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A new serotype 14 variant of the pneumococcal Spain^{9V}-3 international clone detected in the central region of Argentina

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The penicillin-resistant Spain^{9V}-3 clone of *Streptococcus pneumoniae* is widespread and presents different serotype variants originating from recombination of the capsular genes. In this work, the genetic relatedness of 29 invasive pneumococci isolated from the central region of Argentina (Cordoba, Buenos Aires, Santa Fe and La Pampa provinces) was assessed by multilocus sequence typing (MLST). All of the penicillin-non-susceptible isolates studied (21/29) belonged to a serotype 14 variant of the Spain^{9V}-3 clone. This clone was predominant, suggesting that it was responsible for the penicillin resistance spread in this region. Interestingly, this serotype 14 variant (named Cordoba S14V) could be differentiated from the European one by its *pbp1a* gene, suggesting a different recombinational replacement of the capsular genes. The putative recombination sites were analysed, resulting in the proximal crossover point being clearly localized in the *spr0309* gene, with the distal site restricted to the *recU* gene, confirming a different recombination event. Analysis of the *dexB*, *cpsB*, *aliA* and *pbp1a* genes from these strains showed a high similarity with the corresponding genes of the Spain^{14}-5 clone, suggesting that the capsular genes were provided by this international clone. Analysis of the genetic polymorphisms of the *pbp1a* (nt 1473–1922) and *spr0309* (nt 1–790) genes is proposed as an epidemiological tool to help recognize the Cordoba S14V of the Spain^{9V}-3 clone. On the other hand, BOX-repeat-based PCR and MLST analyses of serotype 14 strains revealed a divergent epidemiology of the Cordoba S14V, suggesting a non-recent dissemination in the paediatric population. It is suggested that this molecular epidemiology work will be a reference for monitoring the evolution of S14Vs of Spain^{9V}-3, the emergence of new clones and the impact of pneumococcal vaccination programmes in Argentina.

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INTRODUCTION

Worldwide, *Streptococcus pneumoniae* is one of the main human pathogens, being the causal agent of infections such as otitis and sinusitis, as well as severe infections such as pneumonia, bacteraemia and meningitis. In developing countries, more than 1 million children under 5 years of age are estimated to die annually from pneumococcal pneumonia (Greenwood, 1999). The major epidemiological concerns about pneumococcal infection are determining the origin of emergence and the dissemination of drug-resistant strains. Penicillin-resistant isolates were first detected in the late 1960s, and their prevalence increased rapidly during the 1980s. Since 1989, the incidence of penicillin-resistant pneumococci has remained fairly constant at about 20–30 % of all isolates (Lynch & Zhanel, 2005). Molecular characterization of penicillin-resistant strains from around the world has highlighted an important diversity among isolates, but has also identified a number of successful pneumococcal clones with a high

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Abbreviations: BOX-PCR, BOX-repeat-based PCR; MLST, multilocus sequence typing; ST, sequence type.

level of penicillin resistance, some of which have spread globally (Crook & Spratt, 1998). Countries such as Spain, South Africa and Hungary reported high frequencies of penicillin-resistant pneumococcal infections in the mid-1970s (Fenoll *et al.*, 1998), followed later by France, Iceland and the USA in the mid-1980s to early 1990s (Breiman *et al.*, 1994; Lefevre *et al.*, 1995). The increasing number of penicillin-resistant clones, the diversity of the genotypes and the serotype exchange make it difficult to assign isolates to these clones. It is now recommended that new genotype assignments should be made only after the strain has undergone careful PFGE, BOX-repeat-based PCR (BOX-PCR) or multilocus sequence typing (MLST) analysis (McGee *et al.*, 2001). One of the best-characterized clones is Spain^{9V}-3, which appears to have emerged in Spain during the 1980s (Fenoll *et al.*, 1991). This clone has been prevalent in France for the last 15 years and has been found in many other countries including Germany, the Netherlands, Poland, Denmark, Uruguay, Argentina, Brazil, Taiwan and South Africa (Castanheira *et al.*, 2004; Coffey *et al.*, 1999; Corso *et al.*, 1998; Hermans *et al.*, 1997a, b; Shi *et al.*, 1998).

Strains that are highly related by MLST may express different serotypes. This phenomenon is caused by a recombinational replacement occurring in neighbouring regions of the capsular (*csp*) genes. The Spain^{9V}-3 clone has been isolated as serotype 9A, 9V, 14 and 19 variants (McGee *et al.*, 2001). For the European serotype 14 variant (S14V) of this clone (isolated in Uruguay, Denmark and Spain), the proximal recombination site was detected in the *cspA* gene, whereas the distal site was localized in the *pbp1a* gene (Coffey *et al.*, 1999).

