

THE USE OF ENZYME-LINKED IMMUNOSORBENT
ASSAYS TO DETECT ANTIBODIES OF
THE COLORADO TICK FEVER VIRUS

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Charleen Ann Harmon

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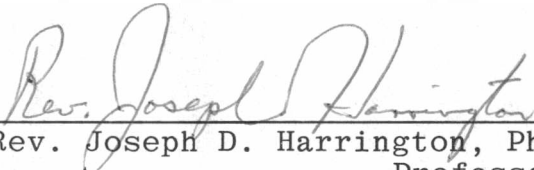


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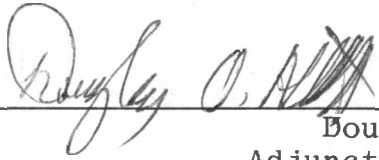
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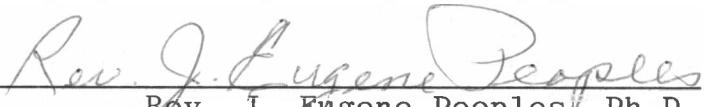
This thesis for honors recognition has been
approved for the Department of Biology by:



Rev. Joseph D. Harrington, Ph.D., Advisor
Professor of Biology



Douglas O. Abbott, Ph.D.
Adjunct Professor in Biology
Bureau Chief
Microbiology Laboratory Bureau
Montana Department of Health and
Environmental Sciences



Rev. J. Eugene Peoples, Ph.D.
Associate Professor, Theology

TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	iii
ABSTRACT.....	iv
LIST OF TABLES.....	v
LIST OF FIGURES.....	vi
INTRODUCTION.....	1
LITERATURE REVIEW.....	5
MATERIALS AND METHODS.....	16
RESULTS.....	25
DISCUSSION.....	30
LITERATURE CITED.....	36

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Last, and certainly not least, my parents deserve a multitude of thanks and love in return for the constant support and encouragement they've given to me.

ABSTRACT

An enzyme-linked immunosorbent assay was attempted to determine antibody titers of serum from potential Colorado tick fever victims. An exemplary assay of influenza A monoclonal antibodies worked very well. After turning to the elucidation of optimal reagent purifications and titrations, a high degree of non-specificity was found. The numerous non-specific reactions which were observed necessitate the need for further development of the technique by the State of Montana Health and Environmental Sciences virology department.

LIST OF TABLES

Table	Page
1. Results of conjugate titration using Influenza A antigen and monoclonal antibody.....	26
2. Results of antigen titration using Colorado tick fever virus antigen and reference serum pool.....	27
3. Results of conjugate titration using Colorado tick fever virus antigen and reference serum pool.....	29

LIST OF FIGURES

Figure	Page
1. Homogenous enzyme immunoassay: Antibody-induced reactivation of enzyme.....	6
2. Homogenous enzyme immunoassay: Antibody-induced inhibition of enzyme.....	7
3. Heterogenous enzyme immunoassay: Indirect method for assay of antibody.....	9

INTRODUCTION

Colorado tick fever is a tick-borne disease caused by an arbovirus. The virus is a member of the family Reoviridae and of the Orbivirus genus (6,22,28).

The virus is composed of double-stranded RNA segments which are synthesized and mature in the cytoplasm of infected cells of the vector. The major vector is the wood tick, Dermacentor andersoni. According to a study of the ecology of the virus carried out in Rocky Mountain National Park, two of the major animal hosts are Eutamias minimus, the least chipmunk, and Spermophilus lateralis, the golden-mantled ground squirrel (6). These viremic rodents serve as the source of infection for infected larval and nymphal ticks. Hibernating ticks remain infective until the following spring and reinitiate the transmission cycle. Colorado tick fever virus has the ability to invade erythrocytes, thereby protecting the virus from antibody action (15,29). The affected geographical region, the mountainous regions of western United States and Canada, corresponds with the living habitats of the vector and hosts (6). Recent findings suggest that a virus identical or similar to the Colorado tick fever virus is prevalent in northwestern-westcentral California (23).

Infected persons are usually outdoors people who frequent areas populated by rodents, which are infected by immature and adult stages of the wood tick. However, disease by blood transfusion has occurred; the first time CTF was known to have been transmitted via a transfusion was at a Red Cross Blood Center in Montana.

The infection caused by the virus is characterized by fever, chills, headache, muscle aches, myalgia, lethargy, and leukopenia (1,27,28). A rash may be present which may make it difficult to distinguish from Rocky Mountain spotted fever. Recovery is usually complete and lifelong immunity is then enjoyed.

Hemorrhagic or encephalitic conditions can occur in children, but this occurs rarely. Teratogenic effects have been produced in mice after Colorado tick fever virus was inoculated in the second week of pregnancy. These effects included increased incidence of still births and neonatal deaths (36).

In Montana, between five and twenty cases of Colorado tick fever are reported every year. Over one hundred cases of Colorado tick fever are reported annually in Colorado and California. Still, California epidemiologists claim Colorado tick fever is the most underreported infectious disease in the state. Seldom reported diseases, such as Colorado tick fever, or those reported only optionally by physicians to the health authorities, should not be dismissed lightly. Lack of medical awareness and alert-

ness result in misdiagnosis, needless suffering, and fatalities that are easily avoided if correct treatment is prescribed early (15).

Present serologic diagnosis of Colorado tick fever at the Montana Department of Health and Environmental Sciences is accomplished by fluorescent antibody techniques which determine antibody titers of acute and convalescent sera. An increased titer greater than fourfold is evidence of infection. Complement fixation tests are also used to determine titers. Disadvantages of complement fixation include development of anti-complementary activity, subjectivity of interpretation of results, and the brief stability of reagents.

It was believed by the Montana State Laboratory that enzyme-linked immunosorbent assay techniques could be employed to diagnose Colorado tick fever with more simplicity, more objectivity, sensitivity, cost-effectiveness, and speed. The proper technique, once determined, can be highly automated and can be used efficiently to screen large numbers of serum samples.

