A Hemagglutinating Virus Producing Encephalomyelitis in Baby Pigs

by A. S. Greig[1], D. Mitchell[1], A. H. Corner[1], G. L. Bannister[1], E. B. Meads[2], and R. J. Julian[3]

A viral type of encephalomyelitis in baby pigs has been known to occur in Ontario for at least three years (1, 2). Studies have been undertaken in an effort to identify the cause or causes of this apparently infectious condition (3, 4). During the course of this work feces and brain tissue from piglets showing histopathological evidence of viral polioencephalomyelitis have been seeded into monolayer cell cultures of pig kidney origin in an attempt to isolate an infectious agent. Some of the results have been previously reported in regard to the isolation of enteroviruses (4).

Recently three outbreaks of encephalomyelitis in different parts of Eastern Ontario have been investigated. In each case a cytopathogenic microorganism markedly different to the enteroviruses previously described (4) has been isolated. These agents proved to be hemagglutinating viruses and were shown to be pathogenic for baby pigs. This report describes preliminary investigations conducted with these hemagglutinating encephalomyelitic viruses (HEV) and discusses their relationship to porcine encephalomyelitis in Ontario.

Materials and Methods

Tissue culture: Monolayer cell cultures, prepared from the cortices of kidneys from piglets two to eight weeks of age, were used throughout this work. The methods of trypsinization and culture production were the same as described for use in studies on porcine enteroviruses (4).

Growth medium consisted of a solution of 0.5% lactalbumin hydrolysate (enzymatic), 0.1% proteose peptone No. 3 (Difco) and 10% inactivated (56°C.) bovine serum in Hank's balanced salt solution. The maintenance medium used during virus growth trials was filtered bovine amniotic fluid (BAF), adjusted to pH 7.4-7.6 with sodium bicarbonate.

Most of the work was done in cultures grown as monolayer wedges in 18 x 150 mm. glass tubes. These were prepared and incubated initially in stationary racks, but after inoculation were transferred to roller drums. For the production of antigen for hemagglutination, Roux bottle cultures containing 50 ml. of fluid overlay on well developed cell sheets were used.

Histopathology: Tissues from field and experimental cases of the disease were fixed in ten per cent formalin in physiological saline solution. Tissue specimens usually consisted of one-half of the cerebrum and cerebellum together with the medulla oblongata and various levels of the spinal cord and attached paravertebral ganglia. Numerous sections were cut from various levels, using the paraffin method, and stained with hematoxylin and eosin.

Hemagglutination and Hemagglutination-inhibition tests: Hemagglutination (HA) antigens were prepared in pig kidney cell cultures. Following seeding with virus, the cultures were observed microscopically each day for signs of cytopathic changes. From the second day onward the fluid overlay was tested for HA activity.

1. Animal Pathology Laboratories, Health of Animals Division, Canada Department of Agriculture, Animal Diseases Research Institute, Hull, Quebec.
2. Regional Veterinary Laboratory, Kemptville, Ontario.
3. Regional Veterinary Laboratory, Brighton, Ontario.

The procedure used was that suggested by the Committee on Standard Serological Procedures in Influenza Studies (5). Two-fold dilution of antigen in 0.5 ml. amounts were placed in 13 x 75 mm. round-bottom tubes and 0.5 ml. of 0.5 per cent washed, chicken erythrocytes was added to each dilution. The tubes in racks were shaken to mix the reagents and kept at room temperature for 20-30 minutes. The last tube showing agglutination was considered to contain the dilution of antigen equivalent to 1 HA unit.

In the hemagglutination-inhibition (HI) test, four HA units were used as a constant amount of antigen with varying dilutions of sera. Since in the HI test only 0.25 ml. of antigen dilution was used in each tube, the required units of antigen were calculated by dividing the reciprocal of the antigen titre by a factor of eight. The resulting figure represented the reciprocal of the dilution necessary to give four HA units per 0.25 ml. antigen.

All sera in the HI test were heated to 56°C. for 30 minutes prior to use, and were diluted in two-fold steps starting with 1:2 in 0.25 ml. amounts. Serum dilutions and antigen were mixed by shaking and were held at room temperature for 30 minutes before adding 0.5 ml. of erythrocyte suspension to each tube. The HI reactions were read in 20 to 30 minutes after addition of the erythrocytes. The titres of sera were recorded as the highest dilutions capable of inhibiting four HA units of virus. Some sera were treated with trypsin to remove non-antibody inhibitor substances (6).

