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Association of Toll-like receptor 2 Arg753Gln and Toll-like receptor 1 Ile602Ser single-nucleotide polymorphisms with leptospirosis in an argentine population

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ABSTRACT

Toll-like receptor 2 (TLR2), a member of the Toll-like receptor family, plays an important role in the recognition of and subsequent immune response activation against leptospirosis in humans. The genetic polymorphism in TLR2 of an arginine to glutamine substitution at residue 753 (Arg753Gln) has been associated with a negative influence on TLR2 function, which may, in turn, determine the innate host response to *Leptospira* spp. This bacterium signals through TLR2/TLR1 heterodimers in human cells. The aim of the present study was to investigate the Arg753Gln single-nucleotide polymorphism (SNP) of the TLR2 gene, and the isoleucine to serine transversion at position 602 (Ile602Ser) of the TLR1 gene (previously associated with Lyme disease), in leptospirosis patients compared to healthy controls, carrying out a retrospective case/control study. The TLR2 polymorphism adenine (A) allele was observed in 7.3% of leptospirosis patients but was not found in the control group, whereas the guanine (G) allele of the TLR1 polymorphism was found in 63.6% of patients and 41.6% of controls. Susceptibility to leptospirosis disease was increased 10.57-fold for carriers of the TLR2 G/A genotype ($P = 0.0493$) and 3.85-fold for carriers of the TLR1 G/G genotype ($P = 0.0428$). Furthermore, the risk of developing hepatic insufficiency and jaundice was increased 18.86- and 27.60-fold for TLR2 G/A carriers, respectively. Similarly, the risk of developing jaundice was increased 12.67-fold for TLR1 G allele carriers (G/G and T/G genotypes). In conclusion, the present data suggest that the TLR2 Arg753Gln and TLR1 Ile602Ser SNPs influence the risk of developing leptospirosis and its severity.

1. Introduction

Leptospirosis is an important global zoonosis that is more frequent in tropical and subtropical areas, caused by pathogenic spirochetes of the genus *Leptospira* (Faine and Bolin, 1999). Historically, the transmission of leptospirosis has mainly been associated with exposure of individuals to wild or farm animals (Faine and Bolin, 1999; Levett, 2001). Humans usually become infected through contact with urine-contaminated soil and water or with infected animal tissues (Lecour et al., 1989; de Faria et al., 2008). However, in the last decades, it has become a re-emerging disease prevalent in cities with sanitation deficits and large populations of urban rodent reservoirs (Ko et al., 1999). Therefore, leptospirosis is a neglected tropical disease with an endemoepidemic pattern associated with slum settlements, where deficiencies in the sanitation infrastructure are maximal (Maciel et al., 2008) and where differences in socioeconomic status contribute to the risk of *Leptospira* infection (Reis et al., 2008).

Innate immunity is the first line of defense against pathogenic microorganisms, and its activation is initiated by the recognition of microbial structures by pattern recognition receptors (PRRs) (Janeway and Medzhitov, 2002). The first and most well studied class of PRRs is that of the Toll-like receptors (TLRs), type

Abbreviations: A allele, adenine allele; Arg753Gln, arginine to glutamine substitution at position 753; CI, confidence interval; dbSNP, single nucleotide polymorphism database at the National Center for Biotechnology Information (NCBI); G allele, guanine allele; HWE, Hardy–Weinberg equilibrium; LD, linkage disequilibrium; OR, odds ratio; RFLP, restriction fragment length polymorphism; SNP, single-nucleotide polymorphism; T allele, thymine allele; TLR1, Toll-like receptor 1; TLR2, Toll-like receptor 2.

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I glycoproteins expressed on the cell surface or intracellularly in the endoplasmic reticulum and endosomes. Ten human TLRs have been identified so far, with important roles in host defense against bacteria, viruses, parasites and fungi (Akira et al., 2006; Kawai and Akira, 2010). Their sequence contains an ectodomain, which is involved in ligand recognition and interaction with co-receptors, a transmembrane region and a Toll/IL-1R domain (TIR) for intracellular signaling (Doyle and O'Neill, 2006).

