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ENZYME-LINKED IMMUNOSORBENT ASSAYS FOR DETECTING ANTIBODY TO *RICKETTSIA AUSTRALIS* IN SERA OF VARIOUS ANIMAL SPECIES

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Abstract—New endemic areas of spotted fever-like rickettsial disease have been found in south-eastern Australia (Gippsland, Victoria and Flinders Island, Tasmania). The rickettsia responsible is currently unknown although it may be *Rickettsia australis*. To investigate serological evidence of rickettsial exposure in various wild animal species, a competitive ELISA was developed which detected antibodies to *R. australis*. It was based on inhibition of an indirect ELISA detecting antibody to *R. australis* in guinea pig sera. Pre- and post-infection sera from 2 dogs, 2 rabbits, 5 mice and 6 rats, experimentally infected with *R. australis*, were tested by competitive ELISA. The results showed that all pre-infection sera were negative and all post-infection sera positive for antibody to *R. australis*. To test the utility of the competitive ELISA for detecting natural rickettsial infection in non-laboratory animals, 51 dog sera, negative for rickettsial antibody by immunofluorescence (IF) and 20 IF positive dog sera (collected from various locations on the east coast of Australia) were tested. Compared to the IF test the competitive ELISA was 90% sensitive and 96% specific. This new test has potential for detecting antibody to *R. australis* in the sera of different wild animal species.

Key words: Rickettsia australis, antibody, competitive ELISA, immunofluorescence, spotted-fever, wild animals.

Résumé—De nouvelles régions endémiques de fièvres de maladies rickettsiennes comparables à une fièvre hémorragique ont été découvertes dans le sud-est de l'Australie (Gippsland, Victoria et l'île Flinders, Tasmanie). La rickettsie responsable est actuellement inconnue bien qu'elle puisse être *Rickettsia australis*. Pour chercher des traces sérologiques d'une exposition rickettsienne dans différentes espèces animales sauvages, un test ELISA par compétition a été mis au point pour détecter les anticorps contre la *R. australis*. Il a été fait pour sur l'inhibition d'une technique ELISA indirecte détectant les anticorps sériques dans les serums de cobaye contre *R. australis*. Les serums avant et après infection de 2 chiens, 2 lapins, 5 souris et 6 rats infectés experimentalement par *R. australis* ont été testés par le test ELISA par compétition. Les résultats ont montré que tous les serums avant infection étaient négatifs et que tous les serums après infection étaient positifs pour les anticorps à *R. australis*. Pour apprécier l'utilité du test ELISA par compétition pour détecter infection rickettsienne naturelle chez les animaux autres que de laboratoires, 51 serums de chiens, négatifs aux anticorps antirickettsiens par immunofluorescence (IF) et 20 serums de chiens positifs aux anticorps antirickettsiens par IF (récoltés dans des différentes zones de la côte est de l'Australie) ont été testés. Comparés au test IF, le test ELISA par compétition était sensible à 90% et spécifique à 96%. Ce nouveau test peut être utilisé pour détecter les anticorps contre *R. australis* dans les serums de différentes espèces animales sauvages.

Mots-clefs: Rickettsia australis, anticorps, test ELISA par compétition, immunofluorescence, fièvre hémorragique, animaux sauvages.

INTRODUCTION

In Australia, the only currently known rickettsial disease of the Spotted Fever Group (SFG) is Queensland Tick Typhus (QTT), caused by *Rickettsia australis*, which occurs in Queensland and NSW, as far south as Sydney [1]. However, in recent years cases of a spotted fever-like disease with serological evidence of rickettsial etiology have been detected in Gippsland (Victoria) and Flinders island (Tasmania), both well south of Sydney. To date, the rickettsia responsible remains to be isolated and identified although it may be *R. australis*. SFG rickettsia are usually tick-transmitted, from one mammalian host to another. To clarify the natural transmission cycle of the disease, it is necessary to investigate serological evidence of rickettsial exposure in various wild animal species that are potential hosts for the tick and rickettsia. A direct method of detecting antibody to *R. australis* in wild animals, using a commercial latex agglutination test for *R. conorii*, was unsuitable in our hands. Due to the unavailability of species-specific enzyme conjugated immunoglobulins, indirect ELISA could not be used to detect antibody to *R. australis* in wild animal sera. Consequently, we have developed a competitive ELISA to detect antibody to *R. australis* in dogs, rabbits, mice and rats based on an indirect ELISA for detecting antibody to *R. australis* in guinea pig sera. This test has potential for investigating rickettsial serology in wild (non-laboratory) animals.