Animal Inoculation. Baby pigs from two local premises were used as experimental subjects for infectivity trials. Other than attempting to obtain strong healthy piglets from clinically normal dams, no special selection or treatment of these animals was made. Their ages in most cases were between five and nine days at the time of inoculation. All were born naturally and suckled the sow until removed for experimental study.

Intracranial, subcutaneous and oral routes of inoculation were used in different subjects. All experimental animals were bled prior to inoculation and the serum stored at -20°C. Post-infection or convalescent samples of serum were harvested where possible. After inoculation the piglets were housed individually in Horsfall units and fed a commercial orphan pig diet. In some cases daily rectal temperatures were recorded. When clinical symptoms developed some piglets were slaughtered; others were allowed to die and tissues were removed for histological study and virus isolation.

In addition, two 3-month old pigs were inoculated, one intracranially and the other subcutaneously in the axillary region. Three 6-month old calves and four rabbits were also inoculated subcutaneously.

Tissue Culture Inoculum. Infectious material used for virus isolation consisted of brain and spinal cord from field cases or experimentally inoculated animals. The brain and cord were removed from the affected piglets and either held at -40°C or used immediately to provide inoculum. In all cases portions of cerebrum, cerebellum and, when available, spinal cord, in approximate total amounts of one gram were macerated in a glass tissue grinder and suspended in ten ml. of BAF. The suspension was then centrifuged for ten minutes at 2000 rpm. The opalescent supernatant fluid, further diluted 1:10 in BAF, constituted the inoculum. Penicillin and streptomycin were added to give a concentration of 100 units or micrograms in each ml. of inoculum. Aureomycin was used to treat one inoculum at a level of 200 micrograms per ml. Inoculum was used to replace completely the growth medium on well developed cultures.

Results

Isolation of Virus Strains. Three virus strains have been isolated from different field outbreaks. Lacking final identification at this stage of the investigation, the three viruses have been tentatively identified as HEV-1, HEV-2 and HEV-3.

HEV-1 was isolated from the brain of an eight-day old piglet. This animal was one of a litter of 11 animals which remained clinically normal until six days of age. On that day the owner noticed that the piglets were reluctant to suckle and on the seventh day they were obviously sick. They were shivering, huddling in a corner, squealing and showing some ataxia but no marked nervous symptoms. They exhibited only slight inappetence and appeared to be constipated. The specimen from which virus was obtained was autopsied on the eighth day. By the 10th day all but two of the litter had died.

These two survivors were hand fed and although both showed a marked impairment in weight gain and one was seen to vomit, both recovered. Two other sows on the premises farrowed normal litters one and three months respectively after the birth of the affected litter. The three sows and one of the survivors were bled four months after the outbreak.

HEV-2 was isolated from the brain of a seven-day piglet. All of the piglets in this litter had developed similar symptoms to those described above when five days old but in the terminal stages they became recumbent and exhibited paddling movements. The piglet from which virus was isolated had a temperature of 104°F. prior to euthanasia and autopsy.

HEV-3 was isolated from the brain of an eight-day old piglet, one of a litter of eight which developed symptoms at five days of age. The initial clinical picture was similar to that described under HEV-1 but when seen three days later, the five animals which were still alive showed marked incoordination, stiffness, hyperesthesia, varying degrees of posterior paralysis and temperatures varying from 99 to 104°F. The virus isolation was made from one of three brains submitted, all of which showed histopathological changes of viral encephalomyelitis.

On the same premises a four-week old litter developed a similar but milder illness about the same time. The only symptoms observed were slight inappetance, shivering, loss of condition and some vomiting but no deaths occurred. Seven to ten days before this, several pigs in two separate litters which were two and a half and three months old respectively had shown transient inappetance, loss of condition and vomition. All but one of these animals recovered after a few days with only a slight interruption in growth rate.

