A New Serovar in the Grippotyphosa Serogroup Comprising Leptospiral Isolates from Different Regions

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Pulsed-field gel electrophoresis (PFGE) studies performed with leptospiral isolates led us to suspect the existence of a new serovar in the Grippotyphosa serogroup. The results obtained with reference serological techniques used in leptospiral identification, including cross-agglutination absorption and monoclonal antibody techniques, confirmed the existence of a new serovar exemplified by strain Dadas I. Four other isolates from different regions of the world were submitted for identification by PFGE and produced *NotI* restriction patterns similar to that of strain Dadas I. Our data demonstrate the power of PFGE for identifying leptospiral isolates. The name *dadas* is proposed for the new serovar.

Pathogenic leptospires are spirochetes that were first isolated and identified by Inada et al. in 1915 (9). These microorganisms are responsible for a zoonanthroposis of worldwide distribution, the symptoms of which were classically described by Weil in 1886 (20). The main advance in our knowledge of spirochete phylogeny came from the results of 16S RNA sequence determinations. Spirochetes represent one of the oldest bacterial phyla within the eubacteria (21). The phylogenetic separation of these organisms is based on specific morphological characteristics, including a helical form and the presence of an internal locomotor organelle (2). The family *Leptospiraceae* within the spirochetes (8) contains two genera, *Leptospira* and *Leptonema* (17). The genus *Leptospira* can be differentiated into two nomenclatures, the nonpathogenic taxon *Leptospira biflexa* sensu lato and the pathogenic taxon *Leptospira interrogans* sensu lato (10). The G+C contents of the DNAs of these organisms range from 36 to 39% mol% (17, 19). On the basis of DNA-DNA hybridization results, 10 genomic species have been identified within the two nomenclatures (pathogenic and nonpathogenic) (18, 22).

Early in this century, leptospires were classified by using serological criteria. By using the agglutination-lysis reaction developed by Martin and Pettit (16), it was possible to discriminate *Leptospira* strains having different antigenic properties. Within the nomenclature *L. interrogans*, more than 200 serovars can be distinguished; these serovars are grouped into 23 serogroups (13). The nomenclature *L. biflexa* contains 65 serovars divided into 38 serogroups (13). The grouping of serovars into serogroups is based on antigenic similarities between serovars and is used for practical reasons.

The serovar is considered as the basic taxon. When the microscopic agglutination and cross-agglutinin absorption tests are used (10), two serovars are considered to be differentiated if, after cross-absorption with adequate amounts of heterologous antigen, 10% or more of the homologous titer regularly remains in at least one of the two antisera (5). Serological identification of *Leptospira* strains by these reference proce-

dures is long and time consuming. To circumvent such difficulties, different workers have chosen to prepare mouse monoclonal antibodies (MAbs) directed against specific epitopes of reference strains (14, 15, 19). This technique, which depends on the use of a collection of carefully chosen MAbs, gives results which correlate well with cross-absorption results. Another strategy is to use a restriction endonuclease and pulsed-field gel electrophoresis (PFGE) (6, 7). This technique permits identification of any serovar which possesses a specific and clear banding pattern when its DNA is cut with restriction endonuclease *NotI*; the patterns are called large restriction fragment patterns (LRFP). Clinical isolates can be identified by comparison with a data bank consisting of the LRFPs obtained with numerous serovars.

In the course of a study of new isolates obtained from mammals (7), one strain belonging to the Grippotyphosa serogroup, Dadas I, was found to produce a specific DNA restriction pattern with endonuclease *NotI* (7). Strain Dadas I, which was isolated from a bull in Turkey, was studied further. In this paper, we describe serovar identification of strain Dadas I by the cross-absorption procedure, the MAb technique, and PFGE. Four other mammal isolates belonging to the Grippotyphosa serogroup (Kornilov, Bernkopf, Kizilchaman, and Karakabay) produced LRFPs and MAb patterns similar to those of strain Dadas I.

All strains were cultivated in EMJH or Korthof's media (4, 11, 12). A DNA restriction analysis by PFGE was performed as previously described (1, 7). Antisera for cross-absorption were produced in paired rabbits by using the eight reference serovars in serogroup Grippotyphosa and strain Dadas I (3, 5, 12). Strain Dadas I was tested against the serogroup Grippotyphosa reference antisera, while the serogroup Grippotyphosa reference serovars were tested against antiserum to strain Dadas I by the microagglutination test (12, 16). Thirteen MAbs, which were produced as previously described (14, 19), were used to discriminate among reference serovars of serogroup Grippotyphosa and to compare these taxa with strain Dadas I.

