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Analysis of 16S rDNA sequences from pathogenic *Leptospira* serovars and use of single nucleotide polymorphisms for rapid speciation by D-HPLC

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ABSTRACT

Leptospira have a worldwide distribution and include important zoonotic pathogens yet diagnosis and differentiation still tend to rely on traditional bacteriological and serological approaches. In this study a 1.3 kb fragment of the *rrs* gene (16S rDNA) was sequenced from a panel of 22 control strains, representing serovars within the pathogenic species *Leptospira interrogans*, *Leptospira borgpetersenii*, and *Leptospira kirschneri*, to identify single nucleotide polymorphisms (SNPs). These were identified in the 5′ variable region of the 16S sequence and a 181 bp PCR fragment encompassing this region was used for speciation by Denaturing High Performance Liquid Chromatography (D-HPLC). This method was applied to eleven additional species, representing pathogenic, non-pathogenic and intermediate species and was demonstrated to rapidly differentiate all but 2 of the non-pathogenic *Leptospira* species. The method was applied successfully to infected tissues from field samples proving its value for diagnosing leptospiral infections found in animals in the UK.

1. Introduction

Leptospira are an important group of spiral-shaped bacteria which include major animal and zoonotic pathogens that are widely distributed in the environment and a wide range of wild and domestic animals. Human infections can result from contact with contaminated soil, water, or body fluids (especially urine) of infected carrier animals such as wild rodents (Bahaman and Ibrahim, 1988; Levett, 2001) and can cause potentially life threatening disease, particularly of the hepatonephritic type (Weil's disease) which has mortality rates approaching 15% (Faine et al., 1999). In the UK leptospirosis is an endemic disease in animals and an important cause of infertility, abortion, stillbirth and weak neonates in cattle and pigs (Dhaliwal et al., 1996a; Smyth et al., 1999) and milk drop in cattle (Dhaliwal et al., 1996b).

The *Leptospira* genus consists of a heterogeneous group of pathogenic and saprophytic species belonging to the order *Spirochaetales*. The genus *Leptospira* comprises 20 genomospecies, based on phylogenetic analysis of 16S rRNA (Morey et al., 2006; Matthias et al., 2008; Slack et al., 2008, 2009) and these have been broadly divided into pathogenic, saprophytic and intermediate types according to molecular typing and pathogenicity factors.

Serology, using microscopic agglutination tests (MAT), is currently the primary diagnostic tool for current and past infections. In reference laboratories cross-absorption agglutination test and monoclonal antibodies are used for serovar identification (Faine et al., 1999; Feresu et al., 1994). These methods use viable bacterial cultures, which can take between two to twelve weeks to grow, due to the fastidious nature of these organisms. However, interpretation of MAT results can be subjective.

Molecular approaches may be used when organisms are likely to be present. Several PCR methods have been developed (Stoddard et al., 2009; Fearnley et al., 2008; Fernandes et al., 2008; Slack et al., 2006a; Gravekamp et al., 1993) for detection whilst numerous molecular typing methods have been described including restriction fragment length polymorphism (Djadid et al., 2009; Woodward and Redstone, 1993), variable number tandem repeats (Slack et al., 2006b; Majed et al., 2005; Perolat et al., 1993), PFGE (Herrmann et al., 1992; Galloway and Levett, 2008), ribotyping (Perolat et al., 1994), arbitrarily primed-PCR (Perolat et al., 1994), multiplex PCR (Richtzenhain et al., 2002), 16S rRNA gene sequencing (Morey et al., 2006), *ompL1* species specific PCR (Reitstetter, 2006), multilocus sequence typing (Ahmed et al., 2006) and *wzx* gene sequence variation (Wangroongsarb et al., 2007).

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Table 1
Leptopira strains from the VLA panel and GenBank *Leptopira* strains included for comparison and DHPLC.

