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ORIGINAL ARTICLE

Molecular epidemiology of *Paenibacillus larvae larvae* and incidence of American foulbrood in Argentinean honeys from Buenos Aires province

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SUMMARY

Paenibacillus larvae larvae, the causative agent of American foulbrood disease of honey bee larvae occurs throughout the world and is found in all beekeeping areas of Argentina. Microbiological analysis of 394 honey samples obtained from bee hives from Buenos Aires province (Argentina) from three years of sampling (1999–2001) yielded 219 positive cases (55.6%). The incidence of *P. l. larvae* infected honey samples for 1999 was 68.1% (n = 160), for 2000 47.1% (n = 102), and 46.2% for 2001 (n = 132). The mean values of spore contamination for the three-year study showed a continuous reduction, probably due to good practices of disease management by beekeepers by breeding bees for hygienic behaviour and reduction of antibiotic treatments for control of AFB.

P. l. larvae populations isolated from honey were characterized on the basis of DNA fingerprints using the repetitive-sequence-based polymerase chain reaction technique (rep-PCR) with BOX- and REP- sequence-specific primers. Four distinctive patterns, named A, B, C, and D, were distinguishable among the isolates. Genotype D was not observed in previous studies; this finding could be correlated with a new introduction of the disease in Argentina since 1997 when only three genotypes (A, B, and C) were confirmed. The rep-PCR fingerprint patterns obtained were compared with the patterns generated by a world-wide collection of *P. l. larvae* strains. The same 4 genotypes patterns were found within a collection of strains from 18 different countries of the world. It is important to point out that pattern C was only found in Argentina and in one sample from Uruguay located in the border line, suggesting that genotype C could have been derived from genotype A and disseminated to Uruguay from Argentina. These findings support the hypothesis that American foulbrood disease is exposed to a limited selective pressure from climatic and environmental sources.

Keywords: AFB, *Paenibacillus larvae larvae*, honey, honey bees, *Apis mellifera*, PCR, DNA fingerprinting, molecular epidemiology, Argentina

INTRODUCTION

Argentina is the lead country in honey exports with an annual production of 80 000 t resulting in revenues of about US$104 million. Sixty percent of the total is produced in Buenos Aires province where there are about 400 000 beehives owned by 3000 beekeepers.

One of the most important factors that affects the development of the colonies and decreases production is the sanitary condition of the beehives. In this respect, American foulbrood (AFB) disease caused by the spore-forming Gram-positive bacterium *Paenibacillus larvae larvae* (Heyndrickx *et al.*,1996) (formerly *Bacillus larvae*) is the most serious disease of bacterial origin affecting the larval and pupal stages of honey bees (*Apis mellifera*). AFB is one of the few bee diseases capable of killing a colony and possesses unique problems for prevention and control because the spores can remain viable for long periods of time and survive environmental adversities (Matheson & Reid, 1992; Shimanuki & Knox, 1991). AFB is a disease of high socioeconomic importance and significance in international trade, with contaminated honey being one of the most important factors spreading the disease. The examination of honey for viable spores is a useful tool for determining the prevalence of AFB spores in beekeeping areas (Hornitzky & Clark, 1991; Hornitzky, 1999). In many countries, including Argentina, colonies are treated with antibiotics that suppress clinical signs by controlling only vegetative cells, but bacterial spores accumulate in the hive and contaminate honey, remaining infective for many years.

Molecular techniques have been developed for identification and characterization of the causative agent (Alippi & Aguilar, 1998a; Alippi & Aguilar, 1998b; Alippi *et al.*, 2002; Alippi *et al.*, 2004; Bakonyi *et al.*, 2003; Dobbelaere *et al.*, 2001; Govan *et al.*, 1999; Lauro *et al.*, 2003). In addition, genotypic characterization based on DNA fingerprinting generated by the polymerase chain reaction (PCR) is commonly used for the study of microbial communities from different environments. Families of repetitive

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DNA sequences REP, ERIC, and BOX (collectively known as rep-PCR) (Versalovic *et al.*, 1994) are dispersed throughout the genome of diverse species, and they are utilized to produce typical DNA fingerprint patterns that may reveal the inter- or intra-species diversity of different bacterial genera (De Bruijn, 1992; Louws *et al.*, 1994). This technique has been successfully used with *P. l. larvae* (Alippi & Aguilar, 1998a; Alippi & Aguilar, 1998b; Genersch & Otten, 2003), but there is little information on molecular epidemiological studies on AFB. For these reasons, the aims of this study were to determine the presence of viable *P. l. larvae* spores in honeys from Buenos Aires province (Argentina) and to compare the isolated populations using DNA fingerprints generated by rep-PCR with those from a worldwide collection.

