Studies of the epidemiology, causation and transmission of Potomac Horse Fever.

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Dedicated to the memory of

EDWARD WOOTTON

(1926 - 1986)

who provided me with all the support, encouragement and inspiration I could ever have wished for.

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DECLARATION

I hereby declare that this thesis, presented to the University of Edinburgh for the degree of Doctor of Veterinary Medicine and Surgery, has been composed entirely by myself.

I also declare that the work described herein was performed by myself as a member of a research group, for which I acted as chairman, and provided a substantial contribution. The contributions of myself and of other members of the research group are clearly identified in the thesis.

B.D. Perry, B.V.M.&S., D.T.V.M., M.Sc., M.R.C.V.S.
June 19, 1987

I hereby declare that I have read this thesis prepared by B. D. Perry, and, as a member of the research group which carried out these studies, can attest to his substantial contribution to this work and to the fact that the nature and extent of his contributions are accurately described in the thesis.

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ABSTRACT.

A newly recognized disease of horses, called Potomac Horse Fever (PHF), emerged during the late 1970's in a region close to the Potomac River near Washington, D.C., United States of America. The studies which were carried out by a research group to define the epidemiology and establish the cause of the disease are described. Further studies examined the possible route of transmission of the causative organism, and the role of farm animals and wild rodents in the epidemiology of the disease.

Results of a case-control study conducted in 1983 characterised the disease as a non-contagious, infectious seasonal disease of horses of all age, sex and horse-use categories. The study found a lack of association with most of the variables studied, and assisted in discounting some of the numerous aetiologies proposed at the time. The results showed positive associations with a few variables, which included the previous presence of the disease in a barn, the presence of other livestock and the presence of habitats favourable for arthropod breeding and development.

The disease was established experimentally in ponies following blood transfusion from natural cases of PHF, an Ehrlichia was isolated from the white blood cells of the experimentally infected ponies and this organism subsequently reproduced the disease on inoculation into susceptible ponies. The experimental disease was consistent with that seen in field cases. Pathological studies on the disease were carried out, and the causative Ehrlichia was identified on the wall of the large intestine of affected animals.

Experiments established that the intradermal route was effective in transmitting the disease to ponies. A serological survey of farm animals and wild rodents from affected farms showed no indication of previous exposure to the causative Ehrlichia in 98% of samples tested. None of the mammalian species studied appears to serve as a reservoir of the infection.

CHAPTER ONE

INTRODUCTION

ORIGIN OF THE INVESTIGATION.

The Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, is located in Blacksburg in southwestern Virginia, USA, approximately 250 miles southwest of the capital, Washington, D.C. The College has a large animal practice in the area covered in a 35-mile radius from Blacksburg. In addition, it offers a consulting service for the investigation of disease outbreaks or impaired productivity of animals within the states of Virginia and Maryland.

In August 1982, at the request of the Head of the Department of Veterinary Science, University of Maryland, a field investigation into the cause of an unusually large number of cases of diarrhoea in adult horses in Montgomery County, Maryland, was initiated. The disease was given the name Potomac horse fever (PHF). The investigation took the form initially of a survey of veterinary practitioners and diagnostic services to acquire background information on the nature and extent of the problem. In 1983, a structured case-control study was carried out to characterise the disease. In 1984, further field studies were carried out, and experimental studies in ponies were initiated, culminating later that year in the discovery of the cause of PHF. The investigation then turned to aspects of the transmission of the disease, on which subject they are still in progress. This thesis

provides a critical account of these studies.

HISTORY OF POTOMAC HORSE FEVER.

The history of PHF is indeed short. During the latter half of the last decade, the disease was first recognised by veterinary practitioners in five veterinary practices located in Montgomery County, Maryland, USA (Figure 1).

FIGURE 1. THE LOCATION OF MONTGOMERY COUNTY, MARYLAND, IN THE UNITED STATES OF AMERICA.

During the initial field investigation of 1982, the following description[1] of the disease was given by representatives of the veterinary practices mentioned.

1. Affected horses would go off their feed, and develop a fever of 101-105°F (38.4-40.5°C).
2. A leucopaenia would be detectable by a complete blood count carried out at first examination.
3. The disease had a characteristic sudden onset. Some cases were very severe, reminiscent of an intestinal torsion; others were mild.
4. Diarrhoea developed 2 - 5 days after anorexia was detected. Some horses were initially presented with diarrhoea.
5. The diarrhoea varied in consistency and duration.
6. Laminitis was a sequel in some cases.
7. The prognosis was extremely variable.

One of the practitioners[2] reported seeing the first case he recognized in 1976. This horse developed severe diarrhoea, became dehydrated and died. Another practitioner[3] reported that he first observed the disease in 1979 during the late summer, when he recorded two cases. Subsequent cases seen in that veterinary practice up to the initiation of studies reported here are shown in Table 1. Other practices started

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[1]The description is from hand-written notes taken by the author at the time.

[2]Dr. Roger Scullin, Damascus Equine Associates, Damascus, Maryland.

[3]Dr. Chet Anderson, Peachtree Veterinary Hospital, Beallsville, Maryland.

recording cases from 1979 onwards.

The disease was reported sporadic in its occurrence, and cases were strictly seasonal, reportedly occurring during the summer months. Equine salmonellosis was considered the main differential diagnosis, but Salmonella spp. were not isolated from affected horses.

TABLE 1. CLINICAL DIAGNOSES OF POTOMAC HORSE FEVER IN A MARYLAND VETERINARY PRACTICE, 1979-1982.

Year	Number of Clinical Cases	Number of Deaths
1979	2	1
1980	8	3
1981	15	5
1982	42	14

Equine zoography of Montgomery County, index focus of Potomac horse fever.

Montgomery County, Maryland, lies on the Potomac River in Maryland, borders on Washington, D.C., and occupies an area of approximately 500 square miles. The Potomac River forms the southwestern boundary of the county, flowing in a general southeasterly direction.

The exact horse population of the county is unknown. However, during a vaccination campaign for Venezuelan equine encephalomyelitis

conducted in 1972, approximately 7,000 horses were vaccinated (State of Maryland Department of Agriculture, 1973). Many of the local residents commute to their employment in the city of Washington, D.C. and most horses in the county are kept for recreational riding. A wide variety of horse breeds are present, but prior to this study, no specific data were available.

STRUCTURE OF THE INVESTIGATION.

The investigation into Potomac horse fever by the author and colleagues which followed can be retrospectively divided into three stages.

1. Epidemiological study carried out prior to the determination of the cause of the disease. This was a structured case-control study carried out in 1983 in Montgomery County, Maryland, to characterise the epidemiology of the disease, and to attempt to identify risk factors associated with the presence of the disease.
2. Studies on the causation and pathogenesis of the disease. These were carried out in experimentally infected ponies under controlled conditions at the Virginia-Maryland Regional College of Veterinary Medicine in Blacksburg, Virginia in 1984 and 1985.
3. Studies on the transmission of PHF. These comprised field and laboratory studies on the role of arthropods in the transmission of the disease, and studies on the source of infection, by the examination of the role of horses, other

domestic animals and wild animals as reservoirs of the causative organism. They were carried out in 1984 and 1985.

Author's contributions to the investigation.

The investigations described are those of a research group. The author was co-instigator of the investigations, and the principal investigator and author of six of the seven funded research projects which supported these studies. The author is a veterinary epidemiologist and has been the chairman of the Potomac horse fever research group at the Virginia-Maryland Regional College of Veterinary Medicine since its inception in 1984.

The contributions of the author are identified in bold type in the Materials and Methods and Results sections of each chapter to differentiate them from contributions of other members of the research group, which are displayed in italics.

CHAPTER TWO

EPIDEMIOLOGICAL STUDIES OF POTOMAC HORSE FEVER

INTRODUCTION.

Potomac horse fever (PHF) was first recognized and described in the late 1970's by the veterinary practitioners of Montgomery County, Maryland. At first there was considerable confusion as to whether this was a distinct disease entity, and if so, whether it was in fact salmonellosis which could not be confirmed by the diagnostic laboratories concerned. By 1982, it became fairly clear that it was indeed a distinct disease entity, and that several different laboratories had been unable to causally associate it with Salmonella spp. There followed a plethora of suggested aetiologies, proposed by veterinarians, researchers, horse-owners, members of the public and others. These included arthropod-borne viruses, intestinal protozoa, clostridial enterotoxins, pesticides, toxic plants, aflatoxins and sewage slurry spread on to fields. At the time, the basis for suggesting the existence of a causal relationship between PHF and any proposed aetiology was diagnostic laboratory confirmation on specimens submitted from sick horses. The testing of a causal hypothesis by other means, such as by comparing the epidemiology of PHF with that of any of the proposed aetiologies was difficult, as there was extremely limited information available on the structure and characteristics of the horse population of the region. In addition, the intensive screening in diagnostic laboratories of specimens from affected horses for potential pathogens meant that certain agents

would inevitably be identified, but without reference to the possible presence and distribution of such agents in the horse population at large, their mere identification was difficult to interpret. Such was the case with a coronavirus-like agent which was isolated from some affected horses (Huang et al., 1983), but subsequently found also to be present in other horses as well as those suffering from PHF.

Given the plethora of aetiologies proposed for PHF at the time, and the lack of substantive data on the structure and characteristics of the horse population of the affected region of Maryland, it was decided to carry out a structured epidemiological study. The objective of this study was to carefully characterise the pattern of the disease in the horse population, so that in the event that a cause was not discovered during that year or soon after, there would exist data on which the validity of proposed causal hypotheses could be tested from the epidemiological viewpoint.

In addition, a structured epidemiological study could identify risk factors associated with the presence of the disease, and therefore act as an aid to experimental studies to determine the cause of PHF. For this reason, a case-control study was performed. This technique allows the study of diseases of low incidence, and permits the estimation of risk associated with variables studied by the calculation of an odds ratio (McMahon and Pugh, 1970; Schlessman, 1982; Thrusfield, 1986a). This chapter describes the design, execution and analysis of the case-control study. The author was the instigator and director of the study, and was responsible for the data analysis and interpretation.

At the time of this epidemiological study, no aetiological agent had

been identified. Subsequently, however, Ehrlichia spp. were detected in the wall of the large colon of experimentally infected ponies (Rikihisa et al., 1984; 1985), and the causative ehrlichial organisms were subsequently isolated in tissue culture (Holland et al., 1984; 1985a; Rikihisa and Perry, 1984; 1985) and reinoculated into susceptible ponies, reproducing the disease (Dutta et al., 1985; Holland et al., 1985a; Rikihisa and Perry, 1985; Palmer et al., 1986). The causative organism has been named Ehrlichia risticii (Holland et al., 1985b). The author's contribution to the causation studies carried out at the Virginia-Maryland Regional College of Veterinary Medicine in Blacksburg are described in Chapter 3.

MATERIALS AND METHODS.

Study design.

The target population selected for the case-control study was all the horses on farms served by five veterinary practices in Montgomery County, Maryland. At the time, over 90% of reports of PHF originated from these practices. Case subjects for the study population were identified throughout the summer of 1983 following the clinical diagnosis of PHF by the attending veterinarian. Cases were designated at two levels, which were the affected horse and the affected premises. The owner of each affected horse was then requested to answer questions contained on two personal interview questionnaires, which are shown in Appendices A and B. The first, designated Form A, pertained to

the horse and the second, designated Form B, pertained to the premises at which the horse was kept. Questions covered management procedures of the horses at pasture and in the stable, movement of horses off the premises, contact with PHF, feeding practices, medical history and medication received. Form B requested a full inventory of horses on the premises, and included questions on human health problems recognized, to investigate any possible zoonotic implications of the disease.

A total of 135 questions were asked. The interrogators were veterinary students and faculty members.

Affected premises were then matched with unaffected premises, which were designated control subjects for the study population. The matching of case and control premises was based on the following criteria:

a) the same veterinary practice;

b) approximately the same number of horses;

c) the same type of premises (for example, boarding stables, breeding farm); and

d) the same geographic region of the county.

On each of the control premises, up to five horses were identified and matched with affected horses for age and sex. These were designated control subjects. In addition, up to five unaffected horses were identified as control subjects on affected premises, using the same matching criteria of age and sex.

Horse questionnaires were then completed for all control horses and farm questionnaires for all control premises.

The study thus comprised cases and controls at the level of premises and individual horses. In addition, there were two groups of control

horses, those on affected premises and those on control premises. The study design is illustrated in Table 2.

TABLE 2. DESIGN OF THE CASE-CONTROL STUDY.

	Cases		Controls	
Level	Description	Number	Description	Number
Premises	Affected farms (AF)	21	Unaffected farms (CF)	20
Horses	Affected horses on affected farms (AA)	68	Unaffected horses on affected farms (UA)	84
	---		Unaffected horses on unaffected farms (UC)	108

Serum and faecal samples were collected for each affected and unaffected horse. *Faecal samples were subjected to routine bacteriological analysis and to analysis for Clostridium perfringens type A enterotoxin and for C. difficile cytotoxin.* Sera were retained at -40°C for future reference.

Following the identification of the causative agent of PHF and the development of a serological test, the indirect fluorescent antibody test (IFAT) (Rikihisa and Perry, 1984; 1985), sera were tested to substantiate the clinical classification of cases and controls.

A total of 77 feed samples, both grain and hay, were taken from affected and unaffected premises, *and analyzed for gross abnormalities.*

(Analysis performed by National Animal Disease Laboratory, Ames, Iowa.)

Data on the daily temperature and rainfall for a site in Montgomery County near the town of Potomac, and lying on the Potomac River, were collected (Source: Washington Suburban Sanitary Commission, Hyattsville, Maryland) and charted as mean monthly temperature and mean monthly rainfall.

Data analysis.

Data from the two questionnaires were processed on an IBM 3084 mainframe computer. Epidemiological characteristics related to variables considered important and for which sufficient responses were obtained were quantified. Each horse-level variable was analyzed by both AA/UA and AA/UC comparisons, and each farm level variable was analyzed by AF/CF comparisons. Odds ratios were computed (Fleiss, 1981) and evaluated for significance using the chi-square analysis using Statistical Analysis System (SAS, Carey, North Carolina). Data were expressed as proportions of each study group positive for the variable where numbers were sufficient to effectively perform the chi-square analysis.

In addition, analysis of age, sex and horse-use distribution was made by the comparison of AA horse data for these variables with those from the total population of horses on AF and CF farms (derived from the responses to questions 18, 19 and 20 of Form B; see Appendix B). The chi-square analysis was performed on the tables of data so generated.

A discriminant analysis was subsequently performed in collaboration with Mr. David Morris, Department of Statistics, Virginia Polytechnic

Institute and State University, on a subset of 12 variables selected on the basis of a statistically significant odds ratio, possible suitability as predictor variables of clinical PHF and conforming with the assumptions of the discriminant analysis. The primary assumption was that the variables have at least approximately normal distribution and so categorical variables were excluded. The procedures of STEPDISC, CANDISC and DISCRIM (SAS, 1982) were used. The variables were examined initially by both discriminant and canonical discriminant analysis. Discriminant analysis classified horses as affected or unaffected based on a model using the selected variables. In contrast, canonical discriminant analysis found a linear combination of the variable that best summarized the differences between affected and unaffected horses. Next a parsimonious model was sought with a step-wise discriminant procedure which searched for an optimal subset of variables on the basis of squared partial correlations. These discriminant procedures were run with both AA/UA and AA/U (UA + UC) comparisons.

RESULTS.

General.

The location of the premises in Montgomery County at which cases of PHF occurred during 1983 is shown in Figure 2. The temporal distribution of these cases by month is shown in Figure 3. The mean monthly temperature and rainfall data are presented in Figure 4.

A summary of the results of AA/UA horse comparisons for selected

variables is given in Table 3; that for AA/UC comparisons is presented in Table 4.

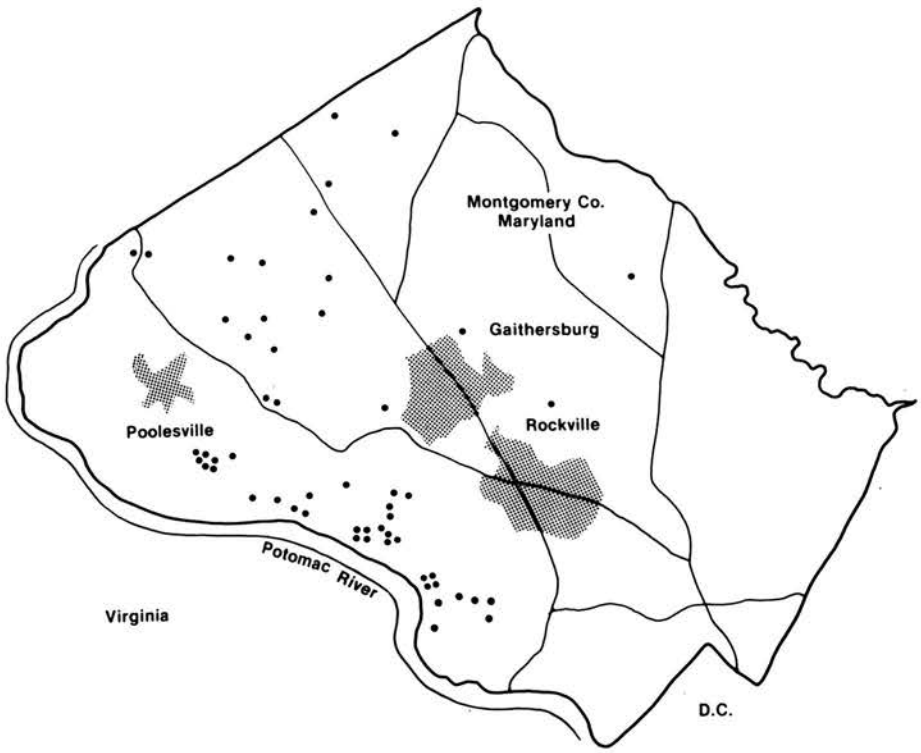

FIGURE 2. SPOT MAP OF MONTGOMERY COUNTY, MARYLAND SHOWING THE LOCATION OF PREMISES AT WHICH PHF CASES OCCURRED DURING 1983.

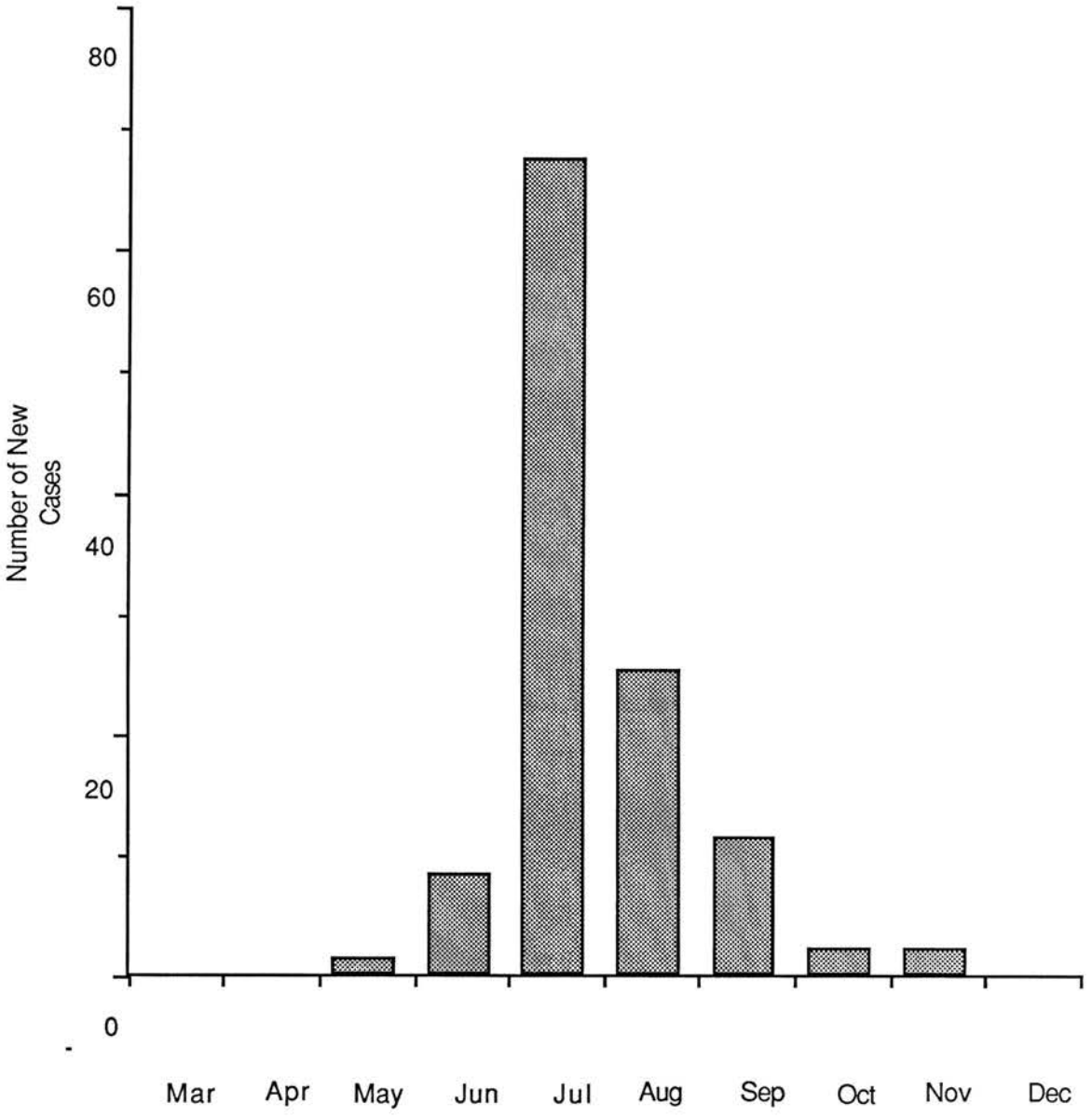

FIGURE 3. THE REPORTED MONTHLY INCIDENCE OF POTOMAC HORSE FEVER IN MARYLAND, 1983

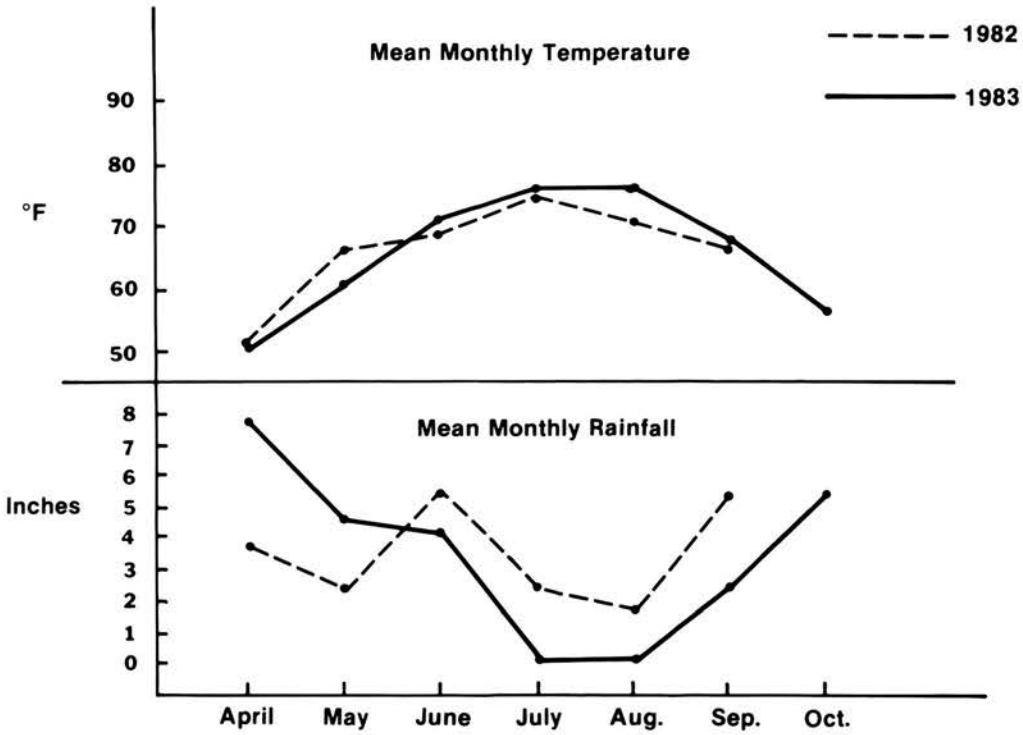

FIGURE 4. METEOROLOGICAL DATA, MONTGOMERY COUNTY, MARYLAND.

