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A SIMPLIFIED METHOD FOR DETECTION OF AFRICAN SWINE FEVER ANTIBODIES BY IMMUNOELECTROOSMOPHORESIS

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Newly designed equipment and procedures have been developed that permit more rapid detection of African swine fever virus precipitating antibody. The methodology is also applicable to hepatitis diagnosis and other electrophoresis operations. Costs are greatly reduced, making these methods readily available to small laboratories.

Introduction

African swine fever (ASF) is rapidly becoming a grave threat to international livestock raising and pork production (*1*). During 1978 new foci of ASF appeared in Latin America, Malta, and Sardinia (*2*). The Latin American outbreaks do not appear to have followed the course of a previous outbreak on Cuba (*3*), during which the disease was eliminated relatively fast. Instead, ASF now appears established endemically in the Western Hemisphere.

Protection against endemic ASF must rely upon prompt and accurate diagnosis. Unfortunately, endemic ASF may no longer resemble the "textbook" disease (*4*) that produces high mortality and virtual self-elimination. The existence of carrier swine, together with establishment of the virus in susceptible ticks, has made eradication of the chronic and endemic forms problematic.

To compound the problem, the newer subclinical and chronic forms of ASF are hard to distinguish from hog cholera and other diseases (*5*). Even laboratory diagnosis may be difficult. Some strains cannot be identified by the usually reliable hemadsorption test (*6*). Also, the virus may be hard to isolate; and the fluorescent antibody (FA) test does not detect it in many cases (*7*).

Fortunately, complement-fixing (CF) and precipitating antibodies appear early in almost every infected animal. High levels of these antibodies are particularly apt to be found in animals with chronic and subclinical infections—precisely those animals in which the virus and viral antigens may be elusive. These antibody levels remain high, even in the presence of the virus, and they persist when the virus can no longer be detected (*8,9*).

Probably the most expedient and sensitive method for detecting ASF antibody is the immunoelectroosmophoresis (IEOP) test. It is much more sensitive than the agar gel double-diffusion precipitin test and is even more sensitive than the CF test (*10*).

While demonstrating the IEOP test over the past several years in foreign animal disease courses, we found no laboratories that were using the test, although many expressed interest. Laboratory directors were usually reluctant to invest in the equipment needed to conduct this single test.

Recently, visits by Plum Island Animal Disease Center scientists and technicians to veterinary laboratories in North and South America found few equipped to perform the

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test. And personnel in recent foreign animal disease classes—from overseas and from U.S. diagnostic laboratories—report that inflationary pressures have made the cost of the needed equipment and some supplies a definite deterrent to use of the IEOP test. For example, none of the laboratories involved ordinarily stocked agarose, and its price was considered by some to be prohibitive.

Over the past few years efforts have been directed at developing simpler procedures that would allow a laboratory to perform the IEOP test with improvised equipment and available supplies costing much less than the conventional system. Most recently, stimulated by the ASF upsurge and the desire of veterinary authorities to equip small, mobile laboratories (*11*) for ASF diagnosis, such efforts have been aimed at designing, building, and testing ultra-simplified equipment that could use very cheap supplies—with the goal of making the IEOP and related tests available to any laboratory or classroom. The results of those efforts are reported here.

Materials and Methods

Reagents

Preparation of the soluble antigen required for the IEOP test has been described previously (*10*). Antisera from experimental and field cases were used. Normal hog sera and hog sera obtained from swine infected with other diseases were used as controls.

Conventional IEOP Test Procedures

The basic procedures involved have previously been described (*10*). A highly detailed guide for production of soluble antigen and a detailed account of the IEOP methodology have also been published in manual and microfiche forms (*12*); these are available from the Director of the Plum Island Animal Disease Center.

To summarize briefly, the conventional test requires soluble antigen (at present available only from the Plum Island center if the local laboratory does not produce it) and positive and normal hog antisera for controls. The test was standardized for use with Gelman[2,3] equipment—including a power supply, immunoframes, holders, tanks, and other accessories. In testing ASF antisera, from one to three immunoframes covered with 0.6 per cent agarose in IEOP buffer were subjected to between 300 and 450 volts of direct current for 30 minutes. Depending upon the particular pattern and punch size, the 3 mm-diameter wells were spaced 10 to 12 mm apart.