MATERIALS AND METHODS

Honey samples

During the years 1999, 2000 and 2001, 394 honey samples were collected from different apiaries from the Buenos Aires province, the major honey producing state in Argentina. Samples were stored at 4 °C until analysis. The sampling number was chosen according to the following formula: $n = (K/c)^2 pq$ (Cantatore de Frank, 1980), where n is the size of sample randomly selected from the population, K is the area under normal curve corresponded to the desired confidence level value, c is the desired confidence level value, p is the chance of detecting the event, q is the chance of not detecting the event. We selected a confidence level of 0.95 in which n corresponds to a minimum of 386 samples. The samples were taken at random from different apiaries of the same region but were all different samples. No data about clinical signs of apiaries were registered in this study so no correlation between clinical signs and spore numbers were made.

Culture of honey for *P. l. larvae*

For isolation of viable *P. l. larvae* spores from honey, the method of Alippi (1995) was used with modifications. Briefly, honey samples were homogenized in a water bath kept at 40 °C, 10 ml of honey was mixed with an equal volume (1:1) of 0.01 M sodium phosphate buffered saline pH 7.2 (PBS), and centrifuged at 3500 G for 45 min at 5 °C. The supernatant was discarded leaving approximately 3 ml per tube that was then vortex-mixed for 1 min to resuspend the pellet, then heated at 85–90 °C for 15 min in a water bath in order to kill vegetative cells of bacteria and yeasts. The samples were high speed vortex-mixed again for 2 min and 100 µl of the sediment-fluid mixture poured on MYPGP plates (Dingman & Stahly, 1983) containing nalidixic acid (9 µg/ml) and pipemidic acid (20 µg/ml) (Sigma) to inhibit most of spore-forming bacteria present in honey samples (Alippi, 1995). The mixture was spread over the surface of the medium by using a sterile Drigalski spatula. Four plates were prepared for each honey tested. The plates were incubated at 37 ± 1 °C in an atmosphere of 10% CO_2 and examined after seven and 14 days. Colony forming units (cfu) of *P. l. larvae* were counted at 14 days. The highest number of cfu per plate that is possible to count with a Quebec colony counter is 1000 cfu. For control of growth of other spore-forming bacteria, one plate of tryptic soy agar (TSA) per sample were inoculated with 100 µl and incubated in aerobic conditions at 30 °C.

For isolation of *P. l. larvae* strains from larvae exhibiting clinical signs of AFB, a previously described technique was used (Alippi & Aguilar, 1998a).

Bacterial strains

A total of 382 *P. l. larvae* strains from diverse origins were used in this study and are listed in table 1. The collection includes: 219 isolates from honey samples obtained from the three year study; 99 strains from different geographical origins characterized in a previous study (Alippi & Aguilar, 1998a) including five strains from Culture Collections (Alippi & Aguilar, 1998a); 22 isolates from larvae showing clinical signs of AFB from different outbreaks in Argentina after 1997; 38 strains from other countries isolated from honey or diseased larvae or received as a culture after 1997; and four strains from other Culture Collections: ATCC 9545, ATCC 25747, ATCC 25748, and NRRL B-3555 (reference strain).

Identification of *P. l. larvae* isolates

P. l. larvae isolates were primarily identified by their distinct colony morphology and catalase reaction (Alippi, 1991; Alippi, 1992). Only one colony per honey sample from the four replicates was randomly selected for further study. The presumptive *P. l. larvae* cultures were grown on MYPGP agar plates to check purity and stored frozen at –80 °C in liquid MYPGP plus 20% glycerol (v/v) as cryoprotectant and also in sterile tap water at room temperature.

Identification of selected strains was confirmed by standard techniques as described by Gordon *et al.* (1973) and Alippi (1991) including shape and position of the endospore, lack of growth in nutrient agar (NA), growth at 20 °C, and reduction of nitrate to nitrite. In addition, sensitivity/resistance of all isolates to bacteriophage PPL1c by the spot test technique was tested as described by Stahly *et al.* (1999).

