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# A unique strain of *Leptospira* isolated from a patient with pulmonary haemorrhages in the Andaman Islands: a proposal of serovar portblairi of serogroup Sehgali

P. VIJAYACHARI[1], R. A. HARTSKEERL[2], S. SHARMA[1], K. NATARAJASEENIVASAN[1], S. ROY[1], W. J. TERPSTRA[2] AND S. C. SEHGAL[1]*

[1] *National Leptospirosis Reference Centre, Regional Medical Research Centre, Indian Council of Medical Research, Post Bag No. 13, Port Blair 744 101, Andaman & Nicobar Islands, India*
[2] *KIT – Biomedical Research, WHO/FAO Collaborating Centre for Reference and Research on Leptospirosis, Royal Tropical Institute, Meibergdreef 39, 1105 AZ Amsterdam, The Netherlands*

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## SUMMARY

Leptospirosis is endemic in the Andaman Islands, often occurring as outbreaks during the post-monsoon period. Pulmonary involvement is common and associated with high morbidity and mortality. During the investigation of an outbreak in North Andaman in 1996 an isolate was recovered from the blood of a patient with fever, headache, body aches and haemoptysis with respiratory distress as presenting symptoms. The isolate was characterized using the cross-agglutination absorption test (CAAT) and monoclonal antibodies (mAbs). The isolate showed typical morphology and characteristic motility of the genus *Leptospira*. Growth was inhibited at 13 °C and in the presence of 8-azaguanine. The isolate could not be identified with grouping sera representing 25 serogroups, CAAT and mAbs. A new serovar of a new serogroup is proposed. Genetic characterization using polymerase chain reaction (PCR) followed by sequencing of the PCR product and randomly amplified polymorphic DNA fingerprinting (RAPD) showed that the isolate was genetically similar to *L. interrogans sensu stricto*.

## INTRODUCTION

Leptospiral infections usually cause mild disease but in a substantial number of cases several organs are affected leading to a high case-fatality rate. Leptospires have been grouped into two species, namely pathogenic *L. interrogans* and non-pathogenic *L. biflexa* based on growth at 13 °C and resistance to the purine analogue 8-azaguanine [1]. Serological classification is based on antigenic characters and the serovar is regarded as the basic taxon. Serological classification has certain inherent limitations. To overcome some of these limitations, classification based on genetic similarities has been used in recent years [2].

The cross-agglutination absorption test (CAAT), factor sera analysis and typing with monoclonal antibodies (mAbs) are some of the serological techniques currently employed, while DNA–DNA hybridization, restriction fragment length polymorphism, randomly amplified polymorphic DNA (RAPD) fingerprinting and polymerase chain reaction (PCR) followed by sequencing of the PCR product are examples of genetic methods used to characterize leptospires [3–9].

Leptospirosis is endemic in the Andamans with regular annual outbreaks in areas where agricultural activities are the major occupation of the population. Serovars of serogroup Grippotyphosa are most commonly isolated from clinical specimens followed by those of serogroups Icterohaemorrhagiae, Australis, Sejroe and Hebdomadis [9, 10].

* Author for correspondence.

During investigation of an outbreak of leptospirosis in North Andaman in 1996, four isolates were obtained from the blood of patients. Three isolates were identified as belonging to serogroup Grippotyphosa serovar valbuzzi but the fourth could not be identified with conventional serological techniques. Here, we report on the serological and genetic characterization of this isolate.

## MATERIALS AND METHODS

### Isolate DS2

The isolate, coded DS2, was obtained from the blood of a 30-year-old female patient living rurally who was admitted to the Community Health Centre, Diglipur, North Andaman, with complaints of fever, generalized body ache, headache and massive haemoptysis with respiratory distress.

### Microscopy and staining

The morphological characters of the isolate were studied using dark-ground (Olympus-CH 40, Tokyo, Japan), electron microscopy (cryo-electron microscopy, Soft Imaging Systems, Münster, Germany) and silver staining technique (Fontana's method) [11]. Length, diameter, wavelength and amplitude were measured using software supplied with the electron microscope.

### Tests for pathogenic status – species level identification

*Growth at 13 °C and in the presence of 8-azaguanine*

To establish the pathogenic status as well as the species identification, the isolate DS2 was tested for growth at 13 °C [12] and in the presence of 8-azaguanine (Sigma, St. Louis, MO, USA) with a concentration of 225 $\mu$g/ml [13]. The pathogenic strains Jez Bratislava and Wijinberg and the non-pathogenic strains Patoc I and Veldrat Semarang were included as controls. Growth was judged by estimating the densities of the cultures using a bacterial counting chamber (Hawksley, Lancing, UK) on alternative days up to 21 days of incubation.

### Antisera and monoclonal antibodies

Rabbit antisera against leptospires were raised as recommended by the International Committee on Systematic Bacteriology, Sub-Committee on the Taxonomy of *Leptospira* (TSC) [14]. Monoclonal antibodies were developed as described earlier [15].

