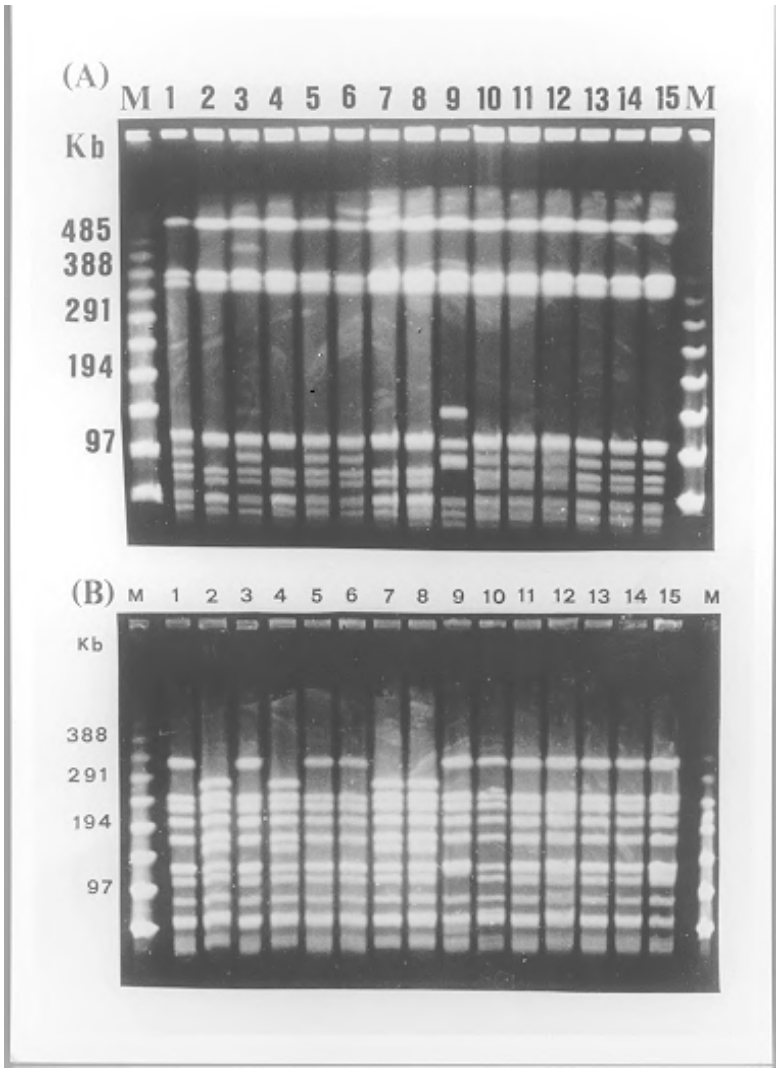
(A)
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 M
Kb
485
388
291
194
97
(B)
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 M
Kb
388
291
194
97

FIGURE 1.

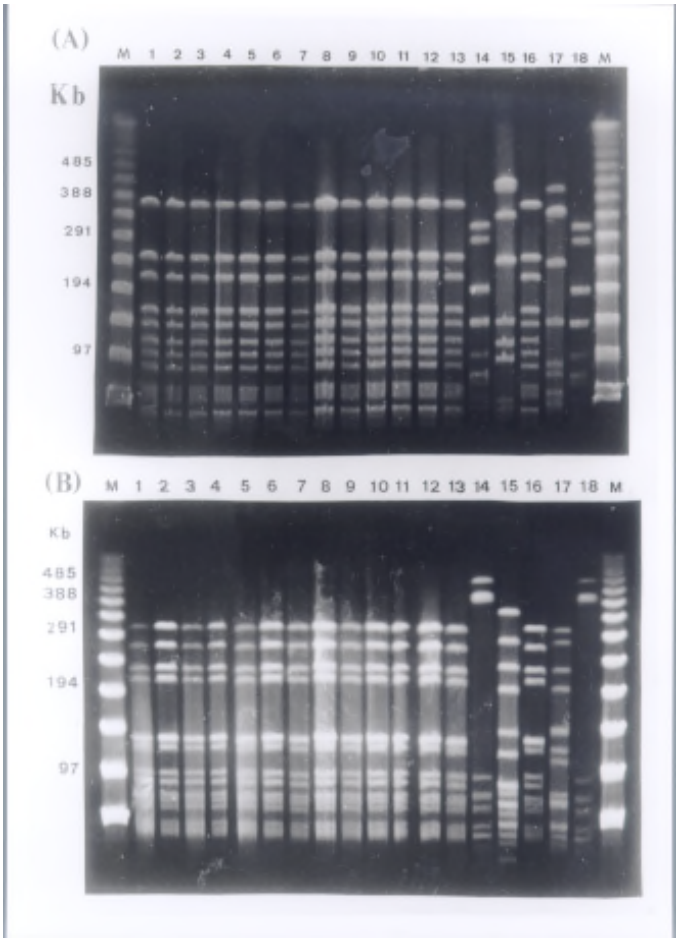
(A)
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 M
Kb
485
388
291
194
97
(B)
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 M
Kb
485
388
291
194
97