Identification of other spore-forming species

Other aerobic-spore forming species of the genera *Bacillus, Paenibacillus* and *Brevibacillus* that developed in the isolation plates (TSA controls and occasionally on the semi-selective medium) were identified on the basis of Gram reaction, colony morphology, and microscopic examination of bacterial smears by lipid globule staining procedure (Harmon *et al.*, 1991) and determination of presence of crystalline inclusions (Hannay, 1953). Presence of spores, as well as shape, location, and size of vegetative cells were also evaluated. Bacterial cultures were tested by catalase reaction, production of lecithinase, tyrosinase activity, anaerobic utilization of glucose, haemolysis, and starch hydrolysis according to the criteria of Gordon *et al.* (1978) and Lancette & Harmon (1980). When necessary, API 50 CH strips plus API 50CHB medium (Biomerieux, Buenos Aires, Argentina) were used for further identification.

Data analysis

The 394 honey samples were randomly selected during years 1999, 2000 and 2001 from all the honey producing counties of Buenos Aires province. Values of cfu obtained – calculated as the average of the four replicates for each honey sample – were ranked into a six-level scale developed for this purpose as follows: level 0 (0 detectable cfu per plate; level 1 (between 1 and 20 cfu per plate; level 2 (between 21 and 50 cfu per plate; level 3 (between 51 and 199 cfu per plate; level 4 (between 200 and 999 cfu per plate; and level 5 (1000 cfu per plate. Pearson Chi Square test was used to determine significant differences ($P > 0.05$) between spore contamination levels in honey in the three years of study.

DNA preparation

Bacterial cells from active growing cultures were grown in MYPGP agar in aerobic conditions for 48 h. For bacterial DNA preparation, a rapid procedure by using whole cells from plates was employed (Alippi & Aguilar, 1998a). After centrifugation to remove bacterial debris and resin, the supernatant was used as DNA template.

rep-PCR fingerprinting The rep-PCR method with BOXA1R, REP (REP1R-I and REP2-I) and ERIC (ERIC1R and ERIC2) primers was used (Versalovic *et al.*, 1994). PCRs were carried out in a final volume of 25 µl according to the protocols of Louws *et al.* (1994) using 5 µl of the supernatant of crude DNA preparation as template source (Alippi & Aguilar, 1998b).

The reaction mix was overlaid with a drop of mineral oil and incubated in a thermal cycler (Biometra Uno Thermoblock) according to the specifications of Louws *et al.* (1994) for each primer. Five microliters of the PCR reactions were run on a 1.6% agarose gel in TBE buffer 0.5 × and observed under UV light after being stained with ethidium bromide (Ausubel *et al.*, 1992) and photographed using a digital image capture gel documentation system (Kodak digital Science 1D, model DC120). Fingerprints generated were compared visually. In addition, digital images of PCR profiles were analysed with Gelcompar v. 4.1 software (Applied Maths, Kortrijk, Belgium) using the UPGMA clustering algorithm and DICE coefficient for a combined gel.

RESULTS

Detection and identification of *P. l. larvae* and other aerobic spore-forming bacteria in honey samples

From the 394 honey samples collected from Buenos Aires province, 219 were found to contain viable *P. l. larvae* spores (55.6%), ranging from the detection of only a single colony of *P. l. larvae* (level 1) to overgrown plates (level 5). 298 honeys (76% of samples) also contained other spore-forming bacteria as observed on duplicate TSA plates that have been inhibited by the incorporation of nalidixic acid and pipemidic acid in the semi-selective MYPGP agar. Some of these TSA plates contained more than one bacterial species, the most prevalent being *Paenibacillus alvei* (40%), *Bacillus cereus* (28%), and *Bacillus megaterium* (14%). On the other hand, *Brevibacillus laterosporus, Bacillus thuringiensis, Bacillus sphaericus, Bacillus mycoides, Bacillus subtilis, Bacillus circulans*, and *Bacillus pumilus* were also found in a minor proportion (a total of 6%).

Regarding MYPGP semi-selective isolation plates, 14 samples (4%) were overgrown by other spore-forming species such us *B. circulans* and *B. sphaericus* that are able to grow in the presence of nalidixic acid and pipemidic acid at the concentrations used. In addition, some resistant isolates of *B. laterosporus, B. licheniformis, B. pumilus,* and *P. polymyxa* were detected. Nevertheless, the efficiency of recovery of this method with the use of semi-selective medium was high; from 394 honey samples analysed, 219 isolates of *P. l. larvae* were obtained and 161 samples yielded negative results without any bacterial contamination.

