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Molecular and immunological characterisation of a *Leptospira* strain isolated from kidney of rats (*Rattus rattus*) in India

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ABSTRACT

Kidneys from field rats trapped in the Izatnagar area of Uttar Pradesh, India, were inoculated in EMJH medium. One of the samples yielded *Leptospira* organism which was designated as SR 20.Serogrouping of the isolate (SR 20) was done by microscopic agglutination test. The isolate belonged to serogroup Javanica. Cross agglutination absorption tests revealed that SR 20 did not belong to the known serovars of this group, viz. ceylonica, coxi, mengma, sofia, zhenkang, dehong and yaan. Further serological relationships based on reaction obtained with a panel of monoclonal antibodies showed that the isolate was closely related to dehong. The genomic profile of Not I digested chromosome of SR 20 was compared with serovars zhenkang, yaan, mengma, dehong, sofia, coxi and ceylonica. The profile of SR 20 appeared identical to dehong. The isolate was considered to be a new serovar (proposed name Izatnagar) closely related to dehong.

Key words: Isolation, *Leptospira*, Rat

Leptospirosis is prevalent in animals in various regions of India (Srivastava *et al.* 1990, Sehgal *et al.* 1994). During a seroepidemiological study undertaken in buffaloes in an area of Izatnagar, Uttar Pradesh, India, a high percentage of animals (cattle and buffaloes) revealed antibodies to various leptospiral serovars (Mallick *et al.* 1988). To find out the possible source of infection, field rats (natural carriers) and water samples from local ponds in that area were collected and tested for the presence of *Leptospira*.

MATERIALS AND METHODS

Isolation

Field rats (*Rattus rattus*) were trapped alive and killed at laboratory. The kidneys were removed aseptically and homogenised in EMJH medium containing 5-fluorouracil (100 µg/ml) (Johnson and Rogers 1964). One ml of each of ten-fold dilutions starting at 1:10 dilutions of the homogenised tissue suspension was inoculated into tubes containing 9 ml of the EMJH medium with 0.1% agar. The tubes were incubated at 28°C for 3 months and checked for growth at every 2-3 weeks by dark field microscopy.

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Indirect test of pathogenicity

The pathogenicity of the isolate was determined by 2 methods. First method: The isolate was inoculated into EMJH containing 8-azoguanine at a final concentration of 0.1 mg/ml as described by Johnson and Harris (1967); and second method: The isolate was cultured at 13°C. Unlike saprophytic strains, pathogenic strains do not grow under these conditions.

Cross agglutination and agglutinin absorption tests

Prior to cross agglutination and agglutinin tests with relevant serovars, the isolate was grouped by using the microscopic agglutination test (MAT) with reference rabbit antisera against reference strains representing the following serogroups: Australis (serovars *australis* and *bratislava*), Autumnalis (serovars *banginang*, *butembo*, *carlos* and *rohmati*), Balium (serovar *balium* and *kenys*), Bataviae (serovar *bataviae*), Canicola (serovar *canicola* and *scheffneri*), Celledoni (serovar *celledoni*), Cynopteri (serovar *cynopteri*), Djasiman (serovar *grippotyphosa* and *huanuco*), Hebdomadis (serovars *hebdomadis* and *worsfold*), Icterohaemorrhagiae (serovars *ictohaemorrhagiae* and *coopenhaegni*), Javanica (serovar *poi*), Mini (serovar *mini*), Pomona (serovar *pomona*), Pyrogenes (serovar *pyrogenes*, Sarmin (serovar *rio weaveri*), Sejroe (serovars *hardjo* and *saxkoebing*), Shermani (serovar *shermani*) and Tarassovi (serovars *bakeri*, *mogden*, *rams* and *tarassovi*).

Cross-agglutination and cross-absorption tests were carried out according to the method of Dikken and Kmety (1978). In

brief, cross agglutination tests of relevant rabbit antisera and reference strains with strain and antiserum SR20 were performed to establish the relationship within the relevant serogroup. Only those reference strains whose antisera reacted with SR 20 to 25% or more (MAT) of the titre for the homologus strains were used for the cross agglutinin absorption test, monoclonal antibody analysis and pulsed field gel electrophoresis.

Monoclonal antibody analysis

Those reference strains that showed a close antigenic relationship with strain SR 20 microscopic agglutination tests with monoclonal antibodies (MCAs), were performed with the same serovars for the cross agglutinin absorption tests.

Monoclonal antibodies were produced as per Terpstra *et al.* (1985). Titres of MCAs in the ascitis fluid were also determined by MAT. Six MCAs of fusion 98 were selected for the identification of Javanica group reference strains and the isolate SR 20.