Cytopathological Effect in Tissue Culture. The first sign of infection visible microscopically in the unstained cell cultures occurred in 12 to 18 hours. This consisted of scattered round, homogenous areas containing numerous centrally-located round or oval refractile bodies about the size and shape of cell nuclei. In stained cultures these areas were seen to be a type of large multinucleated giant cell. These syncytia at first contained 10 to 40 or more randomly situated, distinct nuclei, the majority appearing in the centre of a large mass of pale cytoplasm. Between 18 and 48 hours the nuclei became pyknotic and then fragmented, giving the giant cells a foamy appearance. Still later, most of the cells in the syncytia died and many cells in the remainder of the monolayer became rounded with heavily stained pyknotic nuclei, giving the cell sheet an appearance of general degeneration. However, even after four or five days incubation many normal appearing cells remained, although most of these had lost their original epithelial appearance. Possibly these remaining cells are predominantly fibroblastic. May-Grunwald-Giemsa stain failed to demonstrate inclusion bodies, although in some giant cells irregularly shaped pink-staining bodies were seen in the cytoplasm. These bodies have not appeared consistently.

Trials undertaken in cell lines of bovine embryo kidney, lamb embryo kidney and rabbit embryo kidney have failed to demonstrate virus growth.

Animal Inoculation. Five separate experimental attempts to infect pigs were carried out, four with tissue culture propagated material and one with the original brain tissue from a field case of the disease. A total of 27 pigs were employed; 25 were between five and nine days of age at the time of inoculation and two about three months of age. The results of these experiments are tabulated in Table I and summarized in Table 2.

In experiment one, six 5-day old pigs (PI-P6), were inoculated intracranially or orally with the seventh tissue culture passage of strain HEV-1. All of these animals developed signs of infection in three to six days and two died before the seventh day. Two others were killed when death appeared imminent. The remaining two appeared to recover but one died on the 12th post-inoculation day. The other (P2) was exsanguinated and killed 22 days post-inoculation at which time it appeared completely normal. Three of the five animals in this experiment examined histologically showed a viral encephalomyelitis. Three of the brains yielded virus in tissue culture.

In the second experiment, seven 6-day old piglets (P7-P13) were used. The sixth tissue culture passage of strain HEV-2 provided the inoculum. Four were inoculated intracranially and three orally. One (P9) died shortly after inoculation and is not included in the summary. Of the remaining

TABLE 1

Results of the inoculation of baby pigs with HEV

Experiment Number	Pig No.	Inoculum	Route[1]	Incubation[2] period	Time of[3] death	Histopathology[4]	Virus re-isolation[5]
1	P1	HEV-1	I.C.	3	D13	Neg.	Neg.
	P2	Pass. 7	I.C.	3	K22	Neg.	Neg.
	P3	"	I.C.	3	K4	Pos.	Pos.
	P4	"	I.C.	3	K4	Pos.	Pos.
	P5	"	Oral	6	D7	Pos.	Neg.
	P6	"	Oral	5	D6	ND	Pos.
2	P7	HEV-2	I.C.	2	D2	Pos.	Pos.
	P8	Pass. 6	I.C.	4	K4	Pos.	Pos.
	P9	"	I.C.	Died shortly after inoculation			
	P10	"	I.C.	2	D4	Pos.	Pos.
	P11	"	Oral	4	K4	Neg.	ND
	P12	"	Oral	5	K7	Pos.	Pos.
	P13	"	Oral	4	D5	Neg.	Pos.
3	P14	HEV-2	I.M.	NS	K27	ND	Neg.
	P15	Pass. 5	I.M.	6	K7	Pos.	Pos.
	P16	"	S.C.	NS	K27	ND	Neg.
	P17	"	Oral	7	D20	Neg.	ND
	P18	"	Oral	7	K9	Pos.	Pos.
	P19	"	Oral	7	D22	Neg.	ND
4	P20	HEV-2	I.C.	NS	All animals challenged with virus of Teschen disease.		
	P21	Original	I.C.	NS			
	P22	brain	I.C.	NS			
	P23	"	Oral	NS			
	P24	"	Oral	NS			
	P25	"	Oral	NS			
5	P26	HEV-1	I.C.	6	K36	ND	Neg.
	P27	Pass. 2	S.C.	NS	K36	ND	Neg.

1 — Route: I.C. — intracranial; I.M. — intramuscular; S.C. — subcutaneous
2 — Incubation period: in days; N.S. — animals not susceptible or no visible signs of infection
3 — Time of death; Dx — Died on day x after inoculation; Kx — Killed on day x
4 — Histopathology: Pos. — Histological changes in brain; Neg. No changes seen; ND — not done
5 — Virus re-isolation: Pos. — Virus isolated; Neg. — Virus not isolated; ND — not attempted.

