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A COMPARATIVE ASSESSMENT OF FOUR SEROLOGICAL METHODS
USED IN THE DETECTION AND MEASUREMENT OF ANTI-PARASITE
ANTIBODIES IN THE SERUM OF THE AMPHIBIAN, BUFO VIRIDIS

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Abstract—Ingram G. A. and Al-Yaman F. 1988. A comparative assessment of four serological methods used in the detection and measurement of anti-parasite antibodies in the serum of the amphibian, Bufo viridis. International Journal for Parasitology 18: 371-377. Antibodies against Crithidia fasciculata choanomastigotes were detected in green toad (Bufo viridis) sera by direct agglutination, indirect haemagglutination (IHA), complement-fixation test (CFT) and enzyme-linked immunosorbent assay (ELISA). Correlation coefficients (r) were calculated for comparisons between each of the techniques and regression formulae derived in order to convert antibody levels as determined by one immunological method to that of another. The highest mean titre obtained by ELISA was approximately 1.5-3.5 times greater than those obtained by the other techniques whilst CFT gave the lowest values. IHA and ELISA titres were affected by different preparations of the crithidial antigen extracts. Highly significant r values were determined for control sera when IHA was compared to ELISA (r > 0.79), and to both CFT and ELISA with immune animals (r > 0.96). ELISA would seem most applicable for screening other lower vertebrates for anti-parasite antibodies especially in areas of human disease prevalence.

INDEX KEY WORDS: Green toads; Bufo viridis; amphibians; Crithidia fasciculata choanomastigotes; serum antibodies; agglutinins; indirect haemagglutination; complement-fixation test; complement-fixing antibodies; ELISA.

INTRODUCTION
Amphibians are frequently parasitized by various protozoans present in body fluids and tissues and in some cases the parasites cause serious and debilitating diseases (Abrams, 1969; Roudabush & Coantney, 1937). Trypanosomatid flagellates, in particular trypanosomes, have been reported in and isolated from the blood of frogs, toads and newts (Bardsley & Harmsen, 1973; Woo & Bogart 1986).

Throughout the investigations into trypanosomatid infection in humans and mammals, sera have been examined for both anti-parasite antibodies and parasite antigens using various immunodiagnostic techniques (Strickland & Hunter, 1982; Tiru & Hennessen, 1985). However there is a dearth of information pertaining to the use of serodiagnostic methods to detect kinetoplastid infections and resultant antibody production in poikilothermic vertebrates with only reptiles having been studied (Dollahon, Hager & Hua, 1983; Ingram & Molyneux, 1983a, b, 1984a, b, c).

In this paper we present the results of a comparative assessment of four serological tests (direct agglutination, indirect haemagglutination, complement-fixation test and ELISA) used to detect and to determine the levels of antibodies in the sera of green toads (B. viridis), used as experimental models. These amphibians had been injected with the choanomastigotes of C. fasciculata, a trypanosomatid flagellate. The parasites were chosen because of their ease of culture under laboratory conditions and more importantly because of their presence in the blood and alimentary canal of ranid anurans under natural conditions (Smyth & Smyth, 1980). To the authors' knowledge, this is the first report of the use of an immunoenzyme method to detect antibodies in amphibians. Furthermore, there are no previous data concerning anti-parasite antibody detection in amphibian serum.

