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Research article

Serovar distribution of a DNA sequence involved in the antigenic relationship between *Leptospira* and equine cornea

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Abstract

Background: Horses infected with *Leptospira* present several clinical disorders, one of them being recurrent uveitis. A common endpoint of equine recurrent uveitis is blindness. Serovar *pomona* has often been incriminated, although others have also been reported. An antigenic relationship between this bacterium and equine cornea has been described in previous studies. A leptospiral DNA fragment that encodes cross-reacting epitopes was previously cloned and expressed in *Escherichia coli*.

Results: A region of that DNA fragment was subcloned and sequenced. Samples of leptospiral DNA from several sources were analysed by PCR with two primer pairs designed to amplify that region. Reference strains from serovars *canicola, icterohaemorrhagiae, pomona, pyrogenes, wolffi, bataviae, sentot, hebdomadis* and *hardjo* rendered products of the expected sizes with both pairs of primers. The specific DNA region was also amplified from isolates from Argentina belonging to serogroups Canicola and Pomona. Both *L. biflexa* serovar *patoc* and *L. borgpetersenii* serovar *tarassovi* rendered a negative result.

Conclusions: The DNA sequence related to the antigen mimicry with equine cornea was not exclusively found in serovar *pomona* as it was also detected in several strains of *Leptospira* belonging to different serovars. The results obtained with *L. biflexa* serovar *patoc* strain Patoc I and *L. borgpetersenii* serovar *tarassovi* strain Perepelicin suggest that this sequence is not present in these strains, which belong to different genomospecies than those which gave positive results. This is an interesting finding since *L. biflexa* comprises nonpathogenic strains and serovar *tarassovi* has not been associated clinically with equine uveitis.

Background

Leptospirosis is a widespread disease that affects wild and domestic animals as well as humans [1]. Animals which have recovered from acute leptospirosis may develop a carrier condition, shedding leptospires in their urine [2].

Horses infected with *Leptospira* present several clinical disorders, one of them being recurrent uveitis or iridocyclitis [3,4]. In equine recurrent uveitis (ERU), episodes of acute anterior uveitis are separated by quiescent periods of variable duration [5]. Acute signs include blepharospasm, lacrimation, photophobia, myosis, corneal edema and

vascularisation, aqueous flare and hypopyon, although no single case shows these signs all together. With repeated attacks, the severity of the lesions becomes more pronounced. The inflammatory process may lead to anterior or posterior synechiae, cataract, iris atrophy, retinal detachment, lens luxation and corneal opacity [3,5].

A common endpoint of ERU is blindness. Horses with uveitis associated with leptospiral seroreactivity are at increased risk of developing blindness with respect to horses which uveitis was attributable to other causes [6]. Serovar *pomona* has often been incriminated, although others have also been reported.

Parma *et al.*[7] showed that equine cornea and *Leptospira* share partial antigenic identity. In fact, corneal opacity was provoked by inoculating horses either with killed *Leptospira* or equine cornea. Based on these findings, ERU is considered an organ-specific autoimmune disease [8].

The epitopes shared between *Leptospira* and equine cornea belong to a protein structure located inside this bacterium [9]. A leptospiral DNA fragment that encodes cross-reacting epitopes was cloned and expressed in *Escherichia coli* as a β-galactosidase-fusion protein [10]. This clone, isolated by expression screening with a polyclonal serum raised against equine cornea proteins, encodes a 90 kDa protein of serovar *pomona*. Antibodies directed against this leptospiral antigen recognized a 66 kDa equine corneal protein. These findings suggest that an immune response to that leptospiral antigen participates in pathogenesis of equine uveitis.

Therefore, it would be interesting to investigate whether this region is spread in the genomes of several serovars of *Leptospira*.

Before 1989, taxonomy of the leptospires distributed these bacteria between two species, *Leptospira interrogans* (pathogenic) and *L. biflexa* (saprophytic) comprising over 200 serovars [11] on the basis of surface agglutinins. For convenience, antigenically related serovars are organized into serogroups.

