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Genetic diversity of *Burkholderia contaminans* isolates from cystic fibrosis patients in Argentina

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ABSTRACT

One hundred twenty *Burkholderia cepacia* complex isolates collected during 2004–2010 from 66 patients in two cystic fibrosis reference centers in Argentina were analyzed. *B. contaminans* was the species most frequently recovered (57.6%) followed by *B. cenocepacia* (15%), a species distribution not reported so far. The *recA*-PCR-based techniques applied to the *B. contaminans* isolates revealed that 85% of the population carried the *recA*-ST-71 allele. Our results showed the utility of BOX-PCR genotyping in analyzing *B. contaminans* diversity. This approach allowed us to address a clonal transmission during an outbreak and the genetic changes occurring in infecting bacteria over the course of chronic infection.

Burkholderia cepacia complex species are capable of causing chronic and often severe respiratory-tract infections in cystic fibrosis (CF) patients and other types of infections in immunocompromised patients. Although many CF patients remain infected by these bacteria and relatively healthy for prolonged periods, others either have a severe decline in their pulmonary status, or die shortly after the initial colonization (2). In Argentina, the first reports of *B. cepacia* complex bacteria infecting CF patients started around 1990. At that time *Burkholderia* spp. were recovered sporadically and with a very low prevalence (<0.1%). The prevalence started to increase over the last decade from 0.2 to 3.6% depending on the medical center. In early 2004 an outbreak involving main Argentine CF-care centers occurred, and the proportion of patients from whom *B. cepacia* complex species could be recovered ranged from 19 to 36% (5, 18, 22). Currently, through strict infection-control procedures, the prevalence of *Burkholderia* spp. in local patients has decreased to approximately 10%. Recent worldwide surveillance studies concerning the distribution of *B. cepacia* complex species in CF patients have cited as the most frequently recovered species *B. multivorans* and *B. cenocepacia*. These species account for approximately 80% of the infected patients, and the prevalence between them is geographically and temporally dependent (13, 15, 19). Unlike this worldwide situation, in Argentina a relatively high prevalence of *B. contaminans* followed by *B. cenocepacia* has been encountered (18, 22). To our knowledge, no other geographical region has been reported in the literature with such a high occurrence of *B. contaminans* infecting CF patients. In view of this particular scenario coupled with the lack of biodiversity information available for *B. contaminans* species, we decided to study this local population by *recA*-PCR-based techniques and by repetitive element sequence-based PCR (rep-PCR) approaches. These methods allowed us to address the genetic diversity of *B.*

contaminans isolates recovered during that outbreak along with the genetic changes occurring in the infecting bacteria over the course of a chronic infection.

Distribution of *Burkholderia cepacia* complex species in CF patients. This study involved the analysis of a total of 120 clinical *Burkholderia cepacia* complex isolates recovered from 66 CF patients from 2004 through 2010 and 13 reference strains (Table S1, Supplementary Material). The isolates were recovered from sputum samples of CF patients treated at 2 main CF reference centers of Argentina (Hospital de Niños Sor María Ludovica, La Plata [HNLP], and Hospital Santísima Trinidad, Córdoba [HST], located 800 km from La Plata to the north; plus one hospital (Hospital Dr. Pedro Moguillanski, Cipoletti, Río Negro [HNRN], 1,200 km from La Plata to the south. Twenty-two isolates (indicated with a "v" in Table S1, Supplementary Material) were withdrawn from patients infected during the *B. cepacia* complex outbreak, and a cohort of 51 isolates was recovered from 21 patients chronically infected with *B. cepacia* complex bacteria. Patients were considered chronically infected when three positive cultures of *B. cepacia* complex strains were isolated within a 6-month period (24). Bacterial isolation was performed on *B. cepacia* selective agar medium (BCSA, Lab. Britania S. A., Argentina) according to clinical microbiology practice recommendations for respiratory-tract specimens from CF patients (9, 16) and were phenotypically characterized by means of conventional biochemical techniques (9, 16, 23, 33). The species-level identification was achieved by *recA*-PCR-based techniques. The *recA* gene (1040 bp) was amplified through the use of BCR1 and BCR2 primers (21) and the amplified products subjected to restriction-fragment-length-polymorphism (RFLP) analysis with the restriction enzyme *Hae*III (Promega, Inc.; 21). The restriction-PCR patterns obtained were analyzed and compared with those reported in the literature (11, 12, 21, 29). The amplified products were purified through the use of the QIAquick PCR-purification kit (Qiagen Inc., CA, USA), and sequenced on both strands with the BigDye Terminator Cycle Sequencing Ready Reaction