TLR2 identification, molecular characterization, and cloning were first published in 1998 (Rock et al., 1998). The increased expression of TLR2 in humans is limited to a small number of cell types that are primarily involved in acute host defense, with strong expression in leukocytes, particularly neutrophils (Kurt-Jones et al., 2002), followed by lung cells (Texereau et al., 2005), vascular endothelial cells, intestinal cells, fat cells and cardiomyocytes (Rehli, 2002; Kabelitz, 2007). TLR2 plays a key role in the innate immune response because it has the capacity to form functional heterodimers with other TLRs and interacts with a large number of non-TLR molecules, allowing the recognition of a great variety of pathogen-associated molecular patterns (PAMPs) from bacteria, viruses, fungi, and parasites, including lipoproteins and lipopeptides, peptidoglycans, lipoteichoic acid, lipopolysaccharides (LPS), porins, glycolipids, zymosan and hemagglutinin (Akira and Takeda, 2004; Zahringer et al., 2008; Oliveira-Nascimento et al., 2012). Upon ligand recognition, TLR2 forms either homodimers or heterodimers with TLR1 or TLR6, triggering the activation of nuclear factor (NF)-κB, a central transcription factor of the inflammatory response, and its translocation to the nucleus (Ozinsky et al., 2000; Hajjar et al., 2001; Takeuchi et al., 2001). Although classified as Gram-negative bacteria, *Leptospira* spp. express an unusual LPS with a unique Lipid A structure (Que-Gewirth et al., 2004) that is recognized through murine TLR4, but not through human TLR4, nor human or murine TLR2 (Nahori et al., 2005). However, the whole LPS is recognized through human TLR2/TLR1 (but not TLR6), whereas TLR2 but also TLR4 contribute to activation in murine cells (Werts et al., 2001; Nahori et al., 2005). Leptospiral major lipoprotein LipL32, and leptospiral outer membrane protein extracts, have also been described as TLR2 agonists in human cells (Werts et al., 2001; Yang et al., 2006), making of TLR2 a key receptor for recognition and defense against *Leptospira interrogans* in humans.

The human *tlr2* gene is located on chromosome 4q32 and consists of three exons. The first two are non-coding exons (not translated), while the entire open reading frame is located in exon 3 (Haehnel et al., 2002) (GenBank accession number AF051152). SNPs are DNA sequence variations that occur when a single nucleotide in the sequence genome is altered. Many SNPs have no effect on the function of the cells, either because they do not result in amino acid changes (synonymous SNPs), or because the amino acid change does not affect the protein structure or its characteristics (non-synonymous SNPs). There have been 175 SNPs reported for the *tlr2* gene, but only 17 are located in the third exon (Texereau et al., 2005; Brown et al., 2010) and only nine of them are non-synonymous.

Two of the aforementioned non-synonymous polymorphisms have been linked to a reduced NF-κB activation and to an increased risk of infections (Kirschning et al., 1998; Akira et al., 2001). The first non-synonymous *tlr2* SNP, which was only found in a small European population (Ioana et al., 2012), consists of a C > T substitution at nucleotide 2029 from the start codon of *tlr2*, which is predicted to replace arginine (Arg) with tryptophan (Trp) at position 677. The second functional *tlr2* variant consists of a G > A substitution at nucleotide 2258 from the start codon of *tlr2*, and is predicted to replace arginine with glutamine (Gln) at position 753. This polymorphism, within a group of highly conserved amino acids at the C-terminal end of *tlr2*, has been the most thoroughly investigated because of its prevalence in the Caucasian population (Lorenz et al., 2000; Brown et al., 2010).

As mentioned above, TLR1 is a key co-receptor for the recognition of leptospiral LPS, and it is expressed at higher levels in the spleen and peripheral blood cells (Zarember and Godowski, 2002). The human *tlr1* gene is located on chromosome 4p14 and consists of six exons (GenBank accession number AB445617). Seventeen SNPs have been identified in the coding region of the *tlr1* gene sequence, seven non-synonymous and ten synonymous, with seven of them in the extracellular domain and two in the intracellular domain (Hawn et al., 2007). One of the most common polymorphisms is a T > G transversion at position 1805, located between the transmembrane and intracellular domain of the receptor (Hawn et al., 2007), predicted to replace isoleucine (Ile) with serine (Ser) at position 602. This polymorphism would be expected to have a substantial impact on function and disease expression because of its location in the transmembrane domain of the receptor and its high frequency in Caucasian populations.

To test the hypothesis that patients with the Arg753Gln SNP of the *tlr2* gene and/or the Ile602Ser SNP of the *tlr1* gene have an increased susceptibility to leptospirosis, we studied these polymorphisms in DNA samples collected from 2007 to 2011 in Santa Fe, Argentina.

2. Materials and methods

2.1. Ethics statement

This study was approved by the Institutional Review Board of the National Institute of Respiratory Diseases (INER, ANLIS/UNL, Santa Fe, Argentina), and was carried out at the Institute of Biotechnology and Molecular Biology (IBBM, CONICET-UNLP, La Plata, Buenos Aires, Argentina). Samples were selected from a preexisting INER database and randomized for this study.

