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Serologic Test Development

July 1, 1975—June 30, 1976

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SEROLOGIC TEST DEVELOPMENT ANNUAL REPORT

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ABSTRACT

The indirect Enzyme-Labeled Antibody (ELA) hog cholera test has been successfully tested on a 640-sample, computer-coded serum bank. A greater than 99% correlation with the hog cholera serum neutralization test was observed. A successful field trial of the ELA hog cholera test was carried out at the Hog Cholera Task Force Laboratory, Trenton, NJ. The serum factor present in packinghouse swine responsible for the high level of false positive ELA trichinosis reaction has been localized. This factor appears to be immunologically nonspecific. Investigation of the properties of various enzyme substrates has been done. Design for fabrication of preprototype, semiautomatic equipment has been completed.

I. INTRODUCTION

Satisfactory progress in the development of the Enzyme-Labeled Antibody (ELA) disease detection system has continued. Test development for the applications of ELA to virus disease detection has been successfully completed with hog cholera as the prototype disease. Parameters developed for the hog cholera test have also proven useful to our work on trichinosis and will also apply to almost any disease we work on. Some insight has been gained about the false positive problem we have encountered in the trichinosis ELA test. A search for a sensitive and commercially available substrate has proved successful. Instrumentation for the semiautomation of the test is now being fabricated.

This report is organized into (1) several sections that describe work done in the various areas of test applications of interest to the United States Department of Agriculture (USDA), (2) a section that describes test refinements, and (3) a section that describes progress in instrumentation development.

II. HOG CHOLERA

Hog cholera has been the prototype virus disease for detection by ELA. The successful solving of the numerous problems we have encountered in hog cholera test development will make application of ELA to other important virus diseases (both animal and man) a much easier task when the need arises. A paper describing details of the hog cholera test development has been accepted for publication in the "American Journal of Veterinary Research."[1]

A. Hog Cholera Serum Bank Trial

Following our return from the Amarillo Hog Cholera Task Force Laboratory, we reran the Veterinary Services Laboratory (VSL) hog cholera serum bank (made up of mixed hog cholera positive and negative sera) incorporating the use of noninfected cells as a built-in control for each serum. The ELA results correlated at better than 99% to hog cholera serum neutralization (HCSN) results on the same sera. False positive and negative results were minimal with both tests -- Tables I and II.

However, it was seen that ELA shares with HCSN the problem of cross-reactivity between hog cholera (HC) and bovine viral diarrhea (BVD) -- Table III. Endpoint analysis may eventually be applied to distinguish between these two antibodies.

The March 1976, New Jersey hog cholera emergency provided a good chance to test the efficiency of the ELA hog cholera test under field conditions. Antigen plates were prepared at the Los Alamos Scientific Laboratory (LASL) and shipped by Federal Air Express to reach the Trenton Laboratory within 24 hours. Plates were stored at -70°C until used. To increase the longevity of the antigen trays, a minor change in the fixation procedure was instituted in the antigen trays shipped from LASL. To the 20% acetone saline fixative (pH 7.2), we added 200 mg bovine serum albumin/liter of fixative. After fixation, the trays were allowed to air dry without any rinse following removal of the fixative. Previous experience has shown that the antigenicity of the trays remained for only about 10 days after preparation, even when stored at -20°C. The addition of a small amount of protein has proved to be a good idea; trays prepared on the 29th of March still showed excellent antigenicity (stored at -20°C, wrapped in plastic) during the first week of June. During the next year, trays will be removed from storage periodically to test for antigenicity. We have observed that the condensation, which forms on the trays after they have been removed from the freezer, must be allowed to dry thoroughly before the tray is used; otherwise, the cells have a tendency to slough off during processing. This sloughing of cells was observed early in Trenton, and when we discovered the cause, it was corrected and we had no further problem of this type.

One other problem that occurred in Trenton involved the step using the tap water rinses, following the serum and conjugate incubation steps. The background staining level was considerably higher than observed at the LASL. Since the Trenton tap water was quite cloudy, it was concluded that the water was causing the higher background staining. When deionized filtered water was substituted for tap water, the problem disappeared.

TABLE I

FREQUENCY OF POSITIVE TESTS AMONG

SERUMS KNOWN NEGATIVE FOR HOG CHOLERA

Group	Number of Positive Tests[a] ELA	HCSN[b]
Control Pigs (80)[c]	1	1
Erysipelas Pigs (92)	0	0
HC Pigs, preinnoculation (36)	0	0
BVD Pigs, preinnoculation (22)	0	0

[a]The serum dilution for ELA was 1:20 and for HCSN was 1:4.

[b]Data obtained by Diagnostic Virology Section, VSL, APHIS, USDA, Ames, IA.

[c]The number of samples per group is in parentheses.

TABLE II

FREQUENCY OF NEGATIVE TESTS AMONG

SERUMS FROM HOG CHOLERA INFECTED PIGS

Group[a]	No. of Samples	Number of Negative Tests[b] ELA	HCSN[c]
HCSN Negative and 1:2	26	26	25
HCSN 1:4 and 1:8	18	4[d]	1[d]
HCSN $\geq$ 1:16	204	0	1

[a]Serum neutralization titers were determined on serum bank samples when they were collected.

[b]The serum dilution for ELA was 1:20 and for HCSN was 1:4.

[c]Data obtained by Diagnostic Virology Section, VSL, APHIS, USDA, Ames, IA.

[d]Not statistically significant by chi square analysis ($p > 0.20$).

TABLE III

FREQUENCY OF POSITIVE TESTS USING HOG CHOLERA

ANTIGEN ON SERUMS FROM BVD-INFECTED PIGS

Group	No. Samples	No. with Pos.[a] HCSN Titer	No. Pos. by ELA
BVDSN Titers[a] 1:8 - 1:32	6	0	0
BVDSN Titers $\geq$ 1:64	78	66 (1:2 to 1:256)[b]	64

[a]BVDSN and HCSN titers were determined at the time serum bank was collected.

[b]Range of HCSN titers.

Once these problems were worked out, one individual could keep up with the volume of samples submitted for screening. It appeared that one person doing ELA could screen the same number of samples as three people doing the HCSN. The most tedious part of the test was reading the samples after processing. A small, portable and completely automated readout system would make work much easier and daily throughput could increase significantly.

The comparison of ELA results with HCSN obtained at the Trenton Field Laboratory are presented in Table IV. Considering the conditions and the volume of samples processed, the correlation is quite good.

The successful outcome of this field trial should allow adoption of the ELA procedure as the screening test of choice for hog cholera. To aid in the adoption of ELA for field use in the event of another HC outbreak, a list of materials and equipment has been included in Appendix A to this report, so that a field kit can be created.

The ELA hog cholera test was designed primarily as an efficient screening method. However, with a little work, ELA should also be useful in determining endpoints as well. Appendix B shows the results of a comparison of HCSN and ELA endpoints on most of the hog cholera positive serum bank samples. This data has been submitted to Dr. Merrill Swanson of VSL, Ames, Iowa, for mathematical evaluation. It appears that ELA titers are significantly higher than HCSN titers during the early stages of the immune response, but become equal to, or slightly less than, HCSN titers later in the response. This difference presumably reflects the difference in the specificity of the antibodies being measured. The HCSN test detects antibodies specific to protein involved in the binding of the virus to the cells. The ELA test measures specificity against all viral proteins whose antigenicity is preserved during the fixation process. The rate and amount of production of antibodies against the different specificities being measured by the two tests may vary considerably.

TABLE IV

ELA DATA SUMMARY HC TASK FORCE LABORATORY

TRENTON, NJ

1. ELA done on 36 of 46 cases.
2. ELA done on 2,148 of 2,560 sera submitted.
3. In HCSN negative cases 334 of 336 sera were ELA negative. 99.4% correlation
4. 47 of 49 sera[a] with HCSN titers $\geq$ 1:64 were ELA positive. 95.9% correlation
5. 34 of 39 sera[a] with HCSN titers of 1:16 were ELA positive. 87.2% correlation
6. In infected herds, ELA has 56 additional positive results on HCSN (at 1:16) negative samples, but only 2 positive results in HCSN negative herds.

[a]From HC confirmed herds.

One final piece of data was obtained as a result of the New Jersey outbreak. In cooperation with epidemiologists, 110 blood samples obtained from rats killed on HC-infected premises were tested for HC antibody at the LASL. All 110 animals were negative for hog cholera antibodies as determined by ELA.