Elimination of nonspecific precipitin lines is sometimes necessary. Certain batches of antigen, in combination with certain normal sera, may produce apparent precipitin lines. These do not usually resemble true IEOP lines, although on rare occasions such "false" lines may cause difficulties for an inexperienced operator.

However, our experience indicates that all such "false" lines can be readily removed by immersing the immunoframe in 2 per cent sodium chloride solution for between 15 minutes and 3 hours. This saline treatment causes all the nonspecific lines to disappear and the specific lines to appear stronger than ever. In addition to applying this saline treatment, experiments were carried out with both normal and ASF antisera, some of which were left untreated while others were immersed in a water bath at 56°C for 30 minutes—a treatment not conventionally required for the test.

Experiments Using a Variable-Voltage Power Supply and Glass Slides

A very large share of the IEOP equipment cost is eliminated if a homemade power pack

[2] The Gelman Instrument Company, Ann Arbor, Michigan 48106, U.S.A.

[3] Specification of trade names or proprietary products does not constitute any guarantee of that product by the U.S. Department of Agriculture, nor does it imply approval of the product to the exclusion of other products that may also be suitable for the intended purpose.

or other variable DC source is used that is capable of supplying 2 to 3 amperes of current at 200 to 500 volts. Such a power supply was constructed using electrodes prepared from the carbon rods of used dry-cell batteries; staining dishes were used instead of the conventional chamber (*12*).

Standard microscope slides and lantern-slide cover glasses were coated with agarose or agar for use with this improvised system. In numerous trials and demonstrations to classes, we used 2.5 × 7.5 cm single glass microscope slides, "double" microscope slides measuring 3.8 × 7.5 cm, and large standard lantern-slide cover glasses measuring 8.2 × 100 cm. The proper amounts of hot agar or agarose needed to coat these were determined, together with the voltages and times required. Concurrent tests were also carried out with commercial equipment.

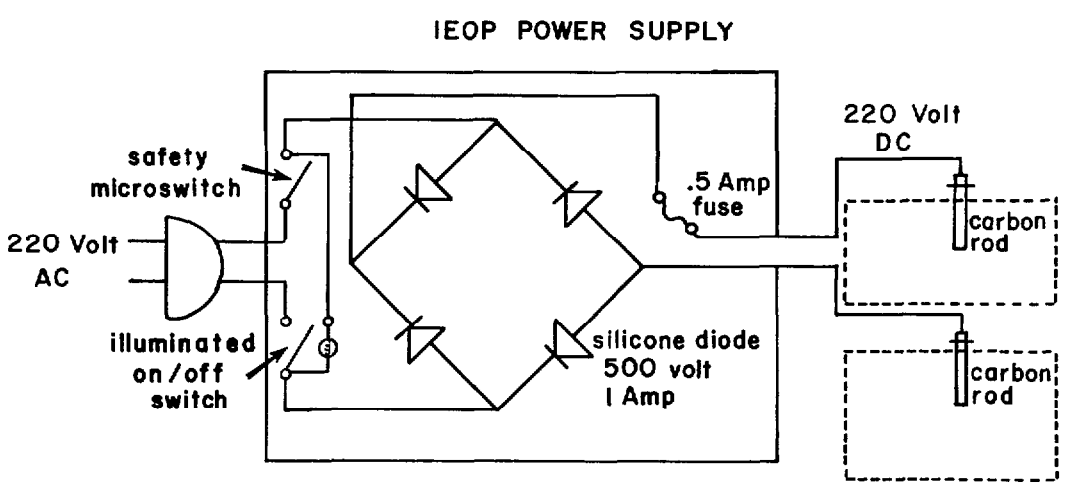
Experiments Using a Simplified Power Supply