FIGURE 2.

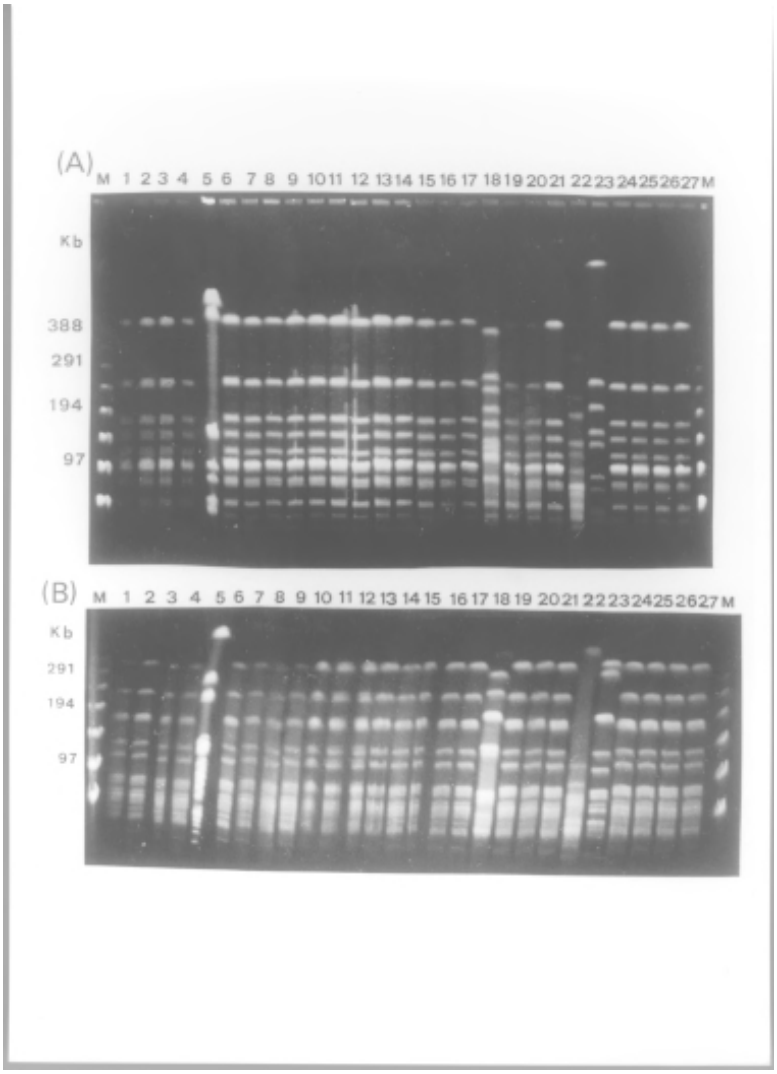
(A)
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 M
Kb
388
291
194
97
(B)
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 M
Kb
291
194
97

FIGURE 3.

FIGURE 4