kit (Applied Biosystem, Foster City, CA, USA) at Macrogen, Inc. (Seoul, Korea). A final identification was achieved by *recA*-gene-DNA-sequence analysis and *gyrB DNA* fragment (1,900 bp) sequencing when an identification remained ambiguous. The *recA*-sequence type (*recA*-ST) within each species was obtained by comparing the sequence of a 398 bp *recA* fragment against the Multi Locus Sequence Typing (MLST) database (http://pubmlst.org/bcc/) (2).

The analysis of species distribution among the patients studied showed that 8 out of the 17 species described for the *B. cepacia* complex were infecting the local CF population, with a remarkably high representation of *B. contaminans* that accounted for 57.6% of the infected patients. The data on the species of the first isolate available from each patient indicated that *B. cenocepacia* was the second most frequent species, it encountered at a frequency of almost 15% of the patients, followed by *B. cepacia* at 7.5%, *B. multivorans* at 6.0%, *B. stabilis* and *B. vietnamiensis* at 5%, *B. seminalis* at 3.0 %, and *B. ambifaria* at 1.5%.

In order to examine the genetic diversity among the *B. contaminans* isolates, sequence analysis of a 900 bp fragment of the *recA* gene was performed over the 79 *B. contaminans* isolates (Table S1, Supplementary Material). The phylogenetic relationship among them was investigated through the use of the MEGA version 4.0 software (http://www.megasoftware.net; Fig. 1**)**. Isolates were clearly separated into one large homogenous cluster that grouped 85% of the *B. contaminans* population under study along with four small clusters containing only 5 isolates. When the sequence of the 398 bp DNA fragment from the r*ecA* gene was compared against the MLST database (2), all the bacteria included in the main cluster showed to belong to the *recA*-ST-71 allele; while 4 of the isolates grouping outside this cluster displayed different *recA*-ST alleles (ST-64, -166, and -243). The isolates grouped in the main cluster had a *recA*-*Hae*III-restriction pattern corresponding to the K-type, whereas the isolates outside this group gave profiles of K,

AT, and J (Table S1, Supplementary Material). Interestingly, one of the isolates (HNLP 1001) exhibited the J *recA*-RFLP pattern which was reported to be representative of the species *B. cenocepacia*, *B. stabilis, B. lata, B. seminalis*, and *B. arboris* (21,27, 28), but so far not documented for *B. contaminans*. It is notable that none of the *recA* sequences corresponding to isolates that grouped in the main cluster was represented by the *recA* reference sequences available at GenBank. It should also be remarked that two reference sequences displaying the *recA*-ST-71 allele, those belonging to two isolates recovered from CF patients in Brazil (*B. contaminans* R18428 and R9929), clustered at a short distance from the homogenous cluster (Fig. 1). A further exploration of a possible regional incidence of *B. contaminans* among isolates circulating in Brazil should prove interesting. In this regard, the incidence of RFLP K pattern strains in CF patients seen at two different hospitals in Brazil (1, 12) might also be indicating the existence of a regional spreading of *B. contaminans* between bordering countries.