On the basis of studies on DNA homology, polymorphic patterns and rRNA typing, the taxonomy of *Leptospira* has been recently reorganized into 17 genomospecies: *L. interrogans* sensu stricto (hereafter called *L. interrogans*), *L. biflexa* sensu stricto (hereafter called *L. biflexa*), *L. borgpetersenii*, *L. weilii*, *L. noguchii*, *L. santarosai*, *L. inadai*, *L. wolbachii*, *L. meyeri*, *L. parva*, *L. kirschneri*, *L. fainei* and 5 new genomospecies one of which was named *L. alexanderi*[12–15]. However, until simpler DNA-based identification methods are developed and validated, it will be necessary for clinical laboratories to retain the serological classification of pathogenic leptospires for the foreseeable future [16].

In the present study, we began with the analysis of serovars of *Leptospira* by PCR with two primer pairs designed to amplify the region antigenically related to equine cornea.

Results and Discussion

A region of the leptospiral DNA fragment that encodes epitopes involved in the antigenic cross-reactivity between this bacterium and equine cornea was subcloned and sequenced. The obtained sequence was deposited in the GenBank database under accession no. AY046585. Two primer pairs were designed from the sequence to amplify that region by PCR: S3a/S3b and S4a/S4b, which produce 253 and 152 bp PCR amplicons, respectively.

Several samples of leptospiral DNA, from reference strains and isolates from Argentina, were analysed by PCR with these primers (Table 1). Reference strains from serovars *canicola*, *icterohaemorrhagiae*, *pomona*, *pyrogenes*, *wolffi*, *bataviae*, *sentot*, *hebdomadis* and *hardjo* rendered products of the expected sizes with both pairs of primers (Fig. 1). The specific DNA region was also amplified from isolates from Argentina belonging to serogroups Canicola and Pomona. A single product was obtained in all cases of positive amplification.

Both L. biflexa serovar patoc and *L. borgpetersenii* serovar *tarassovi* rendered a negative result when analysed with the two pairs of primers S3a/S3b and S4a/S4b (Fig. 1).

Another set of primers (G1/G2 and B64-I/ B64-II) previously described [17,18] was used as a positive control of leptospiral DNA (results not shown). With these primers, *L. borgpetersenii* and all *L. interrogans* strains here mentioned were amplified, suggesting that the failure to amplify the sequence related antigenically to equine cornea from *L. borgpetersenii* serovar *tarassovi* was not due to template degradation or to the presence of PCR inhibitors Therefore, this strain is not likely to carry this sequence or presents variations in DNA fine structure that prevent PCR primer annealing.

As primers G1/G2 and B64-I/ B64-II are specific for pathogenic leptospires, the presence of leptospires in the aliquot taken from the culture of *L. biflexa* serovar *patoc* was determined by microscopic observation.

These results suggest that in the nonpathogenic strain analysed, the sequence related to the antigen mimicry with equine cornea is not present. In addition, this sequence could not be detected either in one of the pathogenic strains. Interestingly, it belongs to a different genomospecies (*L. borgpetersenii*) than the other pathogenic strains

