Two New Leptospiral Serovars in the Hebdomadis Serogroup Isolated from Zimbabwe Cattle

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Four strains belonging to the genus *Leptospira* serogroup Hebdomadis were isolated from Zimbabwe cattle at slaughter. These isolates were subjected to cross-agglutinin absorption tests and to restriction fragment length polymorphism and pulsed-field gel electrophoresis analyses of their genomic DNAs. One of these strains represents a new serovar, for which the name *mhou* is proposed; strain SBF 40 is the reference strain of this serovar. The other three strains belong to a second new serovar, for which the name *marondera* is proposed; the reference strain of this serovar is strain SBF 5. The three strains of serovar *marondera* could be differentiated by their restriction fragment polymorphism and pulsed-field gel electrophoretic patterns.

Pathogenic *Leptospira* strains are currently divided into seven officially recognized species (*Leptospira borgpetersenii*, *Leptospira inadai*, *Leptospira interrogans*, *Leptospira kirschneri*, *Leptospira noguchii*, *Leptospira santarosai*, and *Leptospira weilii*) on the basis of the results of DNA-DNA hybridization studies (33, 42). The identification of a pathogenic strain as a member of one of these seven species is, however, insufficient for epidemiologists since it does not reflect any host-leptospire relationship. Furthermore, the techniques used for hybridization are still too complicated and not ideally suited for routine clinical laboratories, while phenotypic characteristics that have been proposed as characteristics which can be used to differentiate the seven species are limited and cannot be used for routine reliable identification. As a result, the serovar is still the recognized and generally accepted taxon for classification of members of the genus *Leptospira* at the subspecies level (23). Each serovar is represented by a reference strain to which its description is attached. "Two strains are said to belong to different serovars if after cross-absorption with adequate amounts of heterologous antigen, more than 10% of the homologous titer regularly remains in at least one of the two antisera in repeated tests" (23).

For practical purposes, strains belonging to separate but closely related serovars are grouped together into serogroups. The allocation of serovars into serogroups is essentially a consequence of the technique of serological typing and does not appear to have any other biological significance (23).

The serovar classification method used for leptospire is unsatisfactory because it is laborious and time consuming, requires maintenance of numerous reference strains and their corresponding rabbit immune sera and depends on an arbitrarily chosen numerical limit to distinguish serovars within a particular serogroup, which makes the method subjective and not reproducible. In addition, this method may delineate artificially closely related strains on the one hand while it fails to distinguish genetically different but antigenically similar strains

on the other hand (36, 44). Furthermore, serological groups do not appear to corroborate DNA relatedness groups.

In an effort to replace the serological classification system with a natural subspecies classification system based on phylogenetically determined relationships, a variety of modern methods for genetic analysis have been introduced. These methods include chromosomal DNA restriction fragment length polymorphism (RFLP) analysis (6, 10, 11, 13, 25, 36), DNA hybridization with total DNA probes (26, 27, 35), restriction analysis by Southern blotting with recombinant DNA probes (24, 28, 37, 41, 43, 44), restriction analysis of chromosomal DNA by pulsed-field gel electrophoresis (PFGE) (14–16), rRNA gene RFLP analysis (17, 19, 29, 30), and PCR fingerprinting (1a, 2, 12, 18, 31, 32). Although useful, these methods have so far failed to produce a coherent picture which allows researchers to develop a natural and logical classification system based on genetic traits. Moreover, the differences revealed by these methods have not yet been able to describe the desired host-leptospire relationships required for epidemiological studies.

Pending the development of a new classification system based on the new molecular typing methods, the International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Leptospira* recommended that new field isolates should still be typed by the recognized standard method, the cross-agglutinin absorption test (CAAT), and given serovar status; however, other valid methods can be used for identification. The recommended methods include analysis with monoclonal antibodies or factor sera, analysis of RFLP or rRNA gene restriction fragment patterns, and PFGE analysis (21).

