DETECTION OF ANTI-VIA ANTIBODIES IN CATTLE SERA BY COUNTERIMMUNOELECTROPHORESIS

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SUMMARY

The counterimmunoelectrophoresis (CIEP) technique was adapted for the study of antibodies to the VIA (virus-infection-associated) antigen in cattle sera in order to offer a new highly sensitive and consistent technique suitable for detecting those antibodies in low titer sera avoiding false negative results.

This study utilized sera from animals that had been intranasally infected with attenuated-foot-and-mouth disease virus, with titers varying from strongly positive to weak and including negatives according to previous tests by double immunodiffusion (DID) in agar; cattle sera from an FMD-free country were also used.

To increase the sensitivity of the technique, the sera were tested against several dilutions of a standardized preparation of VIA-antigen; it was shown that most of them reacted with one or more of the 1/30, 1/50, and 1/75 dilutions of the standard VIA-antigen preparation.

Two agaroses were assayed, one of low and one of high $-m_r$ (cathodic mobility coefficient), in order to provide two alternatives for technique performance; the optimum electrophoretic conditions of both supporting media are described.

Sensitivity, specificity and consistency of CIEP and DID, as well as their correlation, were studied statistically; it was found that although both techniques are highly specific and their consistency is comparable, CIEP is much more sensitive and detects twice as many positive sera from infected cattle.

The epidemiological usefulness of a sensitive technique such as CIEP, its speed of performance, economy of reactants and yield in number of samples processed simultaneously, are emphasized.

INTRODUCTION

The technique of counterimmunoelectrophoresis (CIEP) was introduced in 1959 by Bussard (*3*), for the purpose of detecting specific antibody and antigen reactions. He called the technique electrosyneresis. The simplicity and sensitivity of the method led to its use by many investigators and for different systems. It is frequently found in the scientific literature under many other names such as electro-immunoprecipitation, immuno-electro-osmophoresis, etc.

This technique has been commonly applied in the laboratory for the diagnosis of hepatitis B infection (*6, 8, 13*) and of a variety of viral (*5*), fungal and parasitic diseases. It has also been used for the serotyping of adenovirus (*9*), Coxsackie and ECHO[2] viruses (*11*), estimation of antibodies against native and denaturated DNA (*1, 14*), to detect activation of C3 component of the complement (*2*), etc.

Basically, it consists of a combination of the double immunodiffusion (DID) reaction in agar and electrophoresis. In the DID in agar, the reactants are placed in individual wells from which they diffuse toward each other until they meet forming a precipitation band or line (one or more, according to the heterogeneity of the system) whose position, characteristics and time of formation will depend on the nature and concentration of the antigens and antibody. In CIEP, the reactants practically do not diffuse, but are electrophoretically "pushed" toward a common reaction zone.

In ideal conditions, the antigen-antibody complexes precipitate to form a band equidistant from the point of origin of each component.

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The sensitivity of this technique is greater than that of the DID in agar since both antigen and antibodies migrate as a whole thus, maintaining a high concentration.

The studies carried out to determine the most suitable electroendosmotic conditions for a system constituted of the VIA (virus-infection-associated)-antigen (*4*) of the foot-and-mouth disease (FMD) virus and its homologous antibodies, are described in this paper with the purpose of offering a technique of high sensitivity, great consistency and simplicity that permits the detection of very low concentrations of bovine anti-VIA antibodies in relatively short periods of time.

MATERIALS AND METHODS

Antigens

Two preparations, identified as VIA-1 and VIA-2, were utilized in this study. The first was produced in accordance with the technique developed by the Diagnostic and Reference Laboratory of the Pan American Foot-and-Mouth Disease Center (*18*), and the second was prepared following the method at the Plum Island Animal Disease Research Center, U.S.A. (*17*) with some modifications: i.e., the final concentration was obtained not by ammonium sulphate precipitation but through negative osmotic pressure using PEG 20,000[2]. The final volume generally corresponded to 1/200th of the original virus culture fluid.