2.2. Study subjects

DNA samples from leptospirosis patients, mostly from Caucasian origin, were provided by the National Institute of Respiratory Diseases (INER, Santa Fe, Argentina). The case definition criteria for leptospirosis used to classify the samples was based on the regulations of the National Epidemiological Surveillance System by Argentinian Laboratories (SIVILA), which states that confirmed cases include those that are clinically compatible with a seropositive microagglutination test (MAT) seroconversion or titer >1/200, culture, or real-time PCR (targeting leptospiral LipL32 or ribosomal RNA *16s*). The control group included blood samples from healthy donors ethnically, geographically and age-matched to the patient group.

2.3. DNA extraction

DNA from patients' blood samples was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA), following the manufacturer's instructions. For the controls, total DNA was extracted from blood samples by mechanical homogenization in 500 μl of lysis buffer (50 mM Tris–HCl pH 8.0; 1 μM EDTA, 1% Triton X-100, 0.5% Tween-20; 1% SDS) containing 2 μg/ml proteinase K, and then incubated for 2 h at 56 °C. One volume of phenol (pH 8.0) was added and centrifuged at 12,000 × *g* for 15 min at 4 °C. The aqueous phase was extracted once more with phenol and then four times with 0.5 volumes of chloroform/isoamyl alcohol (24:1), centrifuging at 12,000 × *g* for 10 min at 4 °C between each step. DNA was precipitated overnight at −20 °C by the addition of 100 μl of 3 M sodium acetate (pH 5.2) and absolute ethanol up to 1 ml,

and then centrifuged at 12,000 × *g* for 20 min at 4 °C. The DNA pellet was washed twice with 70% ethanol, resuspended in TE buffer (10 mM Tris–HCl pH 7.5, 1 mM EDTA) and incubated at 55–60 °C for 15–20 min. DNA was stored at −20 °C until use.

2.4. Amplification of the tlr2 and tlr1 genes

The *tlr2* gene was amplified with specific primers (5′–3′) CAT TCC CCA GCG CTT CTG CAA GCT CC and GGA ACC TAG GAC TTT ATC GCA GCT C designed to amplify a 129-bp fragment within exon 3 (Lee et al., 2008). The *tlr1* gene was amplified with specific primers (5′–3′) CTA CCC GGA AAG TTA TAG AGG AAC C and TTT GGC AAT AAT TCA TTC TTC ACC designed to amplify a 303-bp fragment (Oosting et al., 2011). Amplification of β-actin with the primers (5′–3′) GGC TGT ATT CCC CTC CAT CG and CCA GTT GGT AAC AAT GCC ATG T was used as an internal control. To 100 ng genomic DNA, 1× PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM of each dNTP, 10 pmol of each primer and 1 unit of Taq DNA polymerase (EmbioTec, Buenos Aires, Argentina) were added, and the final volume was adjusted to 25 μl with distilled water. Following initial denaturation at 94 °C for 14 min, the samples were subjected to 35 cycles of denaturation at 94 °C for 30 s, 56 °C for 30 s and extension at 72 °C for 30 s, with a final 10 min extension step at 72 °C.

2.5. Restriction enzyme digestion of the TLR2 fragments

To 15 μl of each PCR product 1× enzyme reaction buffer and 10 units of MspI restriction enzyme (Thermo Fisher Scientific Inc., USA) were added. The final volume was adjusted to 20 μl with distilled water and then incubated at 37 °C for 16 h. Each reaction product was mixed with 4 μl 6× gel loading dye (30% sucrose, 0.05% bromophenol blue, 0.05% xylene cyanol), loaded onto a 10% native polyacrylamide gel and run at 75 volts for 2.5 h in 1× electrophoresis TBE buffer (Tris–HCl 25 mM, glycine 200 mM, SDS 0.1%). The gel was then stained in TBE buffer containing 0.5 μg/ml ethidium bromide for an hour. Images were captured with the *Electrophoresis Documentation and Analysis System 120* software (Kodak).

2.6. Sequencing of PCR products

PCR products were purified and sequenced by external contractors: Instituto de Biotecnología (CICVyA-INTA, Castelar, Buenos Aires, Argentina) and MACROGEN (Seoul, Rep. of Korea). Sequences were aligned and analyzed with the program Vector NTI Advance® v11.5 (Life Technologies). Chromatograms were analyzed with Chromas v2.4.3 (Technelysium Pty Ltd.).