III. TRICHINOSIS

Work on trichinosis has concentrated on the false positive problem, which was discussed in detail in the 1974-1975 Annual Report.[2] Our approach to an understanding of and possible solutions to this problem has been: (1) to attempt to identify the serum component(s) responsible for the false positive reaction by fractionation of known T. spiralis negative, positive, and false positive sera; (2) to attempt to remove those components of the antigen, which react with the false positive serum factors by fractionation of the T. spiralis antigen preparation; (3) to attempt to balance the trichinosis antigen with an indifferent antigen as in the hog cholera system.

A. Fractionation of Swine Sera by Ion Exchange Chromatography

Sera were fractionated on Diethylaminoethyl (DEAE)-cellulose columns. The fractions obtained were analyzed for serological activity in the ELA test and for precipitin activity in agar double diffusion tests.

Sera were applied to 2.2 x 30 cm DEAE columns, eluted with a sequence of 7 buffers of increasing molarity and decreasing pH.[3] Fractions of 7 ml each were collected and their absorbance at 280 nm read in a spectrophotometer. Figure 1 shows the protein elution pattern obtained. All sera fractionated gave the same pattern of peaks I through IX, with some variation in the amount of protein in the peaks. Fractions from the peaks were pooled to give 9 major

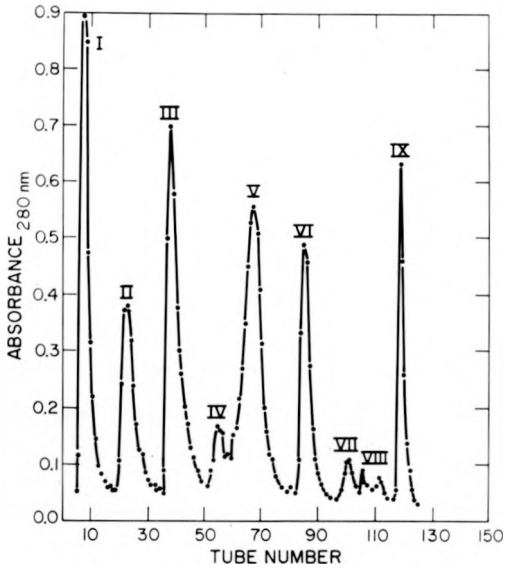

Fig. 1. Fractionation of swine serum on DEAE cellulose.

fractions. After determination of protein,[4] the pools were concentrated by per-evaporation, dialyzed against saline, and lyophilized.

B. Analysis of Pools for Activity in the ELA Test

Lyophilized fractions were reconstituted to contain, in the case of positive sera 4/75 and Pig C, the same amount of protein as was calculated to be present in that fraction of whole serum, or in the case of negative (SPF and Miles) and false positive (Pig 59 and the false positive pool), 4 times the concentration of that fraction in whole serum. The reconstituted fractions were tested for serological activity in the ELA test. The results of these tests are shown in Table V. In general, under the conditions used here, DEAE fractions I and II are almost pure 7S IgG, while 19S IgM is recovered primarily in fractions VI, VII, and VIII.[3]

TABLE V

ACTIVITY OF SERUM FRACTIONS IN ELA TEST AND AGAR DOUBLE DIFFUSION TEST

Serum	I	II	III	IV	V	VI	VII	VIII	IX[4]	Concentration Relative to Whole Serum
SPF	-	-	-	-	-	-	-	-	-	4 X
Miles[1]	-	-	-	-	-	+N	-	+N	-	4 X
4-75[2]	+P	+P	+P	+N	-	-	-	-	-	1 X
Pig C[3]	+P	+P	+P	+N	+N	+N	+N	+N	-	1 X
False Positive #59	-	-	-	-	-	+N	+N	+N	-	4 X
False Positive Pool	-	-	-	+N	-	+N	+N	+N	-	4 X

+ = Specific activity (ELA units/mg protein) of 20 or more.

- = Specific activity of less than 20.

1) "Normal Swine Serum," obtained from Miles Laboratories, described as pooled packinghouse sera.

2) Serum taken more than 1 year after infection to T. spiralis larvae.

3) Serum taken 52 days after infection to T. spiralis larvae.

4) Peak IX contains no protein as measured by the Lowry technique.

N = No precipitin found in agar double diffusion test.

P = Precipitin found in agar double diffusion.

Most of the ELA activity present in false positive sera was found in fractions VI, VII, and VIII; however, some activity was present in fraction IV of the false positive pool sample. Sera taken late in T. spiralis infection, i.e., 4/75, showed activity in fractions I, II, III, and IV only; while early serum, i.e., Pig C, had significant activity in fractions V, VI, VII, and VIII, as well as in fractions I, II, III, and IV, presumably due to synthesis of high molecular weight, 19S immunoglobulins as well as 7S immunoglobulins, early in the infection. The fact that the false positive serum component is probably of a different immunological class than T. spiralis antibody means that it may be possible to prepare a T. spiralis antigen that reacts only with the antibody and not with the false positive factor. It is of theoretical interest to note that there is no ELA activity with the present T. spiralis antigen in any fraction of SPF swine serum.

C. Precipitin Reaction of Whole Sera and Fractions in Agar Double Diffusion Tests

While whole T. spiralis positive sera, e.g., 4/75 and Pig C, give clear precipitin bands when tested with the T. spiralis antigen in agar double diffusion tests, it has never been possible to show precipitin activity with false positive sera. One of the purposes of fractionation of the false positive sera was to obtain concentrated fractions that would allow larger amounts of serum proteins to be used in the agar double diffusion test.

As shown in Table V, precipitin bands, due to interaction of diffusing antigen and antibody molecules, were formed by fractions I, II, and III of positive sera. No bands were formed with any fraction of the false positive sera, even in experiments in which the protein concentrations of the fractions were 10 times those in whole serum. It seems highly unlikely, therefore, that the false positives could actually be due to low levels of specific T. spiralis antibody; but presence of a weak cross-reacting precipitin in the false positive sera cannot be ruled out.

D. Progress Toward Identification of the False Positive Serum Factor

1. Cross-reaction between T. spiralis Gram-negative Bacteria. Cross-reactions between T. spiralis antigen and antibody to S. typhi, and between S. typhi antigen and antibody to T. spiralis have been reported in the literature[5] and are frequently referred to in review articles. In addition, there has been considerable interest in the so-called "natural antibody" to Gram-negative bacteria in pigs,[6] which has usually been reported to be of the IgM or 19S class.[7] In view of the fact that the false positive serum factor appears to be IgM, on the basis of its behavior on DEAE, a careful study of the possibility that this factor is antibody to Gram-negative cell wall antigens, which cross-reacts with T. spiralis antigen, was undertaken in an attempt to answer the following questions:

1. Does swine antiserum to T. spiralis react with Gram-negative bacterial cell wall antigen in the ELA test?

2. If so, is the false positive serum factor found in packinghouse pig sera antibody to Gram-negative bacteria?

A manuscript for publication concerning the details of these experiments is in preparation. The major conclusions reached as a result of this study are as follows:

1. Neither swine or rabbit anti-T. spiralis sera or swine false positive sera cross-react in the ELA test with a heat-stable E. coli antigen prepared in this laboratory or with S. typhi somatic antigen sent to us by Dr. Billie Blackburn, Ames, USDA, VSL.

2. Rabbit anti-S. typhi serum, sent to us by Blackburn, does not cross-react in the ELA test with our T. spiralis antigen preparation.

3. Antibody to E. coli and S. typhi antigens can easily be detected by the ELA test in swine sera, including SPF and the packinghouse false positive sera; however, this antibody is present in DEAE fraction I and not in fractions VI, VII, or VIII.

4. Antibody to E. coli or S. typhi, which reacts in the ELA test, is removed from swine or rabbit sera by absorption with E. coli or S. typhi antigen, but not by absorption with T. spiralis.

5. Antibody to T. spiralis, which reacts in the ELA test and in the agar double diffusion test

for precipitin antibodies, is removed from swine T. spiralis positive or false positive swine sera by absorption with T. spiralis larvae, but not by E. coli or S. typhi antigen preparations.

In summary, the serum factor found in fractions VI, VII, and VIII of false positive swine serum is not "natural antibody" to Gram-negative bacteria.

2. Heat Stability of the False Positive Serum Factor. In most systems which have been studied, IgG antibody has been shown to be more resistant than IgM to inactivation in serological tests by heat. Attempts to demonstrate that the serologically active material in DEAE fraction VI of false positive sera is more sensitive to inactivation in the ELA test by heat (65 and 70°C for 30 min) than the antibody in fraction I have been equivocal. Under most of the experimental conditions tried, heating of whole false positive serum increased the ELA activity; however, heating of fractions VI and VII decreased ELA activity.