Results obtained in these experiments guided efforts to devise the simplest possible equipment suitable for the IEOP test. Figure 1 shows the wiring diagram of a device constructed entirely of parts purchased from a small radio supply store. A full-wave bridge rectifier able to convert 220-volt alternating current to 220-volt direct current was built with four silicone rectifier diodes rated at 500 volts and 1 ampere. The DC side of the circuit was protected with a 0.5-ampere fuse and a potentiometer with a range of 0 to 100,000 ohms. This last component, regarded as optional, kept the circuit from shorting out if the electrodes accidentally touched. Carbon rods from old dry cells, with wires soldered to their brass caps, were used as electrodes. Alternatively, platinum wires provided by the authors were used; these proved highly satisfactory, but such wire may not be generally available.

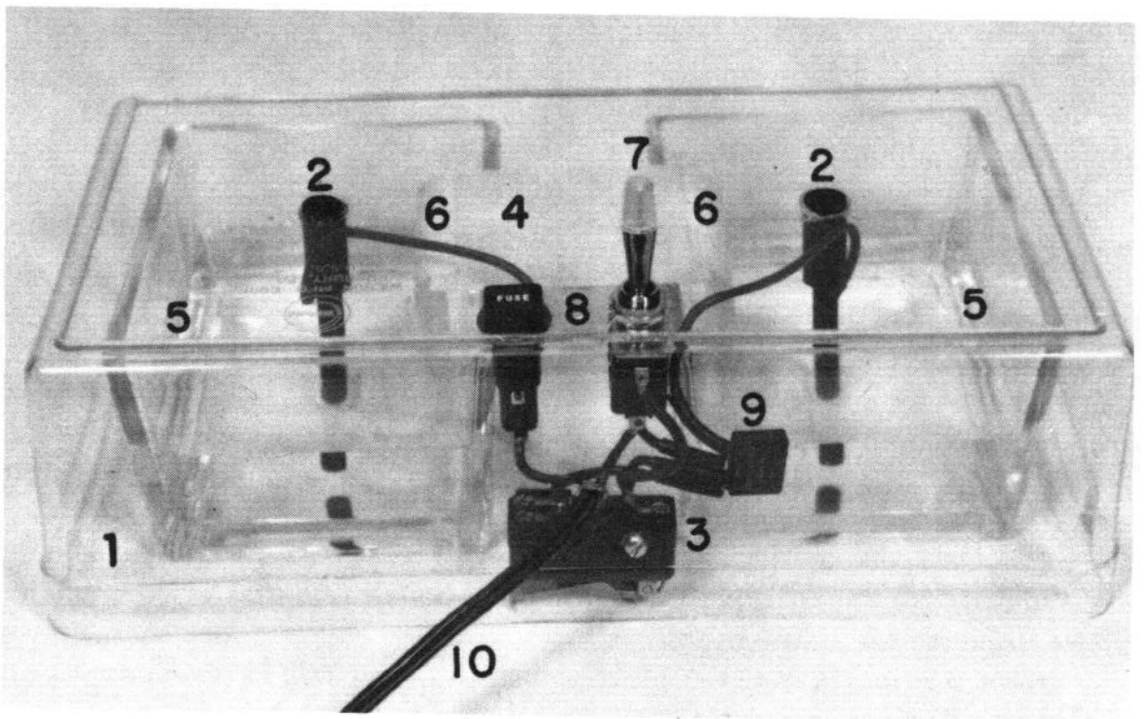
In initial trials, two staining dishes were used to hold the IEOP buffer; the slide was placed across them, and the electrodes were taped to the sides of each dish. Later, a plastic shoe-box was used to cover the buffer dishes; the electrodes were then mounted on plastic posts, and a safety microswitch was fastened so that the device operated only when the cover was in place (see Figure 1).

Preliminary trials were made using the

Figure 1. Wiring diagram of the power supply for the simplified immunoelectroosmophoresis test device. The components were obtained from a small radio supply shop and old dry-cell batteries. The total cost was less than US $10.00. A safety microswitch was incorporated into the cover, so that when the cover is removed the current is shut off.