2.7. Statistical analysis

Statistical analysis was carried out using PLINK v1.07 software (Purcell et al., 2007). The goodness of fit to Hardy–Weinberg Equilibrium (HWE) was performed using a Chi-square (χ^2) test. Allele and genotype frequencies were determined by direct counting and then divided by the number of chromosomes to produce an allele frequency or by the number of subjects to produce the genotype frequency. To assess associations of interest, allele or genotype frequencies were compared between patients and controls in different models. The models were the allelic model, the genotypic model, the additive model (Cochran–Armitage trend test), the dominant model and the recessive model. Pairwise linkage disequilibrium between the TLR2 and TLR1 SNPs was also calculated. All test statistics were distributed as Fisher's exact test. Odds ratios (OR) and 95% confidence interval (CI) were calculated with GraphPad Prism v6.01 for Windows (GraphPad Software, Inc.). A *P*-value of <0.05 was considered significant.

3. Results

The study population consisted of 55 confirmed leptospirosis patients (45 men, 10 women, 35.7 ± 15.3 years, range 1–65 years) and 60 healthy donors (39 men, 21 women, 34.3 ± 17.0 years, range 2–68 years) that were used as controls.

Restriction fragment length polymorphism (RFLP) analysis of the Arg753Gln SNP of TLR2 was carried out with specific primers to amplify a 129-bp fragment of exon 3, followed by digestion with the restriction enzyme MspI. The Arg753Gln single-nucleotide polymorphism changes the codon CGG (arginine) to CAG (glutamine), whereby the wild-type phenotype of TLR2 is G/G and the variants may be G/A or homozygous A/A. MspI recognizes the sequence (5′–3′) CCGG and cleaves the DNA after the first cytosine. In the absence of the polymorphism, digestion resulted in two fragments of 104- and 25-bp (not observable), but when the polymorphism was present, the enzyme did not make the cut, and the 129-bp DNA fragment remained intact. The digestions were separated in 10% native polyacrylamide gels and stained with ethidium bromide to visualize the bands (Fig. 1A). All PCR products were sequenced to confirm the presence of the TLR2 polymorphism (Fig. 1B).

Because *L. interrogans* signals through TLR2/TLR1 heterodimers in human cells, we searched for the TLR1 Ile602Ser SNP in both the patients and control groups, by sequencing a 303-bp DNA fragment of the *tlr1* gene amplified by PCR with specific primers. The Ile602Ser single-nucleotide polymorphism changes the codon ATC (isoleucine) to AGC (serine), whereby the wild-type phenotype of TLR1 is T/T and the variants may be T/G or homozygous G/G (Fig. 2).

Allele and genotype frequencies (Table 1) of the TLR2 Arg753Gln and TLR1 Ile602Ser polymorphisms in leptospirosis patients and controls were compared to determine susceptibility to *L. interrogans* infection. Both groups displayed HWE ($P \geq 0.4874$) for both SNPs. TLR2 Arg753Gln and TLR1 Ile602Ser SNPs were not in linkage disequilibrium ($r^2 = 0.042$).

The minor allele frequency of the TLR2 Arg753Gln SNP was higher in leptospirosis patients (3.8%) compared with controls (0%; $P = 0.0508$, OR 10.18, 95% CI 0.54–191.50); however, the *P*-value was not significant. Analysis using Fisher's exact test showed slightly a significant difference between leptospirosis patients and controls in genotype frequencies of this SNP ($P = 0.0493$, OR 10.57, 95% CI 0.56–201.20). Association with leptospirosis was evaluated with different models (Table 2). In this case, the dominant model of association ($P = 0.0493$) is equal to the genotypic model because there are no homozygous carriers of the TLR2 Arg753Gln SNP.

The minor allele frequency of the TLR1 Ile602Ser SNP was higher in leptospirosis patients (41.8%) compared with controls (25%; $P = 0.0078$, OR 2.16, 95% CI 1.23–3.78). The genotype frequencies of this polymorphism were different in leptospirosis patients when compared to controls. The G/G genotype was more significantly associated with leptospirosis ($P = 0.0428$, OR 3.85, 95% CI 1.17–12.68) than the T/G genotype ($P = 0.1031$, OR 2.10, 95% CI 0.94–4.72). The association of the TLR1 Ile602Ser SNP with leptospirosis was confirmed in the genotypic ($P = 0.03952$), additive ($P = 0.0110$), and dominant ($P = 0.0249$) models.

Patients presented fever (90.9%), jaundice (14.5%), headache (63.6%), myalgia (60.0%), respiratory symptoms (27.3%), hepatic insufficiency (18.2%), renal insufficiency (12.7%), meningeal signs (3.6%), hemorrhages (12.7%), and leukocytosis (40%). Thirty-two (58.2%) patients required hospitalization (severe cases) and the mortality rate was 10.9%. When Fisher's exact test was performed for evaluation of the differences in the ratios of different symptoms

Table 1
Allele and genotype frequencies of TLR2 Arg753Gln and TLR1 Ile602Ser SNPs in confirmed leptospirosis patients and controls.

