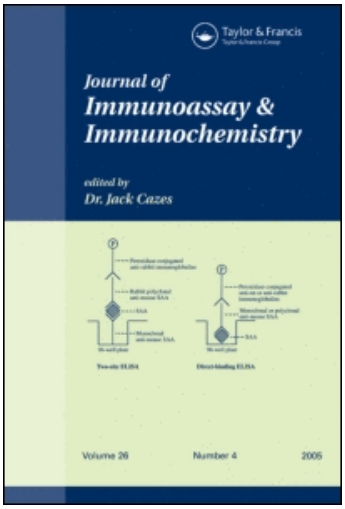

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Quantitation of Antibodies to *Toxoplasma gondii* in Swine Sera by Enzyme-Linked Immunosorbent Assay

Junko Takahashi[a]; Eiji Konishi[a]
[a] Department of Medical Zoology, Kobe University School of Medicine, Kobe, Japan

To cite this Article Takahashi, Junko and Konishi, Eiji(1986) 'Quantitation of Antibodies to *Toxoplasma gondii* in Swine Sera by Enzyme-Linked Immunosorbent Assay', Journal of Immunoassay and Immunochemistry, 7: 4, 257 — 272
To link to this Article: DOI: 10.1080/01971528608060471
URL: http://dx.doi.org/10.1080/01971528608060471

JOURNAL OF IMMUNOASSAY, 7(4), 257-272 (1986)

QUANTITATION OF ANTIBODIES TO TOXOPLASMA GONDII IN SWINE SERA BY ENZYME-LINKED IMMUNOSORBENT ASSAY

Junko Takahashi and Eiji Konishi

Department of Medical Zoology, Kobe University School of Medicine, Kobe 650, Japan

ABSTRACT

An enzyme-linked immunosorbent assay (ELISA) was developed for quantitation of antibodies to Toxoplasma gondii in swine sera. Because a commercial anti-swine IgG conjugate was directed also against swine IgM, the conjugate was absorbed with the IgM fraction to eliminate the interference of naturally occurring IgM antibodies that appeared consistently in sera collected from slaughtered pigs at an abattoir. The ELISA values of 0.2 or more observed in most of the sera successfully decreased to less than 0.2 by the use of absorbed conjugate. An attempt to use a protein A conjugate has failed. Evaluation of this system by comparing it with the latex agglutination test provided a high significant correlation, indicating its usefulness for serodiagnosis of swine toxoplasmosis. (KEY WORDS: Toxoplasma gondii, Enzyme-Linked Immunosorbent Assay, Antibody Quantitation, Swine)

INTRODUCTION

Toxoplasmosis is a worldwide parasitic zoonosis important both for medical and veterinary sciences. Domestic animals used for meat for human consumption mainly become infected by ingestion

 0197-1522/86/0704-0257$3.50/0

of sporulated oocysts eliminated in the cat feces, and they showed 0-86% of antibody prevalence in previous surveys reviewed by Remington and Desmonts (1). The cyst form of Toxoplasma gondii in the edible flesh of these animals was suggested as a source of infection in men who consumed undercooked or raw meat (2, 3). In Japan, swine and cattle are the main animals slaughtered in abattoirs, and Kobayashi (4) reported their seropositivities to be 24% and 8%, respectively. The prevalence of 5-20% demonstrated by isolation of Toxoplasma from muscle of pigs may relate to the 59-78% prevalence among abattoir workers, which is by far higher than 21.7% in the general population with a similar mean age. He also mentioned the great epidemiological importance of swine population for estimation of oocyst contamination in the soil of the survey area.

Enzyme-linked immunosorbent assay (ELISA) is well recognized as superior to the conventional serological tests for detecting Toxoplasma antibodies, such as the dye (DT), the indirect fluorescent antibody (IFA), the indirect hemagglutination (IHA), the latex agglutination (LA), etc. In contrast to cumulative studies of ELISA for serodiagnosis of human toxoplasmosis (1, 5-10), little literature has been published on its use for swine (11-13). Waltman et al (13) described significant correlations between ELISA and other tests including DT, IFA and IHA, but the cutoff values for ELISA titer (1:64) and %ELISA (27%) were relatively high, indicating considerable reactions occurring even in negative sera. The aim of this study is to develop a more accurate ELISA system without such reactions, which will be useful for antibody quantitation in swine sera.