The simplified immunoelectroosmophoresis device in operation. The rectifier-powered fixed line-voltage device has the following parts: (1) a plastic shoe-box cover to which major components are attached. (2) carbon electrodes from used dry cells. (3) a safety microswitch that turns the current off when the cover is raised. (4) a glass lantern-slide cover glass coated with agar or agarose; pairs of wells hold reagents, with the antigen being placed nearest the cathode. (5) glass dishes holding buffer. (6) wicks (two layers of filter paper) providing communication between the agar or agarose and the buffer. (7) an illuminated "on-off" switch. (8) a fuse container. (9) rectifying diodes. (10) the 220-volt power line.



simplified device on an ordinary 220-volt line; tests using the same reagents and conventional equipment were done concurrently. These trials tested numerous sera from pigs infected experimentally with ASF virus, sera from recent field cases in Brazil, and sera from other field cases. Block titrations with both systems were done using antiserum and antigen dilutions ranging from 2:1 to 128:1. (The antigen had been diluted previously for optimum diagnostic use.)

Comparative Tests with Agarose and Washed Agars

Because agarose,[4] a costly supply item, may be beyond the budget of smaller laboratories, numerous tests were conducted with washed agars. These washed agars included ones used for bacteriologic purposes, Special Noble Agar,[5] and Ionagar.[6] The agars were prepared at concentrations of 2.4 per cent, cut into 1.5 cm squares, washed in tap-water for 2 days, and given a final rinse in distilled water. Agarose and washed agars were prepared for pouring at final concentrations of 0.6 per cent in an IEOP buffer solution (ionic strength 0.1, pH 8.6) made by combining 13.38 g of sodium barbitol, 8.83 g of sodium acetate ($3H_2O$), 2.25 ml of 10 per cent sodium azide, and enough distilled water to bring the volume to 1.50 liters; the solution's pH was adjusted to 8.6 with concentrated hydrochloric acid.

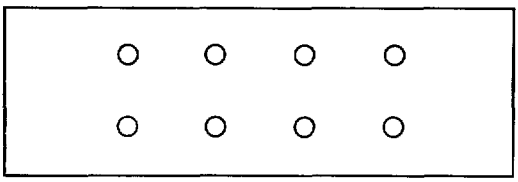
When the agarose or agar solution had

[4] VSR Scientific Inc., New York, New York 10025, U.S.A.

[5] DIFCO, Detroit, Michigan 48106, U.S.A.

[6] Colab Laboratories, Inc., Chicago Heights, Illinois 60411, U.S.A.

Figure 2. A pattern made to scale for cutting wells. The inside diameter of each well is approximately 2.5 mm if punched with a 12-gauge canula (3 mm is also satisfactory). Wells are punched about 10 mm apart, edge to edge. Plugs are easily removed by mouth aspiration with a rubber hose attached to a Pasteur pipette.



cooled, the plates were placed in a refrigerator for at least 30 minutes. Wells were then cut in the gel (see Figure 2) with a standard 3 mm punch or a sharpened 12-gauge canula. Tests with agarose and washed agars were performed using both the conventional and simplified IEOP systems.

Additional Tests

Tests using Gelman chambers with and without ammeters were carried out by placing crushed ice around the latter to serve as a coolant at high voltages. In addition, frozen cubes of IEOP buffer were made to serve as a coolant in the simplified device by adding them to the unfrozen buffer. Finally, simple transformers were used to operate the simplified device at 440 as well as 220 volts.

