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RAPID DIAGNOSIS OF BOVINE CYSTICERCOSIS BY COUNTERIMMUNOELECTROPHORESIS

by

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Summary — The procedural details of counterimmunoelectrophoresis (CIEP) for the diagnosis of bovine cysticercosis were worked out under experimental conditions. A buffer-gel system yielding migration ratio (-mr) range from 0.3 tot 0.4 gave ideal results. Borate buffer-agarose system was found optimum for the purpose.

The intensity of metacestode burden of the animals had direct monitoring influence on the first appearance of precipitins in the host so that, depending on the level of their infection, the first precipitins were detected between two and 16 weeks post-infection. The animals harbouring less than 50 metacestodes usually failed to reveal any precipitins in their sera. CIEP is found somewhat more sensitive and less specific when compared with immunoelectrophoretic method though the former is less demanding, rapid and needs smaller quantities of samples.

KEYWORDS : Cysticercosis, Experimental; *Taenia saginata*; Cattle; Counterimmunoelectrophoresis; *In vivo* Diagnosis.

Introduction

Counterimmunoelectrophoresis (CIEP) was initially used in the early seventies for the detection of hepatitis associated antigen and antibodies in human beings. The procedure is now increasingly being tried for serodiagnosis of various other infections. It is also reported that CIEP is ten times as sensitive as the micro-method of double diffusion in agarose for detecting serum antigen (Gocke and Howe, 1970). More recently CIEP has been used for the serodiagnosis of various helminthic infections of human beings and animals (Beltran and Gomez-Priego, 1973; Despommier *et al.*, 1974; Hillyer, 1975; Kelkar and Kotwal, 1975; Lopez-Lemes and Varela-Diaz, 1975; Phillips and Draper, 1975; Desowitz and Una, 1976; Mahajan *et al.*, 1976; Ardehali *et al.*, 1977 and Desowitz *et al.*, 1977).

The *in vivo* diagnosis of bovine cysticercosis remaining largely unresolved (Geerts, Kumar and Vercruysse, 1977), it has been emphasised that the value of CIEP, *inter alia*, should be assessed for this purpose (Bull. W. H. O., 1976). Earlier, immunoelectrophoretic studies have shown that within certain defined limits specific *in vivo* diagnosis of this condition can be achieved (Geerts, Kumar and Aerts, 1979). This latter procedure, however, is time consuming and unsuitable for routine purposes. CIEP, a relatively rapid, simple and inexpensive procedure, has been standardised by the authors for the diagnosis of bovine cysticercosis under experimental conditions and the details are reported.

TABLE 1

The first appearance of precipitins in the sera of calves harbouring experimental *T. saginata* cysticercosis by CIEP

Lightly infected (< 100 metacestodes)			Moderately infected (100 to 1,000 metacestodes)			Heavily infected (> 1,000 metacestodes)		
Animal No.	No. of metacestodes recovered	First precipitins detected at (WPI)	Animal No.	No. of metacestodes recovered	First precipitins detected at (WPI)	Animal No.	No. of metacestodes recovered	First precipitins detected at (WPI)
2	3	— (*)	1	990	4	3	1,730	3.5
7	2 (V)	— (*)	4	356 (V)	3.5	11	6,510	2
8	0	— (*)	5	468 (V)	4.5	12	9,455	3
9	50 (V)	15	6	158 (V)	4.5	13	5,285	2
21	87	16	18	373	7	14	1,610	4
24	47 (V)	6	19	232 (V)	5.5	15	3,150	2
			20	106	10	16	2,555	4
			22	296 (V)	7	17	3,695	3
			23	185 (V)	6			
			25	109 (V)	7			

Weeks post-infection abbreviated as WPI.
(*) No precipitins were detected even at 17 WPI when the animals were autopsied.
Unless otherwise indicated by a letter (V), the majority of the metacestodes were dead.

Materials and Methods

Serum samples

24 healthy and parasite-free calves were infected with the eggs of *Taenia saginata.* These eggs were used for infection between two and four months of their harvest from the gravid proglottides of the cestode. Calves No. 1, 3, 4, 17, 19 and 24 (vide Table I) received single infection doses of 4,000 to 150,000 eggs *per os* while the remainder received this infection in two, three or four divided doses. During the entire course of experimental study, the calves were bled at weekly intervals and their serum samples were frozen at − 20 °C until used.

