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Serological studies on British isolates of the Sejroe serogroup of leptospira

II. An evaluation of the factor analysis method of identifying leptospires using strains belonging to the Sejroe serogroup

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SUMMARY

Twelve British isolates of leptospira belonging to the Sejroe serogroup were examined using a series of six factor sera prepared by a number of different absorption methods. Ten of the isolates were identified as *Leptospira interrogans* serovar *hardjo* and two as *L. interrogans* serovar *saxkoebing*. These isolates had previously been identified using the cross agglutination absorption method.

INTRODUCTION

Improvements in methods for the isolation of leptospira and in particular the introduction of a medium containing bovine albumin and Tweens such as EMJH medium (Johnson & Harris, 1967) and also the use of 5-fluorouracil (Johnson & Rogers, 1964) as a selective agent have made it possible to isolate large numbers of strains of leptospira. For epidemiological purposes accurate identification of strains is essential, particularly when attempting to determine the maintenance or reservoir host of strains which may be affecting man and domestic animals or when introducing control of infection by vaccination.

The classical and only internationally recognized method for identifying leptospira is the cross agglutinin absorption test (CAAT). This time-consuming and costly method is not suitable for the rapid examination of large numbers of isolates. Also it is apparent that there are discrepancies in the identification of strains between laboratories (Kmety, 1974; Torten, 1979) due to the lack of standard procedures for the production of antisera and method of absorption. To overcome some of these problems, Kmety (1966, 1974) proposed an alternative method of serological classification which he called factor analysis.

Kmety (1974) defined factor analysis as a method to distinguish serovars on the basis of the arrangement of their main antigens. He defines a main antigen as an antigen which elicits in rabbits antibodies responsible for the homologous titre and the high co-agglutinations of closely related serovars. Antibodies were considered to reflect the presence of a main antigen if there remained, in the absorbed sera of all the serovars showing this main antigen, residual antibodies of at least five titre steps higher than against the absorbing strain.

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Factor analysis has been carried out on the following serogroups: Australis (Kmety, 1960), Javanica and Celledoni (Kmety 1963), Icterohaemorrhagiae, Canicola, Ballum and Pyrogenes (Kmety, 1967), Grippotyphosa (Kmety & Lataste-Dorelle, 1973) and most recently Hebdomadis (Kmety, 1977) and a summary of the results of this work has been published (Dikken & Kmety, 1978). The Pomona serogroup has been similarly examined by Manev (1976) and the proposed new serogroup, Manhao, has been examined by Qin Jincai *et al.* (1981). Production of specific factor antisera allows the rapid provisional identification of isolates whose identity can be confirmed by a single cross agglutinin absorption test if necessary (Kmety, 1974).

The Hebdomadis serogroup was initially subjected to antigenic analysis by Borg-Petersen (1944) when it consisted of only five serovars. Manev & Yanakieva (1973) in Bulgaria were able to produce four monospecific sera which were used to identify 83 field isolates of the Hebdomadis serogroup as either *sejroe*, *saxkoebing* or *balcanica* and the results were confirmed using the classical cross agglutinin absorption test. Kmety (1977) however, examined all 29 serovars belonging to the Hebdomadis serogroup and proposed, on the basis of the distribution of the main antigens, that the serogroup should be divided into three separate serogroups, Hebdomadis, Sejroe and Mini. He further suggested that the Hebdomadis serogroup should be subdivided into Hebdomadis and Borincana subgroups and the Sejroe serogroup into Sejroe, Saxkoebing and Wolffi subgroups.

The only reported use of factor analysis as described by Kmety has been by Manev (1971) who used the method to identify strains of the Icterohaemorrhagiae and Australis serogroup and Manev *et al.* (1980) who identified a large number of Australis serogroup isolates from wild life.

The recent isolation of large numbers of Sejroe serogroup strains from cattle in Britain (Ellis, O'Brien & Cassells, 1981, Orr & Little, 1977, Hathaway & Little, 1983),and from wild life (Salt & Little, 1977) has resulted in a need for a simpler and more rapid method of identification than the CAAT and the production of specific factor sera appears to be a practical approach.

Using twelve strains of the Sejroe serogroup which have been previously identified using the CAAT (Little, Stevens & Hathaway, 1986) it was decided to investigate the use of factor analysis to identify British isolates.

MATERIALS AND METHODS

Leptospiral strains

The following reference strains were used in this study and were all obtained from the Leptospirosis Reference Laboratory, Colindale, UK: *sejroe* strain M84, *balcanica* strain 1627 Burgas, *polonica* strain 493 Poland, *istrica* strain Bratislava, *saxkoebing* strain Mus 24, *haemolytica* strain Marsh, *ricardi* strain Richardson, *medanensis* strain Hond HC, *wolffi* strain 3705, *hardjo* strain Hardjoprajitno, *recreo* strain LT 957, *trinidad* strain LT 1098, *gorgas* strain LT 829, *roumanica* strain LT 294.