The basic problem underlying implementation of the technique was the determination of proper procedures to obtain reagents and determination of usable dilutions of reagents. Since the disease affects a relatively small region of the country and is rarely fatal, commercial procedures and reagents have not been adapted or processed for laboratory use. Therefore, it was necessary to attempt

to adapt to our own needs, methods which have proven successful in diagnosing similar agents.

LITERATURE REVIEW

Various techniques of enzyme immunoassay are presently used to detect substances in body fluids. Basically, there are two divisions of enzyme immunoassays: homogenous assays and heterogenous assays.

Homogenous assays involve conjugation of enzyme to hapten. Once the conjugate reacts with antibody, the enzyme's activity is altered; either the enzyme is induced (Fig 1, p. 6) or inactivated by antibody reaction (Fig 2, p. 7) (34). With either procedure the enzyme activity is directly proportional to the concentration of free antigen (usually a hapten) in the sample. Homogenous assays do not require separation of bound and free reagent; but, unfortunately, the assays are restricted to low-molecular weight substances, i.e., drugs (49).

The second division, heterogenous assays, is synonymous with enzyme-linked immunosorbent assay, the name given to solid-phase systems employing separation of free and bound labelled molecules (34). Antigens are adsorbed onto a suitable surface, i.e. polystyrene well, while retaining immunological specificity. Incubation of test serum in the well allows antibody to react with available antigen. Binding of enzyme-labelled immunoglobulins,

Homogenous Enzyme Immunoassay

Antibody-induced reactivation of enzyme

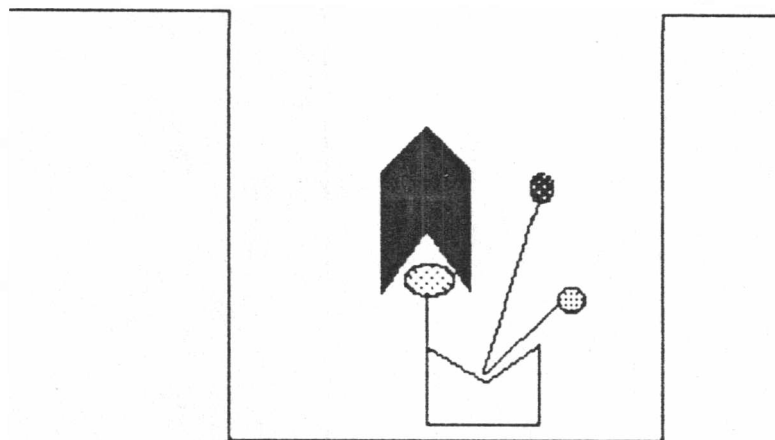
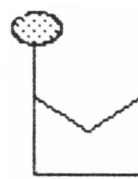


Figure 1a. Antibody present in sample.
Hapten binds to antibody, enzyme is reactivated.



Conjugate



Antibody



Product



Substrate

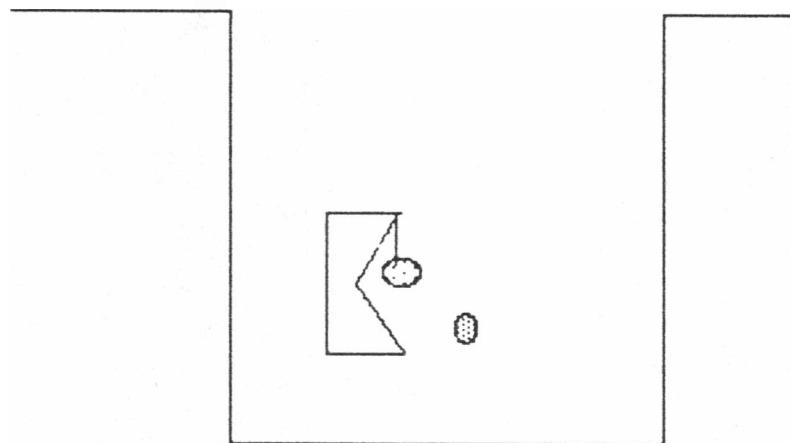


Figure 1b. No antibody present in sample.
Hapten inhibits enzyme's ability to bind substrate.
No product upon addition of substrate.