MATERIALS AND METHODS
Amphibians and parasites. Adult green toads, of mean weight ± standard error of the mean (S.E.M.) 13.5 ± 1.5 g (range 3.7-29.7 g) and mean length 123 ± 4.1 mm (90-148 mm), were obtained from reservoirs in the Hashemia region near Zarka, Jordan. The animals were kept in aquarium tanks at 26-27°C and fed on worms and flies. C. fasciculata was obtained from stock cultures at the Liverpool School of Tropical Medicine. The parasites were maintained on a medium which comprised nutrient agar (28 g l-1 distilled water) to which was added 10% human blood. The overlay consisted of NaCl (137 mm); KCl (2.70 mm); CaCl2 (1.80 mm); glucose (13.9 mm) and
for possible non-specific reactions in the toad sera. The material was then centrifuged to remove cell debris and previously (Ingram & Alexander, 1976) and the immunoglobulin titres of the antisera obtained were estimated by an immunoglobulin/antigen substrate system. Furthermore, the gut contents and PBS extracts of randomly selected insects and worms respectively were also examined microscopically and by culture for the presence of trypanosomatids. The chlamydomastigotes in culture overlay were centrifuged at 250 X g for 5 min. The overlay was removed and the parasites were washed three times in phosphate buffered saline PBS, pH 7.3 (145 mM-NaCl, 7.53 mM-Na2HPO4 and 1.50 mM-NaH2PO4, 2H2O in de-ionized water). The number of parasites was counted and the suspension adjusted with PBS to give the required dose for injection purposes.

Injection and sera. Before injection of the parasites, a small sample of blood was taken by cardiac puncture from each of the toads and inspected for any current infection (with naturally-occurring trypanosomatid flagellates) by smear, wet mount preparation and slope culture as described elsewhere (Ingram & Molyneux, 1983a, b). In addition, blood smears were also examined by an immunoenzyme method reported previously by Ingram and Molyneux (1984b, c) but using a rabbit anti-C. fasciculata serum/swine anti-rabbit immunoglobulin/antiserum/ peroxidase-rabbit antiperoxidase/amino-ethylcarbazole substrate system. Furthermore, the gut contents and PBS extracts of randomly selected insects and worms respectively were also examined microscopically and by culture for the presence of trypanosomatids. The chlamydomastigotes in culture overlay were centrifuged at 250 X g for 5 min. The overlay was removed and the parasites were washed three times in phosphate buffered saline PBS, pH 7.3 (145 mM-NaCl, 7.53 mM-Na2HPO4 and 1.50 mM-NaH2PO4, 2H2O in de-ionized water). The number of parasites was counted and the suspension adjusted with PBS to give the required dose for injection purposes. Toads were given a single intraperitoneal (IP) injection of 16 X 106 chlamydomastigotes in PBS. Control animals consisted of those given PBS IP and normal, un.injected toads. The toads were anaesthetized, bled, killed and their weights and lengths noted. The parasite- and PBS-injected animals were bled at 7-day intervals. The uninjectected controls were sampled at random intervals throughout the duration of the experiment. In order to detect parasite infection, blood was examined in a similar manner to that obtained from previously injected animals and the uninjected controls. The sera were isolated and stored at -20°C.

Specificity. The promastigotes and procyclids of Leishmania hertigi hertigi and Trypanosoma Brucei brucei respectively, both kinetoplastid flagellate species related to C. fasciculata, were used in the antibody assays to examine for possible non-specific reactions in the toad sera.

Antisera preparation. Rabbit anti-toad serum was produced by an immunization schedule as described previously (Ingram & Alexander, 1976) and the immunoglobulin titres of the antisera obtained were estimated by either countercurrent electrophoresis or ELISA (Ingram & Molyneux, 1983a).

Antigen extract. Parasite antigen extracts were produced in two ways for use in the immunological techniques. The chlamydomastigotes were centrifuged at 1700 X g for 10 min in cold PBS, pH 7.3. The pellet formed was resuspended in PBS and then washed and centrifuged a further three times. Prior to use, the cells were subjected to either freezing and thawing (F&T) or sonication (Son) treatments. In the former case the pellet was resuspended in chilled PBS, broken up by mixing and the parasites F&T at 3-min periods for 20 min. The material was then centrifuged to remove cell debris and the supernatant protein in concentration measured by the Lowry method. Alternatively, after addition of cold buffer, the pellet was disrupted by ultrasonication for three 4-min intervals whilst the mixture was kept chilled. The sonicated material was then left overnight at 5°C to further remove any protein. It was then centrifuged at 2900 X g for 15 min and the amount of soluble protein in the supernatant determined.

