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Achromobacter xylosidans respiratory tract infection in Cystic Fibrosis patients

Antonietta Lambiase¹, Maria Rosaria Catania¹, Mariassunta del Pezzo¹, Fabio Rossano¹, Vito Terlizzi², Angela Sepe², Valeria Raia²

¹Department of Cellular and Molecular Biology and Pathology “Luigi Califano” and
²Department of Pediatrics, Regional Cystic Fibrosis Center,
Medicine School,
University “Federico II”,
Naples, Italy

*Corresponding author
E-mail address: alambias@unina.it

Abstract
Aims of this study were to evaluate the frequency of Achromobacter xylosidans infection in a cohort of Cystic Fibrosis patients, to investigate antimicrobial sensitivity, to establish possible clonal likeness among strains, and to address the clinical impact of this infection or colonization on the general outcome of these patients.

The study was undertaken between January 2004-December 2008 on 300 patients receiving care at the Regional Cystic Fibrosis Centre of Naples University “Federico II”. Sputum samples were checked for bacterial identification. For DNA-fingerprinting, pulsed-field electrophoresis was carried out.

Fifty-three patients (17.6%) had at least one positive culture for A. xylosidans. 6/53 (11.3%) patients were defined as chronic infected and all were co-colonized by P. aeruginosa. 18.8% of patients persistently carried multi-drug resistant isolates.

Macrolestriction analysis showed the presence of seven major clusters. DNA fingerprinting also showed genetic relationship among strains isolated from the same patients at different times.

Results of DNA-fingerprinting indicate evidence of bacterial clonal likeness among enrolled infected patients. We found no significant differences in FEV₁ and BMI comparing the case group of A. xylosidans chronic infected patients with the control group of P. aeruginosa chronic infected patients.

Key words: Cystic Fibrosis, emerging pathogens, antibiotic-resistance, PFGE.
Introduction

Over the past 20 years, the epidemiology of bacteria involved in acute infections in Cystic Fibrosis (CF) has become increasingly complex. Although *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Haemophilus influenzae* have been the most common pathogens in the lower airways of CF patients, with improved survival, new emergent pathogens such as *Burkholderia cepacia* complex (Bcc), *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, *Aspergillus* spp, Nontuberculous Mycobacteria and respiratory viruses have been detected in the last few years [1-12]. Other unusual bacteria such as *Acinetobacter* spp, *Bordetella* spp, *Moraxella* spp, *Comamonas* spp, *Rhizobium* spp, *Herbaspirillum* spp, and *Inquilinus limosus* have recently been described [13], and also Italian studies have reported Gram-negative non fermentative bacteria such as *Chryseobacterium meningosepticum*, *Chryseobacterium indologenes*, *Chryseobacterium gleum*, *Sphingobacterium spiritovorum* and *Sphingobacterium multivorum* in sputum samples of CF patients [14-15]. Moreover, mycetes such as *Scedosporium apiospermum*, *Penicillium* and *Exophiala* have also been recognized [16-19]. The pathogenic role of several of these microorganisms has not yet been clarified.

*Achromobacter xylosoxidans* is an aerobic, oxidase and catalase positive, non-lactose fermenting, gram-negative bacillus widely distributed in the natural environment. Its taxonomic position has been considered uncertain during the last decades: the genus was named *Achromobacter*, then *Alcaligenes* and then again *Achromobacter*. The phylogenetic analyses of 16S rRNA, besides a difference of more than 10% in GC content of DNA, demonstrated that *Achromobacter xylosoxidans* and *Alcaligenes faecalis* (type species of the genus *Alcaligenes*), belong to two distinct genera, *Achromobacter* and *Alcaligenes*, respectively [39].

*Achromobacter xylosoxidans* is an opportunistic human pathogen capable of causing a variety of infections, including bacteraemia, meningitis, pneumonia and peritonitis, particularly in immunocompromised hosts and patients with underlying diseases [20]. Nosocomial outbreaks attributed to disinfectant solutions, saline solutions and diagnostic tracers contaminated with this species have been described [21-22].

In patients with CF, an increasing prevalence of *A. xylosoxidans* isolates has recently been reported [23-27], but clinical significance of *A. xylosoxidans* infection is still unclear.