MATERIALS AND METHODS

Sera

A total of 250 sera collected from 6-month-old swine June through October in 1985 at an abattoir in Kobe, Japan, were provided by Dr. S. Anada of the Meat Inspection Office of Kobe City. Four hyperimmune sera were supplied by Dr. S. Ito of the National Institute of Animal Health, who obtained them from 2 pigs infected orally with 1 x 10^6 oocysts of the O-1 strain of Toxoplasma (14) and from 2 pigs primed orally with 1 x 10^6 oocysts of Hammondia hammondi followed by boosting with 2 x 10^2 or 2 x 10^4 oocysts of the O-1 strain. One out of the former 2 hyperimmune sera with an LA titer of 1:256 was used for ELISA as the positive control at a dilution of 1:1,000 and also for analysis on sucrose density gradient. The immunoglobulin (Ig) fraction was obtained by precipitation with ammonium sulfate at 50% saturation.

2-Mercaptoethanol (2-ME) Treatment

To destroy IgM antibodies (15) an 0.4 ml aliquot of the Ig fraction was mixed with an equal volume of 0.2M 2-ME in 0.01M phosphate-buffered saline (PBS). After 1 h incubation at 37°C, the Ig fraction was extracted twice with 5 ml of cold aceton, dried in vacuo and then restored in 0.4 ml of PBS.

Fractionation by Sucrose Density Gradient Centrifugation

The Ig fraction with or without 2-ME treatment was diluted in PBS and an 0.5 ml amount corresponding to 0.1 ml of the original

serum was layered onto 4 ml of a 10-40% (w/w) sucrose gradient prepared in PBS. After centrifugation at 100,000 x g for 18 h, 16 to 17 fractions were collected dropwise from the bottom of the tube, and were diluted 20 times in the ELISA diluent (PBS containing 1% bovine serum albumin, 0.05% Tween 20 and 0.02% NaN_3) for subsequent testings by ELISA.

ELISA for Antibody Quantitation

The magnetic processing ELISA method in our previous report (9) was followed, excepting the enzyme-substrate system. Conjugates used were the peroxidase conjugated IgG fraction of rabbit anti-swine IgG (heavy and light chains specific: Cappel Laboratories Inc., Cochranville, PA) and the protein A-horseradish peroxidase conjugate (E-Y Laboratories Inc., San Mateo, CA). These were diluted 1:2,000 and 1:1,000 respectively, in ELISA diluent without NaN_3. The substrate was 0.4 mg of o-phenylenediamine dihydrochloride per ml and 0.003% H_2O_2 in 0.1 M citrate-phosphate buffer (pH 4.8). The enzyme reaction was stopped by adding 3N H_2SO_4 to the reaction mixture. Absorbance values at 490 nm obtained in duplicate were averaged and adjusted with the value for the constant positive control as 1.0, to minimize interplate variations.

ELISA for Estimation of Ig Levels

The direct method was essentially based on the previous description (16). Solid phase iron beads were sensitized at 4°C overnight with 1:10 to 1:10,000 dilutions of test specimens in 0.1 M sodium

carbonate buffer (pH 9.6). The first reaction with anti-swine IgG conjugate was performed at 37°C for 1 h, followed by the second reaction with the substrate at 37°C for 10 min. Absorbances obtained with 1:10 diluted specimens were recorded for estimation of Ig levels. When the absorbance exceeded 1.5, the reliability limit of the microplate spectrophotometer, the result obtained from one of the remaining dilutions (1:100, 1:1,000 or 1:10,000) was adjusted with the standard dose-dependent absorbance curve in this system.