TABLE 3. RESULTS OF THE COMPARISON OF STUDY VARIABLES BETWEEN AFFECTED AND UNAFFECTED HORSES ON AFFECTED FARMS (AA/UA).

Variable Studied	Proportion of Study Group Positive for Variable (%) AA	UA	Odds Ratio	Statistical Significance*
ORIGIN OF HORSE:				
1. Born on farm where currently kept	7.3	10.8	0.65	NS
2. Born in the Potomac area	21.7	27.6	0.73	NS
CHARACTERISTICS OF PASTURE:				
1. Currently on pasture of 10 acres or less	55.2	46.9	1.39	NS
2. Currently pastured with 5 or more other horses	44.7	48.1	0.87	NS
3. Presence of little pasture where currently grazing	14.9	11.1	1.4	NS
4. Use of fertilizer on currently grazed pasture	53.8	58.0	0.84	NS
5. Use of this pasture for other livestock species for grazing	31.3	18.3	2.04	NS
6. Occurrence of PHF in previous years on currently used or adjacent pastures	44.7	73.9	1.0	NS
CHARACTERISTICS OF STABLE:				
1. Currently housed in barn with 5 or more horses	74.6	83.8	0.56	NS
2. Use of communal watering facilities	29.1	37.0	0.7	NS
3. Use of sawdust bedding	33.1	46.8	0.57	NS
4. Painting of stalls vs. use of creosote	48.0	28.0	2.37	NS
5. Occurrence of PHF in currently used barn in previous years	56.9	29.8	3.1	0.002

*NS = not significant (P > 0.05)

continued...

TABLE 3, continued

Variable Studied	Proportion of Study Group Positive for Variable (%) AA	UA	Odds Ratio	Statistical Significance*
ANIMAL MOVEMENTS:				
1. One or more movements from the premises during the previous months of 1983:				
January	10.0	10.0	1.11	NS
February	8.3	16.2	0.47	NS
March	17.1	12.1	1.49	NS
April	22.7	16.4	1.54	NS
May	24.4	19.4	1.34	NS
June	22.7	18.1	1.33	NS
July	28.6	13.0	1.9	NS
2. Purpose of excursions away from premises during 1983:				
Horse show	59.5	75.7	0.47	NS
Three-day event	6.2	4.7	1.32	NS
Training	12.8	6.5	2.11	0.02
Breeding	21.5	13.1	1.83	0.02
3. Use of horse transport vehicle other than that owned by premises	47.1	32.3	1.87	NS
PRESENCE OF CLOSTRIDIAL TOXINS:				
1. Presence of C. difficile cytotoxin in faeces	3.8	11.1	0.32	NS
2. Presence of C. perfringens A enterotoxin in faeces	33.0	27.2	1.3	NS

*NS = not significant ($P > 0.05$)

TABLE 4. RESULTS OF THE COMPARISON OF STUDY VARIABLES BETWEEN AFFECTED HORSES ON AFFECTED FARMS AND UNAFFECTED HORSES ON UNAFFECTED FARMS (AA/UC).

Variable Studied	Proportion of Study Group Positive for Variable (%) AA	UC	Odds Ratio	Statistical Significance*
ORIGIN OF HORSE:				
1. Born on farm where currently kept	7.3	8.3	0.87	NS
2. Born in the Potomac area	21.7	9.5	2.64	0.05
CHARACTERISTICS OF PASTURE:				
1. Currently on pasture of 10 acres or less	55.2	65.1	0.66	NS
2. Currently pastured with 5 or more other horses	44.7	40.7	1.14	NS
3. Presence of little pasture where currently grazing	14.9	35.8	0.31	NS
4. Use of fertilizer on currently grazed pasture	53.8	65.4	0.62	0.002
5. Use of this pasture for other livestock species for grazing	31.3	11.7	3.46	0.001
CHARACTERISTICS OF STABLE:				
1. Currently housed in barn with 5 or more horses	74.6	72.7	1.1	NS
2. Use of communal watering facilities	29.1	29.5	0.98	NS
3. Use of sawdust bedding	33.1	14.7	2.9	0.004
4. Painting of stalls vs. use of creosote	48.0	59.6	0.62	NS

*NS = not significant (P > 0.05)

continued...

TABLE 4, continued

Variable Studied	Proportion of Study Group Positive for Variable (%) AA	UC	Odds Ratio	Statistical Significance*
ANIMAL MOVEMENTS:				
1. One or more movements from the premises during the previous months of 1983:				
January	10.0	14.1	0.68	NS
February	8.3	14.9	0.52	NS
March	17.1	16.2	1.06	NS
April	22.7	26.0	0.86	NS
May	24.4	35.7	0.58	NS
June	22.7	38.4	0.47	NS
July	28.6	25.0	1.67	NS
2. Purpose of excursions away from premises during 1983:				
Horse show	59.5	64.7	0.8	NS
Three-day event	6.2	8.7	0.69	NS
Training	12.8	13.1	0.97	NS
Breeding	21.5	13.5	1.76	0.017
3. Use of horse transport vehicle other than that owned by premises	47.1	37.5	1.49	NS
PRESENCE OF CLOSTRIDIAL TOXINS:				
1. Presence of C. difficile cytotoxin in faeces	3.8	3.0	1.32	NS
2. Presence of C. perfringens A enterotoxin in faeces	33.0	15.1	2.8	NS

*NS = not significant ($P > 0.05$)

The results of AF/CF farm comparisons are shown in Table 5.

TABLE 5. RESULTS OF THE COMPARISON OF STUDY VARIABLES BETWEEN AFFECTED AND UNAFFECTED FARMS (AF/CF).

Variable Studied	Proportion of Study Group Positive for Variable (%) AF	CF	Odds Ratio	Statistical Significance*
PHF PRESENCE IN AREA:				
1. Presence of neighbour with horses within 0.4 Km	52.3	71.4	0.44	NS
2. Known presence of PHF on neighbour's farm within 0.4 km	17.6	29.4	0.51	NS
HORSE MOVEMENTS:				
1. Addition of horses to the farm over the last 12 months	72.7	85.7	0.44	NS
2. On farms where horses were added, the addition of 5 or more horses to the farm over the last 12 months	44.0	61.1	0.49	NS
3. Temporary removal of horses over the last 12 months	83.4	90.5	0.67	NS
CHARACTERISTICS OF PASTURE AND FEED:				
1. Keeping of horses full time at pasture (never stabled)	66.7	57.9	1.45	NS
2. Presence of excellent and lush pasture	33.3	5.5	8.5	0.03

*NS = not significant (P > 0.05)

continued...

TABLE 5, continued

Variable Studied	Proportion of Study Group Positive for Variable (%) AF	CF	Odds Ratio	Statistical Significance*
3. Presence of weed, seed or insect damage in concentrate horse feed used	13.1	10.2	1.28	NS
PRESENCE OF ANIMALS OTHER THAN HORSES:				
1. Ratio of other domestic livestock on the premises-cattle:dogs:cats: chickens	3:2.6:2.2:1	1:2.2:2.6:3	---	0.0001
2. Recent occurrence of illness in domestic animals other than horses	19.0	9.5	0.45	NS
3. Farm frequented by wild animals	72.2	82.4	0.56	NS
4. The recognized presence of rodents in the barn	71.4	65.0	1.34	NS
5. The recognized presence of rodents on the pasture	78.9	50.0	3.75	0.05
HUMAN HEALTH:				
1. The presence of unexplained illness in people living or working on the premises	11.1	9.5	1.19	NS

*NS = not significant ($P > 0.05$)

Figure 5 shows the proportion of horses in each study group turned out to pasture and Figure 6 gives the average number of hours spent at pasture, by month.

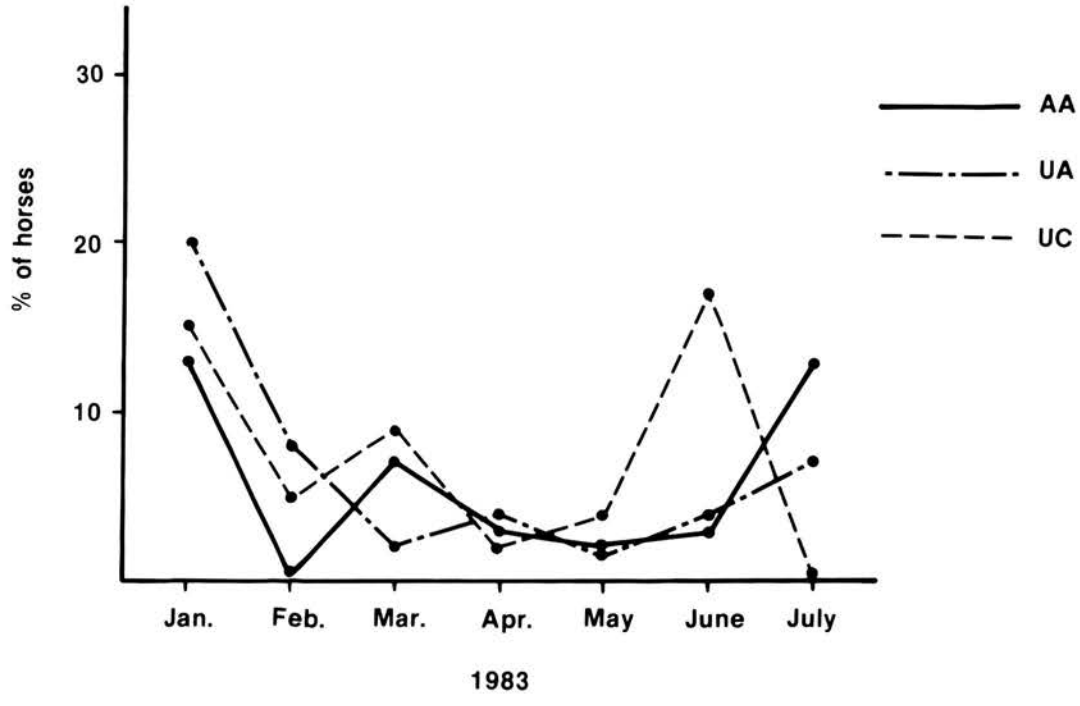

FIGURE 5. PROPORTION OF HORSES TURNED OUT TO PASTURE, BY MONTH.

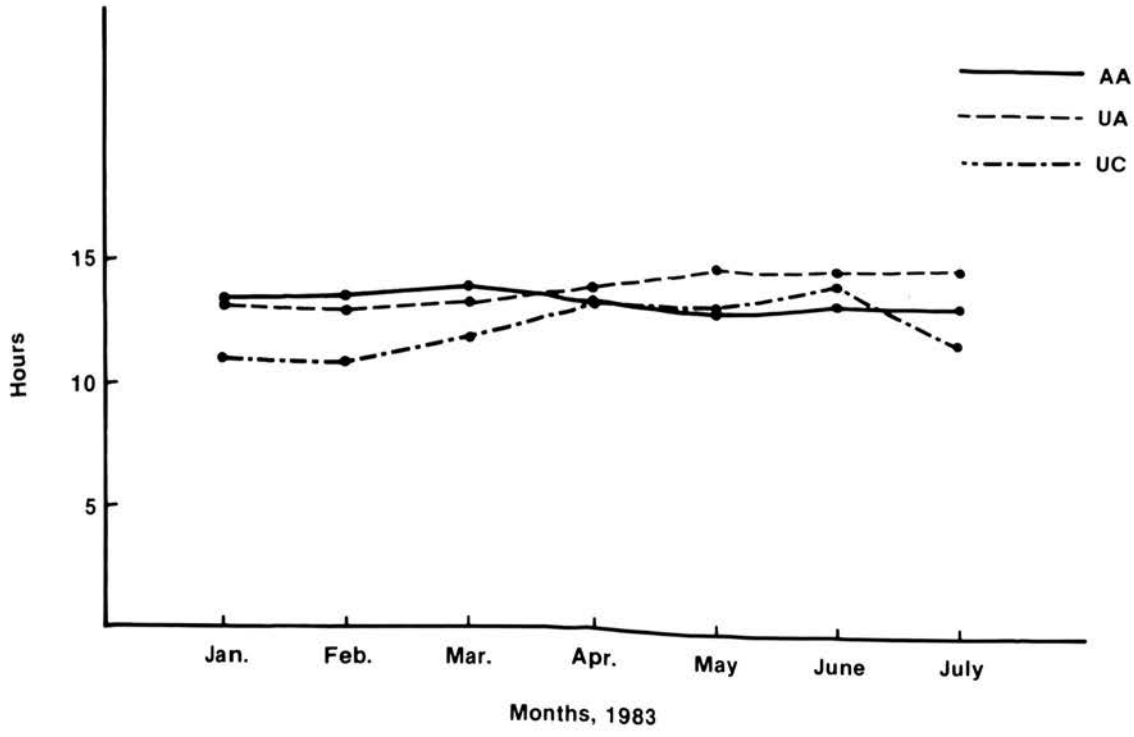

FIGURE 6. AVERAGE NUMBER OF HOURS PER DAY SPENT AT PASTURE, BY MONTH.

TABLE 6. DETECTION OF ABNORMALITIES IN 77 FEED SAMPLES EXAMINED FROM HORSE PREMISES IN MARYLAND.

	Affected Farms	Unaffected Farms
Feed samples with gross abnormalities	6[1]	4[2]

[1] Whole barley: weevil infestation and severe weed seed contamination
Whole oats: severe weed seed contamination
Whole and cracked maize: meal moth infestation
Sweet feed: weevil infestation
Sweet feed: weevil infestation and severe weed seed contamination
Oats: old and discoloured

[2] Sweet feed: moderate weed seed contamination
Pelleted feed: presence of Gentian violet
Sweet feed: foreign material
Sweet feed: moderate to severe weed seed contamination

Of the affected study farms, 14 (70%) had experienced a single case of PHF. Three farms (15%) had reported two cases of PHF, two farms had reported three cases, and two farms reported four and five cases of the disease respectively. No farm reported more than five cases.

At pasture, 79% of the affected horses (AA) had no other PHF cases on the same pasture, and 53% of unaffected horses on affected farms (UA) had one or more PHF cases on the same pasture.

In the barn, 67% of the affected horses (AA) were the only case in the barn. Of the affected premises, 58% had no recognized PHF cases in the barn during previous years. Of affected horses, 9% had reportedly been affected with the disease previously.

Using the UA group of horses as the control group for comparison, there was no significant risk, as measured by the odds ratio, associated with all but three of the study variables analyzed. The variables with a significant risk association were the occurrence of PHF in the currently used barn in previous years, and excursions away from the premises during 1983 which were associated with training or breeding.

Using the UC group of horses as the control group for comparison, significant risk associations were found between the presence of the disease and five of the variables analyzed. These were birth within the Potomac area, the use of pasture for grazing by other livestock species, the use of sawdust bedding, excursions away from the premises during 1983 which were associated with breeding, and a negative association with the presence of little pasture where currently grazed.

In the comparison of affected (AF) and unaffected (CF) farms, positive associations with the disease were found with three variables analyzed:

the ratio of other domestic livestock on the premises, the presence of excellent and lush pasture, and the recognized presence of rodents on the pasture.

When the distributions of age, sex and horse-use within the AA group of horses were compared to those of all the horses on the 41 premises studied, no significant differences were detected. The results of this comparison are shown in Tables 7, 8 and 9.

TABLE 7. COMPARISON OF THE SEX DISTRIBUTION OF AFFECTED (AA) HORSES WITH ALL THE HORSES ON 41 STUDY FARMS.

	Stallion	Mare	Gelding
Total horses on surveyed farms (%)*	7	43	50
Affected (AA) horses (%)	8	43	49

*No significant difference between the two distributions.

TABLE 8. COMPARISON OF THE AGE DISTRIBUTION OF AFFECTED (AA) HORSES WITH ALL HORSES ON 41 STUDY FARMS.

	<3	4-6	7-10	11-15	16-20	>20
Total horses on surveyed farms (%)*	18	15	27	22	13	5
Affected (AA) horses (%)	13	16	35	21	13	2

*No significant difference between the two distributions.

TABLE 9. COMPARISON OF THE HORSE USE DISTRIBUTION OF AFFECTED (AA) HORSES WITH THAT OF ALL HORSES ON 41 STUDY FARMS.

	Plea-sure	Show	Event	Race	Polo	Breed-ing	Young
Total horses on survey farms (%)*	49	13	0	3	3	25	7
Affected (AA) horses (%)	34	37	6	1	1	14	7

*No significant difference between the two distributions.

Faecal microbiology.

Microbiology assay of faecal samples did not reveal any significant isolations. Salmonella spp. were isolated from only two horses in the UC group. No Salmonella spp. were isolated from affected horses.

Retrospective Serology.

All UC horses were seronegative to the IFAT for the causative Ehrlichia.

Discriminant analysis.

The canonical discriminant analysis on the variables in Table 10 gave a canonical R^2 value (analogues to the R^2 of multiple determination in regression) of 0.24 for the AA/UA grouping and 0.19 for the AA/U grouping. In terms of placement accuracy in discriminant analysis, the AA/U grouping placed affected and unaffected horses with 63 and 78% success, respectively, whereas the AA/UA grouping had 74 and 70% success, respectively. A summary of the stepwise analyses in Table 11 indicates that variables 1, 2, 9, 10 and 12 are among the best discriminators for both groupings. Further trials with variable subsets, using the five mentioned above as a core, led to the model using variables 1, 2, 5, 9 and 10. This model provided a canonical R^2 of 0.21 for the AA/UA grouping.

TABLE 10. DISCRIMINANT ANALYSIS: VARIABLES STUDIED.

Variable Number and Title	12 Variable Model	5 Variable Model
1. Size of current pasture	X	X
2. Number of other horses at pasture	X	X
3. The use of current pasture by other livestock	X	
4. The presence of abundant forage on current pasture	X	
5. Occurrence of PHF on currently used pasture or adjacent pasture in previous years	X	X
6. Number of other horses in barn	X	
7. The occurrence of PHF in barn in previous years	X	
8. The use of sawdust bedding	X	
9. Previous occurrence of PHF in horse studied	X	X
10. Number of horses introduced since January 1983	X	X
11. Number of horses introduced during last 30 days	X	
12. Number of horses introduced during last 10 days	X	

As a final exploratory procedure, a frequency distribution was calculated using only AA and UA horses. The groupings were based on the canonical variable score from CANDISC. Frequencies of horses in each class were then printed for each group of scores. Horses with extreme scores were analyzed using DISCRIM. Perfect placement of these horses was obtained. The low sample size resulting from this selective procedure limits the interpretation of this section of the analysis.

TABLE 11. DISCRIMINANT ANALYSIS: STEPWISE ANALYSIS SUMMARY.

Stage	AA/U			AA/UA		
	Variable number entered	Partial R^2	$P < F$	Variable number entered	Partial R^2	$P < F$
1	10	0.1015	0.0001	10	0.0792	0.0028
2	3	0.1018	0.0001	12	0.0320	0.0614
3	8	0.0817	0.0004	1	0.0336	0.0563
4	9	0.0473	0.0124	2	0.0463	0.0253
5	12	0.0516	0.0085			
6	1	0.0320	0.0545			
7	2	0.0379	0.0321			

DISCUSSION.

Case-control studies have not been widely used as field investigation techniques in veterinary medicine, despite their extensive use in human medicine, although the frequency of their use has increased considerably in recent years. Thrusfield (1986b) recently commented on the sparsity of epidemiological studies in general in equine veterinary medicine.

The case-control study is an observational study used to identify risk indicators and to estimate the quantitative effects of the various components that contribute to the occurrence of disease (Thrusfield, 1986a). Their most frequent use in veterinary medicine has been for the retrospective analysis of hospital clinic data (e.g., Dorn and Priester, 1976; Goggin, 1970; Hayes, 1974; Thrusfield, 1985; Willeberg, 1975) and of slaughterhouse data (Aalund et al., 1976; Willeberg, 1978). Recently the use has expanded to analyze relationships between diseases (Doohoo and Martin, 1983) and to study complex multifactorial diseases (Pritchard et al., 1983).

The technique relies on the comparison of proportions of animals with the disease in question in groups exposed or unexposed to variables which may be causal, and to the frequency of exposure to these variables in animals unaffected by the disease. The odds ratio calculated is a measure of the relative risk associated with each study variable. Values of greater than one indicate the possibility of a positive association with the variable, and those less than one indicate a possible negative association. The principal merit of the case-control study is that it allows the study of diseases of low incidence, by the purposive selection

of each case which occurs, and the comparison of study variables affecting each case with those affecting non-diseased members of the population. The major disadvantage of the technique is that in order to avoid the demonstration of spurious associations with common confounding variables such as age, sex and breed, controls are selected from the population, and the potential confounding variables matched with those of the cases, thus limiting the representation of the study population of the population at large.

In this study, the case-control technique was chosen due to the low attack rate in the resident horse population (estimated at 19 per 1000), the lack of available data on the characteristic features of that population, and the unknown aetiology of the disease at the time. The control subjects were selected from the horse population at large within the limited geographical area of Montgomery County and not from a limited group of hospital admissions, thus providing considerably more representation, and hopefully relevance when extrapolating results to the general horse population than the conventional case-control studies which rely on hospital or slaughterhouse data. Selection bias (Sackett, 1979) was therefore probably kept to a minimum.

The decision to select two control groups from the horse population (UA and UC) was made for two reasons. Firstly, and most important, to control for possible misclassification bias as in the absence of a definitive diagnostic test at the time, controls could not be easily defined. Secondly, the selection of unaffected premises ruled out the calculation of the odds ratio by the comparison of attack rates in the AA and UC groups for certain parameters relating to the presence or

previous presence of the disease, as the latter group had been selected on the basis of never having experienced PHF. In these instances, AA/AU comparisons alone were made. The problem of misclassification in the identification of controls was of course extended to the identification of cases. It was decided to use the clinical classification of the five practices, based on the presence of fever, anorexia, leucopaenia, typical decreased borborygmal sounds and diarrhoea, which was supplemented by the failure to isolate Salmonella spp. In retrospect, the clinical criteria proved accurate, and subsequent serological confirmation of control classifications was made.

A potential disadvantage of the technique was the use of a questionnaire format for much of the study, which inevitably limited the depth of information obtained. However, this method was the only one available whereby the information required could be obtained, and its accuracy was enhanced by ensuring that data were obtained by conducting personal interviews on the farm.

The results clearly characterise the disease as one which is not associated with horse numbers and horse density, nor is it associated with contact with other horses which have the disease. There is no association with movement of horses on and off premises to attend competitive events, nor any association with exposure to a common source of infection or contamination in water or feed. The results clearly indicate a lack of association with most of the variables analyzed, at least at the depth permitted by the questionnaire. These findings were particularly important in eliminating some of the various aetiologies which had been suggested at the time, especially in relation to common source ingested agents or

toxins. With hindsight knowledge of the ehrlichial causation of the disease, the absence of such associations is upheld.