Homogenous Enzyme Immunoassay

Antibody-induced inhibition of enzyme

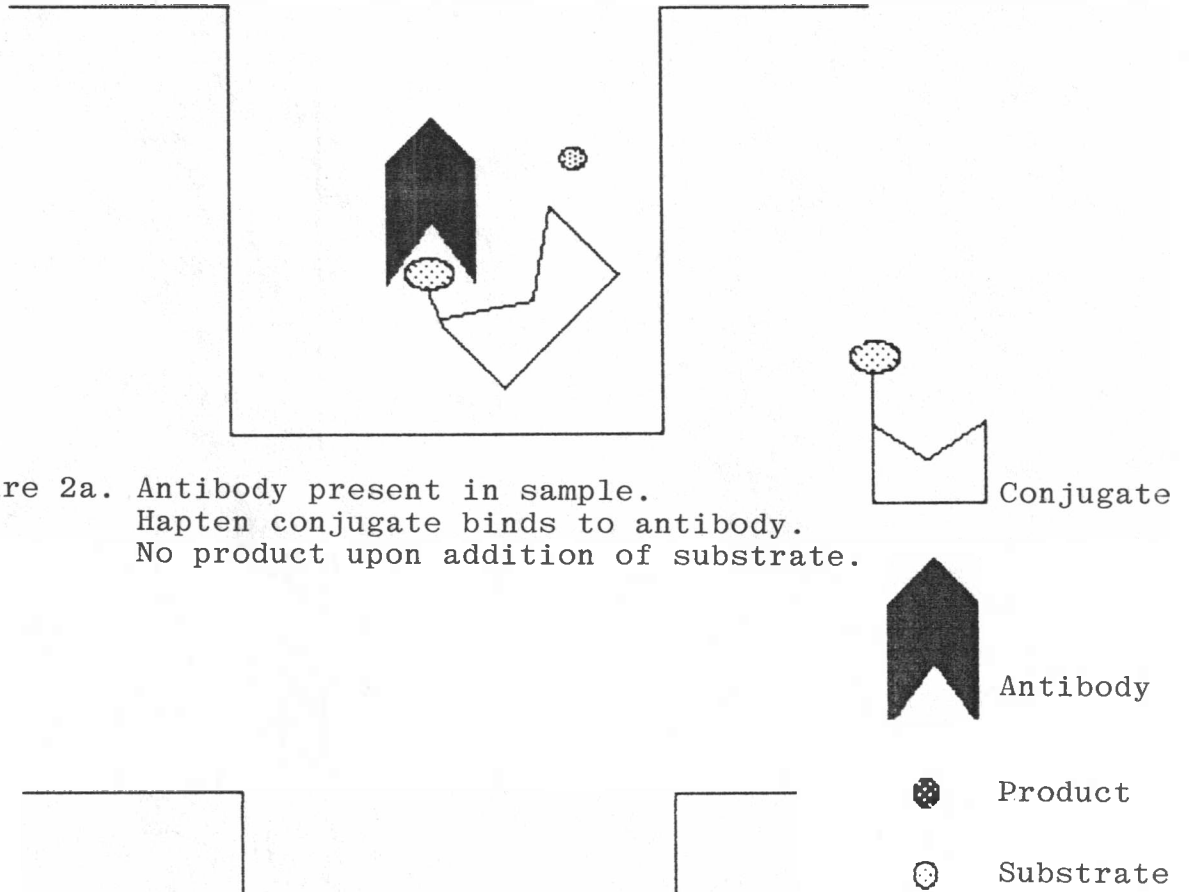


Figure 2a. Antibody present in sample.
Haptene conjugate binds to antibody.
No product upon addition of substrate.

Figure 2b. No antibody present in sample.
Addition of substrate results in product.

followed by chromogenic substrate incubation, provides for detection of antibody through spectrophotometric methods (Fig 3, pp. 9-10). The following procedures refer to various techniques used in the implementation of heterogeneous assays.

An essential step of the process is the binding of antigen to the solid phase without loss of immunological activity. Once bound to the solid phase support, bound and free reagents can be easily separated. The wells of polystyrene microplates can be sensitized by passive adsorption with antigens through incubation at 37C for three hours. For convenience, overnight coating of 4C is often used with satisfactory results (49).

Recent studies have shown notable variations in reagent binding due to plates composed of different plastics, different manufacturer's plastics, and different lots of plates of the same manufacturer, all of which would account for variation between simultaneously tested plates. Within a single plate, uneven attachment of antigen is demonstrated by well-to-well variation and the edge effect. The edge effect is exhibited by a more pronounced well-to-well variation in outer wells than in overall plate variation. Such uneven binding decreases reproducibility and reliability of the tests. Edge effect can be minimized by incubating closed plates in a moist chamber at 37C. Another possible solution is the incubation of water in all outer wells to assure even temperature distribution.

Heterogenous Enzyme Immunoassay
Indirect method for assay of antibody

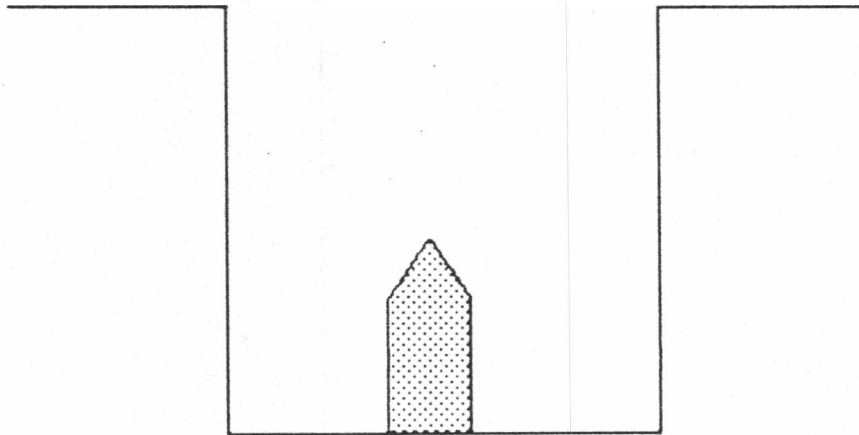


Figure 3a. Antigen adsorbed to plate.

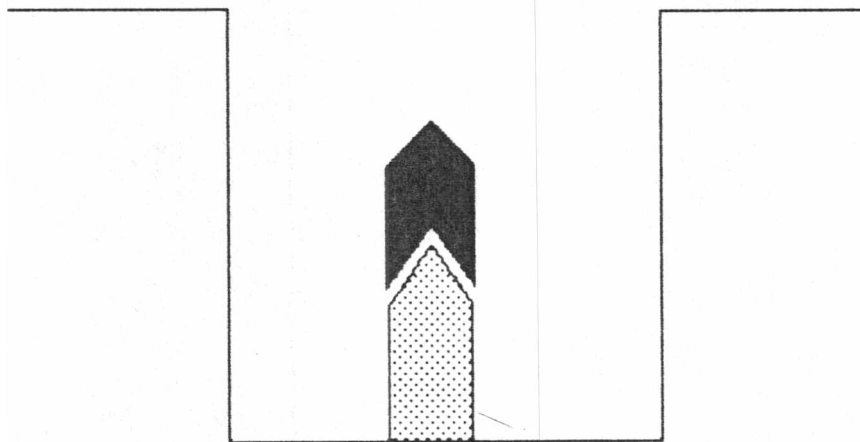


Figure 3b. Addition of serum sample.
Any specific antibody attaches to available antigen.