Pathogenic *Leptospira* strains are divided into more than 220 serovars, which are grouped into 23 serogroups (23). Until recently, the Hebdomadis serogroup was the largest and geographically most widespread of all serogroups. This serogroup became so large that some of its serovars showed very little serological relationship to one another. Using the factor analysis method, Kmety (22) proposed that this serogroup should be divided into three autonomous serogroups (the Hebdomadis, Mini, and Sejroe serogroups) on the basis of the distribution of main antigens. This proposal was adopted in 1982 (20). However, the members of these groups still exhibit clear intergroup relationships and cross-react during cross-agglutination tests.

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TABLE 1. CAAT results for Zimbabwe strains and antisera

Antiserum	Absorbing strain	Reciprocal of titer				Reciprocal of homologous titer (% of unabsorbed serum titer)
		Before absorption		After absorption		
		Homologous strain	Absorbing strain	Homologous strain	Absorbing strain	
SBF 5	SBF 21	12,800	12,800	100	100	0.78
SBF 21	SBF 5	6,400	6,400	100	100	1.56
SBF 5	SBF 40	12,800	3,200	3,200	100	25
SBF 40	SBF 5	6,400	3,200	800	100	12.5
SBF 5	SBF 50	12,800	6,400	100	100	0.78
SBF 50	SBF 5	12,800	12,800	400	100	3.125
SBF 21	SBF 40	6,400	3,200	1,600	100	25
SBF 40	SBF 21	3,200	3,200	800	100	25
SBF 21	SBF 50	6,400	3,200	100	100	1.56
SBF 50	SBF 21	12,800	6,400	100	100	0.78
SBF 40	SBF 50	6,400	3,200	1,600	100	25
SBF 50	SBF 40	6,400	3,200	1,600	100	25

The new Hebdomadis serogroup contains 12 recognized serovars which are genetically very diverse and whose members fall into at least six genospecies (*L. borgpetersenii*, *L. interrogans*, *L. kirschneri*, *L. santarosai*, *L. weilii*, and an unnamed genospecies) (1, 12, 33, 42). The majority of the reference strains of these serovars were initially isolated from humans (23). Only strain Bovino 131 of serovar *goiano* was isolated from the kidney of an apparently healthy cow in Brazil (34). The reference strains of serovars *kabura*, *kambale*, *jules*, and *nona* were first isolated from patients in Zaire (38–40). Serogroup Hebdomadis strains have also been isolated from humans in Kenya (3, 4).

The isolation and serogrouping of four strains belonging to serogroup Hebdomadis obtained from Zimbabwe cattle have been described previously (8). In this paper we describe identification of these strains to the serovar level, which was accomplished by performing CAAT, RFLP, and PFGE analyses of genomic DNA.

MATERIALS AND METHODS

Antisera from reference strains belonging to serogroup Hebdomadis serovars *borincana*, *goiano*, *hebdomadis*, *jules*, *kabura*, *kambale*, *kremastos*, *longnan*, *man-zhuang*, *maru*, *nona*, *sanmartini*, and *worsfoldi*, serogroup Mini serovars *beye*, *georgia*, *mini*, *perameles*, *nuparupae*, *szwajizak*, and *tabaquite*, and serogroup Sejroe serovars *balcanica*, *caribe*, *dikkeni*, *geyaweera*, *gorgas*, *guaricura*, *haemolytica*, *hardjo*, *istrica*, *medanensis*, *nyanza*, *polonica*, *recreo*, *ricardi*, *roumanica*, *saxkoebing*, *sejroe*, *trinidad*, and *wolffi* and from the new isolates for use in the CAATs were produced in paired rabbits and pooled before use as described previously (9). The strains used are strains that are in the reference collections at the National Reference Laboratory at the National Disease Center (Ames, Iowa) and the World Health Organization/Food and Agriculture Organization Reference Laboratories at The Royal Tropical Institute (Amsterdam, The Netherlands) and Institut Pasteur (Paris, France).

The four serogroup Hebdomadis isolates obtained from Zimbabwe cattle were designated SBF 5, SBF 21, SBF 40, and SBF 50. These isolates and their antisera were tested in cross-agglutination tests against each other and against the serogroup Hebdomadis, Sejroe, and Mini reference strains and their antisera.