SNP	Controls ($n=60$)	Leptospirosis patients ($n=55$)	P-value[a]	OR (95% CI)
TLR2 Arg753Gln				
A	0(0%)	4(3.8%)	0.0508	10.18 (0.54–191.50)[c]
G	120(100%)	106(96.2%)		
G/A	0(0%)	4(7.3%)	0.0493[b]	10.57 (0.56–201.20)[c]
G/G	60(100%)	51(92.7%)		
TLR1 Ile602Ser				
G	30(25%)	46(41.8%)	0.0078[b]	2.16 (1.23–3.78)
T	90(75%)	64(58.2%)		
G/G	5(8.3%)	11(20.0%)	0.0428[b]	3.85 (1.17–12.68)
T/G	20(33.3%)	24(43.6%)	0.1031	2.10 (0.94–4.72)
T/T	35(58.4%)	20(36.4%)		

[a] Fisher's exact test.
[b] $P<0.05$.
[c] OR and CI calculated with Haldane's correction; OR, odds ratio; CI, confidence interval.

in the patients group, TLR2 Arg753Gln G/A carriers were found to be more significantly associated with the development of hepatic insufficiency ($P=0.0165$, OR 18.86, 95% CI 1.71–207.90) and jaundice ($P=0.0079$, OR 27.60, 95% CI 2.38–318.20) than G/G carriers (Table 3). TLR1 Ile602Ser G allele carriers (G/G + T/G genotypes) were also found to be more significantly associated with higher risk of jaundice ($P=0.0406$, OR 12.67, 95% CI 0.69–232.60) than carriers of the T/T genotype (Table 3).

4. Discussion

Sequencing has revealed that there are about 14 million SNPs in the human genome, of which 200,000 occur in coding sequences, demonstrating extensive genetic variability and its possible effects on susceptibility to diseases and individual responses to medical treatments (Texereau et al., 2005).

Here we report, to the best of our knowledge, the first association study of the TLR2 Arg753Gln (rs5743708) and TLR1 Ile602Ser (rs5743618) SNPs with leptospirosis in an Argentine population. For Arg753Gln, slightly a significant difference was found between patients with leptospirosis and healthy donors in the genotype distributions, whereas the P-value for the allelic distributions was over the significance limit, probably due to the small sample size of our study population and, consequently, lower statistical power. It was interesting to find that the allelic frequency of the A allele was 0% in the control group. The reported global MAF (minor allele frequency) for this SNP is 0.0068/34 or 0.68% (dbSNP NCBI). For Ile602Ser, a significant difference was found between patients with leptospirosis and healthy donors in both allelic and genotypic distributions.

We found that susceptibility to leptospirosis disease for subjects with the G/A genotype of the TLR2 Arg753Gln polymorphism was 10.57-fold higher than for carriers of the G/G genotype. Furthermore, the risk of developing hepatic insufficiency and jaundice was significantly increased 18.86- and 27.60-fold, respectively, for patients that carried the G/A genotype. Additionally, carriers of the TLR1 Ile602Ser SNP G/G genotype were 3.85-fold more susceptible to leptospirosis than carriers of the T/G and T/T genotypes. The risk of developing jaundice was also 12.67-fold higher for G allele carriers (G/G and T/G genotypes) than for T/T genotype carriers.

The frequencies of TLRs polymorphisms vary between populations of different ethnic and geographic origins. The three best-known non-synonymous TLR2 polymorphisms, namely, Pro631His (1892C > A), Arg677Trp (2029C > T) and the one studied in our work, Arg753Gln (2258G > A), have only been found in European populations (Ioana et al., 2012). Similarly, the most-studied non-synonymous TLR1 SNP, Ile602Ser (1805T > G), was found in 75% of European–American Caucasians, in 25% of African–Americans and was almost absent in East Asians (Hawn et al., 2007). Not surprisingly, we have found the Arg753Gln and Ile602Ser polymorphisms in our samples, considering that Argentineans carry a large fraction of European genetic heritage in their Y-chromosome (94.1%) and autosomal (78.5%) DNA (Corach et al., 2010).