Results

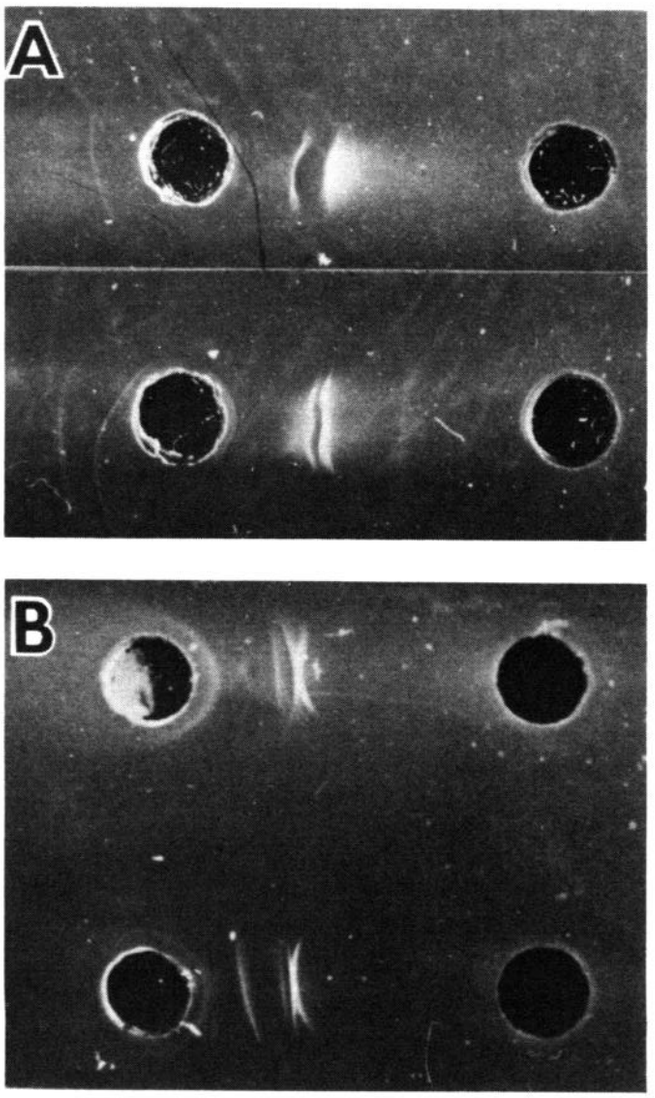
We found that the best volumes of agar or agarose to use for coating slides and cover glasses were as follows: 2.3 ml for a single microscope slide 2.5 × 7.5 cm (area: 19 cm^2); 3.5 ml for a "double" microscope slide 3.8 × 7.5 cm (area: 29 cm^2); and 10.0 ml for a lantern-slide cover glass 8.2 × 10.0 cm (area: 82 cm^2).

When glass slides were used in the conventional chamber with the commercial power supply, 220 volts were applied to a single lantern-slide cover glass and 200 volts were applied to two of them—so as to avoid burning out the ammeter on our Gelman DeLuxe chamber, which had a 50 milliampere limit. By using crushed ice as a coolant beneath another chamber with no ammeter, 220 volts could be applied to three plates. The coated glass slides had greater resistance than the conventional immunoframes. The same number of tests (24) could be carried out on one lantern-slide cover glass as could be performed on one immunoframe. Figure 2 shows the pattern generally used to punch wells for four tests; however, the size of the wells could be reduced and the distances between them varied by 2 to 4 mm without making any appreciable difference in the results.

When the simplified device was operating, measurement showed the line voltage to be 220 volts and the current to be 15 to 18 milliamperes. In both the conventional and simplified devices, resistance increased as the test proceeded.

The accompanying photograph shows comparative results of tests carried out with conventional (A) and simplified (B) equipment. There may be considerable variation in the appearance of the lines when the conventional system is used, but an experienced operator usually has little difficulty determining a specific reaction. In more than 100 tests made in the same manner as those used to test field samples, the simplified device (used with a 220-volt line current) gave results that were within the same parameters as those obtained with conventional equipment. In some trials, one system or the other yielded lines that were "better" (i.e., longer, heavier, or straighter); but in all trials with both systems, all the lines produced with positive antisera were discernable as typical positive IEOP reactions. "False positive" lines arose in tests with both the simplified and conventional systems, but these were removed by saline treatment. Some antigens had a tendency to produce such false positive lines with certain normal sera. Inactivation of normal sera and antisera at 56°C

A comparison of results obtained with the conventional apparatus (A) and the simplified device (B). Identical antigens (in right wells, nearest the cathode) and antisera (in left wells, nearest the anode) were used in these trials. The conventional apparatus was operated at 350 volts for 30 minutes, and the simplified device was operated at 220 volts for 15 minutes. The results of both tests were positive. The lines were not removed by overnight immersion in 2 per cent saline.



removed about a third of such false reactions without changing the positive reactions.