MATERIALS AND METHODS

Rickettsial strain and cell culture

R. australis (strain PHS) obtained from the Queensland State Health Department, was grown in tissue culture, using Buffalo Green Monkey Kidney (BGMK) cells. The medium, Eagle's (modified) Minimum Essential Medium, with 20 mM HEPES, contained 10% foetal calf serum (heat-inactivated) but no antibiotics.

Soluble R. australis antigen for ELISA

Actively growing cultures of *R. australis* were harvested by freezing and thawing. The cell suspension was inactivated at 56°C for 30 min followed by sonication twice for 2 min each in a MSE sonicator at 16 μm. The cell suspension was centrifuged for 30 min at 14,600 ***g*** and the supernatant discarded. The pellet was resuspended in phosphate buffered saline pH 7.4 (PBS), centrifuged at 365 ***g*** for 10 min, the supernatant collected and further centrifuged at 18,800 ***g*** for 30 min. The supernatant was discarded and the pellet resuspended in PBS. Optical density (O.D.) at 600 nm was adjusted to 1.15. This suspension was designated "crude *R. australis* suspension". One volume of crude *R. australis* suspension was mixed with three volumes of lysis buffer (0.15 M NaCl, 12 mM sodium deoxycholate, 15 mM Tris, 0.1% sodium dodecyl sulphate, 0.01% Triton X-100), incubated at 37°C in a water bath for 30 min, sonicated three times, each for 4 min in a SONIFIER 450 (Branson, U.S.A.) at 160 W, followed by centrifugation at 5000 ***g*** for 10 min at 4°C. The supernatant was collected and adjusted to an O.D. of 0.35 at 280 nm. The protein concentration of the supernatant was 200 μg/ml as detected by the Coomassie Blue method [2]. This supernatant was designated "soluble *R. australis* antigen" for use in the ELISA assay.

Animal sera

Serum samples were collected from 46 guinea pigs (English Short Hair), 73 dogs (mixed breed), 2 rabbits (New Zealand white cross), 5 mice (C57 BL/10 Sc Sn), 6 rats (Sprague–Dawley) and 40 humans. Immune sera were prepared by intraperitoneal inoculation of experimental animals with viable *R. australis* (unspecified titre) followed by bleeding 4–8 weeks later. The two control dogs were inoculated 2 and 3 times respectively, but all other animals received one inoculation only.

Latex agglutination test for detecting antibody to R. conorii in the sera of various animal species

The commercial kit, Latex-*R. conorii* (Mediterranean Spotted Fever), was purchased from Integrated Diagnostics Inc, Baltimore, U.S.A. The test was performed according to the manufacturer's instruction.

Microimmunofluorescence test (IF) for detecting antibody to R. australis in guinea pigs, dogs and mice

Guinea pig, dog or mouse serum (10 μl at a 1/64 dilution) was spotted onto *R. australis* antigen which had previously been acetone fixed onto glass slides. After reacting for 30 min at 37°C in a moist atmosphere, the slides were washed 3 times with PBS (pH 7.4), and air dried; then 10 μl of fluorescein labelled goat anti-guinea pig immunoglobulins (Organon Teknika Corporation, Pennsylvania, U.S.A.) or sheep anti-dog IgG (Selenus Laboratory, Australia) or goat anti-mouse IgG (Sigma, U.S.A.) at the appropriate dilution (usually 1/80) was added to each spot, and reacted for 30 min at 37°C. The slides were washed 3 times in PBS, air dried, a fluorescence-enhancing mounting fluid added followed by a coverslide, and examined immediately for immunofluorescence (IF). A positive test showed fluorescing *R. australis* within the cytoplasm of the BGMK tissue-culture cells. Sera positive at 1/64 were subsequently diluted out and retested to determine the antibody titre.