The origin and identification by the CAAT of the British isolates used in this study have been previously described (Little, Stevens & Hathaway, 1986) Strains M204, L43, K1, 12/5, 44/471, S76, P442, S1201 and B215 were *hardjo* strains, D38 and 766V were *saxkoebing* and OW305/4 closely resembled *saxkoebing*.

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Antisera

Antisera was prepared as described by Little, Stevens & Hathaway (1986).

Preparation of factor sera

The following factor sera were prepared using the Bratislava technique described by Dikken & Kmety (1978): Sj-2,3; Sj-21; Sj-22; Sj-10; Sj-14,15; and Sj-13.

Where Dikken & Kmety (1978) describe a number of alternative absorbing antigens for the preparation of each factor sera, each was used to determine which produced the more suitable sera for use in Britain. A number of further modifications were suggested by Kmety (personal communication). The amount of antigen required was adjusted to reduce unwanted cross-reactions to less than 1/400 but not to over-absorb causing a large reduction in the titre to the specific antigen.

Determination of serovar by factor analysis

A systematic approach to factor analysis is essential (Kmety 1977) and the factor sera must be used in a specific order, the method effectively being a dichotomous key.

To identify isolates in the Sejroe serogroup it was first necessary to determine to which subgroup they belong. This was done using factor sera Sj-2,3 and Sj-21. Any isolates belonging to the subgroup Saxkoebing were tested with factor serum Sj-22. Those isolates belonging to subgroup Wolffi were tested with factor sera Sj-10, Sj-14,15 and Sj-13. All reference strains and isolates were tested with the operator unaware of their identity. Standard controls were included in each test.

RESULTS

The results are presented in Tables 1–5. Table 1 shows the results of the different methods of preparing factor serum Sj-2,3. When antiserum to *sejroe* was absorbed with *medanensis* unwanted cross reactions to *saxkoebing* and *hardjo* still occurred at higher titres than described by Kmety (1977). When *wolffi* was used, instead of *medanensis* (Dikken & Kmety 1978), even more unwanted cross reactions occurred. Absorbing antisera to *sejroe* with *medanensis* and *saxkoebing* removed unwanted cross reactions to *saxkoebing* but the resulting factor serum still had a titre to *hardjo* and also a low titre to *balcanica*. The optimum result was when antisera to *sejroe* and *balcanica* were mixed in equal amounts and then absorbed with *saxkoebing*, *medanensis* and Kl, a recent *hardjo* isolate.

Preparation of factor serum Sj-21 using the recommended method of absorbing antiserum to *saxkoebing* with *polonica* gave a sera that showed several unwanted cross reactions (Table 2). Also different batches of this factor serum gave very variable results. Following the recommendation of Kmety (personal communication) *istrica* was substituted for *polonica* and the resulting factor serum Sj-21 proved to be satisfactory. When factor serum Sj-22 was prepared *istrica* was again substituted for *polonica* and used together with *ricardi* to absorb antiserum to *saxkoebing*.

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Table 1. *Agglutination of reference strains of the Hebdomadis (Sejroe) serogroup with factor serum Sj-2,3 prepared by various methods*

Reference strain serovar	Antisera to Sejroe absorbed with: *medanensis*	*wolffi*	*medanensis* and *saxkoebing*	Antisera to *sejroe* and *balcanica*, mixed and absorbed with *medanensis*, *saxkoebing* and K1
sejroe	12800*	12800	1600	6400
balcanica	3200	6400	800	1600
polonica	6400	12800	6400	1600
istrica	3200	6400	3200	3200
saxkoebing	3200	12800	—	—
haemolytica	—†	—	—	—
ricardi	—	400	—	—
medanensis	—	3200	—	—
trinidad	—	6400	—	—
recreo	—	—	—	—
gorgas	—	—	—	—
hardjo	400	800	400	—
wolffi	—	—	—	—
roumanica	—	—	—	—

* Results expressed as reciprocal titres.
† —, less than 1/400.

Table 2. *Agglutination of reference strains of the Hebdomadis (Sejroe) serogroup with factor serum Sj-21 prepared by various methods*

Reference strain serovar	Antisera to *saxkoebing* with *polonica*	*istrica*
sejroe	1600*	—†
balcanica	400	—
polonica	—	—
istrica	400	—
saxkoebing	12800	6400
haemolytica	400	1600
ricardi	800	1600
medanensis	1600	—
trinidad	3200	—
recreo	—	—
gorgas	—	—
hardjo	—	—
wolffi	—	—
roumanica	—	—

* Results expressed as reciprocal titres
† —, less than 1/400.

