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Article *in* International Journal of Systematic and Evolutionary Microbiology · April 2006

DOI: 10.1099/ijs.0.63278-0 · Source: PubMed

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New *Leptospira* serovar Sokoine of serogroup Icterohaemorrhagiae from cattle in Tanzania

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The prevalence of leptospirosis is generally high in domestic animals and rodents in Tanzania. Identification of *Leptospira* isolates from cattle was carried out to establish prevalent *Leptospira* serovars. Serological typing was done based on monoclonal antibodies and the standard cross-agglutination absorption test. Molecular typing involved pathogenic- and saprophytic-specific PCRs and a PCR specifically amplifying DNA from the species *Leptospira kirschneri*. DNA fingerprinting with primers derived from sequences of insertion elements IS*1500* and IS*1533* was carried out. Both serological and molecular characterization indicated that one of the *Leptospira* isolates, coded RM1, represents a new serovar of the species *L. kirschneri* of serogroup Icterohaemorrhagiae. The serovar name Sokoine is proposed for this new *Leptospira* isolate.

Relatively few cases of leptospirosis have been recorded from the African continent, mainly due to difficulties in diagnosis in both human and animals; hence the disease is not well investigated. However, the prevailing climatic and socio-economic environments of the African continent are favourable for a high incidence of this disease. Previous findings in Tanzania (Machang'u *et al.*, 1997, 2003, 2004) showed that leptospirosis is much more common than generally thought. Currently, 20 *Leptospira* serovars from Africa have been described. Eleven of these serovars belong to the species *Leptospira kirschneri*, with eight being found in the Democratic Republic of Congo (Zaire), two in Kenya and one in Ghana (Faine *et al.*, 1999). This suggests that *L. kirschneri* may be the prevalent *Leptospira* species, especially in Central and East Africa. Areas of high prevalence, such as Tanzania, might be harbouring many serovars which have not yet been described. Consistent with this assumption, we describe in this paper the isolation and identification of strain RM1 from cattle in Tanzania representing a new *L. kirschneri* serogroup Icterohaemorrhagiae proposed serovar, Sokoine.

Leptospira isolates and known pathogenic and saprophytic strains used in this study are listed in Table 1. Serovar Copenhageni, strain Wijnberg was used as reference pathogen, whereas serovars Semaranga and Patoc served as typical saprophytic reference strains. EMJH and Fletcher culture media were used to isolate and grow the leptospires (Faine, 1982). Leptospires were isolated from cattle in a slaughterhouse in Morogoro, Tanzania. Urinary bladders were aseptically punctured to obtain 0·5 ml aliquots of urine, which were then used to inoculate 5 ml Fletcher's medium, supplemented with 5-fluorouracil (200 μg ml^{-1}) as a selective inhibitor of contaminating micro-organisms (Faine, 1982). Cultures were incubated at ambient temperatures (25–30 °C) and examined weekly over a period of 8 weeks using dark-field (DF) microscopy (Faine, 1982; Machang'u *et al.*, 1997).

The pathogenic status of the isolates was determined by conventional methods and pathogen-specific PCR. The conventional methods consisted of determining *Leptospira* growth rates in EMJH medium at 13 °C and in EMJH medium containing 8-azaguanine according to Johnson & Harris (1967) and Johnson & Rogers (1964), respectively.

Briefly, 0·1 ml culture of isolate RM1 was used to inoculate 5 ml EMJH medium in four tubes and duplicates were incubated at 13 and 30 °C to determine the growth rates at low and high temperatures. EMJH medium (5 ml) with or without 8-azaguanine (225 μg ml^{-1}) was inoculated in 'duplicate' with 0·1 ml isolate RM1 and then incubated at 30 °C to determine inhibition (reduction) of growth in the presence of 8-azaguanine. *Leptospira* growth was determined under DF microscopy and by measuring culture density at 420 nm spectrophotometrically (DU Series 62;

Published online ahead of print on 4 November 2005 as DOI 10.1099/ijs.0.63278-0.

Abbreviations: CAAT, cross-agglutination absorption test; DF, dark-field.

Supplementary figures showing determination of the pathogenic status of isolate RM1 (serovar Sokoine) by PCR and serotyping of leptospires with mAbs are available as supplementary material in IJSEM Online.