In previous reports, the TLR2 Arg753Gln SNP was associated with tuberculosis (Ogus et al., 2004), pneumonia (Telleria-Orriols et al., 2013), higher risk of *Staphylococcus aureus* infection (Lorenz et al., 2000), development of reactive arthritis after *Salmonella enteritidis* infection (Tsui et al., 2008), infectious endocarditis (Bustamante et al., 2011), higher risk of cytomegalovirus (HHV-5) infection (Brown et al., 2009; Kang et al., 2012), and with increased rates of recurrence of infection with Gram-positive bacteria and septic shock (Lee et al., 2011). The other most studied polymorphism, Arg677Trp, was associated with lepromatous leprosy (Kang and Chae, 2001; Kang et al., 2002; Bochud et al., 2003) and tuberculosis (Ben-Ali et al., 2004). On the other hand, reports about the TLR1 Ile602Ser SNP are diverse depending on the pathogen studied

Table 2
Models of association for TLR2 Arg753Gln and TLR1 Ile602Ser SNPs in confirmed leptospirosis patients compared to controls.

SNP	Controls ($n=60$)	Leptospirosis patients ($n=55$)	Genotypic model P-value[a]	Additive model[b] P-value[a]	Dominant model P-value[a]	Recessive model P-value[a]
TLR2 Arg753Gln						
G/A	0(0%)	4(7.3%)	0.0493[c]	N/A[d]	0.0493[c]	N/A[d]
G/G	60(100%)	51(92.7%)				
TLR1 Ile602Ser						
G/G	5(8.3%)	11(20.0%)	0.03952[c]	0.0110[c]	0.0249[c]	0.1046[c]
T/G	20(33.3%)	24(43.6%)				
T/T	35(58.4%)	20(36.4%)				

[a] Fisher's exact test.
[b] Cochran–Armitage trend test.
[c] $P<0.05$.
[d] N/A, not applicable (no homozygosity).

Table 3
Genotype distributions of TLR2 Arg753Gln and TLR1 Ile602Ser SNPs for symptoms observed in confirmed leptospirosis patients.

Symptoms	Present		Absent		Present		Absent	
	TLR2 Arg753Gln SNP				TLR1 Ile602Ser SNP			
	G/A carriers	G/G carriers	G/A carriers	G/G carriers	G/G + T/G carriers	T/T carriers	G/G + T/G carriers	T/T carriers
Fever	4	46	0	5	34	16	1	4
P-value[a]	1.0000				0.0532			
OR (95% CI)	1.07 (0.05–22.55)[b]			8.50 (0.88–82.36)				
Jaundice	3	5	1	46	8	0	27	20
P-value[a]	0.0079[c]				0.0406[c]			
OR (95% CI)	27.60 (2.38–318.20)			12.67 (0.69–232.60)[b]				
Headache	3	32	1	19	22	13	13	7
P-value[a]	1.0000				1.0000			
OR (95% CI)	1.78 (0.17–18.38)			0.91 (0.29–2.87)				
Myalgia	3	31	1	20	21	13	14	7
P-value[a]	1.0000				0.7791			
OR (95% CI)	1.94 (0.19–19.94)			0.81 (0.26–2.53)				
Respiratory symptoms	2	13	2	38	12	3	23	17
P-value[a]	0.2975				0.2076			
OR (95% CI)	2.92 (0.37–22.92)			2.96 (0.72–12.14)				
Hepatic insufficiency	3	7	1	44	7	3	28	17
P-value[a]	0.0165[c]				0.7311			
OR (95% CI)	18.86 (1.71–207.90)			1.42 (0.32–6.23)				
Renal insufficiency	2	5	2	46	6	1	29	19
P-value[a]	0.0745				0.4019			
OR (95% CI)	9.20 (1.05–80.33)			3.93 (0.44–35.31)				
Meningeal signs	0	2	4	49	2	0	33	20
P-value[a]	1.0000				0.5286			
OR (95% CI)	2.20 (0.09–53.26)[b]			3.06 (0.14–67.01)[b]				
Hemorrhage	2	5	2	46	4	3	31	17
P-value[a]	0.0745				0.6960			
OR (95% CI)	9.20 (1.05–80.33)			0.73 (0.15–3.66)				
Leukocytosis (>10,000/mm^3)	3	19	1	32	16	6	19	14
P-value[a]	0.2904				0.3912			
OR (95% CI)	5.05 (0.49–52.13)			1.97 (0.61–6.30)				

[a] Fisher's exact test.
[b] OR and CI calculated with Haldane's correction.
[c] $P < 0.05$; OR, odds ratio; CI, confidence interval.

