A new leptospiral serovar, ngavi, in the Tarassovi serogroup isolated from Zimbabwe oxen

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INTRODUCTION

Tarassovi is currently the largest and most diverse serogroup of the genus Leptospira as it comprises 21 serovars with two others listed in the annex of the revised list (23). The reference strains of these serovars were isolated from wild animals, humans, pigs and settled human sewage (23). None was isolated from cattle.

In Africa, titres to the Tarassovi serogroup have been detected in humans (4, 11, 18, 20), wildlife (7, 18, 21) and cattle (1, 3, 7, 21, 26, 27). Antibodies to serogroup Tarassovi have also been detected in Zimbabwe cattle (12, 19). Reference strains for serogroup Tarassovi serovars kisuba, kanana and tunis were isolated from a patient in Zaire, a rodent in Kenya and a pig in Tunisia, respectively (2, 9, 37).

The pathogenic leptospires are currently divided into seven species based on DNA relatedness studies (30, 39). The identification of a pathogenic strain as belonging to one of these species, however, is not very useful to the epidemiologist as it does not reflect any host–leptospire relationship. As a result, the taxonomy of the genus Leptospira at the subspecific level is still based on serovars, although other valid methods can be used for identification (22). Such methods include analyses with mouse mAbs, factor sera, RFLP of chromosomal DNA, PFGE and rRNA gene restriction fragment patterns.

‘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 titre regularly remains in at least one of the two antisera in repeated tests’ (23). The cross-agglutinin absorption test (CAAT) used for serovar classification is laborious, subjective, inconsistently reproducible and lacks the sensitivity required to identify intra-serovar differences which may be important in epidemiological studies (10).

mAbs have been used in a complementary serological method for identifying field strains of the genus Leptospira (24, 33). The mAb technique depends on the detection of surface antigens and on the careful selection of antibodies that discriminate between serovars. mAbs usually confirm serological differences between serovars and may sometimes allow for
differentiation of strains within serovars (23). However, like CAAT, this method does not corroborate DNA relatedness groupings.

RFLP analysis was first proposed as an alternative method for classifying leptospires by Marshall et al. (25). Since then most of the reference strains have been examined and the bulk of the information is now readily available. RFLP has an advantage over CAAT in that it allows for further differentiation between strains of the same serovar which may be of importance in epidemiological studies (10). It has, however, failed to distinguish between antigenically different serovars in some cases, such as serovars tarassovi, guidae, tunis and moldaviae in serogroup Tarassovi (41).

RFLP analysis of chromosomal DNA often yields large numbers of fragments and the patterns are smeared as the molecular size of the fragments decreases. Consequently, analysis of RFLPs is difficult and pattern differentiation is based only on the high-molecular-mass fragments. Southern blotting with repetitive sequence genetic probes, which highlight specific DNA restriction site heterogeneities, results in simpler banding patterns which allow for easier identification of bands and for direct identification of serovars from clinical samples (28, 36, 38, 40, 41). However, the method is still cumbersome, even when non-radioactive probes are used (34).

The isolation and serogrouping of two isolates belonging to serogroup Tarassovi from Zimbabwe oxen at slaughter was reported previously (13). This paper describes the identification of these strains to serovar level using CAAT, mAbs, RFLP of genomic DNA, and Southern blotting and hybridization of the RFLPs with a probe synthesized from a repetitive sequence element cloned from serovar hardjo strain Hardjo-bovis.

**METHODS**

**CAATs.** Antisera of reference strains of serovars belonging to serogroup Tarassovi (atchafalaya LSU 1013, atlantae LT 81, bakeri LT 79, bravo Bravo, chagres 1913 K, darien 637 K, gatuni 1473 K, guidae RP 29, kanana Kanana, kaup LT 64-68, kisuba Kisuba, langati M 39090, mogdeni Compton 746, navet TRVL 109873, rama 316, tarassovi Perepelitsin, tunis P 2/65 and vuglia LT 89-68) and of the two isolates (SBF 16 and SBF 19) for use in CAATs were produced in paired rabbits and pooled before use as previously described (14). The reference strains are part of the reference collections at either the National Reference Laboratory, National Animal Disease Center (Ames, IA, USA) or the World Health Organization/Food and Agriculture Laboratory (Amsterdam, The Netherlands). The isolates and their antisera were tested in reciprocal tests against each other and against the reference strains and antisera of serogroup Tarassovi using the microscopic agglutination test (6).

The two isolates were cross-absorbed against each other's antisera. Only those reference strains which had a reciprocal titre of at least 1:800 with the isolates were used in CAATs. The CAATs were performed using the Bratislava technique as described by Dikken & Kmet (8) at the laboratory in Ames, and the results were confirmed by workers at the Amsterdam laboratory. Reference strains for the remaining five serovars were not included as they had either not yet been confirmed by a reference laboratory (banna A 31, genoves M 48, mengenius L 82 and vuglia LT 1100) or were confirmed but not yet published (sulzerae LT 82) and therefore were not part of the Ames reference collection at the time of the study.