Table 1. List of *Leptospira* strains used in this study

Species	Serogroup	Serovar	Strain	Reference
L. kirschneri	Icterohaemorrhagiae	Sokoine	RM1-cattle	Described in this paper
Unknown	Grippotyphosa	Isolate RM4	RM4-cattle	Unpublished
Unknown	Grippotyphosa	Isolate RM7	RM7-cattle	Unpublished
L. borgpetersenii	Ballum	Kenya	Sh9-giant rats	Machang'u *et al.* (2004)
L. borgpetersenii	Ballum	Kenya	Sh25-giant rats	Machang'u *et al.* (2004)
L. biflexa	Semaranga	Patoc	Patoc I	Brenner *et al.* (1999)
L. meyeri	Semaranga	Semaranga	Veldrat Semarang 173	Brenner *et al.* (1999)
L. interrogans	Icterohaemorrhagiae	Copenhageni	Wijnberg	Brenner *et al.* (1999)
L. kirschneri	Icterohaemorrhagiae	Mwogolo	Mwogolo	Brenner *et al.* (1999)
L. kirschneri	Icterohaemorrhagiae	Ndambari	Ndambari	Brenner *et al.* (1999)
L. kirschneri	Icterohaemorrhagiae	Ndahambukuje	Ndahambukuje	Brenner *et al.* (1999)
L. kirschneri	Icterohaemorrhagiae	Bogvere	LT 60-69	Brenner *et al.* (1999)
L. kirschneri	Icterohaemorrhagiae	Dakota	Grand River	Brenner *et al.* (1999)
L. interrogans	Icterohaemorrhagiae	Icterohaemorrhagiae	RGA	Brenner *et al.* (1999)
L. interrogans	Icterohaemorrhagiae	Copenhageni	M20	Brenner *et al.* (1999)
L. interrogans	Icterohaemorrhagiae	Lai	Lai	Brenner *et al.* (1999)
L. interrogans	Icterohaemorrhagiae	Birkini	Birkin	Brenner *et al.* (1999)
L. interrogans	Icterohaemorrhagiae	Gem	Simon	Brenner *et al.* (1999)
Unknown	Icterohaemorrhagiae	Hongchon	18 R	Kmety & Dikken (1993)
L. interrogans	Icterohaemorrhagiae	Mankarso	Mankarso	Brenner *et al.* (1999)
L. interrogans	Icterohaemorrhagiae	Naam	Naam	Brenner *et al.* (1999)
L. interrogans	Icterohaemorrhagiae	Smithi	Smith	Brenner *et al.* (1999)
L. borgpetersenii	Icterohaemorrhagiae	Tonkini	LT 96-68	Brenner *et al.* (1999)
Unknown	Icterohaemorrhagiae	Yeonchon	HM 3	Kmety & Dikken (1993)
Unknown	Sarmin	Cuica	RP 88	Kmety & Dikken (1993)
L. santarosai	Sarmin	Machiguenga	MMD 3	Brenner *et al.* (1999)
L. santarosai	Sarmin	Rio	Rr 5	Brenner *et al.* (1999)
L. weilii	Sarmin	Sarmin	Sarmin	Brenner *et al.* (1999)
L. interrogans	Sarmin	Waskurin	LT 63-68	Brenner *et al.* (1999)
L. santarosai	Sarmin	Weaveri	CZ 390	Brenner *et al.* (1999)

Beckman Instruments). Pathogenic strain Wijnberg and saprophytic strain Patoc I (Table 1) were included in the test for comparison of growth responses.

DNA was extracted from fully grown cultures using the Anansa Fast 'n' Easy Genomic DNA purification kit (Tebu-Bio Laboratories) and the method described by Boom *et al.* (1990). The quantity of extracted DNA was estimated by electrophoresis in a 1·5 % agarose gel stained with ethidium bromide by comparing the intensity of the genomic DNA bands with a standard DNA size marker (100–1000 bp, Smart ladder; Eurogentec). To determine the pathogenic status of the *Leptospira* isolate, PCR analysis was performed as described by Murgia *et al.* (1997) and Perolat *et al.* (1998). Primer pairs used included Lepat 1 and Lepat 2, which specifically amplify a 330 bp fragment from pathogenic leptospires, and Sapro 1 and Sapro 2, which specifically generate a 240 bp product from saprophytic leptospires (Murgia *et al.*, 1997). Other primer pairs were LP1 and *a*1190 and LU and rLP, which amplify DNA from pathogenic leptospires producing specific amplicons of 1008 and 420 bp, respectively (Perolat *et al.*, 1998).

PCR procedure described by Gravekamp *et al.* (1993) was used to determine whether isolate RM1 belonged to the species *L. kirschneri*. Primer pairs G1 and G2 that specifically amplify a 285 bp DNA fragment from all pathogenic *Leptospira* species, except *L. kirschneri*, and B64I and B64II that specifically amplify a 563 bp from *L. kirschneri* were used. Reference saprophytic and pathogenic strains were used as controls.

DNA fingerprinting was carried out as described previously (Zuerner *et al.*, 1995; Zuerner & Bolin, 1997).