Agglutination assay. Two-fold serial dilutions of toad sera, inactivated by heating at 48°C for 20 min to destroy naturally-occurring complement activity, were prepared with PBS, pH 7.3 (containing 248 mm-NaCl). To each dilution was added an equal volume of chlamydomastigotes (15 X 106 cells ml-1) and the mixtures incubated at 30°C for 45 min. The direct agglutination (DA) end point titre was regarded as that dilution in which visible agglutination was observed when compared to the PBS-parasite controls. Normal toad sera were examined for the presence of natural haemagglutinins against sheep erythrocytes (ShE) before commencing the IHA test as described by Weir (1978). PBS washed ShE were tanned with 2.3 X 10-5 mM tannic acid and coated with either F&T or Son antigen extract containing 0.9 mg ml-1 protein. Doubling dilutions of inactivated sera were made and to each was added the same volume of 2% tanned and coated ShE. The test samples were incubated at 37°C for 45 min followed by overnight at 4°C. The samples were then examined and the degree of haemagglutination assessed. Untanned, tanned and antigen-coated ShE were used as controls.

Complement fixation test (CFT). The determination of any anticomplementary or haemolytic activities of the antigen and the titrations of the haemolytic serum and complement have been reported previously (Ingram & Alexander, 1977). Prior to examination, all sera were inactivated as above to destroy endogenous complement. The Son extract was used at a protein concentration of 10 mg ml-1, haemolysin at a dilution of 1:400 and ShE at 2%, all in CFT buffer, pH 7.2. A pooled, normal and fresh B. viridis serum (BVS) and commercially available guinea pig complement (GPC) were used as sources of complement at dilutions of 1:45 and 1:30, respectively. Antigen and anti-sera and either BVS or GPC were mixed together and fixation allowed to occur overnight at 5°C. Sensitized ShE were then added and the mixtures incubated at 37°C for 60 min after which the end-points were scored. Following further incubation for 12 h at 5°C, the samples under test were re-examined. The complement-fixing antibody (CFA) titre was taken as that dilution which gave 50% haemolysis.

ELISA. A modification of the method of Chandler, Cox, Healey, MacGregor, Premier & Hurrell (1982) was used. The concentrations of antigen, rabbit anti-toad antisera and enzyme-conjugate used for the ELISA were determined by checkerboard titration. The F&T and Son antigen extracts (containing 1 mg ml-1 protein) were prepared in 0.053 M-carbonate/bicarbonate coating buffer pH 9.6. C. fasciculata was adjusted to 2.5 X 106 cells ml-1 in coating buffer and the three antigen preparations were separately dried onto the plates by overnight incubation at 30°C. After washing with PBS (containing 0.05% Tween 20) pH 7.3, a range of two-fold serial dilutions of control and immune sera were added to the appropriate wells and the plates were reincubated at 37°C for 30 min. They were washed again, rabbit anti-toad antisera (ELISA titre 1:1024) at a dilution of 1:75 was added and the plates were incubated as before. After re-washing, sheep anti-rabbit IgG immunoglobulin conjugated to urease, diluted 1 in 120, was dispensed into the wells and the plates were similarly incubated. After a final three washes with PBS/Tween followed by four washes with distilled water, urease substrate (SeraLab, Crawley, U.K.) was added to the wells. The plates were subjected to a final incubation at 37°C for 30–40 min and the reaction halled by the addition of 1% thiomersal solution (v/v). The end point antibody titre was considered to be that dilution which was visually different from the appropriate reference controls included with each experimental run.
RESULTS

The mean toad serum antibody titres, S.E.M. and ranges in the control and C. fascicularis-injected groups, together with the number of sera-containing detectable antibodies, are given in Table 1. The mean value, range in titres and number of animals in which antibodies were detected were lowest for the CFT and highest by ELISA. Of the 29 control and 38 parasite-injected toads, 93 and 92%, respectively contained antibodies detectable by ELISA. When all four immunological assays were applied to each individual serum, in all cases antibodies were detected by at least two or more of the methods used. However the background 'positive antibody' titres in the control animals ranged from 0 to 2^{-4} depending on the technique employed. Therefore values higher than 2^{-4} were considered to be positive for the parasite-injected toads. Furthermore comparisons between control and immune sera for each method revealed significant differences in antibody titres (P < 0.01; Student's t-test). Moreover, 'antibody' levels were not significantly different between the two control groups (P > 0.05).