Pulsed field gel electrophoresis

Pulsed field gel electrophoresis (PFGE) was performed at the Institute Pasteur, Paris, using *NotI* digests of leptospiral chromosome (Herrmann *et al.* 1992). DNA extraction and restriction enzyme digestion were performed on plugs containing leptospires before electrophoresis on counter clamped homogenous electric field (CHEF) using Pharmacia apparatus.

RESULTS AND DISCUSSION

Leptospires were isolated from kidney of a rat (*Rattus rattus*) and was denoted SR 20 (Satish-Rishendra 20). Strain 20 did not grow either at 13°C or in medium containing 8-azaguanine, indicating that this strain belonged to the group of pathogenic leptospires. Agglutination of SR 20 with rabbit reference antisera representing different serogroups, was observed with the related Javanica, Sarmin and Celledoni serogroups representatives.

An isolate was considered to be a serovar within an existing serogroup, when it cross agglutinated to at least 6% of the homologous titre with reference antisera to members of that serogroup. SR 20 complied with these criteria. Strain SR 20 reacted with most antisera from the Javanica serogroup to this level (Fig. 1). No significant MAT titres were found with antisera from the Sarmin and Celledoni serogroup (data not shown). The closest relationship was found with the reference strains for serovars *dehong* and *yaan*, rabbit antisera to serovars *ceylonica, coxi, menama, sofia* and *zhenkang* reacted to more than 25% with strain SR 20. Other Javanica group antisera and reference strains (*fluminense, javanica, mengla, menoni, mengrun, poi, sorexjalna* and *vargonicas*) reacted to strain and antiserum SR 20 to a much lesser extent.

This relationship was confirmed when the analysis was continued by carrying out agglutinin absorption tests with

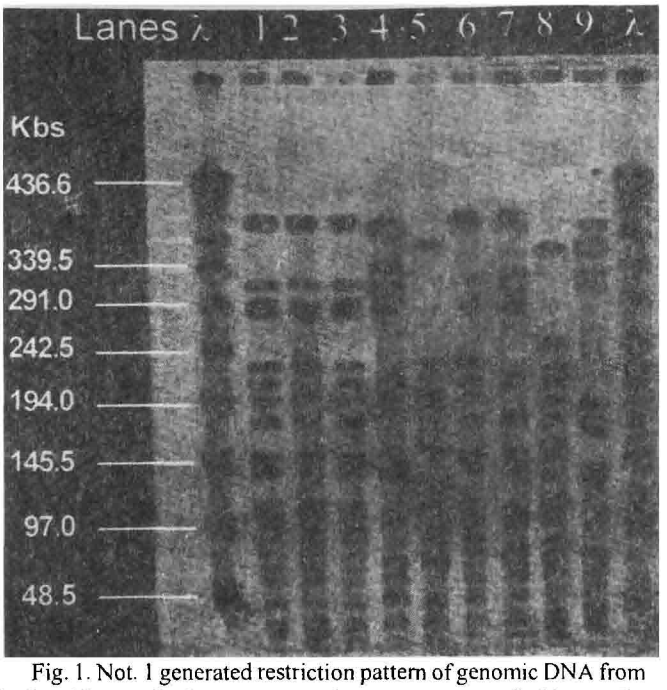

Fig. 1. Not. I generated restriction pattern of genomic DNA from isolate SR20 and other representative serovars as studied by PAGE (Lane 1 = SR20; 2 = Zhenkang; 3 = SR20; 4=Yaan; 5=Mengma; 6=Dehong; 7=Sofia; 8=Coxi; Lanes T contain T phage DNA.

Table 1. MAT titres expressed as percentage of the reciprocal titre for the homologus strain, after absorption tests of Javanica serogroup antisera with strain SR 20 and vice-versa

Antiserum	Absorbed with strain	Titres
ceylonica	SR 20	12.5.25
SR 20	*ceylonica*	25
coxi	SR 20	12.5-25
SR 20	*coxi*	100
dehong	SR 20	1.6
SR 20	*dehong*	25
mengma	SR 20	12.5-50
SR 20	*mengma*	
sofia	SR 20	12.5-50
SR 20	*sofia*	50
yaan	SR 20	1.6-6.2
SR 20	*yaan*	12.5
zhenkang	SR 20	25
SR 20	*zhenkang*	100
SR 20	SR 20	0

the same serovars (Table 1). The absorption tests of antisera and strains to serovars (*ceylonica, coxi, mengma, sofia* and *zhenkang* with strain and antiserum SR 20 indicated that SR 20 did not belong to any of these serovars. The results with serovars *dehong* and *yaan* showed that, when antiserum SR 20 was absorbed with these serovars, more than 10% of the antibodies reacting with the homologous strain remained in

their antisera. These results indicate that SR 20 is a new serovar, closely related to serovar *dehong* and *yaan*.