The antigens were titrated by the Laurell electro-immunodiffusion technique (*10*), taking VIA-1 as reference and using a hyperimmune guinea pig serum obtained from the Serology laboratory. Alternatively, the DID in agar method as described by McVicar and Sutmöller (*12*) was employed. Subsequently, VIA-2 was diluted to a value equal in activity to that of the VIA-1 taken as standard.

[2]PEG 20,000 Polyethylene Glycol 20,000 J.T. Baker Chemical Co., Phillipsburg, N.J., USA.

Effects of pH and buffers composition on the electrophoretic mobility

Two buffers were first studied with respect to the influence of their components on the movement of the serum proteins during electrophoresis and the consequent formation of the antigen-antibodies precipitation bands in the agarose. The compositions of the buffers were the following:

a)	Barbituric Acid	0.01 M
	Sodium Barbiturate	0.05 M
	Sodium Acetate	0.05 M
	Acetic Acid 1 N to adjust pH	
b)	Barbituric Acid	0.026 M
	Sodium Barbiturate	0.084 M
	HCl 1 N to adjust pH	

The effect of the pH on the mobility of the antigen-antibody system was also studied using the described buffers and varying their pH at discreet intervals from 8.0 to 8.6. Thus, the optimum value was determined for both antigen and antibody to be displaced at a similar speed in the electrophoretic field.

In order to obtain maximum displacement of the immunoglobulins with minimum distortion, cattle serum was studied electrophoretically in 1% agarose. The pH was maintained constant and the ionic strength of the buffer in which the agarose was dissolved was varied from 0.075 to 0.0125.

Supporting media

Two types of agarose were utilized in this study. One was Seakem[3], and the other HEEO[4] of low and high electroendosmotic activity, respectively.

In both cases the agarose was prepared at 1% (w/v) in the following manner: 1 g of agarose was slowly added to a 125-ml Erlenmeyer flask containing 60 ml of distilled water, while the medium was magnetically stirred. The mouth of the

[3]Colloid Marine Colloid Industries, USA.
[4]Miles Laboratories Inc. Elkhart, Indiana, USA.

container was covered with aluminum foil to prevent evaporation. The mixture was then heated to the boiling point while continuously stirred and kept in these conditions for two minutes to obtain a perfect solution. Once this stage was reached, 50 ml of buffer were added, either of barbital-sodium barbital or barbital-sodium acetate, 0.11 ionic strength. The solution was briefly heated, cooled to about 50°C and poored onto the slides as described later on. It is advisable to prepare the agarose solution just prior to use.

Electrophoresis

A Gelman[5] unit with frames that support six slides (2.5 x 5.0 cm) in two rows of three was used in this study. Thirty ml of agarose cooled to approximately 50°C were poured ever each frame in which the slides, previously cleaned with alcohol, had been placed. The process was conducted on a leveled table.

The frames were placed in a cooler at 4°C for five minutes to ensure good gelling. Using a gel punch[6] with ten cutters of 3 mm in diameter, 20 wells were made in the agarose layer according to the model shown in Fig. 1. For that purpose it was first necessary to cut 10 cavities per slide, then move the gel punch longitudinally to the desired distance between wells, where a second cut was made. The agarose fragments were removed by means of a section needle (2.5 mm external diameter); one of its ends was applied to the surface of the agarose which was to be removed and the other end connected to a light suction source (rubber bulb or an aspirator water pump connected to a faucet).

To investigate the optimal distance for formation of precipitation bands in the shortest possible electrophoresis time span, even with the use of low titer sera, several plates were made up. The space between the edges of the antigen and antibody wells varied from 3 to 5 mm.

Beginning from the left, the wells (with a volumetric capacity of 14 μl) were alternately filled with undiluted sera, either problem or control. The remaining wells were filled with dilutions of VIA-antigen. At first, the VIA dilutions were made in an arbitrary manner, starting with the undiluted preparation and continuing with dilutions (1/10, 1/20, 1/30, 1/40, 1/50, 1/60, 1/70, 1/80, 1/90 and 1/100) in order to find the dilution or dilutions that would optimally react with a given group of sera. With respect to the sera, a group was chosen from blood samples taken at different intervals from infected cattle. It was thus possible to experiment with a range of values varying in degrees of positivity from strong to weak and negatives. All these sera had previously been studied by the DID technique in agar. The results were recorded as S, strong, when the precipitation bands were very evident, and W, weak, when they were visible without great effort; and finally, as VW, very weak, when they were observed with some difficulty or appeared only after being stained with a Ponceau solution.