The presence of viable *P. l. larvae* spores in honey samples for the three successive years was determined and found to be 68.1% (n = 160) for year 1999, 47.1% (n = 102) for 2000, and 46.2% for 2001 (n = 132) (fig. 1 and table 2). In all years, the maximum level (5) and minimum level (0) of spore contamination were found. The means of spore contamination levels during the three-year study showed a continuous decrease (table 2). Pearson Chi square test showed significant differences in the levels 1, 3, 4, and 5 in the 3 years (table 3), with 1999 being the year with highest spore contamination levels (table 3 and fig. 1).

All the *P. l. larvae* isolates obtained from the honey sampling were Gram positive, catalase negative, able to reduce nitrate to nitrite,

TABLE 1. Genomic fingerprint patterns (named A, B, C, and D) among *Paenibacillus larvae larvae* populations and origins of the strains examined. Data are from this study and from Alippi & Aguilar (4).

Country	Fingerprint				Total per country
	A	B	C	D	
Argentina[1]	258	6	21	9	294
Belgium[2]	2	0	0	0	2
Brazil[1]	1	0	0	0	1
Canada[1]	2	1	0	0	3
Czech Republic[3]	0	4	0	0	4
Chile[4]	0	3	0	0	3
France[5,6]	6	3	0	3	12
Germany[7]	3	0	0	0	3
Italy[1,8]	4	4	0	0	8
Japan[9]	2	0	0	0	2
New Zealand[10]	1	8	0	0	9
Poland[11]	4	2	0	0	6
Spain[4]	1	4	0	0	5
Sweden[12]	1	3	0	0	4
Tunisia[1]	1	0	0	0	1
United Kingdom[13]	0	3	0	0	3
USA[1,14,15,16]	12	0	0	2	14
Uruguay[17]	7	0	1	0	8
Totals	305 (79%)	41 (11%)	22 (6%)	14 (4%)	382 (100%)

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Fig. 1

and unable to grow in NA or at 20 °C. As honey samples were taken at random from different apiaries, some of the same region but others from different areas, it was considered that each isolate (one per honey sample selected) corresponds to a different strain of *P. l. larvae*. Two hundred and sixteen out of 219 strains (99%) of *P. l. larvae* tested for sensitivity to PPL1c were found to be sensitive. Concerning nitrate reduction test, all the *P. l. larvae* isolates from Argentina were positive, whereas comparing with the worldwide collection only four strains from France and one from the USA (NRRL B3555) were negative (1% of the total examined).

TABLE 2. Descriptive statistics: number of honey samples by year and values of spore contamination levels.

Year	Number of cases	Mean value of spore contamination levels
1999	160	2.1
2000	102	1.5
2001	132	0.8

rep-PCR amplification and analysis of amplification products

DNA fingerprint analysis of the *P. l. larvae* collection by using, respectively, primers BOX- and REP- revealed four genotypes named A, B, C, and D (figs 2 and 3), each consisting of a variable number of bands: between nine and 11 bands with molecular sizes ranging from 100 bp to 2.1 kb for BOX-PCR, and 12 and 15 bands ranging from 150 bp to 2.1 kb for REP-PCR. On the other hand, we found a unique fingerprint ERIC- pattern of about 14 bands ranging from < 100 bp to 2 kb (fig. 2) within the collection of 382 *P. l. larvae*, so only data from BOX- and REP- primers were selected to construct the dendrogram shown in figure 3. Analysis of the combined BOX- and REP- revealed that all strains clustered together in four groups at levels of 100% within each one (fig. 3). While clusters D and B clustered together at a level of 85%, clusters A and C clustered together at a