Indirect method (continued)

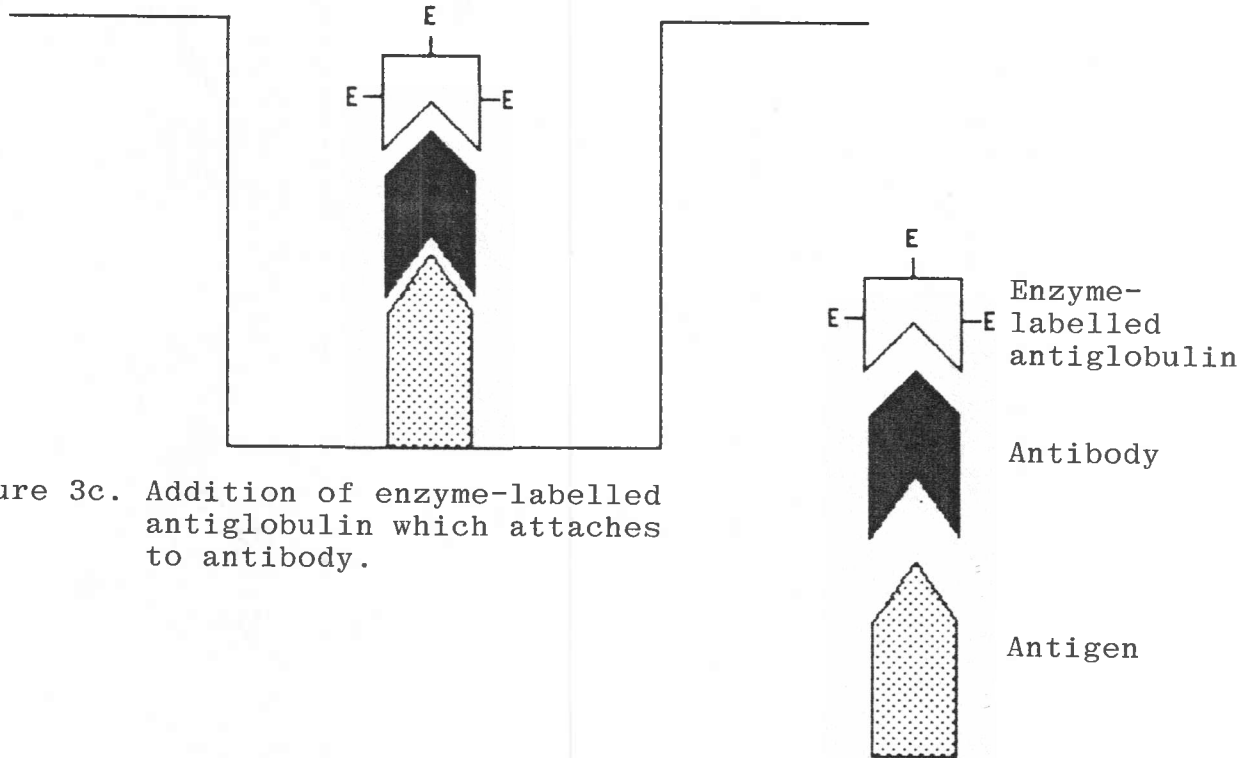


Figure 3c. Addition of enzyme-labelled antiglobulin which attaches to antibody.

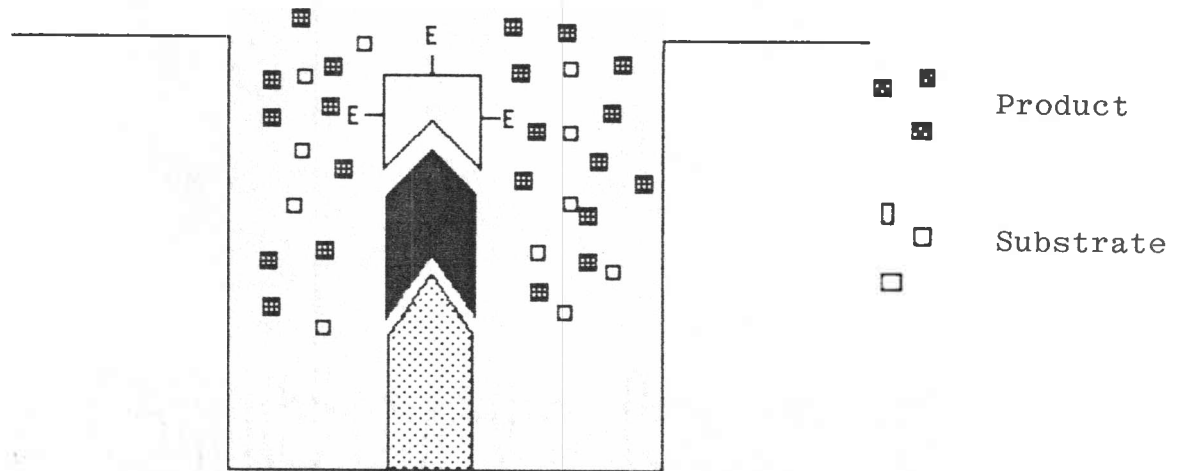


Figure 3d. Addition of substrate
Product obtained due to presence of
enzyme-labelled antiglobulin.

To avoid misinterpretation of results due to well-to-well variation, samples should be run at least in duplication, especially if a single dilution is being tested (43).

Several different methods have been adopted to increase antigen binding to wells, a few are cited. To detect specific antibody to measles virus, particular measles virus-infected cells have been used to sensitize wells. By using infected cells the antigen preparation is simplified while still providing antigen-coated plates which are stable for several months. The successful use of infected cells demonstrates major virus-specific determinants are present in these preparations. One would question the possibility of non-specific reaction between test serum and cells, but Rice maintains the titers measured by ELISA in this manner have correlated well with complement-fixation results and immunofluorescent antibody results (39).

To enhance rotavirus antibody assay sensitivity, rotavirus particles have been fragmented by chaotropic agents (NaSCN or guanidine hydrochloride) before adsorption of antigen to solid phase (16). This method was adopted when direct adsorption of rotavirus antigen didn't give reliable results. To bypass unreliability, others have tried indirect binding accomplished by preadsorption of guinea pig rotavirus antibody to the plate (52 in 16).

Skurrie advocates that passive adsorption of rubella antigens is variable also. By using albumin as a bridge,

the rubella antigen can be reliably attached to the wells. In particular, indirect binding techniques are especially useful when low-levels of antibody are being detected (45).