The plates were placed in an electrophoresis chamber and connected to the buffers with nylon wicks according to the technique described in the Gelman Instruments Co. Manual. 160 volts were applied during varying periods of time to obtain the minimum necessary time span to produce the immuno-precipitation bands. The electrophoresis chamber contained the same buffer employed in agarose preparation but with an ionic strength of 0.11. The electroendosmosis phenomenon was studied in both agarose preparations. With the HEEO agarose (High-Electro-Endosmosis) it was observed that at optimal pH the antigen migration speed and that of the antibodies toward their respective poles were virtually the same; the precipitation band was formed at an equal distance from the two cavities when the antigen-antibody ratio was optimum. As the $-m_r$ values (cathodic mobility ratio) (*9*) of the Seakem agarose were lower than those of the HEEO, it could be observed that the VIA moved to the anode faster than the immunoglobulins to the cathode; therefore, the precipitation band was formed closer to the serum well, creating some difficulty in the reading and consequently in the positivity evaluation. To

[5] Gelman Instruments Company, Ann Arbor, Michigan, U.S.A.

[6] Gelman Instruments Co. Gel punch, Catalog No. 71686.

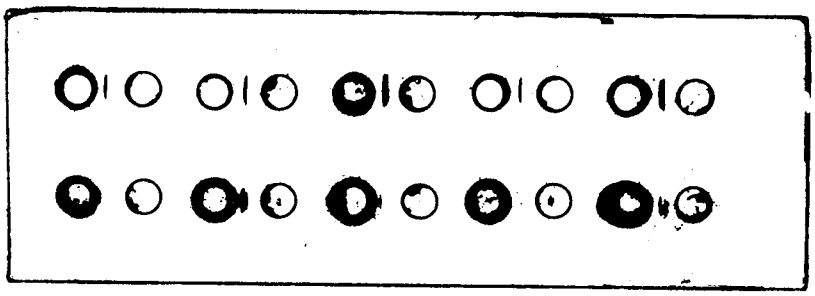

FIGURE 1. *Precipitation bands obtained with the CIEP. The picture shows the distribution of wells in the slides. The antigen was applied in the wells of the right (cathode) and the sera on the left side wells (anode).*

overcome this inconvenience the sera were first run at 260 volts for 30 minutes; then the cavities were filled with the antigen and the whole system was again run, at 160 volts for an additional 90 minutes. In this particular circumstance, when the antigen-antibody ratio was optimum the precipitation bands were formed equidistant from both cavities, ie., that which contained the antigen and that which contained the antibodies.

When the electrophoresis was finished, the plates were observed and the absence or presence of precipitation bands was recorded. To prevent unspecific precipitations due to the low solubility of the bovine gamma globulins in low ionic strength media, the cavities were refilled with a phosphate buffer (Na_2HPO_4 0.15 M, NaCl 0.40 M, KH_2PO_4 0.03 M, pH 7.8) 50 minutes after the run was finished and the plates were read again 10 or 20 minutes later, taking as reference the moment when any precipitate previously observed in the negativity controls region had disappeared. The bands were recorded according to their intensities. To prevent the plates from becoming dried, they were always kept inside a humid chamber; a second reading was made 24 hours later.