TABLE 2

Summarized results on experimentally inoculated susceptible piglets (Experiments 1 to 3)

Route of* Inoculation	Number Inoculated	Incubation period (days)	Histopathology positive	Virus re-isolated	Recovery from infection	No signs of infection
I.C.	7	3—4	5	5	2	0
Oral	8	4—7	3	4	2	0
I.M.	2	6	1	1	0	1
S.C.	1	—	0	0	0	1

*I.C. — intracranial; I.M.— intramuscular; S.C. — subcutaneous

six piglets, all developed symptoms in two to five days and either died or were killed within seven days after exposure to the virus. Four of the piglets showed histological evidence of viral encephalomyelitis and virus was recovered in tissue culture from five of the brains.

In experiment three, six piglets (P14-P19), nine days of age, were inoculated with tissue culture fluids of the fifth passage of HEV-2. All the piglets received one ml. of material. P14 and P15 were given the virus intramuscularly, P16 subcutaneously and P17, P18 and P19 orally.

P15 and P18 showed signs of infection on the sixth and seventh days respectively. P15 was killed when near death on the 7th day and P18 similarly on the 9th day. From the brain and cord of each pig virus was re-isolated in tissue culture, and each showed histological evidence of viral encephalomyelitis.

P17 and P19 remained normal until the 20th and 22nd days when each died following an acute rise in body temperature. Although the actual cause of death was not determined, there was no histological evidence of viral encephalomyelitis. The two remaining pigs, P14 and P16 showed no evidence of infection at any time and were bled and killed on the 27th day after inoculation. Each of their sera showed a low but significant HI titre against the virus.

Six piglets (P20-P25) from a single four-day old litter, constituted the subjects of experiment four. In this trial, the piglets were exposed to original brain material from which HEV-2 had been previously isolated. Three of them, P20-P22, were inoculated intracranially with 0.2 ml. of an approximate 1:100 suspension of the brain in BAF, while the other three were given one ml. each of the suspension by the oral route. The inoculum was also tested for the presence of the virus by seeding into pig kidney tissue cultures.

Twenty-four hours after seeding, the tissue cultures showed typical cytopathic effects attributable to the growth of the hemagglutinating virus.

At the time of inoculation blood samples were taken for serum from each piglet. When tested subsequently for HI activity, each sample was found to be positive, showing titres ranging between 1:8 and 1:32. In the following two weeks none of the animals showed any evidence of infection, and it is concluded that the titres indicated a degree of immunity adequate to protect the piglets. Since the piglets were very young, the immunity must have been of a passive nature derived from colostrum antibodies. Subsequent testing of the dam's serum, however, showed no detectable HI titre. Seventeen days after inoculation with HEV, all the piglets in the litter were bled for serum, then, along with a control pig, were challenged by intracranial inoculation with the Reporyje strain of Teschen disease virus. Within ten days after challenge, all the piglets including the control died from Teschen disease. The sera taken prior to challenge showed little or no change in HI titre over the pre-inoculation samples.

Experiment five involved two three-month old pigs. One (P26) was given an intracranial inoculation of 0.2 ml. of the second passage of HEV-1. The other (P27) received one ml. of the same material subcutaneously. P26 showed signs of mild incoordination and anorexia between the sixth and eighth days post inoculation. These signs disappeared rapidly after the eighth day and no further evidence of disease developed. The other pig showed no evidence of infection at any time. Both pigs underwent euthanasia on the 36th day after inoculation at which time blood was taken for serum. Neither yielded virus from the brain in tissue culture, but both developed high HI titres in the serum.

Three calves were each inoculated subcutaneously with sixth passage HEV-1. None showed any significant signs of infection. Serum taken 36 days following inoculation from one calf did not show HI antibody.

Two rabbits were inoculated subcutaneously with the sixth tissue culture passage of HEV-1 and two were similarly inoculated with the original brain suspension of HEV-1. All four rabbits remained normal.

Histopathology. Histopathological studies were conducted on portions of the central nervous system of six field cases representing the three outbreaks from which the virus isolations were made. In addition, specimens were examined from 15 of the piglets inoculated experimentally with tissue culture fluids containing the infectious agent.