The main purpose of this work was to assess the genetic relatedness of invasive pneumococcal strains isolated from children in Cordoba, Argentina, using genotypic methods such as MLST, BOX-PCR and RFLP analysis of the penicillin-binding protein (*pbp*) genes. Our results showed that all penicillin-non-susceptible strains belonged to a new S14V of the Spain^{9V}-3 clone; therefore, we decided to investigate whether this variant was similar to others described previously by analysing the recombinational replacement of the capsular genes and their flanking regions. We also examined whether this S14V was disseminated among other provinces in Argentina.

METHODS

Bacterial strains and antimicrobial susceptibility testing. All of the pneumococcal strains were obtained from invasive infections of paediatric patients and were collected from public children's hospitals of different provinces (Cordoba, Buenos Aires, La Pampa, Santa Fe and Buenos Aires City) during the period 2003–2005. The original Spain^{9V}-3 clone (ATCC 700671) and the Spain^{14}-5 clone (ATCC 700902) were utilized as references. Serotyping was performed using the Quellung reaction with sera produced by the Statens Serum Institut, according to the manufacturer's instructions. For antimicrobial susceptibility testing, strains were grown at 37 °C in an atmosphere of 5 % CO_2 on Mueller–Hinton agar with 5 % defibrinated sheep blood. Penicillin MICs were determined by agar dilution following a CLSI protocol (CLSI, 2007).

RFLP analysis of *pbp* genes. *pbp* genes were amplified by PCR using the following primers: F1a/R1a for *pbp1a*, F2b/R2b for *pbp2b* and F2x/R2x for *pbp2x* (Table 1). The reaction mixtures contained 2 mM $MgCl_2$, 0.5 μM each dNTP and 2 U *Taq* DNA polymerase (Invitrogen). PCR was performed using a Bio-Rad Gene Cycler for 30 cycles of 95 °C for 1 min, 53 °C for 30 s and 72 °C for 45 s. Purified PCR product (1 μg) was digested with *Mse*I/*Dde*I (3 U each enzyme) or 2 U *Hin*fI for 2 h at 37 °C and analysed by 7 % non-denaturing PAGE.

PCR conditions. For amplification of the *pbp1a* DNA fragments, the primer pairs F1a/R1a$_2$, F1aS/R1aS, F1aM/R1aM and F1aF/R1a were utilized. An internal fragment of the *cpsB* gene was amplified using the primers cpsB-down (Coffey *et al.*, 1998) and cpsS3 (Kong & Gilbert, 2003). These PCR products were all amplified using the following PCR parameters: initial denaturation at 94 °C for 4 min, 35 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 30 s and elongation at 72 °C for 1 min, and a final extension at 72 °C for 10 min. A 639 bp internal fragment from the *dexB* gene and an 842 bp internal fragment from the *aliA* gene were amplified using the primer pairs Fdex/Rdex and Fali/Rali, respectively. The PCR parameters were initial denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 52 °C for 45 s and 72 °C for 1 min. The *spr0309* and *recU* genes were amplified using the primer pairs Fspr/Rspr and FrecU/RrecU, respectively. All primer sequences are shown in Table 1. Chromosomal DNA was isolated using a Wizard Genomic DNA purification kit (Promega) following the manufacturer's instructions. The PCR products were amplified from chromosomal DNA and sequenced in both directions using the primers used for amplification. The *cpsB* sequences from the Baltimore strains were kindly provided by Dr Lee Harrison (McEllistrem *et al.*, 2004).

MLST. MLST was performed as described previously (http://www.mlst.net). The internal fragments from the *aroE*, *gdh*, *gki*, *recP*,