Table 1. *MAT results of isolate DS2 against 39 'group-specific' representative rabbit antisera of 25 serogroups*

| | Serovar | Strain | Titre |
|---|---|---|---|
| 1. Australis | australis | Ballico | <20 |
| 2. Australis | bratislava | Jez Bratislava | 40 |
| 3. Autumnalis | bangkinang | Bangkinang I | <20 |
| 4. Autumnalis | butembo | Butembo | <20 |
| 5. Autumnalis | carlos | 3C | 40 |
| 6. Autumnalis | rachmati | Rachmat | <20 |
| 7. Ballum | ballum | Mus 127 | 40 |
| 8. Ballum | kenya | Njenga | 20 |
| 9. Bataviae | bataviae | Swart | <20 |
| 10. Canicola | canicola | H.Utrecht IV | 160 |
| 11. Canicola | schueffneri | VI.90 C | <20 |
| 12. Celledoni | celledoni | Celledoni | <20 |
| 13. Cynopteri | cynopteri | 3522 C | <20 |
| 14. Djasiman | djasiman | Djasiman | <20 |
| 15. Grippotyphosa | grippotyphosa | Moskva V | 40 |
| 16. Grippotyphosa | huanuco | M 4 | <20 |
| 17. Hebdomadis | hebdomadis | Hebdomadis | <20 |
| 18. Hebdomadis | worsfoldi | Worsfold | <20 |
| 19. Icterohaem. | copenhageni | M 20 | 160 |
| 20. Icterohaem. | icterohaem. | RGA | 160 |
| 21. Javanica | poi | Poi | 160 |
| 22. Louisiana | louisiana | LSU 1945 | <20 |
| 23. Manhao | manhao | L 60 | <20 |
| 24. Mini | mini | Sari | <20 |
| 25. Panama | panama | CZ 214 K | <20 |
| 26. Pomona | pomona | Pomona | <20 |
| 27. Pyrogenes | pyrogenes | Salinem | 160 |
| 28. Sarmin | rio | Rr 5 | <20 |
| 29. Sarmin | weaveri | CZ 390 | 160 |
| 30. Sejroe | hardjo | Hardjopraj. | <20 |
| 31. Sejroe | saxkoebing | Mus 24 | 20 |
| 32. Shermani | shermani | 1342 K | <20 |
| 33. Tarassovi | bakeri | LT 79 | 20 |
| 34. Tarassovi | mogden | Compton | <20 |
| 35. Tarassovi | rama | 316 | 20 |
| 36. Tarassovi | tarassovi | Perepelitsin | 20 |
| 37. Ranarum | ranarum | ICF | 40 |
| 38. Undesignated | sichuan | 79601$^T$ | <20 |
| 39. Hurstbridge | hurstbridge | BUT6$^T$ | <20 |

### Serological characterization

*MAT (microscopic agglutination test) with group sera (rabbit antisera)*

As a first step to identify the isolate up to serogroup status, MAT was performed on the isolates using a panel of 39 anti-*Leptospira* rabbit anti sera (Table 1) representative of 25 serogroups, following the standard procedure [3]. Subsequently, the isolate was tested with rabbit antisera directed against all

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reference serovars/strains [6, 16] within the 25 serogroups as listed in Table 2.

*CAAT*

The test was performed as a two-step procedure of cross-agglutination and absorption as described by Kmety & Dikken [3], KIT, WHO/FAO Collaborating Centre for Reference and Research on Leptospirosis, Amsterdam and at the National Leptospirosis Reference Centre, Regional Medical Research Centre (ICMR), Port Blair.

*Typing with mAbs*

Serological typing with mAbs was done by MAT using panels of mouse mAbs belonging to serogroups Bataviae, Canicola, Grippotyphosa, Hebdomadis, Icterohaemorrhagiae, Javanica, Mini, Pyrogenes, Shermani and Sarmin (Table 3) following the procedure described earlier [9].

**Genetic characterization**

*Reference strains*

Ten reference strains belonging to six species (*L. interrogans* serovar icterohaemorrhagiae strain RGA, serovar australis strain Ballico, *L. kirschneri*, serovar grippotyphosa strain Moskva V, serovar ratnapura strain Wumalasena, serovar cynopteri strain 3522C, *L. borgpetersenii* serovar poi strain Poi, serovar mini strain Sari, *L. noguchii*, serovar louisiana strain LSU1945, *L. meyeri* serovar ranarum strain ICF and *L. santarosai* serovar canalzonae strain CZ188) along with the isolate DS2 were included for genetic analysis.

*Isolation of DNA*

Strains and the isolate were grown at 30 °C in EMJH (Ellinghausen–McCullough–Johnson–Harris) medium and harvested by centrifugation during late logarithmic phase. DNA was isolated as described by Broom et al. [17].

*PCR and DNA sequencing*

PCR was performed on the isolate using two sets of primers G1/G2 and B64-I/ B64-II as reported earlier [9]. Primer set G1/G2 amplifies DNA from all pathogenic species except *L. kirschneri* and primer set B64-I/B64-II amplifies DNA from genomospecies *L. kirschneri* [18]. One strand of the G1/G2-generated PCR product was sequenced on an ABI PRISM model 377 automatic sequencer (Applied Biosystems, Foster City, CA, USA) giving 99% sequence accuracy. DNASIS (Pharmacia LKB, Uppsala, Sweden) software was used for comparison.