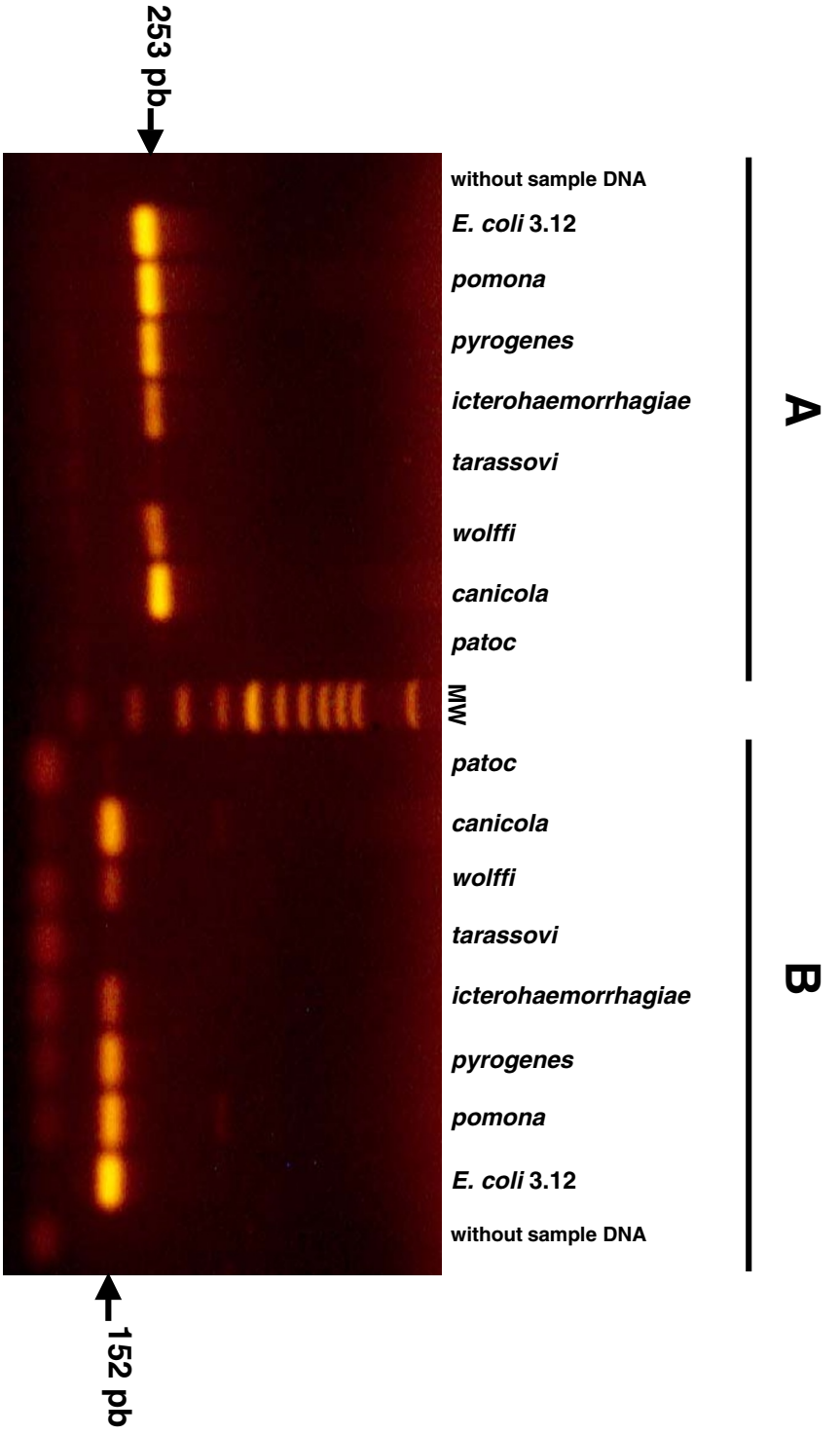

Figure 1
Agarose gel electrophoresis of PCR products amplified from reference strains of *Leptospira* serovars *pomona, pyrogenes, icterohaemorrhagiae, tarassovi, wolffi, canicola* and *patoc* using primers S3a and S3b (A) or S4a and S4b (B). Positive control: recombinant *E. coli* carrying the leptospiral fragment antigenically related to equine cornea (E. coli 3.12). Negative control: distilled water (without sample DNA). Lane MW contains DNA molecular size marker (100 bp DNA Ladder, Promega, Madison, WI). Arrows indicate PCR products.