All of the isolates were cross-absorbed against each other's antisera, while only those reference strains which had a reciprocal titer of at least 1/1,600 with isolates SBF 5, SBF 21, and SBF 50 and of 1/800 with isolate SBF 40 were used in CAATs. The CAATs were repeated three times by using the Bratislava technique (5) at the laboratory in Ames, Iowa, and the results were confirmed by workers at the Amsterdam laboratory.

The RFLP patterns of the four Zimbabwe strains were compared with the patterns of the serovar *balcanica*, *beye*, *dikkeni*, *jules*, *kabura*, *kambale*, *maru*, *medanensis*, *nona*, *perameles*, *saxkoebing*, and *worsfoldi* reference strains. These serovars were selected on the basis of the results of the CAATs. Bacterial DNA extraction, restriction endonuclease digestion, gel electrophoresis, and photography were carried out as described by Thiermann et al. (36).

The DNA *NotI* restriction digestion products of all of the isolates were compared with the digestion products of all of the serogroup Hebdomadis serovar reference strains and the digestion products of serovar *saxkoebing*, *balcanica*,

dikkeni (serogroup Sejroe), and *perameles* (serogroup Mini) reference strains by using PFGE. A restriction analysis in which PFGE was used was performed as described previously by Herrmann et al. (16).

RESULTS

High-level cross-agglutination reactions (>50% of the reciprocal titer of the homologous strain) were observed between the cattle isolates and several serogroup Hebdomadis, Sejroe, and Mini reference strains, indicating that strains SBF 5, SBF 21, SBF 40, and SBF 50 belonged to one of these three serogroups.

The results of CAATs performed with the Zimbabwean strains and antisera demonstrated that strains SBF 5, SBF 21, SBF 40, and SBF 50 belong to two serovars, one represented by strains SBF 5, SBF 21, and SBF 50 and the other represented by strain SBF 40 (Table 1).

Strain SBF 40 had titers higher than 1/1,600 with reference strains representing serovars *kambale*, *maru*, and *nona* (all serogroup Hebdomadis serovars) and higher than 1/800 with reference strains representing serovars *medanensis* and *saxkoebing* (both serogroup Sejroe serovars). The results of CAATs performed with strain SBF 40 and the reference strains indicate that although strain SBF 40 is closely related to serogroup Hebdomadis serovars, it is antigenically different from members of all previously described serovars of this serogroup because more than 10% of the antibodies remained in all of the paired antisera (Table 2). Therefore, on the basis of the current definition of a serovar, strain SBF 40 represents a new serovar in serogroup Hebdomadis.

Although CAATs were carried out with strains SBF 5, SBF 21, and SBF 50, only the results obtained for strain SBF 5, which cross-reacted with the largest number of reference strains of the serogroup Hebdomadis, Sejroe, and Mini serovars, are presented below. Therefore, strain SBF 5 was used as the reference strain for this subgroup. Strain SBF 5 was closely related to serovars *jules*, *kabura*, *kambale*, *kremastos*, *maru*, and *nona* (all serogroup Hebdomadis serovars), serovars *beye* and *perameles* (both serogroup Mini serovars), and serovars *balcanica*, *dikkeni*, and *saxkoebing* (all serogroup Sejroe serovars). Therefore, this strain was cross-absorbed with the reference strains of these serovars (Table 3). The results indicate that strain SBF 5 is most closely related to serogroup Hebdomadis strains but is antigenically distinct from members of all previously described serovars of this serogroup. Therefore, strains SBF 5, SBF 21, and SBF 50 represent a new serogroup Hebdomadis serovar.

TABLE 2. Serovar identification of strain SBF 40 by CAAT

Antiserum	Absorbing strain or serovar	Reciprocal of titer				Reciprocal of homologous titer (% of unabsorbed serum titer)
		Before absorption		After absorption		
		Homologous strain	Absorbing strain	Homologous strain	Absorbing strain	
SBF 40	<i>kambale</i>	3,200	3,200	800	0	25
<i>kambale</i>	SBF 40	12,800	1,600	6,400	200	50
SBF 40	<i>maru</i>	3,200	1,600	1,600	0	50
<i>maru</i>	SBF 40	25,600	1,600	12,800	200	50
SBF 40	<i>nona</i>	3,200	3,200	1,600	100	50
<i>nona</i>	SBF 40	12,800	3,200	6,400	200	50
SBF 40	<i>medanensis</i>	3,200	1,600	1,600	0	50
<i>medanensis</i>	SBF 40	12,800	800	25,600	0	200
SBF 40	<i>saxkoebing</i>	3,200	800	3,200	0	100
<i>saxkoebing</i>	SBF 40	12,800	800	6,400	0	50