LA Test

The LA test was performed as described by Lunde and Jacobs (17) and Tsubota et al. (18) with Toxocheck-MT (Eiken Chemical Co., Ltd., Tokyo, Japan).

RESULTS

ELISA Using Cross-reactive Conjugate

Figure 1 shows the frequency distribution of ELISA values for 250 swine sera collected at an abattoir. The values ranged from 0.151 to 0.832 with a peak of 0.2-0.3, indicating the occurrence of antibody reaction in all serum specimens. The confidence limit calculated from the mean (0.260) and the standard deviation (0.102) by a one-tailed *t*-test at the 0.0005 probability level was 0.596, and only one serum with 0.832 was beyond this limit.

Two sera with ELISA values of 0.832 and 0.508 and one hyperimmune serum with a value of 2.513 were next analyzed on sucrose

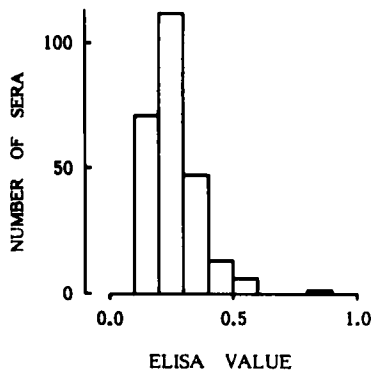

FIGURE 1. Frequency distribution of ELISA values obtained with a commercial conjugate for 250 swine sera collected at an abattoir.

density gradient of their Ig fractions for separation of IgG and IgM classes (Fig. 2A). Antibody reactions to Toxoplasma antigen as well as high Ig levels were distributed in fractions 4 to 7 and 10 to 13. Two peaks detected with one conjugate indicate that the anti-swine IgG conjugate is reactive both to swine IgG and IgM. Fractions of the first peak contained IgM class antibodies that were not observed after 2-ME treatment (Fig. 2B) and therefore those of the second peak resistant for 2-ME included IgG. The IgG antibody level in the fraction 11 depended on the ELISA value of the original whole serum, and one serum with a value of 0.508 showed merely a small reaction. On the other hand, considerable reactions derived from IgM antibodies were found in all three sera. Such IgM antibodies detected on sucrose density gradient were also observed consistently in some other serum samples randomly taken out from the population used in Fig. 1, whether in the presence or absence of IgG antibodies.

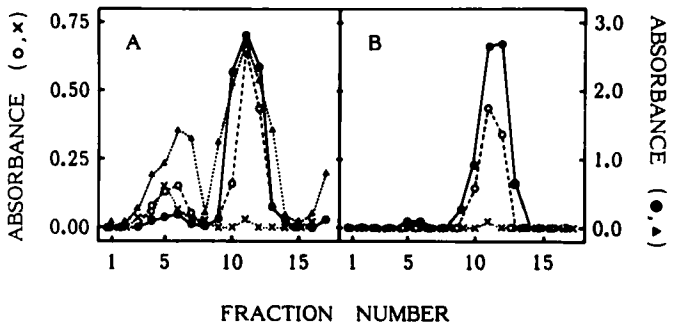

FIGURE 2. Sucrose gradient sedimentation of Ig fractions from three swine sera before (A) and after (B) 2-ME treatment. Antibody levels were determined by ELISA with the commercial conjugate in each fraction obtained from the sera with ELISA values of 2.513 (●), 0.832 (○) and 0.508 (x). As for estimation of Ig levels, a result from one serum with a ELISA value of 2.513 was shown (▲).