Serovar	Serogroup	Reference strain/subtype	Species	GenBank Accession no.
Australis[a]	Australis	Ballico	*L. interrogans*	AM050579
Australis	Australis	Ballico	*L. interrogans*	AY996794
Australis	Australis	Ballico	*L. interrogans*	FJ154556
Autumnalis[a]	Autumnalis	Akiyami A	*L. interrogans*	AM050580
Autumnalis	Autumnalis	Akiyami	*L. interrogans*	AF157069
Autumnalis	Autumnalis	Akiyami	L. interrogans	FJ154543
Autumnalis	Autumnalis	Akiyami A	*L. interrogans*	AY461864
Ballum[a]	Ballum	Musl27	*L. borgpetersenii*	AM050581
Ballum	Ballum	Musl27	*L. borgpetersenii*	AY631884
Ballum	Ballum	Musl27	*L. borgpetersenii*	AY461856
Bataviae[a]	Bataviae	Swart	*L. interrogans*	AM050582
Bataviae	Bataviae	Swart	*L. interrogans*	DQ991469
Bataviae	Bataviae	Van Tienen	*L. interrogans*	AY461865
Bim	Autumnalis	1051	*L. kirschneri*	AY996802
Bratislava[a]	Australis	Jez Bratisalva	*L. interrogans*	AM050583
Bratislava	Australis	Jez Bratisalva	*L. interrogans*	FJ154547
Bratislava[c]	Australis	Isolate HB	*L. interrogans*	AM050584
Bratislava[c]	Australis	Isolate BE	*L. interrogans*	AM050585
Bratislava[c]	Australis	Isolate K5A	*L. interrogans*	AM050586
Bratislava	Australis	Jez Bratisalva	*L. interrogans*	AY461863
Canicola[a]	Canicola	Utrecht V	*L. interrogans*	AM050566
Canicola	Canicola	Hond Utrecht IV	*L. interrogans*	AY461866
Canicola	Canicola	Moulton	*L. interrogans*	X17547
Celledoni[b]	Celledoni	Celledoni ATCC 43285	*L. weilii*	AY631877
Codice[b]	Codice	CDC ATCC 43284	*L.. wolbachii*	AY631879
Copenhageni[a]	Icterohaemorrhagiae	Wijinberg	*L. interrogans*	AM050565
Copenhageni	Icterohaemorrhagiae	Ll-130	*L. interrogans*	AY461869
Copenhageni	Icterohaemorrhagiae	Fiocruz Ll-130 (*rrs*1)	*L. interrogans*	NC005823
Copenhageni	Icterohaemorrhagiae	Fiocruz Ll-130 (rrs2)	*L. interrogans*	NC005823
Grippotyphosa[a]	Grippotyphosa	Moskva V	*L. kirschneri*	AM050567
Grippotyphosa	Grippotyphosa	Moskva V	*L. kirschneri*	AF157067
Grippotyphosa	Grippotyphosa	RM52	*L. kirschneri*	AY461877
Hardjo[a]	Sejroe	Hardjoprajitno	*L. interrogans*	AM050568
Hardjo	Sejroe	Hardjoprajitno	*L. interrogans*	AY461867
Hardjo	Sejroe	Hardjoprajitno	*L. interrogans*	FJ154553
Hardjo	Sejroe	Hardjoprajitno	*L. interrogans*	AY996796
Hardjo	Sejroe	Hardjo Lepto-0184	*L. interrogans*	AY99697
Hardjobovis[a]	Sejroe	Sponslee	*L. borgpetersenii*	AM050569
Hardjobovis	Sejroe	L550	*L. borgpetersenii*	CP000348
Hebdomadis[a]	Hebdomadis	Hebdomadis	*L. interrogans*	AM050570
Hebdomadis	Hebdomadis	Hebdomadis	*L. interrogans*	FJ154551
Hurstbridge[b]	Hurstbridge	BUT6ATCC BAA-1107	*L. fainei*	AY631885
Icterohaemorrhagiae[a]	Icterohaemorrhagiae	RGA	*L. interrogans*	AM050571
Icterohaemorrhagiae	Icterohaemorrhagiae	RGA	*L. interrogans*	AY631894
Icterohaemorrhagiae	Icterohaemorrhagiae	Par	*L. interrogans*	AF157084
Icterohaemorrhagiae	Icterohaemorrhagiae	Jac	*L. interrogans*	AF157073
Javanica[a]	Javanica	Veldrat Bataviae 46	*L. borgpetersenii*	AM050572
Javanica	Javanica	Veldrat Bataviae 46	*L. borgpetersenii*	Z21630
Javanica	Javanica	Veldrat Bataviae 46	*L. borgpetersenii*	FJ154600
Javanica	Javanica	Veldrat	*L. borgpetersenii*	AY461862
Korat[b]	Not designated	Korat-H2	*L. wolffii*	EF025496
Lai	Icterohaemorrhagiae	56601	*L. interrogans*	NC004342
Lyme[b]	Not designated	10 ATCC 43289	*L. inadai*	AY631896
Malaysia[b]	Tarassovi	Bejo-Iso9	*L. kmettyi*	AB279549
Manhao 3[b]	Manhao	L60ATCC 700520	*L. alexanderi*	AY631880
Mini[a]	Mini	Sari	*L. borgpetersenii*	AM050573
Mini	Mini	Sari	*L. borgpetersenii*	FJ154592
Mini	Mini	Sari	*L. borgpetersenii*	AY461859
Mozdoc[a]	Pomona	Pomona 5621	*L. kirschneri*	AM050574
Mozdoc	Mozdoc	5621	*L. kirschneri*	AY461879
Panama[b]	Panama	CZ214 ATCC 43288	*L. noguckii*	AY631886
Pomona[a]	Pomona	Pomona	*L. interrogans*	AM050575
Pomona	Pomona	Pomona	*L. interrogans*	AY996800
Pomona	Pomona	Pomona	*L. interrogans*	FJ154544
Pomona	Pomona	Pomona pomona	*L. interrogans*	AY461872
Ranarum[b]	Ranarum	Iowa City Frog ATCC 43287	*L. meyeri*	AY631878
Sejroe[a]	Sejroe	M84	*L. borgpetersenii*	AM050576
Sejroe	Sejroe	M84	*L. borgpetersenii*	AF157071
Sejroe	Sejroe	M84	*L. borgpetersenii*	FJ154593
Shermani[b]	Shermani	LT821 ATCC 43286	*L. santarosai*	AY631883
Tarassovi[a]	Tarassovi	Perepelicin	*L. borgpetersenii*	AM050577
Tarassovi	Tarassovi	Perepelicin	*L. borgpetersenii*	AY461861
Tarassovi	Tarassovi	Perepelicin	*L. borgpetersenii*	FJ154595
Zanoni[a]	Pyrogenes	Zanoni	*L. interrogans*	AM050578
Zanoni	Pyrogenes	Zanoni	*L. interrogans*	DQ991473
Not designated[b]	Not designated	5399 ATCC BAA-1107	*L. broomii*	AY796065

[a] Strains supplied by KIT (January 1998-October 2005), which were sequenced and used for DHPLC and comprise a panel of 19 antigens used for routine MAT based seordiagnosis.
[b] Strains supplied by KIT (November 2008) which were used for DHPLC (sequences obtained from GenBank).
[c] Strains from VLA culture collection, isolated from infected pig foetal tissue, which were sequenced and used for DHPLC.