Factor serum Sj-10, prepared as recommended by Dikken & Kmety (1978), gave satisfactory results except for the failure of *wolffi* to react with this factor serum. When factor serum Sj-14, 15 was prepared only *hardjo* was used to absorb antiserum to *recreo*. Serum prepared in this way showed no unwanted cross reactions and so the inclusion of *sejroe* was not considered necessary.

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Table 3. *Agglutination of reference strains of the Hebdomadis (Sejroe) serogroup with factor serum Sj-13 prepared by various methods*

Reference strain serovar and isolates	Antisera to *hardjo* absorbed with *wolffi*, *polonica* and *gorgas*	Antiserum to K1 absorbed with *wolffi*
sejroe	—†	3200
balcanica	800*	1600
polonica	800	12800
istrica	NT	800
saxkoebing	—	800
haemolytica	NT	—
ricardi	800	400
medanensis	—	800
trinidad	400	800
recreo	400	400
gorgas	—	—
hardjo	12800	3200
wolffi	—	—
roumanica	—	—
L43	800	3200
K1	800	3200
12/5	1600	3200
44/471	800	6400
S76	800	6400
S1201	800	6400

† —, less than 1/400.
* Results expressed as reciprocal titre.
NT, Not tested.

Table 4. *Methods of preparation of factor sera which gave optimal results*

Factor serum	Antiserum to	Absorbed with
Sj-2,3	*sejroe* and *balcanica**	*medanensis*, *saxkoebing*, K1
Sj-21	*saxkoebing*	*istrica*
Sj-22	*saxkoebing*	*istrica*, *ricardi*
Sj-10	*medanensis*	*balcanica*, *recreo*, *gorgas*
Sj-14, 15	*recreo*	*hardjo*
Sj-13	K1	*wolffi*

* Antisera mixed in equal amounts prior to absorption.

When attempts were made to prepare factor serum Sj-13 using the method of Dikken & Kmety (1978) two problems were encountered. Firstly when *wolffi*, *polonica* and *gorgas* were used to absorb antiserum to *hardjo* the titre of the resulting factor serum to *hardjo* was very variable between batches. Even when a potentially useful factor serum was prepared it reacted with lower titres to recent *hardjo* isolates than to the *hardjo* reference strain. (Table 3). To overcome these problems only *wolffi* was used in the absorptions (Kmety, personal communication) and the antiserum to a recent isolate of *hardjo*, K1, was used instead of antiserum to the type strain Hardjoprajitno. Since identification of the strains using factor sera was carried out in a specific order (i.e. as in a dichotomous key) any reactions

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Table 5. *Agglutination of reference strains and isolates of the Hebdomadis (Sejroe) serogroup with factor sera*

Reference strain serovar and isolates	Factor sera						Serovar isolates from factor analysis
	Sj-2,3	Sj-21	Sj-22	Sj-10	Sj-14,15	Sj-13	
sejroe	6400	—					
balcanica	1600	—					
polonica	3200	—					
istrica	3200	—					
saxkoebing	—	6400	3200				
haemolytica	—	1600	—				
ricardi	—	1600	—				
medanensis	—	—		6400			
trinidad	—	—		12800			
recreo	—	—		—	1600		
gorgas	—	—		—	800		
hardjo	—	—		—	—	3200	
wolffi	—	—		—	—	—	
roumanica	—	—		—	—	—	
M204	—	—		—	—	12800	*hardjo*
L43	—	—		—	—	3200	*hardjo*
K1	—	—		—	—	3200	*hardjo*
D38	—	3200	1600				*saxkoebing*
OW305/4	—	3200	3200				*saxkoebing*
766V	—	3200	1600				*saxkoebing*
12/5	—	—		—	—	3200	*hardjo*
44/471	—	—		—	—	6400	*hardjo*
PS76	—	—		—	—	6400	*hardjo*
442	—	—		—	—	3200	*hardjo*
S1201	—	—		—	—	6400	*hardjo*
B215	—	—		—	—	6400	*hardjo*

of factor serum Sj-13 to strains not in the Wolffi subgroup are irrelevant because of the prior use of factor sera Sj-23 and Sj-21 eliminates these serovars. The use of factor sera Sj-10 and Sj-14, 15 before testing with factor serum Sj-13 eliminates *medanensis*, *trinidad* and *recreo* with which this factor serum also reacts to a low titre. The antisera and absorbing strains finally used to prepare the factor sera are in Table 4.

When the isolates were identified using factor analysis the results were in agreement with those obtained by CAAT (Table 5).