The preinfection serum samples of above mentioned 24 calves as also the serial serum samples of four uninfected healthy calves were used for providing informations on false-positive reaction, if any. Besides, serum samples of cattle and sheep harbouring natural or experimental infections of *Dictyocaulus viviparus*, *Fasciola hepatica*, *Moniezia expansa*, *Cysticercus tenuicollis* and hydatid cyst of *Echinococcus granulosus* were incorporated in the study to supplement informations on cross-reactivity.

Antigen

The non-gravid proglottides of *T. saginata* freshly expelled by Belgian patients, following chemotherapy or spontaneously, were used for preparing the hydrosoluble antigen. To each gram of damp and finely incised pieces of the proglottides, 2 ml of 0.017 Mol NaCl was added and the material stuffed into a X-press disintegrator (Biox, Sweden) precooled to − 30°C. Following the specifications of the manufacturer, the frozen material was pressed four times with the aid of a hydraulic press; each process of pressing was preceeded by precooling to − 30 °C. The available material was thawed by addition of 2 ml of Veronal buffer (pH 8.2, ionic strenght 0.1) and extracted for three hours at 4 °C in a magnetic stirrer. The crude homogenate was spun in a refrigerated ultracentrifuge at 26,000 *g*.

The supernatant, avoiding the top lipid layer, was dialysed against distilled water for 18 hours at 4 °C and lyophilised. This lyophilised material contained an average of 620 μg protein per mg as determined by Lowry's method.

CIEP

In the process of standardization of CIEP procedure the various factors playing vital role were investigated to reach the following optimum working conditions.

4 ml of one per cent molten Agarose A37, Indubiose (Pharmindustrie, Clichy) prepared in borate buffer (pH 8.6, ionic strength ± 0,04 containing 450 ml of 0.1 Mol boric acid and 550 ml of 0.025 Mol sodium tetraborate) was pipetted out onto a clean and dried glass plate, 57 mm × 70 mm × 1 mm, and allowed to solidify. 12 pairs of wells, each well of 4 mm diameter and the opposing well of the pair placed 5 mm apart, were cut in two rows in the agarose matrix (Fig. 1). The cathodic well of each pair was filled with an antigen concentration of 1/300 (weight/volume) and the corresponding anodic well of the pair was filled with the test serum samples of the calves. Electrophoresis was carried out in an assembly (Gelman, model 51156) which was adapted to hold the plate of above mentioned

dimensions. The two ends of the gel were connected with the two reservoirs of the cell, each containing 100 ml of borate buffer of above specifications, by Whatman No. 1 filter paper wicks. A potential difference of 30 V, measured at the two ends of the gel, was applied at room temperature for 40 minutes. The plate was removed after electrophoresis, immersed in physiological saline for two hours and in aqua distillata for 30 minutes before it was dried and stained with Coomassie brilliant blue R 250 or Amido black 10 B.

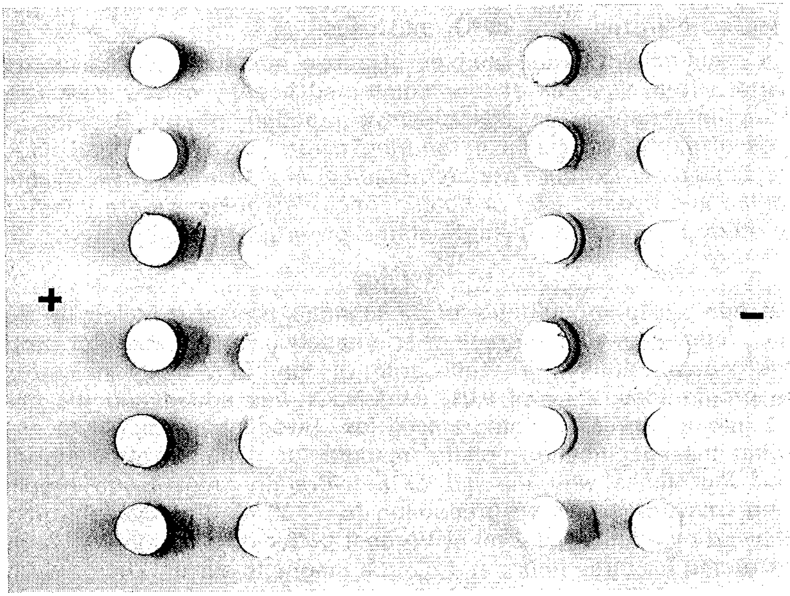

Figure 1.

Visualization of immunoprecipitate by CIEP. The semilunar diffusion fronts seen at the cathodic vicinity of serum wells are to be differentiated from the rather straight immunoprecipitation reaction. In the left row the first pair from top and in the right row the second and fifth pairs from the top do not show precipitation reaction. All the remaining pairs show single or double precipitation reaction. Coomassie brilliant blue R staining.