After this period the plates were washed, dried and stained. For that purpose they were covered with a strip of filter paper for five minutes to remove any excess liquid; once the paper was removed, the cavities were filled with agarose left over from the preparation of the plates. Then the agarose was again covered with filter paper strips over which were placed four or five layers of absorbent paper and then a light weight in order to maintain very close contact between the paper and the agarose surface. After a few minutes the agarose plates were reduced to a thickness of a tenth of a millimeter and were then washed in an LKB tank[7] or similar with phosphate buffer (pH 7.8). The process was aided by magnetic stirring for a minimum period of 3 hours. The plates were subsequently washed with distilled water for 30

[7] LKB tank No. 3948, LKB Produkter AB, Stockholm, Sweden.

minutes. Finally, the wash water was removed by repeating the process as described. The plates were completely dried in an oven or by the heat produced by a 100-watt bulb. To prevent the agarose from coming loose from the slides it is always desirable, before drying the agarose completely, to immerse the slide briefly in a solution of 1% glycerin (v/v) and 7% acetic acid (v/v). The gel will then retain a small amount of moisture, decreasing its fragility and increasing its flexibility.

The dried plates were placed for 30 minutes in a 0.5% Ponceau[8] solution dissolved in 5% trichloroacetic acid (w/v), then washed with 7% acetic acid (v/v) until the excess dye was removed. Finally, the plates were treated with a solution of 1% glycerin (v/v) in 7% acetic acid (v/v) for 30 minutes. The plates were allowed to dry by exposure to the air. In some cases, very weak precipitation bands which could not be observed prior to the dyeing became apparent after this process. The final readings were recorded.

Electro-immunodiffusion

The Laurell method was used (*10*). One gram of LEO[9] (Low-Endo-Osmosis) agarose was dissolved in 100 ml of barbital-sodium acetate buffer the composition of which was previously described, diluted to an ionic strength equal to 0.02 and with its pH adjusted to a value of 8.6. A 5 µl Oxford pipet was used to place the samples in the cavities which had a diameter of 3 mm. The same buffer used in the preparation of the agarose was placed in the electrophoretic chamber. 280 volts were applied for four hours at 4°C in a cold room. The diverse VIA preparations were consequently diluted so that all would migrate the same distance taking as reference the standard VIA-1.

Sera

For this study, 80 sera were obtained from cattle intranasally infected with foot-and-mouth disease attenuated virus. Sera from hyperimmunized guinea pigs and from cattle of known positivity were used for control of positivity. For control of negativity, 40 cattle sera from the Republic of El Salvador (an FMD-free zone) were used.

Antigen controls

In order to eliminate the presence of antibodies against BHK components in animals immunized with vaccines prepared from virus developed in this cell medium and that might produce false positives, a culture of virus-free BHK cells was processed through the same process employed to obtain the VIA antigen. The cytopathic effect was simulated by means of ultrasound. The final product, which was called "false VIA", was then diluted to provide the same protein concentration as the standard VIA. The protein concentration was obtained through the biuret reaction. All sera were simultaneously run against VIA and false VIA.

Biuret reaction

This was performed according to the method of Gornall, Bardawill and David (*7*); an extinction coefficient value of 2.8 was taken, as had been obtained for serum proteins.

Double immunodiffusion (DID) in agar

This method is basically the same as described by McVicar and Sutmöller (*12*). Each system consisted of seven wells: one central well and six wells located around it, 6 mm from its edge. Each well was 4 mm in diameter and 3 mm deep, with a volume of 38 µl. Results were recorded at 24, 72, 86 and 120 hours.

A comparative study was conducted to establish the correlations between the positivities detected by the CIEP and DID techniques. The results are shown in Tables 1, 2 and 3, where the

[8]Ponceau solution, Gelman Instruments, Inc.

[9]LEO Agarose. Miles Laboratories, Inc. USA.

sensitivity, the specificity, consistency and correlation between both techniques are indicated.

Statistical study

A formula from Thorner and Remein (*15*) was used for the statistical comparison of the CIEP and DID sensitivities. Work was done directly with the cell frequencies of a 2 x 2 table where

Test No. 2	Test No. 1 (+)	Test No. 1 (–)	
(+)	a	b	
(–)	c	d	
			n

the difference between sensitivity rates is distributed according to the "t" Student's probability distribution with n-1 degrees of freedom:

$$t = \frac{(b - c) : n}{\sqrt{(b + c) : n^2}}$$

RESULTS

The comparative study of the sodium-barbital-barbital and sodium-acetate-barbital buffers indicated that both showed similar efficiency; no difference in the position, intensity, rate of formation, visibility, etc., of the precipitation bands, etc., was observed. Because neither of them offered any particular advantage with reference to band differentiation or method sensitivity, the less expensive sodium-acetate-barbital buffer was selected.