**Fig. 1.** Electron micrograph of the isolate DS2 at a magnification of 40 500×.

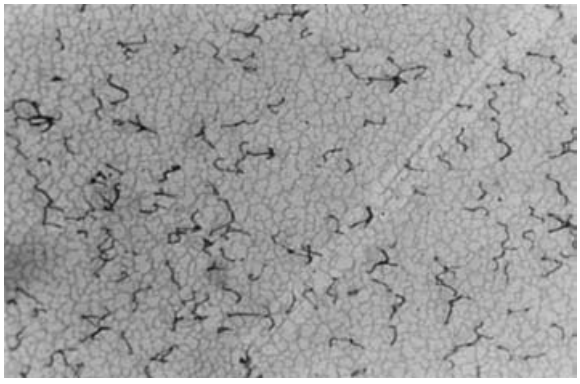

**Fig. 2.** Isolate DS2 stained by Fontana's technique at a magnification of 400×.

*RAPD fingerprinting*

Two types of RAPD fingerprints were generated. In one experiment, primer PB1, 5′-GCG CTG GCT CAG-3′ was used whereas in another experiment, a set of primers B11-CCGGAAGAAGGGGCGCCAT and B12-CGATTTAGAAGGACTTGCACAC were used simultaneously. Both experiments were carried out as described earlier by Brown and Levett [19] and Gerritson et al. [20] respectively using a Thermal Cycler DNA Engine PTC 200 (MJ Research Inc., Waltham, MA, USA). PCR was performed several times and reaction products were electrophoresed on 20-cm long 1% agarose gels, stained with 0·5 μg/ml ethidium bromide (Sigma), viewed under UV light and photographed using a gel documentation system (Vilber Lourmat, France). Dendrograms were generated using Bioprofil software (Vilber Lourmat, Marne-la-Vallée, France).

## RESULTS

**Morphology and motility**

The isolate showed typical morphology and characteristic motility of the genus *Leptospira* under

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Table 2. *Details of rabbit antisera of 233 serovars of 25 sergorups which were used in MAT to screen the isolate DS2*

| Serogroup | Serovars | | | |
|---|---|---|---|---|
| Australis | australis | bajan | bratislava | rushan |
| | fugis | hawain | jalna | Pina |
| | lora | muenchen | nicaragua | soteropolitana |
| | peruviana | ramisi | | |
| Autumnalis | alice | autumnalis | bangkinang | nanla |
| | bim | bulgarica | butembo | weerasinghe |
| | carlos | erinaceiauriti | fortbragg | srebarna |
| | lambwe | mooris | mujunkumi | rachmati |
| Ballum | ballum | ballum 3 (guanggong) | castellonis | peru |
| | kenya | arborea | | |
| Bataviae | argentiniensis | balboa | bataviae | santarosa |
| | brasiliensis | claytoni | djatzi | rioja |
| | kobbe | losbanos | paidjan | |
| Canicola | bafani | benjamini | bindjei | sumneri |
| | broomi | canicola | galton | portlandvere |
| | jonsis | kamituga | malaya | schueffneri |
| | kuwait | | | |
| Celledoni | anhoa | whitcombi | celledoni | hainan |
| | javanica 4 (mengding) | | | |
| Cynopteri | tingomaria | | | |
| Djasiman | agogo | djasiman | gurungi | sentot |
| | huallaga | | | |
| Grippotyphosa | canalzonae | grippotyphosa | huanuco | liangguang |
| | muelleri | ratnapura | valbuzzi | bananal |
| | vanderhoedeni | dadas | | |
| Hebdomadis | borincana | goiano | hebdomadis | nanding |
| | jules | kabura | kambale | manzhuang |
| | kremastos | maru | nona | longnan |
| | sanmartini | worsfoldi | | |
| Icterohaemorrhagiae | birkini | bogvere | copenhageni | honghe |
| | dakota | gem | icterohaem. | yeonchon |
| | lai | mankarso | mwogolo | hongchon |
| | naam | ndahambukuje | ndambari | naaxi |
| | smithi | tonkini | | |
| Javanica | ceylonica | coxi | dehong | zhenkang |
| | fluminense | javanica | a85 (mengla) | mengma |
| | menoni | menrun | poi | yaan |
| | sofia | sorexjalna | vargonicas | |
| Louisiana | louisiana | orleans | saigon | lanka |
| Manhao | lincang | qingshui | lichuan | manhao |
| | lushui | | | |
| Mini | beye | georgia | mini | ruparupae |
| | perameles | swajizak | tabaquite | nanding |
| | hekou | yunnan | | |
| Panama | cristobali | mangus | panama | |
| Pomona | kunming | mozdok | pomona | mozdok type 3 |
| | proechimys | tropica | tsaratsovo | |
| Pyrogenes | abramis | alexi | biggis | nigeria |
| | camlo | guaratuba | hamptoni | menglian |
| | kwale | manilae | myocastoris | varela |
| | princestown | pyrogenes | robinsoni | zanoni |