Table 1. List of primers used in this work

Primer	Sequence (5′→3′)
F1a	CGGCATTCGATTTGATTCGCT
R1a	GGTTGTGCTGGTTGAGGATT
F2b	GATCCTCTAAATGATTCTCAGGTGG
R2b	CAATTATAGCAATAGGTGTTGG
F2x	CGTGGGACTATTTATGACCGAAATGG
R2x	AATTCCAGCACTGATGGAAATAAACATATTA
R1a$_2$	CTGATTCGGTTGTGTTACTTGAAATGGC
F1aS	GCAAGTAGTGAAAARATGGCTGCTGC
R1aS	GACTGTGAAGTTGAACTWTCTGATC
F1aM	CACAGCCGTTGAGACTTTTA
R1aM	GGTCATCATGTAAGCAGTTG
F1aF	TGGACAGGTTATTCGAATCGT
Fdex	AAGATGGAGTTGGTGATTTG
Rdex	TCTCCCCTACTGTCAAGAGA
Fali	ACATTATTGGCGGCGATCAC
Rali	TTGTCCTTCATACTCTTCTCAA
Fspr	CCCATATCTGTCGGTGAAGC
Rspr	CCATGTCCTCCATGGTTCC
FrecU	TGCTTCTGGATGTGAATAGGG
RrecU	CTATTTGTCTCAGGGCTTGG

spi, *xpt* and *ddl* genes were amplified by a PCR utilizing primers described previously (Enright & Spratt, 1998). DNA sequencing was performed by Macrogen and DNA sequences were edited using BioEdit software (Hall, 1999). Alleles and sequence types (STs) were assigned using the database available at http://www.mlst.net.

BOX-PCR typing. The BOX elements were amplified from genomic DNA using the primer BOXA1R (Koeuth *et al.*, 1995). The reaction conditions were 30 cycles of denaturation at 95 °C for 1 min, annealing at 52 °C for 2 min and elongation at 72 °C for 2 min. PCR was performed using a PTC-100 MJR thermal cycler. PCR products were analysed by electrophoresis on a 2 % TAE/agarose gel.

RESULTS

Determination of the genetic relatedness of invasive pneumococcal strains

Initially, 66 invasive pneumococcal strains were isolated from children under 5 years of age attending public children's hospitals in Cordoba, Argentina, during the period 2003–2005, and 20 of these were non-susceptible to penicillin (Table 2). To analyse the genetic relationship of this pneumococcal population by MLST, we selected nine penicillin-non-susceptible isolates with a high penicillin MIC and eight penicillin-susceptible isolates, based on the serotype prevalence in our region. The results showed that all penicillin-non-susceptible strains belonged to the S14V of the Spain^{9V}-3 clone. Seven strains presented an identical allelic profile to ST156 and two strains showed a single-locus variant, ST370 (Table 3). To test the clonal evolution of the Cordoba S14V strains, we used molecular markers that diverge more frequently than housekeeping genes, such as BOX-PCR and RFLP of *pbp* genes. Our Spain^{9V}-3 strains showed five BOX profiles (data not shown), indicating an epidemiological divergence of this clonal complex and a non-recent dissemination in the paediatric population. All nine penicillin-non-susceptible strains isolated from Cordoba showed identical *pbp* RFLP patterns (*pbp1a*/*Alu*I, *Hin*fI or *Mse*I/*Dde*I, *pbp2b*/*Hin*fI and *pbp2x*/*Hin*fI; data not shown), indicating a lower discriminatory power than BOX-PCR.

The penicillin-susceptible strains showed genetic diversity by MLST and were classified into minor international clones (Table 4). Two of the eight strains presented different serotypes to those described originally, namely a serotype 7F variant of the England^{14}-9 clone and a S14V of the Sweden^{15A}-25 clone. We also found two new STs, 2240 and 2241, which we registered in the MLST database.

Table 2. Phenotypic and molecular characterization of non-susceptible strains isolated from Cordoba

Cba, Cordoba; CSF, cerebrospinal fluid; NT, not typable.

Strain	Isolation site	Serotype	BOX profile	Penicillin MIC (μg ml^{-1})
Cba-6	Blood	14	A	>4
Cba-10	Blood	14	A	1
Cba-19	Blood	14	D	2
Cba-22	Pleural fluid	14	A	2
Cba-28	CSF	14	B	4
Cba-33	Blood	14	C	2
Cba-46	Blood	14	E	2
Cba-55	Blood	14	D	2
Cba-62	Blood	14	B	2
Cba-43	Blood	NT	G	1
Cba-44	Blood	19F	B	0.5
Cba-45	Blood	14	B	1
Cba-50	Blood	NT	B	0.12
Cba-52	Blood	14	B	2
Cba-54	Pleural fluid	14	B	1
Cba-56	Blood	23F	I	0.25
Cba-80	CSF	14	A	1
Cba-85	Blood	14	A	0.25
Cba-98	Blood	6B	D	0.75
Cba-112	Blood	14	A	0.25