Genetic diversity among *B. contaminans* isolates with the *recA*–ST-71 allele as investigated by Rep-PCR DNA fingerprinting analysis. The low level of polymorphism in the *recA* gene encountered in the *B. contaminans* population studied here prompted us to explore the genetic diversity among the isolates carrying the *recA*-ST-71 allele by repetitive element sequence-based PCR (rep-PCR; 30). This subset of 75 *B. contaminans* isolates was analyzed by REP-, ERIC- and BOX-PCRs. Amplification reactions were performed in a My Cycler Thermal Cycler (BIO-RAD, USA) with the REP1R-I, REP2-I, ERIC1R-I, and BOX-A1R primers (30). The amplicons generated were electrophoresed in agarose gels and the banding patterns obtained analyzed by means of the GelCompar II version 2.1 software package (Applied Maths, Kortrijk, Belgium). The profiles yielding a band similarity of 85% or greater were considered related clones for all assays. According to this criterion, both REP- and ERIC-banding patterns displayed a very limited diversity. With the REP primers, the isolates showed only four different banding patterns, with one

144 being observed in 92% of the isolates. Similarly, when ERIC-PCR primers were used, 5
145 different patterns were found, with one subtype including 89% of the isolates (data not
146 shown). The highest discrimination power was obtained when BOX-PCR was performed.
147 This fingerprint typing yielded 10 distinct amplification profiles (B1 to B10; Fig. 2). The
148 distribution of the isolates' amplification profiles was as follows: the B8 subtype, 50%; the
149 B1 subtype, 22.9%; the B6 and B7 subtypes, 6.75%; the B9 subtype, 5.4%; and the B2,
150 B3, B7, B5, and B10 subtypes, 1.35%, these comprising only 1 isolate each.
151 Through the use of the first *B. contaminans* isolate recovered from each patient over the
152 period 2004–2010, the incidence of each BOX subtype was determined. Three subtypes,
153 B1, B7, and B8 were the most abundant patterns found, all together accounting for 75% of
154 the isolates. The B6 subtype was observed in 3 patients, while the remaining 6 subtypes
155 were unique fingerprints observed in only one patient each (Fig. 3A). With respect to the
156 geographical distribution, whereas the isolates with B7 and B8 subtypes were found at
157 both references centers at a remarkably high incidence, all the other BOX subtypes were
158 present at only one of the two locations (Fig. 3B). Thus, although the interpatient
159 transmission of *B. contaminans* with the B7 and B8 BOX subtypes could very likely have
160 been a dominant occurrence at both CF centers, other sources of new infections were
161 clearly present. Regarding the prevalence of each BOX subtype over time, during the
162 period of the outbreak (2004–2005), the CF patients were in most instances infected with
163 the B8 (55%), B7 (15%), and B6 (11%) subtypes—but also in a much lower proportion
164 with isolates belonging to other genotypes (B2, B3, B4, B9, and B10; Fig. 3C). We
165 therefore infer that the isolates belonging to the B8 genotype (and to a lesser extent to B7),
166 were efficiently transmitted at the outbreak period to produce a clonal spreading among
167 the patients (Fig. 3B and 3C). Nevertheless, that other different unique BOX subtypes (*e.*
168 *g.*, the B2, B3, B4, B9, and B10 subtypes) were also present should be stressed (Fig. 3C).
169 Hence, as previously reported for different *Burkholderia* spp. outbreaks (4), although a

high number of infections could have been the result of particular BOX subtype patient transmission, other sources of infection—such as through the environment or industrial products—might have accounted for the CF infections within that same time period. Additionally, it should be noted that since 2005 onward, isolates belonging to BOX subtypes B1 and B8 most notably seem to be responsible for new infections in CF patients. The PCR-fingerprinting techniques have been successfully applied previously for *B. cenocepacia, B. cepacia, B. multivorans, B. dolosa*, and *B. pseudomallei* clinical-isolate discrimination (4, 8). In fact, the application of ERIC- and BOX-PCR have proven to be valid technical alternatives to pulsed-field gel electrophoresis (6, 8, 10). In the present work, we were able to demonstrate that BOX-PCR is also a reliable typing method to examine the genetic relatedness among *B. contaminans* isolates so as to enable the identification of a spreading of two different genotypes within a very closely related group of isolates.