A distance of 3 mm between the edges of the antigen and antiserum wells was found to be the most favorable.

With reference to the VIA-antigen concentrations employed, it was observed that the presence and intensity of the precipitation bands depended on the antiserum titer/antigen concentration ratio; the precipitation bands appeared in the zone of equivalence. An analysis of the results indicated that most sera could be ranked within three dilutions of the standard VIA preparation, e.g., 1/30, 1/50 and 1/75 which reacted with strong, medium, weak and very weak sera, respectively.

Among the various ionic strengths tried, the buffer with a value of 0.05 μ was the most efficient and a reasonable displacement of immunoglobulins without distortion was observed. Occasionally, discreet bands were formed in the agarose as a consequence of the low solubility of these cattle serum proteins in that salt concentration. However, no drawback was observed, for as was explained in Materials and Methods the unspecific bands dissolve easily when the ionic strength of the medium is increased after terminating the electrophoresis.

For the buffer in the chamber, a value of 0.11 μ was found to be optimum as it produced a good electrophoretic effect.

The system was run for different period of time. The most efficient time period for HEEO agarose was 2 hours at 160 volts. With a lower -m_r agarose, such as Seakem's, it was first necessary to run the sera for 30 minutes at 260 volts, then add the antigen and run the system again at 160 volts for an additional 90 minutes.

The negative control sera showed no reaction at all, whereas the test sera showed all the alternatives possible, from negatives to strongly positive reactions.

With respect to the control prepared with BHK cells, i.e., the false VIA-antigen, it showed no reaction even when run at several concentrations in the different studies conducted.

Statistical assessment of the results of the comparative study of CIEP and DID techniques showed that although both techniques have the same specificity, the former is much more sensitive and demonstrates nearly twice as many positives. The results are summarized in Tables 1, 2 and 3.

TABLE 1. *Sensitivity and specificity of CIEP and DID for the detection of anti-VIA antibodies*

Techniques		Infection		
		Yes	No	Total
CIEP	Posit.	63	0	63
	Neg.	17	40	57
	Total	80	40	120
	Sensitivity = 78.8 ± 9.0%			
	Specificity = 100%			
DID	Posit.	34	0	34
	Neg.	46	40	86
	Total	80	40	120
	Sensibility = 42.5 ± 10.8%			
	Specificity = 100%			

TABLE 2. *Correlation (ϕ) and comparison of sensitivities (ξ) of CIEP and DID in the detection of anti-VIA antibodies*

	Techniques		
	DID		
CIEP	+	−	Total
+	32	31	63
−	2	15	17
Total	34	46	80

ξ (CIEP) = 78.8%
ξ (DID) = 42.5%
Difference = 36.3% (t = 5.048)
ϕ = 0.32 X^2 = 8.345

TABLE 3. *Consistency of CIEP and DID in detecting anti-VIA antibodies*[a]

Techniques		Determinations		
		First		
		+	−	Total
CIEP	Second +	57	5	62
	Second −	1	17	18
	Total	58	22	80
	(ϕ) = 0.81 X^2 = 52.207 (p < 0.01)			
DID	Second +	32	2	34
	Second −	3	43	46
	Total	35	45	80
	(ϕ) = 0.87 X^2 = 60.956 (p < 0.01)			

[a] In this table the results of two assays with the same components and in similar conditions, are compared.

DISCUSSION

The main object of this work was to adapt the CIEP technique as a sensitive, rapid and relatively simple procedure for investigating anti-VIA antibodies in cattle sera.

At present time, the detection of antibodies to the VIA antigen is done by means of DID in agar as a routine technique which, although possessing a high consistency, is not sensitive enough to detect antibodies in sera with low antibody titers and consequently can yield many false negative results. Conversely, CIEP is a highly sensitive technique which has been widely used for the detection of Australia antigen, antibodies to DNA and many other systems often mentioned in the literature.

