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DNA relatedness of *Leptospira* strains isolated from beef cattle in Zimbabwe

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The DNA relatedness of 17 *Leptospira* strains isolated from beef cattle in Zimbabwe was determined using the hydroxyapatite method. Similarly to previously speciated African strains, all Zimbabwe isolates belonged to either *Leptospira borgpetersenii* or *Leptospira kirschneri*. All serovars within serogroups Pyrogenes (kwale, mombe and a strain closely related to serovar nigeria), Hebdomadis (marondera and mhou), Tarassovi (ngavi) and Sejroe (balcanica and hardjo) were *L. borgpetersenii*. *L. kirschneri* contained all strains in serovars of serogroups Icterohaemorrhagiae (zimbabwe), Australis (fugis), Bataviae (paidjan) and Pomona (a strain closely related to mozdok). The species designations of the Zimbabwe fugis and paidjan strains were different from those of the reference strains of these two serovars, both of which belong to *Leptospira interrogans*.

Keywords: DNA relatedness, *Leptospira borgpetersenii*, *Leptospira kirschneri*

INTRODUCTION

Leptospira traditionally contained two species, *Leptospira interrogans*, which consisted of serogroups pathogenic for man and animals, and *Leptospira biflexa*, with non-pathogenic serogroups (Johnson & Faine, 1984). DNA relatedness studies demonstrated extreme heterogeneity in both of these nomen species. On the basis of these studies, the family *Leptospiraceae* was shown to include the genus *Leptospira* with 12 named and four unnamed species, the genus *Leptonema* with its single species *Leptonema illini* and '*Turneria parva*' (Yasuda *et al.*, 1987; Ramadass *et al.*, 1992; Perolat *et al.*, 1998; Brenner *et al.*, 1999).

Leptospira isolates are still usually identified serologically, to the level of serogroup and/or serovar, although the serological methods used for identification are laborious, subjective and often not reproducible. Based on this concept, pathogenic strains of *Leptospira* are divided into more than 220 serovars which are grouped into more than 30 serogroups (Kmety & Dikken, 1993). A serogroup contains separate but closely antigenically related serovars. Assignment of strains to serogroups is essentially a consequence of the serological typing technique (Kmety & Dikken, 1993). It is now well-known that serovars and serogroups do not equate with species assignation (Yasuda *et al.*, 1987; Ramadass *et al.*, 1992; Brenner *et al.*, 1999). In some cases antigenically similar strains may be genetically diverse (Yasuda *et al.*, 1987; Ramadass *et al.*, 1992; Gravekamp *et al.*, 1993), while in other cases genetic analyses may fail to distinguish between antigenically different strains (Thiermann *et al.*, 1986; Zuerner & Bolin, 1990; Herrmann *et al.*, 1991).

A variety of modern genetic analyses have been applied to *Leptospira* taxonomy in an attempt to reconcile the two approaches. These methods include RFLP of chromosomal DNA (Marshall *et al.*, 1981; Thiermann *et al.*, 1986; Ellis *et al.*, 1991), restriction analysis of chromosomal DNA by PFGE (Herrmann *et al.*, 1991, 1992), DNA hybridization with total DNA probes (Millar *et al.*, 1987; Terpstra *et al.*, 1987; Nielsen *et al.*, 1989), restriction analysis by Southern blotting with recombinant DNA probes (Le Febvre, 1987; Zuerner & Bolin, 1988, 1990; Van Eys *et al.*, 1991; Woodward & Sullivan, 1991; Pacciarini *et al.*, 1992; Zuerner *et al.*, 1993), rRNA gene analysis (Hookey, 1990, 1993; Perolat *et al.*, 1990, 1993) and PCR fingerprinting (Van Eys *et al.*, 1989; Hookey, 1992; Mérien *et al.*, 1992; Corney *et al.*, 1993; Gravekamp *et al.*, 1993; Ralph *et al.*, 1993; de Caballero *et al.*, 1994; Perolat *et al.*, 1994; Zuerner *et al.*, 1995; Murgia *et al.*, 1997; Letocart *et al.*, 1997).