According to the cross-agglutination test results, strain Dadas I belongs to the Grippotyphosa serogroup. On the basis of the results of reference agglutination tests after cross-absorption tests (Table 1), strain Dadas I was found to be closely related to serovars *grippotyphosa*, *valbuzzi*, *vanderhoeni*, *ratnapura*, and *huanuco*. However, when we used

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TABLE 1. Cross-adsorption results with *Leptospira* strains belonging to the Grippityphosa serogroup and strain Dadas I

Immuniserum against strain ^a :	Serovar	Absorbed with:	Microagglutination test with strain:	Agglutination titer (10 ³) ^b
Moskva V	grippityphosa	0	Dadas I	25
		Dadas I	Moskva V	<0.2
Valbuzzi	valbuzzi	0	Dadas I	25
		Dadas I	Valbuzzi	<0.1
CZ 188	canalzonae	0	Dadas I	25
		Dadas I	CZ 188	3
Kipod 179	vanderhoedeni	0	Dadas I	12
		Dadas I	Kipod 179	0.2
Wumalasena	ratnapura	0	Dadas I	25
		Dadas I	Wumalasena	0.1
RM2	muelleri	0	Dadas I	25
		Dadas I	RM 2	3
1880	liangguang	0	Dadas I	25
		Dadas I	1880	12
M4	huanuco ^c	0	Dadas I	<0.1
		Dadas I ^d	0	0
Dadas I	dadas	0	Moskva V	25
		Moskva V	Dadas I	3
Dadas I	dadas	0	Valbuzzi	25
		Valbuzzi	Dadas I	6
Dadas I	dadas	0	CZ 188	25
		CZ 188	Dadas I	12
Dadas I	dadas	0	Kipod 179	1
		Kipod 179	Dadas I	12
Dadas I	dadas	0	Wumalasena	12
		Wumalasena	Dadas I	3
Dadas I	dadas	0	RM 2	12
		RM 2	Dadas I	6
Dadas I	dadas	0	1880	25
		1880	Dadas I	25
Dadas I	dadas	0	M4	6
		M4	Dadas I	6

^a The homologous titers were 12×10^3 to 25×10^3 . Each serological reaction with a heterologous antigen after absorption was negative (titer, $<0.1 \times 10^3$).

^b Antibody titers are expressed by the reciprocal of the serum dilution.

^c This serovar provisionally belongs to serogroup Grippityphosa.

^d Absorption experiment not performed.

immune serum against Dadas I, none of the reference strains used removed antibodies that reacted with the homologous strains (Table 1). This means that strain Dadas I has a main antigen not present in any of the other Grippityphosa sero-

FIG. 1. PFGE of *NorI* restriction fragments from mammal isolates Kizilchaman, Karakabay, Kornilov, and Bernkopf (lanes 1 through 4, respectively) and strain Dadas I (lane 5). The digestion products were separated by electrophoresis at 150 V for 40 h in 1% agarose-0.5× Tris-borate-EDTA. The pulse times were 10 s (10 h), 20 s (12 h), 50 s (14 h), and 100 s (2 h). Lanes S and L contained size markers (*Saccharomyces cerevisiae* and lambda phage DNA, respectively).

group serovars (13). Consequently, strain Dadas I should be considered a member of a new serovar in serogroup Grippityphosa.

Agglutination with a panel of 13 MAb indicated that strain Dadas I produces a unique MAb pattern compared with the patterns obtained for the other reference serovar strains (Table 2). Very similar MAb patterns were obtained for Dadas I and isolates Kornilov, Bernkopf, Kizilchaman, and Karakabay; the single exception was one MAb 71-3, which agglutinated only strain Dadas I (data not shown).

TABLE 2. Agglutination titers obtained with 13 different MAbs for reference strains of serovars of the Grippityphosa serogroup and strain Dadas I

Strain	Serovar	Agglutination titer (10 ³) ^a												
		MAb 71-2	MAb 71-3	MAb 71-9	MAb 71-13	MAb 71-16	MAb 71-17	MAb 164-1	MAb 165-1	MAb 165-2	MAb 165-3	MAb 165-7	MAb 165-8	MAb 165-12
Moskva V	grippityphosa	5	0.6	40	10	40	20	— ^b	10	1	10	5	—	—
RM2		1	0.6	20	10	40	40	—	81	0.6	10	10	—	—
Wumalasena	ratnapura	1	—	5	20	5	20	—	40	0.6	10	2	5	2
Valbuzzi		5	0.6	20	20	0.6	40	—	40	1	20	10	0.3	0.6
Aa 14	bananal	—	—	1	—	—	5	—	2	0.1	2	0.6	—	—
1880		—	—	1	—	—	20	—	20	2	10	1	—	—
Kipod	vanderhoedeni	0.6	—	2	0.08	—	0.6	20	0.08	10	—	1	0.6	0.3
CZ 188		—	—	2	—	—	5	—	—	—	—	0.1	—	—
M4	canalzonae	10	—	—	1	—	—	—	—	—	—	—	—	—
		—	—	—	—	—	—	—	—	—	—	—	—	—
Dadas I	huanuco	0.3	10	81	10	20	40	—	20	1	20	2	10	5

^a Antibody titers are expressed by the reciprocal of the serum dilution.

^b —, <0.020.

The PFGE analysis revealed an unusual LRFP for strain Dadas I (7). Four other isolates, which originated from different regions of the world, including Russia (strain Kornilov), Israel (strain Bernkopf), and Turkey (strains Kizilchaman and Karakabay), were submitted for identification by PFGE and produced the same *NotI* restriction pattern as strain Dadas I (Fig. 1). This finding was corroborated by using another rarely cutting endonuclease, *SgrAI* (data not shown).

In addition to representing a new serovar, strain Dadas I represents a new LRFP in serogroup Grippotyphosa. The name *dadas* is proposed for the new serovar; the type strain of this serovar is strain Dadas I, and this strain is available from the collections of leptospirosis reference laboratories in Amsterdam, Bratislava, and Paris.

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