Nevertheless, the technique must be adapted to each particular system due to the fact that the conditions of the antigen, with respect to its mobility in the electrophoretic field, vary from one substance to another. Furthermore, it is of fundamental importance to establish as thoroughly as possible the optimum ratio between antibody and antigen concentrations in order to avoid the zone phenomenon that may produce false negatives and therefore decrease the sensitivity of the technique.

For the above-mentioned scope, a set of sera was selected from animals intranasally infected with attenuated FMD virus and whose anti-VIA antibody titers varied from strongly positive to negative in accordance with previous tests carried out by DID. These sera were studied by the CIEP technique against a series of VIA-antigen dilutions in order to avoid the zone phenomenon. Finally, it was found that most of the sera reacted with one or more of the 1/30, 1/50 and 1/75 dilutions of the standard VIA-antigen.

Consequently, the sera were studied against those antigen dilutions which enabled the sensitivity of the method to be increased since strong sera would not react with high antigen dilutions and, vice versa, weak sera would not react with high antigen concentrations.

It has been determined (*16*) that an adequate relationship between the mobility of the antigen and the $-m_r$ values of the supporting medium must exist. This value varies for the different brands of agar and agarose and depends on their chemical composition (*9*). In this work, two commercial preparations of agarose were studied, one of low $-m_r$ (Seakem) and the other (HEEO), of high endosmotic properties.

When the former was used, the precipitation bands formed very near to the serum well, which indicated low endosmotic activity and a rapid movement of the antigen. To overcome this drawback, the sera were run first at 260 volts for 30 minutes, allowing a reasonable displacement of the gamma globulins to the cathode; after this, the antigen was added and the run was continued at a lower voltage which allowed the bands to form at approximately the same distance from serum and antigen wells, overcoming in a simple way the drawback of the serum's low cathodic mobility.

There was no such problem with the HEEO agarose because the $-m_r$ values were high enough and, for the conditions of the run, e.g., pH, ionic strength, temperature, etc., the precipitations bands formed equidistantly from the antigen and antiserum wells. In this way, two alternative methods are presented. The choice will depend on the availability of agar or agarose with either high or low electroendosmosis.

Apparently, the components of the buffer used are --within certain limits-- unimportant; both buffers considered in this study yielded practically the same results.

With this method, results can be obtained in a comparatively short time, ordinarily two to three hours after electrophoresis is begun. However, for very weak sera a 20-hour incubation period with a subsequent staining of the precipitation bands is preferred. This enables the very slight, imperceptible bands to be clearly seen after staining, thus increasing test sensitivity. The whole process, from preparation of the supporting medium to the dyeing, takes about 30 hours, which compares favorably with the 120 hours consumed for the DID technique. This fact, in addition to the small slide volume requirements, saves both time and space. The dyed slides may be kept on file if necessary.

The sensitivity, specificity, consistency and correlation between CIEP and DID were analyzed statiscally (Tables 1, 2, 3). A test for statistical significance of the difference between two sensitivity rates should consider the frequency of correlated results. For this study, the application of both CIEP and DID procedures to each serum tends to produce similar responses. Thus, the standard error of the difference between the two ratios must be calculated in a way which eliminates the effect of that correlation. Consequently, an expression was chosen which permitted direct operation with the cell frequencies in a 2 x 2 table (*15*). The value of $t = 5.048$ (79 d.f.) found was highly significant and indicated that the sensitivities percentages (78.8% for CIEP and 42.5% for DID) shown by both methods was substantially favorable to the former.

Thus, the CIEP technique offers great practical advantages for serological surveys for anti-VIA antibody detection because: a) it permits the detection of antibodies to the VIA-antigen in a comparatively short time, always shorter than DID; b) it is much more sensitive than DID and a very low titer of antibodies may be detected; c) a large number of samples may be tested simultaneously and d) the small volume of reactants required in each well (14 μl) and the high dilutions of VIA-antigen employed signify an important economy.

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