Groupings obtained using some of these methods, such as RFLP, PFGE, restriction analysis by Southern blotting with DNA probes and IS*1533*-based PCR assays, were designed to replace serotyping and generally correlate with serovars, while the rest of the methods support taxa generated in DNA relatedness

Table 1. Strains used in the study

Serogroup	Serovar	Strain	Species assignment
Zimbabwe isolates			
Pyrogenes	kwale	SBF 2	*Leptospira borgpetersenii*
	mombe	SBF 20*	*Leptospira borgpetersenii*
	mombe	SBF 28	*Leptospira borgpetersenii*
	mombe	SBF 43	*Leptospira borgpetersenii*
	(nigeria)†	SBF 49	*Leptospira borgpetersenii*
Hebdomadis	marondera	SBF 5*	*Leptospira borgpetersenii*
	marondera	SBF 21	*Leptospira borgpetersenii*
	marondera	SBF 50	*Leptospira borgpetersenii*
	mhou	SBF 40*	*Leptospira borgpetersenii*
Sejroe	hardjo	SBF 27	*Leptospira borgpetersenii*
	hardjo	SBF 41	*Leptospira borgpetersenii*
	balcanica	SBF 47	*Leptospira borgpetersenii*
Tarassovi	ngavi	SBF 19	*Leptospira borgpetersenii*
Icterohaemorrhagiae	zimbabwe	SBF 23*	*Leptospira kirschneri*
Australis	fugis	SBF 3	*Leptospira kirschneri*
Pomona	(mozdok)†	SBF 8	*Leptospira kirschneri*
Bataviae	paidjan	SBF 37	*Leptospira kirschneri*
Reference strains			
Icterohaemorrhagiae	icterohaemorrhagiae	RGA^T	*Leptospira interrogans*
Cynopteri	cynopteri	3522 C^T	*Leptospira kirschneri*
Panama	panama	CZ 214 K^T	*Leptospira noguchii*
Shermani	shermani	1342 K^T	*Leptospira santarosai*
Javanica	javanica	Veldrat Batavia 46^T	*Leptospira borgpetersenii*
Manhao	manhao 3	L 60^T	*Leptospira alexanderi*
Celledoni	celledoni	Celledoni^T	*Leptospira weilii*
Codice	codice	Biflexa CDC^T	*Leptospira wolbachii*
Lyme	lyme	Strain 10^T	*Leptospira inadai*
Ranarum	ranarum	Iowa City Frog^T	*Leptospira meyeri*
Andamana	andamana	CH 11	*Leptospira biflexa*
Undesignated	holland	Waz Holland	*Leptospira* genomospecies 3
Leptonema	illini	3055^T	*Leptonema illini*
Turneria	parva	H^T	'*Turneria parva*'

* Zimbabwe serovar reference strain.

† Closely related to serovar.

studies. The latter methods even show more heterogeneity within DNA related groupings with several classification systems proposed (Perolat *et al.*, 1990, 1993, 1994; Hookey, 1993; Ralph *et al.*, 1993). However, despite these advances, the differences revealed by the molecular methods have not yet been able to describe the desired host–pathogen relationships required in epidemiological studies, or to justify the complete overhaul of the old system based on antigenic structures. Therefore, it is customary to use a variety of these methods in conjunction with serology when characterizing and/or identifying field isolates.

The isolation of 50 *Leptospira* strains from kidneys of beef cattle in Zimbabwe and their serogrouping have been reported previously (Feresu, 1992). The isolates belonged to nine serogroups: Australis, Bataviae, Grippotyphosa, Hebdomadis, Icterohaemorrhagiae, Pomona, Pyrogenes, Sejroe and Tarassovi.

Representatives from each of these serogroups have been characterized using various methods, including the cross-agglutinin absorption test, mAb analysis, RFLP of chromosomal DNA, restriction analysis by Southern blotting with recombinant DNA probes, PFGE and PCR fingerprinting (Feresu & Bolin, 1991; Feresu *et al.*, 1993, 1994, 1995, 1996, 1998, 1999). These studies identified five new serovars: zimbabwe (Icterohaemorrhagiae), mombe (Pyrogenes), mhou and marondera (Hebdomadis) and ngavi (Tarassovi).