Table 1: Reference strains of *Leptospira* tested in this study

GENOMOSPECIES (*)	SEROGROUP	SEROVAR	STRAIN
L. interrogans	Canicola	*canicola*	Hond Utrecht IV
L. interrogans	Icterohaemorrhagiae	*icterohaemorrhagiae*	RGA
L. interrogans	Pomona	*pomona*	Pomona
L. interrogans	Pyrogenes	*pyrogenes*	Salinem
L. borgpetersenii	Tarassovi	*tarassovi*	Perepelicin
L. interrogans	Sejroe	*wolffi*	3705
L. biflexa	Semaranga	*patoc*	Patoc I
L. interrogans	Bataviae	*bataviae*	Van Tienen
L. interrogans	Djasiman	*sentot*	Sentot
L. interrogans	Hebdomadis	*hebdomadis*	Hebdomadis
L. interrogans	Sejroe	*hardjo*	Hardjoprajitno

(*) as indicated in *Leptospira* Molecular Genetics Server [http://www.pasteur.fr/recherche/Leptospira]

studied which are all *L. interrogans*. It should be noted that, so far as we know, serovar *tarassovi* has not been associated with ERU.

According to Faine [2], there has been a tendency to extrapolate and generalize a conclusion to all leptospires from observations on one or few strains studied. But nowadays, newer advances on genetic groupings dictates that much of the conventionally accepted knowledge has to be studied critically again with modern techniques to ascertain the extent of genotypic and phenotypic variation. This study contributes to the knowledge of the distribution of a DNA sequence, which is present in *L. biflexa* serovar *pomona*, among different serovars of *Leptospira*.

Additional studies with different strains of *L. borgpetersenii* will be necessary to know if this sequence is not present in this genomospecies. It would be also interesting to investigate whether corneal epitopes crossreactive with this bacterium are present in different breeds of horses and in other species of the family Equidae (as ERU is also the leading cause of blindness in mules).

Conclusions

A DNA sequence of sevovar *pomona* related to the antigen mimicry with equine cornea was detected in several strains of *Leptospira* belonging to different serovars, including reference strains and isolates from Argentina. Therefore, this sequence is not exclusively present in serovar pomona.

The results obtained with *L. biflexa* serovar *patoc* strain Patoc I and *L. borgpetersenii* serovar *tarassovi* strain Perepelicin suggest that this sequence is not present in these strains, which belong to different genomospecies than those which gave positive results. This is an interesting finding since *L. biflexa* comprises nonpathogenic strains and serovar *tarassovi* has not been associated clinically with ERU.

Materials and Methods

Bacteria and culture conditions

Reference strains of *Leptospira* used in this publication are listed in Table 1. Six clinical isolates from Argentina were also studied (3 belonging to serogroup Canicola and 3 to Pomona). Leptospires were maintained in Fletcher medium [19].

Sample preparation

Aliquots were taken from cultures, diluted 1/10 in water and boiled for 10 min. Five microliters were used as the template for PCR amplification.

Primers

Primer sequences for the indicated region were as follows.

S3a (sense):

5' GCGGATATGGGAAGCTTAGAAACT 3'

S3b:

5' CCGAAACTGTAGCCGAAGAAGAAA 3'

S4a (sense):

5' TCCTTTTGGCGATTTAGCAGAA 3'

S4b:

5' CGTGTCCGGAGTAGAAGTGAATGT 3'

PCR amplification

PCR was performed in a final volume of 25 µl containing 50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% (v/v) Triton X-100, 1.5 mM $MgCl_2$, 200 µM each dNTP, 0.5 µM each primer (either S3a and S3b or S4a and S4b), 1U *Taq* DNA polymerase.

PCR amplifications were performed as follows: initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 1 $^1/_2$ min, annealing at 66°C (primer pair S3a/S3b) or 57°C (primer pair S4a/S4b) for 1 $^1/_2$ min, extension at 72°C for 2 min and a final extension at 72°C for 5 min. Reaction products were analysed in a 1.5% agarose gel stained with ethidium bromide.

Another set of primers (G1/G2 and B64-I/ B64-II) previously described [17,18] was used as a positive control of leptospiral DNA. The reaction mix was constituted by 50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% (v/v) Triton X-100, 2 mM $MgCl_2$, 0.01% (m/v) gelatine, 250 µM each dNTP, 0.5 µM each of the four primers, 0.5 U *Taq* DNA polymerase in a final volume of 25 µl. PCR amplifications were performed as described [18].

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