Because of the relevance of bacterial lung infections in CF patients, the aims of our study were 1) to evaluate the frequency of *A. xylosoxidans* infections in a cohort of CF patients, 2) to investigate the antimicrobial sensitivity of isolates, 3) to establish possible clonal likeness among strains, as well as 4) to elucidate possible clonal likeness among strains isolated from the same patients during the study period, and 5) to address a possible clinical relevance of this infection on the general outcome of these patients.
Patients and methods

Study population

A retrospective chart review of microbiological samples obtained from 300 CF patients (145 males and 155 females; mean age
16.21 years; range 0.5-50 years) regularly attending the Regional Referral CF Center of Naples, Italy, was undertaken. Sputum
samples were collected during the period January 2004-December 2008. CF was diagnosed on the basis of standard methods
(sweat chloride test above 60 mmol/l by pilocarpine and two relevant CF transmembrane regulator mutations).

For each patient, data about anthropometric parameters, pancreatic status, mean number of pulmonary exacerbations requiring
intravenous antibiotics during the previous 12 months were obtained from our existing patient database.

Patients over 6 years of age had at least one lung function evaluation during each year of observation as measured by forced
expiratory volume in 1 sec (FEV₁), expressed as a percentage of predicted values for the relevant age, sex, ethnic background,
weight and height.

Despite the central role that pulmonary exacerbations play in CF patient care and research, no standardized definition about
pulmonary exacerbations exists. Some of the characteristics most strongly associated with a pulmonary exacerbation include
increased cough, increased sputum production, decreased exercise tolerance, decline in weight-for-age percentile, reduced
appetite, hemoptysis, and new sounds on examination of the chest [28-29]. In our patients, pulmonary exacerbations were
defined according to these suggestions.

Sputum samples for microbiological studies were obtained from each patient at least every 3 months at clinical examinations.
Patients infected by A. xylosoxidans were characterized for age, age of acquisition of first infection, co-infection, lung function
and death. Chronic infection was defined as persistence of 3 positive cultures for at least 6 consecutive months, intermittent
infection was defined as the presence of 3 non consecutive positive cultures per year, and sporadic infection when less than 3
cultures were positive per year [30]. Co-infection was defined as sputum culture positive for more than one microorganism.
Isolates were defined multi-drug resistant organisms (MDROs) according to the most recent definition given by CDC [31].

In order to correlate chronic A. xylosoxidans infection and chronic P. aeruginosa infection to lung function, clinical features of
chronically A. xylosoxidans infected patients (case group) were compared with those of chronically P. aeruginosa infected
patients (control group): thus, two groups were matched for age, gender, body weight, FEV₁ and P. aeruginosa infection status.
A. xylosoxidans had never been isolated from any patient of the control group. Nutritional status was calculated as body mass
index (BMI, Kg/m²). Data were compared for the period from one year before to one year after the onset of A. xylosoxidans
infection. t-test was used for analysis of continuous variables and chi-square test for categorical variables. A p value of less than
0.05 was considered statistically significant.

Processing of sputum samples, culture of microorganisms and phenotypic analysis
Sputum samples, obtained from all patients during the study period, were mixed with equal volumes of 1% dithiothreitol before incubation at 37°C for 30 min. All specimens were examined microscopically and plated on several agar media, including Mac Conkey agar, CNA agar, BCSA, Sabouraud agar, at 37°C for 24 up to 72 h.

All isolates were identified by the Phoenix system (Becton Dickinson); the API 20 NE identification system (bioMerieux) was used to confirm the identification of A. xylosoxidans isolates.

All A. xylosoxidans isolates were cryopreserved at -80°C for subsequent investigations.

**Analysis for 16S rDNA gene based identification**

To validate the phenotypic identification, a PCR assay was performed. DNA was prepared by heating one or more colonies (picked from an overnight growth plate) at 95°C for 15 min in 20 μl of lysis buffer containing 0.25% sodium dodecyl sulfate and 0.05 NaOH. After lysis, 180 μl of sterile distilled water was added to the lysis buffer.