3. Comparison of Anti-IgG with Anti-IgM Conjugates. A heavy chain specific, anti-swine IgM serum was sent to us by Dr. Stanley Stone (Ames, ARS). One of us (GCS) prepared a horseradish peroxidase conjugate from this anti-serum. The activity of this conjugate appears to be considerably less than that of the anti-swine IgG conjugate regularly used in the ELA test. In the course of evaluation of the efficiency of the two conjugates in a direct ELA test; i.e., using swine sera as antigen, a clear "prozone effect" was observed for serum dilution through 1:2000 for the anti-IgG conjugate and through 1:1000 for the anti-IgM conjugate.

The data from experiments comparing the activity of the two conjugates in ELA tests of DEAE fractions I and II of positive sera and fractions VI and VII of false positive sera were difficult to interpret, due to differences in activity of the conjugates. These data are, however, consistent with the hypothesis that the anti-IgG conjugate cross-reacts with IgM, i.e., contains antibody to the light chain of the IgM molecules; and the anti-IgM conjugate is specific for the heavy chain of IgM, i.e., does not cross-react with IgG. If this is true, it appears that the false positive serum factor in DEAE fractions VI, VII, and VIII is indeed IgM. In this case, it may prove possible to eliminate the false positive reactions by use of a conjugate specific for the heavy chain of IgG. Further experiments along these lines are in progress. For this aspect of the work, it is important to locate a reliable source of highly specific immunological reagents.

E. Pre-test Treatment of Sera

Heating of sera before testing and testing at acid pH have been reported to eliminate nonspecific false positive reactions in certain serological tests.[8,9] Heating false positive sera at 56°C for 30 min or 60°C for 60 min increased the ELA readout, as did testing sera diluted in acetate buffer at pH 3.

Heterophil, or Forssman antigen has been reported to be present in T. spiralis larvae, while swine are heterophil negative;[10] therefore, false positive sera were absorbed with sheep red blood cells, which remove heterophil antibody. This treatment had no effect on the ELA activity of either positive or false positive sera in the single experiment done. It seems unlikely that the false positives are due to presence of heterophil antibody, but additional experiments would be required to rule this possibility out unequivocally.

F. Antigen Fractionation

Work on fractionation of the T. spiralis antigen was begun in April of this year. The goal of this work is to isolate, if possible, a fraction with high activity for the 7S T. spiralis specific antibody and low activity for the false positive serum factor and/or a fraction with high activity for the false positive factor and low activity for the T. spiralis specific antibody. This work has not yet progressed to a point where a judgment as to the likelihood of success can be made. It is, however, obvious that this is a formidable task due to the extreme heterogeneity of the crude antigen preparation and the extreme sensitivity of the ELA test to very small amounts of antigen. The fact that relatively pure fractions of T. spiralis specific antibody and false positive serum factor can be used in exploratory work with antigen fractions may simplify this task.

G. Continued Testing of Packinghouse Swine Sera

In November 1975, Dr. W. W. Bishop (APHIS, USDA) spent three days in our laboratory learning the ELA test procedure for trichinosis. We have been collaborating with Bishop in further testing of packinghouse pig sera he has collected from animals sent to slaughter in Tennessee packinghouses. In April of this year, we tested 114 sera sent to us by Bishop from animals found by him to be negative

by the Zimmerman digestion procedure.[11] The distribution of ELA readouts for these sera are shown in Fig. 2. These results are similar to those for packinghouse pigs previously tested,[2,12] originating in Iowa, Kansas, and Nebraska. Thus, the false positive problem in packinghouse pigs is not unique to animals raised in the midwestern states. A similar problem with "ELISA," an enzyme-labeled antibody test similar to ELA, when applied to testing "conventionally raised" swine for trichinosis has been reported recently by Ruitenberg[13] working in the Netherlands.

At the same time that the Tennessee sera were tested, i.e., April 1976, several sera from digestion positive animals, which had been fed 100 or 500 larvae each in an experiment carried out with Dr. W. Zimmerman (Ames, Iowa) two years ago, were re-tested using the ELA test exactly as done on the packinghouse sera, with results as shown in Fig. 2. It is evident that in the absence of a pre-infection serum sample, these low-titer sera cannot be distinguished from most negative sera. Preliminary data from DEAE fractionation of pooled sera from this experiment indicate that sera from the laboratory-raised pigs used in this experiment do not contain ELA activity in fractions VI, VII, and VIII.

H. Antigen Balancing Experiments

In an attempt to apply the knowledge gained from the ELA hog cholera development to the false positive problem in trichinosis, various concentrations of different proteins were dried on alternate rows of Microtiter trays to compare with trichinosis antigens of various concentrations placed in the other wells of the trays. When it appeared that a balance had occurred when testing a pair of negative field serum against an antigen pair, several of the false positive sera were also tested. Using bovine fibrinogen at a concentration of 0.5 mg/ml and against trichinosis crude antigen at a dilution of 1:750, the false positive rate on 143 packinghouse sera dropped from 15% to just over 2%. Using this same combination against 67 sera that were digestion positive with at least 1 cyst/g of diaphragm, all 67 were ELA positive. However, 3 of these sera were just above the threshold of a signal-to-noise (S/N) ratio for positivity of 1.5 (1.5 - 1.7), thus suggesting that the possibility exists of eventually missing a digestion positive animal. Perhaps it might be more desirable in this type of system to set the threshold at a S/N of 1.3 and accept a higher rate of false positive reaction to avoid significant numbers of false negatives.

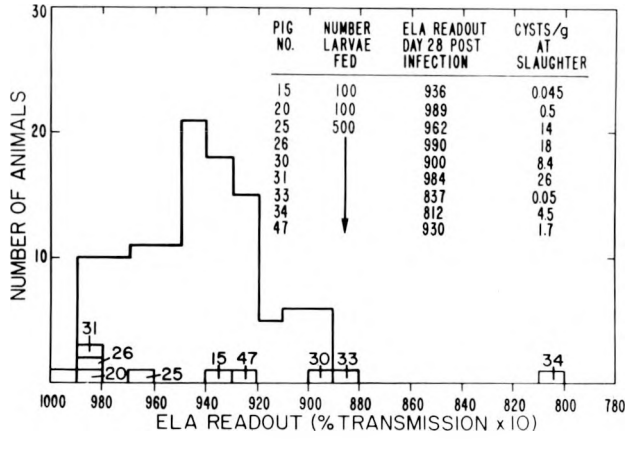

Fig. 2. Distribution of ELA readouts for sera from 114 negative and 9 digestion positive swine. The solid line shows readouts for the negative sera.

IV. BRUCELLOSIS (BOVINE)

Work on brucellosis was limited to an evaluation of the effect of antigen concentration on the ELA test performed on known positive and negative sera and an evaluation of this antigen concentration on 60 mixed serum samples supplied by Dr. Donald Pietz, VSL, Ames, IA.

A. Antigen Concentration

We found that higher concentrations of antigen markedly inhibited binding of brucellosis positive bovine sera (Table VI). This has been the first time we have seen such an effect while we have been developing ELA procedures. However, the phenomenon has been reported in other test systems. The table illustrated that a satisfactory S/N ratio occurs at an antigen dilution of 1 to 1000. A similar curve was also seen with positive and negative swine brucellosis sera.

B. Probe of 60 Assorted Bovine Sera

Pietz sent 60 bovine sera of different types for the purpose of tuning the ELA test for a future blind serum bank trial. These sera consisted of: (1) naturally infected animals, (2) nonspecific

TABLE VI

EFFECT OF SOLUBLE BRUCELLA ANTIGEN CONCENTRATION ON THE ELA RESPONSE OF A BRUCELLA-POSITIVE BOVINE SERUM

Dilution	Positive Serum[a]		Negative Serum[a]		Signal/Noise
	Individual	Sum	Individual	Sum	
10^{0}	19, 17	36	4, 4	8	4.5
10^{-1}	46, 36	82	6, 4	10	8.2
10^{-2}	209,216	425	15,15	30	14.2
10^{-3}	212,224	436	16,18	34	12.8
10^{-4}	77, 79	156	16,24	40	3.9
10^{-5}	42, 34	76	16,22	38	2.0

[a]Sera diluted 1:25; conjugate diluted 1:400; 5 minutes sera, 5 minutes conjugate and 5 minutes substrate.

"tube test" reactors, (3) animals vaccinated as calves and then artifically challenged with Brucella abortus, (4) animals not vaccinated that were artificially challenged, and (5) animals from categories 3 and 4 that were challenged but did not become infected. The raw data for this experiment can be found in Appendix C. The results of ELA and several other tests done on the same sera are tabulated. The ELA results were very good. Naturally infected animals were positive; nonspecific reactors were negative; vaccinated and nonvaccinated challenged animals that became infected were positive; vaccinated and nonvaccinated animals that did not become infected were negative.