Tests of hog antisera against disease agents other than ASF virus were negative, as were numerous tests of normal hog sera.

Table 1 shows typical results of a block titration test using progressively doubled dilutions of antigen and antiserum. The simplified system, employing 220 volts, typically showed a positive result at dilutions about one step lower than the conventional system—which was using 350–450 volts. In a few trials where the simplified equipment was used with a step-up transformer at 440 volts, there were no differences between the systems.

As expected, because agarose contains no ionizing agaropectin it proved superior to any of the agars. Most ordinary agars used for bacteriologic plating yielded no lines before washing and only very faint or unsatisfactory lines after thorough washing; weak antisera tested on gel made with these agars gave no reaction. Results with Special Noble Agar were poor before washing and only fair after washing. Results with Ionagar were also indifferent before washing. However, Ionagar gave satisfactory results after thorough washing, and in many trials the results were as good as those obtained with agarose.

Results of numerous tests with both the conventional and simplified systems showed that anomalies—in the form of odd, weak, or absent lines—could occur. This might happen if the reagents were frozen and thawed many times, if the buffer was used at the wrong pH or was used too many times, or if similar variables were not controlled.

Discussion

There are many variables in the IEOP test, and meticulous attention to detail is required if the test performed is to be effective. When the conventional test is done, the appearance of the precipitin lines can vary in many ways. The lines can be faint and may only be visible when stained; they can vary from 1 to 6 in number; they can be straight, slanting, wavy, or curved toward one well or the other; and they can be either precise and sharp or (rarely, with weak reagents) soft and indistinct. These variations are related to many factors—including the precision with which the plates were poured, dessication of the agar or agarose after pouring, the age and pH of the buffer, the quality and storage temperature of the antigen (which keeps best at

Table 1. Typical results of a block immunoelectroosmophoresis titration test using both the conventional and simplified systems.

Antiserum dilutions	Antigen dilutions*							
	u	1:2	1:4	1:8	1:16	1:32	1:64	1:128
				Conventional system				
u	+	+	+	+	−	−	−	−
1:2	+	+	+	+	+	−	−	−
1:4	+	+	+	+	+	−	−	−
1:8	+	+	+	+	+	−	−	−
1:16	−	+	+	+	+	−	−	−
1:32	−	−	−	−	−	−	−	−
1:64	−	−	−	−	−	−	−	−
1:128	−	−	−	−	−	−	−	−
				Simplified system				
u	+	+	+	+	−	−	−	−
1:2	+	+	+	+	−	−	−	−
1:4	+	+	+	+	+	−	−	−
1:8	+	+	+	−	−	−	−	−
1:16	−	−	−	−	−	−	−	−
1:32	−	−	−	−	−	−	−	−
1:64	−	−	−	−	−	−	−	−
1:128	−	−	−	−	−	−	−	−

*The antigen was previously diluted 1:6 for field use.
+ = Positive with typical precipitin lines.
− = Negative with no precipitin lines.
u = "Undiluted" (actually pre-diluted 1:6 for field or bench use).

− 70°C or lower), and the quality and storage temperature of the antisera. Storage temperature for the antisera should not be higher than − 20°C.

Repeated freezing and thawing of the reagents will have a detrimental effect upon the test. Both the antigen and positive antisera used as reagents in the test should be distributed in small aliquots and frozen. After testing, we subdivide the properly diluted antigen into aliquots of 0.3 ml and store it at − 70°C. Certain highly reactive ASF antisera are likewise subdivided into aliquots of 0.5 ml and held at − 20°C for use as positive antisera in the test. Normal hog serum is similarly divided into aliquots and held at − 20°C for use as a normal control serum.