The 16S rDNA gene (163 bp) was amplified using the primers AX-F1 and AX-B1, respectively, for the target 5’ and 3’ ends of the 16S rDNA gene locus (AX-F1, sequence 5’-3’ GCAGGAAGAAACGTCGCGGGT, nucleotide positions 427-448; AX-B1, sequence 5’-3’ ATTTCACTTCTTTCTTTCCG, nucleotide positions 576-595) [32]. PCR was performed in a total volume of 100 μl containing 0.5 μg DNA, 25 mM MgCl₂, 25 mM dNTPs, 1 μM concentration of each oligonucleotide primer and 5U/μl Taq-polymerase. After initial heating at 94°C for 1 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 45 sec and extension at 72°C for 1 min were performed. The final extension step was at 72°C for 10 min. Control strain (A. xylosoxidans ATCC 27061) and negative control PCRs were employed for every experiment.

**Antimicrobial susceptibility test**

To assess the sensitivity to aztreonam, piperacillin, piperacillin-tazobactam, cefotaxime, cefepime, ceftazidime, ciprofloxacin, levofloxacin, chloramphenicol, imipenem, meropenem, trimethoprim-sulfamethoxazole, gentamicin, netilmicin, rifampin, tetracycline, an agar-diffusion method (Kirby-Bauer) and the microbroth dilution assay (Phoenix system) were used. A total of five non-CF- A. xylosoxidans isolates (one isolate per year of study) was included for antimicrobial susceptibility analysis. These isolates were obtained from several biological samples, i.e. sputum, blood, vascular catheter, burn wound, urine.

Interpretative criteria for susceptibility for all the methods used in the study were in accordance with CLSI criteria [33].

**PFGE genotyping**

Isolates were grown overnight on nutrient agar and suspended in SE buffer (75 mM NaCl, 25 mM EDTA, pH 7.5). The cell suspension (4 McFarland) was mixed with an equal volume of 1.6% low-melting point agarose, molded into plugs at 4°C, and lysed with lysis buffer (1% N-lauryl sarcosine, EDTA 0.5 M, pH 8.00) with the addition of Proteinase K [34]; the DNA inserts were digested with DraI (Promega), at 37°C for 19 h. Macrorestriction fragments were separated using CHEF DR III (Biorad)
at 10°C for 20 h, with start time of 5s and end-pulse time of 35s, at a field strength of 6V/cm. A concatemer ladder of lambda phage DNA was used as a size marker. Fragment patterns were compared according to Tenover’s criteria [35]. Besides, to interpret the molecular patterns, a dendrogram was generated using Phoretix 1D-Pro software (Totallab). To examine whether each patient persistently carried the same A. xylosoxidans strain during the study period, from two up to six isolates for each patient per year were analyzed. A total of five non-CF- A. xylosoxidans isolates (one isolate per year of study) was included for PFGE analysis (obtained from sputum, blood, vascular catheter, burn wound, urine).

Results

Prevalence of isolates, chronically infected patients and co-infection with other microorganisms.

Over the study period, a total of 5315 sputum samples were obtained from 300 patients. In table 1, total number of isolates, number and percentage of total MDROs for each type of bacteria isolated in the study period are reported. A total of 276 isolates of A. xylosoxidans was recovered and results obtained by PCR assay were in agreement with biochemical identification. A total of 53 patients (17.6%) had at least two positive cultures for A. xylosoxidans (males 24 and 29 females; mean age 21.14 years; range 6.25-48 years).

Among these, 6/53 (11.3%) were defined as chronically infected patients (males 3 and 3 females, mean age 11.5 years, range 9.9-21.4 years, mean colonization period 2.7 years) and were co-colonized by P. aeruginosa (mean age at P. aeruginosa chronic infection 9.5 years, range 6-13.7 years).

Patients considered with intermittent infection by A. xylosoxidans were 7/53 (13.2%) (males 4 and 3 females, mean age 30 years, range 21-51.9 years), while patients with sporadic infection were 40/53 (75.5%) (males 18 and 22 females, mean age 17.3 years, range 6.2-35.7 years).

In general, co-infection by P. aeruginosa was found in 17/53 patients (32%) with sporadic/intermittent infection: particularly, 4/17 patients were co-infected by A. xylosoxidans and P. aeruginosa, while 13/17 patients were co-infected by A. xylosoxidans, P. aeruginosa and other microorganisms (S. aureus, H. influenzae, B. cepacia complex, S. maltophilia, P. mirabilis, C. indologenes, and A. fumigatus). Co-infection by C. albicans was found in 23/53 patients (43.3%).