The titres against L. hertigi and T. brucei varied from 0 to 2^{-3} and from 0 to 2^{-4} in the control and immune sera, respectively.

The results for each technique were compared in turn with those for the other methods and the half matrix of the Pearson product-moment correlation coefficients (r) calculated for all experimental animals (Table 2). The significance of each r value was tested by the t-test. In the case of the control sera, r values ranging from 49 to 89% (P < 0.01) were calculated whilst overall higher correlations were determined for sera from immune animals which varied from 82 to 99% (all P < 0.001).

In order that the results of the present study can be used by other investigators for comparative purposes with different experimental models, regression formulae to convert the antibody titres as determined for each technique to those of another are given in Table 3. The regression equations, based on the rectilinear relationship \( Y = mX + c \), were only calculated for the highest mean titre found for each of the four immunological methods.

DISCUSSION

The mean antibody titres against C. fascicularis and the number of control and immune sera containing detectable antibodies were the lowest for DA and CFT, intermediate for IHA and highest for the ELISA method (Table 1). These findings may reflect the different sensitivities of the immunological techniques used (Voller & De Savigny, 1981).

The classical agglutination test has often been used for antibody titration in amphibian immunobiological studies (Cooper, 1976). Significant correlations (P < 0.01) were obtained for both control (range 49–76%) and immune sera (83–92%) when the DA titres were compared to the values found for the other methods. Although DA is simple to perform, preconditions of the test include antigen-type specificity, the non-immobilization and non-autogauglulation of parasites and usually the use of living cells. In the current study, loss of cell motility was observed in some instances and the possibility of inclusion of non-viable cells in others cannot be ruled out. Nevertheless caution must be taken in the interpretation of natural 'antibody' levels in view of the detection of 'antibodies' in normal BVS against L. hertigi and T. brucei with titres within the range found for C. fascicularis. Positive results for normal BVS would suggest the presence of low amounts of specific immunoglobulins induced by a current infection with or previous exposure to C. fascicularis parasites. However, the low levels of naturally-occurring 'antibodies' in normal BVS may also have been stimulated by the environmental presence of micro-organisms or other trypanosomatid flagellates which non-specifically cross react with shared cell wall carbohydrate antigenic determinants (Andrews, Reilly, Ferris & Hanson, 1965; Schnaidman, Yoshida, Gorin & Travassos, 1986; Sharabi & Gilboa-Garber, 1980). In the present study, the low levels of 'antibody' in sera from the control groups are not likely to be caused by a current infection because no increase in titres were found throughout the 10-week duration of the experiment.

The lack of detection of C. fascicularis in blood microscopically or by culture coupled with the finding of parasite antigen(s) in blood up to 2 weeks post-injection by the immunoenzyme method suggests that B. viridis possesses an efficient immune system responsible for the rapid elimination of antigen. Therefore it was not possible in the work reported here to correlate the level of parasitaemia and antibody titres. However the exposure of B. viridis to C. fascicularis evoked a specific immune response and resulted in significantly increased levels of serum antibody. Under normal environmental situations, it is feasible that naturally-occurring immunoglobulins in BVS restrict parasite numbers to below a potential infectious threshold. The finding of serum antibody titres above those of normal background levels in amphibians or other animal hosts in similar habitats or areas endemic for certain diseases would indicate current infection with parasites, other infectious agents or pathogens within a population.