***B. contaminans* diversity in chronically infected patients.** CF patients are known to be susceptible to respiratory-tract chronic infection by certain *Burkholderia* species (3). Both the persistence of the same strain and the colonization with a different strain during the course of long-term infection have been reported (3, 7, 20). In 8 out of the 21 chronically infected patients analyzed in this work during the 7-year period, more than one *Burkholderia* species was recovered during the course of the infection (Table 1, rows 1 to 3), either as a consequence of coinfection or through subsequent colonization by different species (so-called "species replacement"). In contrast, in the remaining 13 chronically infected patients only one species (*B. contaminans*) was obtained (Table 1, rows 4 and 5). Upon consideration of the BOX subtypes of serially recovered isolates during the infection of each of these 13 patients we found that 5 patients remained infected with isolates carrying the same BOX subtype, whereas the other 8 evidenced a change in their isolate's BOX subtype during chronic infection. The latter result again could have occurred either

196 as a consequence of a "strain replacement" or through coinfection with different isolates
197 being collected—completely by chance—at the different samplings (31). In addition, the
198 BOX-PCR diversity among the isolates recovered throughout the course of prolonged
199 infection demonstrated that those clones isolated during the first year or years of infection
200 belonged to different BOX subtypes (B6–B8) with the B8 subtype being predominant—
201 that finding probably since the sample collection overlapped with the outbreak period
202 (2004–2005). In contrast, after a long-lasting infection (more then three years of being
203 colonized by *B. contaminans*), either the B1 or B8 BOX subtypes became predominant,
204 and were likely coexistent (31). Therefore, we conclude that the BOX-subtype diversity of
205 the infecting strains responsible for the chronic cases investigated seemed to decrease over
206 the time of infection, with B1 and B8 subtypes being the ones that mostly persisted—and
207 apparently replacing the initially infecting strains. This type of occurrence was found to be
208 common in *P. aeruginosa* infections; where after a period of recurrent colonization, the
209 CF patients became colonized permanently with a single lineage (17, 25). Once this *P.*
210 *aeruginosa* lineage became adapted to the hostile environment of the CF lung (*i. e.*, as the
211 "adapted dominant epidemic strain"), that bacterium could persist for several decades so
212 as to overcome both the host defense mechanisms and any intensive antibiotic therapy (14,
213 17, 26, 32).

214 In conclusion, the species *B. contaminans* was indeed able to develop chronic
215 colonization in CF patients. Especially isolates belonging to the B8 and B1 BOX subtypes
216 persisted during the 7 years of our surveillance, either infecting CF patients for the first
217 time (Fig. 3C) or colonizing CF patients chronically. We therefore can infer that the
218 aforementioned genotypes can survive in certain ecological niches or industrial products,
219 spread among patients, and most readily adapt to the lungs of CF patients. This research
220 has enabled a deeper understanding of the diversity of the *B. contaminans* isolates
221 recovered from CF patients treated in Argentina. Since a knowledge of the currently

222 infectious strains is critical in prescribing patient treatment and making prognoses, isolates
223 characterized by the B1 and B8 subtypes have now become the object of ongoing
224 investigations in our laboratory. This and the forthcoming information will provide deeper
225 insights into the strategies employed by *B. contaminans* to adapt and persist within CF
226 patients.
227
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235

Table 1. *Burkholderia cepacia* complex species recovered from 21 patients over the time period of their chronic infections.

***Burkholderia cepacia* complex species recovered from the initial sample** [a]	**Species recovered in subsequent samples**	**Number of patients**
B. contaminans	*B. cenocepacia*	4
B. cepacia	*B. vietnamiensis*	2
B. cenocepacia	*B. contaminans, B. cenocepacia*	2[b]
B. contaminans	*B. contaminans*	5[c]
B. contaminans	*B. contaminans*	8[d]

[a] Identification of the first available clinical isolates recovered from the chronic patients.

[b] Species different from the initial one was detected and then, in later cultures, the initial species was found again, indicating probable species co-infection.

[c] The same BOX-subtype was detected in subsequent cultures.