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Table 2 (*cont.*)

| Serogroup | Serovars | | | |
|---|---|---|---|---|
| Ranarum | evansi | ranarum | pingchang | |
| Sarmin | machiguenga<br>waskurin | rio<br>weaveri | sarmin | cuica |
| Sejroe | balcanica<br>geyaweera<br>haemolytica<br>medanensis<br>recreo | caribe<br>gorgas<br>hardjo<br>nyanza<br>ricardi | dikkeni<br>guaricura<br>istrica<br>polonica<br>roumanica | jin<br>wolffi<br>trinidad<br>sejroe<br>saxkoebing |
| Shermani | aguaruna<br>shermani | babudieri | luis | carimagua |
| Tarassovi | atlantae<br>chagres<br>guidae<br>kisuba<br>rama<br>tunis | bakeri<br>darien<br>kanana<br>langati<br>sulzerae<br>vughia | bravo<br>gatuni<br>kaup<br>navet<br>tarassovi<br>darien | banna<br>yunxian<br>mogdeni<br>mengpeng<br>gengma<br>atchafalaya |
| Undesignated | sichuan | | | |
| Hurstbridge | hurstbridge | | | |

Table 3. *List of monoclonal antibodies used in MAT against the isolate DS2*

| Serogroup | Monoclonal antibodies |
|---|---|
| Icterohaemorrhagiae | F12C3, F20C3, F20C4, F52C1, F52C2, F70C4, F70C7, F70C13, F70C14, F70C20, F70C24, F70C26, F82C1, F82C2, F82C7, F82C8, F89C3, F89C12 |
| Canicola | F152C1, F152C2, F152C5, F152C7, F152C8, F152C10, F152C11, F152C13, F152C14, F152C17, F152C18 |
| Bataviae | F129C2, F129C3, F129C4, F129C6, F129C7, F129C9, F129C15, F129C18, F129C19, F129C20, F129C24, F129C25, F129C6 |
| Grippotyphosa | F71C2, F71C3, F71C9, F71C13, F71C16, F71C17, F164C1, F165C1, F165C2, F165C3, F165C7, 165C8, 165C12 |
| Hebdomadis | F35C10, F38C13, F38C20, F38C24, F50C3, F106C1, F106C5, F106C9 |
| Javanica | F12C3, F20C3, F20C4, F70C20, F98C4, F98C5, F98C8, F98C12, F98C17, F98C19, F98C20 |
| Mini | F35C10, F38C13, F38C20, F38C24, F50C3, F106C1, F106C5, F106C9 |
| Pyrogenes | F134C1, F134C2, F134C4, F134C5, F134C6 |
| Shermani | F151C1, F151C6, F151C7, F151C8, F151C9, F151C13, F151C17, F151C19, F151C20 |
| Sarmin | F12C3, F20C3, F20C4, F70C7, F70C20, F70C20, F98C4, F98C5, F98C12, F98C17, F98C19, F98C20 |

dark-field microscopic examination. Under the electron microscope the cells were helical in shape with the dimensions of 9–12 $\mu$m length, 0·13–0·14 $\mu$m diameter, 0·48–0·52 $\mu$m wavelength and 0·12–0·14 $\mu$m amplitude (Fig. 1).

### Cultural characters

The strain grew well in aerobic conditions both in EMJH and Fletcher's media. It did not grow in Tripticase soy broth in which *Leptonema* grow well. The optimum temperature for growth was 28–30 °C and the optimum pH was 7·2–7·4, culture characteristics of *Leptospira*.

### Staining

Cells were difficult to stain by Gram's method but stained well by silver impregnation techniques such as Fontana's method (Fig. 2).

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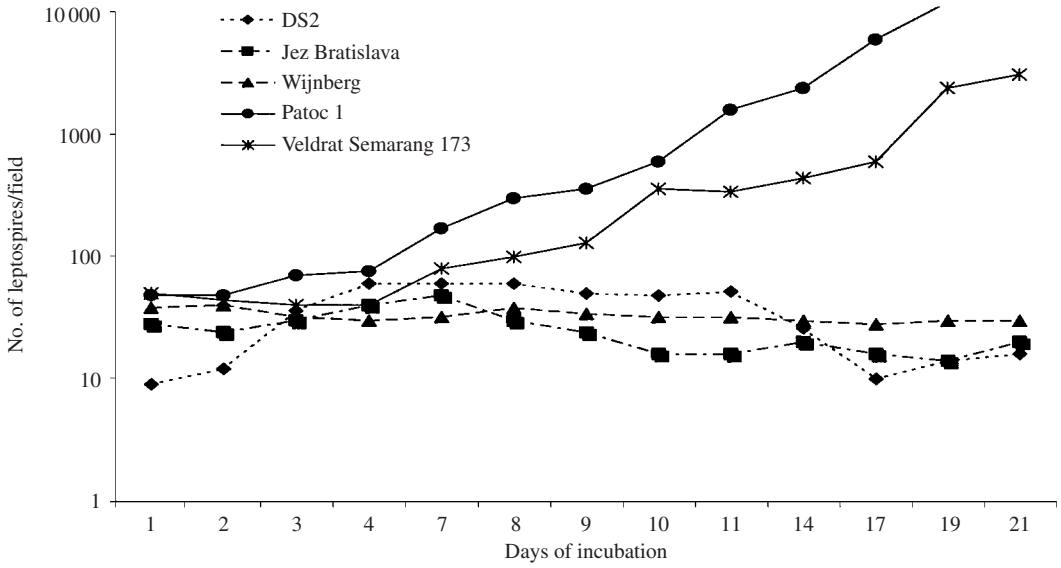