No haemolytic anti-complementary activities were detected in normal BVS against the antigen extracts unlike previous reports for amphibians (Gigli & Austen, 1971). Whereas the CFT is frequently non-specific and inconvenient for handling many samples, it can utilize crude, soluble parasite antigen extracts. As with DA cross reactivity can often lead to false positive results. When the results obtained by CFT were compared with each other and with IHA and ELISA, the lowest range of correlations, although significant and similar to those determined for the DA comparisons, were found for control BVS (50–77%;
Table 1—The mean antibody titres ± S.E.M. (ranges) in combined control and anti-C. fasciculata sera together with the number of toad sera containing antibodies

|                     | CFT          | IHA          | ELISA         | Whole cells |
|---------------------|--------------|--------------|---------------|-------------|
|                     | DA*          | GPC          | BVS           | F&T         | Sonicated   | F&T          | Sonicated   | ny          |
| Control             |              |              |               |             |             |              |              |             |
| (No. = 29)†        | 1.53 ± 0.22  | 0.83 ± 0.17  | 1.55 ± 0.21   | 1.86 ± 0.23 | 2.33 ± 0.26 | 2.07 ± 0.23  | 2.38 ± 0.25 | 2.65 ± 0.23 |
| No. Aby.‡          | 23           | 15           | 23            | 23          | 25          | 25           | 25           | 27          |
| Parasite-injected   |              |              |               |             |             |              |              |             |
| (No. = 38)†        | 6.45 ± 0.42  | 4.95 ± 0.46  | 6.50 ± 0.46   | 7.99 ± 0.48 | 7.13 ± 0.49 | 10.11 ± 0.71 | 10.61 ± 0.73 | 11.21 ± 0.76 |
| No. Aby.‡          | 30           | 26           | 32            | 32          | 29          | 33           | 34           | 35          |

*Abbreviations for each method designated as per the text.
†Number of toads in each group.
‡Number of sera containing detectable antibodies (Aby).

Table 2—Correlation coefficients and probabilities† determined for comparisons between the serological methods applied to B. viridis combined control (CC), and immune (IMM) sera obtained from C. fasciculata-injected amphibians

|                   | CFT‡         | IHA          | ELISA         | Whole cells |
|-------------------|--------------|--------------|---------------|-------------|
|                   |              |              |               |             |
|                   | GPC          | BVS          | F&T           | Sonicated   | F&T         | Sonicated   |             |
| DA                |              |              |               |             |             |             |             |
|                   | 0.49         | 0.55         | 0.72          | 0.73        | 0.76        | 0.71        | 0.69        |
|                   | IMM          | 0.83         | 0.86          | 0.92        | 0.85        | 0.87        | 0.89        | 0.90        |
| CFT (GPC)         |              |              |               |             |             |             |             |
|                   | 0.50         | 0.89         | 0.61          | 0.66        | 0.85        | 0.85        | 0.85        | 0.90        |
| CFT (BVS)         |              |              |               |             |             |             |             |
|                   | 0.50         | 0.77         | 0.89          | 0.91        | 0.85        | 0.82        | 0.82        | 0.90        |
| IHA (F&T)         |              |              |               |             |             |             |             |
|                   | 0.87         | 0.94         | 0.80          | 0.79        | 0.95        | 0.96        | 0.96        | 0.99        |
| IHA (Sonicated)   |              |              |               |             |             |             |             |
|                   | 0.84         | 0.82         | 0.84          | 0.82        | 0.90        | 0.89        | 0.89        | 0.92        |
| ELISA (F&T)       |              |              |               |             |             |             |             |
|                   | 0.91         | 0.96         | 0.91          | 0.96        | 0.97        |             |             |
| ELISA (Sonicated) |              |              |               |             |             |             |             |
|                   | 0.94         |              | 0.94          |             |             |             |             |