Monoclonal antibody analysis

A panel of 6 monoclonal antibodies was used to investigate the relationship of isolate SR 20 with Javanica of isolate SR 20 with Javanica group serovars. Strain SR 20 showed different agglutination profiles when this isolate was compared with serovars *ceylonica, coxi, mengma, sofia, yaan* and *zhenkang*. Strain 20 mostly resembled serovar *dehong*.

This result supports the finding with the cross agglutination absorption test where SR 20 also showed a close relationship to this serovar.

Pulsed field gel electrophoresis (PFGE)

Not I generated (Fig.1) restriction enzyme patterns of genomic DNA from isolate SR 20 were compared with serovars *zhenkang, yaan, mengma, dehong, sofia, coxi* and *ceylonica*. The profile of SR 20 appeared identical to that of *dehong* and closely resembled to that of *zhenkang*, differing slightly in the position of one band (130 kb). Similarity of the pattern of SR 20 with that of *dehong* is consistent with the close similarity found by serological methods. Such identity of PFGE patterns from 2 distinct serovars has already been observed in *icterohaemorrhagiae* and *copenhageni* serovars (Herrman *et al.* 1991).

The present work was undertaken to find out the possible source of leptospiral infection to cattle and buffaloes in the Izatnagar area of India especially in relation to the murine reservoir problem which is well recognised throughout the world. The present study resulted in the isolation of a strain belonging to serogroup Javanica from kidneys of a field rat.

Serologically, there is ample evidence to conclude that the infection due to serovars from Javanica serogroup is prevalent in cerain areas in India, especially in Uttar Pradesh (Rajasekhar and Keshavmurthy 1976). The isolation of serovar *javanica* has been reported from rats and bandicoots (Adinarayanan and James 1980, Rajasekhar and Keshavmurthy 1976) and serovar *menoni* from *Bandicoota bengalensis* (Dikken *et al.* 1981) from areas different from the one reported in this study. This study reports on the isolation and characterisation of a new serovar which is closely related but not identical to *dehong*. A new serovar, *Izatnagar* is proposed for the isolate SR 20 within the serogroup Javanica. There is particularly a relationship between SR 20 and *dehong* (isolated from rat in Yunnan province in China 1981), *yaan* Sichun province, Chinain 1989, and at the DNA-level, *zhenkang* (from a house rat, *Rattus flavipectus*, in Yunnan 1986). Although China is located about 2000 miles away from this place, it might be possible that the same serovar of China origin might be existing in the country which had not been detected so far.

The first Javanica group serovar, *javanica*, was isolated for the first time on the island of Java (Indonesia) with 5 more serovars added by 1969. At present the serogroup Javanica consists of 14 serovars, most of them have originated from various parts of Asia (Cao *et al.* 1984, Li Chi-zhi *et al.* 1988). Results of cross agglutination and agglutinin absorption tests showed a relationship between the isolate SR 20 and serovars of the Javanica group, in particular with serovars *yaan* and *dehong*.

However, after cross absorption of the SR 20 rabbit antiserum with these 2 serovars, more than 10% of the homologous titres remained in repeated experiments (Table 1). This finding was confirmed by another laboratory (Dr Kmety, WHO/FAO collaborating Laboratory for the Epidemiology of Leptospirosis, Bratislava). Therefore it is justified to conclude that SR 20 is a new serovar of the Javanica group (TSC 1986).

A close relationship between SR 20 and *dehong* was also found by comparing reactivity patterns with MCAs and by comparison of *Not I* generated PFGE profiles. In these two tests, only little similarity was found with serovar *yaan*, suggesting a more distant relationship with this serovar. Interestingly, the PFGE patterns of isolate SR 20 was almost identical to that of serovar *zhenkang*, whereas, only little relationship between these serovars was revealed by both the serological methods. Apparently, SR 20 and *zhenkang* share a high degree of DNA identity that express different antigens profiles. A possible explanation for these apparently conflicting observations might be that the 2 serovars originate from the same host but have adapted phenotypically to different host or environment resulting in the expression of different genes. Further research on the host-parasite relation of both serovars will be needed to find support for the suggested differences in gene expression.

Taken the results of the serological and genetic typing methods together, strain SR 20 is closely related to serovar *dehong* of the Javanica group but, according to the definitions of the taxonomic subcommittee (TSCL 1978) SR 20 should be considered as a new serovar. We named this new serovar *izatnagar* after the area of isolation. Further research on the host parasite relation of both the serovars will be needed to find support for this supposition. Based on the results of the 3 tests we may conclude that strain SR 20 shows closest relationship with the reference strain for serovar *dehong*. However, serologically it is a distinct serovar.

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