Migration ratio

The migration ratio (− mr) provides informations on the extent of electroendosmosis available in a buffer-gel system and thus on the net mobilities of the reactants. The procedure of − mr determination described here was adapted from the account elaborated in a brochure issued by Millipore Corp. U. S. A. (Cat. No. LPM 713).

An electrophoretically neutral 1 per cent solution of vitamin B_{12}* and a 1 per cent solution of albumin** tagged with bromo-thymol-blue dye were incorporated in the buffer-gel system and allowed to migrate in an

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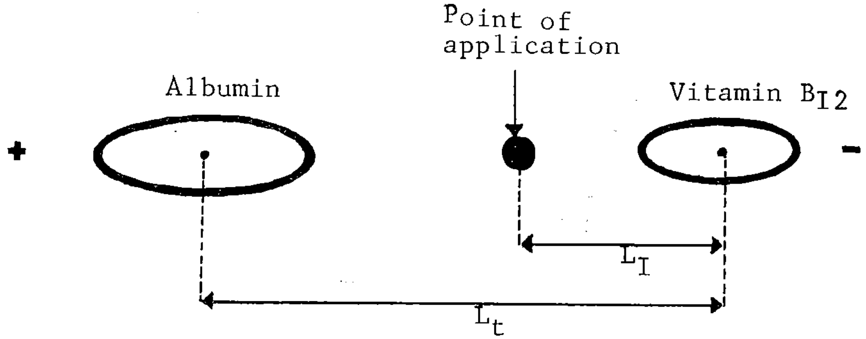
(*) Vitamin B_{12}, biochemical grade (Cyanocobalamin), E. Merck, Darmstadt.
(**) Albumin, biochemical grade (from bovine blood), E. Merck, Darmstadt.

electrical field of specified voltage and duration as given above. The $-$ mr value was calculated by the following formula :

$$- mr = \frac{- L_1}{L_t}$$

where L_1 is the distance of cathodic migration of vitamin B_{12} from the point of its application and L_t is the total distance between the two points of migrations of vitamin B_{12} and albumin as illustrated below.



Results

Conditions of CIEP

The net mobilities of reactants in a matrix of gel in opposing fronts, *viz.*, the anodic migration of antigens and the cathodic migration of antibodies, in CIEP procedure are determined by a variety of factors present in the buffer-gel system. These factors together with others which had vital bearing on the success of the procedure were investigated for their suitability and are considered below.

(i) Buffer-gel system :

Veronal and borate buffers of varying pH and ionic strengths were used for preparing gels of HEEO Agarose (High Electroendosmosis Agarose, Miles Laboratories Ltd, England) and Agarose A37, Indubiose. On each occasion, either of these two agaroses were used in one per cent concentration and to cover the glass plates of aforementioned dimensions volumes of 4 ml or 8 ml were poured on the plates to yield matrices of two thicknesses.

It was found that the $-$ mr value of a given gel matrix is strongly influenced by the composition of the buffer employed and the electroendosmotic properties of the agarose. The four available combinations of buffer-gel systems yielded the following $-$ mr values.

Buffer-gel system	$-$ mr values
Veronal-Agarose A37	0.12 - 0.15
Veronal-HEEO Agarose	0.22 - 0.31
Borate-Agarose A37	0.33 - 0.37
Borate-HEEO Agarose	0.40 - 0.46

We have observed that the combinations of buffer-gel systems giving the $-$ mr values ranging from 0.30 to 0.40 produced ideal and reproducible precipitation reaction. Accordingly, Veronal-HEEO Agarose and borate-Agarose A37 combinations were found most suitable for detecting the precipitins in calves against metacestodes of *T. saginata.* In our hands, however, the best results were obtained by incorporating borate-Agarose A37 system. The Veronal-Agarose A37 and borate-HEEO Agarose systems did not provide satisfactory results because of either too low or too high electroendomosis. Significantly, the clarity of the precipitation reaction was best maintained when 4 ml of the molten borate-Agarose A37 gel was used to cover the plate.

(ii) Antigen concentration :

Solutions of lyophilised hydrosoluble extracts of *T. saginata* in concentrations ranging from 1/50 to 1/800 (weight/volume) were evaluated. A concentration of 1/300, which corresponded to 2 mg protein per ml, gave optimum precipitation reaction.

(iii) Voltage and duration of current :

The suitability of electrical potentials of 20 V to 60 V, measured at the two ends of the gel, was investigated for different durations of run of the current. A potential difference of 30 V in the gel for 40 minutes gave satisfactory results.