Significant positive risk associations at the horse comparison level were found between the presence of the disease and its previous presence in the barn, the presence of other livestock on the pasture, the presence of abundant forage on the pasture, the use of sawdust as bedding and the birth of a horse within the Potomac area. Although there was no association identified with the movement of horses off premises, when the reasons for travel were analyzed, there was a greater proportion of horses which went away for training and breeding in the AA group (12.8% and 21.5% respectively) than in the UA group (6.5% and 13.1% respectively), and this significant difference was also identified with respect to breeding in the AA/UC comparison (21.5% vs. 13.5%).

In the comparison of affected and unaffected farms, there was a significantly greater proportion of dogs and cattle on affected premises than unaffected premises. Significant risk associations were also found with the presence of excellent and lush pasture, and with the recognized presence of rodents on the pasture. Current knowledge of the disease provides an explanation for some of these associations, and also raises several questions meritworthy of further investigation.

The significant association with the previous identification of the disease may be explained by the possible persistence of infection with the organism in horses, other mammals or in vector species on a premises or within a region. The role of horses as carriers of E. risticii following recovery is still unclear. Although most studies have

failed to isolate E. risticii from the blood of recovered horses, the organism is present at high levels in the blood concurrently with clinical signs in the horse (Rikihisa and Perry, 1985). It has also been found in a recovered pony on day 39 following experimental infection (see Chapter 3). The study also indicated a significant association with the disease and the presence of other animals in the AA/UA comparison, a non-significant association (odds ratio of 3.04) in the AA/UA comparison, and a significant association with greater proportions of cattle and dogs on the premises in the AF/CF comparison. Secondary hosts have been identified for several rickettsial diseases (Scott, 1977). In addition, other species of livestock may serve as attractants for potential arthropod vectors, thereby increasing the risk of infection in the primary host, in this case, the horse. The role of other mammals in the epidemiology of PHF is considered in Chapter 4 of this thesis.

The results show an epidemiological pattern characteristic of an infectious disease which is transmitted by an arthropod vector. The lack of association with contact with or proximity to previous cases of PHF indicate that the disease is not contagious and that a "point source" infection related to ingestion is unlikely. Faecal-oral transmission of PHF probably does not therefore occur in the field. Subsequent transmission studies using whole blood (A.L. Jenny, 1984; Whitlock et al., 1984) and agent isolation and identification (Holland et al., 1984, 1985a; Rikihisa and Perry, 1984, 1985) confirm the infectious nature of the disease.

Many of the rickettsial diseases of man and animals are arthropod-borne; E. canis, the cause of canine ehrlichiosis, is transmitted by the

brown dog tick, Rhipicephalus sanguineus (Donatien and Lestoquard, 1935), and Cytoecetes (Ehrlichia) phagocytophila, the cause of tick-borne fever, is transmitted by the sheep tick Ixodes ricinus (MacLeod and Gordon, 1933). Adult tick infestations of horses in the region are exclusively the American dog tick, Dermacentor variabilis (Carroll and Schmidtmannn, 1986; Fletcher, 1987), and pastures with abundant forage are potentially more likely to support significant tick populations, offering a possible explanation of the associating with this variable. The larval and nymphal stages of D. variabilis feed on small rodents (Sonenshine et al., 1966) offering a possible explanation of the significant association with the presence of rodents on the pasture; a study of this possible association will be reported in Chapter 4.

The significant association with the use of sawdust bedding in stables identified in the AA/UC comparison is more difficult to explain. However, Fletcher (1987) has pointed out that sawdust is recognized as a promoter of the larval development of Stomoxys calcitrans, the stable fly, in manure piles (Bishopp, 1927), and this species is also a potential vector of PHF.

The discriminant analysis was performed in an attempt to eliminate the effect of possible confounding variables. However, using variables with a significant odds ratio and others with potential as predictor variables, poor discrimination between affected and unaffected horses was obtained, unless the number of variables was reduced to five, and the comparison run only between AA and UC horses. In this instance, however, the sample size was reduced to 26, and all the control horses represented came from three farms.

All of the discriminant analyses require that the variables follow

a multivariate normal distribution, at least approximately. The present data might not support that assumption. Furthermore, the data used to build the discriminant models were used to validate the models as well. Obviously this resulted in some upward bias in any measures of model adequacy, such as R^2 and percent of successful placement.

In summary, the rather laborious technique of discriminant analysis did not enhance the results of this study, possibly due in part to the difficulty in acquiring adequate information on variables following a multivariate normal distribution from a questionnaire, which provides predominantly categorical data.

A report of this study has been published as follows:

Perry, B.D., Palmer, J.E., Troutt, H.F., Birch, J.B., Morris, D., Ehrich, M. and Rikihisa, Y. (1986) A case-control study of Potomac Horse Fever. Preventive Veterinary Medicine, 4, 69-82.

A copy of this publication is attached to this thesis as an appendix.

CHAPTER THREE

STUDIES OF THE CAUSATION AND PATHOGENESIS OF POTOMAC HORSE FEVER

INTRODUCTION.

The clinical signs of PHF are almost indistinguishable from those of salmonellosis in individual horses, but the failure to isolate Salmonella spp. from affected horses, and the unusual epidemiology for a disease of the gastrointestinal tract, described in the previous chapter, clearly indicated a cause other than Salmonella spp. Other causes of acute diarrhoea in the horse, the characteristic clinical feature of PHF, have been described by Whitlock (1986) and include colitis X, antibiotic-associated diarrhoea and endotoxic shock. Colitis X, which often follows stress or debilitation, is of unknown aetiology, although Weirup (1977) associated the disease with an enterotoxaemia caused by Clostridium perfringens Type A. For this reason, faecal samples from the case-control study described in the previous chapter were screened for this enterotoxin and for a cytotoxin of C. difficile during 1983. In September, 1983, workers at the National Animal Disease Laboratory, Ames, Iowa, and the University of Pennsylvania demonstrated that PHF could be reproduced by the intravenous inoculation of whole blood taken from a clinical case into susceptible horses (Whitlock et al., 1984). One of these workers, Dr. A. L. Jenny, was carrying out an extensive serological study on affected and recovered horses in Maryland to many of the

recognized infectious agents including Babesia equi, B. caballi, Leptospira spp. (12 serovars), equine rhinopneumonitis, equine coital exanthema, equine viral arteritis, equine adenovirus, Venezuelan, Western and Eastern equine encephalomyelitis, equine infectious anaemia, African horse sickness and Chlamydia spp. He reported no evidence of seroconversion to any of these agents (Jenny, 1984). However, serum samples submitted to Dr. Miodrag Ristic for screening for antibodies to Ehrlichia equi were also tested for antibodies to the related organisms E. canis and E. sennetsu. Some recovered horses demonstrated seroconversion to Ehrlichia spp. as assayed by the fluorescent antibody technique (Jenny, 1984). The seroconversion was greatest to the antigen of E. sennetsu, with lower levels of seroconversion to E. canis and no seroconversion to E. equi (Jenny, 1984; Holland et al., 1985).

Following the indication of a possible rickettsial association with PHF, studies were initiated at the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM) to experimentally reproduce the disease in ponies, to isolate and identify the causative agent and to investigate the pathogenesis of the disease.

The author was the initiator and coordinator of this study, which was carried out by a team of investigators. The work of transmitting the disease experimentally to ponies and the subsequent clinical evaluation of affected ponies was designed and carried out by the author, with the assistance of Dr. Donald Cordes. Data collection and evaluation were carried out by the author. Histopathology was carried out by Dr. Donald Cordes and gross pathology performed under the direction of Dr. Cordes with the author as a collaborator. Clinical pathology

assays were performed by Dr. William Chickering. Cell culture of E. risticii and electron microscopy of tissues from affected ponies was carried out by Dr. Yasuko Rikihisa.

MATERIALS AND METHODS.

Examination for clostridial toxins.

Faecal samples were obtained form 108 horses in the case-control study carried out in 1983. A total of 51 specimens were from horses clinically diagnosed as having PHF and 57 were from clinically normal horses on affected and unaffected premises, matched with cases with respect to age, breed and sex. Some horses were matched with more than one control. Faecal specimens (92 $\geq$ 10 g; 16 $<$ 19 g) were collected from the rectum of horses, placed in plastic bags and any excess air was removed. Specimens were stored frozen until the day of assay. *Assays for C. perfringens Type A enterotoxin and C. difficile cytotoxin were carried out at the VMRCVM by Dr. Marion Ehrich.*

Results were analyzed for visible differences between affected and unaffected horses. Numbers in some categories were too small to apply the chi-square test.

Examination for serum antibodies to Rickettsia rickettsii.

Forty-nine of the serum samples taken from PHF-affected and unaffected horses in the case-control study were examined for antibodies

to Rickettsia rickettsii, the causative agent of Rocky Mountain spotted fever (RMSF). This disease is prevalent throughout the study region. Sera were held at -40°C until assay. *They were examined using the Indirect Fluorescent Antibody Test (IFAT) by Dr. Farhang-Azad, Department of Microbiology, School of Medicine, University of Maryland.* Sera with reciprocal titres of 1:40 or greater were considered positive. Results were tabulated and analyzed for visible differences between affected and unaffected horses.

Establishment of experimental infection in ponies.

Approximately 350 ml of whole blood was taken into sterile blood transfusion bags containing citrate phosphate dextrose anticoagulant solution USP (Fenwall Laboratories, Deerfield, Illinois, USA) from each of two field cases in the acute stage of PHF before the initiation of therapy in early August, 1984. Both cases occurred in Montgomery County, Maryland. One of the cases subsequently died three days later and the other recovered.

The blood samples from each case were transported at approximately 4°C in styrofoam cool boxes to the VMRCVM where each sample was inoculated into two separate recipient ponies (1 and 2) 24 to 26 hours after collection.

Maintenance of experimental ponies.

Twenty-seven ponies of mixed breed, age and sex were purchased

locally in southwest Virginia and transported to the VMRCVM. Ponies were clinically examined and acclimatized for 3 to 28 days in a paddock, where they were fed pasture and hay. All ponies were shown to be negative to equine infectious anaemia (immunodiffusion test), Babesia equi and B. caballi (complement fixation test) by submission of sera for *testing to the National Animal Disease Laboratory, Ames, Iowa.* Ponies 1 to 13 received no anthelmintic or antibiotic prior to experimental infection. Ponies 18 to 40 were dewormed with ivermectin (Ivermec, Hoechst, USA) at a dose of 0.2 mg per kg by the oral route.

At the time of experimental infection, ponies were moved to individual indoor accommodation. Each pony was housed in a room of dimensions 4 x 4.5 m. Access to the pony was from a central corridor through an anteroom where disinfection of footwear was carried out on entry and departure in a footbath. Windows in the pen were screened to exclude insects.

Maintenance of experimental infection of ponies.

Following the development of clinical disease in ponies 1 and 2, these animals became donors of infected blood to two further ponies. Thus, a system of passage of infection was established from infected to susceptible ponies, which is illustrated in Table 12.

TABLE 12. ESTABLISHMENT AND MAINTENANCE OF EXPERIMENTAL POTOMAC HORSE FEVER IN PONIES.

Study Pony Number	Source of Infection	Incubation Period (days)*	Outcome**
1	Field case of PHF	15	Euthanasia, d.4
2	Field case of PHF	12	Euthanasia, d.2
3	Pony 2	8	Euthanasia, d.3
4	Pony 1	13	Died, d.11
5	Pony 3	11	Euthanasia, d.8
6	Ponies 4,7	not determined	Recovered
7	Pony 5	13	Euthanasia, d.4
8	Ponies 6,7	12	Euthanasia, d.2
9	Pony 8	--	Transfusion reaction, died d.4 post inoculation
12	Pony 8	10	Euthanasia, d.5
13	Pony 8	8	Euthanasia, d.14
18	Culture of E. risticii	10	Euthanasia, d.6
19	Culture of E. risticii	10	Recovered
20	Pony 16	12	Euthanasia, d.14
24	Pony 20	11	Euthanasia, d.7
25	Pony 24	14	Died, d.4
27	Pony 25	9	Euthanasia in extremis, d.8
28	Pony 27	8	Euthanasia, d.17
29	Pony 28	11	Euthanasia, d.18
33	Pony 30	7	Died, d.8
34	Normal histiocyte cells	--	Euthanasia, 47 d. post inoculation
35	Pony 33	12	Euthanasia, d.4
36	Pony 35	11	Euthanasia, d.9
37	Pony 36	11	Euthanasia, d.23
38	Pony 37	12	Euthanasia, d.6
40	Pony 38	8	Euthanasia, d.11
C	Normal histiocyte cells	--	Euthanasia

*Number of days from infection until first appearance of fever, anorexia, depression or diarrhoea.

**Indicates fate of pony and day (d) following the onset of clinical signs on which euthanasia or death occurred.

On each occasion, approximately 350 ml of whole blood was taken as previously described from the affected pony in the early stages of clinical disease, which was generally approximately 14 days following infection. The blood was then transfused into the subsequent susceptible pony in the series within 30 minutes.

Clinical observations.

Clinical observations of each pony were made at least once daily starting 1-3 days prior to infection. Records were made of rectal temperature, pulse, respiration, demeanour, abdominal sounds, state of hydration and faecal consistency. Mean values and standard deviations were calculated by standard methods for the day of onset of four major clinical signs for 12 ponies in which comprehensive observations were recorded. Blood samples were collected at two-day intervals for serum and also into Vacutainer (Becton Dickenson) tubes containing ethylene-diamine tetraacetic acid (EDTA) for a complete blood cell count.

The mean incubation period and standard deviation, recorded as the number of days from infection until the first observation of one of the major clinical signs of fever, depression, anorexia or diarrhoea, was calculated by standard methods for 21 ponies in which infection was induced by whole blood transfusion.

Mean and standard deviation daily rectal temperatures following infection were calculated by standard methods for the same 21 ponies.

Total white blood cell counts, and differential cell counts for neutrophils and lymphocytes were carried out by Dr. William Chickering.

Changes in these values following infection were plotted for selected individual ponies. Mean values and standard deviations for each day of sampling were calculated by standard methods for total white blood cells and lymphocyte counts for 10 ponies in collaboration with Dr. William Chickering.

Culture of ehrlichial organisms.

Blood samples of approximately 350 ml were collected into sterile blood transfusion bags containing citrate phosphate dextrose solution, USP (Fenwall Laboratories, Deerfield, Illinois) from each of three ponies (ponies 8, 13, and 24) infected by blood transfusions and two ponies (ponies 18 and 19) infected by cell cultures of the causative Ehrlichia subsequent to its isolation and identification. Samples were taken 13 to 18 days following infection. *Subsequent isolation of ehrlichial organisms and examination of infected cell cultures by electron microscopy was performed by Dr. Yasuko Rikihisa, and has been described by Rikihisa and Perry (1985).*

Infection of ponies with pure cultures of ehrlichial organisms.

Histiocyte cell cultures (from 1 x 10^7 to 4 x 10^7 cells in 3 to 6 ml of RPMI 1640 medium (GIBCO Laboratories, Grand Island, New York) prepared by Dr. Rikihisa, heavily infected with ehrlichial organisms and free of any contamination by other microorganisms and virus (determined by Diff-Quick (Harleco, Gibbstown, New Jersey) and Gram staining of

aliquots, blood agar culture and electron microscopy) were harvested and held at 4°C for 1 to 20 hours before inoculation. The organisms were inoculated intravenously into ponies 18 and 19. Two animals (ponies 34 and C) were inoculated with non-infected histiocyte cultures to serve as controls.

Reisolation of ehrlichial organisms.

From each of the four ponies described above, 350 ml was obtained from the jugular vein of experimental ponies in citrate phosphate dextrose 3 to 18 days following the inoculation of histiocyte cell cultures. *The samples were subsequently processed for isolation of ehrlichial organisms by Dr. Rikihisa as previously described.*

Immunofluorescent antibody assay of pony sera.

A limited study of the serological response to infection was carried out in ponies 6, 19, 28, 29, 36 and 37. Serum was separated from the blood collected as previously described. Sera were retained at -80°C until testing. *Detection of antibodies to PHF was carried out by Dr. Yasuko Rikihisa using the Indirect Fluorescent Antibody Test (IFAT) developed at the VMRCVM.* Fluorescence at serum dilutions of 1:80 or greater was considered positive. Antibody titrations by time were charted for ponies 6 and 19.

Pathological studies.

Twenty-one of the twenty-seven experimental ponies were destroyed using T61 euthanasia solution (American Hoechst Corporation, Sommerville, New Jersey) at stages ranging from 2-23 days following the onset of clinical signs. Routine post mortem examinations were carried out within 5-15 minutes following euthanasia. These examinations were carried out under the direction of Dr. D. O. Cordes, with the author as collaborator. The gastrointestinal tract was removed and examined systematically. After the contents were examined, noted and removed, samples of stomach, duodenum, jejunum, ileum, caecum, large colon and small colon were selected and fixed in 10% neutral buffered formalin *for subsequent paraffin embedding, sectioning and staining with haematoxylin and eosin according to standard methods. Sections so produced were examined under a light microscope.*

Further samples of adjacent tissue from the same organs were cut into 3 mm cubes and placed in a mixture of 2.5% paraformaldehyde, 5% glutaraldehyde and 0.03% trinitrophenol in 0.1 M cacodylate buffer (pH 7.4) and left overnight at 4°C. *Further tissue processing, sectioning, and examination with a JEM 100 CX11 electron microscope was carried out by Dr. Yasuko Rikihisa as described by Rikihisa et al. (1985).*

RESULTS.

Examination for clostridial toxins.

The results of analysis for Clostridium perfringens type A enterotoxin and C. difficile cytotoxin are given Tables 13 and 14.

TABLE 13. ANALYSIS OF FAECAL SAMPLES FROM HORSES SAMPLED IN THE CASE-CONTROL STUDY FOR THE PRESENCE OF CLOSTRIDIUM PERFRINGENS TYPE A ENTEROTOXIN.

Presence of C. Perfringens Enterotoxin	Horse Group			Total
	AA	UA	UC	
+	14*	5*	5*	24
-	28	13	27	68
Total	42	18	32	92

*No apparent difference between prevalence rates in the three study groups.

TABLE 14. ANALYSIS OF FAECAL SAMPLES FROM HORSES SAMPLED IN THE CASE-CONTROL STUDY FOR THE PRESENCE OF CLOSTRIDIUM DIFFICILE CYTOTOXIN.

Presence of C. Difficile Cytotoxin	Horse Group		Total
	AA	UA and UC	
+	1*	2*	3
-	50	55	105
TOTAL	51	57	108

*No apparent difference between prevalence rates in the two study groups.

Examination for serum antibodies to Rickettsia rickettsii.

Antibodies to R. rickettsii were found in 5/15 (33.3%) sera taken from control horses, 5/14 (35.7%) affected horses sampled on day 2 of clinical PHF and 3/20 (15%) affected horses sampled 21 days after the onset of clinical PHF. These results are displayed in Table 15.

TABLE 15. PREVALENCE OF ANTIBODIES TO RICKETTSIA RICKETTSII IN ACUTE AND CONVALESCENT SERA OF HORSES WITH PHF AND UNAFFECTED CONTROL HORSES.

Presence of Antibodies to R. rickettsii	Horse Group			
	Control	Day 2	Day 21	Total
+ ($\geq$ 1:40 titre)	5*	5*	3*	13
- ($<$ 1:40 titre)	10	9	17	36
Total	15	14	20	49

*No apparent difference between prevalence rates in the three study groups.

Geometric mean titres of seropositive horses by group were:

Control horses:	1:184
Affected horses (day 2):	1:108
Affected horses (day 21):	1:160

Experimental reproduction of the disease.

Potomac horse fever was experimentally induced by blood inoculation in 22 ponies. The disease was reproduced by the intravenous inoculation of a pure culture of the causative Ehrlichia in two ponies. One pony died as the result of a transfusion reaction (pony 9) four days after inoculation. Two ponies were inoculated with uninfected histiocyte cell cultures as controls.

Three of the ponies infected by blood transfusion (ponies 4, 25 and 33) died. One pony infected by transfusion (pony 6) and one pony infected by Ehrlichia in cell culture (pony 19) were allowed to survive.

Clinical characteristics of PHF.

The clinical disease reproduced in experimental ponies was similar to that recorded in field cases, and was characterised by fever (Figure 7), depression (Figure 8), anorexia, and diarrhoea (Figure 9). The calculated mean incubation period was 10.86 $\pm$ 2.17 days, with a range of 7-15 days. Severe dehydration was a common sequel to infection, easily demonstrated by the delayed reaction of skin in the skin-pinch test (Figure 10). A summary of the presence, time of onset and duration of these signs in 12 ponies are shown in Table 16.

TABLE 16. SUMMARY OF CLINICAL SIGNS IN 12 PONIES WITH EXPERIMENTAL PHF.

Clinical Sign	PONY IDENTIFICATION 1	2	3	4	5	6	7	8	12	13	20	24	TOTAL (%) Mean Period (days)
FEVER*:													
Presence	+	+	+	+	+	-	+	+	+	+	+	+	11/12 (91.6%)
Time of onset**	15	12	8	13	11	-	13	12	10	8	12	11	11.4 ± 2.1
DEPRESSION:													
Presence	+	+	-	+	+	+	+	+	+	+	+	+	11/12 (91.6%)
Time of onset**	15	12	-	13	11	10	13	12	10	9	13	11	11.7 ± 1.7
ANOREXIA:													
Presence	+	+	-	+	+	-	+	+	+	+	+	+	10/12 (83.3%)
Time of onset**	15	11	-	13	11	-	14	12	13	9	14	11	12.3 ± 1.6
DIARRHOEA:													
Presence	+	+	+	+	+	-	+	+	+	+	-	-	9/12 (75.0%)
Time of onset**	17	12	10	13	14	-	14	12	13	12	-	-	13.0 ± 1.9

* Rectal temperature above 38.5°C.
** Days following injection.