**Fig. 3.** Results of 13 °C test on the isolate DS2 and control strains.

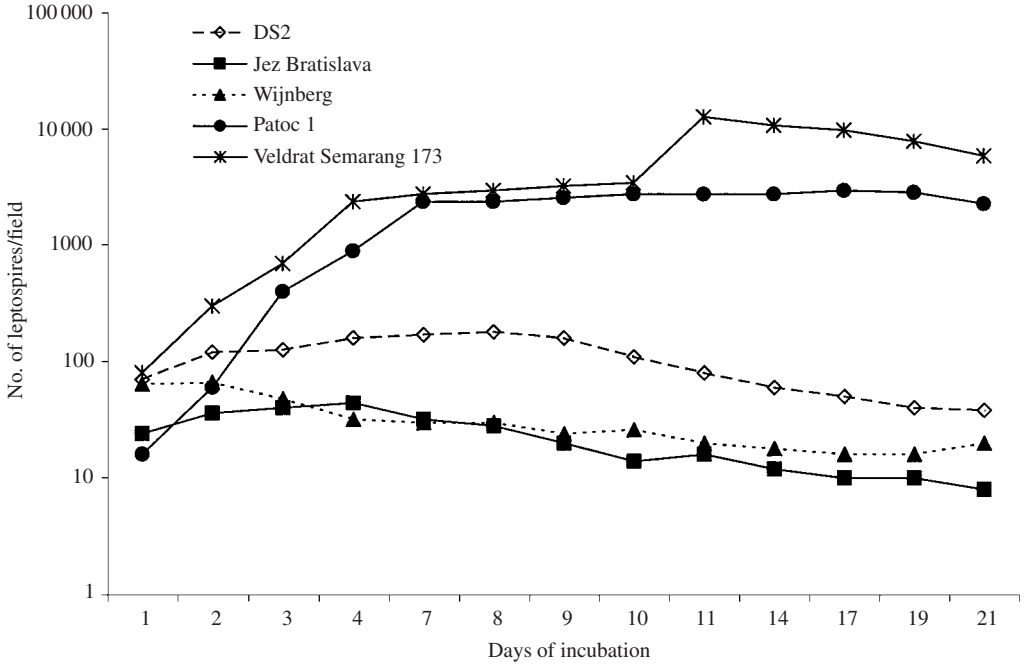


**Fig. 4.** Results of the azaguanine test on the isolate DS2 and control strains.

### Pathogenic status

The saprophytic strains Veldrat, Semarang and Patoc I reached a maximum density within 21 days at 13 °C and in the presence of 8-azaguanine within 14 days of incubation. In contrast, the growth of DS2 as well as the pathogenic reference strains Wijnberg and Jez Bratislava was inhibited at 13 °C, and in the presence of 8-azaguanine even after 21 days of incubation (Figs 3 and 4) indicating the pathogenic nature of the isolate.

### Serological characterization

Agglutination of the isolate with group sera (Table 1) and with all serovar-specific reference rabbit antisera within 25 serogroups (Table 2) failed to identify the isolate conclusively. However, the isolate gave titres

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Table 4. *MAT titres of DS2 against serovars of 25 serogroups* (*showing only serovars which gave a titre of 80 or more*)

| Antiserum | Serogroup | Titre |
|---|---|---|
| Santarosa | Bataviae | 320 |
| Canicola | Canicola | 320 |
| Vanderhoedeni | Grippotyphosa | 1280 |
| Manzhuang | Hebdomadis | 1280 |
| Jules | Hebdomadis | 1280 |
| Copenhageni | Icterohaemorrhagiae | 160 |
| Icterohaemorrhagiae | Icterohaemorrhagiae | 160 |
| Poi | Javanica | 320 |
| Fluminense | Javanica | 1280 |
| Szwajizak | Mini | 1280 |
| Rabinsoni | Pyrogenes | 320 |
| Nigeria | Pyrogenes | 160 |
| Manilae | Pyrogenes | 160 |
| Aguaruna | Shermani | 320 |
| Weaveri | Sarmin | 160 |
| Andamana | Andamana | 80 |
| Patoc | Semaranga | 80 |

ranging from 160 to 1280 against 15 serovars belonging to 10 serogroups (Table 4).

Results obtained by cross-agglutination with all the serovars belonging to those 10 serogroups showed more than 10% relation in the case of serovars fluminense, vanderhoedeni, manzhuang and szwajizak (Table 5). The results obtained after absorption with these four serovars failed to identify the isolate as belonging to any of them (Table 6).

The isolate was screened against a panel of mAbs (Table 3) that generate characteristic agglutination profiles for various serovars of serogroups Grippotyphosa, Icterohaemorrhagiae, Canicola, Bataviae, Hebdomadis, Javanica, Mini, Pyrogenes, Sarmin and Shermani. The use of mAbs was restricted to these serogroups because rabbit antisera against one of more reference strains of these serogroups could agglutinate the isolate DS2. However the isolate did not react against any of the mAbs in MAT indicating that it does not belong to any of the serovars of these 10 serogroups.

### Genetic characterization

The DNA of the isolate DS2 was not amplified in PCR using primer pairs B64-I/B64-II, whereas the primer set G1/G2 amplified a 285-bp product indicating the isolate does not belong to the genospecies *L. kirschneri*.

