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Isolation and classification of sixteen strains of saprophytic leptospires

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SUMMARY

Sixteen strains of leptospires were isolated from surface water (14 in England and 2 in Italy) and were classified. They were all saprophytic, and nine of them belonged to known serovars: the others were found to represent new serovars within already known serogroups.

INTRODUCTION

Fourteen strains of organisms belonging to the genus Leptospira were isolated during the space of a number of years from external sources in England. Preliminary tests indicated that they all belong to the species of Leptospira known as *Leptospira biflexa* which consists of nonpathogenic strains commonly found in water and occasionally in animals (Cinco & Ivanov, 1978; Subcommittee on the Taxonomy of Leptospira, 1978). After purification, the isolates were sent for identification and classification to the Institute of Microbiology of the University of Trieste, Italy.

In addition two other saprophytic strains, viz. L. tap-water and AM3 both originating in Trieste in 1942 are included in this investigation.

MATERIAL AND METHODS

Source of isolates

The earlier isolates were obtained by Dr Khera in 1966 from canal, river and ditch water near Pyrford in Surrey, England. They are identified as Khera 9, 10, 11 and 12. Three others were contaminants of 3 different batches of uninoculated Korthof's medium in a London laboratory.

They are Wild A, Wild B and Wild C, and were isolated in May, July and August 1967.

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During 1977 the staff of the Microbiology Department of the Agricultural Research Council's Institute for Research on Animal Diseases at Compton, Berkshire conducted an investigation into sewage effluent as a possible source of leptospires, financed by the Thames Water Authority. Samples of sewage were examined at different stages before and during treatment from two different plants: Plant D which received effluent from a cattle market in Oxfordshire; and Plant F, a large urban sewage treatment plant receiving material of mainly human origin.

Many isolates were obtained, and preliminary tests carried out at the Leptospira Reference Laboratory, Colindale, indicated that with one possible exception not yet identified, they all belonged to the Biflexa complex. A representative number were sent to Dr Cinco for identification. They were Compton nos 393, 433, 521 and 636 from final effluent of sewage which contained waste material from cattle; Compton nos 421 and 699 from digested sewage of human origin; and Compton 481 from mixed human and animal sewage during digestion of solid sludge.

Method of isolation

Samples of final effluent were reduced from an initial volume of 20 ml to 5 ml by centrifugation at 800 ***g*** for 15 min. Depending upon their consistency 1 ml or 1 g of the sample was mixed with 5 ml of phosphate-buffered saline and filtered through sterile muslin. Suspensions were then centrifuged at 800 ***g*** for 30 secs, and 5 ml supernatant was filtered through a 0·45 μm membrane (Millipore) in a Hemming filter centrifuged at 800 ***g*** for 15 min. The filtrate was then inoculated into Ellinghausen & McCullough's semi-solid medium (1965) with the addition of 1% rabbit serum, 100 μg per ml 5 fluorouracil (Johnson & Rogers, 1964*a*) and 50 μg per ml amphotericin B (Jones & Matthews, 1975).

Classification

The preliminary biological tests which separate the biflexa strains from those that are pathogenic to man and animals are (*a*) the resistance of the saprophytic strains to 260 μg per ml of the purine analogue 8-azaguanine (2-amino-6-oxy-8-aza-purine) added to the medium (Johnson & Rogers, 1964*b*; Braun & McCulloch, 1968), (*b*) the ability of saprophytic strains to grow at a temperature of 13 °C and (*c*) the failure of saprophytic strains to be agglutinated by antiserum prepared against representative strains of all serogroups of pathogenic leptospires.

Identification of serogroups and serovars (serotypes) was carried out according to the rules laid down in the Report of the World Health Organization (1967), and confirmed by the members of the Subcommittee on the Taxonomy of Leptospira of the International Committee on Systematic Bacteriology at their meeting in Munich in September 1978.

New isolates were compared by their cross reactions in microscopic agglutination (MAT) and agglutinin-absorption (AAT) tests with serovars representative of the *L. biflexa* species. The selection of standard serovars for this purpose was based on their main serogroup and serovar antigens that had been previously determined

by factor analysis (Cinco, 1977; Cinco & Dougan, 1975; Cinco, Dougan & Stefanelli, 1977; Cinco & Stefanelli, 1977).