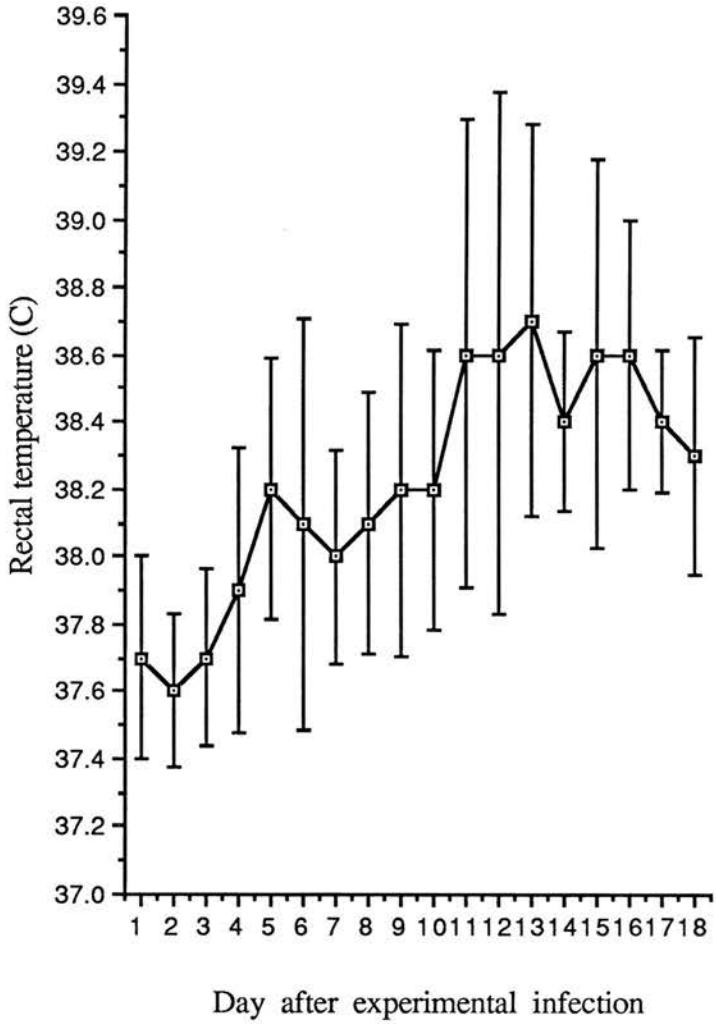

FIGURE 7. MEAN RECTAL TEMPERATURE IN 21 PONIES INFECTED WITH PHF

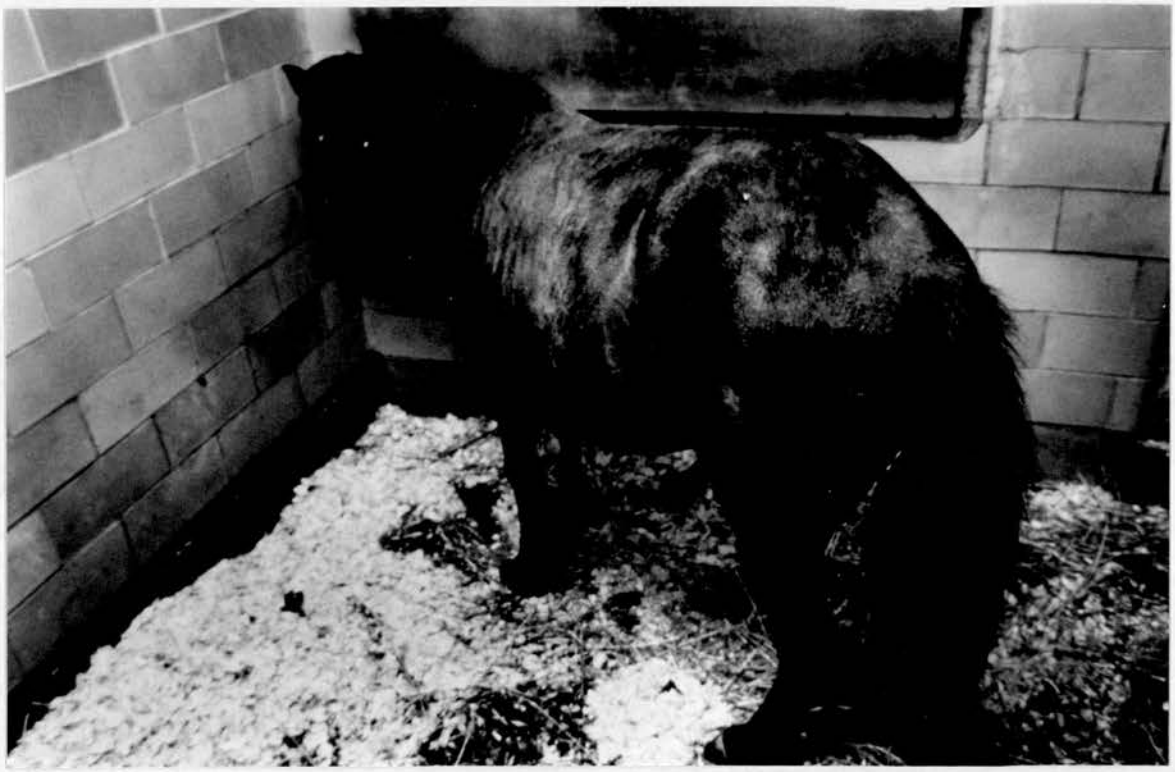

FIGURE 8. AN EXPERIMENTAL PONY SHOWING ACUTE DEPRESSION IN THE EARLY CLINICAL STAGES OF PHF.

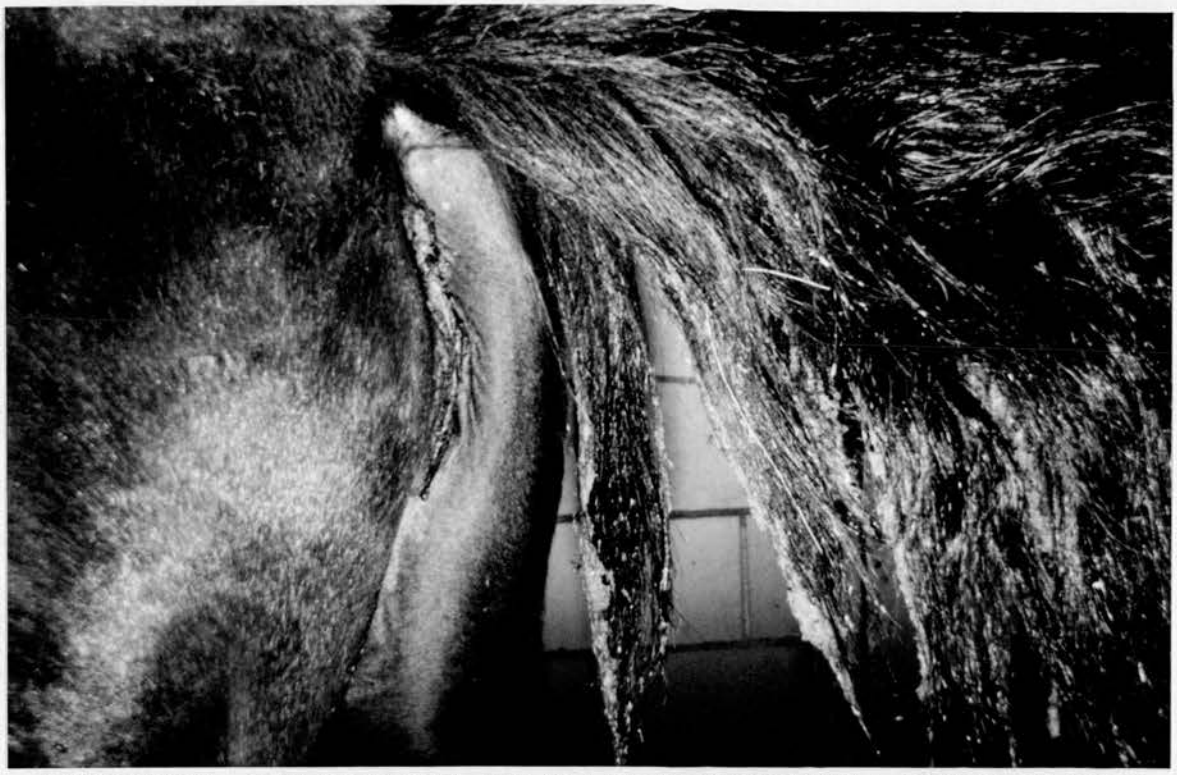

FIGURE 9. DIARRHOEA IN AN EXPERIMENTAL PONY IN THE EARLY CLINICAL STAGES OF PHF.

FIGURE 10. DEHYDRATION DEMONSTRATED IN A PONY WITH CLINICAL PHF BY THE DELAYED RETRACTION OF SKIN OF THE NECK IN THE SKIN-PINCH TEST.

Mean total leucocyte counts by day following infection in 10 ponies infected by whole blood transfusion are shown in Figure 11. Mean white blood cell differential counts for lymphocytes by day following infection for the same group of animals are given in Figure 12.

Clinical profiles of three ponies representative of the group in which blood-transfusion infection was induced (ponies 1, 2 and 4) and one pony infected by the causative Ehrlichia in pure culture (pony 18) are given in Figures 13, 14, 15 and 16.

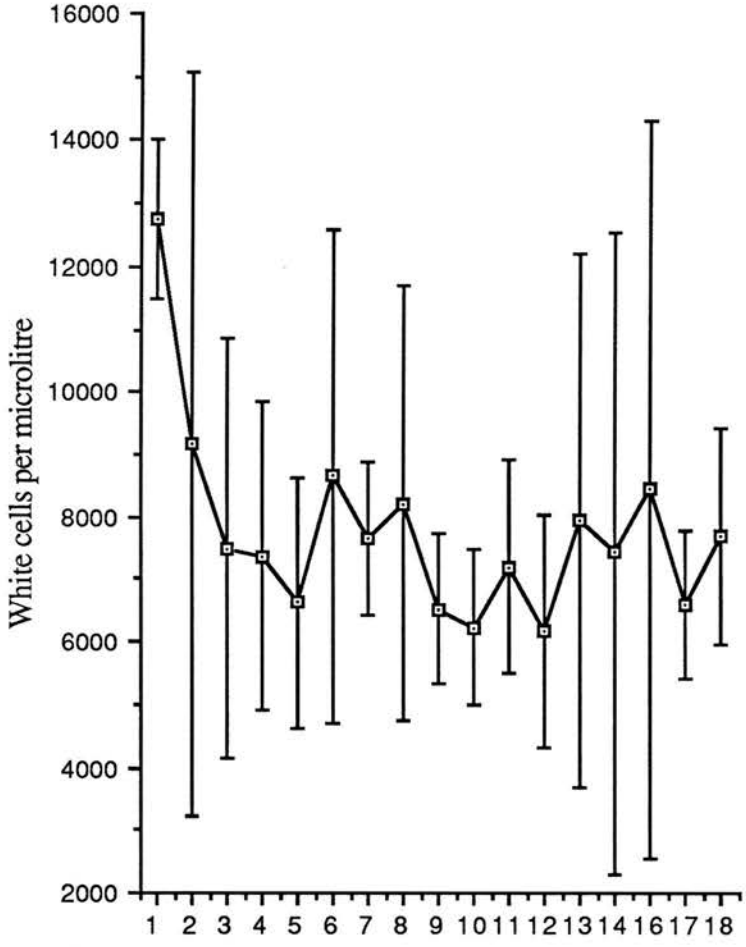

FIGURE 11. MEAN LEUCOCYTE COUNTS IN EXPERIMENTAL PHF

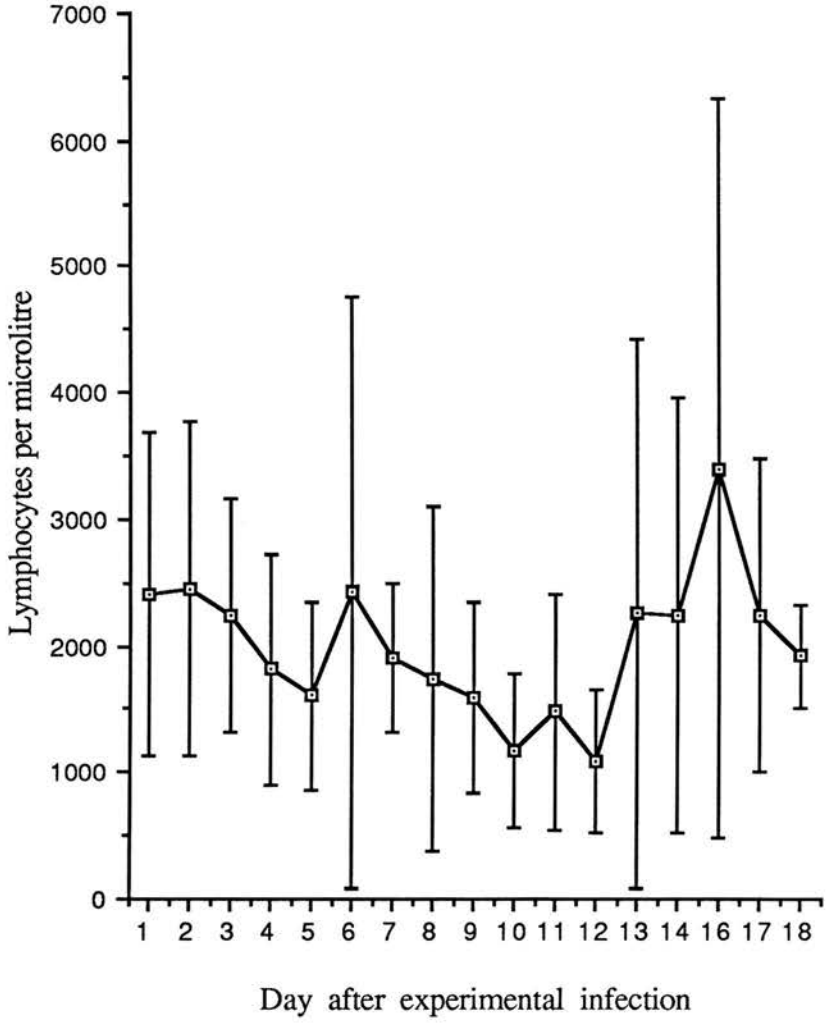

FIGURE 12. MEAN LYMPHOCYTE COUNTS IN EXPERIMENTAL PHI

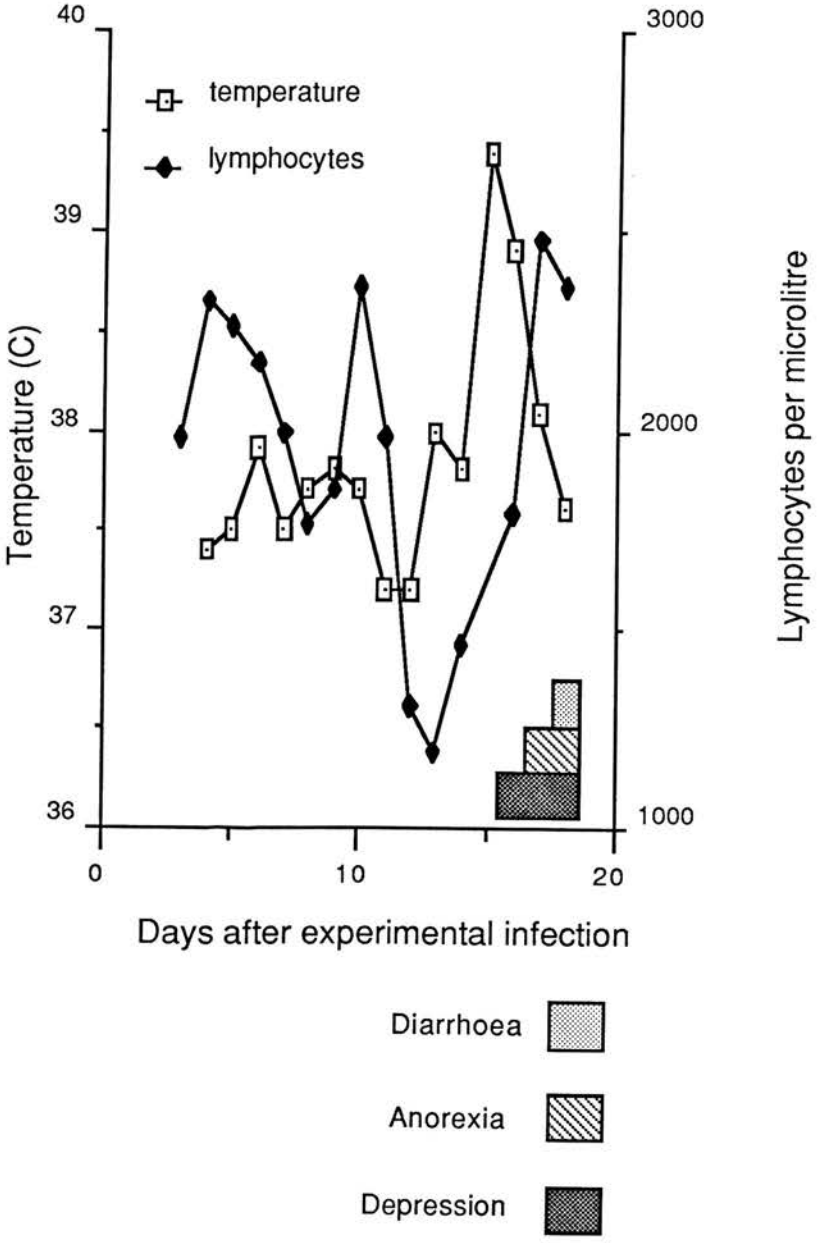

FIGURE 13. CLINICAL PROFILE OF PONY 1

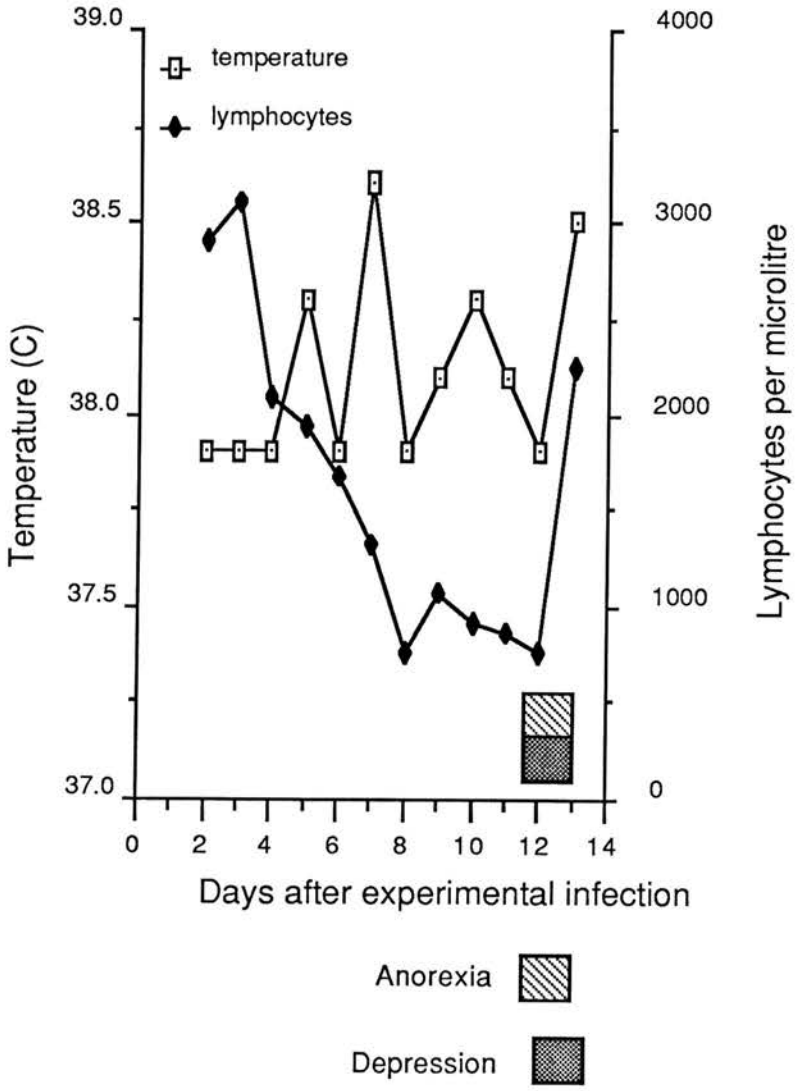

FIGURE 14. CLINICAL PROFILE OF PONY 2

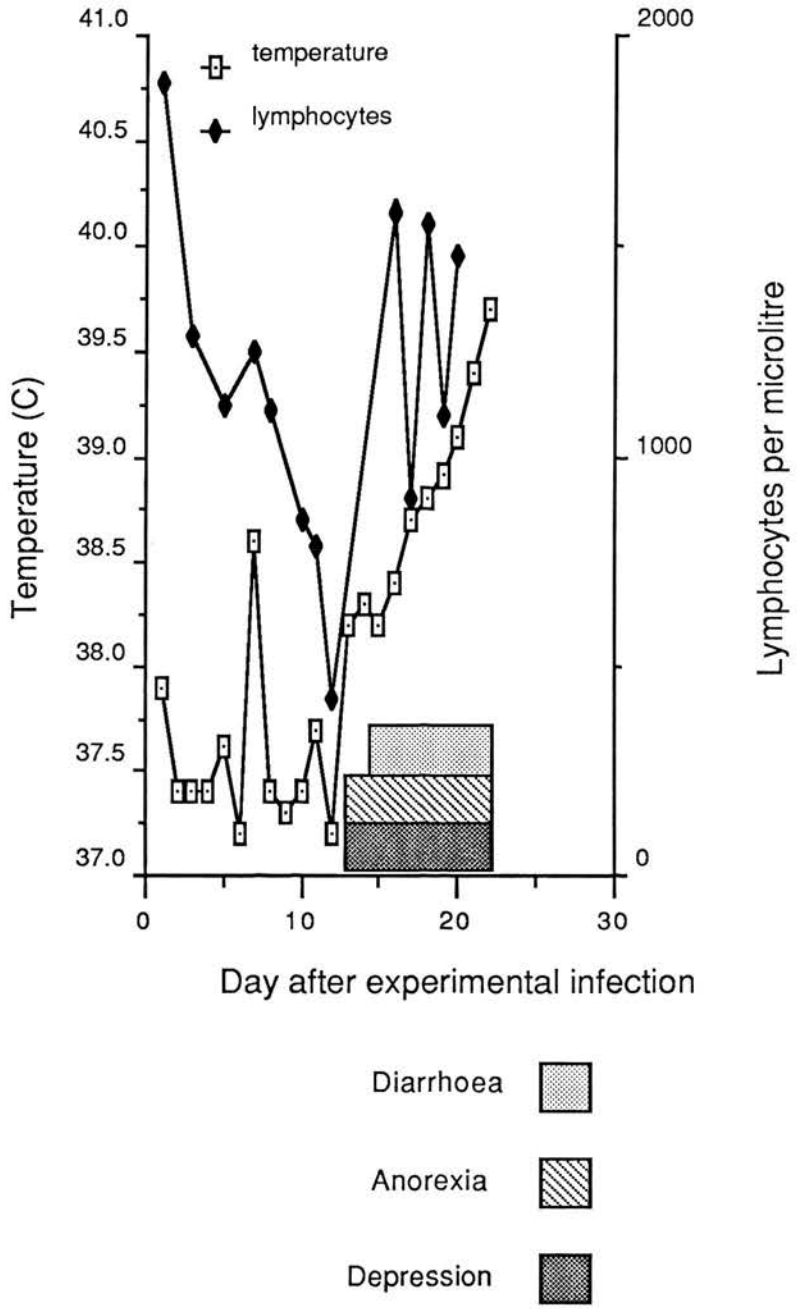

FIGURE 15. CLINICAL PROFILE OF PONY 4

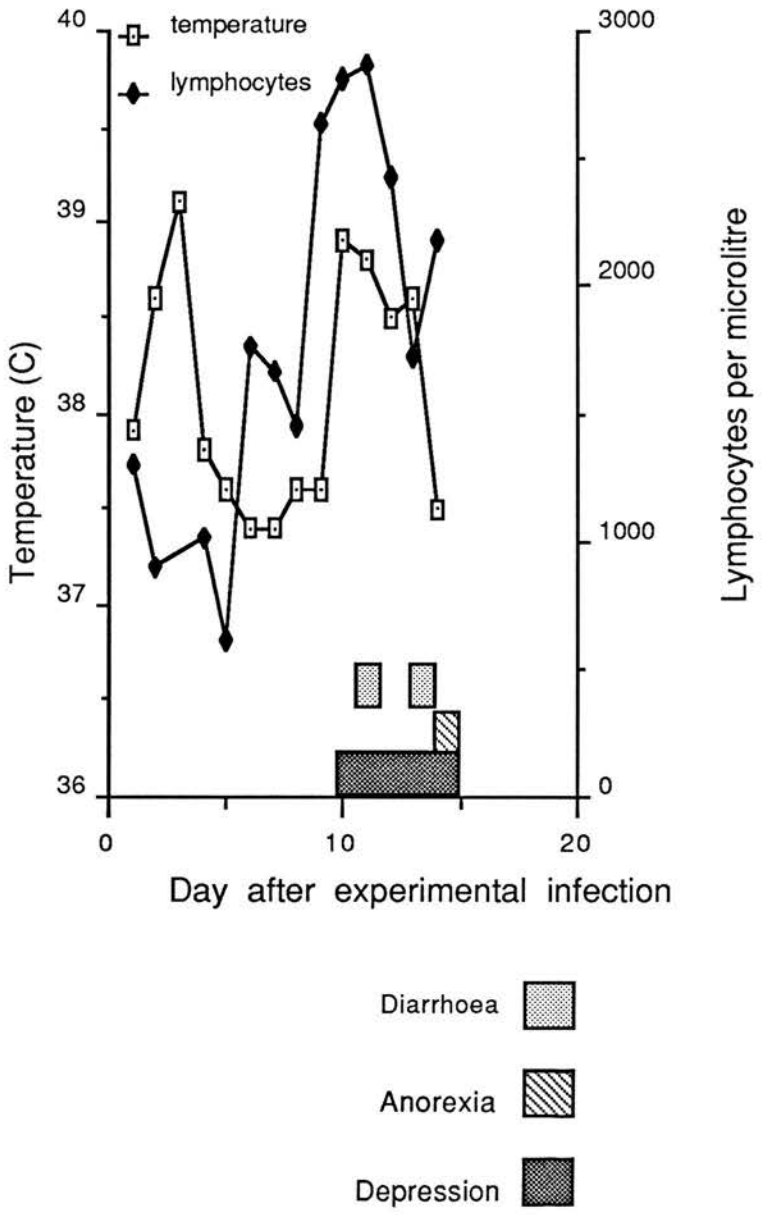

FIGURE 16. CLINICAL PROFILE OF PONY 18

Isolation of the causative agent of PHF.

