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Rapid Micromethod of Screening for Antibodies to Disease Agents Using the Indirect Enzyme-Labeled Antibody Test

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We have developed a rapid and simple indirect enzyme-labeled antibody procedure suitable for screening for antibodies to viral, bacterial, and parasitic antigens. Disposable microplates were utilized as antigen carriers and test vehicles. The test worked equally well with serum or whole unclotted blood. Using an automatic pipettor, 96 tests could be performed in 30 to 60 min. We report on the development of enzyme-labeled antibody procedures for the detection and surveillance of animal diseases.

The United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) and the Los Alamos Scientific Laboratory have been cooperating in an effort to develop a rapid test system for the detection of antibodies in animals to a multiplicity of disease agents. Such a system must be sensitive, yet environmentally safe, because of its potential use in such places as slaughter plants. Reagents must be inexpensive, stable, and simple to use. The method must be amenable to automation to keep up with slaughter rates of about 600 animals/h. The indirect enzyme-labeled antibody (ELA) test appears to adequately meet these criteria.

Others (3, 4, 6, 10-13) have reported on the principle of using immunoenzyme techniques for the detection of serum antibodies and antigens. However, these procedures as reported are not ideally suited for large-scale economic screening of animal and human sera for antibodies (or antigens) because of the fairly long incubation times required (60 min to several hours), types of reaction vehicles employed (such as individual polystyrene tubes or culture dishes), or the relatively large amounts of reagents required in macrotechniques. We have previously reported (8, 9) an ELA test system based on the use of cellulose acetate filters as the antigen vehicle. This system is fairly rapid and sensitive, but has the following drawbacks: (i) washing of excess reagents from the filter after the incubation steps is time-consuming and often incomplete; (ii) elution of the reaction product from the disk is often incomplete, and eluted reaction products are not stable because of the conditions of elution; (iii) handling of the individual disks is somewhat cumbersome; (4) binding of virus-infected cells (cell-virus antigens) to the filters is not possible. The microplate method described herein eliminates these problems without apparent loss of sensitivity.

MATERIALS AND METHODS

Antigens. A lipid-free saline extract of *Trichinella spiralis* larvae was prepared in this laboratory according to the method described by Chaffee et al. (2). Soluble *Brucella abortus* antigen, prepared from autoclaved culture filtrate, was supplied by R. D. Angus, Veterinary Services Laboratory (VSL), APHIS, USDA, Ames, Iowa. Hog cholera virus was grown in PK-15 cells in this laboratory as described by Carbrey et al. (1).

ELA (conjugate). Horseradish peroxidase (type VI, Sigma Chemical Company, St. Louis, Mo.)-labeled rabbit antiporcine gamma globulin was prepared by the method of Nakane and Kawaoi (5). The conjugate utilized in these experiments contained an average molar ratio of 4 mol of enzyme to 1 mol of gamma globulin. The sterile filtered conjugate was stored in 1-ml aliquots at 4 C. No loss of activity has been noticed over a 1-year period. Addition of phenol to a final concentration of 0.5% to nonsterile conjugate does not appear to adversely affect activity. Depending on the antigen system, the conjugate was used at dilutions ranging from 1:100 to 1:500. Dilutions are made in 0.5 M NaCl containing 1% Tween 80 (Fisher Scientific, Fair Lawn, N.J.) adjusted to pH 7.4 with 0.1 M K_2HPO_4. The high salt concentration and Tween 80 reduce nonspecific binding.

Sera and whole blood. Control sera and sera from swine infected with hog cholera virus or *B. abortus* were supplied by D. E. Pietz, VSL, APHIS, USDA, Ames, Iowa. Sera from swine infected with *T. spiralis* were supplied by W. Zimmerman, Veterinary Medical Research Institute, Iowa State University, Ames. Packinghouse sera and citrated porcine blood were supplied by C. L. Campbell, USDA, APHIS, Veterinary Services, through the courtesy of the Schwartzmen Packing Company, Albuquerque, N.M. Blood and sera were routinely diluted between

1:5 and 1:20 in the same fluid used for conjugate dilutions.