Additional tuning of the test (antigen, serum, conjugate, indifferent antigen, etc.) will be attempted with the sera, so that we can perform the blind serum trial with high expectations of success.

V. ENZYME-LABELED ANTIBODY (ELA) TEST REFINEMENTS

The details describing the adaptation of the test to Microtiter trays have been published in the "Journal of Clinical Microbiology."[12] Several significant improvements to the test have been developed and investigated during the past year. These include: (1) the investigation of available commercial readout instrumentation, (2) the investigation of commercially available substrates, (3) the covalent binding of antigens to polystyrene microtiter trays, (4) control of each serum by use of an indifferent antigen.

A. Commercial Readouts

ELA readout units are presently derived from a transmission measurement. As seen in Fig. 3, absorbance is a linear function of concentration, and transmission approximates a linear curve only to 60% transmission (ELA reading of 400). Absorbance is the preferred measuring parameter, because it is a linear function of reaction product. When transmission values no longer approximate a linear curve, as can happen with 5 AS and with other substrates with molar extinction coefficients greater than that of 5 AS, the transmission values no longer accurately represent the amount of product present. We talked to six companies that manufacture spectrophotometers that will give a digital readout of the absorbance. These companies also have equipment that will automatically pull up a sample from a series of samples in test tubes, read it, and print the sample number and its absorbance. The companies are Bausch & Lomb, Varian, Beckman, Gilford, McPherson, and Hitachi. These instruments are good for any lab manually doing the ELA test and desiring a readout.

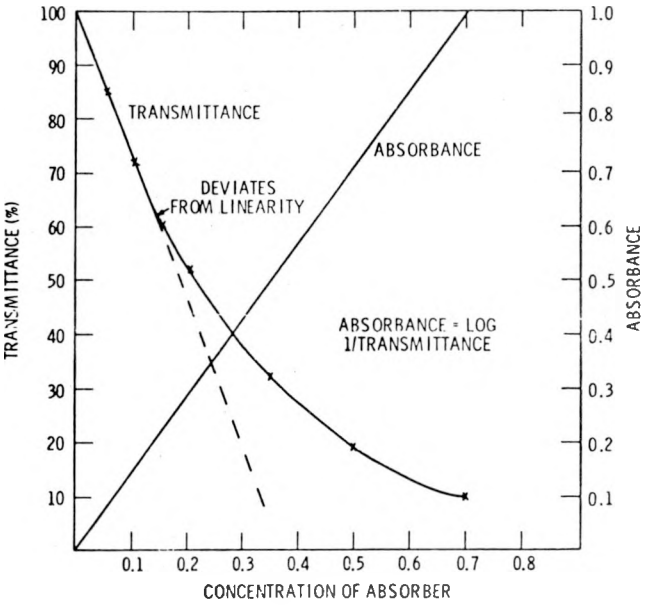

Fig. 3. Curves showing the relationship between absorbance and concentration, transmission and concentration, and absorbance and transmission.

We tested another type of readout in our lab. This is a Brinkman Probe Colorimeter shown in Fig. 4. Instead of transferring the solution to a measuring compartment, which is done in the readouts previously discussed; one transfers the measuring probe shown in the operator's left hand, to the solution. The instrument consists of an analog readout shown on the left, which contains a white light source with a selection of six filters giving six wavelength bands of about 20 nm. The 490-nm filter was used. The instrument also has a digital readout shown on the right, and a printer that prints the sample number and the absorbance or transmittance of the sample. The printer can be triggered by the operator as soon as the reading has stabilized. Because the probe can easily be wiped between samples, the contamination of one sample to the next is less than with the other readouts. Although the Brinkman literature says there is no warm-up time, we found a 2% transmittance drift per hour, which settles to approximately 0.25% transmittance per hour after about 75 minutes; so a 75-minute warm-up time was found necessary. We also found that with the probe and the printer, the readings can be obtained about twice as fast as with the ELA readout. The Brinkman readout is more sensitive, because its light source has a narrow band width (20 nm) centered near the absorption maximum of the product (490 nm); whereas, the LASL instrument has a 100-nm bandpass, centered at 560 nm.

Fig. 4. Photo of the Brinkman Probe Colorimeter being used at the LASL.

TABLE VII

RESULTS OF TWO READOUTS FOR S/N OF POSITIVE & NEGATIVE TB SERA

Dilution of Conjugate	ELA Reading LASL Instrument + Serum	- Serum	S/N	Beckman Spectophotometer 500 nm + Serum	- Serum	S/N
1/50	441	295	1.49	.368	.176	2.09
1/100	407	254	1.6	.34	.145	2.34
1/250	284	110	2.58	.165	.053	3.11
1/500	226	60	3.77	.118	.031	3.81

Table VII illustrates the advantages of using a very narrow band width light source (Beckman spectrophotometer with 1.3-nm band width centered at 500 nm) and absorbance measurements instead of transmission. The table shows the effect of conjugate dilution on the signal-to-noise (S/N) of a positive and a negative TB serum. The 1/50 dilution of conjugate has a higher S/N when using the Beckman because absorbance is measured, and the ELA reading no longer accurately represents the amount of product present. The same solutions were read in both instruments. Qualitatively, the conclusions are the same; namely, that a 1/500 dilution of conjugate gives a better S/N, but quantitatively speaking, the Beckman readings give better S/N ratios for all four dilutions of conjugate.

B. Investigation of Various Substrates for Horseradish Peroxidase (HRP)

Various substrates were investigated in order to find one of greater stability and sensitivity than 5 aminosalicylic acid HCl (5 AS), and at the same time, commercially available in a pure form. Greater stability is desired so that the substrate can be stored for longer periods. Greater sensitivity will result in greater signals for the same amount of antibody antigen reaction.

In last year's annual report,[2] it was noted that when using 5 AS as substrate, dilution of the product in 1 M NaOH doubles the sensitivity. However, it was found that the absorbance versus time after starting the enzyme reaction is not linear. In fact, it is very unusual in that the absorbance after early reaction times is higher than the absorbance after later reaction times. This phenomenon has been repeated. The results can be interpreted as the formation by HRP of more than one oxidation

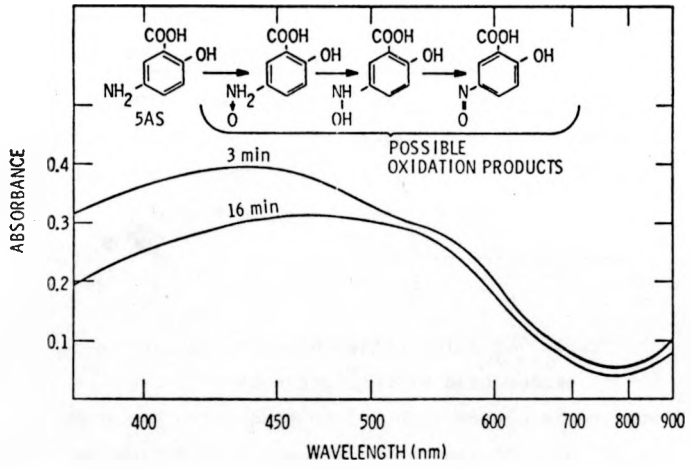

Fig. 5. Absorption spectra in 1 M NaOH and chemical formulae of the oxidation products of 5 AS.

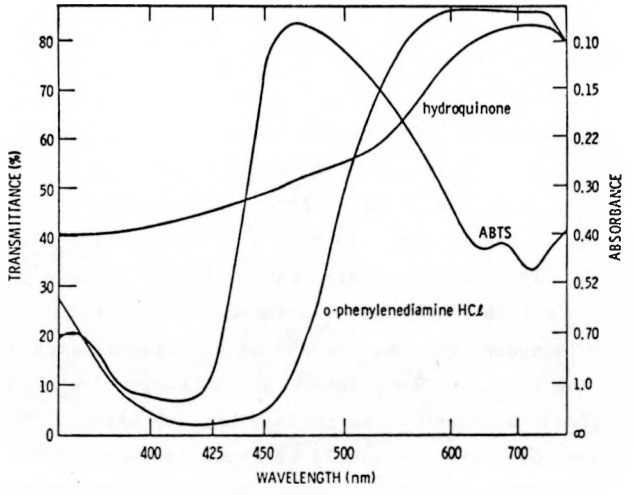

Fig. 6. Transmission spectra of the oxidation products of three substrates.

product. To verify this, the absorption spectra of the product at 3 min and at 16 min were taken. These are shown in Fig. 5. The shapes of the spectra are different, and this verifies the existence of more than one product. The possible chemical formula of these oxidation products are shown in Fig. 5. Because of the existence of more than one oxidation product seen with 5 AS and with other substrates, one must be very careful in varying any parameters of the enzyme assay.