Cultures made from leucocyte fractions of whole blood of ponies 8, 13 and 24 revealed the presence of intracellular microorganisms consistent with the genus Ehrlichia. *These organisms were detected from day 6 to day 12 of culture by Diff-Quick staining and immuno-fluorescent antibody labelling (Table 17).*

TABLE 17. SUCCESSFUL ISOLATION OF EHRLICHIAL ORGANISMS FROM PONIES EXPERIMENTALLY INFECTED WITH PHF BY WHOLE BLOOD INOCULATION.

Pony	Days Following Infection
8	13
13	11
24	18

The microorganism found in culture was further characterised by electron microscopy as being consistent with the ultrastructure and intracytoplasmic location of members of the genus Ehrlichia. An electron micrograph of the organism in pure culture taken by Dr. Rikihisa is shown in Figure 17.

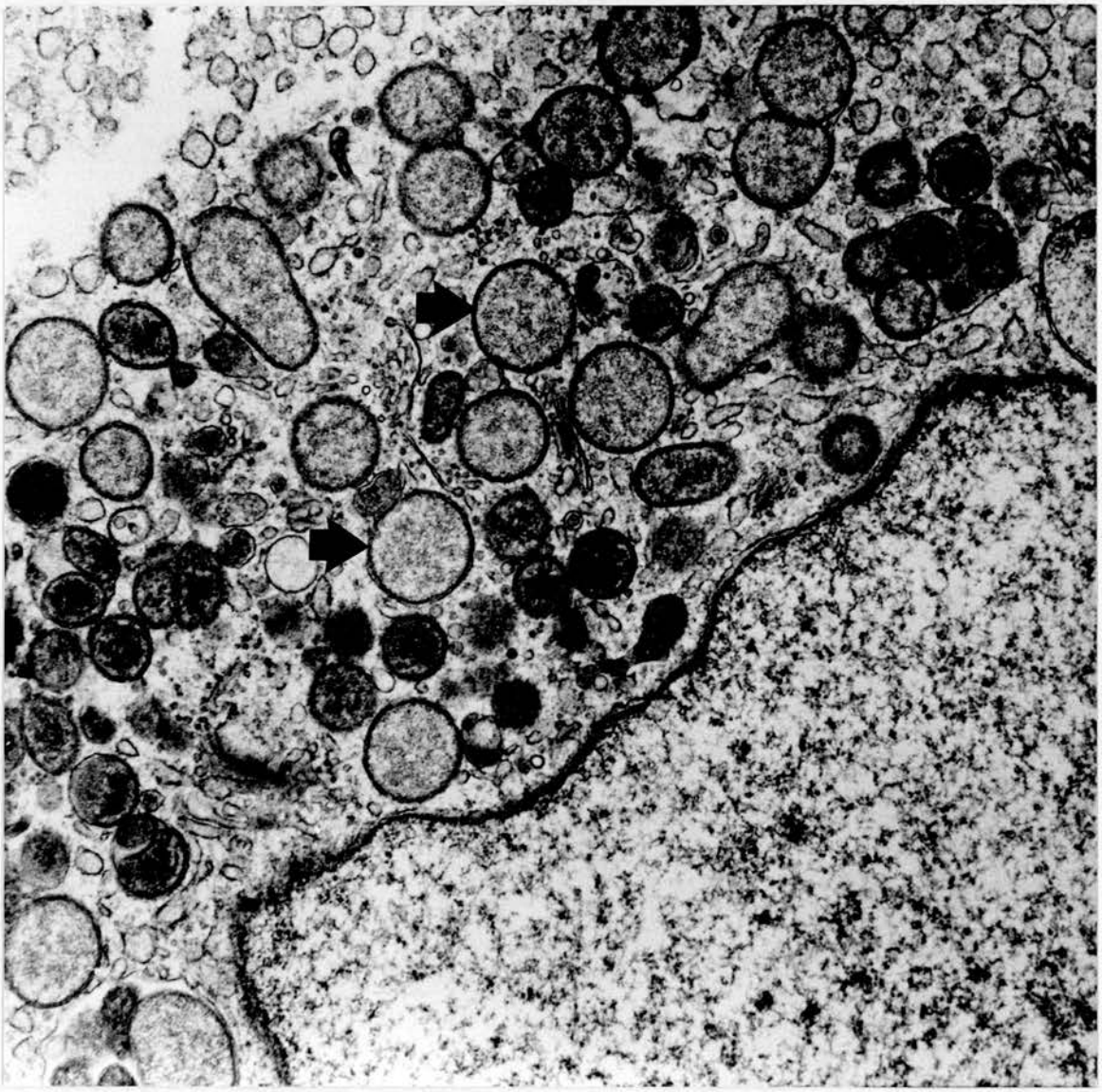

FIGURE 17. ELECTRON MICROGRAPH OF EHRLICHIA RISTICII (Arrows) IN PURE CULTURE (FROM RIKIHISA AND PERRY, 1985).

Reisolation of ehrlichial organisms from ponies infected by pure cultures.

Ponies 18 and 19 both developed clinical signs of PHF following inoculation with 1 x 10^7 histiocytes infected with ehrlichial organisms. The clinical profile of one of these animals, pony 18, is shown in Figure 16.

Attempts to reisolate ehrlichial organisms from these ponies on days 9, 10, 11 and 13 following infection were successful. *The identity of organisms so isolated in pure culture was confirmed by examination of cultures by electron microscopy.* The organisms were also isolated from pony 19 on day 39 after infection, at which time the animal had made a clinical recovery.

Serum antibody assays in experimental infection.

Serum antibodies to PHF measured by the IFAT were initially detected in the six ponies studied on days 9, 12, 12, 13, 14, and 20 respectively following infection (median 12.5 days). Antibodies persisted at high levels of up to a titre of 1:1280 until the study ponies were destroyed or the study terminated. The humoral responses of ponies 6 and 19 as measured by the IFAT are shown in Figures 18 and 19. Pony 6 showed a distinct and sustained seroconversion despite having exhibited virtually no clinical manifestations of PHF.

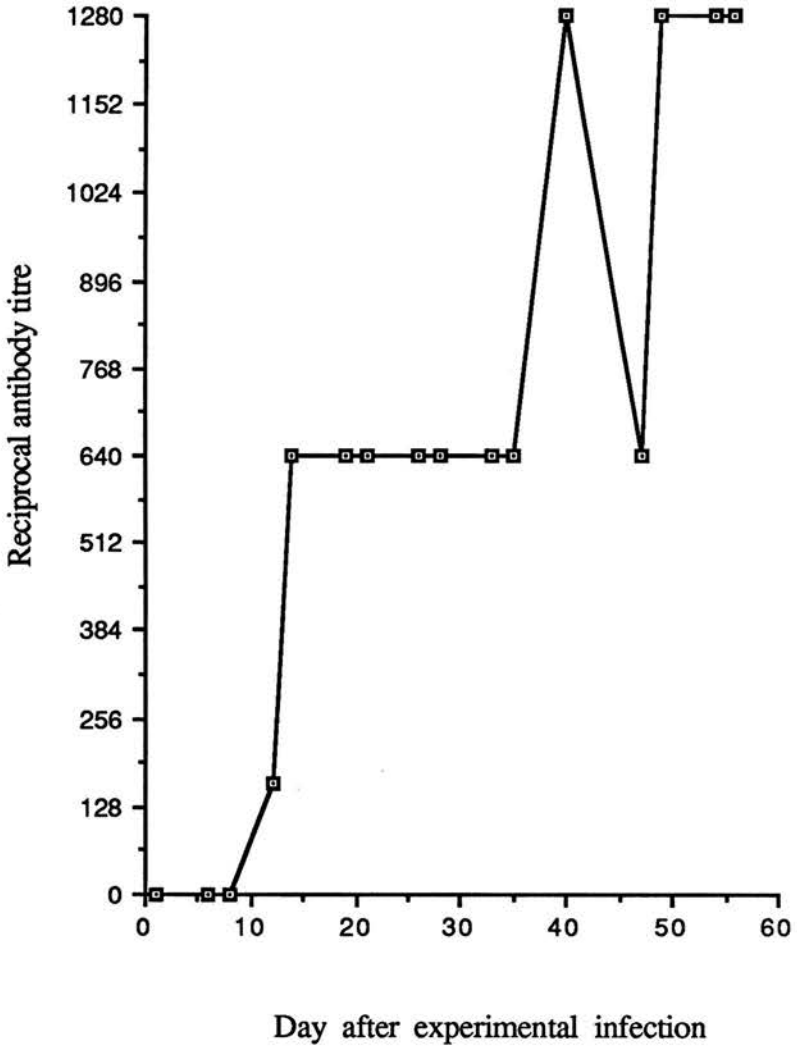

FIGURE 18. ANTIBODIES TO E. RISTICII IN PONY 6

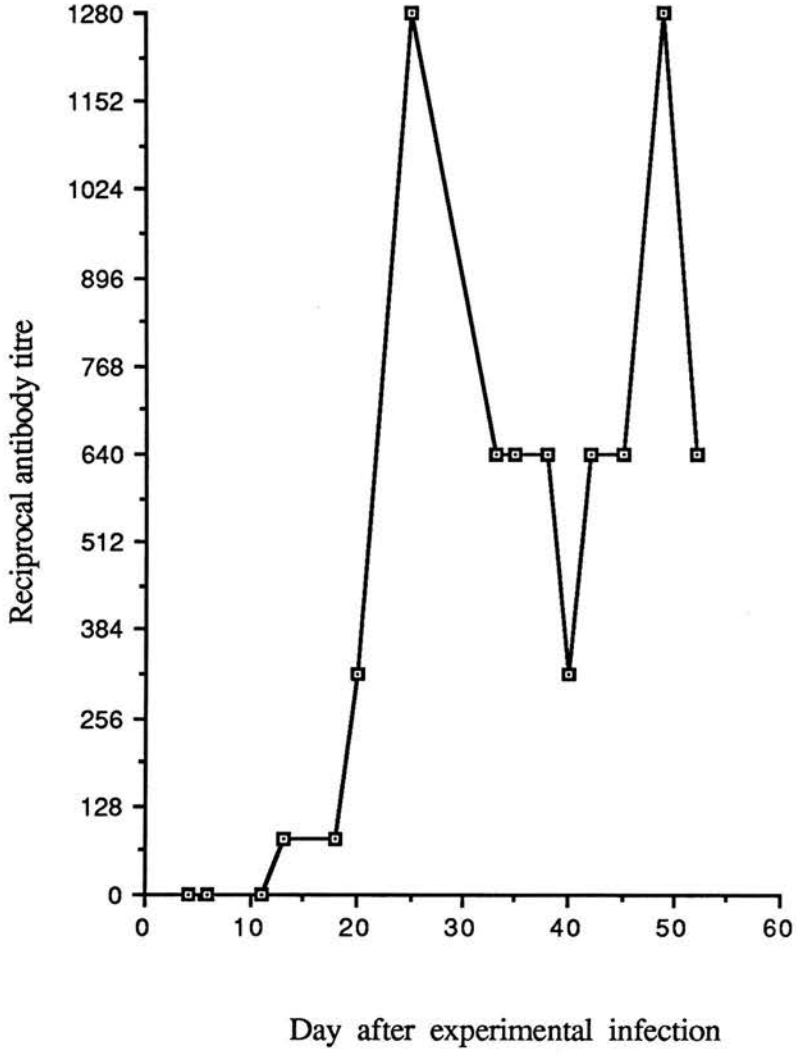

FIGURE 19. ANTIBODIES TO E. RISTICII IN PONY 19

Gross pathology findings.

The specific gross lesions in the digestive tracts of 14 of the experimentally infected ponies have been tabulated by Dr. D. O. Cordes and reported by Cordes, et al., 1986.

In general, the most consistent gross finding was that of a fluid consistency of the approximately normal volume of intestinal contents. In some cases, the contents were extremely watery. The alteration in consistency of intestinal contents was most frequently observed in the large colon, small colon and caecum, and to a lesser degree in the small intestine. Watery contents were also seen in the above organs in ponies which had not shown clinical diarrhoea.

Gross changes in the wall of the intestinal tract were not regularly observed and there was no gross disruption of the integrity of the intestinal mucosa. In those animals with visible intestinal lesions, these were most commonly observed in the large colon and caecum, occasionally in the small intestine and rarely in the small colon. Such lesions consisted of focal patches of hyperaemia, occasional petechiation of the mucosa and rarely discrete focal areas of ecchymoses. The lesions described are illustrated in Figures 20, 21 and 22.

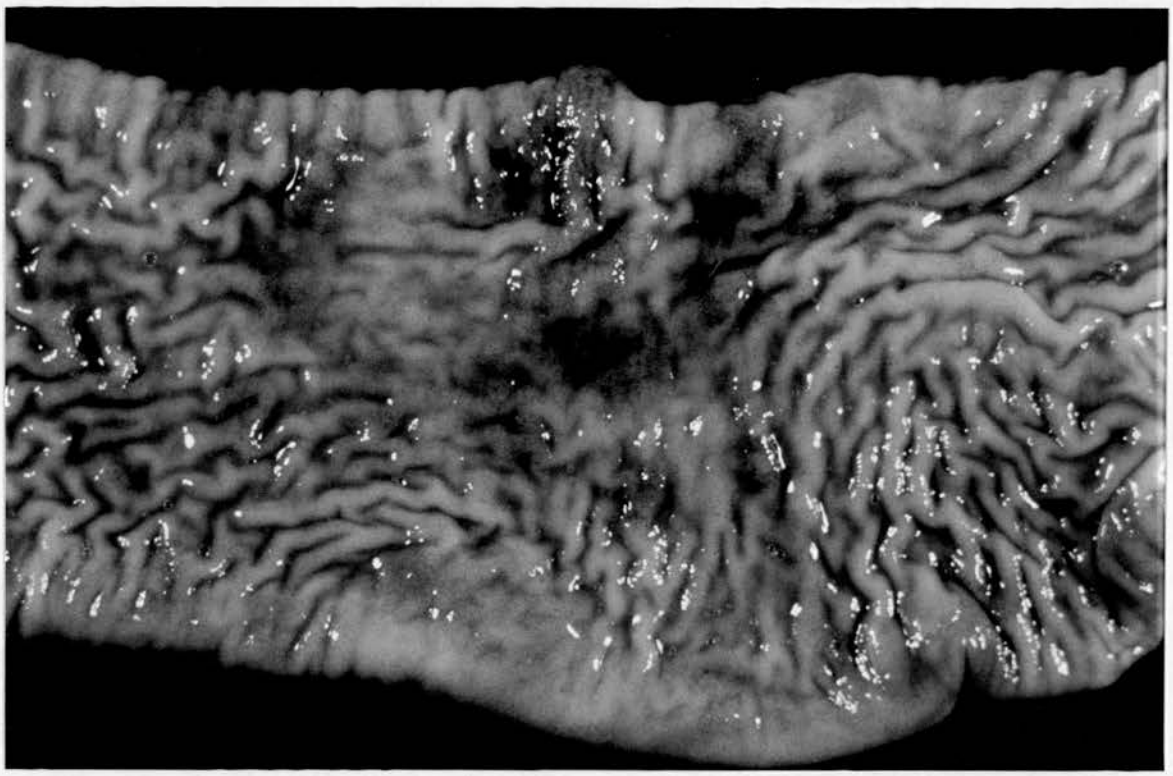

FIGURE 20. MUCOSAL SURFACE OF THE JEJUNUM OF PONY EXPERIMENTALLY INFECTED WITH PHF, SHOWING FOCAL PATCHES OF HYPERAEMIA.

FIGURE 21. MUCOSAL SURFACE OF THE CAECUM OF PONY EXPERIMENTALLY INFECTED WITH PHF, SHOWING FOCAL HYPERAEMIA AND PETECHIAE (Arrows).

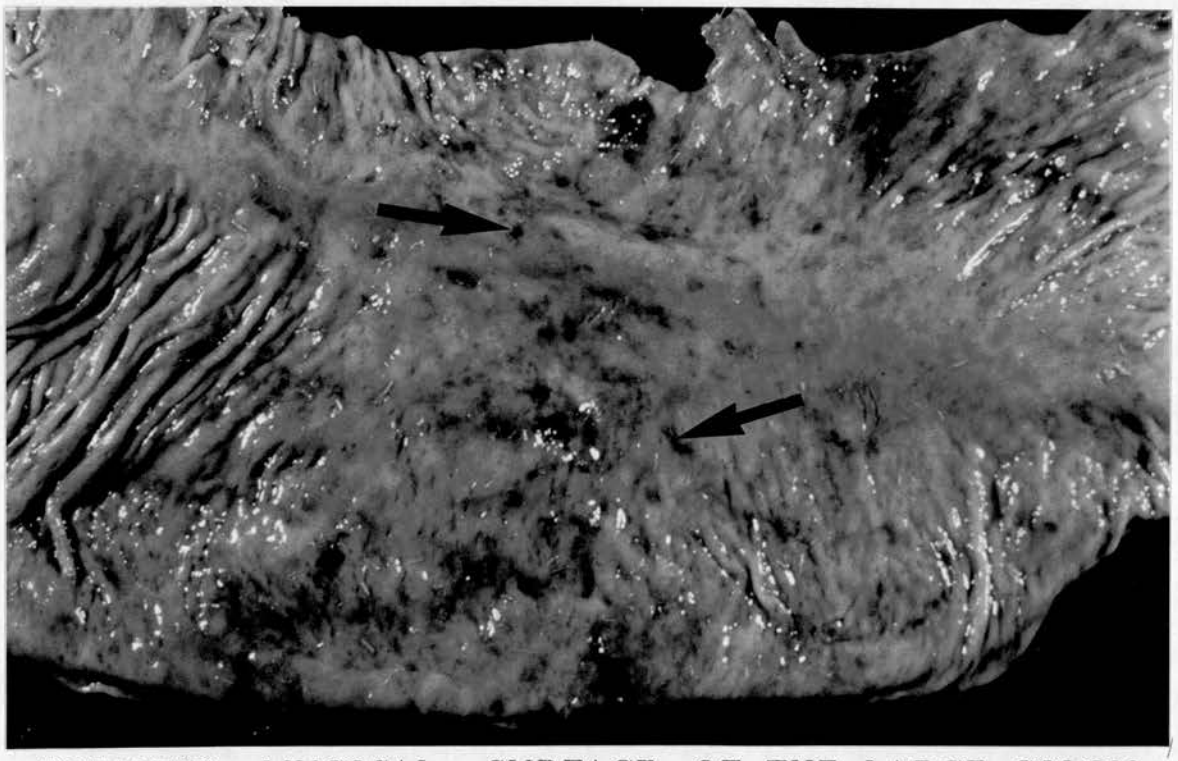

FIGURE 22. MUCOSAL SURFACE OF THE LARGE COLON OF PONY EXPERIMENTALLY INFECTED WITH PHF, SHOWING FOCAL AREAS OF HYPERAEMIA (Arrows).

Histopathology and ultrastructural findings.

Histopathological findings were observed and recorded by Dr. D. O. Cordes. Lesions were mostly confined to the caecum and large colon. The foci of hyperaemia observed were seen by the light microscope as accumulations of erythrocytes in capillaries or within the tissues of the superficial zone of the lamina propria. In general, there was minimal disruption of the integrity of the epithelium in the intestinal tract, even in the ponies with severe diarrhoea and/or watery contents of the caecum and large colon. A mixed population of moderate to large numbers of inflammatory cells was present in the lamina propria and in some cases in the submucosa. The population included eosinophils, plasma cells, lymphocytes, macrophages, mast cells and neutrophils. Ultrastructural findings were observed and recorded by Dr. Yasuko Rikihisa. Rickettsial microorganisms were consistently found by her in the wall of the large colon of infected ponies (Table 18).

TABLE 18. IDENTIFICATION OF EHRLICHIAE IN ORGANS OF PHF-INFECTED PONIES BY ELECTRON MICROSCOPY (FROM RIKIHISA, PERRY AND CORDES, 1985).

	Detection of Ehrlichiae in Pony Number[a]						
Organ	1	2	3	5	7	8	13
Duodenum	/	/	/	/	/	-	-
Jejunum	+	+	+	/	/	/	/
Ileum	/	/	/	/	+	-	/
Caecum	-	/	-	-	+ +	+ +	+
Large colon	+ + +	+ + +	+ + +	+ +	+ +	+ +	+
Small colon	/	+ +	+ +	/	+	-	-
Stomach	-	-	-	/	-	-	-
Liver	-	-	-	/	/	/	/
Spleen	-	-	-	/	-	-	-
Lymph nodes							
Mesenteric	-	-	-	/	/	/	-
Ileal	/	/	/	/	/	-	/
Caecocolic	/	/	/	/	+	/	/
Cerebrum	-	/	-	/	/	/	/
Cerebellum	-	/	-	/	/	/	/

[a] + = Ehrlichiae detected
- = Ehrlichiae not detected
/ = Specimens not examined

Large numbers of ehrlichial organisms were present in the cytoplasm of deep glandular epithelial cells (Figure 23), in macrophages in the lamina propria and submucosa, in exfoliating epithelial cells and in the cytoplasm of mast cells in the lamina propria and submucosa. Despite the large numbers of microorganisms they contained, most host cells were morphologically intact except for the presence of swollen mitochondria. At least two morphological forms of the pleiomorphic ehrlichial organisms were observed by Dr. Rikihisa. One form was a small electron-dense structure (0.2-0.4 μm in diameter), found mostly in loose host membrane vacuoles. This form of the organism was seen undergoing binary fission in glandular epithelial cells. The other form was a larger less electron-dense organism (0.6-1.5 μm in diameter) which was individually enveloped by a very tight host membrane.