(iv) Size of well and inter-well distance :

Well diameters of 4 mm each having capacity for 7 μl of the reactants were found adequate for the purpose. Although a precipitation reaction could be visualised at any edge to edge inter-well distance of 3 mm to 10 mm, the clarity and reproducibility of the reaction was best maintained when 5 mm inter-well distance was used.

Sensitivity of CIEP

Since the intensity of metacestode burden of the calves had discernible monitoring effects on the time of appearance of precipitins in the host, the levels of their infection are graded in three categories. The calves harbouring fewer than 100 metacestodes are referred to as lightly infected, the ones harbouring from 100 to 1,000 metacestodes are referred to as moderately infected while the others harbouring more than 1,000 metacestodes are referred to as heavily infected. The first appearance of precipitins in these three categories of calves is outlined in Table I.

In the group of heavily infected animals the first precipitins were demonstrable relatively early between two and four weeks post-infection. Among the moderately infected animals, the first precipitins appeared between three and a half and 10 weeks post-infection. In the group of calves harbouring light infection, the precipitins could be detected only in those harbouring more than 50 metacestodes with the exception that in case of calf No. 24, which yielded 47 metacestodes, the precipitins could be detected much earlier at six weeks post-infection. In one case the first precipitins appeared much later at 16 weeks post-infection.

The host's precipitin response to the metacestode remained highly variable eventhough the intensity of their metacestode burden remained in quite identical range. While the calf No. 24 harbouring 47 metacestodes showed the first precipitins much earlier at six weeks post-infection, the calf No. 21 which harboured 87 metacestodes showed the precipitins as late as 16 weeks post-infection. The same variable situation was seen, among others, in the moderately infected calves No. 4 and No. 18. The quantum of metacestode burden of the host did not provide any spectacular difference in the intensity of precipitation reaction. Generally speaking, although the development of single precipitation line was more a feature of lightly and moderately infected animals, among the heavily infected animals two and less frequently three precipitation lines were seen in most of the cases (Fig. 1). When compared with immunoelectrophoretic method, CIEP could detect precipitins from a week to a month earlier among calves No. 14, 17, 19, 20, 23 and 25.

The precipitins were found present until the time of autopsy of the animals four to five months post-infection. Even if the majority of the metacestode burden of some of these animals had undergone degenerative processes of caseation or calcification, there was no indication of alteration in precipitating antibody response of the host in that these antibodies were persistently present. In one case, where the serum of a cattle was obtained 10 months post-infection from elsewhere, the precipitins were still found present.

Specificity of CIEP

None of the preinfection serum samples of the 24 experimental calves or the serial serum samples of four uninfected healthy animals maintained for a period of about five months showed a false-positive precipitation reaction. In order to evaluate the cross-reactivity with other helminthic infections, serum samples of *D. viviparus* and *F. hepatica* infected homologous cattle hosts were used. In the absence of availability of *M. expansa*, *C. tenuicollis* or hydatid cyst infected serum samples from homologous host, serum samples of sheep harbouring these infections were utilised.

TABLE 2

Data on specificity of CIEP

	Uninfected control	*Fasciola hepatica*	*Dictyocaulus viviparus*	*Moniezia expansa*	*Cysticercus tenuicollis*	Hydatid cyst of *Echinococcus granulosus*
Number of animals tested	29	10	4	2	1	10
Number showing false positive or cross reaction	0	0	2	0	1	1

The data on the specificity of CIEP procedure are summarised in Table 2. Two out of four *D. viviparus* infected, one out of 10 hydatid cyst infected and the only one *C. tenuicollis* infected serum samples reacted to show cross-reacting precipitins.

Discussion

The sensitivity of immunoprecipitation reaction in gel visualized through CIEP is influenced not only by the union of antigen and antibodies in optimum proportions to provide a visible reaction but also by a delicate balancing of two opposing forces driving the reactants to unite in the gel. While union of antigen and antibody in optimum proportion is accomplished by simple titration studies, the forces which govern the movement of the reactants in an electrical field are principally affected by the electro-endosmotic properties of the matrix which, in turn, is the function of buffer-gel system employed. The speed of anodic mobility of a given antigen would be vital in the adjustment of a balance of the two opposing forces in gel.