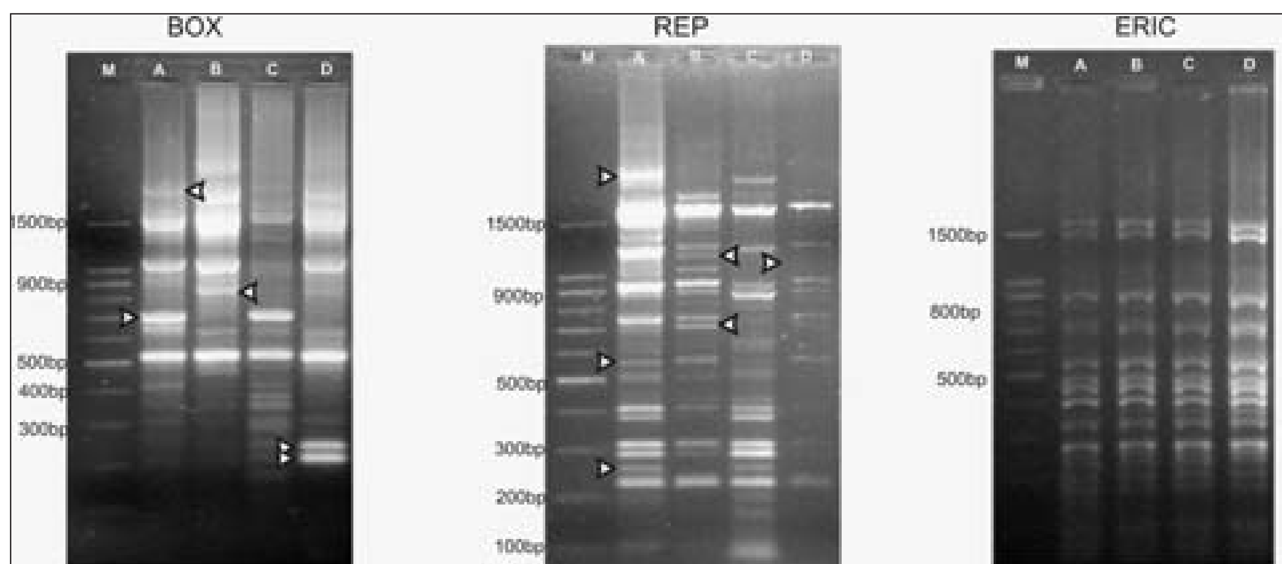
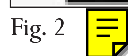


Fig. 2 

level of about 78%, and the four groups clustered together at a level of about 72% (fig. 3) revealing a closer relationship between genotypes A and C and genotypes B and D, respectively.

When we examined the whole collection of *P. l. larvae* isolates from Argentina (*n* = 294) (Alippi & Aguilar, 1998a and this study), a higher prevalence of pattern A was observed over the rest of the genotypes (A (88%, B (2%, C (7% and D (3%); when we investigate only the *P. l. larvae* population isolated from Argentinean honeys in Buenos Aires province in the three-year period of sampling, the prevalence of pattern A was also observed (A = 93.4%; B = 0.3%; C = 4.3% and D = 2%).

DISCUSSION

Each *P. l. larvae* colony produced on a plate is equivalent to three viable spores per ml of honey, corresponding to approximately two spores per g honey with 1 ml honey being estimated at 1.4 g. These values represent the minimum level of detection of the technique used involving the employment of the semi-selective medium. The highest number of cfu per plate possible to count with a Quebec colony counter is 1000 cfu which corresponds to about 2000 viable spores per g honey.

As only 6% of spores germinate in culture media (Dingman & Stahly, 1983), our value of two viable spores per g honey corresponds to about 33 total spores per g honey, and our value of 2000 viable spores per g is equivalent to about 33 333 total spores per g honey. The 6% value of spore germination in culture medium obtained by Dingman & Stahly (1983) was corroborated by Reynaldi (2001) by performing spore counts by direct microscopic counting in a Petroff-Hausser counting chamber and compared with the number of colony forming units obtained by plating different *P. l. larvae* spore dilutions on PBS mixed with honey on the surface of MYPGP plates with the addition of both antibiotics.

The presence of *P. l. larvae* viable spores in honey samples for the three successive years was found to be 68.1% for 1999, 47.1% for 2000 and 46.2% for 2001, reflecting a reduction of the number of spore counts since 1999 in spite of the high levels of spore contamination found in honey (table 3 and figure 1). This may be due to better practices of disease management by the beekeepers, including reduction in the number of antibiotic treatments and incorporation of honey bee lines with better hygienic behaviour (Spivak & Gilliam, 1998a, 1998b; Del Hoyo *et al.*, 2000).