Table 5. *Cross-agglutination test results* (*showing only serovars which gave more than 10% relation*)

| Antiserum | Antigen | Homologous titre | Heterologous titre | % |
|---|---|---|---|---|
| Vanderhoedeni | DS2 | 10240 | 1280 | 12·5 |
| DS2 | Vanderhoedeni | 40960 | 80 | 0·19 |
| Manzhuang | DS2 | 10240 | 1280 | 12·5 |
| DS2 | Manzhuang | 40960 | 80 | 0·19 |
| Fluminense | DS2 | 10240 | 1280 | 12·5 |
| DS2 | Fluminense | 40240 | 1280 | 3·1 |
| Szwajizak | DS2 | 10240 | 1280 | 12·5 |
| DS2 | Szwajizak | 40240 | 160 | 0·39 |

Sequence analysis of the PCR products generated with primer set G1/G2 from the isolate and from those 41 strains belonging to five genomic species revealed a highest per cent identity of 97·5–99·6% with the G1/G2 products generated from various strains belonging to *L. interrogans sensu stricto*. Percentages of identity shared with the sequence of other species varied from 91·9% with *L. noguchii* serovar proecchimys strain 1161 U, to 82·8% with *L. borgpetersenii* serovar poi strain Poi. Sequence alignment of PCR products obtained with primer set G1/G2 from the isolate and reference strains belonging to five species are shown in Figure 5.

Figures 6 and 7 show RAPD fingerprints and dendograms of isolate DS2 and members of six genomic species. Fingerprints generated using primer PB1 (Fig. 6) revealed that the isolate DS2 shared the highest per cent identity (85%) with the two strains Ballico and RGA which belong to genospecies *L. interrogans*. The shared genetic similarity of DS2 with members of the other five species ranged from 23 to 48%. The fingerprints generated with the primer set B11 and B12 (Fig. 7) also showed that the isolate DS2 had the highest genetic similarity of 87% with the strains Ballico and RGA, further substantiating the evidence that DS2 is genetically similar to *L. interrogans sensu stricto*.

## DISCUSSION

Serological characterization of leptospires is a complex and time-consuming exercise. There are 25 serogroups and each serogroups contains several serovars. A representative group serum is expected to react strongly with all the serovars in that serogroup, but in practice may cross-agglutinate with strains belonging

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Table 6. *Agglutination absorption test results of isolate DS2*

| Antiserum | Absorbing strain or serovar | Titre of heterologous strain before absortion | Titre of homologous strain before absortion | Titre of homologous strain after absortion | % |
|---|---|---|---|---|---|
| Fluminense | DS2 | 1280 | 10 240 | 5120 | 50·0 |
| Vanderhoedeni | DS2 | 1280 | 10 240 | 10 240 | 100·0 |
| Manzhuang | DS2 | 1280 | 10 240 | 5120 | 50·0 |
| Szwajizak | DS2 | 1280 | 10 240 | 5120 | 50·0 |