In the present study, 17 representatives of eight of these serogroups were further characterized for DNA relatedness and assigned to species.

METHODS

Bacterial strains. Table 1 presents the 17 Zimbabwe strains which were studied and their serological assignments. All isolates were obtained from stock cultures maintained in

Table 2. Percentage DNA relatedness of Zimbabwean and reference strains

R, Relatedness; D, divergence; ND, not done.

Source of unlabelled DNA serovar/strain	Source of labelled DNA											
	hardjo Hardjo-bovis SBF 41			zimbabwe SBF 23			mombe SBF 28			paidjan SBF 37		
	R (%) at 55 °C	D (%)	R (%) at 70 °C	R (%) at 55 °C	D (%)	R (%) at 70 °C	R (%) at 55 °C	D (%)	R (%) at 70 °C	R (%) at 55 °C	D (%)	R (%) at 70 °C
SBF 2	96	0·5	98									
SBF 20	95	0·5	97									
SBF 43	93	1·0	94									
SBF 49	95	2·0	97									
SBF 5	86	1·0	92									
SBF 21	92	1·5	96									
SBF 50	95	2·0	100									
SBF 40	97	1·5	100									
SBF 27	93	0·0	97									
SBF 41	100	0·0	100									
SBF 47	96	1·0	98									
SBF 19	93	2·0	97									
SBF 28							100	0·0	100			
SBF 23				100	0·0	100						
SBF 3				96	2·0	90						
SBF 8	45			88	0·0	90						
SBF 37	49									100	0·0	100
icterohaemorrhagiae	52									78	7.0	50
cynopteri	45			88	1·0	90				86	1·5	85
panama	32									60		
shermani	53									36		
javanica	90	2·5	89				82	2·0	90	ND		
manhao 3	74	7·0	60				70	7·0	ND	60		
celledoni	73	8·5	56				66	8·0	51	46		
codice	4									5		
lyme	13									10		
ranarum	7									6		
andamana	3									5		
holland	6									4		
illini	6									6		
parva	6									5		

liquid nitrogen as part of the reference collection of the National Reference Laboratory at the National Animal Disease Center (Ames, IA, USA). The serovar identity of these strains had been previously verified by the National Reference Laboratory at Ames and confirmed by the WHO/FAO Collaborating Centre for Reference and Research on Leptospirosis (Amsterdam, The Netherlands) (Feresu *et al.*, 1993, 1994, 1995, 1996, 1998, 1999).

Reference strains for the family *Leptospiraceae* (Table 1) were obtained from the stock culture collection of the Special Bacteriology Reference Laboratory in the Meningitis and Special Pathogens Branch at the Centers for Disease Control and Prevention (Atlanta, GA, USA). These strains were maintained in polysorbate albumin medium (PLM-5; Armour Pharmaceutical).

DNA relatedness studies. All strains were grown in polysorbate albumin medium at 30 °C and harvested by centrifugation during their stationary phase of growth. The methods used to extract and purify DNA and the hydroxyapatite method for determining levels of DNA relatedness have been described previously (Brenner *et al.*, 1982). Labelled DNAs from strains SBF 41, SBF 23, SBF 28 and SBF 37 were prepared enzymically *in vitro* with [^{32}P]dCTP by using a nick reagent kit (Bethesda Research Laboratories) as instructed by the manufacturer. Hybridization reactions were carried out at an optimal reassociation temperature of 55 °C and at a stringent incubation temperature of 70 °C (Yasuda *et al.*, 1987).

Levels of relatedness were expressed as percentage values, and levels of divergence of related sequences were determined by calculating the decreases in thermal stability in heteroduplexes (reassociated labelled DNAs from two different strains) compared with the thermal stability in homoduplexes (reassociated labelled and unlabelled DNAs from the same strains). The assumption was that each 1 °C decrease in heteroduplex DNA stability was due to approximately 1% unpaired bases within the related DNA (Brenner *et al.*, 1982), and therefore represents 1% divergence. Divergence was calculated to the nearest 0·5%.