Strains SBF 5 and SBF 21 had identical RFLP patterns when they were digested with endonucleases *Hha*I (Fig. 1) and *Eco*RI (data not shown). Strain SBF 50 had a slightly different pattern; it lacked several fragments in the 4.5- to 5.6-kb size range. Although strain SBF 40 shared some bands with strains SBF 5, SBF 21, and SBF 50, its RFLP pattern was more distinct.

All of the cattle strains had patterns which were different from the patterns of all of the reference strains.

The PFGE results are shown in Fig. 2. Although isolates SBF 5, SBF 21, and SBF 50 had similar PFGE patterns, the restriction patterns of strains SBF 21 and SBF 50 had two fragments, one 140 kb long and the other 320 kb long, which were not present in the restriction pattern of strain SBF 5. In addition, the patterns of strains SBF 5 and SBF 21 had a similar largest fragment (length, >500 kb) which was not pres-

ent in the strain SBF 50 pattern. Instead, the largest fragment in the strain SBF 50 pattern was approximately 400 kb long.

Strain SBF 40 had a unique profile which was different from the profiles of the other three cattle isolates.

The PFGE restriction patterns obtained for strains SBF 5, SBF 21, SBF 40, and SBF 50 were quite different from the patterns obtained for most of the serogroup *Hebdomadis* serovars, including serovars *borincana*, *goiano*, *hebdomadis*, *kambale*, *kremastos*, *sanmartini*, and *worsfoldi*, the patterns obtained for serogroup *Sejroe* serovars *balcanica*, *dikkeni*, and *saxkoebing*, and the pattern obtained for serogroup *Mini* serovar *perameles* (data not shown), but had several fragments in common with the patterns obtained for serogroup *Hebdomadis* serovars *kabura*, *jules*, *maru*, and *nona* (Fig. 2). The restriction patterns of strains SBF 5, SBF 21, SBF 40, and SBF 50 were most similar to those of serovars *jules* and *nona*.

TABLE 3. Serovar identification of strain SBF 5 by CAAT

Antiserum	Absorbing strain or serovar	Reciprocal of titer				Reciprocal of homologous titer (% of unabsorbed serum titer)
		Before absorption		After absorption		
		Homologous strain	Absorbing strain	Homologous strain	Absorbing strain	
SBF 5	<i>jules</i>	12,800	3,200	6,400	100	50
<i>jules</i>	SBF 5	6,400	1,600	3,200	100	50
SBF 5	<i>kabura</i>	12,800	3,200	6,400	200	50
<i>kabura</i>	SBF 5	6,400	3,200	3,200	100	50
SBF 5	<i>kambale</i>	12,800	3,200	800	0	6.25
<i>kambale</i>	SBF 5	12,800	6,400	6,400	400	50
SBF 5	<i>kremastos</i>	12,800	1,600	6,400	0	50
<i>kremastos</i>	SBF 5	12,800	3,200	6,400	200	50
SBF 5	<i>maru</i>	12,800	6,400	3,200	200	25
<i>maru</i>	SBF 5	51,200	12,800	6,400	400	12.5
SBF 5	<i>nona</i>	12,800	6,400	6,400	400	50
<i>nona</i>	SBF 5	12,800	6,400	12,800	200	100
SBF 5	<i>beye</i>	12,800	1,600	12,800	200	100
<i>beye</i>	SBF 5	12,800	1,600	6,400	200	50
SBF 5	<i>perameles</i>	12,800	1,600	6,400	100	50
<i>perameles</i>	SBF 5	12,800	3,200	12,800	200	100
SBF 5	<i>balcanica</i>	12,800	1,600	6,400	0	50
<i>balcanica</i>	SBF 5	6,400	1,600	6,400	100	100
SBF 5	<i>dikkeni</i>	12,800	1,600	6,400	400	50
<i>dikkeni</i>	SBF 5	6,400	1,600	3,200	200	50
SBF 5	<i>saxkoebing</i>	12,800	3,200	6,400	0	50
<i>saxkoebing</i>	SBF 5	12,800	3,200	6,400	100	50