Binding of antigens. Disposable microtiter trays (Linbro Chemical Co., New Haven, Conn.), types IS-FB-96 and IS-FB-96-TC, are used as test vehicles for soluble and cell-virus antigens, respectively. Soluble antigens are bound in the following manner. First the trays are rinsed in distilled water. Then 0.05 ml of a 2.5% solution of fetal bovine serum (FBS) (Rheis Chemical Co., Phoenix, Ariz.) in saline is added to each well of the tray. Several trays are prepared at a time, and a 96-channel automatic pipettor is used (Cooke Engineering Co., Alexandria, Va.) to add the FBS. The trays are allowed to air-dry, and then 0.05 ml of 0.25% glutaraldehyde in phosphate-buffered saline (adjusted to pH 7.0 with 0.1 M K_2HPO_4) is added to each well to fix the FBS to the plastic surface. After 30 min of fixation, the trays are washed four times in distilled water. The FBS treatment provides a suitable binding surface for some of the soluble antigens we have worked with. The *T. spiralis* antigen and the hog cholera virus system do not require this preliminary step for successful binding, but initial experiments with new antigens should probably employ it.

For each FBS-treated tray, 5.5 ml of saline containing 0.02 mg of *T. spiralis* or other protein antigen is prepared, and 0.05 ml of this solution is added to each well. After the antigen solution has air-dried, the plates are rinsed in distilled water to remove unbound antigen. We have found it impossible to remove bound antigen by ordinary wash procedures. Antigen-bound plates may be stored in a dry, clean environment for at least 1 year without loss of activity.

An example of binding a cell-virus antigen follows. Preliminary binding of FBS to the tray is not necessary. PK-15 cells confluent in 250-ml T-75 flasks (Falcon Plastics, Oxnard, Calif.) are infected with 10^6 plaque-forming units of end point hog cholera virus. Two days later, the cells are trypsinized, washed, and transferred in 16 ml of fresh medium to microtiter trays, 0.05 ml per well. Each T-75 flask provides enough infected cells to fill three trays. After the cells become confluent in the wells of the tray (24 to 48 h), the trays are rinsed three times with saline. The cells are then fixed for 10 min with a mixture of 20% acetone in saline brought to pH 7.4 with 0.1 M K_2HPO_4. Greater concentration of acetone inactivates the antigen. After fixation, the trays are rinsed three times with distilled water. When dry, the trays are stored in a vacuum at room temperature until used. Hog cholera antigen remains stable for aproximately 2 weeks. Reasons for loss of antigen stability are currently under investigation.

ELA test protocol for soluble antigens. Standard dilutions (usually 1:10 or 1:20) of test sera are prepared. At least one known positive and negative sample should be run per group. When large numbers of samples are to be tested (more than 10), we use an automatic pipettor to add reagents. To accomplish this, aliquots of the serum dilutions, conjugate dilution, or substrate solution are added to untreated microtiter trays; the pipettor is then used to transfer the reagents to the tray containing antigen. A series of three pipettor heads are required for a complete test. If only one head is available, it must be thoroughly washed and blown dry between steps. To each test well, 0.05 ml of serum is added. The sera are incubated for 5 min on a horizontal shaker (Eberbach, Ann Arbor, Mich.) at 60 cycles/min. The tray is then washed rapidly six times with a stream of wash fluid (0.15 M NaCl containing 0.5% Tween 80). After the rapid washes, two washes of 2 min each are done on the shaker. After excess wash fluid has been shaken from the wells, 0.05 ml of conjugate dilution is added per well. After a 5-min incubation, the tray is washed as before and 0.05 ml of substrate solution per well is added. (Substrate stock solutions consist of 2.25 mg of 5-aminosalicyclic acid hydrochloride per ml [Matheson, Norwood, Ohio] in water, and 0.05% hydrogen peroxide in water. Working solutions are prepared by adding enough 1% NaOH to 9 ml of 5-amino-salicyclic acid solution to bring the pH to 6.0 and then adding 1.0 ml of the H_2O_2 solution. This mixture is stable for several hours, but gradually darkens.) After 10 min, 1 drop of a 0.3% sodium azide solution is added to each well to stop the enzyme reaction. The substrate-reaction product solution is transferred with a Pasteur pipette into tubes containing 1 ml of 0.005 N H_2SO_4 (the H_2SO_4 stabilizes the reaction product). This solution is transferred to a cuvette, where a reading of transmitted white light is obtained in an instrument built in this Laboratory (9). Light transmitted (no interference filters are used) through the sample is carried by fiber optics to a photodiode. Resistance of the photodiode is proportional to the amount of light transmitted through the sample and is read by a digital ohmmeter. Absorbance at 450 nm has been used by others to quantitate the product formed in the horseradish peroxidase-catalyzed reaction (11). The reading of a blank, consisting of substrate and sodium azide in 0.005 N H_2SO_4, is subtracted from the sample reading.