Indirect ELISA for detecting antibody to R. australis in guinea pigs

Optimal concentration of antigen ("soluble *R. australis* antigen"), serum (from immune guinea pigs), and conjugate (peroxidase-labelled rabbit anti-guinea pig serum) were determined by checkerboard titration. The diluent for serum and conjugate was obtained from AMRAD Corporation (Australia). The wells of a 96-well, U-bottom, polystyrene microtiter plates (Nunc, Denmark) were coated with 100 μl of soluble *R. australis* antigen diluted 1:50 in 0.06 M sodium carbonate coating buffer (pH 9.6). After incubation overnight at 4°C, the wells were coated with 100 μl 0.2% casein in coating buffer at room temperature for 60 min. The plates were then washed 3 times in PBS with 0.05%. Tween 20 (PBS-Tween) using a Titertek, Microplate Washer 120 (Flow Laboratories, U.K.). To each duplicate well, 100 μl of 1:200 diluted guinea pig anti-*R. australis* serum was added and incubated for 60 min at 37°C. The plates were washed 5 times and 100 μl peroxidase labelled rabbit anti-guinea pig immunoglobulin (Dako, Denmark), diluted 1:10,000, was added to each well. After 60 min incubation at 37°C, plates were washed four times and 100 μl/well of peroxidase substrate containing 0.01% tetramethylbenzidine (Sigma, U.S.A.) in acetate buffer pH 6.0 was added. After 30 min at room temperature, the reaction was stopped with 100 μl/well of 1 N sulphuric acid. The plates were read against air at dual wavelength of 450 and 650 nm in an Automated Microplate Reader Model EL310 (Bio-Tek, U.S.A.).

A result was considered positive when the O.D. was two standard deviations above the mean of known negative samples.

Competitive ELISA for detecting antibody to R. australis in animal species other than guinea pigs

This test was the same as the indirect ELISA described above except that instead of adding guinea pig anti-*R. australis* serum to each well, a mixture of 50 μl of 1:200 diluted known positive guinea pig anti-*R. australis* serum and 50 μl of undiluted unknown animal serum was added to duplicate wells (a total volume of 100 μl per well). Pooled normal dog serum was used as the negative control serum irrespective of which animal species was being tested. However when testing human sera, pooled normal human serum was used as the control. The result of the competitive ELISA was expressed as a percentage inhibition by using the following equation:

$$\text{Percentage inhibition (\%)} = \frac{\text{O.D. (positive control)} - \text{O.D. (unknown sample)}}{\text{O.D. (positive control)}} \times 100.$$

RESULTS

Detection of antibody to R. conorii by latex agglutination

IF positive serum specimens collected from 5 humans, 3 dogs, 3 mice and 3 guinea pigs were tested with a commercial kit for *R. conorii* antibody. All five human specimens were positive while all 9 of the other animal serum specimens were negative. Accordingly, we proceeded to develop our own competitive assay to detect antibody to *R. australis* in animals.

Development of an indirect ELISA for use in a competitive assay

IF negative serum samples (titre < 1:32) collected from 42 normal guinea pigs and IF positive serum samples (titre > 1:64) collected from 4 *R. australis* infected guinea pigs were tested. The results showed that the O.D. mean $\pm$ SD was 0.06 ± 0.02 for IF negative serum samples and 1.12 ± 0.26 for the 4 IF positive serum samples. Therefore we considered any O.D. greater than 0.10 (i.e. 2 SD above the mean of the negative sera) to be positive. All samples that were negative by IF were also negative by ELISA and all 4 samples that were positive by IF were also positive by ELISA (Table 1).