RESULTS

A species is defined as a group of strains that exhibits levels of DNA relatedness at the optimal reassociation temperature of 70% or more and whose related sequences exhibit 5% or less divergence (Wayne *et al.*, 1987). The DNA relatedness results for the Zimbabwe strains are given in Table 2 and the species designations are in Table 1. The 17 strains belonged to two species, *Leptospira borgpetersenii* and *Leptospira kirschneri*.

All the serogroup Pyrogenes, Hebdomadis, Sejroe and Tarassovi strains were 86 % or more related at both the optimal and stringent incubation temperatures with 2% or less divergence within related sequences. These 13 strains were *L. borgpetersenii*, since the two of

them that were tested (hardjo SBF 41 and mombe SBF 28) were in the same relatedness group as serovar javanica strain Veldrat Batavia 46^T, the type strain for *L. borgpetersenii* (Yasuda *et al.*, 1987), displaying 82% or more relatedness with this strain at both temperatures with 2·5% or less divergence. At 55 °C, strains SBF 41 and SBF 28 were 66–74% related to reference strains of serovars manhao 3 and celledoni but showed less relatedness, 51–60%, at the more stringent (70 °C) temperature, and exhibited 7–8·5% divergence. Thus the 13 strains, including those belonging to four of the new serovars, mombe (Pyrogenes), marondera and mhou (Hebdomadis) and ngavi (Tarassovi), all belonged to *L. borgpetersenii*.

The remaining four strains from serogroups Icterohaemorrhagiae, Australis, Pomona and Bataviae were all 88% or more interrelated, and strains SBF 23 and SBF 37 from this group were 86% or more related to serovar cynopteri strain 3522 C^T, the type strain for *L. kirschneri* (Ramadass *et al.*, 1992), at both temperatures with less than 2·0% divergence within related sequences. Although strain SBF 37 from this group was 78% related to *L. interrogans* at 55 °C, divergence was 7% and relatedness fell to 50% in 70 °C reactions. Therefore this group, composed of the reference strain for the new Icterohaemorrhagiae serovar, zimbabwe, strains belonging to serovars fugis and paidjan, and a strain closely related to serovar mozdok, belonged to *L. kirschneri*.

DISCUSSION

Relatedness values obtained using the hydroxyapatite method reflect degrees of overall similarity between genomic DNAs of strains and hence define bacterial species in phylogenetic terms (Wayne *et al.*, 1987). It was possible to assign all the Zimbabwe cattle isolates belonging to serogroups Icterohaemorrhagiae, Pyrogenes, Hebdomadis, Tarassovi, Sejroe, Pomona, Bataviae and Australis to already existing species of *Leptospira*. The isolates all belonged to either *L. borgpetersenii* or *L. kirschneri*.

Members of serogroup Icterohaemorrhagiae are found in five species: *L. interrogans*, *L. borgpetersenii*, *Leptospira inadai*, *L. kirschneri* and the unnamed *Leptospira* genomospecies 5 (Yasuda *et al.*, 1987; Gravekamp *et al.*, 1993; Brenner *et al.*, 1999). Similar to the other three Icterohaemorrhagiae reference strains of serovars of African origin (mwogolo, ndambari and ndahambukuje), the reference strain of our new serovar, zimbabwe, belongs to *L. kirschneri* (Feresu *et al.*, 1993; Gravekamp *et al.*, 1993). Although strain SBF 23 was antigenically very similar to reference strains of serovars birkini, gem, lai and mwogolo (Feresu *et al.*, 1993), all these other reference strains, except that of mwogolo, belong to *L. interrogans* (Gravekamp *et al.*, 1993; Brenner *et al.*, 1999).

Five of the isolates studied were members of the Pyrogenes serogroup. Three of the strains belong to our new serovar mombe, one strain belongs to serovar kwale and another strain is very closely related to serovar nigeria (Feresu *et al.*, 1994). Pyrogenes serovars generally are found in *L. interrogans*, *L. borgpetersenii*, *Leptospira noguchii*, *Leptospira santarosai* and *Leptospira weilii* (Yasuda *et al.*, 1987; Gravekamp *et al.*, 1993; Brenner *et al.*, 1999). All the Zimbabwe isolates together with the reference strain for serovar kwale, which is of Kenyan origin, belong to *L. borgpetersenii*. The reference strain of the only other recognized Pyrogenes serovar of African origin, nigeria, has not yet been assigned to a species.