For a given antigen, establishing whether a sample is positive or negative requires experimentation with several known positive and negative sera. In most of our work we have considered a signal-to-noise ratio (S/N), obtained by dividing the reading of a suspect serum by the reading of the negative standard, of 1.5 as being positive. Testing of 100 negative and 50 hog cholera serum-neutralization-positive sera (from Diagnostic Reagents Section, VSL, APHIS, USDA, Ames, Iowa) showed that no negative serum had a S/N greater than 1.4, and no positive serum a S/N of less than 1.5. In the trichinosis test system, the S/N has not been as useful in distinguishing positive from negative sera because of the wide variation in ELA signal units obtained with different negative sera.

ELA test protocol for cell-virus antigens. The protocol for testing sera against cell-virus antigens follows the same sequence as that for soluble antigens. However, the serum incubation with antigen is 10 min; conjugate incubation remains at 5 min. There are three wash steps of 3 min each after the

six quick washes after both serum and conjugate incubations. Substrate incubation requires a period of 10 to 20 min. The substrate-reaction product solution is read as above to obtain a numerical value.

RESULTS

Soluble bound antigens. The data in Table 1 are representative of those obtained with swine experimentally infected with *T. spiralis*, noninfected packinghouse swine, and pooled sera from conventionally raised and specific-pathogen free *T. spiralis*-negative swine. Of 250 packinghouse animals tested, 37 (15%) were judged serologically positive; i.e., had S/N values of well over 1.5. Diaphragms of 11 of these animals were tested by R. Furrow, Agriculture Research Center, Beltsville, Md., and no *T. spiralis* larvae were found; therefore, these animals are presently considered to be false-positives.

TABLE 1. *Detection of Trichinella spiralis antibodies in swine*

Animal[a] no.	Day post-infection	ELA signal units (sera diluted 1:20)[b]	(S/N)[c]	ELA diagnosis[d]
6501M	0	25, 30: 27.5 ± 3.5	1.0	Negative
	7	37, 40, 36: 37 ± 2.1	1.45	Negative
	21	55, 44: 49.5 ± 7.8	1.9	Positive
	42	116, 103, 114: 111 ± 7.0	4.2	Positive
M5	0	18, 10: 14 ± 5.7	0.54	Negative
M11	0	38, 36: 37 ± 1.4	1.4	Negative
M31	0	80, 79: 79.5 ± 0.7	2.9	Positive
N	0	24, 30, 23, 24, 27, 26: 26 ± 2.6	1.0	Negative
SPF	0	15, 14, 12, 16, 16, 12: 14 ± 1.8	0.54	Negative

[a] Animal 6501M was fed 2,500 *T. spiralis larvae* and contained 0.01 cyst/g of diaphragm at the time of slaughter. Serum was provided by W. Zimmerman, Veterinary Medical Research Institute, Ames, Iowa. Animals M5, M11, and M31 were packinghouse swine. M31 was negative as determined by testing of the entire diaphragm by R. Furrow, Agriculture Research Center, Beltsville, Md. N and SPF represent pooled sera from conventionally raised negative swine and specific pathogen-free swine respectively.

[b] ELA signal units are given for two or three duplicate determinations, followed by the mean ± standard deviation of the duplicate determinations.

[c] Obtained by dividing the sample readings by reading of the negative pool, N.

[d] ELA diagnosis based on S/N (signal-to-noise ratio) greater than 1.5 as positive.

Cell-virus-bound antigens. Data obtained from swine experimentally infected with hog cholera virus are presented in Table 2. Hog cholera-infected swine can be serologically detected by ELA as early as 2 weeks after infection. Controls consisted of groups of both noninfected swine and swine infected with erysipelas.

Whole blood versus serum. Table 3 illustrates that dilutions of citrated blood (heparinized blood is also suitable) appear to work just as well as dilutions of serum. Whole blood is used at one-half the dilution of serum to account for the volume difference between blood and serum. The example compares a trichina-infected pig to its preimmune serum and to other negative animals.