The correlation among clinical features of chronically A. xylosoxidans infected patients (case group) and chronically P. aeruginosa infected patients (control group) was carried out. As shown in table 2, no significant difference was found between case and control groups in relation to the parameters considered.

Antimicrobial susceptibility test

Considering one isolate per patient, and considering that each isolate showed the same antimicrobial profile during all the study period, 10/53 (18.8%) A. xylosoxidans isolates were multi-drug resistant, showing resistance to aztreonam (MIC>16
µg/mL), to cephalosporins, including cefepime (MIC>16 µg/mL), ceftazidime (MIC>16 µg/mL) and cefotaxime (MIC>32 µg/mL), to carbapenem (imipenem MIC>8 µg/mL; meropenem MIC>8 µg/mL), to aminoglycosides (gentamicin MIC>8 µg/mL), to ciprofloxacin (MIC>2 µg/mL); to levofloxacin (MIC>4 µg/mL) and trimethoprim-sulfamethoxazole (MIC>2/38 µg/mL). On the other hand, these 10 isolates were sensitive to piperacillin (MIC<4 µg/mL) and piperacillin/tazobactam (MIC<4/4 µg/mL). All six chronically infected patients carried multi-drug resistant isolates.

Forty-three patients carried isolates that were resistant to aztreonam and sensitive to ceftazidime (MIC<8 µg/mL), to carbapenem (imipenem MIC<2 µg/mL; meropenem MIC<1 µg/mL), to ciprofloxacin (MIC<2 µg/mL), to levofloxacin (MIC<24 µg/mL), trimethoprim-sulfamethoxazole (MIC<0.5/9.5 µg/mL) and cloramphenicol (MIC<16 µg/mL). Also these isolates were sensitive to piperacillin and piperacillin/tazobactam. Table 3 shows the results of antimicrobial susceptibility testing of A. xylosoxidans isolates (n=53), besides the antimicrobial profile of P. aeruginosa co-infected isolates (n=23).

**Genome macrorestriction analysis**

PFGE analysis (fig. 1 and fig. 2) showed the possibility to group into clusters more than half of strains (39/53; 73.58 %). Seven major clusters were found (A-G clusters). Strains grouped in each cluster showed a diversity (distance level) less than 10% (or homology level >90%). Cluster A groups 9/53 strains, cluster B groups 3/53 strains, cluster C groups 8/53 strains, cluster D groups 3/53 strains, cluster E groups 4/53 strains, cluster F groups 6/53 strains and cluster G groups 6/53 strains. Other 14 strains showed a unique macrorestriction profile. Sequential strains obtained in the study period from the same patient showed identical macrorestriction profiles.

**Discussion**

Survival of CF patients is now increased (median age>30 years) and consequently new pathogens have emerged in the CF lung. As underlined by Tan [36], the intensive use of antibiotics may be one factor that increases the likelihood of opportunistic infections with resistant microorganisms.

We found in our cohort a high prevalence of A. xylosoxidans infection (17.6% of the 300 enrolled patients), if data are compared with those of Magni [37] where a prevalence infection of 8.8% is indicated (enrolled patients= 450). Our data indicate a high prevalence of infection also if they are compared with European studies such as that conducted by Kanellopoulou [26], where the authors show a prevalence of A. xylosoxidans infection of 12.6% (enrolled patients= 71). The reason for this high prevalence in our population is likely linked to a higher mean age of patients.

The clinical impact of A. xylosoxidans infection is not clear, as well as its lung colonization. Our data are not indicative of increased morbidity linked to this infection/colonization. Besides, our study design does not indicate effects on clinical status from chronic/intermittent/sporadic A. xylosoxidans infection. We found no significant difference in FEV₁ and BMI comparing chronically A. xylosoxidans infected patients with chronically P. aeruginosa infected patients during the study period. The mild
lower mean FEV₁ observed in patients with chronic *A. xylosoxidans* infection could also have been influenced by other covariates such as diabetes.