†Probabilities obtained when the t-test was applied to the r values to determine the levels of significance for the various correlations; *P < 0.01, **P = 0.002. Values without asterisks indicate P < 0.001.
‡Abbreviations as designated in the text.
Table 3—Formulae for the regression of $Y$ on $X$ and $X$ on $Y$ for all pair-wise comparisons to convert one method of antibody determination to another for combined control and immune toad sera

| Method   | DA  | CFT  | HAA  | ELISA |
|----------|-----|------|------|-------|
| Control  | 0.3 + 0.05 (CFT) | 0.92 + 0.48 (DA) | 0.53 + 0.26 (CFT) | 0.63 + 0.03 (ELISA) |
| Immune   | 0.02 + 0.14 (CFT) | 0.80 + 0.72 (DA) | 0.13 + 0.63 (CFT) | 0.78 + 0.08 (ELISA) |

Abbreviations as designated in the text.
P > 0.01) with higher values (82–94%; P < 0.001) for immune BVS. This suggests that DA and CFT show comparable sensitivities in the detection of low levels of antibody against C. fasciculata choanomastigotes or related trypanosomatid flagellates in normal BVS. Amphibians produce predominantly IgM which is an efficient complement fixer and also a good agglutinin (Atwell & Marchalonis, 1976; Yamaguchi, Kurashige & Mitsushashi, 1973). However, the ‘antibodies’ in normal BVS would be present in limited amounts, fix complement less effectively and hence result in low background CFA values. This could also account for the lowest correlations found when the CFA titres were compared to those of the other methods for the controls.

The source of complement seems to be important for the efficient fixation of toad antibodies. The use of toad serum as homologous complement source gave a higher mean value and usually slightly higher individual endpoint titres in both control and immunized animals compared to the antibody levels obtained with heterologous GPC. Homologous serum has proved a reliable source of complement for the fixation of immunoglobulins in other anurans (Alexander & Steiner, 1980; Sekizawa, Fujii & Katagiri, 1984). However, the use of commercially available GPC is also known to initiate good fixation in amphibian species (Lallone, Chambers & Horton, 1984; Romano, Geczy & Steiner, 1973). In the current study, 50% correlation (P < 0.01) and 92% (P < 0.001) were found for control and immune sera, respectively when the different complement sources were compared. Low (50–77%, controls) and high (86–94%, immunized) but significant (P < 0.01) r values were determined in comparisons between IHA and CFT. IHA is prone to lack specificity in some cases and, in contrast to the CFT, usually requires highly purified antigen preparations but is easier to perform.

ELISA gave the highest percentage positive titres in all the samples examined and appears to be the most sensitive of the techniques used to detect anti-crithidial antibodies in toad sera. ELISA was easy to perform, specific, reproducible and accurate in the detection of anti-parasite antibodies especially in normal BVS where some of the other methods proved negative. ELISA has only been used recently in antibody detection in other lower vertebrates (Cossarini-Dunier, 1986; Ingram & Molyneux, 1984a) and has been adapted for use on large numbers of samples in field diagnoses with visual determination of results (Ho, Leeuwenburg, Mbugua, Wamachi & Voller, 1983; Pappas, Hajkowski, Tang & Hockmeyer, 1985). In this laboratory the use of a urease/urease substrate system has given distinct, unequivocal end point titres. Furthermore, urease is not found in amphibian cells and no background enzyme activity was observed when urease-immunoglobulin conjugates were used to detect antibodies in toad serum. This enzyme/substrate system may be worth extending to other lower vertebrate species.

An important factor which affects the values of the antibody titres is the preparation of the antigen extract (Crouch & Raybould, 1983; Pappas, Hajkowski, Cannon & Hockmeyer, 1984). Son antigen preparations gave higher titres than F&T antigen extracts. However, the coating of ELISA plates with whole cells produced the highest values. In the work reported here, similar batches of antigen were used thus reducing potential variations due to different preparations. It is of interest to note that a comparison between IHA and ELISA control titres revealed 79–89% (P < 0.001) significant correlations and similar numbers of positive animals. This implies that the above two techniques have similar sensitivities in the screening of normal BVS for anti-parasite antibodies. Nevertheless the method of antigen preparation may not be a salient criterion for antibody estimation in immune sera because correlation values of over 89% (P < 0.001) were found when IHA titres were compared to those of ELISA.