Analysis of the capsular gene region to detect recombinational replacements

It has been reported that a S14V of the Spain^{9V}-3 clone isolated in Baltimore (MD, USA) could be differentiated from the original Spain^{9V}-3 clone (serotype 9V) by *cpsB* gene polymorphisms (in the 72–548 region) and from the Uruguayan/European variant by the *pbp1a* gene (in the 1498–1710 region) (McEllistrem *et al.*, 2004). To determine whether our S14V was derived from the other S14Vs that have been described, the *cpsB* (72–548 region) and *pbp1a* (1–2160 region) genes were amplified and sequenced. All nine Cordoba S14V strains showed identical *cpsB* sequences to the Baltimore S14V and presented one polymorphism at position 446 compared with the European S14V. However, there was a 17 % divergence (82 out of 476 bp) from the original Spain^{9V}-3 clone (Fig. 1). For the *pbp1a* gene, from position 1473 to 1922,

Table 3. Molecular characterization of the penicillin-non-susceptible Cordoba S14Vs of the Spain^{9V}-3 clone

Strain*	MLST allele number							ST
	aroE	*gdh*	*gki*	*recP*	*spi*	*xpt*	*ddl*	
Cba-6	7	11	10	1	6	8	1	156
Cba-10	7	11	10	1	6	8	1	156
Cba-19	7	11	10	1	6	8	1	156
Cba-22	7	11	10	1	6	8	1	156
Cba-28	7	11	10	1	6	3	1	370
Cba-33	7	11	10	1	6	8	1	156
Cba-46	7	11	10	1	6	8	1	156
Cba-55	7	11	10	1	6	8	1	156
Cba-62	7	11	10	1	6	3	1	370

*Cba, Cordoba.

Table 4. Phenotypic and molecular characterization of penicillin-susceptible strains isolated from Cordoba

Strain	Isolation site	Serotype	Antibiotic susceptibility*	MLST allele number							ST	Clonal complex
				aroE	*gdh*	*gki*	*recP*	*spi*	*xpt*	*ddl*		
Cba-2	Blood	NT	S	7	2	1	10	27	4	14	148	
Cba-5	Blood	18C	S	7	2	1	1	10	1	21	113	Clone 36
Cba-8	Pleural fluid	NT	S	8	9	2	1	6	1	17	191	Clone 39
Cba-21	Blood	9A	S	15	17	4	1	6	1	17	2240†	
Cba-23	Blood	6B	S	7	47	29	1	6	1	80	2241†	
Cba-24	Blood	7F	S	1	5	4	5	5	1	8	9	England^{14}-9
Cba-25	Pleural fluid	6B	S	7	47	29	1	6	1	80	2241†	
Cba-32	Blood	14	S	2	5	36	12	17	21	5	782	Sweden^{15A}-25

NT, Not typable.
*Susceptibility to erythromycin, rifampicin, trimethoprim/sulfamethoxazole, vancomycin and chloramphenicol.
†New STs registered in the MLST database.

the Cordoba S14V was identical to the Baltimore S14V (McEllistrem *et al.*, 2004) and to the Spain^{14}-5 clone, but had 60 bp differences compared with the original Spain^{9V}-3 clone and the European S14V (Coffey *et al.*, 1999) (Fig. 2). From position 1473 to 1872, our strains were also different to the Polish S14V (Coffey *et al.*, 1999) with 48 polymorphisms, but were identical to the Polish and Baltimore S14Vs from position 1873 to 1922. A single polymorphism at position 2045 was present only in the Cordoba variant and the Spain^{14}-5 clone, although the DNA sequence of this *pbp1a* region was not available for the Baltimore S14V (Fig. 2). All of these results suggested that the Baltimore S14V and our S14Vs had similar clonal origins, and that both had a different clonal origin to the European S14V, also suggesting a different capsular replacement. It has been proposed that serotype variants of penicillin-non-susceptible clones have arisen by recombinational exchanges at the capsular biosynthetic locus, *cps*. Molecular evidence in support of this hypothesis was reported for the European S14V, which arose by large