All chronically *A. xylosoxidans* infected patients were co-colonized also by *P. aeruginosa* and, generally, 43.3% of patients presents also *P. aeruginosa* infection. In the study of Van Daele [38], there is indicated the strong tendency by *A. xylosoxidans* to install itself in a lung already infected by *P. aeruginosa*; but, in this study, only patients colonized by *P. aeruginosa* were enrolled. Our data also indicate this tendency by *A. xylosoxidans* but, differently from the study of Van Daele, our population also included patients not co-colonized by *P. aeruginosa* (56.6%; 30 non-co-infected by *P. aeruginosa* /53). Thus, we can affirm that *A. xylosoxidans* can infect a lung also not previously colonized by *P. aeruginosa*.

In our cohort, none of the patients had a transplantation during the study. About 10% of patients (5/53) died in the examined period, two were chronically infected by *A. xylosoxidans* and all these five patients were chronically infected by *P. aeruginosa*; however, it is not possible to link these deaths to *A. xylosoxidans* infection/colonization. 

Because of the emergence of new gram-negative microorganisms among CF patients, bacterial misidentification is especially problematic and presents a challenge to effective infection control in CF, to antimicrobial therapy and patient prognosis.

There is evidence of weakness of commercial systems for phenotypic identification of non-fermentative gram-negative bacteria recovered from CF patients. Saaiman, in a report of 2001 [25] indicated that in a group of 106 isolates obtained from 78 patients from 49 CF Centres in the United States and all identified as *A. xylosoxidans*, 89% was correctly identified, whereas 11% was misidentified: thus, 10 isolates were found to be *P.aeruginosa*, one isolate *S. maltophilia* and one *B. cepacia* complex. We have demonstrated that the phenotypic identification, carried out by Phoenix and API 20 NE systems, was confirmed by PCR analysis.

As a consequence of the increasing use of antibiotics concomitant to acute pulmonary exacerbations in CF patients due to *P. aeruginosa* infection, *A. xylosoxidans* as well as other non-fermentative gram-negative bacteria are showing growing drug resistance. Our data support this evidence because of frequent previous colonization with *P.aeruginosa*.

It is well known that for most of the non-lactose fermenting, gram-negative rods, the disk diffusion antibiogram is not validated by the CLSI. In fact, there are several interpretation problems, such as unclear inhibition zone borders. Consequently, in the present study also a microbroth dilution assay was carried out and no differences were found between the two methods.

With the emergence of antimicrobial resistance of new pathogens, the *scenario* has changed with respect to the availability and susceptibility of antimicrobial agents. In our study, about 20% of *A. xylosoxidans* isolates showed a multi-drug resistant profile. This percentage of isolates was resistant to aztreonam, cephalosporins, including cefepime, cefazidime and cefotaxime, to carbapenem, aminoglycosides, quinolones, and trimethoprim-sulfamethoxazole. These same isolates were sensitive to piperacillin with or without tazobactam. Cefazidime, carbapenems, quinolones and trimethoprim-sulfamethoxazole, besides piperacillin and piperacillin/tazobactam, were active against non multi-drug resistant isolates. *A. xylosoxidans* isolates recovered from non-CF-patients were resistant to molecules such as ceftazidime, carbapenems, and
levofloxacin. This finding indicates that isolates of *A. xylosoxidans* can be resistant to many antimicrobial agents, independently from the study population.

Currently, reports for patient-to-patient spread are controversial. Several studies rule out the possibility of *A. xylosoxidans* by inter-human contacts, or at least they indicate little evidence of person-to-person transmission, such as the study of Vu-Thien [21]. In our study, PFGE indicates that more than half of *A. xylosoxidans* can be grouped into seven different clusters, suggesting the possibility of patient-to-patient transmission.

Results of PFGE have been interesting for several reasons. First, all six chronically infected patients carried strains of two different clusters (four strains of cluster “A” and two strains of cluster “C”); besides, among five cases of death, one patient carried strain “A” and four patients carried strains with a unique macro-restriction profile. At last, any strain with a unique macro-restriction profile was considered MDRO, whereas were considered MDRO four strains of clone “A”, two of clone “B” and four of clone “C”.

These results are supported by a recent study of Kanellopoulou [26] where the Author indicated five CF patients colonized by genetically related strains. Our results certainly suggest a common-source of contamination and this is highly probable because of frequent social contacts that occur among patients of a single care centre.