Taking into account the total of *P. l. larvae* spores in honeys from Buenos Aires province, from 394 samples collected 219 were positive (55.6% incidence), ranging for the detection of a single colony (level 1) to completely overgrown plates (level 5). Hornitzky & Clark (1991) correlated the score of colony forming units per isolation plate and the AFB disease status of the beekeeper, finding that a score of one (equivalent to our 1 level) gives 60% chance of finding the disease in beehives of origin; a score of two (equivalent to our level 2) gives a 80% chance of finding the disease, and a score of three (equivalent to our levels 3, 4 and 5) raises the probability to near certainty. The reasons why a honey culture test could prove positive in colonies with no clinical signs of AFB could include recently-robbed contaminated honey, disease about to occur, disease recently cleared up, or disease symptoms masked by antibiotics (Hornitzky & Clark, 1991).

TABLE 3. Comparison of the levels of infection during three sampling years.

	Number of samples for year			Total	*P* value
	1999	2000	2001		
Level 0	51	54	71	176	0.138
Level 1	29	6	35	70	< 0.0005
Level 2	14	8	14	36	0.368
Level 3	21	11	9	41	0.049
Level 4	30	21	2	53	< 0.0005
Level 5	15	2	1	18	< 0.0005

80 90 100

PI 310
PI 174
PI 155
PI 127
PI113
PI361
PI312
NRRL B-3555
ATCC 25748
PI 173
PI 224
PI 257
PI 280
PI 322
PI 331
PI 346
PI 351
ATCC 9545
ATCC 25747
PI 114
PI 120
PI 134
PI 144
PI 178
PI 185
PI 234
PI 251
PI 225
PI218
PI279
CCM 44833
PI200