Absorption of Cross-reactive Conjugate with IgM Fraction

The IgM fraction was obtained by sucrose density gradient centrifugation from 3 swine sera with ELISA values of less than 0.2. Fractions 5 and 6 of these sera were combined and diluted serially from 1:10 to 1:10,000 in ELISA diluent. An 0.5 ml aliquot of each dilution was mixed with an equal volume of the anti-swine IgG conjugate at a 1:1,000 dilution. After 1 h incubation at 37°C, the mixture was further incubated at 4°C overnight, and then centrifuged at 8,000 x *g* for 1 h. The supernatant was examined for conjugate activity in the *Toxoplasma* antibody-detecting ELISA system where appropriate dilutions of the IgM and IgG fractions obtained from the hyperimmune serum were used as the first antibody (Fig. 3). The IgM fraction mixed with the conjugate had no effect on its reactivity to IgM at dilutions of 1:100 or more, but the reactivity

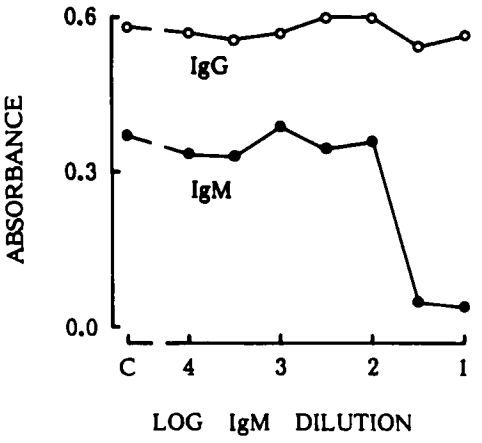

FIGURE 3. Absorption of the cross-reactive conjugate with the swine IgM fraction. Abscissa indicates dilutions of the IgM fraction mixed with the 1:1,000 diluted conjugate. The ELISA diluent was used as the control (C). Ordinate indicates ELISA absorbance values representing conjugate activity of the mixture, which were obtained with the IgG (○) and IgM (●) fractions of the hyperimmune serum as the first antibody.

remarkably decreased at dilutions of 1:10 and 1:30. The reaction to IgG was constant at any concentration of IgM used here. The 1:30 dilution of the IgM fraction was therefore chosen for absorption in subsequent experiments.

ELISA Using Absorbed Conjugate

The peak responsible for IgM antibodies was not observed on sucrose density gradient when the absorbed conjugate was used (Fig. 4A). But patterns for IgG antibodies in three sera were similar to those in Fig. 2. Figure 4B shows ELISA reaction of the protein

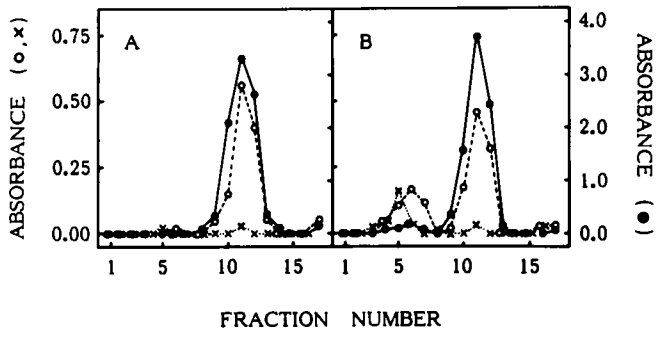

FIGURE 4. Sucrose gradient sedimentation of Ig fractions from three swine sera with ELISA values of 2.513 (●), 0.832 (○) and 0.508 (×). Antibody levels were determined by ELISA with the absorbed conjugate (A) and the protein A conjugate (B).

A conjugate, which in contrast to the absorbed conjugate, contributed to the detection not only of IgG but also IgM antibodies. This phenomenon was also observed in another lot of the protein A conjugate.

Frequency distribution of ELISA values obtained with the absorbed conjugate for 250 swine sera (Fig. 5) revealed a binominal pattern with a peak at 0.0-0.1, and most samples (93.6%) had values of less than 0.2. The mean ELISA value was 0.0820 with a standard deviation of 0.0818. The confidence limit obtained by the same manner as described above was 0.351, and two sera with 0.824 and 0.472 were beyond this limit.