Multiple disease screening. Table 4 shows that screening for antibodies to more than one disease agent in a single test is possible. In this case, both *Brucella* and trichina antigens were bound to the microtiter tray. Swine containing antibody to either agent are positive in the ELA test. To discriminate the antigen to which an individual positive animal has been exposed, the serum must be retested against each antigen. However, since the number of positive animals in a large population will be fairly small, the multiple-screening concept could prove to be quite economical, since the majority

TABLE 2. *Detection of hog cholera infection in swine by ELA*

Animal pool[a]	Weeks post-infection	ELA signal units[b]	S/N	ELA diagnosis[c]
Preinfection		47 (48, 46)	0.9	Negative
Hog cholera	2	83 (80, 86)	1.6	Positive
Hog cholera	4	133 (125, 141)	2.6	Positive
Hog cholera	6	195 (182, 208)	3.8	Positive
Erysipelas	6	53 (52, 53)	1.0	Negative
Noninfected controls		40 (39, 41)	0.8	Negative
Standard negative		51 (47, 54)	1.0	Negative

[a] Each pool represents at least five animals; the sera were diluted 1:20.

[b] Mean of the two replicates. Individual readings are in parentheses.

[c] S/N of ≥1.5 considered positive.

of sera will have to be tested only once in a well containing several antigens.

DISCUSSION

Results of the ELA test in terms of absolute signal units are strongly influenced by very small variations in incubation times and conjugate dilution. Automation of the test is expected to minimize these effects.

Experience with the ELA test for antibodies to *T. spiralis* has shown that a significant number of sera from conventionally raised swine contain a material that reacts with our antigen preparation to give a false-positive result. Similar findings have recently been reported by Ruitenberg et al. (7). Reasons for this apparent high false-positive rate are currently under investigation in this laboratory. We anticipate that an acceptable solution will be found in purification of our reagents, in particular the highly heterogeneous antigen preparation.

TABLE 3. *Results of ELA determinations using whole blood and serum*[a]

Animal identification	ELA units[b]		S/N		ELA diagnosis with whole blood or serum
	Whole blood	Serum	Whole blood	Serum	
E[c] (preinfection)	30	35	1.0	1.3	Negative
E (17 days postinfection)	75	72	2.6	2.7	Positive
S-10 (not infected)	33	30	1.1	1.1	Negative
B-16 (not infected)	30	31	1.0	1.1	Negative
S-16 (not infected)	28	30	1.0	1.1	Negative
Negative pool	29	27	1.0	1.0	Negative

[a] Sera were diluted 1:10; whole-blood samples were diluted 1:5.
[b] Mean of two replicates.
[c] *T. spiralis* larvae (12,000) were fed to pig E.

However, the present test system appears to be adequate for detection of antibodies in experimental infections where a preimmune serum is available for comparison with postinfection samples.

The reliability of the ELA test for the detection of hog cholera antibody has been confirmed in a double-blind study. Of 640 swine sera tested by ELA, there was a greater than 99.5% correlation with the serum neutralization (1) results on the same sera. A detailed report concerning these data is in preparation.

Other diseases that appear amenable to diagnosis by ELA (as a result of preliminary experiments or probes by the authors) include brucellosis, tuberculosis, and virus diarrhea in swine and cattle, and cysticercosis in cattle.

Other potential applications of immunoenzyme technology include the detection of microbial toxins and low-molecular-weight haptenic substances (carcinogens, pesticides, and drug residues). The sensitivity of the system appears to be quite high; we have been able to detect 10^{-3} μg of *Staphylococcus* A enterotoxin in a total test time of 20 min (8).

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TABLE 4. *ELA screening for two diseases in a single test*[a]

Serum type	Antigen bound to tray								
	Brucella and trichina			*Brucella*			Trichina		
	ELA[b] units	S/N	ELA[c] result	ELA units	S/N	ELA result	ELA units	S/N	ELA result
Brucellosis postive	126	3.5	Positive	90	3.9	Positive	35	0.9	Negative
Trichinosis positive	87	2.4	Positive	30	1.3	Negative	89	2.2	Positive
Negative standard	36	1.0	Negative	23	1.0	Negative	41	1.0	Negative

[a] Sera diluted 1:20.
[b] Mean of two replicates.
[c] Positive result is a S/N $\geq$1.5.

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