The precipitins against the metacestodes of *T. saginata* were effectively revealed by CIEP when a buffer-gel system of rather high — mr value was used. The hydrosoluble extract of *T. saginata* used as antigen in the present case apparently had high anodic mobility. Accordingly, a buffer-gel system of — mr value range of 0.30 - 0.40 was found ideal to slow down this mobility. It can be conceived that a buffer-gel system of lower — mr value than this range would help drag the antigen too far towards the anode while a — mr value higher than this optimal range would have undesirable inhibitory influence on the anodic migration of the antigen. There are no previous records of observations on these aspects of *T. saginata* antigen for direct comparison with the present findings but it appears probable that the method of antigen extraction could eventually affect the speed of anodic mobility of antigen.

The sensitivity of CIEP in the present case is essentially comparable to the earlier immunoelectrophoretic observations reported elsewhere (Geerts *et al.*, 1979) excepting that in the former case it was possible to detect the precipitins upto one month earlier in one-fourth of the animals examined. If these observations can provide any index about the relative sensitivity of CIEP and immunoelectrophoretic procedures, CIEP, in general, can be claimed slightly more sensitive. Nevertheless, in cases where the metacestode burden of the host was lower than 50, both these procedures failed to reveal any detectable precipitins. This problem of insensitivity at low levels of infection leading to false negative findings presents a major handicap and will remain open for scrupulous enquiry not only by immunoprecipitation methods but by other immunodiagnostic procedures as well.

The specificity of CIEP for detecting precipitins against the metacestodes appears to be somewhat lower when compared with immunoelectrophoretic observations. Lopez-Lemes and Varela-Diaz *(loc. cit.)* and Rickard (1979), while discussing on the immunodiagnosis of hydatidosis, had also pointed out their inability to attain the degree of specificity with CIEP that immuno-electrophoresis can offer. In the present case, while no cross-reactions were revealed with *M. expansa* infected serum by either of these two procedures, *C. tenuicollis* infected serum cross-reacted by both these procedures and hydatid cyst infected serum cross-reacted only once by CIEP and never by immunoelectrophoresis.

A curious observation was that two out of four *D. viviparus* infected sera cross-reacted by CIEP although, according to the earlier observations of the authors (Geerts *et al.*, 1979), there were no common antigenic determinants present in *D. viviparus* antigen. It is not quite certain if the lungworm infection alone was responsible for revealing this cross-reactivity since the circumstancial evidence would also appear to indicate these two animals having been sensitized to other antigen possibly sharing common antigens with *T. saginata.* Immunoelectrophoretic procedure never revealed any cross-reaction with *D. viviparus* infected sera.

The outstanding merits of CIEP procedure are that it does not involve an elaborate laboratory method, needs less of antigen and time of performance and several samples can be handled at one time. When compared with immunoelectrophoretic method, CIEP is somewhat more sensitive although less specific. Nevertheless, CIEP warrants its evaluation for seroepizootiological work in relation to bovine cysticercosis.

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Diagnostic rapide de la cysticercose bovine par contre-immunoélectrophorèse.

Résumé — Les conditions optimales ont été déterminées pour le diagnostic de la cysticercose d'animaux, infectés expérimentalement, à l'aide de contre-immunoélectrophorèse. Un système de gel-tampon avec un rapport de migration entre 0,3 et 0,4 donnait les meilleurs résultats. La combinaison de tampon borate et d'agarose était optimale.

Le nombre de metacestodes présents chez l'animal, avait une influence directe sur le moment d'apparition des précipitines.

Selon le degré d'infection, les lignes de précipitation apparaissaient entre 2 et 16 semaines après l'infection. Des resultats faussement négatifs ont été obtenus avec les sérums d'animaux, porteurs de moins que 50 cysticerques. CIEP est un peu plus sensible et moins spécifique que l'immunoélectrophorèse, mais a surtout l'avantage que c'est un test simple, rapide et économique

Snelle diagnose van cysticercose bij runderen b.m.v. tegenstroom-immunoelectroforese.

Samenvatting — De counterimmunoelectrophoresistest (CIEP) werd gestandardiseerd voor de diagnose van cysticercose bij exeperimenteel besmette runderen. Een buffer-gel systeem met een migratie-ratio van 0,3 tot 0,4 gaf de beste resultaten. De kombinatie van boraat buffer met agarose was optimaal.

De grootte van de metacestode populatie had een direkte invloed op het tijdstip waarop de precipitatielijnen verschenen. Naargelang de graad van besmetting konden de eerste precipitines aangetoond worden tussen 2 en 16 weken na de infektie.

Dieren, die minder dan 50 metacestoden telden, konden gewoonlijk niet opgespoord worden met deze test. CIEP is iets meer gevoelig maar minder specifiek dan immunoelectrophoresis, maar heeft het voordeel dat hij vlugger en gemakkelijker uit te voeren is, en veel kleinere hoeveelheden serum en antigeen nodig heeft.

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