Four aminosalicylic acid HCl (4 AS) was also tested as a substrate. It is commercially available in a purer form than 5 AS, the substrate currently used. However, when 4 AS and 5 AS were tested at pH 6 and pH 7, 5 AS proved to be more sensitive.

Other compounds were investigated. The transmission spectra of three of these: hydroquinone, o-phenylenediamine HCl, and 2, 2' azino-di-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) are shown in Fig. 6. It should be pointed out that this is a transmission plot, and the low transmissions of o-phenylenediamine 2 HCl and ABTS in the 415-nm range correspond to absorbances in the range of 1 to infinity, i.e., very high absorbance. Hydroquinone has much less absorbance; and indeed, when tested at pH 6 and pH 7 was not very sensitive. At pH 8, it was not stable enough to use as a substrate. O-phenylenediamine 2 HCl, when tested at pH 4, was 10 times more sensitive than 5 AS; but its solutions were not quite as stable as 5 AS.

ABTS was investigated more extensively because not only is it a substrate of high sensitivity, as shown by Fig. 6, but solutions of it are very stable. Monitoring the production of product by the absorbance at 414 nm, the enzyme activity was tested at various pH's. As shown by Fig.7, pH 3 and pH 4 give the best reaction rate. This is surprising because pH 6 appears to be preferred.[14]

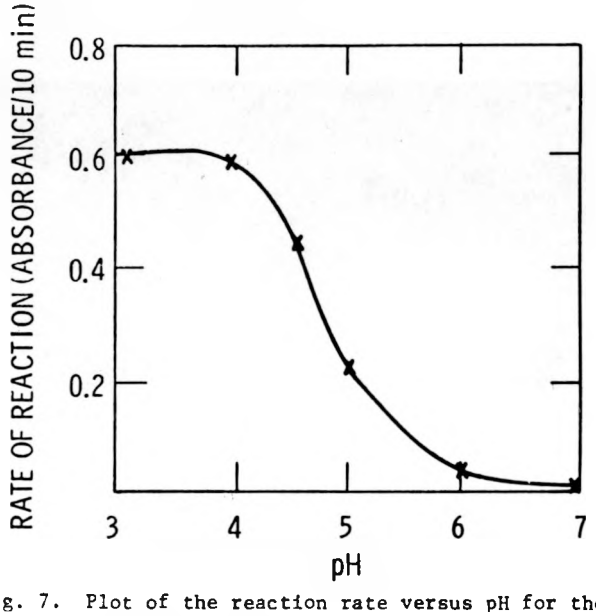

Fig. 7. Plot of the reaction rate versus pH for the substrate ABTS.

Figure 8 is a plot of the absorbance of 5 AS and ABTS versus time of reaction. The same enzyme concentration was used for both curves. The concentration of 5 AS was near its limit of solubility, 11 mM. ABTS at 0.2 mM has about three times more absorbance than 5 AS. ABTS solutions up to 56 mM can be made, which would further increase the sensitivity. This greater solubility results in a further advantage of ABTS over 5 AS; namely, that ABTS can be stored in a concentrated solution and diluted into a buffer the right pH for the enzyme reaction, thereby eliminating the pH meter, which is needed for doing the ELA test with 5 AS.

However, a solution that stops the enzyme reaction, and at the same time stabilizes the blue product, has not yet been found. NaN_3 and Na_2S both cause fading of the blue colored oxidation product. H_2SO_4 and trichloroacetic acid cause an increase in the blue color. Table VIII shows the effects of various concentrations of HF and HCl. Increasing the concentration of HF from 0.03 to 0.3 M increases the stability of the reaction product solution.

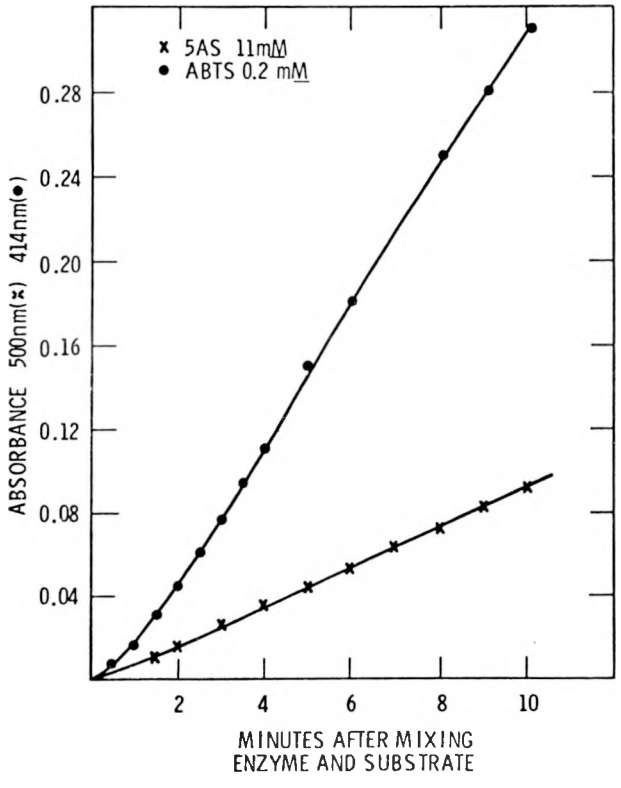

Fig. 8. Measurement of the absorbance of 5 AS and ABTS versus time after mixing enzyme and substrate. Both curves were at the same enzyme concentration.

TABLE VIII

EFFECT OF TWO STOPPING REAGENTS

ON THE HRP-ABTS SYSTEM

Stopping Reagent	Absorbance Change in 10 Minutes at 414 nm
0.03 M HF	0.06
0.3 M HF	0.02
0.55 M HF	0.04
0.1 M HCl	0.05
1.0 M HCl	0.02

However, further increasing the HF to 0.55 M did not stop the absorbance change; in fact, it increased it. Other chemicals will be investigated. However, ABTS can still be used without a stopping reagent, if the solution is read after a definite time period, or if the ABTS is withdrawn from the Microtiter well and thereby separated from the enzyme, which is bound to the Microtiter well.

C. Covalent Binding of Antigens to Microtiter Trays

Covalent binding of a protein to the Microtiter wells was investigated. This would permit reuse of the antigen - an important factor in a case where the antigen has to be purified. The various chemical reactions that were followed are shown in Appendix D. Even though polystyrene is reported to soften only at 85°C, the 70°C treatment in Step 2 deformed the microtiter plate. Reducing the temperature to 60°C produced better results. The procedure appears to form many reactive groups on the polystyrene, as shown by their reaction with β-naphthol; however, these reactive groups combine very slowly with protein.

Another consideration in the reuse of the Microtiter-antigen plates is the washing material. The antigen antibody bond is strong and not too many chemicals successfully dissociate it. Four M KSCN even with sonication was not successful. Seven M guanidine HCl and 1 M HCl for 30 min appeared to denature the antibody and the conjugate, and caused them to stick in the wells instead of removing them. Sodium dodecyl sulfate appears to be the best washing material. Following the reactions in Appendix

D, Part 1, and using sodium dodecyl sulfate to wash in between tests, enough antigen was bound to the Microtiter tray to produce 120 ELA readout units of product. Following the gluteraldehyde modification, (Appendix D, Part 2) enough antigen was retained to produce 60 ELA readout units of product. Further chemical modifications will be tried to increase the bound antigen.

D. Control of Each Serum by Use of an Indifferent Antigen

During the North Texas hog cholera outbreak of July-August 1975, we observed that the binding of individual negative field serums to the infected cells varied tremendously. Within a group of 30 samples, it was common to observe a 10-fold difference in binding. This degree of variability made it impossible to utilize a "standard" negative serum as a control. Using the reasoning that most of the binding was nonspecific, we decided to infect only alternate rows of the Microtiter trays with virus, leaving the noninfected cells to remain as a binding control for each serum. Serum with hog cholera antibody would presumably evoke a darker reaction in infected cells than it would in the noninfected cells; but a serum with a high degree of nonspecific binding would result in equally dark substrate in both infected and noninfected wells. The idea proved to work very well and is now an integral part of the ELA test for hog cholera. Extensive hog cholera data are presented in Section II.

Using the same reasoning (nonspecific binding) to at least partially explain the degree of false positive trichinosis reactions observed from serum obtained at slaughter plants, we have attempted to devise an indifferent antigen to use as a balance with trichina antigen. Early results of experiments have been quite promising, and they are discussed in Section III-H.