Primers derived from insertion element sequences IS*1533* and IS*1500*, consisting of EPL-2 and EPR-2 and iP1 and iM16, respectively, were used separately and in combination. The generated DNA fingerprints of isolate RM1 and other *Leptospira* strains were compared following separation by electrophoresis in a 1·5 % agarose gel stained with ethidium bromide.

Isolate RM1 was subjected to serological microagglutination with 42 rabbit sera representative of all pathogenic and four

saprophytic serogroups. The rabbit sera were prepared as described by Faine (1982) and the microagglutination test was carried out as described by Cole *et al.* (1973). Monoclonal antibody (mAb) typing was done using a panel of mAbs (F12C3-10, F20C3, F20C4-1, F52C1, F52C2, F70C4-1, F70C7-8, F70C13-1, F70C14-6, F70C20-3, F70C24-14, F70C26-1, F82C1-3, F82C2-2, F82C7-3, F82C8-4, F89C3-3 and F89C12-4) that characteristically agglutinate serovars belonging to serogroups Icterohaemorrhagiae and Sarmin as described by Korver *et al.* (1988). Reference serovars of Icterohaemorrhagiae and Sarmin groups (Table 1) were included in this test for comparison of their agglutination patterns with that of isolate RM1.

Cross-agglutination absorption test (CAAT), the gold standard test for serological classification of *Leptospira* serovars, was repeatedly carried out by the Sokoine University of Agriculture (Tanzania) as described elsewhere (Stallman, 1987; Dikken & Kmety, 1978). CAAT results were confirmed by the WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis of the Royal Tropical Institute, Amsterdam, The Netherlands.

Results from this study revealed that isolate RM1 is a pathogenic *Leptospira* as indicated by suppressed growth both at 13 °C and in the presence of 8-azaguanine. PCR with pathogenic primers Lepat 1 and Lepat 2 gave a DNA product of 330 bp (Supplementary Fig. S1 available in IJSEM Online). No DNA product was generated using the saprophytic *Leptospira*-specific primers Sapro 1 and Sapro 2 (data not shown). In addition, a 1008 bp PCR product was obtained with the primers LP1 and *a*1190 (Supplementary Fig. S2 available in IJSEM Online) and a 420 bp DNA product was obtained with primer pair LU and rLP (data not shown). These findings support the pathogenic status of RM1. Strain RM1 was PCR-positive with an *L. kirschneri*-specific primer pair (B64I and B64II) by generating an amplicon of 563 bp, while no product was generated with primers G1 and G2 (data not shown). This suggests that RM1 is a strain of the species *L. kirschneri* (Gravekamp *et al.*, 1993). PCR-based fingerprinting with primer iM16 derived from IS*1500* produced a pattern from RM1 DNA which was closely related to that of serovar Ndahambukuje and somewhat related to *L. kirschneri* and *Leptospira interrogans* strains (data not shown). Distinct DNA patterns were obtained from serovar Ndambari and Bogvere that produced few bands for comparison. Apparently, RM1 is genotypically closely related to serovar Ndahambukuje. However, the iM16-based fingerprinting was not informative enough to explain the genetic relatedness of these serovars. DNA fingerprinting with primers derived from IS*1533* (EPL-2 and EPR-2) did not yield DNA profiles with multiple bands from *L. kirschneri* strains included in the analysis to enable comparison (data not shown). This probably suggests that there are only a few copies of the IS*1533* element in these strains.

Sero-agglutination was observed with sera specific for serogroups Icterohaemorrhagiae, Canicola and Sarmin. Further agglutination tests with rabbit sera against individual serovars of each of the three groups revealed highest agglutination titres with 13 of 17 serovars of the Icterohaemorrhagiae group, one of six serovars of the Sarmin group and one of 13 serovars of the Canicola group. These findings suggest that RM1 most likely belongs to the serogroup Icterohaemorrhagiae or to the serologically closely related serogroup Sarmin.

Table 2. CAAT between selected reference serovars of the Icterohaemorrhagiae group and serovar Sokoine (isolate RM1)

Antiserum against serovar:	Absorbing serovar/strain	Homologous titre before absorption	Homologous titre after absorption	Remaining homologous titre (%)
Sokoine	Birkini	1 : 10240	1 : 5120	50
Sokoine	Bogvere	1 : 10240	1 : 5120	50
Sokoine	Gem	1 : 10240	1 : 5120	50
Sokoine	Lai	1 : 10240	1 : 5120	50
Sokoine	Ndahambukuje	1 : 10240	1 : 5120	50
Sokoine	Ndambari	1 : 10240	1 : 160	1·5*
Sokoine	Mwogolo	1 : 10240	1 : 5120	50
Birkini	Sokoine	1 : 2560	1 : 1280	50
Bogvere	Sokoine	1 : 5120	1 : 1280	25
Gem	Sokoine	1 : 2560	1 : 1280	50
Lai	Sokoine	1 : 5120	Not tested	Not tested
Ndahambukuje	Sokoine	1 : 2560	1 : 320	12·5
Ndambari	Sokoine	1 : 1280	1 : 640	50

*Homologous titre of $<10\%$ remaining in both forward and reverse reactions indicates the same serovar. Serovar Sokoine (1·5 %) and Ndambari (50 %) are therefore different as one has $>10\%$ homologous antibodies remaining.