[d] Different BOX subtype patterns were detected in subsequent sputum cultures.

LEGENDS TO THE FIGURES

Fig. 1. Phylogenetic tree of the 79 *B. contaminans* CF isolates (Table S1 in Supplementary Material) based on the analysis of a 900 bp *recA* fragment sequence using the neighbor-joining method. Bootstrap values greater than 70% are shown for 1,500 replicates. Five *B. contaminans* reference sequences obtained from the GenBank sequence database were included in the analysis (ascension numbers indicated in brackets). *RecA*-gene-sequence types (*recA*-STs) were obtained by comparing the sequence of a 398 bp *recA* fragment against the public MLST database (http://pubmlst.org/bcc/). *B. xenovorans* was used as the outgroup.

Fig. 2. Dendrogram showing the relatedness among representative BOX-PCR fingerprinting patterns obtained from *Burkholderia contaminans recA*-ST-71 clinical isolates. The dendrogram was produced by the UPGMA method. The scale bars indicate the percent similarity. Clusters were delineated with an 85%-similarity cut-off value as indicated by the heavy vertical line. One isolate representative of each pattern is indicated. For the key to the abbreviations used for the medical centers, *cf.* the text.

Fig. 3. Distribution of BOX-PCR subtypes (B1–B10) of *B. contaminans* isolates with the *recA*-ST-71 allele obtained over a 7-year period with only the first isolate recovered from each patient being considered: A) Incidence; B) Geographical distribution; C) Temporal distribution.