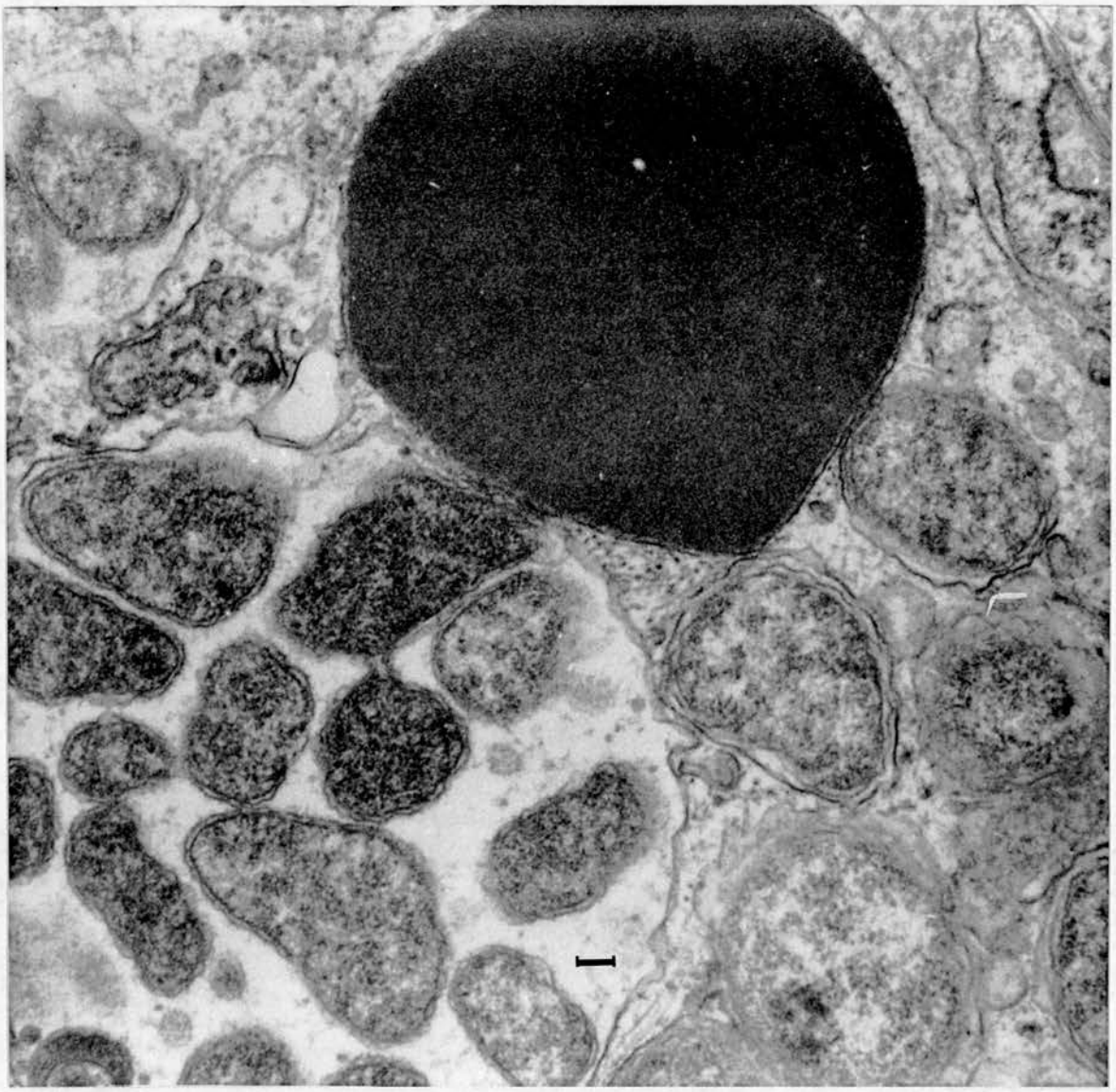

FIGURE 23. EHRLICHIAL ORGANISMS IN BINARY FISSION IN THE CYTOPLASM OF GLANDULAR EPITHELIAL CELLS OF THE LARGE COLON OF A PONY EXPERIMENTALLY INFECTED WITH PHF (Bar, 0.1 μM). (FROM RIKIHISA, PERRY AND CORDES, 1985).

DISCUSSION

The studies described in this chapter examined potential causal hypotheses on the aetiology of PHF, culminating in the isolation and identification of an Ehrlichia from affected ponies, the experimental reproduction of the disease in susceptible ponies using the isolated organism in pure culture, and the initial characterisation of the pathogenesis of the disease. The causative Ehrlichia was subsequently isolated in pure culture from experimentally infected ponies, fulfilling the criteria contained in the postulates of Robert Koch on the verification of a causal relationship between an infectious agent and a disease.

The first study described in this chapter investigated the possible role of clostridial enterotoxins. Although it was clearly demonstrated that C. perfringens Type A enterotoxin can be found in horses with PHF, there was no real difference between the prevalence of the enterotoxin in these horses and in unaffected horses. This finding also illustrates the limitation of this procedure in the laboratory confirmation of cases of colitis-X (Weirup, 1977), as the mere presence of these toxins is not indicative of any causal relationship.

Following the demonstration of the infectious, non-contagious nature of the disease by epidemiological (Perry et al., 1984; 1986) and whole blood transmission experiments (Whitlock et al., 1984) and the demonstration of apparent seroconversion of affected horses to the antigen of E. sennetsu, sera of affected and unaffected horses were examined to study the possible role of Rickettsia rickettsii, a recognized rickettsial infection transmitted by the American dog tick, Dermacentor variabilis, in much of the

eastern United States. However, although antibodies to R. rickettsii were detected in affected horses, there was no real difference between the prevalence of antibodies in PHF-affected and unaffected horses. Nevertheless, it was an indication that horses in the region were being exposed to sufficient challenge by D. variabilis to give an overall prevalence of antibodies to R. rickettsii in the study horses of 26.5%.

Following the establishment and maintenance of experimental PHF infection in ponies at the VMRCVM, it was possible to characterise the clinical disease so produced, and compare the results with those described from field cases. Experimentally infected ponies exhibited all of the major clinical signs of fever, anorexia, depression and diarrhoea. There was, however, a considerable variation in clinical signs between ponies, consistent with reports from the field (Knowles et al., 1984; Whitlock et al., 1984; 1985; Perry, in press). The clinical signs of colic, laminitis and oedema of the limbs, reported from a proportion of field cases, were not observed in the experimental disease.

The disease was characterised by a mean incubation period of approximately 11 days, and a biphasic fever. Anorexia, seen in 83.3% of infections, soon followed. Diarrhoea was observed in 75% of induced infections. One animal (pony 6) showed only transient depression as a clinical sign, but subsequently developed high levels of circulating antibodies. The case-fatality rate in the experimental infection, calculated as the number of ponies which died naturally before being destroyed as a proportion of all the ponies infected, was 4/24 (16.7%). However, several of the study ponies were destroyed in the early stages of the disease, possibly artificially lowering this figure. The

case-fatality rate in the field has been reported as 25%, although during 1986 this reportedly fell to about 10%, due in part to the extensive use of oxytetracycline therapy initiated during that year (Perry, in press).

The complete blood counts showed a leucopaenia in the range of 4000-5000 per µl during the latter stages of the incubation period. This was principally a result of an often dramatic lymphopaenia, on occasion recorded as low as 400 per µl just prior to the onset of clinical signs. There was, however, considerable variability in the clinical pathology parameters measured, which is consistent with other reports (Ziemer et al., 1987).

This clinical response seen here is similar to that of several other rickettsial diseases with respect to incubation period, fever, depression, effect upon appetite and effect upon the leucocytic series of blood cells (Scott, 1977). However, the almost pathognomonic involvement of the gastrointestinal tract, particularly the large intestine resulting in clinical diarrhoea, is certainly not typical of rickettsial diseases.

Following the culture of the buffy coat fraction of blood from PHF-infected ponies in human histiocytes, an organism with the cultural and structural properties of members of the genus Ehrlichia was consistently isolated in pure culture. Following the inoculation of these cultures into susceptible ponies by the intravenous route, the disease was reproduced, and the same organism was subsequently isolated in pure culture from recipient ponies. This demonstrated the ehrlichial cause of the disease. Similar results were obtained in independent studies carried out at the same time at the University of Illinois (Holland et al., 1984; 1985a)

and subsequently at the University of Maryland (Dutta et al., 1985) and the University of Pennsylvania (Whitlock and Palmer, 1986).

The organism has subsequently been characterised as being antigenically similar to E. sennetsu, the cause of a mononucleosis-like disease called sennetsu fever of man in Japan and parts of the Far East (Holland et al., 1985a), and the newly identified cause of PHF has been named Ehrlichia risticii in honour of the contributions to studies of leucocytic rickettsia made by Dr. Miodrag Ristic of the University of Illinois (Holland et al, 1985b). The tribe Ehrlichieae contains three genera: Ehrlichia, Cowdria and Neorickettsia (Kreig, 1984). Until the identification of E. risticii, there were four members of the genus Ehrlichia according to Bergey's Manual of Systematic Bacteriology (Kreig, 1984), these being E. canis, E. phagocytophila, E. equi and E. sennetsu. Ehrlichia equi (Gribble, 1969), which parasitises cells of the granulocytic series, particularly neutrophils (Ristic and Huxsoll, 1984; Scott, 1977) has also been identified in horses in the United States and is found principally in California. The clinical disease of equine ehrlichiosis caused by E. equi is distinct from and generally milder than that caused by E. risticii. There is no antigenic relationship reported between the two organisms (Holland et al., 1985a; 1985b).

Ehrlichia risticii also has been shown to share antigenic characteristics with E. canis (Holland et al., 1985a). The latter organism is principally a parasite of monocytes and is widespread in the United States and in much of Africa, the Middle East and the Far East, causing canine ehrlichiosis and tropical canine pancytopaenia in dogs. It is transmitted by the brown dog tick, Rhipicephalus sanguineus. This organism also

shares antigenic characteristics with E. sennetsu (Ristic et al., 1981). Recently, antibodies to E. canis have been found in human patients initially thought to be suffering from Rocky Mountain spotted fever (Maeda et al., 1987; Fishbein et al., in press). The significance of this finding and the obviously complex relationship between these organisms has not been elucidated. Other closely related organisms have been identifed and assigned to the genera Ehrlichia or Cytoecetes, but their phylogenetic status is far from clear (Marchette, 1982). These include E. bovis (Donatien and Lestoquard, 1936), E. ovina (Lestoquard and Donatien, 1936) and Cytoecetes ondiri (Haig and Danskin, 1962).

The pathological features of experimentally induced PHF were generally undramatic, with the exception of the sometimes liquid contents of much of the large intestine. The features seen in this study were fewer than those described earlier by Knowles et al. (1984). However, earlier descriptions were from field cases in which autolysis and the effects of prolonged therapy probably complicated the range and extent of lesions seen.

The remarkable feature seen under light and electron microscopy was the general maintenance of the integrity of mucosal and submucosal layers of affected organs, and the widespread and consistent presence of ehrlichial organisms in the epithelial cells, macrophages and mast cells of the lamina propria and the submucosa. The organism was identical to that identified in culture, and both the smaller electron dense forms and the larger less electron dense form were observed. The presence of large numbers of ehrlichial organisms in the large colon suggests an organotropism for that organ. The main feature of

the clinical disease is an apparent progressive accumulation of fluid in the large intestine, suggestive of a failure in the normal fluid reabsorption mechanism. With the absence of gross damage to the lamina propria and submucosa, or to the host cells in which ehrlichial organisms were found, it is possible that interference with epithelial cell function may be caused biochemically as seen in cholera infection in man (Mims, 1982) by organisms multiplying in epithelial cells, macrophages and mast cells at that site.

Epidemiological findings indicate that faecal-oral transmission of PHF does not occur. In the absence of major disruption of the mucosal layers of the large intestine, it is unlikely that large numbers of organisms are released into the intestinal lumen, and thus ultimately into the faeces. Furthermore, all rickettsial organisms are intracellular and closely cell-associated, so it seems likely that even if ehrlichial organisms do find their way into the intestinal lumen, the resulting cell destruction with consequent exposure of organisms to intestinal contents is unlikely to be conducive to their survival. This suggests therefore that although the multiplication of E. risticii in the wall of the large colon plays a primary and important role in the pathogenesis of the disease, its presence in the intestinal tract is of no significance in the transmission of the disease, which is probably the result of transmission of organisms in infected monocytes in the blood stream, most likely effected by an arthropod vector.

Since it was initially reported in the late 1970's, this disease has received a number of names, including Potomac Horse Fever and Acute Equine Diarrhoea Syndrome (Knowles et al., 1984). Since the identification

of its cause, this list has been supplemented by Equine Ehrlichial Colitis (Rikihisa and Perry, 1984) and Equine Monocytic Ehrlichiosis (Holland et al., 1984).

Reports of the studies reported in this chapter are the subject of the following publications, which are attached as appendices to this thesis.

Ehrich, M., Perry, B.D., Troutt, H.F., Dellers, R.N. and Magnusson, R.A. (1984). Acute diarrhoea in horses of the Potomac River Area: Examination for clostridial toxins. Journal of the American Veterinary Medical Association, 185, 433-435.

Cordes, D.O., Perry, B.D., Rikihisa, Y. and Chickering, W. (1986). Enterocolitis caused by Ehrlichia sp. in the horse. (Potomac Horse Fever). Veterinary Pathology, 23, 471-477.

Rikihisa, Y., Perry, B.D. and Cordes, D.O. (1984). Rickettsial link with acute equine diarrhoea. Veterinary Record, 115, 390. (Photocopy attached, permission obtained.)

Rikihisa, Y. and Perry, B.D. (1984). Causative agent of Potomac Horse Fever. Veterinary Record, 115, 554. (Photocopy attached, permission obtained.)

Rikihisa, Y., Perry, B.D. and Cordes, D.O. (1985). Ultrastructural study of ehrlichial organisms in the large colon of ponies infected with Potomac Horse Fever. Infection and Immunity, 49, 505-512.

Rikihisa, Y. and Perry, B.D. (1985). Causative ehrlichial organisms in Potomac Horse Fever. Infection and Immunity, 49, 513-517.

CHAPTER FOUR

STUDIES OF THE TRANSMISSION OF POTOMAC HORSE FEVER

INTRODUCTION.

Potomac horse fever is markedly seasonal in its occurrence, with 69.4% of the 904 cases which have been recorded in Maryland during the years 1982-1986 occurring during the months of July and August. Epidemiological studies described in Chapter 1 showed the non-contagious pattern of disease occurrence, and the demonstration of the ehrlichial cause of PHF and its transmission by whole blood inoculation strongly suggest that the disease is arthropod-borne.

The majority of rickettsias known to man are arthropod-borne (Traub and Jellison, 1981). Of those which parasitize domestic animals, more than half of them are known also to parasitize ticks (Scott, 1977). Within the genus Ehrlichia, arthropod vectors have been identified for two of the species listed in Bergey's Manual of Systematic Bacteriology (Kreig, 1984). These are E. canis, for which the vector is the brown dog tick Rhipicephalus sanguineus (Donatien and Lestoquard, 1935) and Cytoecetes (Ehrlichia) phagocytophila, for which the vector is the sheep tick Ixodes ricinus (MacLeod and Gordon, 1933). The ticks Hyalomma aegyptium and R. bursa have been incriminated as vectors of E. bovis and E. ovina respectively (Donatien and Lestoguard, 1936; Lestoquard and Donatien, 1936) but definitive substantiation of the role of these or other ticks as vectors of these organisms is not available (Ristic and Huxsoll, 1984). The vectors of E. equi and E.

sennetsu have not been identified.

Some rickettsial organisms such as R. rickettsii appear to have a comparatively wide vertebrate host range, permitting several species to provide a source of infection to their vector in nature (Traub and Jellison, 1981). Although most of the species of ehrlichial organisms will infect more than one species of vertebrate host, their host range is relatively narrow, but there is considerable variation within the genus (Marchette, 1982); Cytoecetes (Ehrlichia) phagocytophila infects sheep, cattle, goats and several species of deer (Foggie, 1951; Hudson, 1950; McDiarmid, 1965). Clinical disease is reported principally in domestic livestock, particularly sheep, and the role of deer in the epidemiology of the disease is unclear (Marchette, 1982). Cytoecetes ondiri affects bushbuck, cattle and sheep (Snodgrass et al., 1975). Ehrlichia canis is generally restricted to canids and foxes (Ewing et al., 1964; Amyx and Huxsoll, 1973; Huxsoll, 1976). Although E. equi has been shown to infect sheep, goats, dogs and monkeys under experimental conditions (Gribble, 1969; Lewis et al., 1975), natural infection is thought to be restricted to the horse. Thus, species other than the primary hosts have been implicated in the epidemiology of some of the ehrlichial diseases, whereas others, such as E. canis and E. equi infection are characterised by their narrow natural host range. In the majority of rickettsial diseases, a carrier state following recovery from clinical disease is reported to develop in the primary host (Scott, 1977), which can itself therefore play a prominent role in providing a source of infection to potential vector species of arthropods.

In this chapter, two simple studies of the transmission of PHF are

described. These investigate the intradermal route of infection of ponies, which would be the most likely route of an arthropod vector, and the existence and significance of E. risticii infections of vertebrate hosts other than horses in the endemic region of Maryland. This chapter also contains a discussion of published data on the transmission of PHF, including studies carried out by Mr. Michael Fletcher, Ph.D. candidate in the Department of Entomology at Virginia Polytechnic Institute and State University, under the supervision of the author.

MATERIALS AND METHODS.

Intradermal transmission of E. risticii to ponies.

Ehrlichia risticii was cultured on human histiocyte cells by Dr. Yasuko Rikihisa as previously described (Rikihisa and Perry, 1975). Infected cells were harvested and infection was confirmed by examining Giemsa-stained smears of cultured cells. Infected cells were counted using a haemato-cytometer, and 4 x 10^7 infected cells suspended in 5 ml of RPMI 1640 medium were pipetted into sterile centrifuge tubes and sealed.

One ml aliquots of the cell suspension were inoculated by the intradermal route into three susceptible ponies, kept under identical conditions to those described in Chapter 3. Five distinct inoculation sites were chosen on each pony. These were located on the neck, flank and rump. Whole blood in anticoagulant (350 ml) was collected from the jugular vein of pony 26 on day 19 following infection. *Leucocytes were aseptically separated and cultured as previously described (Rikihisa and Perry, 1985).* Blood was taken in Vacutainer tubes (Becton Dickenson) from all three ponies on alternate days following infection, and serum was removed and stored at -80°C. *Serum was subsequently tested by the IFAT for antibodies to E. risticii as previously described (Rikihisa and Perry, 1985).* Reciprocal titres of 1:40 or greater were considered positive. Clinical observations were made on each pony, including daily rectal temperatures and complete blood cell counts on blood collected on alternate days into Vacutainer tubes containing EDTA.

Following the death of pony 26 on day 20 post infection, a full post mortem was carried out by Dr. G. Saunders and the author in the manner described in Chapter 3.

Detection of antibodies to E. risticii in non-equine mammals.

This study was designed to examine the sera of wild, peridomestic and domestic non-equine mammals in the endemic area of Maryland for evidence of antibodies to PHF. It was initiated in an attempt to identify potential reservoirs of E. risticii, and to clarify the relationships of ticks or other arthropod vectors that may be involved in the natural transmission of PHF in horses. The author was responsible for the design and collection of serum samples from wild rodents and domestic farm animals and the general analysis and interpretation of results. The collection of serum samples from peridomestic rodents was carried out by Dr. E. T. Schmidtmann, Agriculture Research Service, Beltsville, Maryland. Serological testing was carried out by Dr. R. Rice, Walter Reed Army Institute of Research, Washington, D.C., and Dr. Yasuko Rikihisa, VMRCVM, Blacksburg, Virginia.

This study was conducted between February and September 1985 on 20 different premises in Montgomery County, Maryland and on one farm in Howard County, Maryland. Seventeen of the premises kept horses, of which 10 of these farms had previously reported suspected cases of PHF. Blood samples were taken in Vacutainer tubes from cattle, sheep, pigs and goats on various farms. Serum was separated and stored at -70°C or -40°C until tested.

The peridomestic rodents, Norway rats (Rattus norvegicus) and house mice (Mus musculus) were captured by Dr. Schmidtmann in live traps set in horse barns on 11 premises between 15 April and 15 August 1985. Traps containing a rat or mouse were returned to Dr. Schmidtmann's laboratory where they were humanely euthanized in a CO_2 chamber and bled by cardiac puncture. Rat sera were separated by centrifugation and mouse blood from each animal was placed on a piece of filter paper. All samples were stored at -70°C until tested.

White-footed mice (Peromyscus leucopus Rafinesque) and meadow voles (Microtus pennsylvanicus Ord.) were trapped on three horse farms at regular intervals on six occasions during the period February to September 1985 in collaboration with Dr. Jorgen Hansen of the VMRCVM and Dr. E. C. Turner and Mr. M. G. Fletcher, Department of Entomology, Virginia Polytechnic Institute and State University. Pre-baiting of rodent runs using peanut butter and apple was carried out in the late afternoon. Folding aluminum traps (Sherman Traps, Inc., Tallahassee, Florida) of dimensions 7.6 x 8.9 x 22.9 cm were laid 24 hours later and left overnight. Trapped rodents collected the following day were anaesthetized in CO_2 chambers and bled out from the axilla. Serum was separated using standard techniques and stored at -70°C until tested.

All sera collected were examined by Drs. Rice and Rikihisa using the IFAT as previously described (Dutta et al., 1985; Rikihisa and Perry, 1985). Positive control sera were produced in Dr. Rice's laboratory by infecting laboratory reared white-footed mice, meadow voles, and ICR strain laboratory mice with non-tissue culture propagated E. risticii.

Positive control sera were not available for the other species tested.

RESULTS.

Intradermal transmission of E. risticii to ponies.

One of the inoculated ponies (pony 26) developed clinical signs consistent with those of PHF following an incubation period of nine days. Signs consisted of a fever of 38.3°C accompanied by progressive depression. Fifteen days after inoculation it stopped eating completely. Diarrhoea developed 18 days after inoculation and the pony died two days later (Figure 24).

The other two inoculated ponies developed mild (pony 31) or inapparent (pony 32) PHF.

All ponies seroconverted to E. risticii from 7-19 days following infection.

From cultures made from leucocyte fractions of pony 26 on day 19 following infection, a heavy growth of E. risticii was identified from day 7 of culture. The identity of the organism was confirmed by Dr. Rikihisa by immunofluorescent microscopy and electron microscopy as previously described (Rikihisa and Perry, 1985).

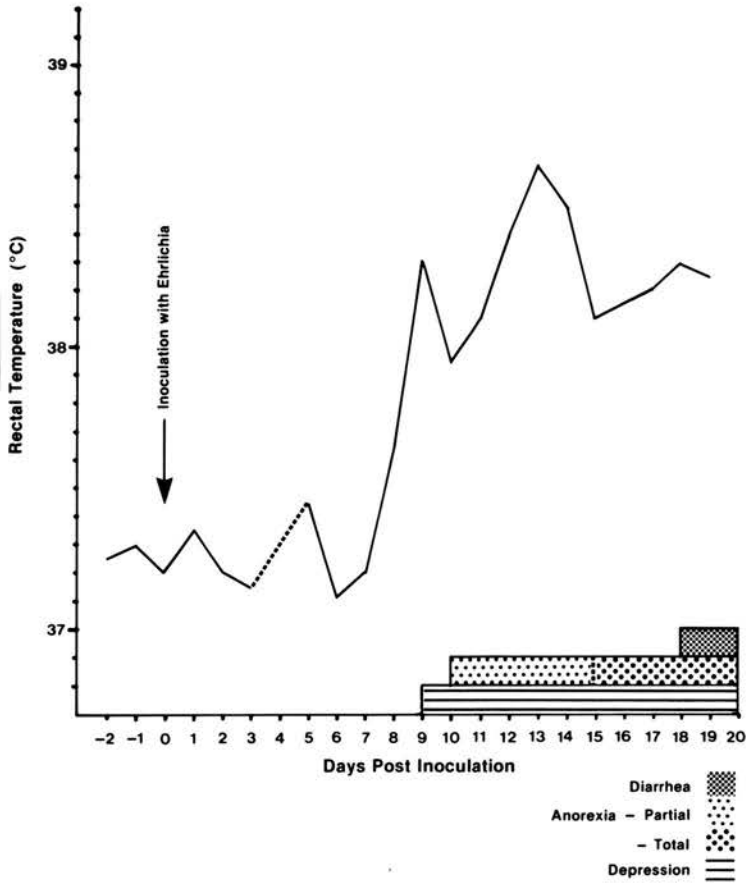

FIGURE 24. CLINICAL RESPONSE IN A PONY TO THE INTRADERMAL INOCULATION OF A PURE CULTURE OF E. RISTICII.