Before a test with either system begins, the investigator needs to become completely familiar with the equipment to be used and with the proper voltages and times required. Since the simplified equipment is operated at lower voltages than those generally used for the conventional test, it is especially important to determine the time required for the test under local conditions. We found failure to be frequent when the voltage fell below 200. At 220 volts we usually obtained satisfactory results in 15 minutes; increasing the time to 20 or 25 minutes sometimes resulted in stronger lines.

Using either system, different batches of soluble antigen were found to have slightly different characteristics. In our experience, antigen made from Vero cells gave fewer or no false positive lines and better true precipitin lines. The dilution of the antigen is very important. When a new batch is received, before carrying out the recommended dilution for bench use it is desirable to do a block titration similar to that shown in Table 1. In this way the optimum dilution for the local laboratory situation can be determined.

The quality of any agar used for the IEOP

test should also be determined. If agarose is too costly for general use, a small amount might be kept for purposes of comparison. Tests of different thoroughly washed agars against agarose will reveal which agars can be substituted for agarose.

The condition of the sera to be tested is also important. Many sera obtained from the field need to be centrifuged, so that contaminating erythrocytes, debris, and bacteria in the pellet can be discarded. It is probably best to routinely inactivate sera at 56°C for 30 minutes.

In discussing the IEOP test with personnel from the few laboratories using it, we found that disappointment with the test was usually related to imprecise methodology, details of which were not always available in the literature. However, we have recently learned of some supposedly normal hog sera that yielded IEOP lines not removed with saline treatment (*13*). It is theoretically possible that swine vaccinated with hog cholera antigens made from the same type of cell culture used to prepare the soluble ASF antigen might give precipitin lines in the test that are not specific for ASF antibodies. Therefore, the history of any antisera tested should be obtained.

Concluding Remarks

We have indicated three basic methods that will enable a laboratory to use the IEOP test. The first and most convenient is purchase and use of conventional equipment. The second is construction or procurement of equipment including a 500-volt, 125-milliampere power supply, platinum or carbon electrodes, glass chambers improvised from beakers or staining dishes, and agar-coated glass slides. The third is employment of 220-volt line current (or 110-volt line current and a step-up transformer) with our simplified device. To protect any U.S. Government interests, we have applied for a patent on the device, but we encourage laboratories in need of the IEOP test to construct the device and try it.

Using this simplified system, we believe that any laboratory will be able to perform routine IEOP tests on diagnostic ASF-suspect serum samples. The equipment can be put together by any electrician or amateur radio operator, and the supplies can be obtained in almost any laboratory. We do not know the range of possible uses for this recently designed equipment, nor do we suggest that it can take the place of more sophisticated equipment for research purposes.

We also wish to emphasize that the direct use of line current can be dangerous. Hence, it is very important to take every precaution in testing and using locally constructed equipment. Among other things, a safety device should be incorporated into the design and construction of this or any similar current rectifier.

SUMMARY

This article describes the construction and operation of simplified equipment for detection of African swine fever (ASF) antibodies by immunoelectroosmophoresis (IEOP).

A full-wave bridge rectifier to convert 220-volt alternating current to 220-volt direct current was constructed; four silicone rectifier diodes rated at 500 volts and 1 ampere were used for this purpose. Besides an inexpensive rectifier, the system made use of microscope slides or lantern-slide cover glasses coated with either agarose or agar and either 220-volt line current or 110-volt current provided by passing 220-volt current through a step-up transformer. A safety microswitch was incorporated into the design.

This system proved capable of performing the

IEOP test using less time and lower voltages than systems incorporating conventional IEOP power sources, chambers, and immunoframe accessories. At dilutions used for conventional tests, the simplified equipment performed nearly as well as commercial IEOP systems. Also, one washed agar yielded results nearly as good as those obtained with more expensive agarose, though other agars proved less suitable. The total cost of the simplified equipment was less than US $10.00, a fact which makes the IEOP procedures described feasible and affordable for small laboratories.

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