Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

International Journal for Parasitology Vol. 18, No. 3, pp. 371–377, 1988.
Printed in Great Britain.

0020-7519/88 $3.00 + 0.00
Pergamon Press plc

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*

GEORGE A. INGRAM* and FADWA AL-YAMAN†

*Department of Biological Sciences, University of Salford, Salford, Lancashire M5 4WT, U.K.
†Department of Biological Sciences, Yarmouk University, Irbid, Jordan

(*Received* 4 *June* 1987; *accepted* 10 *September* 1987)

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 & Coatney, 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); $CaCl_2$ (1.80 mM); glucose (13.9 mM) and

KH_2PO_4 (2.20 mM) in de-ionized water. The slope cultures were inoculated with *C. fasciculata* choanomastigotes and incubated at 25°C for 6–7 days after which period they were sampled and the cells enumerated.

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 immunoglobulins 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 choanomastigotes in culture overlay were centrifuged at $250 \times g$ 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-Na_2HPO_4 and 1.50 mM-$NaH_2PO_4 \cdot 2H_2O$ 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 16×10^6 choanomastigotes in PBS. Control animals consisted of those given PBS IP and normal, uninjected 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 uninjected 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 pre-injected animals and the uninjected controls. The sera were isolated and stored at −20°C.

Specificity. The promastigotes and procyclics 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 choanomastigotes were centrifuged at $1700 \times g$ 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 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 $2900 \times g$ for 15 min and the amount of soluble protein in the supernatant determined.

Agglutination assays. 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 choanomastigotes (15×10^5 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 3.3×10^{-5} mg ml^{-1} 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, toad antisera 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 antiserum and enzyme-conjugate used for the ELISA were determined by chequerboard 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 2.5×10^6 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 re-incubated at 37°C for 30 min. They were washed again, rabbit anti-toad antiserum (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 (Sera-Lab, 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 halted 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. fasciculata*-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. fasciculata* 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, pre-conditions of the test include antigen-type specificity, the non-immobilization and non-autoagglutination 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. fasciculata*. 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. fasciculata* 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. fasciculata* 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. fasciculata* 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-*Crithidia fasciculata* sera together with the number of toad sera containing antibodies

	CFT		IHA		ELISA		
	DA*	GPC	BVS	F&T	Sonicated	F&T	Sonicated	Whole cells
Control	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. = 29)†	(0–3)	(0–3)	(0–3)	(0–4)	(0–4)	(0–4)	(0–4)	(0–4)
No. Aby.‡	23	15	23	23	25	25	25	27
Parasite-injected	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. = 38)	(2–12)	(0–11)	(0–12)	(3–14)	(2–12)	(3–18)	(4–14)	(4–20)
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 *Crithidia fasciculata*-injected amibians

	CFT‡		IHA		ELISA		
	GPC	BVS	F&T	Sonicated	F&T	Sonicated	Whole cells
DA	CC *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)		CC *0.50	0.61	0.66	0.60	**0.55	0.56
		IMM 0.92	0.89	0.86	0.85	0.85	0.90
CFT (BVS)			CC *0.50	0.77	0.58	0.68	0.67
			IMM 0.94	0.91	0.90	0.82	0.90
IHA (F&T)				CC 0.87	0.80	0.79	0.80
				IMM 0.94	0.95	0.96	0.99
IHA (Sonicated)					CC 0.84	0.82	0.89
					IMM 0.90	0.89	0.92
ELISA (F&T)						CC 0.91	0.89
						IMM 0.96	0.97
ELISA (Sonicated)							CC 0.94
							IMM 0.97

†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 FROM ONE METHOD OF ANTIBODY DETERMINATION TO ANOTHER FOR COMBINED CONTROL AND IMMUNE TOAD SERA

Control		Immune	
DA = 0.63 + 0.55 (CFT)*	CFT = 0.92 + 0.48 (DA)	DA = 1.80 + 0.72 (CFT)	CFT = −0.20 + 1.04 (DA)
DA = 0.23 + 0.70 (IHA)	IHA = 0.73 + 0.74 (DA)	DA = 0.53 + 0.74 (IHA)	IHA = 0.61 + 1.14 (DA)
DA = 0.02 + 0.64 (ELISA)	ELISA = 1.17 + 0.79 (DA)	DA = 1.52 + 0.47 (ELISA)	ELISA = −0.34 + 1.69 (DA)
CFT = 0.64 ± 0.55 (IHA)	IHA = 0.78 + 0.66 (CFT)	CFT = −0.76 + 0.91 (IHA)	IHA = 1.71 + 0.97 (CFT)
CFT = 0.35 + 0.55 (ELISA)	ELISA = 1.10 + 0.78 (CFT)	CFT = 0.49 + 0.57 (ELISA)	ELISA = 1.35 + 1.42 (CFT)
IHA = 0.13 + 0.72 (ELISA)	ELISA = 0.78 + 0.86 (IHA)	IHA = 1.24 + 0.63 (ELISA)	ELISA = −1.22 + 1.48 (IHA)

*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 & Mitsuhashi, 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 peform, 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.

Acknowledgements—This work was undertaken whilst one of us (GAI) was on an EEC Funded Project contracted to the British Council, in part subcontracted to the University of Salford, for the development of the Faculty of Science at Yarmouk University, Jordan. GAI would like to thank Dr S. K. Abdul-Hafez for the use of his laboratory facilities for part of the work, Dr N. Ishmail for assistance with the bleeding of the animals and Dr Ellen Lee of the University of Salford Computing Centre for advice on the use of the computer for statistical analysis of the results.

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 anura. *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* **53**: 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.

COSSARINI-DUNIER M. 1986. Indirect enzyme-linked immunosorbent assay (ELISA) to titrate rainbow trout serum antibodies against two pathogens: *Yersinia ruckeri* and Egtved 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.

DOLLAHON N. R., HAGER, R. & HUA T. C. 1983. *Herpetomonas megaseliae*, *Crithidia fasciculata*, and *Leptomonas collosoma* (Kinetoplastida, Trypanosomatidae) in feces 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., MBUGUA 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 *Leishmania 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.

LALLONE 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., GECZY C. I. & STEINER L. A. 1973. Reaction of frog antiserum 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.

SCHNAIDMAN B. 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.

SEKIZAWA A., FUJII T. & KATAGIRI 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. & GILBOA-GARBER N. 1980. Interactions of *Pseudomonas aeruginosa* hemagglutinins with *Euglena gracilis*, *Chlamydomonas reinhardi*, 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. & HENNESSEN W. 1985. *Diagnosis and Vaccines For Parasitic Diseases*. S. Karger, New York.

VOLLER A. & DE SAVIGNY D. 1981. Diagnostic serology of the tropical 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. & BOGART J. P. 1986. Trypanosome infection in salamanders (order: Caudata) from eastern North America with notes on the biology of *Trypanosoma ogawai* in *Ambystoma maculatum*. *Canadian Journal of Zoology* **63**: 121–127.

YAMAGUCHI N., KURASHIGE S. & MITSUHASHI S. 1973. Immune response in *Xenopus laevis* and immunochemical properties of the serum antibodies. *Immunology* **24**: 109–118.