VI. TRAINING, MEETINGS AND PROBES

A. Training

In November 1975, Dr. William Bishop (APHIS, USDA) spent three days in our laboratory to learn the ELA test for trichinosis. In December 1975, we trained three individuals in all aspects of ELA reagent production and testing. Those trained were: Dr. Richard Hidalgo, Department of Veterinary Microbiology, Texas A&M University; Carl Custer, Washington, DC; Fred Ross, Los Angeles, CA.

In December, Dr. Saunders spent a week at VSL, Ames, Iowa, training the Diagnostic Virology Staff in the use of ELA for hog cholera diagnosis.

B. Meetings

Saunders attended the XX World Veterinary Congress, held at Thessaloniki, Greece, and presented a paper co-authored by Clinard and Bartlett, entitled, "Rapid Disease Screening with Enzyme-Labeled Antibodies." Saunders attended the US Animal Health Association (USAHA) Meeting in Portland, Oregon, and gave an invited report to the Swine Disease Committee concerning the development of the ELA test for hog cholera. In June 1976, Clinard presented a paper on serological testing for trichinosis in pigs at the 1976 Congress of the International Pig Veterinary Society in Ames, Iowa. The paper has been published in the proceedings.[15] M. L. Bartlett attended the 1976 American Society of Biological Chemists' Meeting and had the opportunity to discuss readout instrumentation with the various companies (specifically the six companies mentioned in the text), and also discuss the various inert substrates available for affinity chromatography and for covalently binding antigens and the problems associated with these substrates.

C. Short Probes

1. Toxin Detection. While training Carl Custer, who was interested in the use of ELA for toxin detection, we did a short probe into the detection of Staphylococcus A Enterotoxin. A different technique was used than that reported previously.[16] First, a high-titered rabbit antiserum to Staph A Enterotoxin was bound to the Microtiter tray. Solutions containing either small amounts of Staph A Enterotoxin, or Brucella abortus soluble antigen (control) were incubated on the tray for 10 min. Following the wash, the same rabbit antiserum to the Enterotoxin, but labeled with peroxidase, was added to the tray for 5 min. After washing, substrate was added for 10 min. A 0.05 ml of solution containing 5×10^{-11}g (5×10^{-5}µg) of Enterotoxin yielded a fairly strong positive reaction. Additional work into this very promising area should start as soon as possible.

2. Lymphocyte Stimulation. We investigated the feasibility of disease detection via impedance measurements. The disease system we used was PPD stimulation of lymphocyte from a tubercular animal, and we measured impedance changes with a Bactometer. This method should be faster than FMF, since results can be obtained in 12 hours; it involves no staining of cells and the instrumentation is cheaper than FMF, although much less versatile. The Bactometer can continuously monitor impedance changes in a solution. It plots the ratio of the impedance in the sample well to the sum of the impedances in the sample and reference well. It can handle a total of 32 sample and 32 reference wells at one time.

We placed lymphocytes from a tubercular animal in the sample and reference wells and added PPD to the sample well. We tried a variety of lymphocyte samples; namely, lymphocytes isolated with Ficoll-Hypaque solution, preferential lysis of red blood cells, and whole blood. We also varied the media, trying RPMI 1640 with $NaHCO_3$ buffer and with HEPES buffer. The only successful method was lymphocytes isolated with Ficoll-Hypaque and RPMI 1640 with HEPES buffer. The results are shown in Fig. 9. Since the cells were from an animal infected with M. bovis, the results are consistent with a different impedance response caused by PPD-Bovis.

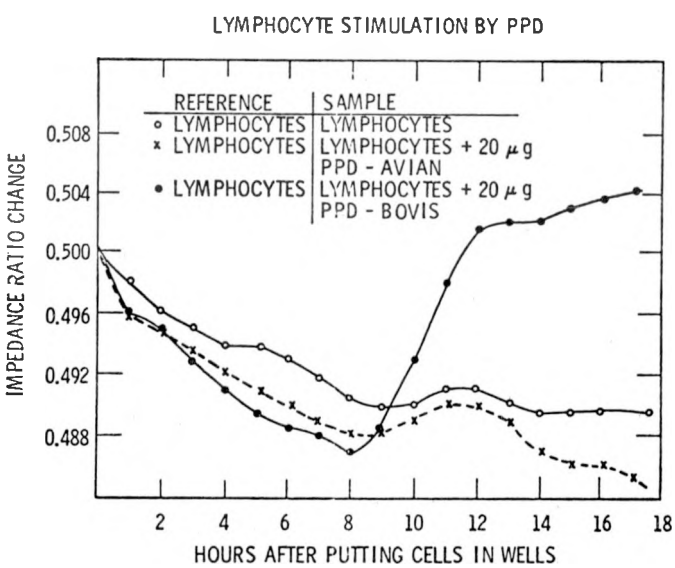

Fig. 9. Lymphocyte stimulation by PPD as measured by impedance.

VII. AUTOMATION OF SEROLOGICAL TEST

To satisfy the requirement for processing 600 whole blood samples/h using the ELA methodology, full automation is necessary. This automation should utilize state-of-the-art computer, electronic, and mechanical technologies. Each of the four basic processing steps (sample transfer, wash, reagent addition, and readout) are being automated individually so that the problems associated with each may be studied and optimally solved. The fully automated process will use the 96-well disposable Microtiter trays; however, only the 24 centrally located wells will be used for these preprototype instruments. Mechanical components have been completed for each of these devices. A microcomputer system, similar to that discussed in Reference 17, will be utilized to test the preprototype instruments and later incorporated as part of the prototype instrument.

The geometry of the 96-well Microtiter tray allows combinations of parallel and serial processing steps. A single well (1 sample), a row (8 samples), a column (12 samples), or the entire tray (96 samples) can be processed in a single operation. Various combinations can be used to accommodate the required high sample processing rate.

A. Automated Sample Transfer Device

It is necessary to simultaneously transfer all the blood samples from the sample tray to the processing tray. The sample transfer device has the following design specifications:

1. Automatic cleanup of the transfer pipettes by flushing with the wash solution.
2. Well-to-well deviations on sample transfer of less than 5%.
3. Total volume of blood transferred 0.2 ml.

A photograph of the preprototype unit is shown in Fig. 10. The processing starts by placing the tray containing blood samples into the transfer device and initiating the automatic withdrawal of blood from each well. This sample tray is removed, and the processing tray, with antigen-coated wells, is placed into the machine; and the blood samples are simultaneously transferred into the processing tray. This tray is then removed for the required incubation time. The sample transfer pipettes are automatically cleaned as indicated above. Adjustments

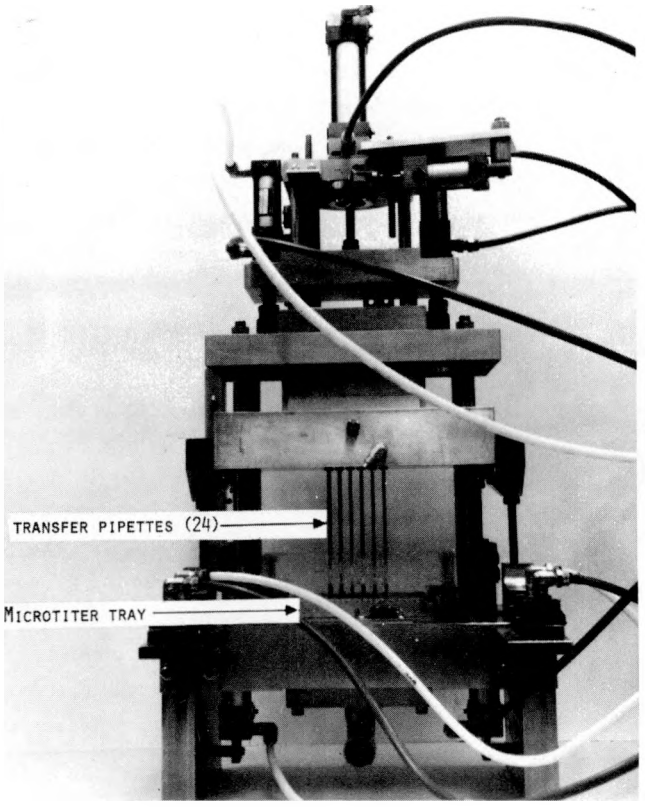

Fig. 10. Automated sample transfer device.