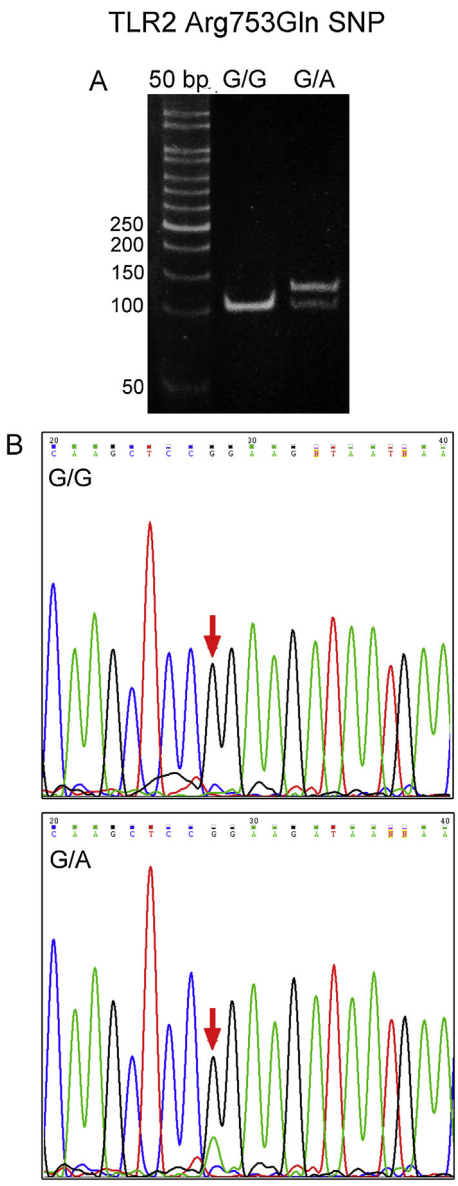

Fig. 1. (A) Representative RFLP analysis of TLR2 showing G/G (104 bp) and heterozygous G/A (129 + 104 bp) genotype carriers of the TLR2 Arg753Gln single-nucleotide polymorphism. 50 bp: 50 base pair ladder. (B) Representative DNA sequencing chromatograms for G/G and G/A genotype carriers. The arrow indicates the position of the SNP.

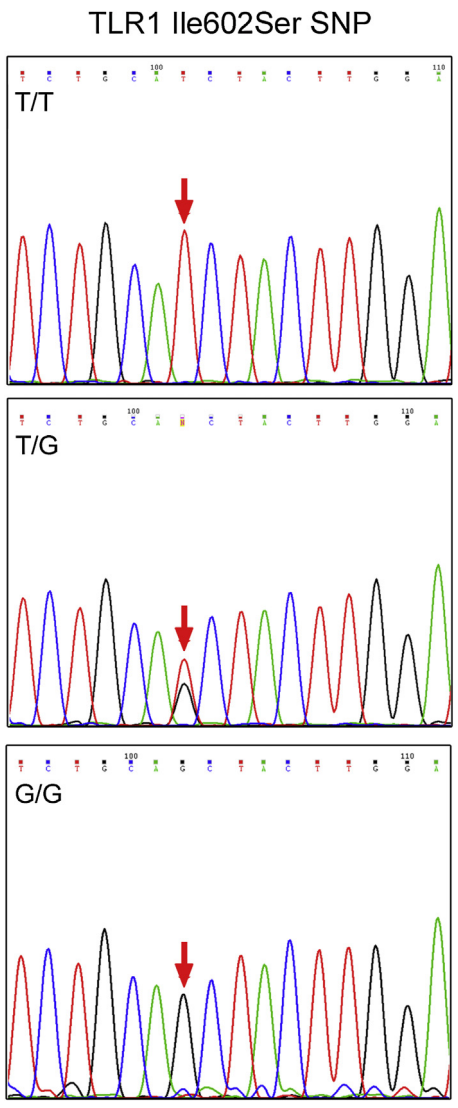

Fig. 2. Representative DNA sequencing chromatograms for T/T, T/G and G/G genotype carriers of the TLR1 Ile602Ser single-nucleotide polymorphism. The arrow indicates the position of the SNP.

and population ethnicity. The G allele (serine) has been associated with protection from tuberculoid leprosy (Johnson et al., 2007; Misch et al., 2008; Wong et al., 2010), but also with susceptibility to candidemia (Plantinga et al., 2012). In addition, both the G allele (Ma et al., 2007) and the T allele (isoleucine) (Uciechowski et al., 2011) have been associated with an increased risk of tuberculosis (Ma et al., 2007). Susceptibility to Lyme disease, caused by the spirochete *Borrelia* spp., was associated with homozygous carriage of three TLR1 SNPs: Ile602Ser and Asn248Ser (80% linkage), and Arg80Thr (Oosting et al., 2011). These data, together with our results, suggest that carrying a SNP in the *tlr2* and/or the *tlr1* genes could be a causative factor for increased susceptibility to some infectious diseases, including leptospirosis.