The following reference strains were used: Patoc 1, CH 11, CAU, RPE, Percedol 1, Basovizza, Aurisina, Farneti, Botanica, Khoshamian, AM6, Piatan, AR18, Bulgaria 16, Friuli 37, Bulgaria 4, Bulgaria 6, Friuli 44, Friuli 48, AM8, Biflexa CDC, Parapatan, M. Bessemans, Nomentano, Ancona Porto, Dindio, Nazare, Vinzent, Iran 1, Abaete, Garcia, Ondina, Sobradinho, Tororo, 3055, B-24, B-2, B 1062, Sidonia, Poona, Muggia. These 41 strains represent 36 serogroups of *L. biflexa* and the single strain of serovar *illini* of the species *L. illini*.

RESULTS

All the new isolates were shown to survive and multiply in the presence of 8-azaguanine and at 13 °C, as do the saprophytic leptospires.

Preliminary agglutination tests revealed the antigenic affinities of the new strains with the known serovars and according to the agglutination titres they were assigned to known serogroups. The strains were then compared by MAT and by AAT, with all other known serovars within the same serogroup. Table 2 shows the serogroup appurtenance of the newly isolated strains.

The results of AAT (Table 1) according to the 10% limit, whereby 'two strains are considered to belong to different serotypes if after cross-absorption with adequate amounts of heterologous antigen, 10% or more of the homologous titre regularly remains in at least one of the two antisera in repeated tests' (World Health Organization, 1967), indicate that half of the new isolates belong to known serovars. In some cases two or three strains belong to the same serovar, for example strains Compton 521, Khera 9 and Khera 12 (serovar *sangiusto* of serogroup Basovizza), L. tap-water, Wild A and AM 3 (serovar *aurisina* of serogroup Aurisina), Wild B and Wild C (two strains of a new serovar *wild* of serogroup Holland).

The identification of the isolates as known serovars of known serogroups confirms the validity of the taxonomy of *L. biflexa*, while new serovars enrich the list of saprophytic leptospires already known. Their taxonomic status and the names given to them is reported in Table 2. A number of interesting facts came to light during the course of this study.

Firstly the validity of screening a new strain against a battery of serogroup-specific antisera was confirmed. These representative antisera are produced in rabbits by strains chosen because of the capability of each to elicit antibodies that react optimally with all serovars within a serogroup, i.e. they contain adequate amounts of the major antigens specific for the group. This is consistent with the requirements of the first stage of factor analysis described by Dikken & Kmety (1978). By this means it was possible in one step to determine the antigenic affinity of each isolate, in other words to determine its serogroup. Secondly, L. tap-water, which was isolated from a drinking water supply, was shown to be of the same serovar of Aurisina as a strain which was isolated from the same source by Babudieri as long ago as 1942 (Babudieri & Archetti, 1947).

Table 1. *Results of Agglutinin-Absorption Tests*

Immune sera	Absorbed with strain	Agglutination titres* with strains: Compton 433	Agglutination titres* with strains: Khoshamian
Compton 433	Khoshamian	50	0
Khoshamian	Compton 433	0	40
		Compton 393	Khoshamian
Compton 393	Khoshamian	10	0
Khoshamian	Compton 393	0	1
		Compton 636	Canela
Compton 636	Canela	50	0
Canela	Compton 636	0	100
		Compton 636	Jequitaia
Compton 636	Jequitaia	NT	NT
Jequitaia	Compton 636	0	25
		Compton 636	Fons
Compton 636	Fons	NT	NT
Fons	Compton 636	0	50
		Compton 636	Bulgaria 6
Compton 636	Bulgaria 6	20	0
Bulgaria 6	Compton 636	0	100
		Compton 521	San Giusto
Compton 521	San Giusto	1	0
San Giusto	Compton 521	0	1
		Khera 9	San Giusto
Khera 9	San Giusto	1	0
San Giusto	Khera 9	0	1
		Khera 12	San Giusto
Khera 12	San Giusto	1	0
San Giusto	Khera 12	0	1
		L. tap-water	Aurisina
L. tap-water	Aurisina	1	0
Aurisina	L. tap-water	0	1
		Wild A	Aurisina
Wild A	Aurisina	1	0
Aurisina	Wild A	0	1
		AM3	Aurisina
AM3	Aurisina	1	0
Aurisina	AM3	0	1
		Khera 10	CAU
Khera 10	CAU	100	0
CAU	Khera 10	0	25
		RPE	Khera 11
Khera 11	RPE	0	25
RPE	Khera 11	25	0
		Isola Sacra	Khera 11
Khera 11	Isola Sacra	0	50
Isola Sacra	Khera 11	100	0