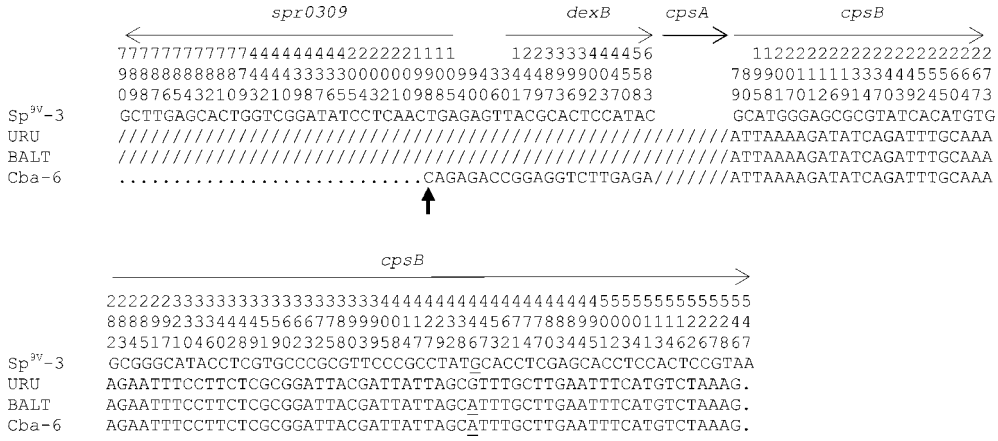

Fig. 1. Comparison of polymorphic sites within the *spr0309–cpsB* region. The DNA sequences shown correspond to the *spr0309–cpsB* region from different serotype variants of the Spain^{9V}-3 clone: Sp^{9V}-3, original Spain^{9V}-3 clone (ATCC 700671); URU, S14V strains of the Spain^{9V}-3 clone isolated in Uruguay (Coffey *et al.*, 1999); BALT, S14V strains of the Spain^{9V}-3 clone isolated in Baltimore (strain I) (McEllistrem *et al.*, 2004); Cba, S14V strains of the Spain^{9V}-3 clone isolated in Cordoba. The Sp^{9V}-3 sequence is shown as a reference and identical sites in other strains are represented by dots, with only polymorphisms being indicated. The nucleotide numbers of the polymorphic sites are indicated vertically and are positioned according to the transcription sense indicated by arrows. The symbol '/' represents unsequenced DNA regions. The solid arrow indicates the proximal crossover point of the recombinational replacement. This site is located at position 198 of the *spr0309* gene from the Cordoba S14V of the Spain^{9V}-3 clone. Position 446 of the *cpsB* gene is underlined. The DNA sequences from the Buenos Aires, Santa Fe and La Pampa strains were identical to that of the Cordoba strains; therefore we used Cba-6 as a representative strain.

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                1111111111111111111111111111111111111111111111111111111111111111111111111111111111111112
                4444444555555555555555555566666666666666667788888888888899999999999999999999999999990
                7788899012344445566677888911223455555778890166677788888990001122233455666677777788999 94
                3624916721025894703628146918032704679160982006925834678694584702928439024501237813036 95
Sp9V-3          ATCCTTACATAGCTACATTCGGACTCAATTGTTCTCTAACTGGCAAGTAAATCTCACCGGCATTCGTCACCATACTGACTATTTAGG
URU             ..........................................................................TACTTTTTCGACTCTGCACCTA.
POL             ....................................................TGCCGTGAGCAGTATAGGCATACTTTTTCGGCTCTGCACCTA.
BALT            CCATCCCAGCTATAGATAATAATTCATGACTCATAGGGTTCTAA....GTGAGCAGTATAGGCA//////////////////////
Cba-6           CCATCCCAGCTATAGATAATAATTCATGACTCATAGGGTTCTAA....GTGAGCAGTATAGGCATACTTTTTCGGCTCTGCACCTAA
Sp14-5          CCATCCCAGCTATAGATAATAATTCATGACTCATAGGGTTCTAA....GTGAGCAGTATAGGCATACTTTTTCGGCTCTGCACCTAA
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Fig. 2. Polymorphic sites within the *pbp1a* gene from different strains. See Fig. 1 legend for details of the different serotype variants; Sp^{14}-5 indicates the Spain^{14}-5 clone (ATCC 700902). From position 1711 to 1874 and from position 2000 to the end of the sequence, our strains were identical to the Spain^{9V}-3 clone, except for a single polymorphism at position 2045, which was found only in the Spain^{14}-5 clone. The DNA sequences from the Buenos Aires, Santa Fe and La Pampa strains were identical to that of the Cordoba strains; therefore, we used Cba-6 as a representative strain. The Uruguayan (URU) and Polish (POL) *pbp1a* sequences were obtained from Coffey *et al.* (1999).