Xiong et al. (2012) demonstrated that the Arg753Gln mutation causes deficient TLR2 signaling by affecting the TLR2/TLR6 heterodimerization, phosphorylation of tyrosine residues of the receptor and recruitment of the adapter proteins MyD88 and Mal, although it does not affect TLR2 expression. As the Arg753Gln mutation is located in the TIR domain at a significant distance from the ligand recognition interface in the ectodomain, it is unlikely that the mutation has a direct impact on the recognition of ligands. For this reason, the authors speculated that signaling is deficient because the polymorphism affects the cooperative interaction between the TIR domains of the TLR2 and TLR6 receptors, by altering the electrostatic potential and the conformation of TLR2. Neither, however, rule out the possibility that this polymorphism affects the traffic and the location of the receptor, analogous to the effect reported with the Pro631His mutation (Etokebe et al., 2010), an important regulatory mechanism of TLR2 signaling (McGettrick and O'Neill, 2010). It is important to note that the arginine residue at position 753 of TLR2 has been described as one of the crucial amino acids for stabilizing the heterodimerization of TLR2, in particular with TLR1 (Gautam et al., 2006).

Regarding the TLR1 Ile602Ser SNP, several studies have shown that compared with homozygous T/T or heterozygous T/G, which are functionally similar, homozygous G/G leads to decreased numbers of TLR1 on the cell surface, impaired downstream signaling, and less cytokine production (Hawn et al., 2007; Johnson et al., 2007; Uciechowski et al., 2011), which lead to a state

of hyporesponsiveness to TLR1/2 agonists, including bacterial lipoproteins and synthetic triacylated lipopeptides. The difference between the TLR1 variants may be attributable to a conformational change induced by the substitution of an isoleucine for a serine within the transmembrane domain. This structural change may impact the extracellular ligand-binding domain or the intracellular TIR signaling domain that binds to adaptor proteins such as MyD88 and TIRAP/Mal.

In our study population, only a small subset (7.3%) of leptospirosis patients carried the Arg753Gln SNP A allele, while 53.6% carried the Ile602Ser SNP G allele. However, other factors of the immune response, including SNPs of other TLRs, might also contribute to increasing susceptibility to leptospirosis. In this regard, although progress has been made in the characterization of the host immune system factors that may affect disease progression and outcome, to date, few other genetic polymorphisms have been associated with susceptibility to leptospirosis, namely, human leukocyte antigen (HLA) Class I alleles (A*24, A*31 and B*08), HLA Class I ancestral haplotype 8.1, interleukin (IL)-4 and IL-4Rα genotypes (Fialho et al., 2009), and also the HLA-DQ6 genotype (Lingappa et al., 2004). Interestingly, HLA alleles have also been associated with susceptibility to other infectious pathogens, including *Plasmodium malariae*, *Mycobacterium tuberculosis* and HIV (Lingappa et al., 2004). With regard to other spirochetes, Lyme disease caused by *Borrelia burgdorferi* has been associated with TLR1 (Strle et al., 2012) and HLA Class II (Wormser et al., 2005) gene polymorphisms; syphilis caused by *Treponema pallidum* was associated with a deletion variant of KIR2DS4 (Zhuang et al., 2012), a killer immunoglobulin-like receptor (KIR); and periodontitis caused by *Treponema denticola* and other "red complex" bacteria has been associated with CD14-260 (Gong et al., 2013), NF-κB (Schulz et al., 2010) and IL-2 (Reichert et al., 2009) gene polymorphisms. These polymorphisms could also be associated with leptospirosis and deserve further investigation.

Argentina is an agricultural country with frequent floods in the centric and north-east subtropical regions; thus, activities associated with rural occupations and exposure to contaminated water are important risk factors for leptospirosis disease (Vanasco et al., 2008). The most prevalent infecting *Leptospira* serogroup is Icterohaemorrhagie (followed by Pomona, Ballum and Canicola), which mainly affects males over 30 years old (Vanasco et al., 2008), consistent with our study population. However, since we only studied a small population, our results should be considered preliminary, and further studies should be performed. If the present data were confirmed in larger populations, they might be used to identify individuals with a higher risk of developing leptospirosis, thus helping public health authorities to design better strategies to prevent the disease.

5. Conclusions

In conclusion, the arginine to glutamine substitution polymorphism at residue 753 and the isoleucine to serine substitution at residue 602 of the Toll-like receptor 2 and 1 proteins, respectively, are candidate alleles that may increase susceptibility to the development, and severity, of leptospirosis.

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