The serogroup Hebdomadis strains included in this study belonged to two new serovars, marondera and mhou (Feresu *et al.*, 1996). Members of serogroup Hebdomadis are found in six species, *L. interrogans*, *L. borgpetersenii*, *L. santarosai*, *L. weilii*, *L. kirschneri* and in *Leptospira* genomospecies 2 (Yasuda *et al.*, 1987; Ramadass *et al.*, 1992; Gravekamp *et al.*, 1993; Brenner *et al.*, 1999). Reference strains of four of the serogroup Hebdomadis serovars, kabura, kambale, jules and nona, are of Zairian origin (Kmety & Dikken, 1993). Those of serovars jules and nona belong to *L. borgpetersenii* while those of serovars kabura and kambale belong to *L. kirschneri* (Ramadass *et al.*, 1992; Brenner *et al.*, 1999). The reference strains for the new serovars marondera and mhou belong to *L. borgpetersenii* similar to serovars jules and nona. A close relationship based on their PFGE restriction patterns had been previously observed among these four serovars (Feresu *et al.*, 1996).

One of the strains, SBF 19, included in the study belongs to the new serovar ngavi, in serogroup Tarassovi (Feresu *et al.*, 1998). Serovars of serogroup Tarassovi are found in five species, *L. borgpetersenii*, *L. inadai*, *L. noguchii*, *L. santarosai* and *L. weilii* (Yasuda *et al.*, 1987; Ramadass *et al.*, 1992; Gravekamp *et al.*, 1993; Brenner *et al.*, 1999). Like the other serogroup Tarassovi strains of African origin (the reference strains for serovars kisuba, kanana and tunis), strain SBF 19 belongs to *L. borgpetersenii* (Gravekamp *et al.*, 1993; Brenner *et al.*, 1999). In earlier studies, strain SBF 19 had RFLP patterns which were very similar to those of reference strains of serovars tarassovi, guidae, tunis and mouldaviae which could not be distinguished by restriction analysis alone (Feresu *et al.*, 1998). The restriction patterns could only be distinguished after their Southern blots had been hybridized with a probe synthesized from a repetitive sequence element cloned from serovar hardjo strain Hardjo-bovis (Zuerner *et al.*, 1993; Feresu *et al.*, 1998). Reference strains for these four closely related serovars also belong to *L. borgpetersenii* (Ramadass *et al.*, 1992; Gravekamp *et al.*, 1993; Brenner *et al.*, 1999).

Most strains (31) isolated from the cattle belonged to serogroup Sejroe serovar hardjo (Feresu, 1992). They were identified as being similar to strain Hardjo-bovis (Feresu & Bolin, 1991). Two representatives of these strains were included in this study. Serogroup Sejroe strains are found in *L. interrogans*, *L. borgpetersenii*, *L.*

santarosai, *L. weilii* and *Leptospira meyeri* (Van Eys *et al.*, 1991; Gravekamp *et al.*, 1993; Brenner *et al.*, 1999). Like all the other Hardjo-bovis strains characterized to date, our two isolates belonged to *L. borgpetersenii*.

One of the Sejroe strains, SBF 47, belongs to serovar balcanica (Feresu & Bolin, 1991). Although the chromosomal DNA of the isolate had a distinct RFLP pattern from that of the balcanica reference strain (Feresu & Bolin, 1991), both strains belong to *L. borgpetersenii*.