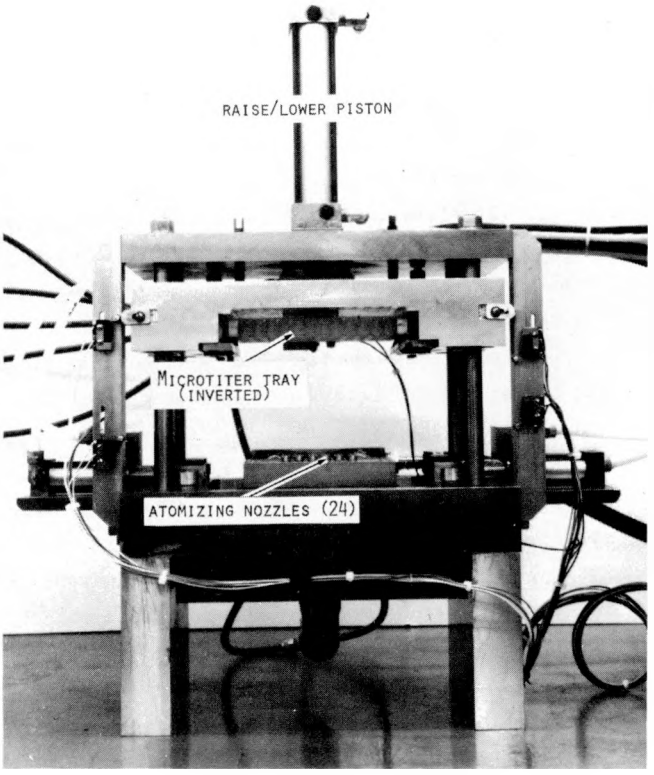

Fig. 11. Automated wash.

are provided for metering the precise amount from each pipette. This unit will be controlled by a microprocessor system.

B. Automated Wash

Present data indicates that the wash process will not be the same for the soluble antigens and for the cell-bound antigens. This fact dictates that some flexibility must be available for this process. The individual wells of the Microtiter tray must be washed gently enough so the bound antigen-antibody molecules are not removed, but turbulent enough so the unbound and excess materials are removed.

Figure 11 shows the equipment for the automatic washing. The tray is placed upside-down onto the washing device, and washing is accomplished by spraying liquid up onto the tray. Since capillary forces frequently cause a random holdup of small amounts of wash water in the individual trays, two methods have been devised for removing this water. One method provides shaking the trays in a vertical motion, and the other provides an air blast from the water nozzles. It is expected that either of these methods or a combination of the two methods will be satisfactory for removing the unwanted wash water.

A number of spray configurations have been investigated. The best results were obtained by using individual, atomizing nozzles for each well. This configuration allows for easy removal of excess water; however, care must be taken to avoid completely drying the coated surfaces. The washing step is mechanically similar between each reagent addition; however, the composition of the wash water is sometimes varied with the separate washes.

C. Automatic Reagent Addition

While the specific reagents are different for each of the steps in the process, the principle of the operation is the same; so a single automatic

reagent addition unit has been built for testing purposes. In a final system, three or more similar units would be required. The reagent addition steps involve the addition of about 0.2 ml of reagent to each well. These additions should be made in easily regulated time intervals of a few seconds. Assuming that each drop contains about 1/20 ml -- 4 drops of reagent are needed for each well.

The device that was designed for testing various features of the reagent addition is shown in Fig. 12. Some of the features that have been studied are the design of a simple flow adjustment valve. A satisfactory valve has been designed that crushes a stainless steel sleeve around the plastic transfer tubes to constrict the flow. This valve seems to be superior to compressing the plastic transfer tube without the sleeve.

We have not studied the problems that may arise from the precipitation of solids from the reagents onto the walls of the pipettes. This problem should not be too serious, unless the precipitation near the tips of the pipettes affects the drop size and therefore the time interval between drops. The capability has been built into the device to keep the tips of the pipettes submerged if this becomes a problem.

D. Automated Readout

A second version of the automated readout is presently being fabricated. The Microtiter tray,

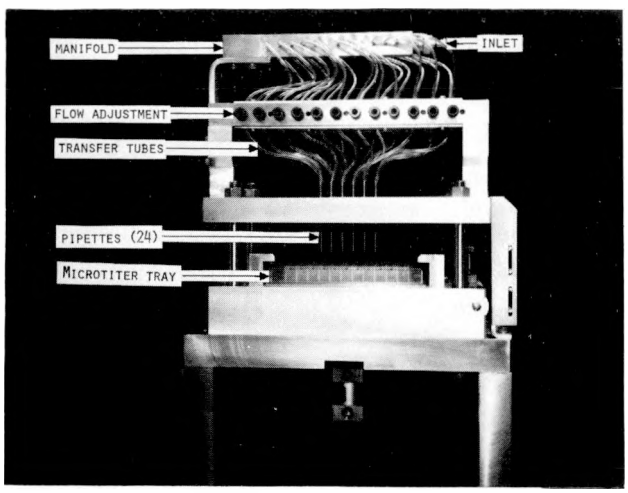

Fig. 12. Automatic reagent addition.

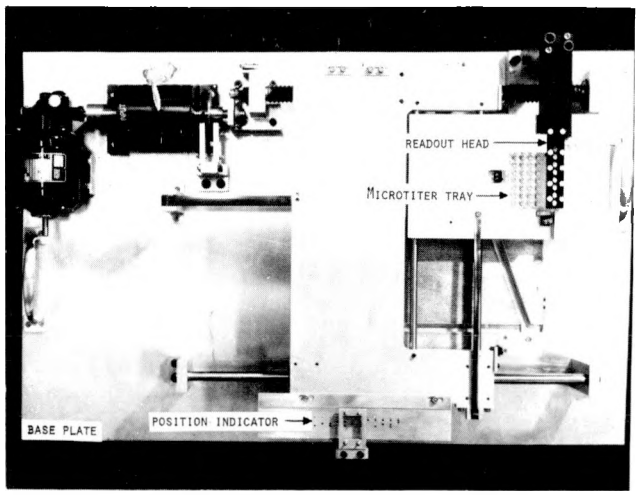

Fig. 13. Automated readout.

shown in Fig. 13, is in the reading position. A load position was also incorporated into the design to accommodate the necessity for a light, tight enclosure. This version will read simultaneously the light absorption from each of 4 wells. In the final system, 8 wells will be read at one time. By reading all 8 wells together, it will be possible to read 1600 samples per hour. Two options are being considered in the automated readout shown. In the option shown, it is presumed that it will be possible to read directly through the sample well. If the quality control of the plastic trays is not sufficient for this type of measurement, it will be necessary to withdraw the fluid into a separate cell to determine the absorption. Since this will complicate the design, it was decided to first investigate the possibility of reading through the trays.

E. Microprocessor Controller

The experience gained with microprocessors on the Electronic Identification and Physiological Monitoring projects has shown that these devices offer the flexibility and reliability necessary for this stage of the development. It is expected that a microprocessor will be incorporated into the initial field experiments, and possibly in the final prototype system. The microcomputer system is similar to the one discussed in Reference 17.

F. Further Developments

After we started on the development of automation, there has been some indication that commercial

automated equipment will be manufactured for ELA processing. Therefore, it was decided to evaluate the LASL equipment, and any commercial equipment that is available during the next 15 months; then decide on the advisability of building the fully automated prototype processor at the LASL. If commercial units could fulfill the requirements of slaughter-house processing, a fully automated system would not be constructed by the LASL.

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APPENDIX A

LIST OF EQUIPMENT, SUPPLIES, AND REAGENTS FOR AN ELA FIELD LABORATORY KIT

ELA Equipment

pH meter - preferably with single electrode
Eberbach horizontal shaker
Magnetic stirrer + stirring bars
Hot plate
Beam balance
Readout instrumentation
Timer (2)
Vortex mixer may be useful

ELA Supplies

Pipettes - 1, 5, 10 ml
Pasteur pipettes - 5-3/4 in.
Rubber bulbs
Linbro trays - IS-FB-96-TC + (Transfer plates and transfer plate holders)
Linbro tray covers for above
2 in. plastic tape
Small diluting tubes
Test tube racks for above
Cornwall-type reagent adders
Beakers - 25-, 50-, 100-, 500-, 1000-ml sizes
Graduates - 100-, 250-, 500-, 1000-ml sizes
Flasks - 125, 250, 1000 ml sizes
Squeeze bottles - 250, 500 ml
Reagent bottles for storage
Marking pens
0.025 ml and 0.05 ml dripper pipettes (Microtiter type)
Weighing spatula
Large (6-10 liter) dispensers

ELA Reagents

Horseradish peroxidase labeled anti-swine gamma globulin
0.5 molar Na_2HPO_4
0.5 molar NaH_2PO_4
10% sodium azide solution
2% sodium hydroxide solution
1 normal H_2SO_4 solution
5 aminosalicylic acid hydrochloride (packets of 225 mg each)
5% hydrogen peroxide - must be fresh
NaCl
Tween 80