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Table 1 (*cont.*)

		Doberdo	Khera 11
Khera 11	Doberdo	0	100
Doberdo	Khera 11	100	0
		AM 13	Compton 481
Compton 481	AM13	0	1
AM 13	Compton 481	1	0
		Wild C	Wild B
Wild C	Wild B	1	0
Wild B	Wild C	0	1
		Wild C	Waz Holland
Wild C	Waz Holland	25	0
		Wild C	AM 13
Wild C	AM 13	50	0
		Wild C	AM 20
Wild C	AM20	50	0
		Wild C	AM6
Wild C	AM6	100	0
		Wild C	Lucaia
Wild C	Lucaia	100	0
		Wild C	Piatan
Wild C	Piatan	50	0
		Wild C	V7/2
Wild C	V7/2	100	0
		Compton 421	B1062
Compton 421	B1062	50	0
		Compton 699	Patoc 1
Compton 699	Patoc 1	1	0
Patoc 1	Compton 699	0	50
		Compton 699	Monte Valerio
Compton 699	Monte Valerio	1	0
Monte Valerio	Compton 699	0	100
		Compton 699	Sao Paulo
Compton 699	Sao Paulo	10	0
Sao Paulo	Compton 699	0	20
		Compton 699	Veldrat S 173
Compton 699	Veldrat S 173	1	0
Veldrat S 173	Compton 699	0	1

* Expressed as percentage value of homologous titre before absorption.
NT = Not tested.

We observed some unusual results by absorbing antiserum to Compton 699 with the various serovars of the Semaranga group. Thus after absorption with Patoc 1, Monte Valerio, Veldrat S 173 and Sao Paulo, the titre of the antiserum against the homologous strain, Compton 699, was considerably lower in each case than 10% of the titre before absorption, whereas the titres to the heterologous strains remained, in general, above that limit (Table 3). This may be due to the

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Table 2. *Proposed classification of the new strains*

Strain	Serovar	Serogroup
Compton 433	*compton**	Khoshamian
Compton 393	*khoshamian*	Khoshamian
Compton 636	*cadore**	Pulpudeva
Compton 521	*sangiusto*	Basovizza
Khera 9	*sangiusto*	Basovizza
Khera 12	*sangiusto*	Basovizza
L. tap-water	*aurisina*	Aurisina
Wild A	*aurisina*	Aurisina
AM 3	*aurisina*	Aurisina
Khera 10	*khera**	CAU
Khera 11	*eleven**	Doberdo
Wild C	*wild**	Holland
Wild B	*wild**	Holland
Compton 481	*tredici*	Holland
Compton 699	*semaranga*	Semaranga
Compton 421	*lugo**	Bebrich

* Indicates a new serovar.

Table 3. *Agglutination titres of antiserum to strain Compton* 699 *before and after absorption with strains of the serogroup Semaranga*

	Antiserum to Compton 699				
		Absorbed with			
Strains	Not absorbed	Patoc 1	Monte Valerio	Sao Paulo	Veldrat S 173
Compton 699	51200	800	400	3200	1600
Monte Valerio	51200	6400	200	6400	12800
Patoc 1	12800	200	400	800	200
Sao Paulo	51200	6400	800	200	12800
Veldrat S 173	51200	800	200	12800	200

fact that the immunogenic power of the antigenic factor specific for serovar Compton 699 is less than that of the other factors present in the strain.

Similar results have been observed by us previously and especially when factor analysis was being undertaken. They may also result from the structure and topographical position of the antigenic determinants on the leptospiral cell that allow some of them to react independently in the agglutination test while other determinants react together resulting in higher titres being manifest.

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