recombinational replacements in the *cpsA–pbp1a* region (Coffey *et al.*, 1999). The *cps* locus contains a series of alphabetically named capsular genes, which are flanked by the conserved genes *dexB* and *aliA* (Fig. 3). In the European S14V, the crossover point of the proximal end was localized to the *cpsA* gene (Coffey *et al.*, 1999). To identify this point in our isolates, the DNA region upstream from the *cpsB* gene was analysed. We found that partial *dexB* sequences of the Cordoba S14V strains were different from that of the original Spain^{9V}-3, indicating that the crossover occurred upstream from *dexB* (Fig. 3). Consequently, the *spr0309* gene (as named in the R6 strain, or *SP0341* in the TIGR4 strain) of three strains (Cba-6, -10 and -28) was amplified and sequenced. The recognition of a divergent block at position 198 of *spr0309* revealed the proximal crossover point of the recombinational exchange that introduced the serotype 14 *cps* locus into our S14Vs (Fig. 1). The distal crossover point of the European S14V was localized in *pbp1a* (Coffey *et al.*, 1999). We could not identify the distal crossover point along the *pbp1a* sequences from Cordoba isolates. Based on the identical sequence homology found in the 5′ region of the *pbp1a* gene and for the complete *recU* gene among our strains and the Spain^{14}-5 and Spain^{9V}-3 clones, we suspected that the distal crossover point could be along this region (Fig. 2). To study the continuity of the extensive recombinational replacement in the *spr0309–recU* region, *aliA*, a gene located approximately in the centre of the replaced DNA region (Fig. 3), was also analysed. Its DNA sequence showed differences from that of the original Spain^{9V}-3, but complete sequence similarity with the same gene described in the Spain^{14}-5 clone (data not shown). This result was in agreement with the other gene analyses and suggested no interruption of the DNA replacement, which apparently was provided by the Spain^{14}-5 clone.

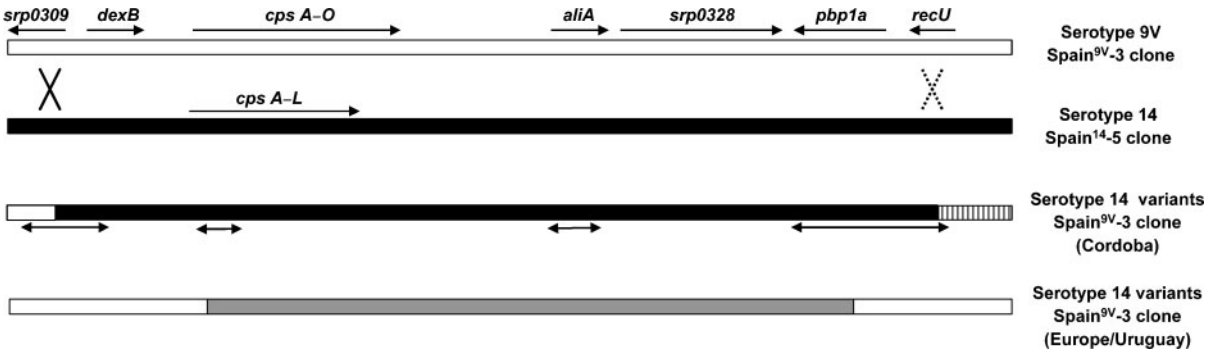

Fig. 3. Graphic representation of the possible recombinational replacements from which the Cordoba S14V of the Spain^{9V}-3 clone may have originated. The *srp0309–recU* region is shown as an open box for the original Spain^{9V}-3 clone, as a filled box for the Spain^{14}-5 clone, and is shaded for an unknown serotype 14 strain. DNA regions that were introduced by recombination from a serotype 14 donor into a serotype 9V receptor are shown as filled or shaded zones. Horizontal arrows below the Cordoba S14V represent the regions that were amplified and sequenced. A solid cross indicates the proximal crossover point identified in the *srp0309* gene. The distal site of the recombinational replacement was not clearly detected. The hatched area represents the zone where the unidentified crossover event is expected to have occurred.