A direct comparison of ELISA systems with the absorbed and unabsorbed conjugates was performed using the 250 samples. Because a large number of sera were distributed in ELISA values below 0.2

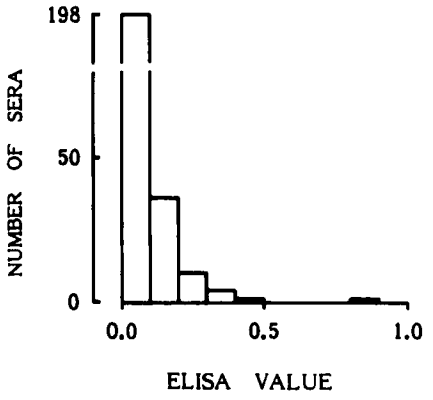

FIGURE 5. Frequency distribution of ELISA values obtained with the absorbed conjugate for 250 swine sera collected at an abattoir.

as measured with the absorbed conjugate, only 35 samples were dotted in Fig. 6 as for this range. Most of the sera showing antibody levels of 0.15-0.42 decreased to 0.0-0.1 by absorption of the conjugate, while a sample with a high level (0.832) had an almost equal result (0.824) in the absorption system. All sera showed smaller results in ELISA with absorption than without absorption, and a significant correlation ($r = 0.806$; $P < 0.001$) was observed between both systems.

The ELISA system was further evaluated by comparing it with the LA test, using 46 serum samples. Because the sera showing high absorbances was small in number, 4 hyperimmune sera and their dilutions were also used in this evaluation. As shown in Fig. 7, sera collected at an abattoir provided no correlation between two methods ($r = 0.198$; $P > 0.05$). While a sample with a high ELISA value of 0.824 had an LA titer of 1:32, 3 sera with 1:64 in LA were

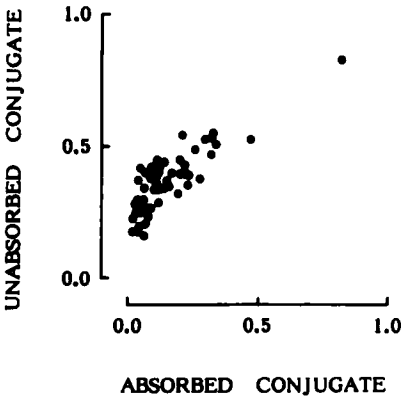

FIGURE 6. Direct comparison of ELISA values obtained with the absorbed and unabsorbed conjugates in 51 swine sera collected at an abattoir. Twenty and 15 sera were randomly sampled out of 198 sera with ELISA values below 0.1 and 36 sera between 0.1 and 0.2, respectively, as determined by ELISA with the absorbed conjugate.

less than 0.3 in ELISA. However, a significantly high correlation ($r = 0.966$; $P < 0.001$) was observed in hyperimmune sera with a regression line of $Y = 0.462X - 0.267$.

DISCUSSION

Antibody reactions to Toxoplasma observed in all swine sera (Fig. 1) turned out to be attributed to the anti-swine IgG conjugate directed also against IgM class antibodies that appeared consistently in the present serum population (Fig. 2). Absorption of the conjugate with an appropriate dilution of the swine IgM fraction (Fig. 3) successfully eliminated anti-IgM activity from the conjugate without any change in anti-IgG activity (Fig. 4A). The use of absorbed conjugate

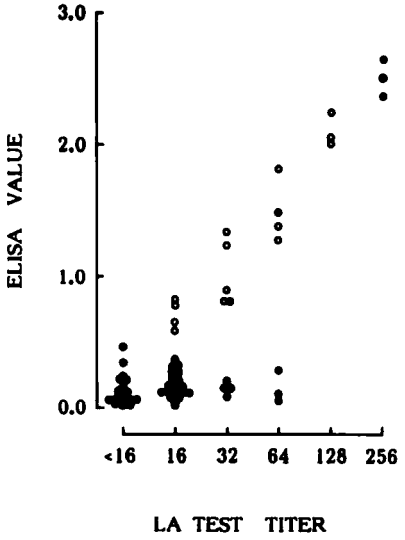

FIGURE 7. Comparison of the ELISA using absorption system and the LA test in 46 swine sera from an abattoir and 4 hyperimmune sera (●), as well as dilutions of these hyperimmune sera (○). Fifteen abattoir samples were randomly selected out of 198 sera with ELISA values below 0.1, and 36 sera between 0.1 and 0.2, respectively.