At the post mortem examination of pony 26, lesions present were consistent with those of PHF. The stomach had a few ulcerations, all less than 1 cm, near the margo plicatus. The small intestine contained only mucus and four areas of haemorrhage were observed, which were located in the duodenum (two), distal jejunum (one) and ileum (one). These were 2 to 5 cm in size and round, stellate or irregular in shape. A mild hyperaemia was evident throughout the mucosa of the small intestine.

The large colon, caecum and small colon contained watery brown fluid. No lesions were seen in the mucosa, except for two discrete ulcers in the caecum.

Antibodies in non-equine mammals.

All sera from wild and peridomestic rodents were negative for antibodies to E. risticii. Two of the four domestic farm animal species tested showed serological evidence of exposure to E. risticii, these being pigs and a goat. The three seropositive pigs were all on the same farm, separated by a distance of 3 kilometers from the farm at which the one seropositive goat was found. Both farms kept horses and had experienced cases of PHF in previous years. The full results are shown in Table 19.

TABLE 19. PREVALENCE OF ANTIBODIES TO EHRLICHIA RISTICII IN NON-EQUINE MAMMALS.

Species	Number of Premises Sampled	Number of Animals Tested	Number of Animals Positive*	Proportion Positive (%)
Cattle	6	75	0	0
Sheep	2	15	0	0
Pig	1	14	3	21.4
Goat	3	3	1	33.3
Barn mice	7	24	0	0
Rats	7	131	0	0
White-footed mice	3	40	0	0
Meadow vole	3	65	0	0

*Positive for antibodies against PHF by IFAT ($\geq$ 1:40).

DISCUSSION.

Previous to this study, experimental transmission of PHF to horses had been achieved by the intravenous transfusion of whole blood from an acutely sick horse to a susceptible horse (Jenny, 1984; Whitlock et al., 1984; Rikihisa et al., 1984; Holland et al., 1984) and by the intravenous inoculation of E. risticii in cell culture (Holland et al., 1985a; Rikihisa and Perry, 1985).

This study demonstrated that acute PHF, with an incubation period apparently indistinguishable from that of intravenous transmission, can be induced by the intradermal route. However, it also indicated that clinical PHF cannot be consistently reproduced by this means, with two of the three study ponies showing evidence of infection as manifest by seroconversion, but little or no evidence of clinical PHF. Unfortunately, ehrlichial cultures from whole blood were not performed on these two animals.

In the field, it seems likely that PHF is arthropod-borne, and these results support this theory. However, epidemiological evidence presented in Chapter 2 suggests that mechanical transmission is unlikely. Mechanical transmission by arthropods is generally the result of interrupted feeding or repeated feeding of arthropods on several hosts, allowing the mechanical transfer of blood from one host to another. This normally requires close juxtaposition of infected and susceptible animals for organisms to remain viable in the transported blood meal. The clustering of cases which results from such transmission is not characteristic of PHF (Perry et al., 1986). A biological transmission by an arthropod is more likely, in which

case it appears that large numbers of E. risticii would have to be inoculated by the arthropod in order to establish infection. Given the sporadic occurrence of PHF on farms, it is likely that arthropod infection rates are low, or that the vector is uncommon.

In the study of non-equine mammals, potential barn or indoor exposure of animals to E. risticii was evaluated by determining if peridomestic rats or mice provided a reservoir of infection to vector species within that environment. Similarly, potential field exposure of horses by the sharing of environment with reservoir species and their ectoparasites was evaluated by examination of white-footed mice, meadow voles, cattle, pigs and goats.

Serum samples were taken from a wide range of animals that may be present on horse farms where PHF has been reported. The complete absence of antibodies to E. risticii in six of the eight species tested on 18 of the 21 farms studied suggests that widespread natural infection of animals other than horses does not occur, in spite of the fact that experimental infections have previously been demonstrated in laboratory mice (Jenkins et al., 1985), dogs (Ristic et al., in press), and non-human primates (Stephenson et al., 1985). Nevertheless, this is consistent with other ehrlichial diseases such as that caused by E. equi infection, where although experimental susceptibility has been documented in several species, natural infection of these species does not appear to occur or to play a significant role in the epidemiology of the disease (Gribble, 1969; Lewis et al., 1975).

Although the vector of PHF has yet to be determined, the American dog tick, Dermacentor variabilis, is a prime suspect and has been the focus of several studies (Carroll and Schmidtmann, 1986; Fletcher, 1987; Miranpuri et al., 1985; Perry et al., 1985; Schmidtmann, 1985; Schmidtmann

et al., 1986). This tick is the primary vector of Rocky Mountain spotted fever (RMSF) in the mid-Atlantic and southeast regions of the United States (Burgdorfer, 1977) and antibodies to RMSF have been identified by several workers in white-footed mice and meadow voles. These species are the principal hosts of the larval and nymphal instars of D. variabilis (Sonenshine et al., 1966). The reported prevalence of R. rickettsii antibodies in these species in endemic RMSF areas has ranged from 16.7 to 21.2% (Magnarelli, 1981; Magnarelli et al., 1983) and as such they may be considered good indicator species of rickettsial activity in the D. variabilis ecosystem. The complete absence of antibodies to E. risticii in the sample population of these species in the present study throws some doubt on the hypothesis that PHF is transmitted by D. variabilis.

More direct studies have also so far been unable to incriminate D. variabilis as the vector of PHF. Adult tick infestations of horses in the affected regions of the mid-Atlantic United States appear to be exclusively D. variabilis (Carroll and Schmidtmann, 1986; Fletcher, 1987) which attach principally in the tail and the mane. Infestation rates per horse are generally low, even during the peak of tick activity in May and June, and rarely exceed five ticks per horse (Carroll and Schmidtmann, 1986; Fletcher, 1987; Schmidtmann, E.T., personal communication). Unengorged adult ticks from pastures of farms which had experienced PHF were fed on susceptible ponies and horses, but these animals were fully susceptible to challenge with PHF 8 to 12 weeks later (Schmidtmann et al., 1986; Fletcher, 1987). Subsequently, larval and nymphal D. variabilis were fed on infected laboratory mice and following

engorgement and moulting, the emergent nymphal and adult ticks were fed on laboratory mice and horses respectively (Fletcher, 1987). There was no evidence of PHF transmission in mice, and recipient horses were susceptible to challenge with E. risticii three months after the ticks had completed their engorgement (Fletcher, 1987). Intra-stadial transmission was also attempted with D. variabilis in one pony in which 300 adult ticks were fed on an infected pony during the early acute stage of the disease (Fletcher, 1987). Feeding was interrupted after four days, and ticks were allowed to re-attach to a susceptible pony to complete their engorgement. The recipient pony remained clinically normal, although did transiently seroconvert to E. risticii from days 10 to 35 following tick attachment. However, it was fully susceptible to challenge with E. risticii on day 47 after tick attachment (Fletcher, 1987). Other potential arthropod vectors have been identified based on the compatibility of their seasonal dynamics with that of the occurrence of the disease (Fletcher, 1987; Perry et al., 1985; Schmidtmann, 1985). These are blackflies (Simulium jenningsi), stable flies (Stomoxys calcitrans) and midges (Culicoides obsoletus and C. varipennis). Experimental studies of their vector potential for E. risticii have not been reported. Schmidtmann (personal communication) has also examined the role of the cat flea (Ctenocephalides felis), which although not commonly found on horses, is abundant on dogs and cats on farms in the endemic region of Maryland. However, he was unable to demonstrate infection of cat fleas with E. risticii under experimental conditions, or to transmit the disease to laboratory mice or horses by means of fleas which had previously engorged on E. risticii infected mice and horses.

The vector of PHF remains unidentified.

A report of the first section of this chapter appears in the following publication, which is attached to this thesis as an appendix.

Perry, B.D., Rikihisa, Y. and Saunders, G.K. (1985). Intradermal transmission of Potomac Horse Fever. Veterinary Record, 116, 246-247. (Photocopy attached, permission obtained.)

A report of the second section of this chapter has been submitted for publication.

Robl, M.G., Perry, B.D., Schmidtmann, E.T., Rice, R., Hansen, J.W., Fletcher, M., Turner, E.C. and Penney, B.E. (in submission). The role of non-equine mammals in the epidemiology of Potomac Horse Fever. Veterinary Record.

CHAPTER FIVE

GENERAL DISCUSSION

The opportunity to search for the cause of a new disease, as described in much of this thesis, is an increasingly uncommon event these days, but when it does occur it induces an extraordinary mixture of enhanced collaboration between researchers (Whitlock et al., 1985) and enhanced competition between research groups. The search for the cause of Potomac Horse Fever was no exception. In the longer term, such a situation hopefully provides an inherent peer review of work being carried out, substantial opportunity for independent corroboration of results and rapid advancement of knowledge unless, in the meantime, researchers disenchanted with the sometimes unusual public forum in which their studies are conducted, decide to turn their attention to other less controversial research pursuits.

The epidemiology study described in Chapter 1 provided the background for much of the subsequent work, eliminated many of the proposed aetiologies, and produced several hypotheses mostly worthy of subsequent testing. It clearly characterised PHF as a non-contagious infectious seasonal disease consistent with it being arthropod-borne. Even before the cause of PHF was known, this provided useful information on which horse-owners and state animal health officials could base decisions relating to the movement of horses to shows and other events, a feature so important and characteristic of the horse industry. Movement restrictions have not been imposed in PHF endemic areas and in spite of the fact that the disease is currently reported from

over 20 states in the United States compared to just three in 1983, this change is undoubtedly due to the recent availability of serological tests (Holland et al., 1985a; Rikihisa and Perry, 1985; Ristic et al., 1986; Dutta et al., 1987) to identify horses which have been exposed to E. risticii, rather than a widespread extension of the disease from the original focus. Indeed, further examination of the epidemiology within the endemic regions in the eastern United States has indicated that infections are generally extremely localized (Palmer et al., 1986) and usually associated with major river courses such as the Hudson River, Ohio River, Susquehanna River, Connecticut River, Snake River and the Potomac River.

The case-control study produced several apparent associations with the disease, described in Chapter 1. Many of these appear to indicate a continued presence of the disease agent on a premises from year to year, an association with animals others than horses, and an association with certain conditions likely to favour the abundance of resident arthropod populations. It has not so far been possible to fully explain these associations with results from experimental studies. This is in part due to the fact that controlled experimental studies with adequate replication in horses are neither cheap nor logistically easy to perform. Nevertheless, limited studies on the persistence of E. risticii in horses following recovery have not been conclusive. In this thesis (Chapter 3), the isolation of E. risticii on day 37 following infection in pony 19 is described. Other workers have reported the failure to transmit PHF by whole blood inoculation from infected horses following clinical recovery to susceptible horses (Ristic, 1985). The development of a carrier state in recovered animals is a unifying

feature of the rickettsial diseases (Scott, 1977). Rickettsial organisms may persist at levels too low to be infectious to arthropods or other vertebrates by whole blood inoculation (Norment and Burgdorfer, 1983) but the presence of persistent serum antibodies in recovered animals, particularly IgM, provides some evidence that a carrier state exists (Woldehiwet and Scott, 1982). This phenomenon has not been adequately investigated in PHF and detailed published reports of testing for circulating antibodies beyond 60 days (Ristic et al., 1986) are not available. Ten of 11 recovered horses and ponies were immune to challenge 8 to 63 weeks following recovery from PHF, and no change in IFAT antibody titre was observed following their re-challenge (Whitlock and Palmer, 1986).

The association of PHF with animals other than horses does not appear to be a result of a reservoir status of E. risticii in these species. The serological study of non-equine mammals described in Chapter 4 was characterised by negative data. The seropositive pigs were in a group which were kept in unusually close contact with horses on the same farm and Potomac horse fever had been reported on the farm every year for the previous three years. However, the association with other species, seen in the epidemiology study at both the horse and farm comparison levels, may be a confounding factor as a result of non-equine mammals serving as attractants to a vector arthropod or arthropods, thereby increasing their chances of infection.

The studies on the causation described in Chapter 2 proved successful within a short period of time, due in part to the focus of attention on a probable aetiology, as a result of the epidemiology study described in Chapter 1 and the serological studies of Jenny (1984) whereby antibodies

to E. sennetsu where identified in recovered horse (Holland et al., 1985a).

However, although providing substantive data on the cause of PHF, the accompanying studies of the pathogenesis of the disease were not conclusive and merit considerable further attention. The principal difficulty encountered was the acquisition of standard animals for experimental infection which were free of other pathogens and were of standard conformation. Although the laboratory mouse is susceptible to E. risticii and can be used for limited studies of pathogenesis and immunity of PHF (Jenkins et al., 1985; Burger et al., 1986) it is of little value in the study of the colitis and diarrhoea so characteristic of the disease in horses.

The identification of E. risticii as the causative agent has not resulted in the rapid identification of the vector of the disease. The tribe Ehrlichieae are not a particularly homogenous group of organisms, with its members parasites of both the monocytic and granulocytic series of white blood cells. Organisms representative of both those groups have been shown to be tick-borne, namely E. canis and C. phagocytophila. However, the vector of Neorickettsia helminthoeca is the salmon trematode Nanophyetus salmincola, and dogs, the only naturally infected vertebrates, contract the disease after eating salmon infected with this trematode (Philip, 1955). Although no vector has been identified for E. sennetsu, the organism most closely related antigenically to E. risticii, human infection has been associated with the consumption of fish (Fukuda et al., 1962: cited in Marchette, 1982), although this work has not been substantiated and other workers (Miranpuri et al., 1985) have suggested that the disease, which is strictly seasonal in Japan, is tick-transmitted.

The failure to incriminate D. variabilis, the commonest tick which infests horses in the endemic PHF region of the United States as the vector of this disease is therefore difficult to interpret. The studies on the transmission of PHF have been carried out under controlled conditions in at least two independent laboratories, and have consisted of experiments which would have been sufficient to demonstrate arthropod transmission of certain similar agents. However, there are several related organisms for which the vector has still not been identified despite many years of study, the most notable of which are E. equi, E. sennetsu, E. bovis, and C. ondiri. It is not difficult to identify potential flaws in studies of the possible role of vector species in the transmission of PHF, the most obvious of which are the timing of feeding by ticks on infected vertebrates, and the suitability to the arthropod of the particular strain of organism with which the vertebrate host was infected. By the same token, with such close relatives as Neorickettsia helminthoeca, there are many potential vectors, arthropods, helminths and others with intimate contact with horses in the endemic region which have not yet even been considered.

This thesis presents a series of closely inter-related studies of the epidemiology and causation of PHF. These studies are still continuing, with their attention currently turning more particularly to the transmission, diagnosis and prevention of the disease. Diagnosis of PHF has relied on clinical criteria and the detection of circulating antibodies by the IFAT. Recently, a more sensitive enzyme-linked immunosorbent assay (ELISA) has been reported (Dutta et al., 1987) which is expected to provide earlier identification of affected horses than the IFAT.

Potomac horse fever has been shown to be sensitive to tetracycline antibiotics (Palmer et al., in press) and the widespread use of oxytetracycline in the endemic region of Maryland in 1986 probably contributed to the considerably lower case fatality rate which was observed.

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APPENDIX A.

FORM A
INDIVIDUAL ANIMAL QUESTIONNAIRE
POTOMAC HORSE FEVER

A1: _____

2.HORSE: _____

3.OWNER: _____ 4.PHONE #: _____

5.ADDRESS: _____

_____ZIP_____

6.VETERINARIAN: _____ 7.PHONE #:_____

8.FARM: _____ 8.FARM QUES.#:_____

10.DATES AFFECTED: Beginning:_____ Ending:_____

All responses should related to 1983 only (Jan to present). Circle correct answer or fill in the blanks where indicated. Explanatory comments are welcome.

SIGNALMENT:

11.Age: _____

12.Sex: a) stallion b) mare c) gelding

13.Breed: a) TB b) STB c) Arabian d) QH e) Trakhener

f) Morgan g) pony (specify breed) _____

h) crossbred (specify cross) _____

i) grade _____

j) other (specify) _____

14.Height: _____hands _____inches

15.Horse use: a) pleasure horse b) show horse c) event horse

d) racehorse e) endurance horse f) polo

g) broodmare/breeding stallion h) foal

i) weanling/yearling-unbroken j) other (specify)_____

PARASITE CONTROL (dates and products used on this horse over the LAST YEAR)

	DATE (15a)	PRODUCT (15b)
1.	_____	_____
2.	_____	_____
3.	_____	_____
4.	_____	_____
5.	_____	_____
6.	_____	_____
7.	_____	_____
8.	_____	_____
9.	_____	_____
10.	_____	_____

16. COMMENT: _____

_____

_____

_____

HOUSING All responses should relate to the months of 1983.

17. PLEASE INDICATE THE AVERAGE NUMBER OF HOURS PER DAY (24 hours) SPENT ON PASTURE AND IN STABLES DURING THE MONTHS OF 1983.

	AVERAGE # OF HOURS PER DAY								
	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP
PASTURE									
STABLED									
ELSEWHERE									

PASTURE (If not on pasture go to Stabling)

18. Size of pasture horse has most recently been turned out on.

a) 1/2 acre b) 1/2-1 acres c) 2-10 acres

d) 10-20 acres e) 20-50 acres f) >50 acres

19. Number of other horses on same pasture:

a) 0 b) 1-5 c) 5-10 d) 10-20 e) >20

20. When was the horse first turned out on this pasture?

DATE:_____

21. Number of horses currently on this pasture who previously have had Potomac fever this year (1983):

a) 0 b) 1-2 c) 3-5 d) >5

22. Number of horses who previously have had Potomac fever this year (1983) on an adjacent pasture with a common fence line:

a) 0 b) 1-2 c) 3-5 d) >5

23. Number of horses currently on this or adjacent pasture who previously have had Potomac fever previous years:

a) 0 b) 1-2 c) 3-5 d) >5

24.Which of the following describes the pasture (may select more than one)

a) low land b) marsh c) hilly d) tall grass e) mowed grass

f) hay out from pasture g) wood land h) fertilized

i) run off from barn yard into pasture j) other _____

25.Amount of forage on pasture:

a) little b) moderate c) abundant d) none

26.Water source for pasture: (circle all that apply):

a) well water b) spring c) stream d) river e) pond

f) city water g) other _____

27.Fertilizer used on pasture: a) none b) type(s):_____

c)when applied:_____

28.Do other livestock graze this pasture? yes / no types:_____

29.Other comments:_____

_____

_____

STABLING: (If not stabled go to next heading)

30.When was this horse first introduced to the barn?

DATE:_____

31.Number of other horses currently using the barn:

a) 0 b) 1-5 c) 5-10 d) 10-20 e) >20

32.Number of other horses in barn affected with Potomac fever this year:

a) 0 b) 1-2 c) 3-5 d) 5-10 e) >10

33. Number of other horses in barn affected with Potomac fever previous years:

a) 0 b) 1-2 c) 3-5 d) 5-10 e) >10

34. Water source for barn: (circle all that apply)

a) well water b) spring c) stream d) river e) pond

f) city water g) other _____ h) common source with home

35. How does the horse receive the water?

a) bucket b) automatic water bucket c) communal trough

d) other (specify) _____

36. Bedding:

a) straw b) shavings c) sawdust d) other (specify) _____

37. The stall construction (circle all that apply):

a) wood b) concrete c) pipe d) cinder block

e) other_____ f) painted

g) stained with _____ h) treated with creosote

i) treated with salt j) treated with other _____

38. Comment:_____

_____

_____

TIME COURSE OF EVENTS

39. Was this horse born on the farm? yes / no unknown

IF NO

a) When was this horse first introduced to this farm?

Date:_____ unknown

b) Was this horse born in the Potomac area? yes / no

c) If this horse was not born in the Potomac area, when was he first introduced to the area? Date:_____ unknown

40. Comment:_____

_____

MOVEMENT:

41. Please indicate the number of times per month THIS HORSE has been off the farm (other than local exercising where no transport is involved).

1983

	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP
NUMBER OF TIMES									
MAXIMUM ONE WAY DISTANCE									
REASON FOR TRAVEL (key below)									

KEY: a) horse show b) endurance ride c) 3 day event d) to be bred

e) racing f) training g) other _____

42. Comment:_____

_____

_____

43. Please indicate the number of OTHER HORSES per month that have been off the farm (other than local exercising where no transport is involved).

1983

	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP
NUMBER OF TIMES									
MAXIMUM ONE WAY DISTANCE									
REASON FOR TRAVEL (key below)									

KEY: a) horse show b) endurance ride c) 3 day event d) to be bred
e) racing f) training g) other _____

44. Comment: _____

_____

_____

45. Number of horses introduced to the farm during since January 1983.

_____ horses

46. Number of horses introduced to the farm during the last 30 days.

_____ horses

47. Number of horses introduced to the farm during the last 10 days.

_____ horses

48. When was last horse introduced to the farm? DATE: _____

49. Transport used for all horses in above questions (indicate all that apply):

a. Own truck/trailer b. commercial van (company _____)

c. Neighbor's truck?trailer (name _____) d. Other ____

EXPOSURE:

50. Has this horse had any contact with an animal suffering from or recovered from Potomac fever? yes / no

51. IF YES

a) When? Date: _____

b) With how many sick horses? _____

c) Type of contact with sick animals:

1) same pasture or barn

2) on the same farm but no direct contact

3) only contact with horses who have contacted a sick animal

4) other_____

52. Has this horse been affected by Potomac fever?_____ When?_____

53. Has this horse been affected by Potomac fever previously? yes / no If so when? _____

54. COMMENT:_____

_____

_____

FEEDING PROGRAM DURING PAST MONTH

CONCENTRATE	A.M.(lbs)	Noon(lbs)	P.M.(lbs)
55.TYPE: _____	_____	_____	_____
Brand _____ Protein _____		TDN _____	
56.TYPE: _____	_____	_____	_____
Brand _____ Protein _____		TDN _____	
57.TYPE: _____	_____	_____	_____
Brand _____ Protein _____		TDN _____	
58.TYPE: _____	_____	_____	_____
Brand _____ Protein _____		TDN _____	

59. Mineral supplement: Brand_____ Amt/day _____

60. Vitamin supplement: Brand_____ Amt/day _____

61. Feed Company_____ Address _____

62. Time of grain feeding _____

Location a) stall b) pasture c) both d) other_____

63. How is the grain fed?

a)bucket b)trough c) on ground d) other_____

64. Hay: (circle % of each)

Alfalfa:	0%	25%	50%	75%	100%
Timothy:	0%	25%	50%	75%	100%
Clover:	0%	25%	50%	75%	100%
Brom grass:	0%	25%	50%	75%	100%
Orchard grass:	0%	25%	50%	75%	100%
Mixed grass:	0%	25%	50%	75%	100%
Other:	0%	25%	50%	75%	100%

65. Time of hay feeding _____

Location: a) stall b) pasture c) both d) other_____

66. How is the hay fed?

a) hay rack/net b) trough c) on ground d) other _____

67. Source of hay: a) local grown (where?_____)

b) hay dealer (who?_____)

c) out of state d) other _____

68. Changes in feeding program or pattern in last month (new hay, different type concentrate, time_____

_____

Past Medical History

69.Major disease problems over past year:

	1982							1983								
	JUN	JUL	AUG	SEP	OCT	NOV	DEC	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP
DIARRHEA																
COLIC																
FOUNDER																
LAMENESS OTHER THAN FOUNDER																
NASAL DISCHARGE																
COUGH																
FEVER > 102°F																
STRANGLES																
OFF FEED > 1 DAY WITHOUT REASON																
OTHER																
OTHER																

70.PLEASE LIST THE TREATMENTS ADMINISTERED

TREATMENT(product):	DATE BEGUN	LENGTH	COMMENTS
1.			
2.			
3.			
4.			
5.			
6.			