Detection of the Cordoba S14V in other provinces

As this new S14V was predominant in Cordoba, we decided to investigate the putative dissemination of this subclone in different geographical regions near this province. Consequently, we requested invasive serotype 14 strains from children's hospitals located in other regions, such as Buenos Aires City and the provinces of Buenos Aires, Santa Fe and La Pampa, covering an approximate area of 800 km^2. We obtained 12 penicillin-non-susceptible strains recovered from blood cultures that corresponded to serotype 14 and these were analysed by MLST (Table 5). Ten isolates were ST156; however, we identified two new sequence types, ST3119 and ST3120, which presented a single locus variant of the allelic profile of the Spain^{9V}-3 clone. To identify whether these strains belonged to the Cordoba S14V, the genetic polymorphisms of the *pbp1a* (nt 1473–1922) and *spr0309* (nt 1–790) genes were analysed and their sequences were found to be identical to those found in the S14V isolated in Cordoba (Figs 1 and 2). These results suggested that the Cordoba S14V is disseminated in the central provinces corresponding to the most densely populated regions of Argentina (Fig. 4).

DISCUSSION

In this work, we determined the genetic background of invasive pneumococcal strains circulating in Cordoba, showing that a new S14V of the Spain^{9V}-3 clone is predominant among penicillin-non-susceptible strains and is disseminated in the central region of Argentina. Previously, a PFGE-based molecular epidemiological study indicated that a S14V of the Spain^{9V}-3 clone was predominant among penicillin-non-susceptible isolates in Argentina (Rossi *et al.*, 1998). Another epidemiological study conducted by the Pan American Health Organization analysed invasive pneumococcal strains isolated from children under 5 years of age in six Latin-American countries from 1993 to 1996 (Tomasz *et al.*, 1998). This PFGE study revealed that the Spain^{9V}-3 and Spain^{23F}-1 clones were highly represented in these countries and that a S14V of the Spain^{9V}-3 clone showed greater than 80 % prevalence in Argentina and Uruguay, with penicillin MICs of greater than 1 μg ml^{-1}. The identical PFGE profile identified in Argentina and Uruguay suggested that the clonal origin was common, and that the importation of this clone in both countries was coincident in time. The majority of these isolations were detected in large cities, sustaining the hypothesis that the spread of these clones has been facilitated by international travel (Tomasz *et al.*, 1998). However, a detailed molecular characterization of the Cordoba S14V revealed that its epidemiological history is different to that of the Uruguayan strains. Coffey *et al.* (1999) described several Uruguayan strains with identical ST profiles and *pbp1a* sequences to those of the European S14V. However, in contrast, we found clear differences between the Cordoba and European S14Vs, suggesting that a different recombinational event has occurred in Cordoba to that which occurred in Uruguayan strains. We found that the proximal crossover point in our strains was located precisely in the *spr0309* gene (Fig. 1), confirming a new recombinational event. When *dexB*, *cpsB*, *aliA* and *pbp1a* sequences were analysed, they were found to be identical to the homologous genes described in the Spain^{14}-5 clone, indicating that the origin of serotype 14 *cps* genes was this international clone and not other serotype 14 strains, as occurring in the European S14V. In contrast to the proximal site, the distal crossover point was not clearly identified. The 1473–1922 region of *pbp1a* was identical between our strains and the Spain^{14}-5 clone, but we found 60 bp differences compared with the European S14V. Also, the *pbp1a* sequences (2000–2160 region) were identical for the original Spain^{9V}-3 clone, the Spain^{14}-5 clone and our

Table 5. Phenotypic and molecular characterization of the Cordoba S14Vs of the Spain^{9V}-3 clone isolated from other provinces

Strain*	Isolation site	Serotype	BOX profile	MLST allele number							ST	Penicillin MIC (μg ml^{-1})
				aroE	*gdh*	*gki*	*recP*	*spi*	*xpt*	*ddl*		
BsAs-1	Blood	14	B	7	11	10	1	6	8	1	156	4
BsAs-2	Blood	14	B	7	11	10	1	6	8	1	156	2
BsAs-3	Blood	14	B	5	11	10	1	6	8	1	3119	2
BsAs-4	Blood	14	B	7	11	10	1	6	8	1	156	2
BsAs-5	Blood	14	B	7	11	10	1	6	8	1	156	2
BsAs-6	Blood	14	B	7	11	10	1	6	8	1	156	2
BsAs-7	Blood	14	B	7	11	10	1	6	8	1	156	2
BsAs-8	Blood	14	B	7	11	10	1	6	8	1	156	2
BsAs-9	Blood	14	B	7	11	10	1	6	8	1	156	2
LaPam	Blood	14	B	7	11	10	1	6	8	1	156	2
Sta Fe-1	Blood	14	B	7	2	10	1	6	8	1	3120	2
Sta Fe-2	Blood	14	B	7	11	10	1	6	8	1	156	2

*BsAs, Buenos Aires; LaPam, La Pampa; Sta Fe, Santa Fe.