Non-specific binding of serum to remaining protein-binding sites of the plate is decreased by the presence of Tween 20 and bovine serum albumin in the diluent of serum samples. A blocking step has also been implemented using PBS with 0.5% to 1.0% bovine serum albumin to attain maximal assay sensitivity. Storage of antigen-prepared plates in PBS-BSA is advocated to obtain maximum stability (47).

The choice of enzyme and the conjugation of the enzyme to the antibody are essential steps to the enzyme immunoassay. The enzyme preferably would have high activity, be relatively low cost, be obtainable in pure form, and have a substrate reaction that is easily measured. The enzymes found most satisfactory at the present time are horseradish peroxidase, glucose oxidase, β -galactosidase, and alkaline phosphatase (49). Commercially prepared peroxidase conjugated antisera are useful in enzyme immunoassays. Conjugates are prepared from IgG fractions of antisera coupled to horseradish peroxidase by a modification of the method of Avrameas (3 in 32).

Substrates should be stable before and after degradation. Chromogenic substrates should be colorless initially and strongly colored after degradation. The determination

of enzyme activity is mainly accomplished by photometry. Fluorogenic, radioactive, chemiluminescence producing substrates are also used to increase sensitivity of assays (14, 31, 20, 19, 50, 48 in 34).

When utilizing peroxidase as the enzyme, substrates needed are substances which become oxidized when the peroxidase catalyzes the release of oxygen from H_2O_2 . The oxidation must be accompanied by a measurable change such as absorption. Orthophenylenediamine has proven to be a satisfactory substrate for peroxidase conjugates; but some prefer 5-aminosalicylic acid and o-toluidene (49).

Conceivably, endogenous peroxidase activity could pose a problem when using peroxidase-labelled gamma-globulins. Endogenous peroxidase is typically present in an acute inflammatory reaction because neutrophils and eosinophils are rich in the enzyme. To eliminate endogenous peroxidase activity, clinical samples can be treated with 0.074% HCL in ethanol for fifteen minutes (4).

Results can be interpreted in a number of ways depending on the ultimate use of the information. Voller, Bidwell, and Bartlett advocate correction of the values of all test samples to the reference positive sample:

$$\text{Corrected sample value} = (\text{measured absorbance of sample}) \times (1.0 / \text{absorbance of reference positive sample})$$

Using this equation the options of reporting results are:

1. Positive/Negative: All samples given an absorbance

value above a threshold level may be considered positive. The "threshold level" is pre-determined by testing a large number of "negative" samples. The "threshold value" is the upper limit of the negative values.

2. Ratios of sample value to mean value of a group of negative samples. Ratios of two or three times negative value are considered positive.
3. As endpoint titers, which involves making serial dilutions of the test sample. The titer is the last dilution which yields a value above that of a group of known negative samples (49).

Voller, Bidwell, and Bartlett also advocate the use of visual readings for diagnostic serology. They claim solutions with absorbance of 0.1-0.2 can be distinguished visually from colorless negative wells (49). With a similar conclusion, Rahaley reported no difficulty in visual assessment of the ELISA endpoint the detection of Brucella ovis antibody in sheep. The majority of the negative sera showed no discernable color change at the lowest dilution and the slight color change detected in a few negative sera was well below the 1+ cut-off point. In the positive sera, the transition from 1+ color change to 0 was generally abrupt, particularly at higher dilutions (38).

Another way to evaluate results is the standard curve. The standard curve method has been described as a simple method to calculate amount of antibody in a large number

of collected sera. Thus far, the sample curve method has been adopted for the assay of IgG antibodies against influenza A virus, respiratory syncytial virus, cytomegalovirus, mumps virus, adenovirus, herpes simplex virus, and rubella virus (9, 24, 40 in 5, 8, 45). Test serum samples are assayed at a single dilution and results are converted to units by reference to a standard serum which is titrated each assay.

For example, in the estimation of rotavirus IgG antibody in human serum samples, the standard serum is used which has an ELISA endpoint titer of 1:128,000, which is arbitrarily assigned a value of 20,000 U of rotavirus Ab/ml. The serum standard is incorporated into each assay at doubling dilutions 1:400 to 1:6400 (50-3.125 U/ml). When optical density is plotted against U/ml, this range corresponds with the linear portion of the curve. The optical density values of the test samples can then be converted to equivalent ELISA units by reference to the standard curve. The positive and negative cutoff value is once again established. By using the standard curve method a good correlation was drawn between ELISA IgG units and CF titers. Advantages would be simplicity, rapidity, reproducibility and the economical use of the serum (5).

MATERIALS AND METHODS

Antigen preparation

Vero cells were cultivated in growth media in several flasks until growth was confluent (4-6 days). One-half of the flasks were inoculated with a large amount of the seed virus. After cell-virus adsorption at 37C for 2-3 hours, the medium was removed from each flask and replaced with maintenance media. The other flasks remained uninoculated and were subjected to the same environmental conditions.

The virus was allowed to grow 2-5 days until significant growth was demonstrated by cytopathic effect. The suspensions were frozen and thawed twice to lyse the cells thereby releasing the virus into the environment. The suspensions were centrifuged at 2,000 rev/min (450 g) for 20 minutes to precipitate cellular debris. Viral antigens and small vero particles remained in solution. The supernatant was frozen until needed.

Positive control serum pool

Positive Colorado tick fever serum samples, as determined by immunofluorescent antibody technique, from the past four years were acquired. The serum samples were tested for CTF virus antibody titers by complement fixation.

Approximately 1 ml was removed from all samples having titers of 1:8 or greater. The resulting serum pool was placed in a centrifuge tube and centrifuged at 2,000 rev/min for 15 min to remove cellular debris. One ml aliquots were drawn, placed in vials, labeled, and frozen until needed.

Negative control serum pool

Serum from Montana State Laboratory personnel was used. The donors could recall no previous infection or exposure to Colorado tick fever virus.

Conjugate

Commercial goat anti-human IgG (heavy and light chains) conjugated to horseradish peroxidase (Miles Scientific Laboratories, Inc., U.S.A.) was used.

Substrate

Enzyme activity was measured with 1.0 ml stock substrate, 99 ml H₂O and 0.1 ml .3% H₂O₂ solution. Stock substrate was composed of 100 mg O-phenylenediamine in 10 ml methanol (40).