Detection of antibody to R. australis in experimentally-infected animals by competitive ELISA

Serum samples were collected from two dogs prior to their being infected with *R. australis*. Two months later the dogs were bled again and the paired samples tested using

Table 1. Comparison of indirect ELISA and immunofluorescence (IF) tests for detecting antibody to *Rickettsia australis* in guinea pig sera

		Indirect ELISA	
		+	−
IF	+	4*	0
	−	0	42

*Positive titres defined as > 1:64 (IF) or optical density > 0.10 (ELISA).

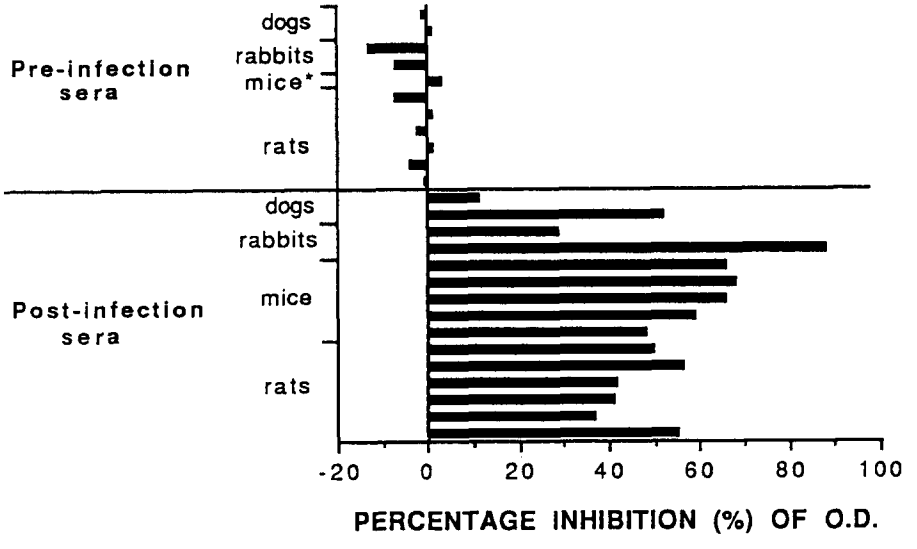

Fig. 1. Comparison by competitive ELISA of normal (pre-infection) and immune sera (post-infection) in animals infected experimentally with *Rickettsia australis*. *Pooled mice sera.

the pre-infection serum as control. The percentage inhibition of the two post-infection dog sera was 11% and 52% (Fig. 1). IF test showed that both pre-infection samples were negative (titre < 1:32), while both post-infection samples were positive (titre 1:128).

Further animal studies involved the testing of sera from 2 rabbits, 5 mice and 6 rats which were experimentally infected with *R. australis*. The pre-infection and post-infection serum samples of the animals were tested using pooled IF negative dog serum as control. The pre-infection sera from 5 mice were pooled. The results showed that all post-infection sera, from all 4 animal species, were positive by competitive ELISA (Fig. 1).

Detection of antibody to R. australis in naturally-infected dogs and humans

We tested 51 IF negative and 20 IF positive dog sera obtained from various locations on the east coast of Australia. Pooled negative dog serum was used as control. The percentage inhibition (mean $\pm$ SD) was $-3.2 \pm 3.4\%$ for IF negative sera (i.e. 3.2% enhancement, rather than inhibition) and $6.3 \pm 3.9\%$ for IF positive sera (i.e. 6.3% inhibition). An inhibition percentage of >3.6% (i.e. 2 S.D. above the mean of the negative sera) was regarded as a definite positive, 2–3.6% as weak positive, and <2% as negative.

Using these criteria, there was good correlation between the competitive ELISA results and IF data for dog sera (Table 2). Compared to the IF test the competitive ELISA was 90% sensitive and 96% specific.