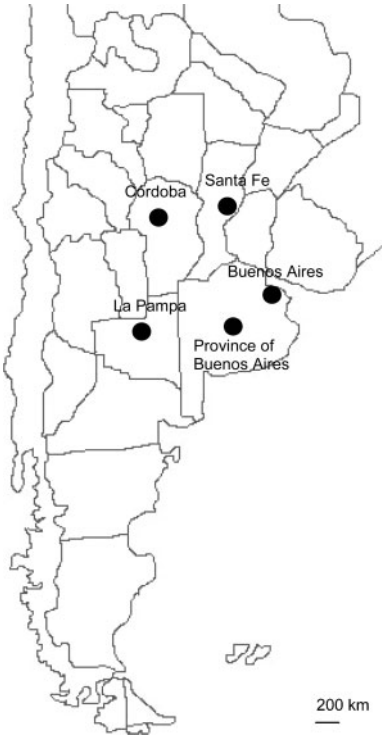

Fig. 4. Map of Argentina indicating sites where the pneumococcal strains were isolated.

clones, except that the Spain^{9V}-3 clone did not have a polymorphism at position 2045. Up to this position, we consider that the replaced DNA region in our strains belongs to the Spain^{14}-5 clone, but after this site, we could not detect a divergent block in the DNA sequences from these clones. For this reason, we assume that the distal crossover occurred in the *recU* region.

Here, we have described a new capsular replacement in the predominant penicillin-non-susceptible Spain^{9V}-3 clone, with this phenomenon also being observed in penicillin-susceptible isolates. We found that the Cba-32 strain was serotype 14 and ST782, belonging to the Sweden^{15A}-25 clone (Table 4). In addition, the Cba-24 strain was serotype 7F and ST9, corresponding to the England^{14}-9 clone. In this case, this capsular replacement has relevance in the application of vaccination programmes, as serotype 7F is currently not included in the vaccine formulation, and this is therefore a risk to be taken into account when considering the pneumococci circulating in our paediatric population.

Due to the geographical proximity with Uruguay, and also the active tourist exchange with our country, we expected to find the same S14V as that observed in Uruguay. However, the Uruguayan S14V presented the same genetic profile as the European S14V (Coffey *et al.*, 1999). From an MLST analysis of our 21 strains belonging to the Spain^{9V}-3 clone, 17 strains were ST156, two were ST370 and two were new STs, ST3119 and ST3120, which were single variants of ST156. By coincidence, a search in the MLST database (http://spneumoniae.mlst.net) showed three STs associated with S14V strains isolated in Uruguay: the original ST156 and two single variants in the *xpt* allele, which were represented by ST164 and ST168. These data revealed that the ST divergence was different in Argentina. Analysis of the BOX regions, an evolutionary marker that diverges more frequently than housekeeping genes, suggested that the Spain^{9V}-3 clone was not disseminated recently in Argentina.

All of our Spain^{9V}-3 strains presented reduced penicillin (Tables 2 and 4) and cefotaxime (data not shown) susceptibility. Castanheira *et al.* (2003) analysed a cefotaxime-resistant population isolated from six Latin-American countries. A major clone, which presented serotype 14 and the same ribotype as Spain^{9V}-3, was present in Argentina, Brazil, Chile and Uruguay. However, the Argentinean and Uruguayan strains showed small differences in the PFGE analysis. Therefore, we may assume that our S14V is the source of the cefotaxime resistance in Cordoba and also in other parts of Argentina, but not in Uruguay.

So far, characterization of the Cordoba S14V has shown the same genetic profile as that of the Baltimore S14V. There are no data about the putative crossover points of the serotype 14 *cps* genes of the Baltimore strains, but when we analysed the published *cpsB* and *pbp1a* sequences (McEllistrem *et al.*, 2004), we also found homology with the Spain^{14}-5 clone, suggesting that the *cps* cassette had the same origin. Here, we have performed a relevant characterization of this new S14V of the Spain^{9V}-3 clone that should be considered for further epidemiological studies to determine whether these S14V strains are disseminated in other countries and also where this clone originated. For this, we propose that the *pbp1a* (nt 1473–1922 region) and *spr0309* (nt 1–790 region) genes should be utilized to help recognize the Cordoba S14V. We suggest that the results of this study will provide a reference for monitoring the evolution of the variants of Spain^{9V}-3, the emergence of new clones and the impact of pneumococcal vaccination programmes in Argentina.

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