It is very interesting to note that in our study, restriction patterns were obtained both by *DraI* and by *SmaI*. Restriction endonuclease *SmaI* generated a lower number of bands with respect those generated by *DraI* (data not shown), consequently, DNA cleavage by this endonuclease was characterized by increased sensitivity.

In conclusion, results of the present study can represent a further step toward the understanding of the epidemiology of these microorganisms and of a possible correlation between microbiological data and clinical outcomes of CF patients.
Table 1. Distribution (number and percentage) of total multidrug-resistant gram-negative isolates during the study period

| Total strains | MDROs |
|---------------|-------|
|               | No    | %   | No   | %   |
| P. aeruginosa | 2340  | 59  | 457  | 19.5|
| S. maltophilia| 750   | 18.9| 176  | 23.4|
| A. xylosoxidans| 276  | 6.9 | 62   | 22.4|
| B. cepacia    | 20    | 0.5 | 5    | 25  |
| B. cenocepacia| 550   | 13.8| 387  | 70.3|
| B. stablis    | 15    | 0.3 | 7    | 46.6|
| B. vietnamiensis| 10   | 0.2 | 4    | 40  |
| B. gladioli   | 3     | 0.07| -    | -   |
|               | 3964  |     | 1098 | 27.6|
Table 2: Clinical features of chronically *A. xylosoxidans* infected patients (case group) compared with control group

|                      | Chronically infected patients by *A. xylosoxidans* (n=6) | Chronically infected patients by *P. aeruginosa* (n=6) | P value |
|----------------------|----------------------------------------------------------|-------------------------------------------------------|---------|
|                      | -1 year | Time 0 | +1 year | -1 year | Time 0 | +1 year |         |
| Mean age             | 10.5    | 11.5   | 12.5    | 11.1    | 12.1   | 13.1    | >0.05   |
| BMI                  | 16.9    | 17.8   | 18.2    | 16.8    | 17.3   | 17.4    | >0.05   |
| Mean FEV₁ (%) (range)| 66.5% (33.6%–86%) | 62% (33.6%–79%) | 61.7% (34.8%–76%) | 63% (45.2%–99%) | 63.2% (38.4%–99%) | 63.7% (38.4%–97%) | >0.05 |
| *P. aeruginosa* co-colonization (no. patients) | 6       | 6      | 6       | -       | -      | -       |         |
| CFRD (no. patients)  | 1       | 1      | 3       | 1       | 3      | 4       | >0.05   |

CFRD=Cystic Fibrosis Related Diabetes
Table 3. Percentage of *A. xylosoxidans* (AX; n=53) and *P. aeruginosa* (PA; n=23) isolates resistant to the antibiotics tested.

| Antibiotics | ATM | FEP | CTX | CAZ | CIP | CHL | GEN | IPM | LVX | MEM | NET | PIP | TZP | TET | SXT |
|-------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| AX          | 100 | 28  | 28  | 18.8| 18.8| 37  | 18.8| 18.8| 41  | 0   | 0   | 73  | 18.8|     |     |
| PA          | 26  | 21  | 82  | 17  | 52  | 91  | 65  | 47  | 56  | 43  | 43  | 43  | 26  | 98  | 100 |

*Isolates recovered in co-infection with Achromobacter xylosoxidans

ATM = *Aztreonam*; FEP = *Cefepime*; CTX = *Cefotaxime*; CAZ = *Ceftazidime*; CIP = *Ciprofloxacin*; CHL = *Chloramphenicol*; GEN = *Gentamicin*; IPM = *Imipenem*; LVX = *Levofloxacin*; MEM = *Meropenem*; NET = *Netilmicin*; PIP = *Piperacillin*; TZP = *Piperacillin-tazobactam*; TET = *Tetracycline*; SXT = *Trimethoprim-sulfamethoxazole*. 
Fig. 1. PFGE of representative strains of *A. xylosoxidans*. The numbers indicate the strains in the study. Molecular size marker (a concatemer ladder of lambda phage DNA) was run in lane ST. Sizes are indicated in kilobases.

Fig. 2. Phylogenetic analysis of digitized PFGE *DraI* profiles of *A. xylosoxidans* analysed in the study. Cluster analysis is based on percentage of distance index. Numbers indicate the strains in study; letters indicate the clusters.
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