Stopping Reagent

8N sulfuric acid (22.4 ml conc. H₂SO₄ + 87.6 ml DH₂O) was used to stop the enzymatic reaction.

Washing Fluid

PBS-Tween-BSA: Phosphate buffered saline, pH 7.4,

containing Tween 20 (5 ml/liter) and .5% bovine serum albumin (Sigma Chemical, St. Louis, MO).

Diluent for sera

Same as above for washing fluid.

Diluent for antigen

Carbonate buffer, pH 9.6, 0.06 M (3.81 gm NaHCO₃, 1.93 gm Na₂CO₃, distilled H₂O to 1 liter).

Solid phase

Plastic flat bottomed Immulon 1 microtitration plates (Dynatech Laboratories).

Microelisa Minireader II

A single wavelength photometer was used for measuring absorbances of samples vertically through microtitration plates. A digital display showed absorbance values to two decimal places. The plate was moved manually under the alignment cone which uniformly engaged the circumference of the well and automatically centered the light beam through the well. The energy of the beam was read by a silicon photodetector and, sequentially, the reading was converted to digital data and printed out.

Absorbance is usually stated in terms of a standard pathlength of 1 cm. However, the pathlength through a solution in a microplate well was less than 1 cm. Since all blanks, controls, and samples are dispensed into identical wells to identical fluid depth, it was not necessary

to correct for 1 cm pathlength. An important point to consider was the precise dispersion of all reagents in order to attain a constant fluid depth.

Approximation of optimal conjugate dilution using ELISA on Influenza A

To approximate the optimal conjugate dilution, an enzyme-linked immunosorbent assay was run on monoclonal antibodies of Influenza A (Centers for Disease Control, Atlanta, Georgia). The procedure was considerably less painstaking due to the fact that several parameters were more solidified than in the CTF assay. The procedure stated below for determination of optimal conjugate dilution was performed except for a few minor changes. The plates were sensitized with cell suspensions diluted 1:4 in bicarbonate buffer (pH 9.6). The conjugate dilutions ranged from 1:400 to 1:12,800.

Determination of optimal antigen dilution

Twofold dilutions of antigen were made extending through the range of 1:25 to 1:1600. Dilutions of normal vero cells were also made and used as a tissue control. In each row of wells of appropriately labelled plates (two CTF virus and two normal vero), 0.2 ml of each dilution was dispensed. In the last row of all plates 0.2 ml carbonate buffer was added as a serum control. The plates were then covered and incubated in 37C incubator for 3 hours.

Serum dilutions, using both positive serum pool and

negative controls, were made with PBS-Tween 20-BSA and extended through a range 1:16 to 1:16,384. When the antigen-sensitized plates were removed from the incubator, antigen was removed by suction and the wells were washed (Dynatech Miniwasher) three times with PBS-Tween 20-BSA. Each wash lasted three minutes. One each of the normal vero-sensitized plates and CTF virus-sensitized plates received positive serum dilutions in the vertical columns of wells. In the same respect, one plate of normal vero-sensitized wells and one plate of CTF virus-sensitized wells received the negative CTF serum dilutions. In the last column of all plates, 0.2 ml PBS-Tween 20-BSA was added as an antigen control. The plates were then incubated at 37C for 35 minutes.

After incubation the serum was moved and the wells washed, in the same manner as above. An appropriate conjugate dilution (in PBS-Tween 20-BSA), as approximated by ELISA on Influenza A, was then added to the wells and incubated 37C for 35 minutes.

The conjugate was removed and the wells were washed. Two-tenths ml working substrate solution was then added to each well. The plates were covered to protect from light and allowed to stand at room temperature for 30 minutes. To stop the enzymatic reaction 0.025 ml 8N H₂SO₄ was added to each well.

The results were read using a Microelisa Minireader II (Dynatech Laboratories) at 490 nm. The optimal dilution

was taken as the highest dilution which gave the maximum reactivity with the positive serum control, minimum reactivity with the negative serum and no reaction in the antigen control columns.

Determination of optimal conjugate dilution

Two plates sensitized with optimal dilutions of CTF antigen were used. Serum dilutions extending through a range 1:16 through 1:16,384 were made in PBS-Tween 20-BSA. The antigen was removed from the plates by vacuum and the wells were washed 3 times in PBS-Tween 20-BSA. In a positively-labelled plate, 0.2 ml of the positive serum dilutions were added to the respective columns. In the negatively-labelled plate, 0.2 ml of the negative serum dilutions were added. In the last column of each plate PBS-Tween 20-BSA was added as a conjugate control. The plates were incubated 37C for 35 minutes.

The serum was removed and washed as above. Conjugate dilutions extending through a range of 1:200 to 1:12,800 were made in PBS-Tween 20-BSA. The conjugate dilutions were added to the plates into the respective rows in both plates. To the wells in the last row of both plates, 0.2 ml PBS-Tween 20-BSA was added as an antigen control. The plates were incubated 37C for 35 minutes.

After incubation, the conjugate was removed and washed as described above. To each well in both plates, 0.2 ml working solution of substrate was added. The plates were

covered and allowed to stand at room temperature for 30 minutes.

The enzymatic reaction was stopped by adding 0.025 ml 8N H₂SO₄. The results were read using a Microelisa Mini-reader II (Dynatech Laboratories) at 490 nm. Optimal dilution of conjugate was taken as the dilution giving maximum reactivity with the positive serum; minimum reactivity with the negative serum and little reactivity with the conjugate controls.

Performance of the test

The optimal antigen dilution in 0.06 M pH 9.6 carbonate buffer and normal vero control was prepared. Two-tenths ml optimal antigen dilution was added to each well in the first 9 columns of 8 wells (1:16-1:4096) in two microtitration plates, except the last three columns in each. In the last three columns of both plates, 0.2 ml normal vero control dilutions (1:16-1:64) were added. The plates were covered and incubated at 37C for 3 hours. The plates were also used after refrigeration (4C) after initial incubation if adequately covered.