**mAb analysis.** Mouse mAbs were produced as previously described (33). A panel of eight mAbs from fusion 151 was tested against the two isolates and the 18 reference strains of serovars of serogroup Tarassovi and the resulting profiles were compared.

**RFLP analysis.** RFLP patterns of the chromosomal DNA of the two strains were compared with those of serogroup Tarassovi serovars bakeri, darien, guidae, kanana, langati, moldaviae, navet, tarassovi, tunis and vuglia. Selection of these serovars was based on the results of CAATs with the exception of guidae and the previously proposed but unrecognized serovar moldaviae, which were included because their RFLP patterns are indistinguishable from those of tarassovi and tunis (41). All strains were examined using the restriction enzymes EcoRI, Hhal and Clal (Bethesda Research Laboratories). The extraction of bacterial DNA, the restriction endonuclease digestion, gel electrophoresis and photography were carried out as described by Thiermann et al. (35).

**Southern blot analysis.** After photography, the restriction fragments were transferred to Hybond-N (31) and the immobilized DNA was hybridized with a digoxigenin-labelled pLI20 probe (42). The non-radioactive labelling and detection kit was purchased from Boehringer Mannheim and reaction conditions for labelling, hybridization and immunological detection were those recommended by the manufacturer. Washing conditions were 2 x 5 min at room temperature with 2 x SSC [(20 x SSC is 3 M NaCl plus 0.3 M sodium citrate (pH 7.0)]] /0.1 % (w/v) SDS; and 2 x 15 min with 0.1 x SSC/0.1 % (w/v) SDS.

**RESULTS**

The two isolates reacted to at least 6% of the homologous titre with sera from most members of serogroup Tarassovi and thus belonged to the serogroup (8). Strains SBF 16 and SBF 19 had cross-absorption titres below 10% in the CAATs and therefore belonged to the same serovar. Although CAATs were carried out with both isolates SBF 16 and SBF 19, only the results for strain SBF 16, which had a broader relationship with other Tarassovi serogroup serovars, will be presented. Strain SBF 16 showed a close relationship to serovars bakeri, darien, kanana, langati, navet, tarassovi, tunis and vuglia and these reference strains were therefore included in the CAATs. The results of the CAATs presented in Table 1 indicate that strain SBF 16 is antigenically different from members of the previously described serovars of serogroup Tarassovi because more than 10% of the antibodies remained in all of the paired antisera. Therefore, on the basis of the current definition of a serovar, strains SBF 16 and SBF 19 represent a new serovar in the Tarassovi serogroup.

Strains SBF 16 and SBF 19 showed similar aggluti-
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**Table 1.** Serovar identification of strain SBF 16 by CAAT

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Absorbing strain</th>
<th>Reciprocal of titre Before absorption</th>
<th>Reciprocal of titre After absorption</th>
<th>Reciprocal of homologous titre (% of unabsorbed serum titre)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Homologous strain</td>
<td>Absorbing strain</td>
<td>Homologous strain</td>
</tr>
<tr>
<td>SBF 16</td>
<td>bakeri</td>
<td>6400</td>
<td>3200</td>
<td>1600</td>
</tr>
<tr>
<td>SBF 16</td>
<td>darien</td>
<td>6400</td>
<td>3200</td>
<td>1600</td>
</tr>
<tr>
<td>SBF 16</td>
<td>kanana</td>
<td>12800</td>
<td>1600</td>
<td>12800</td>
</tr>
<tr>
<td>SBF 16</td>
<td>kanana</td>
<td>6400</td>
<td>800</td>
<td>6400</td>
</tr>
<tr>
<td>SBF 16</td>
<td>vughia</td>
<td>12800</td>
<td>800</td>
<td>12800</td>
</tr>
<tr>
<td>SBF 16</td>
<td>langati</td>
<td>6400</td>
<td>3200</td>
<td>6400</td>
</tr>
<tr>
<td>SBF 16</td>
<td>tunis</td>
<td>6400</td>
<td>1600</td>
<td>800</td>
</tr>
</tbody>
</table>

**Table 2.** Reciprocals of agglutination titres of mAbs with reference strains of serogroup Tarassovi serovars and Zimbabwe strains SBF 16 and SBF 19

<table>
<thead>
<tr>
<th>Serovar or strain</th>
<th>Reciprocal of agglutination titre with mAbs of fusion 151:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C1</td>
</tr>
<tr>
<td>SBF 16</td>
<td>640</td>
</tr>
<tr>
<td>SBF 19</td>
<td>320</td>
</tr>
<tr>
<td>gatuni</td>
<td>2560</td>
</tr>
<tr>
<td>guidae</td>
<td>5120</td>
</tr>
<tr>
<td>vughia</td>
<td>5120</td>
</tr>
<tr>
<td>tunis</td>
<td>5120</td>
</tr>
<tr>
<td>atchafalaya</td>
<td>160</td>
</tr>
<tr>
<td>atlantae</td>
<td>160</td>
</tr>
<tr>
<td>bakeri</td>
<td>2560</td>
</tr>
<tr>
<td>mogdeni</td>
<td>2560</td>
</tr>
<tr>
<td>darien</td>
<td>320</td>
</tr>
<tr>
<td>kaup</td>
<td>1280</td>
</tr>
<tr>
<td>bravon</td>
<td>10240</td>
</tr>
<tr>
<td>chagres</td>
<td>1280</td>
</tr>
<tr>
<td>kisuba</td>
<td>640</td>
</tr>
<tr>
<td>langati</td>
<td>81920</td>
</tr>
<tr>
<td>rama</td>
<td>40960</td>
</tr>
<tr>
<td>kanana</td>
<td>7</td>
</tr>
</tbody>
</table>