Identification of the Zimbabwe serogroup Pomona strain SBF 8 was equivocal (Feresu *et al.*, 1995) as the isolate showed a close similarity to serovars mozdok and proechimys by cross-agglutinin absorption test and to serovar pomona by mAbs but had a unique chromosomal DNA RFLP pattern. Serogroup Pomona strains are found in *L. interrogans*, *L. noguchii*, *L. santarosai* and *L. kirschneri* (Van Eys *et al.*, 1991; Ramadass *et al.*, 1992; Gravekamp *et al.*, 1993; Brenner *et al.*, 1999). Strain SBF 8 was identified as belonging to *L. kirschneri* in agreement with the results obtained using PCR with primer sets G1/G2 and B64-I/B64-II (Gravekamp *et al.*, 1993; Feresu *et al.*, 1995). The reference strains of serovars mozdok, proechimys and pomona, respectively, belong to *L. kirschneri*, *L. noguchii* and *L. interrogans* (Van Eys *et al.*, 1991; Gravekamp *et al.*, 1993; Brenner *et al.*, 1999). Our results confirm strain SBF 8 as a variant within serovar mozdok.

A single strain, SBF 3, belonging to serovar fugis of serogroup Australis was included in the study (S. B. Feresu, unpublished results). Australis strains are found in *L. interrogans*, *L. kirschneri*, *L. borgpetersenii* and *L. noguchii* (Ramadass *et al.*, 1992; Gravekamp *et al.*, 1993; Brenner *et al.*, 1999). The fugis isolate (SBF 3) belongs to *L. kirschneri*, unlike the reference strain of serovar fugis (Fudge), a Malaysian patient isolate, which belongs to *L. interrogans* (Ramadass *et al.*, 1992; Gravekamp *et al.*, 1993). Similar species assignments were obtained for SBF 3 and the Fudge reference strains from both the Atlanta and Amsterdam culture collections using PCR reactions with primer sets G1/G2 and B64-I/B64-II (Gravekamp *et al.*, 1993; S. B. Feresu & A. Whitney, unpublished results). The only other African Australis strain whose species has been determined is the reference strain for serovar ramisi, which also belongs to *L. kirschneri*.

Bataviae serovars are found in five species, *L. interrogans*, *L. noguchii*, *L. santarosai*, *L. kirschneri* and *Leptonema illini* (Gravekamp *et al.*, 1993; Brenner *et al.*, 1999). One of the Zimbabwe cattle strains, SBF 37, belonged to serogroup Bataviae, serovar paidjan (Feresu *et al.*, 1999), species *L. kirschneri*. This was unlike the reference strain for serovar paidjan, a patient isolate from Indonesia, which belongs to *L. interrogans*. Similar species identities were obtained for SBF 37 and the Paidjan reference strains from both the Amsterdam and Atlanta culture collections in PCR reactions using primer sets G1/G2 and B64-I/B64-II (Feresu *et al.*, 1999; S. B. Feresu & A. Whitney, unpublished results). The only other known serogroup Bataviae leptospire which belongs to *L. kirschneri* is the reference strain for serovar djatzi, a human isolate from Puerto Rico (Gravekamp *et al.*, 1993; Brenner *et al.*, 1999). There are no Bataviae reference strains isolated from Africa which could be used for comparison.

Similar to previous observations (Van Eys *et al.*, 1991; Zuerner *et al.*, 1993; Perolat *et al.*, 1994; Brenner *et al.*, 1999), our study has demonstrated a possible correlation between the genotype and geographical origin of a strain. So far all of the 37 African *Leptospira* strains whose DNA relatedness has been determined belong to either *L. kirschneri* (16) or *L. borgpetersenii* (21) (Feresu *et al.*, 1995; Brenner *et al.*, 1999; this study). Ramadass *et al.* (1992) derived a phylogenetic tree in which six of the pathogenic *Leptospira* species were shown to have originated from two ancestors, resulting in two groups: the *L. kirschneri*, *L. interrogans* and *L. noguchii* group and the *L. borgpetersenii*, *L. santarosai* and *L. weilii* group. Thus there appears to be limited species diversity on the African continent as only a single species from each group has been isolated so far.

All the *L. kirschneri* strains identified in this study are either members of a serovar in which the reference strain belongs to *L. interrogans* or are antigenically closely related to serovars which belong to *L. interrogans*. This is interesting as *L. interrogans* is the closest relative of *L. kirschneri* on the phylogenetic tree (Ramadass *et al.*, 1992).

Generally, the African strains within a serogroup belong to the same species; thus, the Hebdomadis serogroup in which the four African serovars belong to either *L. interrogans* or *L. kirschneri* is worth noting.

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