The antigen dilutions were aspirated from the wells and each well was washed with PBS-Tween 20-BSA three times. Each wash lasted three minutes. The sera, including positive and negative controls and patient sera, were diluted 1:4 in PBS-Tween 20-BSA. One-tenth ml of diluted sera was added to appropriate wells in columns 1-10 and 0.5 ml

PBS-Tween 20-BSA was added to all other wells. Twofold serum dilutions were made by diluting sera from column 1-9 with an eight-well automatic pipetter 0.05 ml. The diluting procedure was repeated for columns 10-12. After all dilutions were made 0.15 ml PBS-Tween 20-BSA was added to all wells and the contents were mixed on a mechanical vibrator. The final dilution factor in the first well was 1:16 and extended through the eighth well to 1:4096.

The plates were covered and incubated at 37C for 35 minutes. After incubation, the serum dilutions were aspirated and the wells washed.

After 0.2 ml optimal conjugate dilution was added to each well, the plates were incubated at 37C for 35 minutes. The wells were then washed and 0.2 ml working substrate solution was added to each well.

The plates were then covered and placed in the dark at room temperature for 30 minutes. The enzymatic reaction was stopped with 0.025 ml H_2SO_4 . The results were read with a Microelisa Minireader II (Dynatech Laboratories) set at a wavelength of 490 nm.

The titer of the positive control sera was regarded as the greatest dilution of serum which displayed a distinct color difference from the initial well of the negative control serum dilution series. The test was declared invalid if the positive control sera didn't react with the test antigen at its anticipated titer (or at least with a twofold dilution). Little or no color should have

been expected to develop in any well of the negative control dilution series. If color was displayed in the negative series, the test was declared invalid.

The end point used to designate the quantity of antibody present in a patient's serum was the highest dilution of serum that displayed a distinct color difference from the initial well of the negative control serum dilution series.

RESULTS

Approximation of optimal conjugate dilution using ELISA on Influenza A

From the titration of horseradish peroxidase-labelled anti-IgG in the enzyme-linked immunosorbent assay of monoclonal Influenza A antibody (Table 1, p. 26), it was decided to use the conjugate diluted 1:1600. This dilution not only gave good distinction between positive (+) and negative (-) sera, but was also economical with the conjugate.

The antigen control columns (C) would have indicated any occurrence of reaction between Influenza A antigen in PBS-Tween 20-BSA. Non-specific reaction remained very low in all wells.

The low reaction exhibited in the normal tissue (allantoic fluid) wells was favorable. From the excellent color presented in the positive wells it was surmised that the reagents such as peroxidase, H₂O₂, carbonate buffer, orthophenylenediamine, and HCL were all working in the expected capacities. The microtitration plates appeared to be performing well also.

Determination of optimal antigen dilution

In the enzyme-linked immunosorbent assay of reference serum pools using titrations of antigen (Table 2, p.27),

Table 1. Results of conjugate titration using Influenza A antigen and monoclonal antibody

Antigen Dilution	Conjugate 1:400			Conjugate 1:800			Conjugate 1:1600			Conjugate 1:3200			Conjugate 1:6400			Conjugate 1:12800		
	**																	
	+	-	C	+	-	C	+	-	C	+	-	C	+	-	C	+	-	C
1:4	2.07*	.10	.01	1.84	.02	.01	1.47	.00	.01	.94	.00	.01	.59	.00	.01	.36	.00	.01

*optical density units

**Serum diluted 1:32

Table 2. Results of antigen titration using Colorado tick fever virus antigen and reference serum pool

Conjugate Dilution	Antigen 1:25			Antigen 1:50			Antigen 1:100			Antigen 1:200			Antigen 1:400			Antigen 1:800			Antigen 1:1600		
	** +	-	C	** +	-	C	** +	-	C	** +	-	C	** +	-	C	** +	-	C	** +	-	C
1:1600	.31*	.17	.34	.20	.15	.12	.16	.14	.17	.09	.09	.28	.07	.07	.11	.06	.04	.05	.06	.03	.03

*optical density units

**Serum diluted 1:32

there appears to be a gradient in the reactions of the serial antigen dilutions. Due to the high degree of non-specificity exhibited in the antigen control well (PBS-Tween 20-BSA) the test results were invalidated. The optical densities of the antigen control wells (C) were equal to or greater than the wells of the least dilute positive (+) sera, indicating the PBS-Tween 20-BSA was binding to the surface of the wells and sequentially binding the conjugate.

Numerous attempts were made to decrease non-specific reactions. Table 2 is only an example of many antigen titration attempts. The various methods are discussed in a later section.

Determination of optimal conjugate dilution

In the enzyme-linked immunosorbent assay of reference serum pools using antigen at 1:32 and titrations of the conjugate (Table 3, p. 29), there is the expected gradient between serial conjugate dilutions. It appears 1:1600 would be an appropriate usage. The 1:1600 dilution is an indication that the estimated conjugate dilution used in the optimal antigen determinations was appropriate and was not a source of error for the test. Again, there is the presence of high reactivity in the negative serum wells and antigen control well, for this reason the test results were invalidated. Since the optimal antigen and conjugate titrations could not be determined I could not move onto the actual test serum assays.

Table 3. Results of conjugate titration using Colorado tick fever virus antigen and reference serum pools

Antigen Dilution	Conjugate 1:1600			Conjugate 1:2400			Conjugate 1:3200			Conjugate 1:6400			Conjugate 1:12800		
	**														
	+	-	C	+	-	C	+	-	C	+	-	C	+	-	C
1:32	.47*	.18	.19	.15	.24	.16	.25	.27	.19	.14	.33	.15	.07	.23	.05

*optical density units

**Serum diluted 1:32

DISCUSSION

The enzyme-linked immunosorbent assay of Influenza A monoclonal antibody worked very well. It was possible to determine an approximate conjugate dilution would be 1:1600. There was very little non-specific binding. Besides estimating the appropriate conjugate dilution, using ELISA to determine titers of monoclonal antibodies also served the purpose of familiarizing myself with the correct procedure.