ELISA seems appropriate for use in serodiagnostic surveillance programmes, applied to different amphibian species or other aquatic and semi-aquatic lower vertebrates, to detect antibodies stimulated against diverse parasite environmental pathogens. Furthermore this technique would be of value in screening lower vertebrates for potential carriers or reservoirs of infective kinetoplastid flagellates or indeed other pathogenic micro-organisms. Such information may reflect the health status of animals within a population and aid in the determination of specific epidemiological and aetiological features of zoonoses and epizootics prevalence.

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REFERENCES

Abrams G. D. 1969. Diseases in an amphibian colony. In: Biology of Amphibian Tumors (Edited by Mizell M.), pp. 419–428. Springer-Verlag, Berlin.

Alexander R. J. & Steiner L. A. 1980. The first component of complement from the bullfrog Rana catesbeiana: functional properties of C1 and isolation of subcomponent C1q. Journal of Immunology 124: 1418–1425.

Andrews R. D., Reilly J. R., Ferris D. H. & Hanson L. E. 1965. Leptospiral agglutinins in sera from southern Illinois Herpetofauna. Bulletin of the Wildlife Diseases Association 1: 55–59.
ATWELL J. L. & MARCHALONIS J. J. 1976. Immunoglobulin class of lower vertebrates distinct from IgM immunoglobulin. In: Comparative Immunology (Edited by MARCHALONIS J. J.), pp. 276–297. Blackwell, Oxford.

BARDSLEY J. E. & HARMSEN J. R. 1973. The trypanosomes of anuila. Advances in Parasitology 11: 1–73.

CHANDLER H. M., COX J. C., HEALEY K., MACGREGOR A., PREMIER R. R. & HURRELL J. G. R. 1982. An investigation of the use of urease–antibody conjugates in enzyme immunoassays. Journal of Immunological Methods 187: 187–194.

COOPER E. L. 1976. Immunity mechanisms. In: Physiology of the Amphibia, Vol. 3 (Edited by LOFTS B.), pp. 163–172. Academic Press, London.

CORNARI-DUNIER M. 1986. Indirect enzyme-linked immunosorbent assay (ELISA) to titrate rainbow trout serum antibodies against two pathogens: Yersinia ruckeri and Eged virus. Aquaculture 49: 197–208.

CROUCH C. F. & RAYBOULD T. J. G. 1983. Comparisons of different antigen preparations as substrates for use in passive haemagglutination and enzyme-linked immunosorbent assays for detection of antibody against bovine enteric coronavirus. Journal of Clinical Microbiology 18: 146–149.

DOLLASON N. R., HAGER, R. & HUA T. C. 1983. Herpetomonas megasaeiae, Critithia fasciculata, and Leptomonas collosoma (Kinetoplastida, Trypanosomatidae) in feaces of lizards fed culture forms of the flagellates. Journal of Protozoology 30: 58–62.

GIGLI I. & AUSTEN K. F. 1971. Phylogeny and function of the complement system. Annual Review of Microbiology 25: 309–332.

Ho M., LEEUWENBURG J., MBUGA G., WAMACHI A. & VOLLER R. 1983. An enzyme-linked immunosorbent assay (ELISA) for field diagnosis of visceral leishmaniasis. American Journal of Tropical Medicine and Hygiene 32: 943–946.

INGRAM G. A. & ALEXANDER J. B. 1976. The immune response of brown trout (Salmo trutta L.) to injection with soluble antigens. Acta Biologica et Medica Germanica 35: 1561–1570.

INGRAM G. A. & ALEXANDER J. B. 1977. The primary immune response of brown trout (Salmo trutta) to injection with cellular antigens. Journal of Fish Biology 10: 63–72.

INGRAM G. A. & MOLYNEUX D. H. 1983a. The primary humoral immune response of European green lizards (Lacerta viridis) to Leishmania agamae. Parasite Immunology 5: 95–108.