collection profiles, which only differed in each positive case by a single-step dilution, when tested with the panel of eight mAbs (Table 2). Their patterns were also similar to that of serovar gatuni although the reactions of serovar gatuni were much higher with mAbs C1 and C6 and lower with mAbs C9 and C19. The patterns...
Fig. 1. (a) RFLP patterns of chromosomal DNA from the two Zimbabwe isolates and reference strains of serogroup Tarassovi digested with HhaI. Lanes: M, fragment size markers; 1, strain SBF 16; 2, strain SBF 19; 3–12, reference strains belonging to serovars tarassovi, tunis, moldaviae, guidae, vughia, langati, navet, bakeri, darien and kanana, respectively. (b) Southern blot analysis of RFLP patterns presented in (a) using digoxigenin-labelled probe pLI20.

were different from those of the other 17 reference strains tested (Table 2).
The RFLP patterns of strains SBF 16 and SBF 19 were similar to each other and to those of reference strains for serovars tarassovi, tunis, moldaviae and guidae when their DNAs were digested with enzyme HhaI (Fig. 1a), whereas few differences were observed between the isolates and reference strains with ClaI (Fig. 2a) and more differences could be discerned among all the strains when EcoRI was used (data not shown).

The results obtained when the RFLPs with enzyme HhaI were Southern blotted and hybridized with the pLI20 probe are shown in Fig. 1(b). Although isolates SBF 16 and SBF 19 had similar patterns, they had different size largest fragments in the 1.4–1.6 kb region; strain SBF 16 consistently showed hybridization signals of a higher intensity than strain SBF 19. The two isolates showed several differences to reference strains of tarassovi, tunis, moldaviae and guidae in the 1.3–1.6 kb region and lacked all the fragments > 1.6 kb in length. Their patterns were quite distinct from those of the rest of the reference strains tested.

More differences could be discerned between patterns of the two isolates as well as among patterns of the isolates and all the reference strains including tarassovi, tunis, moldaviae and guidae along the whole range (0.75–9.2 kb) when their ClaI digests were probed with pLI20 (Fig. 2b).

**DISCUSSION**

Using four methods, we have identified a new serovar in serogroup Tarassovi for which the name ngavi is proposed, with strain SBF 16 as its reference strain. The CAAT results indicate that the Zimbabwe isolates are antigenically different from all previously described serogroup Tarassovi serovars.

This is the first reported study in which mAbs have been used to identify the serogroup Tarassovi serovars. The eight mAbs gave different agglutination profiles for the 18 recognized Tarassovi serovars tested, indicating that mAbs could be useful in typing new Tarassovi strains.

The mAb analyses generally confirmed the CAAT results of the two Zimbabwe isolates as the strains displayed very similar agglutination profiles which were generally different from those of all previously described Tarassovi serovars. mAb analyses, however, indicated a similarity between the new serovar and serovar gatuni that was not shown by CAAT. CAAT and mAb analyses, although usually consistent with one another, can lead to different classifications, particularly when dealing with new serovars as the mAbs used are made to and selected by reference strains (32).

Generally, most data obtained using RFLP analyses of chromosomal DNA largely support the serovar concept (17, 35, 41). The Zimbabwe isolates, however, fell into the group of exceptions as their RFLP patterns were very similar to those of reference strains of.
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Fig. 2. (a) RFLP patterns of chromosomal DNA from the two Zimbabwe isolates and reference strains of serogroup Tarassovi digested with Clal. Lanes: M, fragment size markers; 1, strain SBF 16; 2, strain SBF 19; 3–12, reference strains belonging to serogroup Tarassovi serovar tarassovi, tunis, moldaviae, guidae, vughia, langati, navet, bakeri, darien and kanana, respectively. (b) Southern blot analysis of RFLP patterns presented in (a) using digoxigenin-labelled probe pLI20.

The isolates which we examined have now been characterized sufficiently and can be designated members of a new serovar but they still need to be assigned to a species. Serogroup Tarassovi has been demonstrated to be genetically very diverse, with members falling in at least five genospecies (Leptospira borgpetersenii, Leptospira inadae, Leptospira noguchii, Leptospira santarosai and Leptospira weilii) (5, 39). The strains are deposited in the Amsterdam and Ames reference collections.

This study records the fifth new serovar for isolates obtained from kidneys of Zimbabwe beef cattle (15–17). The significance of these new serovars as pathogens is, however, not clear since no clinical syndromes were associated with their isolation.

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