In initial enzyme-linked immunosorbent assays for Colorado tick fever virus antibodies the virus was grown on HeP-2 cells, but it was felt the HeP-2 cells were interfering with the binding of the antigen to the plate. An added problem was the high reactivity of the negative serum and the normal mouse antigen.

With the growth of the virus in monkey kidney cells (vero) and the addition of 0.5% bovine serum albumin in the diluent and washing solution, the non-specificity was significantly decreased. A disadvantage of viral growth on vero cells is the fact that some people have a high antibody titer to monkey kidneys probably from a previous vaccination. This occurrence could possibly explain the high reactivity found in some of the negative

serum controls. Future enzyme-linked immunosorbent assays would have to account for the background count displayed by some people by including normal tissue control wells for each serum sample.

Another possible solution to the cross-reaction caused by the previous vaccination is further purification of the virus. Viral purification procedures available and in use include: ultracentrifugation, millipore filtration, sonication, and sucrose gradient techniques (5, 37, 38, 51). A purification technique was attempted, but the antigen was apparently lost in the procedure. Not only was this verified by erratic ELISA results but, also, in a negative complement fixation reaction.

A disturbing occurrence in virtually every trial was the existence of "hot spots," wells which displayed unrealistic spectrophotometric results. One can project the implications of "hot spots" in several wells in a multitude of plates used for screening of Colorado tick fever. It must be noted that variability during attachment of antigen to solid phase is still the major factor in determining precision of enzyme immunoassay. Presumably a major cause of the "hot spots" is the variability in antigen binding. Another cause of the "hot spots" is the binding of coincidental serum material. In ELISA tests for high molecular weight substances there is often a tendency for materials in samples being assayed to attach non-specifically to the solid phase. This occurrence

is especially prevalent in the case of indirect ELISA tests where the sample is serum, plasma, CSF, etc. (49).

Since the hemagglutination-inhibition antibodies of most members of the Reoviridae family are very pH dependent, it was thought possibly the desired ELISA antibodies were also pH sensitive. An attempt to change the pH of the diluent solution resulted in no change in the occurrence of erratic results.

Another observation was the occurrence of poor or non-existent binding of the antigen in the wells. It was hypothesized that little antigen was binding and was proven true when an ELISA was run on mouse ascitic fluid containing CTF virus antibody. It was felt that if any antigen had been bound to the wells, it would certainly bind to the highly-titered ascitic fluid. The readings from the wells were all erratic and showed no pattern or expected results. The control wells containing no antigen at all had optical densities equal to or greater than the optical densities of the positive serum wells.

An observation of high reactivity in the PBS-Tween 20-BSA wells suggests adsorbance of the albumin to the plates. The albumin may then have acted as a bridge for binding antibody to the plate therefore giving positive results in wells not containing any antigen.

In conclusion, I would hesitate to abandon ELISA for determining titers of CTF antibodies even though I was unable to determine the origin of various problems

I encountered. ELISA has proven to be a convenient, sensitive, and reproducible method of measurement of serum antibodies against a variety of infective agents (45). ELISA, when implemented correctly, has a number of advantages over other serological techniques.

Once the parameters are determined, ELISA is readily adaptable to automation, thus enabling antibody tests for large-scale screening of populations. By using a spectrophotometer the results can be read quantitatively therefore necessitating only one serum dilution and decreasing manipulations and serum volume required.

Enzyme-linked immunosorbent assays have been repeatedly used as immunodiagnostic tools for routine laboratory work in hospitals and for epidemiological purposes.

A number of studies indicate the superlativity of ELISA over other serologic techniques. Results from comparative assays against Epstein-Barr virus indicate ELISA will detect early antibodies which are not evident in the immunofluorescent assay (25). Another example of the superiority of ELISA over other serological tests was shown when enzyme-linked immunosorbent assay and complement fixation tests were compared concerning their ability to detect Brucella ovis antibodies in sheep. It appeared ELISA was much more sensitive in its detection of low quantities of antibody than complement fixation assays. An added advantage was the decrease in the number of false positives. In general, the ELISA was more specific and

more sensitive than the complement fixation test in detecting antibodies in positive sera (38).

Again in another comparative study, a study of ELISA in contrast with hemagglutination-inhibition, complement-fixation, neutralization, and indirect immunofluorescence tests for detection of yellow fever virus antibodies was done. ELISA was found to be more sensitive and more specific than hemagglutination-inhibition and complement fixation tests, while showing similar results as the neutralization tests (11).

The first step toward developing a more effective ELISA for determination of CTF antibody titers would be purification of the virus. Additional purification techniques would include ultra-centrifugation and column chromatography. I would advocate the use of complement fixation tests on each fraction after each step in the purification procedure to determine where the antigen was located. Additional purification would help to decrease the high reactivity in the negative serum samples, thus protecting against the occurrence of false positives. Further purification would also insure stronger reaction with positive samples so that less conjugate and antigen would be used.

A second step would be the build-up of an adequate negative control serum pool. The use of individual serums from people presumed negative for Colorado tick fever was, at best, erratic. I would advocate complement fixation tests on a population of presumed negative serums. Serums

with titers less than 1:2 would be considered negative and added to the pool. The optimal situation would be the use of monoclonal antibodies for Colorado tick fever virus used in the same manner as the ELISA for Influenza A.

The third step taken would be the elimination of "hot spots," which could be attempted by incubation of water in all outer wells thereby insuring even temperature distribution. Added washing steps did not appear to increase or decrease the number of "hot spots" found on each plate. An additional suggestion would be the use of Immulon II microtitration plates (Dynatech Laboratories), a higher quality solid-phase support for increased binding of antigen and more selective binding.

There is no satisfactory way to determine if a particular antigen is suitable for enzyme-linked immunosorbent assays. The best way to find out if the ELISA is possible is through the use of various dilutions of reference sera and conjugate, such as I attempted. Other labs are currently working on developing a good ELISA. These labs have also encountered problems such as a lack of sensitivity as opposed to immunofluorescent antibody results. In addition, the antibodies being detected by ELISA are present 2-3 weeks after the onset of the disease. This time span is not conducive toward diagnosis of the disease. It is evident that in order to unlock the puzzle additional research is necessary.

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