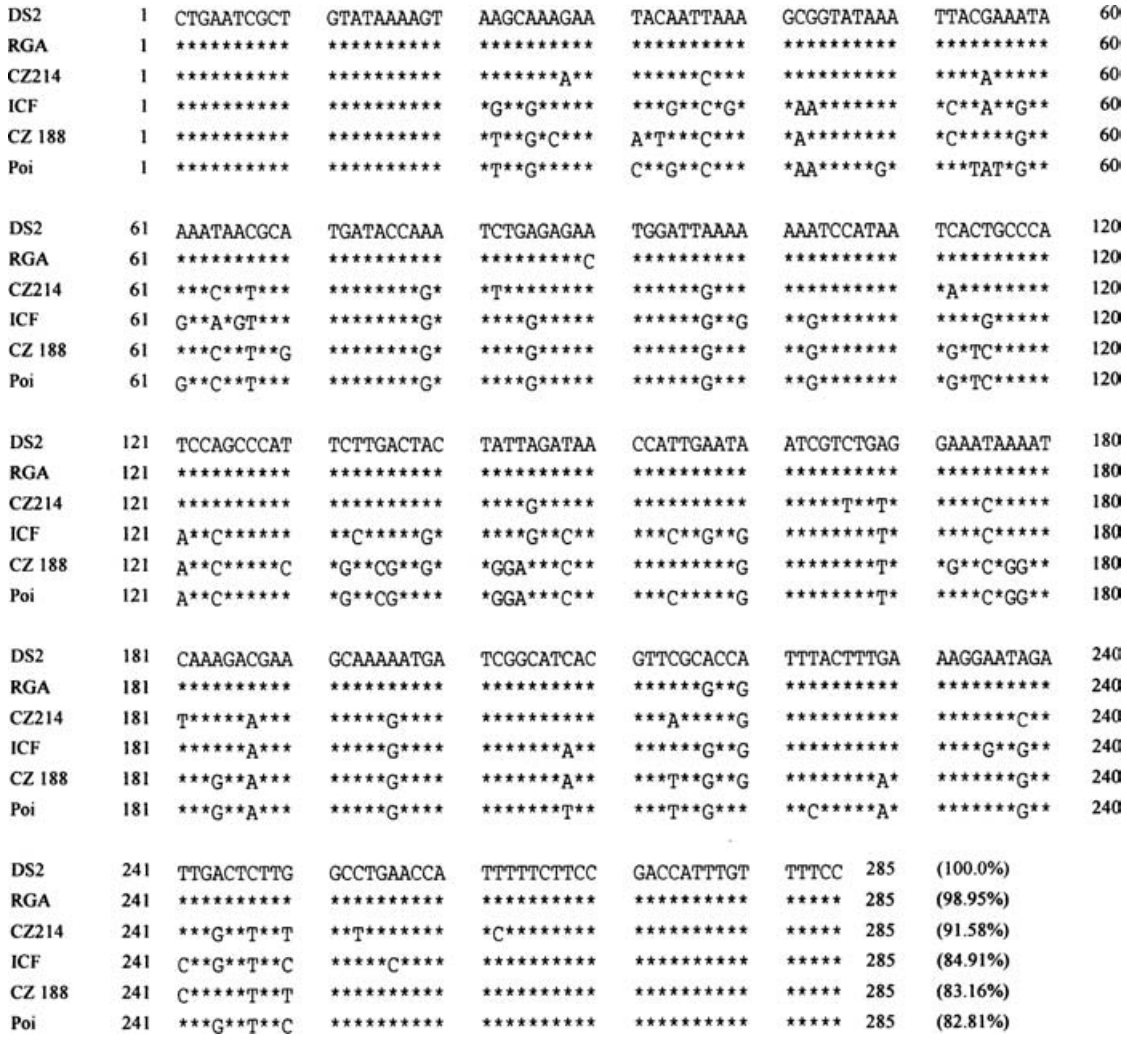


**Fig. 5.** Sequence alignment of 285-bp PCR products obtained with primer set G1/G2 from the isolate DS2 and members of five pathogenic species. *L. interrogans* strain RGA, *L. noguchii* strain CZ 214, *L. meyeri* strain ICF, *L. santarosai* strain CZ188 and *L. borgpetersenii* strain Poi.

to different serogroups. The opposite is also true in practice. An isolate may not give agglutination with an antiserum to the representative serovar of a particular serogroup due to lack of homogeneity or very little serological relationship between serovars of certain serogroups.

Hence, a single representative 'group serum' for each serogroup in a MAT panel may be insufficient to

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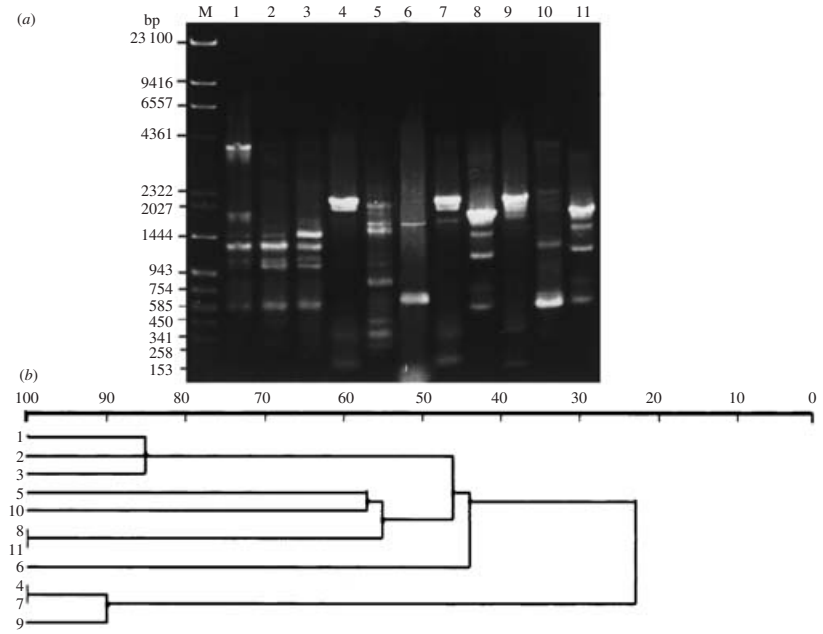


**Fig. 6.** (*a*) RAPD fingerprints of isolate DS2 and reference strains belonging to six species generated with the primer PB1. (*b*) Dendrogram for the similarity (UPGMA). Lane 1, strain Ballico, serovar australis (*L. interrogans*); lane 2, strain RGA serovar icterohaemorrhagiae (*L. interrogans*); lane 3, isolate DS2; lane 4, strain Moskva V serovar grippotyphosa (*L. kirschneri*); lane 5, strain CZ188 serovar canalzonae (*L. santarosai*); lane 6, strain LSU1945 serovar louisiana (*L. naguchii*); lane 7, strain 3522C serovar cynopteri (*L. kirschneri*); lane 8, strain Sari serovar mini (*L. borgpetersenii*); lane 9, strain Wumalasena serovar ratnapura (*L. kirschneri*); lane 10, strain ICF serovar ranarum (*L. meyeri*); lane 11, strain Poi serovar poi (*L. borgpetersenii*); lane M, $\lambda$ DNA, *Hin*d III digest.