C
A
D
B

BOX REP

Fig. 3

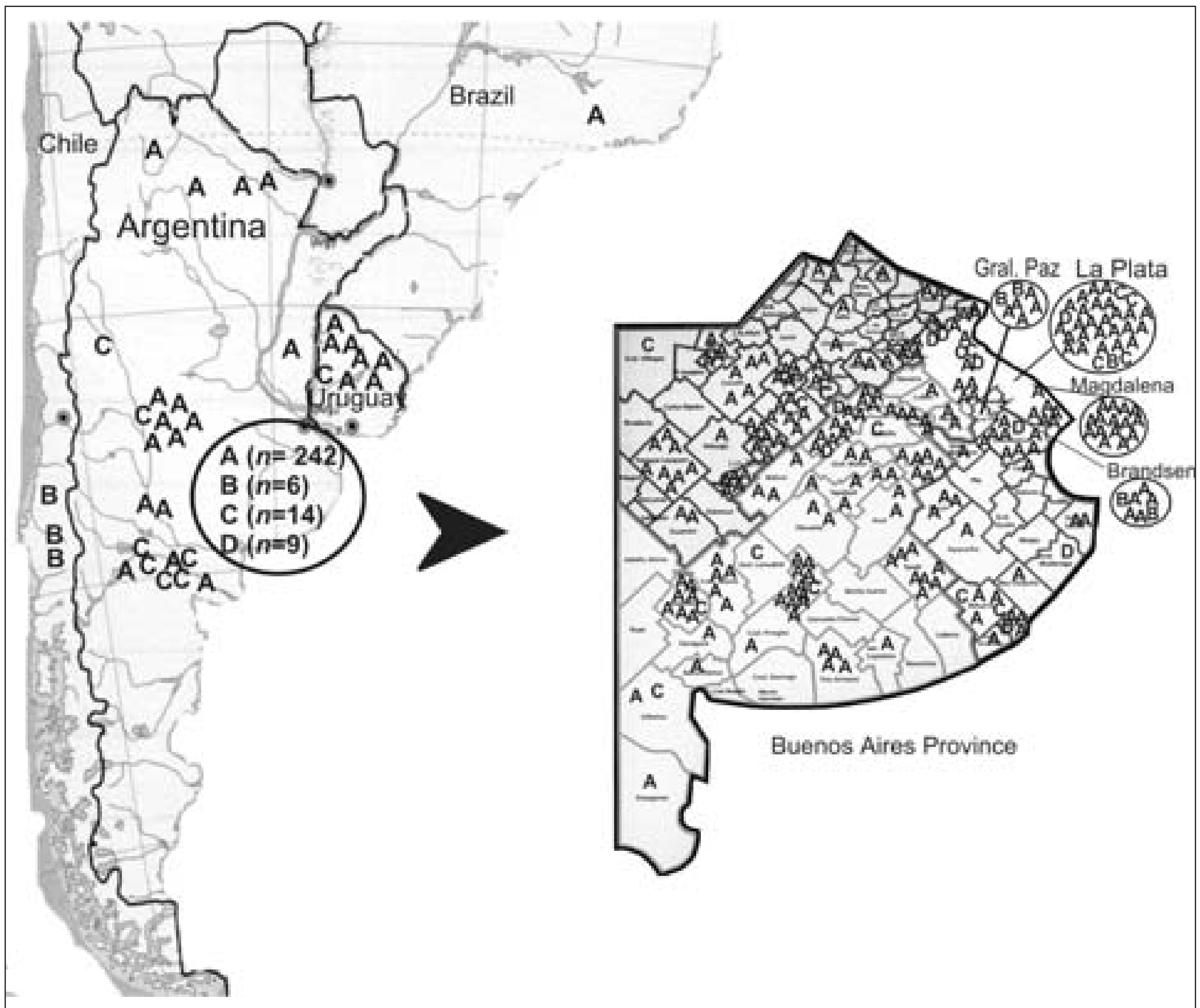


Fig. 4

As observed on TSA control plates, the most prevalent spore-forming species found in Argentinean honeys were *Paenibacillus alvei, Bacillus cereus*, and *Bacillus megaterium*. The high prevalence of *P. alvei* in honeys is in concordance with a previous study (Alippi, 1995) and with observations by authors in Australia (Djordjevic *et al.*, 2000; Hornitzky & Clark, 1991); also, the presence of *B. cereus* and *B. megaterium* spores was observed in honeys from other countries (Snowdon & Cliver, 1996).

In spite of the fact that 4% of samples were overgrown by other spore-forming bacterial species that were not inhibited by both antibiotics, including *Bacillus circulans, Bacillus sphaericus* and some resistant strains of *Brevibacillus laterosporus, Bacillus licheniformis, Bacillus pumilus,* and *Paenibacillus polymyxa* (Priest *et al.*, 1988; Alippi, 1995), the efficiency of recovery of this method was high: from 394 honey samples analysed, 219 isolates of *P. l. larvae* were obtained, and 161 samples yielded negative results without any bacterial contamination. It should be pointed out that no isolates of *Paenibacillus larvae pulvifaciens* were found in any of the studied samples; this subspecies has been reported to cause a minor disease of honey bee larvae called powdery scale (Shimanuki & Knox, 1991). Based on our results we believe that this disease is not present in Argentina.

The results of Gram reaction, catalase production, nitrate reduction, lack of growth in nutrient agar, and growth at 20 °C obtained here and those obtained by microscopic examination of spores and vegetative cells are in agreement with the description of subspecies *P. l. larvae* (Alippi, 1991; Gordon *et al.*, 1973; Heyndrickx *et al.*, 1996).

216 out of 219 strains (99%) of *P. l. larvae* tested for sensitivity to PPL1c were found to be sensitive. When comparing data with those obtained from the worldwide collection, only 2% of strains were resistant to PPL1c (of 382 strains tested, 375 were positive). These results confirm that the PPL1c spot-test is a useful tool for identifying *P. l. larvae* (Stahly *et al.* 1999). Concerning nitrate reduction test, all the *P. l. larvae* isolates from Argentina were positive, whereas comparing with the worldwide collection only four strains from France and one from the USA (NRRL B3555) were negative (1% of the total examined) which reflects the variation reported by Jelinski (1985) for this test.

The results on rep-PCR fingerprinting showed a high homogeneity in the populations of *P. l. larvae* from different geographical areas. In the present study we found a unique ERIC- pattern within the collection of *P. l. larvae*; the same results were obtained by Alippi & Aguilar (1998a, 1998b) and were confirmed with the analysis of 283 new isolates of the foulbrood pathogen collected from 1998 to 2003. This result is not in correspondence with the results obtained by Genersch & Otten (2003) where two ERIC- patterns were revealed within a collection of 105 isolates from Germany. In relation to results obtained by using BOX- and REP- primers, we found four genotypes in both cases, while Genersch & Otten (2003) found three different BOX patterns and two REP patterns (Genersch & Otten, 2003), but their REP pro-

files were obtained by using MBO REP 1 primers instead of REP1RI/REP2-I. As results for REP- subtyping were not comparable because different sets of primers were used, the other differences found with BOX- and ERIC- primers could be due to inter-laboratory reproducibility of rep-PCR or to diversity in populations of the foulbrood pathogen between Argentina and Germany.