APPENDIX B

HOG CHOLERA ELA ENDPOINT DATA

SUMMARY

Serum Bank Sample	HCSN Titer	ELA Titer	Serum Bank Sample	HCSN Titer	ELA Titer
16604-2	4	64	16665-2	16	128
16624-2	4	Negative at 1:16			
16661-2	4	64	16607-2	32	512
			16626-2	32	128
16610-2	8	32	16633-3	32	128
16614-2	8	32			
16627-2	8	128	16602-3	64	512
16630-2	8	16	16604-3	64	256
16642-2	8	32	16614-3	64	512
			16620-3	64	512
16609-3	16	128	16622-3	64	256
16615-2	16	128	16624-3	64	512
16620-2	16	128	16628-3	64	512
16650-2	16	128	16636-3	64	512
16653-3	16	128	16641-3	64	256

Serum Bank Sample	HCSN Titer	ELA Titer
16642-3	64	256
16650-3	64	256
16661-3	64	256
16629-4	128	512
16615-3	128	512
16629-4	128	512
16604-4	128	256
16614-4	128	256
16610-3	128	512
16677-3	128	256
16610-4	256	512
16660-4	256	128
16624-4	256	256
16630-4	256	256
16623-2	256	256
16626-3	256	256
16604-6	256	256
16650-4	256	512
16614-6	256	128
16607-4	256	256
16627-4	256	256

Serum Bank Sample	HCSN Titer	ELA Titer
16603-4	512	1024
16661-5	512	128
16602-4	512	256
16630-6	512	256
16623-2	512	512
16664-4	512	512
16633-5	512	256
16610-6	512	512
16635-5	512	256
16609-4	512	256
16660-5	512	512
16602-5	512	512
16664-5	1024	256
16650-5	1024	512
16642-6	1024	512
16628-4	1024	2048
16641-4	1024	1024
16621-5	1024	256
16665-5	1024	512
16626-5	1024	512
16628-5	1024	512
16620-5	1024	512
16653-4	1024	1024

APPENDIX C

PRELIMINARY ELA RESULTS ON MIXED BOVINE BRUCELLOSIS SERA

Tube No.	I.D. No.	VSL, Ames, IA (4/19/76) Card	Tube	M.E.	Riv.	ELA S/N Result LASL	Comments
1	3384	+	+200	+200	+200	10.2+	STT&ME = 12,800
2	2901	+	+200	I200	+200	7.7+	STT&ME = 6,400
3	3341	N	+200	N25	N25	1.6-	Non-spec.
4	2417	N	I200	N25	N25	1.2-	Non-spec.
5	ELA3	N	N25	N25	N25	1.9-	#2 (3/24/70), V, Inf.
6	ELA4	+	I200	+100	I50	6.2+	(4/7/70)
7	ELA5	+	+200	+100	I100	7.4+	(5/26/70)
8	ELA18	N	N25	N25	N25	0.7-	#24 (5/26/70), V, NI
9	ELA20	$N^{(Tr)}$	+50	+50	+25	7.0+	#32 (2/24/70), V, Inf.
10	ELA21	$\pm$	I100	+50	+25	9.3+	(3/10/70)
11	ELA22	+	+200	+200	I200	11.7+	(5/26/70)
12	ELA23	N	I50	N25	N25	1.3-	#34 (2/17/70), V, NI
13	ELA25	N	+25	N25	N25	1.2-	(5/26/70)
14	ELA32	N	N25	N25	N25	1.3-	#41 (2/10/70), V, Inf.
15	ELA33	+	+200	+200	+100	8.5+	(2/24/70)

Tube No.	I.D. No.	VSL, Ames, IA (4/19/76) Card	Tube	M.E.	Riv.	ELA S/N Result LASL	Comments
16	ELA34	+	+200	+200	+200	11.2+	(5/26/70)
17	ELA41	N	N25	N25	N25	3.9+	#47 (3/24/70), V, Inf.
18	ELA42	N	N25	+25	N25	4.0+	(4/7/70)
19	ELA43	+	+50	+50	I25	7.8+	(4/21/70)
20	ELA44	+	+200	I200	I100	10.5+	(5/26/70)
21	ELA49	N	+25	N25	N25	1.6-	#59 (2/10/70), NV, Inf.
22	ELA50	N	+50	N25	N25	2.4+	(2/24/70)
23	ELA51	N(Tr)	I50	N25	N25	3.0+	(3/3/70)
24	ELA61	N	I50	N25	N25	1.0-	#69 (3/24/70) NV, NI
25	ELA64	N	+50	N25	N25	1.8-	(5/5/70)
26	ELA65	N	I100	N25	N25	1.2-	(5/19/70)
27	ELA66	N(Tr)	I100	N25	N25	2.4+	#73 (2/24/70), NV, Inf.
28	ELA67	N(Tr)	+50	N25	N25	3.2+	#73 (2/24/70), NV, Inf.
29	ELA68	±	+50	I25	N25	5.5+	(3/10/70)
30	ELA69	+	+200	+100	+25	6.8+	(3/17/70)
31	ELA70	+	+200	+200	+200	8.9+	(5/26/70)
32	ELA77	N	I50	N25	N25	2.0+	#75 (2/24/70), NV, Inf.
33	ELA79	N(Tr)	+50	I25	N25	4.8+	(3/10/70)
34	ELA81	+	+50	+50	I25	5.4+	(3/24/70)
35	ELA82	+	+100	+25	I50	7.5+	(4/7/70)
36	ELA84	+	+200	+200	I200	8.0+	(5/26/70)
37	ELA87	N	I50	N25	N25	7.0+	#79 (2/10/70), NV, Inf.
38	ELA88	N	I50	N25	N25	2.4+	(2/24/70)
39	ELA89	N	I50	N25	N25	3.4+	(3/10/70)
40	ELA91	N(Tr)	I50	+50	N25	5.0+	(4/7/70)
41	ELA98	N	I25	N25	N25	1.8-	#89 (2/3/70), NV, Inf.
42	ELA99	N(Tr)	+50	N25	N25	2.3+	(2/10/70)
43	ELA100	+	+200	+100	+50	6.9+	(2/24/70)
44	ELA101	+	+200	+200	I50	13.5+	(5/26/70)
45	ELA105	N	N25	N25	N25	1.8-	#93 (2/24/70), NV, Inf.
46	ELA106	N	+50	N25	N25	2.0+	(3/10/70)
47	ELA107	N	+25	N25	N25	3.0+	(3/24/70)
48	ELA108	+	+200	I100	I50	2.6+	(4/7/70)
49	ELA109	N	N25	N25	N25	3.2+	#94 (2/3/70), NV, Inf.
50	ELA110	N	+50	N25	N25	3.7+	(2/17/70)
51	ELA111	N	+50	N25	N25	5.3+	(3/3/70)
52	ELA112	N	+50	N25	N25	11.9+	(3/17/70)
53	ELA113	+	+200	+200	I100	18.2+	(4/7/70)
54	ELA115	N	N25	N25	N25	2.6+	#97 (2/3/70), NV, Inf.
55	ELA116	+	I200	N25	N25	11.4+	(2/17/70)
56	ELA117	+	I100	I100	I25	15.6+	(5/26/70)
57	3384	+	+200	+200	+200	11.5+	STT&ME = 12,800
58	2901	+	+200	I200	+200	4.2+	STT&ME = 6,400
59	3341	N	+200	N25	N25	1.6-	Non-spec.
60	2417	N	I200	N25	N25	1.0-	Non-spec.

NV = Not vaccinated as a calf

V = Vaccinated with strain 19 as a calf

NI = Not infected

Inf. = Infected

Positive ELA Result = S/N $\geq$ 2.0

APPENDIX D

Part 1. Reaction of Protein with Polystyrene

1. React polystyrene with 47% HNO_3 in H_2SO_4 for 20 minutes at 0°C. Wash with water.
2. React with 6% sodium dithionite in 2 M KOH at 70°C for 5 hours.
3. Diazotize with 0.5% $NaNO_2$ in 0.6 M HCl 0°C for 30 minutes.
4. Wash with 1 M HCl 0°C.
5. Couple to protein immediately.

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Part 2. Gluteraldehyde Modification

1. After Step 2 above, which puts amino groups on the polystyrene, react with 8% gluteraldehyde adjusted to pH 9.4 with 0.2 M $NaHCO_3$ for 30 minutes. Rinse with cold phosphate buffer.
2. React with protein.
3. Reduce with $NaBH_3CN$.