FIG. 1. RFLP patterns for chromosomal DNAs from four Zimbabwe isolates and serogroup Hebdomadis, Mini, and Sejroe reference strains digested with *HhaI*. Lane M, fragment size markers; lane 1, strain SBF 5; lane 2, strain SBF 21; lane 3, strain SBF 40; lane 4, strain SBF 50; lanes 5 to 15, reference strains belonging to serovars *maru*, *nona*, *balcanica*, *dikkeni*, *beye*, *perameles*, *kabura*, *worsfoldi*, *saxkoebing*, *jules*, and *medianensis*, respectively.

DISCUSSION

Identification of serovars is necessary for epidemiological surveillance since some serovars are known to have preferential animal reservoirs (e.g., serovar *icterohaemorrhagiae* occurs in rats, serovar *canicola* occurs in dogs, and serovar *hardjo* occurs in bovines) or are associated with certain clinical forms (e.g., serovar *icterohaemorrhagiae* is associated with icterogenic syndrome and serovar *grippityphosa* is associated with benign meningitis syndrome) (7). Thus, our choice of methods (RFLP analysis of chromosomal DNA and PFGE analysis of *NotI* genomic DNA restriction digestion products) was biased as the data obtained with both of these methods largely support the serovar concept (13–16). In addition, both of these methods allow workers to differentiate strains of the same serovar, which usually correlates with differences in the epidemiology of strains and possibly the pathogenicity of strains (6, 16). Thus, the two methods serve as supplementary typing systems in epidemiological studies.

Another advantage of using RFLP analysis is that most reference strains have already been examined by this method and the bulk of the information is readily available for comparison. PFGE is a complementary method which has the advantage of using larger DNA fragments, thus producing simpler patterns which are easier to interpret (15, 16). Some serovars, however, have similar RFLP and PFGE patterns and are not easily differentiated by these two methods alone (15, 36, 44).

Using three methods, we identified two new serovars in serogroup Hebdomadis which are antigenically and genetically different from all previously described serogroup Hebdomadis serovars. The name *nhou* is proposed for the new serovar represented by strain SBF 40, which also has distinct RFLP and PFGE restriction patterns, and the name *marondera* is

FIG. 2. PFGE of *NotI* restriction fragments from four Zimbabwe isolates and serogroup Hebdomadis reference strains. The digestion products were separated by electrophoresis at 150 V for 25 h in 1% agarose–0.5× Tris-borate-EDTA with a CHEF DR-II apparatus (Bio-Rad Laboratories, Richmond, Calif.). The pulse times consisted of a ramping program from 5 to 40s. Lanes 1 and 12, lambda concatemer size markers; lane 2, strain SBF 5; lane 3, strain SBF 21; lane 4, strain SBF 40; lane 5, strain SBF 50; lanes 6 to 11, reference strains belonging to serovars *nona*, *jules*, *maru*, *kabura*, *hebdomadis*, and *kremastos*, respectively.

proposed for the second new serovar; strain SBF 5 is the reference strain of serovar *marondera*. Within serovar *marondera* we were able to demonstrate that there are three genetic types, represented by isolates SBF 5, SBF 21, and SBF 50.

We believe that all of the isolates which we examined have now been characterized sufficiently so that they can be designated members of new serovars but concede that the methods used for our classification do not reflect the species affiliation of the new strains. The strains have been deposited in the Amsterdam and Paris world reference collections and are available to other researchers for species identification.

An interesting observation is the similarity between the PFGE patterns of our isolates and the patterns of reference strains of serovars *jules* and *nona*, which are also of African origin.

The results of this study once again highlight the existence of many as-yet-undiscovered *Leptospira* types on the African continent.

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