showed a binominal distribution pattern with 93.6% of the serum population below 0.2 in ELISA value (Fig. 5), owing to a marked decrease of antibody levels in most of the sera (Fig. 6). Because anti-swine IgG (γ-chain specific) sera, as far as we know, are not available in markets, direct use of the commercial product for sero-diagnosis in swine inevitably increases ELISA results and brings about subsequent overestimation of the cutoff value, as seen in the ELISA system reported by Waltman et al (13). Fause-negative results may occur under these conditions. Supposing a cutoff value is determined by the confidence limit at the 0.0005 probability level (19), two sera were found positive in ELISA with the absorbed conjugate (Fig.

5), in contrast to ELISA with the unabsorbed conjugate differentiating only one positive case (Fig. 1).

It is not likely that such IgM antibodies resulted from a general immune response to the infection with Toxoplasma. There is almost no possibility that most of 250 sera collected over five months from slaughtered young pigs of different breeding sites were infected in the same stage, where only IgM antibodies were produced but still not IgG antibodies. It is also confirmed by the present state of the prevalence among swine in Kobe, which was reported as 0.0% (0/29,039 heads in 1984) by visual inspection (20), according to the improved sanitary environments for animal breeding. The IgM antibodies are therefore considered as naturally occurring IgM antibodies and are now under study from various angles.

To develop the ELISA system specific for IgG antibodies, production of anti-swine γ-chain serum would be the best method, although good results were also obtained by the absorption of the commercial conjugate with the IgM fraction, which is a more simple way to get a specific conjugate in any laboratory attached especially to abattoirs. An attempt to use the protein A conjugate that was expected to bind only to the swine IgG (21) was unsuccessful, resulting in its cross-reactivity to IgG and IgM antibodies on sucrose density gradient (Fig. 4B). This contradiction may be possibly explained by the difference in detection system.

Waltman et al (13) described positive correlations between ELISA and other serological tests such as DT, IFA and IHA. We evaluated the ELISA system by comparing it with the LA test, the most common

method in Japan (Fig. 7). No correlation and some inconsistencies were observed in swine sera from an abattoir, probably due to the LA system in which all agglutinative antibodies including natural IgM antibodies are detected. However, a significantly high correlation was obtained in dilutions of hyperimmune sera where the effect of IgM antibodies was negligible on LA. These results indicates that the ELISA system with the absorbed conjugate will be useful for quantitation of Toxoplasma antibodies in swine sera.

ACKNOWLEDGMENTS

We are grateful to Drs. S. Anada and S. Ito for their kindness in providing swine sera. Request for reprints should be made to E. Konishi, Department of Medical Zoology, Kobe University School of Medicine, Kobe 650, Japan.

REFERENCES

1. Remington, J. S. and Desmonts, G. Toxoplasmosis. In: Remington, J. S. and Klein, J. O., eds. Infectious Diseases of the Fetus and Newborn Infant, (2nd ed.). Philadelphia: W B Saunders, 1982: 143-263.

2. Desmonts, G., Couvreur, J., Alison, F., Baudelot, J., Gerbeaux, J. and Lelong, M. Étude épidémiologique sur la toxolasmose: de l'influence de la cuisson des viandes de boucherie sur la fréquence de l'infection humaine. Rev. Fr. Étud. Clin. Biol. 1965; 10: 952-58.

3. Schantz, P. M., Juranek, D. D. and Schultz, M. G. Trichinosis in the United States, 1975: increase in cases attributed to numerous common-source outbreaks. J. Infect. Dis. 1977; 136: 712-15.

4. Kobayashi, A. Studies on Toxoplasmosis. Tokyo Jikeikai Med. J. 1977; 92: 614-33 (In Japanese).

5. Voller, A., Bidwell, D. E., Bartlett, A., Fleck, D. G., Perkins, M. and Oladehin, B. A microplate enzyme-immunoassay for toxoplasma antibody. J. Clin. Pathol. 1976; 29: 150-53.