INGRAM G. A. & MOLYNEUX D. H. 1983b. The humoral immune response of the spiny-tailed agamid lizard (Agama caudospinosum) to injection with 1 rixinia agamae promastigotes. Veterinary Immunology and Immunopathology 4: 479–491.

INGRAM G. A. & MOLYNEUX D. H. 1984a. A comparison of selected immunological techniques used to detect anti-leishmanial antibodies in the sera of two reptile species. Journal of Immunological Methods 75: 53–64.

INGRAM G. A. & MOLYNEUX D. H. 1984b. Responses of European green lizards LACERTA viridis following administration of Leishmania agamae promastigotes. Veterinary Parasitology 17: 1–15.

INGRAM G. A. & MOLYNEUX D. H. 1984c. Antigen distribution and humoral response in the lizard, Agama caudospinosum, after injection with Leishmania agamae. Developmental and Comparative Immunology 8: 339–349.

LALONE R. L., CHAMBERS M. R. & HORTON J. D. 1984. Changes in antibody and complement production in Xenopus laevis during postmetamorphic development revealed in primary in vivo or in vitro antibody response. Journal of Embryology and Experimental Morphology 84: 191–202.

PAPPAS M. G., HAJKOWSKI R., CANNON L. T. & HOCKMEYER W. T. 1984. Dot enzyme-linked immunosorbent assay (DOT-ELISA): comparison with standard ELISA and complement-fixation assays for the diagnosis of human visceral leishmaniasis. Veterinary Parasitology 14: 239–249.

PAPPAS M. G., HAJKOWSKI R., TANG D. B. & HOCKMEYER W. T. 1985. Reduced false positive reactions in the Dot-enzyme-linked immunosorbent-assay for human visceral leishmaniasis. Clinical Immunology and Immunopathology 34: 392–396.

ROMANO E. L., GEZZY C. I. & STEINER L. A. 1973. Reaction of frog antisera with guinea pig complement. Immunochemistry 10: 655–657.

ROUDABUSH R. L. & COATNEY G. R. 1937. On some blood protozoa of reptiles and amphibians. Transactions of the American Microscopical Society 56: 291–297.

SCHNEIDMA PR. B., YOSHIDA N., GORIN P. A. J. & TRAVASSOS L. R. 1986. Cross-reactive polysaccharides from Trypanosoma cruzi and fungi (especially Dactylium dendroides). Journal of Protozoology 33: 186 191.

Sekiwa A., Fujii T. & Katahiri C. 1984. Isolation and characterization of the third component of complement in the serum of the clawed frog, Xenopus laevis. Journal of Immunology 133: 1436–1443.

SHARABI Y. & GIBBA-GARBER N. 1980. Interactions of Pseudomonas aeruginosa hemagglutinins with Euglena gracilis. Chlamydomonas reinhardtii, and Tetrahymena pyriformis. Journal of Protozoology 27: 80–83.

Smyth J. D. & Smyth M. M. 1980. Frogs as Host-Parasite Systems I. MacMillan Press, London.

Strickland G. T. & Hunter K. W. 1982. Immunoparasitology: Principles and Methods in Malaria and Schistosomiasis Research. Praeger, New York.

Tiru A. & Hennessey W. 1985. Diagnosis and Vaccines For Parasitic Diseases. S. Karger, New York.

VOLLER A. & De SAVIGNY D. 1981. Diagnostic serology of the trypanic parasite diseases. Journal of Immunological Methods 46: 1–29.

WEIR D. M. 1978. Handbook of Experimental Immunology. Vol. 2. Blackwell, Oxford.

Woo P. T. K. & Bega J. P. 1986. Trypanosome infection in salamanders (order: Caudata) from eastern North America with notes on the biology of Trypanosoma agani in Ambystoma maculatum. Canadian Journal of Zoology 63: 121–127.

Yamaguchi N., Kurashige S. & Mitsushashi S. 1973. Immune response in Xenopus laevis and immunochromatographic properties of the serum antibodies. Immunology 24: 109–118.