The histopathological lesions noted in the central nervous systems of both the field and experimental cases were typical of a viral encephalomyelitis. These changes were characterized by perivascular mono-

TABLE 3

HI test results on sera of some experimentally inoculated piglets

Piglet Number	Inoculum	Route of(1) Inoculation	Pre-inoculation(2) Titre	Post-inoculation(2) Titre	Days after Inoculation
P1	HEV-1-TC-7	I.C.	Neg.	64	11
P2	"	I.C.	Neg.	64	22
P3	"	I.C.	Neg.	Neg.	4
P4	"	I.C.	Neg.	Neg.	4
P8	HEV-2-TC-6	I.C.	Neg.	Neg.	4
P12	"	Oral	Neg.	4	7
P14	HEV-2-TC-5	I.M.	Neg.	2	27
P16	"	S.C.	Neg.	8	27
P18	"	Oral	Neg.	128	9
P22	HEV-2 original brain susp.	I.C.	16	16	17
P25	"	Oral	16	32	17
P26	HEV-1-TC-2	I.C.	Neg.	128	36
P27	"	S.C.	Neg.	32	36

(1)Route — I.C. — intracranial; I.M. — intramuscular; S.C. — subcutaneous
(2)Titres — Neg. — no HI reaction; figure — reciprocal of serum dilution inhibiting 4 HA units o antigen.

nuclear cuffing, the formation of glial nodes and neural degeneration. The changes were essentially similar to those reported previously (2, 3).

The results in the experimentally inoculated cases varied in degree of involvement, but are recorded in Table I as positive where changes were seen, and negative where no changes could be found in the portions examined.

Hemagglutination and hemagglutination-inhibition tests. Hemagglutination occurred when washed chicken red-blood cells were mixed with tissue culture fluids from infected cultures. In three separate trials it was observed that hemagglutinins appeared in inoculated cultures within 24 hours and reached maximum heights in three or four days. None of the titres obtained were very high, the best usually being in the range of 1 unit in 0.5 ml. of 1:16 to 1:64 dilution of tissue culture fluids. Obviously, since four units in 0.25 ml. of dilution are required in the test, a titre of 1:8 was the minimum acceptable. In practice antigens used had titres of 1:16 or better. Bovine amniotic fluid, either fresh or taken from uninoculated pig cell cultures, showed no ability to agglutinate chicken red blood cells.

The results of hemagglutination-inhibition tests on representative serum samples are presented in Tables 3 and 4. All the sera from experimental cases were in paired pre- and post-inoculation samples. It is seen that experimentally inoculated piglets surviving infection developed HI antibodies to clearly significant levels. All the pre-inoculation samples except those in trial four, were negative, although in some cases trypsin treatment was required to remove non-antibody inhibitory effects. The trypsin treatment did not alter the reaction in positive serum samples.

The few field samples available, included in Table 4, provided titres comparable to those in the experimentally inoculated piglets. Also in Table 4 are shown the results of tests using specific antisera to other viruses. No cross reactions were demonstrable.

Discussion

The results of the preliminary studies reported here show that the viruses isolated from the three field outbreaks of encephalomyelitis in baby pigs are pathogenic microorganisms. The three agents are indistinguishable from one another and undoubtedly represent appearances of a single strain of virus in different parts of Ontario.

The experimental data presented support the etiological relationship of the hemagglutinating viruses isolated to the disease observed in the field. This conclusion is based on the following:

TABLE 4

HI test results on field case sera and on sera from other virus diseases

Sample or disease	Animal souce	Result
Field case HEV-3 #1	Sow	64*
#2	"	128
#3	"	64
#4	Recovered piglet	8
Eastern Equine Encephalomyelitis	Quinea pig	Neg.
Newcastle disease	Chicken	Neg.
Fowl plague — Wien strain	Rabbit	Neg.
Hog Cholera	Rabbit	Neg.
Louping ill	Sheep	Neg.
PE-1 enterovirus	Pig	Neg.
Teschen disease — Reporyje strain	Pig	Neg.
Vseicular stomatitis — N.J.	Calf	Neg.
Vesicular exanthema	Pig	Neg.

*Reciprocal of dilution inhibiting 4 HA units of virus.