6. Walls, K. W., Bullock, S. L. and English, D. K. Use of the enzyme-linked immunosorbent assay (ELISA) and its microadaptation for the serodiagnosis of toxoplasmosis. J. Clin. Microbiol. 1977; 5: 273-77.

7. Carlier, Y., Bout, D., Dessaint, J. P., Capron, A., van Knapen, F., Ruitenberg, E. J., Bergquist, R. and Huldt, G. Evaluation of the enzyme-linked immunosorbent assay (ELISA) and other serological tests for the diagnosis of toxoplasmosis. Bull. W.H.O. 1980; 58: 99-105.

8. Lin, T. M., Halbert, S. P. and O'Connor, G. R. Standardized quantitative enzyme-linked immunoassay for antibodies to Toxoplasma gondii. J. Clin. Microbiol. 1980; 11: 675-81.

9. Konishi, E. and Takahashi, J. Reproducible enzyme-linked immunosorbent assay with a magnetic processing system for diagnosis of toxoplasmosis. J. Clin. Microbiol. 1983; 17: 225-31.

10. Franco, E. L., Walls, K. W., Sulzer, A. J. and Soto, J. C. Diagnosis of acute acquired toxoplasmosis with the enzyme—labelled antigen reversed immunoassay for immunoglobulin M antibodies. J. Immunoassay. 1983; 4: 373-93.

11. Yen, C. C. C., Yeh, J. M. and Chang, C. N. Evaluation of enzyme linked immunosorbent assay (ELISA) in the serodiagnosis of toxoplasmosis in swine. J. Chin. Soc. Vet. Sci. 1982; 7: 35-41.

12. van Knapen, F., Franchimont, J. H. and van der Lugt, G. Prevalence of antibodies to toxoplasma in farm animals in the Netherlands and its implication for meat inspection. Vet. Q. 1982; 4: 101-5.

13. Waltman, W. D., Dreesen, D. W., Prickett, M. D., Blue, J. L. and Oliver, D. G. Enzyme-linked immunosorbent assay for the detection of toxoplasmosis in swine: Interpreting assay results and comparing with other serologic tests. Am. J. Vet. Res. 1984; 45: 1719-25.

14. Ito, S., Tsunoda, K., Nishikawa, H. and Matsui, T. Pathogenicity for piglets of toxoplasma oocysts originated from naturally infected cat. Natl. Inst. Anim. Hlth. Q. 1974; 14: 182-87.

15. Caul, E. O., Smyth, G. W. and clarke, S. K. R. A simplified method for the detection of rebella-specific IgM employing sucrose density fractionation and 2-mercaptoethanol. J. Hyg. 1974; 73: 329-40.

16. Konishi, E. and Takahashi, J. Detection of chikungunya virus antigen in Aedes albopictus mosquitoes by enzyme-linked immunosorbent assay. J. Virol. Meth. 1985; 12: in press.

17. Lunde, M. N. and Jacobs, L. Evaluation of a latex agglutination test for toxoplasmosis. J. Parasitol. 1967; 53: 933-36.

18. Tsubota, N., Hiraoka, K., Sawada, T., Watanabe, T. and Ohshima, S. Studies on latex agglutination test for toxoplasmosis. II. Evaluation of the microtiter test as a serologic test for toxoplasmosis in man. Jpn. J. Parasitol. 1977; 26: 286-90.

19. Heck, F. C., Williams, J. D. and Pruett, J. Interpretation of spectrophotometric absorbance values to define results of enzyme-linked immunosorbent assays. J. Clin. Microbiol. 1980; 11: 398-401.

20. The Meat Inspection Office of Kobe City. Annual report from the Meat Inspection Office of Kobe City in 1984. Kobe, 1985.

21. Milon, A., Houdayer, M. and Metzger, J. J. Interactions of porcine IgG and porcine lymphocytes with protein-A sepharose. Dev. Comp. Immunol. 1978; 2: 699-711.