Table 2. Comparison of competitive ELISA and immunofluorescence (IF) tests for detecting antibody to *Rickettsia australis* in dog sera

		Competitive ELISA	
		+	−
IF	+	18	2
	−	2	49

Table 3. Comparison of competitive ELISA and immunofluorescence (IF) tests for detecting antibody to *Rickettsia australis* in human sera

		Competitive ELISA	
		+	−
IF	+	6	14
	−	0	20

Sera from 20 Australian patients with Spotted Fever Group rickettsial disease (i.e. Queensland Tick Typhus or Flinders Island Spotted Fever) and 20 healthy people were tested. Compared to the IF test, the sensitivity of the competitive ELISA was only 30%, even though the specificity was 100% (Table 3).

DISCUSSION

Isolation of rickettsia and detection of antibodies are two important tools in the epidemiological study of rickettsial infection. While isolation of rickettsia is more reliable it is also much more difficult. Several serological methods, such as indirect haemagglutination [3], microimmunofluorescence (IF) [4], latex agglutination [5–7], and ELISA [8–10] have been applied to the diagnosis of human rickettsial diseases and to investigation of rickettsial infection in animals [11].

Latex agglutination is technically simple and, in theory, should be able to detect antibodies across a wide variety of species because the test is direct and, as a result, does not require an anti-species antibody step. Due to the extensive cross-section of antibodies between rickettsia of the spotted fever group, we assessed the applicability of a commercially available latex agglutination assay for *R. conorii* to the detection of *R. australis* antibodies. Although this test detected antibodies in 5/5 human sera positive for *R. australis* by IF, it failed to react with sera from nine experimentally-infected animals. The reason(s) for the species specificity is not clear, but may indicate that the antigen bound to the latex particles is a purified rickettsial antigen which represents the major immunogenic epitopes for humans which is different to those in animals.

To circumvent the difficulties of having non-reactive latex reagents and the lack of various species-specific conjugates for indirect ELISA, we developed a competitive ELISA. The competitive ELISA was based on the competition for limited *R. australis* antigen between known antibody to *R. australis* in guinea pig sera and unknown antibody in the animal sera. We chose an indirect ELISA detecting antibody to *R. australis* in guinea pig serum as detection system because of its sensitivity and specificity compared with the detection of antibody by IF (Table 1). Theoretically, the greater the concentration of antibody to *R. australis* in the animal serum, the higher would be the percentage inhibition of the reaction between guinea pig anti-*R. australis* antibody and *R. australis* antigen.

The competitive ELISA was successful in detecting sero-conversion in four different species of animals experimentally-infected with *R. australis* (Fig. 1). These results correlated with IF in the two infected dogs. Further studies of dogs with this competitive assay involved the testing of 71 randomly collected dog sera to study the incidence of naturally-acquired infections. The competitive ELISA detected antibody in 18/20 IF positive sera and 2/51 IF negative sera, indicating a high sensitivity and specificity of the assay. The use of the competitive ELISA on human sera was less successful than with

animal sera. The assay detected antibody in only 6/20 IF positive sera and in none of 20 IF negative sera. We have no satisfactory explanation as to why the sensitivity of this test was so much lower in human serum than in other animal sera. Possibly it was because of different affinity and avidity of human antibodies compared to those of other animal species.

Among the results of the competitive ELISA several IF-negative sera showed obvious enhancement (inhibition $<0\%$). This phenomenon is probably due to the use of pooled normal dog serum as the control for non-dog species. This was done because in the case of wild animals living in endemic areas it will be impossible to get true negative control sera from these species.

In summary, a competitive ELISA serological test was developed which detected antibody to *R. australis* in the sera of various animal species. It was based on a known guinea pig anti-*R. australis* detection system. By testing negative and positive sera from animals experimentally-infected with *R. australis*, this method was shown to be sensitive and specific for dogs, rats, rabbits and mice. Therefore, this test has potential for detecting antibody to *R. australis* in many different animal species including wild animal species in areas endemic for *R. australis* infection. We are currently using this assay to detect antibodies to *R. australis* in the sera of wild animals from south-eastern Australia.

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