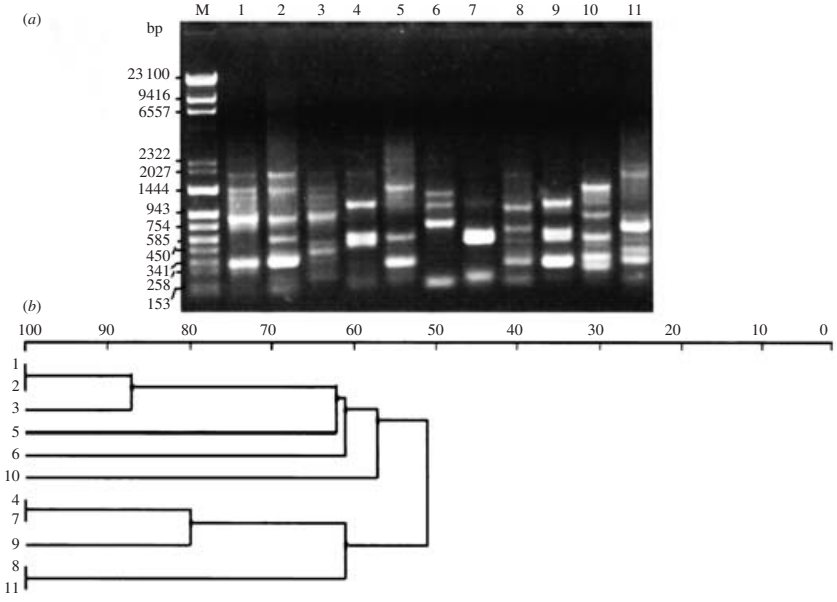


**Fig. 7.** (*a*) RAPD fingerprints of isolate DS2 and reference strains belonging to six species generated with the primer set B11 and B12. (*b*) Dendrogram for the similarity (UPGMA). Lane 1, strain Ballico, serovar australis (*L. interrogans*); lane 2, strain RGA serovar icterohaemorrhagiae (*L. interrogans*); lane 3, isolate DS2; lane 4, strain Moskva V serovar grippotyphosa (*L. kirschneri*); lane 5, strain CZ188 serovar canalzonae (*L. santarosai*); lane 6, strain LSU1945 serovar louisiana (*L. naguchii*); lane 7, strain 3522C serovar cynopteri (*L. kirschneri*); lane 8, strain Sari serovar mini (*L. borgpetersenii*); lane 9, strain Wumalasena serovar ratnapura (*L. kirschneri*); lane 10, strain ICF serovar ranarum (*L. meyeri*); lane 11, strain Poi serovar poi (*L. borgpetersenii*); lane M, $\lambda$ DNA, *Hin*d III digest.

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identify the isolate at serogroup level. This is evident from the fact that some of the original serogroups, that had large number of serovars, showed very little serological relationship to one another. Such serogroups were divided into 2–3 serogroups according to their serological affitinities (e.g. the original serogroup Hebdomadis was divided into three serogroups, namely Hebdomadis, Sejroe and Mini, and serogroup Autumnalis into three serogroups, namely Autumnalis, Djasiman and Louisiana [16]. Therefore, in some circumstances, more than one 'group serum' may be needed for each serogroup to identify isolates up to serogroup status. However selection of the additional 'group serum' of a particular serogroup is difficult without practical experience in a particular geographical region or country. Therefore, our isolate was tested against all the reference antisera belonging to the 25 serogroups (Table 2), that have been described so far, in addition to representative group sera (Table 1). However, the isolate could not be identified. The results obtained using CAAT and mAbs also failed to identify our isolate.

In line with the recommendations of International Committee on Systematic Bacteriology, Sub-Committee on the Taxonomy of *Leptospira* [14] we propose that the isolate DS2 does not belong to any serovar of any serogroup known so far. Therefore we propose a new serovar portblairi and new serogroup Sehgali of *L. interrogans sensu lato*.

Although DS2 is a new serovar of a new serogroup, its genetic characterization employing PCR and sequencing showed relatedness to strains belonging to *L. interrogans sensu stricto* and these findings were further substantiated by RAPD. It is important to note that 87 serovars belonging to 15 serogroups were identified as belonging *L. interrogans sensu stricto* indicating that the species *L. interrogans sensu stricto* has highest collection of serovars of different serogroups [16]. Serovar portblairi of serogroup Sehgali is to be considered as a new entry.

The strain DS2 was recovered from a patient with haemoptysis with respiratory distress, which is a common complication of leptospirosis in the Andaman & Nicobar Islands. Pulmonary involvement in leptospirosis was first observed in India in the Andaman Islands. However this form of presentation has also been seen occasionally in mainland India during recent outbreaks [21]. Pulmonary involvement in leptospirosis has been observed in China and Korea and recently in other countries, e.g. Australia, Nicaragua, etc. [22–24]. In countries like China and Korea, the occurrence of pulmonary haemorrhage has been linked to infection with serovar lai of serogroup Icterohaemorrhagiae. In Australia, pulmonary haemorrhage has been reported in patients infected with serovar australis. In this study, the isolate DS2 of serovar portblairi of serogroup Sehgali was recovered from a patient with massive haemoptysis. All the isolated leptospires causing pulmonary haemorrhages so far have been found to belong to *L. interrogans sensu stricto* but to serovars of different serogroups.

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