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Fig. 1

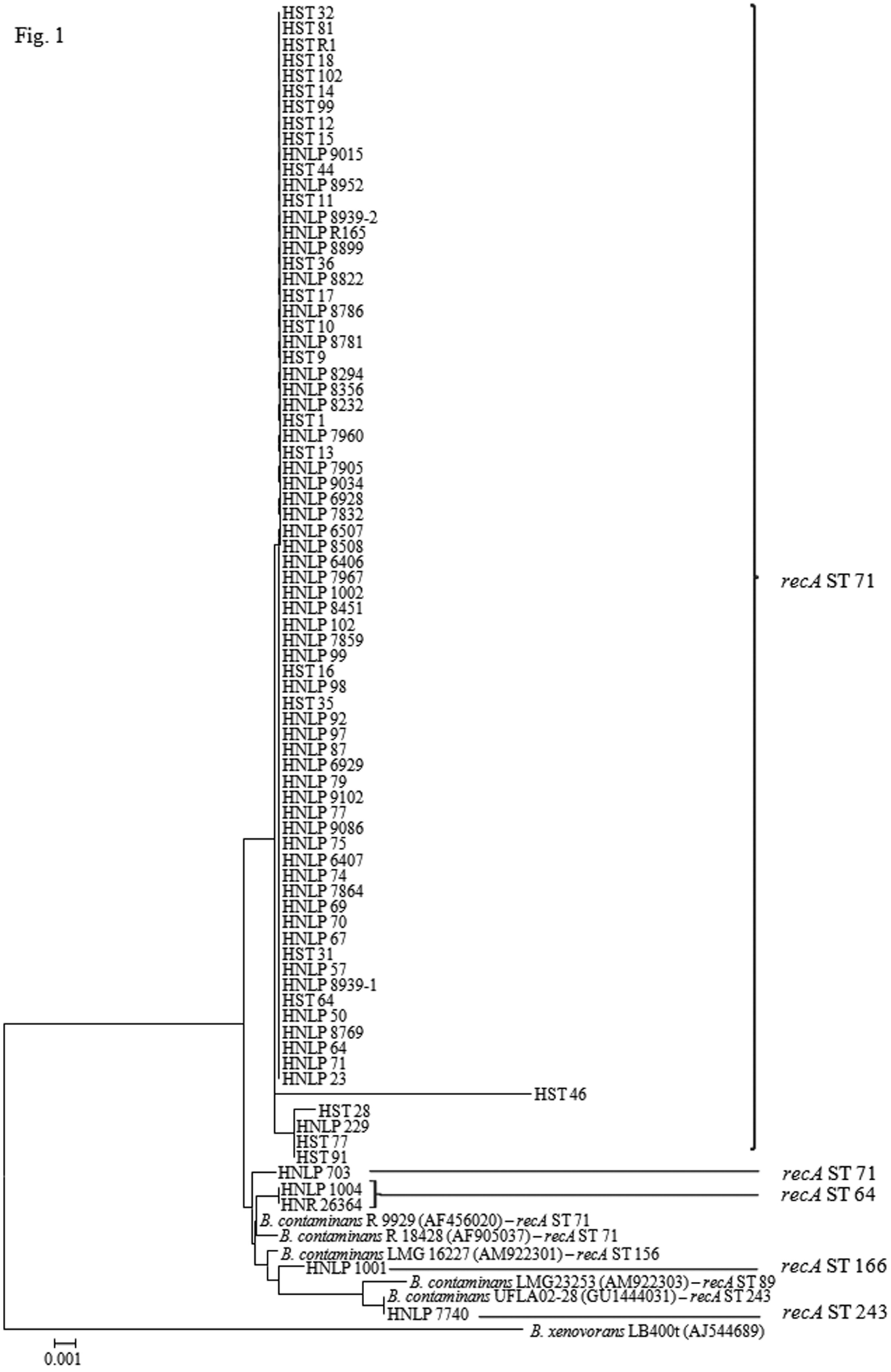

Fig. 2

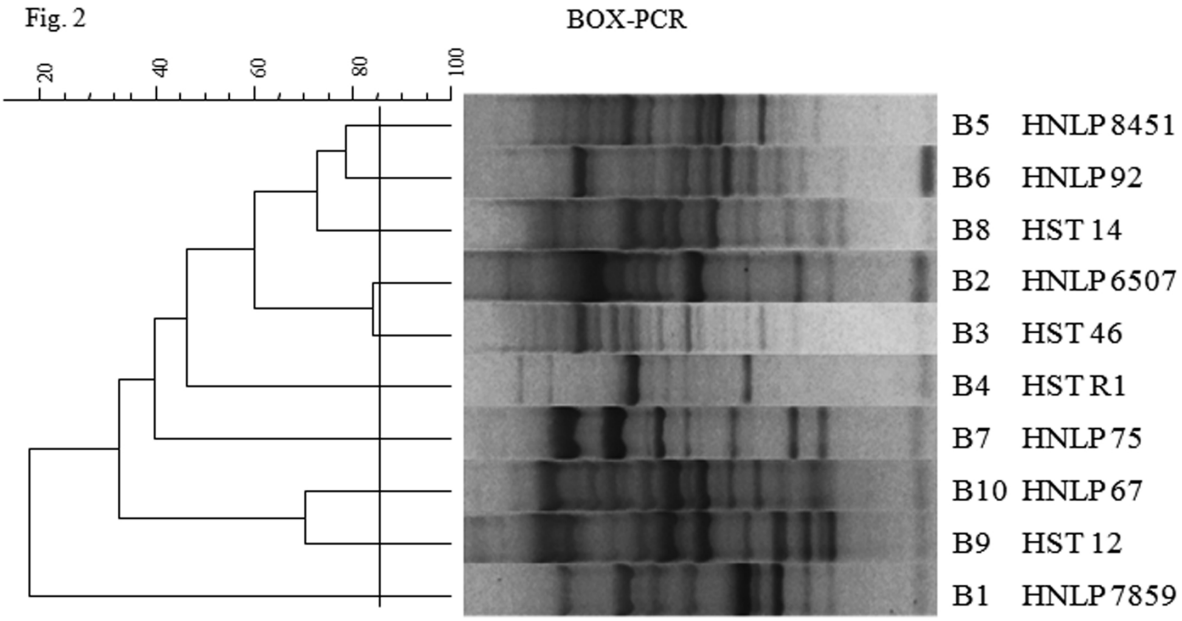

Fig. 3