1. Three identical strains of the virus were isolated in tissue culture from widely separated outbreaks of the disease.
2. These viruses were shown to be highly pathogenic for baby pigs when administered either orally or by parenteral inoculation. Furthermore, demonstration of pathogenicity was not dependent upon the use of colostrum deprived piglets.
3. The symptoms produced in experimental subjects as well as the histopathological findings were closely similar to those observed in field cases.
4. Virus was readily recovered from brain tissue of experimental subjects showing symptoms of the disease regardless of the route of exposure to the virus.
5. In both experimental and field cases which survived the disease, serological evidence of exposure in the form of hemagglutination-inhibition antibodies was readily demonstrated.
6. Piglets showing HI antibody titres prior to inoculation were not susceptible.

Comparative histopathological findings between HEV and porcine polioencephalomyelitis caused by other agents such as Teschen disease, Talfan disease and poliomyelitis suum leads one to the conclusion that all these conditions are virtually indistinguishable with respect to the histological changes seen in the brain and spinal cord.

It is also important to consider the clinical differentiation between HEV infection and infection with the Teschen, Talfan, enterovirus group. Observations to date indicate that HEV is primarily a pathogenic agent for the newborn or very young suckling piglet. The three field outbreaks investigated all involved pigs which were less than a week old at the onset of symptoms. In one case there was evidence of mild disease among older animals, but at present it has not been possible to show any relationship with HEV infection. In the limited number of infectivity trials carried out so far, pathogenicity and mortality were highest in piglets under seven days of age. In experiment three in which nine-day old piglets were used, only two of the six animals showed marked evidence of infection attributable to inoculation. Neither of the three month old pigs inoculated showed anything more than transitory signs of infection.

By comparison, Teschen disease virus strains studied in this laboratory produced encephalomyelitis and death in 30 to 40 lbs. pigs without difficulty (7). Talfan disease (8) and poliomyelitis suum (9) are considered to be mild infections compared to Teschen, but these conditions also often involve pigs three weeks of age and older.

The final identity of HEV has not been determined. It may represent a hitherto unrecognized agent, or a new manifestation of some previously described virus. In any case, studies to date have failed to demonstrate through the HI test any relationship with a number of hemagglutinat-

ing and non-hemagglutinating viruses. In one trial, piglets unsusceptible to HEV were fully susceptible to challenge with Teschen disease virus. Limited trials have also failed to produce infection in either calves or rabbits, or to cause recognizable cytopathic changes in tissue cultures of other than porcine origin.

Summary

Three strains of a virus capable of producing encephalomyelitis in baby pigs were isolated from specimens originating in different parts of Ontario. All three virus strains appeared identical. The virus grew in monolayer cultures of pig kidney cells and produced characteristic cytopathic effects. Tissue culture fluids from infected cultures caused agglutination of chicken erythrocytes, and this effect was inhibited by serum from convalescent animals. The virus proved highly pathogenic for very young suckling pigs which had no HI antibodies at the time of exposure. Final identity of the agent has not been determined.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the technical assistance provided by Mr. R. Hogan and Mr. R. Gatecliffe. Also appreciated was the advice given by Dr. J. F. Frank in the preparation of the manuscript.

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A Critical Evaluation of Bephenium Hydroxynaphthoate as an Antehelmintic in Eight Yearling Hereford Calves

Eight, naturally-parasitized, yearling, Hereford heifers were used to study the anthelmintic efficacy of a 90% preparation of bephenium hydroxynaphthoate. Four calves were dosed at the rate of 250 mg. per kilogram body weight and 4 were untreated. Efficacy was based on a comparison of total worms recovered from treated calves with those recovered from untreated calves. On this basis there was 85% reduction for *Ostertagis ostertagi*, 87% for *Trichostrongylus axei*, 100% for *Oesophagostomum radiatum* and 99% for *Chabertia ovina*. No harmful effects from the drug were noticed in the four treated calves.

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Growth Rate and Other Signs of Infection in Calves Experimentally Infected with Cooperia punctata

Five calves, 8 to 13 weeks old, were each dosed with about 250,000 *C. punctata* infective larvae. Five other calves of approximately the same age and weight were kept as controls. Eggs were observed in the faeces about 12 days after infection. Signs of infection included soft faeces, intermittent or continuous diarrhea, progressive emaciation, reduced feed consumption, weight loss and listlessness. None of the infected calves died during the trial but one was moribund 3½ weeks after infection and was killed. Two of the remaining four infected calves were in poor condition and two were in fair condition 11 weeks after infection. Average weekly gain was 5.9 lbs. for the four infected calves and 9.41 lbs. for the controls.

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