Subsequent typing with a panel of mAbs, which characteristically agglutinate serovars of the serogroups Icterohaemorrhagiae and Sarmin, revealed the highest similarity with serovars from the Icterohaemorrhagiae group, while the relationship with the Sarmin group was virtually ruled out (data not shown). Therefore, we conclude that RM1 represents a serovar of serogroup Icterohaemorrhagiae. The agglutination profile obtained with RM1 was not identical to that of any of the established serovars of serogroup Icterohaemorrhagiae. Best fits were found with the histograms of serovars Ndahambukuje and Ndambari (Supplementary Figs S3 and S4 available in IJSEM Online). These observations suggest RM1 to be a new serovar of the Icterohaemorrhagiae group.

CAAT was performed repeatedly with serovars of serogroup Icterohaemorrhagiae, of which the rabbit sera showed high cross-agglutination titres ($>10\,\%$ compared with the homologous titre) with RM1 and vice versa. CAAT results (Table 2) indicate that none of the rabbit sera, after heterologous absorption, give a remaining titre of less than 10 % in both forward and reverse reactions. According to the definition of the subcommittee on the taxonomy of *Leptospira* (Stallman, 1987), this finding further shows that RM1 represents a new serovar of serogroup Icterohaemorrhagiae.

This study has shown that isolate RM1 represents a new pathogenic serovar of *Leptospira*. It can be classified as a serovar in the serogroup Icterohaemorrhagiae, belonging to the species *L. kirschneri*. We propose the name Sokoine for this new serovar. The proposed serovar Sokoine is serologically related to serovars Ndambari and Ndahambukuje, both isolated from patients in the Democratic Republic of Congo (Zaire). However, the repeated standard CAAT results indicate that there are marked serological differences that justify the recognition of RM1 strain as a new serovar (Stallman, 1987). Additionally, serovar Sokoine is genotypically closely related to serovar Ndahambukuje, although the IS*1500* and IS*1533* fingerprints did not provide enough DNA profiles to conclude on the level of relationships. It might be hypothesized that the observed serological and genotypic relationship between the *L. kirschneri* serovars Ndahambukuje and Ndambari and the proposed serovar Sokoine from Tanzania suggests that these serovars evolved from a common ancestor, composing a prevalent group of related serovars in East and Central Africa and possibly have cattle as a natural host. Further studies will be needed to confirm this hypothesis. These findings agree with the prevalence of *L. kirschneri* in Africa (Faine *et al.*, 1999).

Short description of *Leptospira* serovar Sokoine

The proposed *Leptospira* serovar Sokoine strain RM1 was isolated from cattle in a slaughterhouse in Morogoro, Tanzania. Serovar Sokoine is deposited under its own serovar and strain name (no accession number) in two *Leptospira* culture collection centres: (i) the WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis of the Royal Tropical Institute, Amsterdam, The Netherlands, and (ii) the WHO Collaboration Centre for Diagnosis, Reference, Research and Training in Leptospirosis, Port Blair, Andaman and Nicobar Islands, India. RM1 grows well in *Leptospira* culture media (Fletcher's and EMJH) at ambient temperatures. Growth of this strain is inhibited at 13 °C and by 8-azaguanine, which is consistent with pathogenic status. Genomic DNA of strain RM1 is PCR-positive with primers designed for pathogenic leptospires and with specific primers for amplifying DNA from strains of *L. kirschneri*. Serovar Sokoine is distinct from all other recognized serovars of the Icterohaemorrhagiae group on the basis of the CAAT results. It gives highest titres in agglutination with antibodies to various serovars of the Icterohaemorrhagiae group, and hence can serve as broad antigen for preliminary serological diagnosis of infection with leptospires of the Icterohaemorrhagiae group.

This isolate can be classified as *L. kirschneri* serogroup Icterohaemorrhagiae serovar Sokoine strain RM1. The naming of serovar Sokoine is given in the honour of the Sokoine University of Agriculture in Morogoro, Tanzania, where research on leptospirosis in Tanzania was pioneered.

Acknowledgements

We thank the staff of the Royal Tropical Institute, Biomedical Research Department (The Netherlands) for their technical and material support. We also acknowledge the financial support from NUFFIC (The Netherlands), the Flemish Inter-university Council (Belgium) and the European Union (Ratzooman Project, INCO-Dev ICA4-CT-2002-10056) for additional financial support.

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