The genotype D was not observed in previous studies (Alippi & Aguilar, 1998a; Alippi & Aguilar, 1998b), but in the present work was found in nine isolates from Argentina, three isolates from France, and two from the USA (fig. 2 and table 1). Hence these isolates correspond to a new genotype of *P. l. larvae* with two distinctive bands of about 200 and 250 bp clearly visible when using primers BOX (fig. 2). This finding could be correlated with a new introduction of the disease in Argentina since 1997 where formerly only three genotypes (named A, B, and C) were known (Alippi & Aguilar, 1998a; Alippi & Aguilar, 1998b). Reference strains ATCC 9545 (type strain of *P. l. larvae*), ATCC 25747, ATCC 25748 and NRRL B-3555 from USA showed pattern A (this study), while strains from France (CCT 491) and from Chezch Republic (CCM 4483, CCM 4484, CCM 4485, and CCM 4486) presented pattern B (Alippi & Aguilar, 1998a). Differences between patterns were easily noticeable when using primers BOX in relation to primers REP, but the correlation was maintained (figs 2 and 3). As shown in figure 4, genotypes A and C were found in all beekeeping regions of Argentina, whereas patterns B and D were found only in six and nine samples from Buenos Aires province, respectively (fig. 4). On the other hand, pattern C was found in Argentinean samples from Buenos Aires province and also in Río Negro, Córdoba and Mendoza. It is important to point out that pattern C was only found in Argentina and in one sample from Uruguay located near the border, suggesting that genotype C could have been derived from genotype A and disseminated to Uruguay from Argentina, taking into account that AFB was introduced in Argentina in 1989 (Alippi, 1992) and reported in Uruguay in 2000 (Piccini & Zunino, 2001). The isolates from the first outbreak of AFB in Argentina belonged to genotype A which was introduced into the country by a queen importation from the USA (Alippi, 1992; Alippi & Aguilar, 1998a; Alippi & Nuûez, 1991). The isolates of *P. l. larvae* from Uruguay analysed here belonged to genotypes A and C only (table 1 and fig. 4). AFB was recently detected in Brazil (Tochetto Schuch *et al.*, 2003) and when analysing honey samples from Brazil (n = 12), only one honey sample was contaminated with spores of *P. l. larvae*, the isolated strain belonging to genotype A (this study). AFB could be introduced to Brazil from Argentina or from Uruguay (fig. 4) or with the importation of honey because Brazil mainly imports honey from Argentina (http://www.apiservices.com/database/honeymarket). In Chile, genotype B was found in an outbreak and it is suspected that its introduction was through imported honey from Spain (SAG, personal communication) where fingerprints B and A were present (table 1).

According to the analysis of a limited number of *P. l. larvae* strains from countries other than Argentina (n = 88) (table 1), a prevalence of genotype A was also observed (53%) whereas genotypes B, C and D were represented in 40%, 1%, and 6%, respectively (table 1). Genotype B was found in small proportion in Argentina (2%) but with high prevalence in the rest of the countries analysed here (B (40%), mostly from Europe (table 1). Genotype D (fig. 1) was not observed in previous studies (Alippi & Aguilar, 1998a; Alippi & Aguilar, 1998b, Genersch & Otten, 2003) and is showed by nine isolates from Argentina (all from Buenos Aires province, fig. 4), three from France and two from the USA (table 1).

In conclusion, *P. l. larvae*, the causative agent of AFB, showed considerable genetic homogeneity among isolates of diverse geographical origin. When examining BOX-PCR and REP-PCR fingerprint patterns, only four genotypes showing minor profile variations (fig. 4) were found within a collection of 382 strains from 18 different honey producing countries of the world, while when using ERIC-primers no differences were noticeable (figs 2 and 3). These findings support the hypothesis suggested by Djordjevic and co-workers (1999) that the two bacterial pathogens that cause AFB and European foulbrood diseases are exposed to a limited selective pressure from environmental sources.

Early diagnosis of *P. l. larvae* is important in order to prevent outbreaks of AFB. We believe that the technique we used here for the detection of viable spores of *P. l. larvae* in honey is highly effective and could be useful to trace disease at early stages of infection and ascertain disease prevalence in a determined geographical area.

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