DISCUSSION

The antigenic analysis by Kmety (1977) of the Hebdomadis serogroup, the largest of the serogroups, represents an enormous undertaking and the subdivision of this serogroup into three proposed new serogroups based on the arrangements of the main antigens has greatly simplified the task of those engaged in identification. It has led to the possibility of identifying new isolates without recourse to the large number of cross agglutinin absorption tests which would be required if the classical method was employed. This demonstrates the main advantage of

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the method and it is unfortunate that it has not been more widely introduced. There are no published accounts of the use of the method other than those of its originator and of Manev (1971) and of its use in the examination of two new serovars by Dikken *et al.* (1978).

The production of factor sera is not, however, completely straightforward. This study has shown that the antigens which can be used to produce factor sera give variable results and a number of combinations of methods may be needed to produce antiserum of sufficiently high titre. The difficulty, for example, in providing factor serum Sj-13 when the type strain Hardjoprajitno, was used rather than a recently isolated strain (K1) suggests that the large number of subcultures of the type strain may have resulted in the loss of surface antigens on the type strain. Robinson *et al.* (1982) using bacterial restriction-endonuclease DNA analysis also detected differences between isolates of *hardjo* and the reference strain. Manev (1976), largely based on his experience with the Pomona serogroup, has proposed that the tradition of considering the first strain of any serovar to be isolated as the reference strain should be abandoned in favour of making an active choice from a group of such strains. It may well be that when preparing factor sera recent isolates may give better results than reference strains which have been subcultured regularly for many years.

It was also necessary, as pointed out by Kmety (1967), not to over absorb the factor serum which may lead to non-specific absorption of the specific antibody and it was best to aim to reduce the unwanted cross reactions to a titre of around 100.

The number of absorptions required to produce a number of factor sera may appear somewhat daunting but they are usually less than would be required to type a single isolate using the CAAT and use a similar amount of antigen. Also, although a reference laboratory may require a complete set of factor sera, in a specific geographic location with a known range of serovars, a relatively small number of factor sera may suffice to give a tentative identification.

However, it is essential to realise that factor sera are not totally specific to a main antigen and must be used in the step by step analysis as described by Dikken & Kmety (1978). Thus to identify *hardjo* it is essential to first use Sj-2, 3 and Sj-21 to eliminate strains belonging to the Sejroe and Saxkoebing subgroups and then to use factor Sj-10, Sj-14, 15 and Sj-13 within the Wolffi subgroup. Kmety (1977) pointed out that to utilise Sj-13 alone could erroneously identify some Sejroe subgroup strains as *hardjo* as can be seen in Table 3.

The discovery by Borg-Petersen (1971, 1974) that some leptospires have thermolabile antigens which may lead to confusion in the identification of strains means that Kmety's (1974) recommendation that antiserum for factor serum must be prepared with heat-killed organisms must be followed. Although Kmety (1977) could not demonstrate thermolabile antigens in Sejroe serogroup strains it is essential to follow the standard procedure.

Using the cross agglutinin absorption test (CAAT) about 90% of isolates can readily be identified (Kmety, 1974) but problems arise with a number of strains. Using the CAAT, two strains are considered to belong to different serotypes (serovars) if after absorption with adequate amounts of heterologous antigen 10% or more of the homologous titre regularly remain in at least one of the two antisera

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in repeated tests (World Health Organisation, 1967). The problem arises when results are very close to the 10% criterion which has lead in the past to the description of a number of new serovars which have subsequently been shown to be indistinguishable from well recognized serovars. Another problem which has been stressed by Torten (1979) is that some strains may differ from each other by just a one way CAAT and thus are not identifiable by present criteria. These strains may have been designated sub-serotypes in the past (Kmety 1967).

Strain OW305/4 used in this study clearly demonstrates the problem. It was identified as *saxkoebing* by factor analysis because of the presence of main antigens Sj-21 and Sj-22 and the absence of Sj-2,3. In a previous study (Little, Stevens & Hathaway, 1986) this strain could only be identified as probably being *saxkoebing* as 12·5% of the original titre remained in *saxkoebing* antisera after repeated absorption with OW305/4.

The advantages of factor analysis have been stressed by Kmety (1967, 1974). In principle, each serovar is characterized by the arrangement of its main antigens and the difference between serovars becomes qualitative rather than quantitative. Factor analysis reveals real antigenic relationships which are helpful in circumscribing serogroups. It also requires a more thorough study of new isolates and eliminates the taxonomic group of sub-serotypes which either disappear or get the status of separate serovars (Kmety 1967, 1974).

At the practical level the introduction of factor analysis allows the rapid provisional identification of new isolates whose identity can be confirmed where necessary by a single CAAT. In a further study, factor sera will be used to examine a large number of British isolates (from different hosts) belonging to the Sejroe serogroup.

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