THE FOLLOWING PERTAIN TO THE MOST RECENT DISEASE PROBLEM.

71. Butazolidine used: a) yes b) no c) unknown

dose _____ Length of treatment _____

72. Banamine used? a) yes b) no c) unknown

dose _____ Length of treatment _____

73. Antibiotics used? a) yes b) no c) unknown (AQ139)

Brand Name _____ dose _____

Length of treatment _____

74. COMMENTS: _____

_____

_____

_____

_____

_____

_____

_____

_____

_____

VACCINATIONS DURING THE PAST YEAR:

1. TETANUS TOXOID - TRADE NAME:_____

DATES:_____

2. TETANUS ANTITOXIN-TRADE NAME:_____

DATES:_____

3. EEE/WEE - TRADE NAME:_____

DATES:_____

4. VEE - TRADE NAME:_____

DATES:_____

5. INFLUENZA -TRADE NAME:_____

DATES:_____

6. RHINOPNEUMONITIS - TRADE NAME:_____

DATES:_____

7. STRANGLES - TRADE NAME:_____

DATES:_____

8. BOTULISM TOXOID - TRADE NAME:_____

DATES:_____

9. COMBINATION-TRADE NAME:_____

CONTAINS:_____

DATES:_____

10. COMBINATION-TRADE NAME:_____

CONTAINS:_____

DATES:_____

11. OTHER - TRADE NAME:_____

CONTAINS:_____

CONTAINS:_____

DATES:_____

12. LAST COGGINS TEST: a. DATE _____

b. POSITIVE _____ NEGATIVE _____

APPENDIX B.

FORM B
FARM QUESTIONNAIRE
POTOMAC HORSE FEVER

(FQ1) _____

2. FARM NAME _____ 3. PHONE # _____

4. ADDRESS _____

5. FARM OWNER _____ 6. PHONE # _____

7. FARM MANAGER _____ 8. PHONE # _____

9. VET. #1 _____ 10. PHONE # _____

11. VET. #2 _____ 12. PHONE # _____

13. NUMBER OF HORSES CURRENTLY ON THE FARM _____

14. NUMBER OF HORSES CURRENTLY STABLED _____

15. NUMBER OF HORSES CURRENTLY AT PASTURE (NOT STABLED) _____

16. NUMBER OF ANIMALS AFFECTED BY POTOMAC FEVER THIS YEAR(1983)_____

17. AFFECTED ANIMALS (USE ANOTHER SHEET IF NECESSARY):

	DATE(a)	ID(b)
#1		
#2		
#3		
#4		
#5		
#6		
#7		
#8		

18. TYPE OF HORSES ON FARM (circle all that apply and place number in blank)

a. Pleasure horse _____

b. Show horse _____

c. Event horse _____

d. Race horse _____

e. Endurance horse _____

f. Breeding stock _____

g. Foals _____

h. Weanling _____

i. Yearling _____

j. Other (specify) _____

19. AGE OF HORSES ON THE FARM (number of each)

a. <1 year _____ b. 1 year old _____

c. 2 year old _____ d. 3 year old _____

e. 4 year old _____ f. 5 year old _____

g. 6 year old _____ h. 7 year old _____

i. 8 year old _____ j. 9 year old _____

k. 10 year old _____ l. 11-15 years old _____

m. 16-20 years old _____ n. >20 years old _____

o. Unknown _____

20. SEX OF HORSES ON THE FARM (number of each)

a. Mares _____

b. Stallions _____

c. Geldings _____

21. BREED OF HORSES ON THE FARM (number of each)

a. TB _____

b. STB _____

c. QH _____

d. Morgan _____

e. Arabian _____

f. Ponies (breed _____) _____

g. Crossbred (cross _____) _____

h. Grade _____

i. Other (_____) _____

j. Other (_____) _____

k. Other (_____) _____

l. Other (_____) _____

22. NUMBER OF OTHER DOMESTIC ANIMALS ON FARM

a. CATTLE _____ b. DOGS _____ c. SHEEP _____

d. CATS _____ e. SWINE _____ f. CHICKENS _____

g. DONKEYS _____ h. MULES _____ i. DUCKS _____

j. GOATS _____ OTHER (SPECIFY) _____

23. HAVE ANY OF THE ANIMALS OTHER THAN HORSES BEEN SICK DURING THE LAST 6 MONTHS? YES _____ NO _____

24. EXPLAIN NATURE OF ILLNESS: _____

25. NUMBER OF BARNS ON FARM _____

26. IF POTOMAC FEVER HAS BEEN RECORDED ON YOUR FARM THIS YEAR, HOW MANY BARNS CONTAINED AFFECTED ANIMALS? _____

27. NUMBER OF HORSES IN BOX STALLS _____

28. NUMBER OF HORSES IN STRAIGHT STALLS _____

29. FOR EACH BARN GIVE TOTAL NUMBER OF HORSES PER BARN, AND AFFECTED HORSES IF APPROPRIATE.

	TOTAL (a)	AFFECTED (b)
BARN 1	_____	_____
BARN 2	_____	_____
BARN 3	_____	_____
BARN 4	_____	_____

30. COMMENT_____

_____

31. NUMBER OF ADULT HORSES ADDED TO FARM THIS YEAR:_____

32. NUMBER OF ADULT HORSES TEMPORARILY OFF FARM OVER PAST YEAR (including racing, showing, breeding, hospitalization, etc. but not including local exercise):_____

33. REASON TEMPORARILY OFF FARM IN THE PAST YEAR:

a. showing b. racing c. breeding d. endurance race

e. eventing f. pleasure rides g. hospitalization

h. other:_____

34. CLOSEST NEIGHBOR WITH HORSES:

a. 0-1/4 mile

b. 1/4-1/2 mile

c. 1/2-1 mile

d. 1-2 miles

e. 3-5 miles

f. Over 5 miles

35. TYPE AND USE OF NEIGHBOR'S HORSES:_____

36. CLOSEST NEIGHBOR WITH POTOMAC FEVER RECORDED:

THIS YEAR	OTHER YEARS
a. 0-1/4 mile	a. 0-1/4 mile
b. 1/4-1/2 mile	b. 1/4-1/2 mile
c. 1/2-1 mile	c. 1/2-1 mile
d. 1-2 miles	d. 1-2 miles
e. 3-5 miles	e. 3-5 miles
f. Over 5 miles	f. Over 5 miles

37. NAME AND ADDRESS OF NEIGHBOR WITH POTOMAC FEVER:_____

_____

38. RELATIONSHIP GEOGRAPHICALLY_____

MEDICAL HISTORY OF YOUR FARM THIS YEAR:

39. HAVE YOU HAD ANY OF THE FOLLOWING? (Circle as many as apply)

a. Strangles

b. Flu

c. EIA

d. Foal Diarrhea

e. Diarrhea in Adults

f. Colic (other than associated with this problem)

g. Abortion

h. Difficult to breed mares

i. Unexplained fever

j. Off feed for 2 or more days for unknown reason

k. Other (Describe) _____

1. No health problems

40. GIVE DATES AND EXPLAIN ABOVE _____

_____

_____

_____

_____

_____

PASTURE:

41. TOTAL PASTURE LAND _____ ACRES

42. TOTAL NUMBER OF HORSES ON PASTURE FULL TIME (NEVER STABLED) _____

43. TOTAL NUMBER OF HORSES ON PASTURE PART TIME

(STABLED OCCASIONALLY)_____

44. QUALITY OF FORAGE ON PASTURE

a. None available

b. Poor

c. Fair

d. Good

e. Excellent

f. Lush

g. Variable

h. Other _____

45. CHARACTERISTICS OF PASTURE (Circle all applicable) :

a. Low Land

b. Hilly

c. Flat

d. Marsh land

e. Stream

f. Pond

g. Spring

h. River

i. Woods on pasture

j. Borders woods

k. Hedgerows

l. Dumps

m. Borders highway

n. Other _____

o. Other _____

	TYPE OF PASTURE GRASS:	
KENTUCKY GRASS	0% 25% 50% 75% 100%
FESCUE	0% 25% 50% 75% 100%
ALFALFA	0% 25% 50% 75% 100%
BROOM	0% 25% 50% 75% 100%
TIMOTHY	0% 25% 50% 75% 100%
CLOVER	0% 25% 50% 75% 100%
OTHER	0% 25% 50% 75% 100%
OTHER	0% 25% 50% 75% 100%
UNDETERMINED	0% 25% 50% 75% 100%

46. PASTURE FREQUENTED BY WILD ANIMALS? YES/NO (SPECIFY) _____

47. PASTURE FREQUENTED BY FLOCKS OF BIRDS? YES NO

48. INSECT POPULATION

a. Excessive b. Moderate c. Low d. Rare

49. PROBLEM INSECTS (SPECIFY): _____

50. DO YOU HAVE PROBLEMS WITH

	NONE	FEW	MODERATE	EXCESSIVE
a. ticks.....				
b. mosquitoes.....				
c. biting flies.....				
d. non-biting flies..				
e. gnats.....				

51. RODENTS IN BARN:

a. None

b. Rare

c. Occasional

d. Frequent

e. Excessive

52. RODENTS ON PASTURE

a. None

b. Rare

c. Occasional

d. Frequent

e. Excessive

53. TRAILS USED BY STABLE

a. None

b. Private

c. Shared (With) _____

d. Other _____

54. WHICH HORSE SHOWS OR RACETRACKS HAVE HORSES FROM THE FARM HAVE TRAVELLED TO IN 1983?_____

_____

_____

_____

55. FARRIERS USED ON THE FARM_____

_____

HUMAN HEALTH PROBLEMS:

56. HAVE YOU, YOUR FAMILY, OR OTHER WORKERS ON THE FARM HAD UNEXPLAINED ILLNESS DURING 1983? NO / YES IF YES EXPLAIN _____

57. HAVE YOU, YOUR FAMILY, OR OTHER WORKERS ON THE FARM HAD:

a. Diarrhea

b. Vomiting

c. Stomach cramps

d. Fever

e. Colds

f. Rashes

g. Sore throat

h. Other _____

58. IS THE SOURCE OF WATER FOR YOUR HOUSE COMMON WITH THE:

a. Barn

b. Pasture

c. Both

d. Neither

e. Does not apply.

59. PLEASE INDICATE ANY PESTICIDES, HERBICIDES OR CHEMICAL FERTILIZERS WHICH HAVE BEEN USED ON THE FARM DURING THE PAST 3 YEARS.

PRODUCT	DATE STARTED	PERIOD OF USE
1.		
2.		
3.		
4.		
5.		
6.		
7.		
8.		

GENERAL COMMENTS:

60. Other Details not Covered: _____

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A CASE-CONTROL STUDY OF POTOMAC HORSE FEVER

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ABSTRACT

Perry, B.D., Palmer, J.E., Troutt, H.F., Birch, J.B., Morris, D., Ehrich, M. and Rikihisa, Y., 1986. A case-control study of Potomac Horse Fever. *Prev. Vet. Med.*, 4: 69—82.

A case-control study of Potomac Horse Fever (PHF) was carried out on 260 horses on 41 premises in Maryland. Affected horses were compared with two control groups, one consisting of horses on the affected farms, and one of horses on unaffected farms. Variables studied included data gathered from two personal interview questionnaires, feed analysis, microbiological assay of faecal samples, and subsequent retrospective serological assay following the identification of the causative agent.

The relative merits of this type of case-control study are discussed. Results of multivariable analysis indicated a lack of association with most of the variables studied, but provided quantitative data on the epidemiology of the disease. They clearly demonstrated that the disease was infectious but not contagious, consistent with the involvement of an arthropod vector. Positive associations were found with a few variables including the previous presence of the disease in a barn and the presence of other livestock. The significance of these results in the light of recent findings is discussed.

INTRODUCTION

During the last six years, a disease syndrome characterized by fever, anorexia, depression, leucopaenia, acute diarrhoea and high case fatality has been observed with apparently increasing incidence in horses in an area adjacent to the Potomac river in Maryland and Virginia (Knowles et al., 1984). More recently, the disease has been reported from Pennsylvania, New Jersey, West Virginia, New York, Idaho, Illinois, Ohio, Kentucky and Minnesota (Perry and Rikihisa, unpublished data, 1985; R. Whitlock and M. Ristick, personal communication, 1985). Official statistics on the disease, based on clinical diagnosis, have so far been recorded only in Maryland and Virginia, where the number of cases reported during 1984 was 109 (State of Maryland, Department of Agriculture, and Commonwealth of Virginia,

0167-5877/86/$03.50

Department of Agriculture releases). Approximately 25% of cases over the last three years have proved fatal or were destroyed.

The disease is seasonal, with the greatest incidence during the months of July and August. Cases have been reported only sporadically during the winter months. At the time of this survey (summer, 1983) no aetiological agent had been identified. Recently, however, *Ehrlichia* sp. were found in the colon wall of affected horses (Rikihisa et al., 1984, 1985), and this organism was subsequently isolated in cell culture (Rikihisa and Perry, 1984; Holland et al., 1984) and reinoculated into susceptible horses, reproducing the disease (Holland et al., 1985; Rikihisa and Perry, 1985; Dutta et al., 1985; C.E. Benson and J.E. Palmer, unpublished data, 1985).

As an aid to the determination of the cause of Potomac Horse Fever (PHF), a case-control study was performed to further characterize the epidemiology of the disease. This technique allows the estimation of the relative risk associated with variables studied by the calculation of the odds ratio (McMahon and Pugh, 1970). Preliminary data were reported by Perry et al. (1984) and this paper describes the completed study and its findings.

MATERIALS AND METHODS

The case-control study was carried out in Montgomery County, Maryland with cases identified in five veterinary practices. On the clinical diagnosis of PHF, the owner of each affected horse was requested to answer questions on two personal interview questionnaires: the first related to the horse, and the second related to the premises at which it was kept. Questions covered management procedures at pasture and in the stable, movement of horses, contact with the disease, feeding practices, medical history and medication received. A total of 135 questions were asked. The interrogators were veterinary students and faculty from the two veterinary schools participating in the study.

Affected premises were then matched with unaffected (control) premises, using the following criteria: a) The same veterinary practice; b) Approximately the same number of horses; c) The same type of premises (e.g. boarding stables, breeding farm); d) The same geographic region of the county.

On each of the unaffected (control) premises, up to 5 control horses matched with the affected horses for age and sex were identified. In addition, up to 5 control horses were identified on affected premises using the same matching criteria of age and sex.

Horse questionnaires were then completed for all control horses, and farm questionnaires for all control premises. The study thus comprised two groups of farms and three groups of horses as follows:

Affected farms (AF): 21
Unaffected (control) farms (CF): 20
Affected horses on affected premises (AA): 68
Unaffected horses on affected premises (UA): 84

Unaffected horses on unaffected (control) premises (UC): 108

Serum and faecal samples were taken from affected and control horses. Faecal samples were subjected to routine bacteriological analysis and, in addition, analysis for *Clostridium difficile* and *C. perfringens* Type A enterotoxins. Sera were retained at −40°C for future reference. Following identification of the causative agent of PHF, and the development of a serological test, the indirect fluorescent antibody test (IFAT) (Rikihisa and Perry, 1984), sera were tested to substantiate the clinical classification of cases and controls.

A total of 77 feed samples (grain and hay) were taken from affected and unaffected premises and analyzed for gross abnormalities.

Data from the two questionnaires were computer processed. Epidemiological characteristics related to variables considered important were quantified. Each horse level variable was analyzed by both AA/UA and AA/UC comparisons, and each farm level variable was analyzed by AF/CF comparisons, using the Statistical Analysis System (SAS, Carey, NC), and odds ratios were computed (Fleiss, 1981). In addition, analysis of age, sex and horse-use distribution was made by comparison of AA horse data with those from the total population of horses on AF and CF farms, and χ^2 analysis was performed. Discriminant analysis was subsequently performed on a subset of 12 variables selected on the basis of a significant odds ratio, possible suitability as predictor variables, and conforming with the assumptions of the discriminant analysis. The primary assumption was that the variables have at least approximately normal distributions, and so categorical variables were excluded. The procedures of STEPDISC, CANDISC and DISCRIM (SAS, 1982) were used. The variables were examined initially with both discriminant and canonical discriminant analyses. Discriminant analysis classified horses as affected or unaffected based on a model using the selected variables. In contrast, canonical discriminant analysis found a linear combination of the variables that best summarized the differences between affected and unaffected horses. Next a parsimonious model was sought with a stepwise discriminant procedure which searched for an optimal subset of variables on the basis of squared partial correlations. These discriminant procedures were run with both AA/UA and AA/U (UA + UC) comparisons).

RESULTS

The location of the premises at which cases occurred within the county of Montgomery during 1983 is shown in Fig. 1, and the temporal distribution of these cases is illustrated in Fig. 2. A summary of the results of AA/UA horse comparisons for selected variables is given in Table I; that for AA/UC horse comparisons is presented in Table II, and results of AF/CF farm comparisons are shown in Table III.

At pasture, 79% of the affected horses (AA) had no other PHF cases on

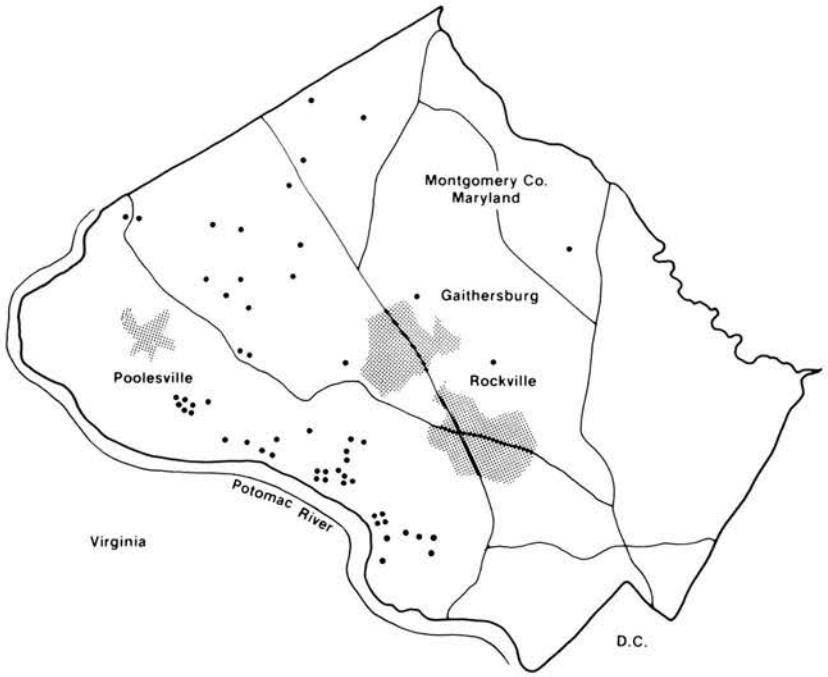

Fig. 1. Spot map of Montgomery County, Maryland showing location of the premises at which PHF cases occurred during 1983.

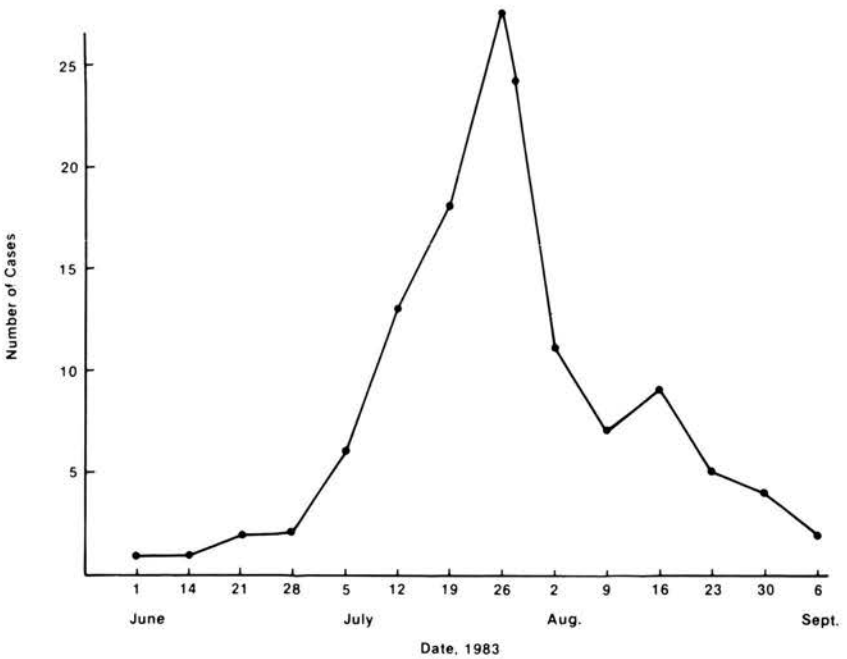

Fig. 2. Potomac Horse Fever: number of new cases in Maryland during 1983, by week.

TABLE I

Results of comparison of selected variables between affected and unaffected horses on affected farms (AA/UA)

Variable studied	Proportion of study group positive for variable (%)		Odds ratio	Statistical significance*
	AA	UA		
1. Born on farm where currently kept	7.3	10.8	0.65	NS
2. Born in the Potomac area	21.7	27.6	0.73	NS
3. Currently on a pasture of 10 acres or less	55.2	46.9	1.39	NS
4. Currently pastured with 5 or more other horses	44.7	48.1	0.87	NS
5. The presence of little pasture where currently grazing	14.9	11.1	1.4	NS
6. The use of fertilizer on currently grazed pasture	53.8	58.0	0.84	NS
7. The use of this pasture by other livestock species for grazing	31.3	18.3	2.04	NS
8. Currently housed in a barn with 5 or more horses	74.6	83.8	0.56	NS
9. The use of communal watering facilities	29.1	37.0	0.7	NS
10. The use of sawdust bedding	33.3	46.8	0.57	NS
11. The occurrence of PHF in previous years on currently used or adjacent pastures	44.7	73.9	1.00	NS
12. The occurrence of PHF in currently used barn in previous years	56.9	29.8	3.1	0.002
13. The presence of *C. difficile* cytotoxins in faeces	3.8	11.1	0.32	NS
14. The presence of *C. perfringens* A enterotoxin in faeces	33.0	27.7	1.3	NS

*NS = not significant at $< P = 0.05$.

TABLE II

Results of comparison of selected variables between affected horses on affected farms and unaffected horses on unaffected farms (AA/UC)

Variable studied	Proportion of study group positive for variable (%)		Odds ratio	Statistical significance*
	AA	UC		
1. Born on farm where currently kept	7.3	8.3	0.87	NS
2. Born in the Potomac area	21.7	9.5	2.64	0.05
3. Currently on a pasture of 10 acres or less	55.2	65.1	0.66	NS
4. Currently pastured with 5 or more other horses	44.7	40.7	1.14	NS
5. The presence of little pasture where currently grazing	14.9	35.8	0.31	0.002
6. The use of fertilizer on currently grazed pasture	53.8	65.4	0.62	NS
7. The use of this pasture by other livestock species for grazing	31.3	11.7	3.46	0.001
8. Currently housed in a barn with 5 or more horses	74.6	72.7	1.1	NS
9. The use of communal watering facilities	29.1	29.5	0.98	NS
10. The use of sawdust bedding	33.3	14.7	2.9	0.004
11. The presence of *C. difficile* cytotoxins in faeces	3.8	3.0	1.32	NS
12. The presence of *C. perfringens* A enterotoxin in faeces	33.0	15.1	2.8	NS

*NS = not significant at $< P = 0.05$.

TABLE III

Results of comparison of selected variables between affected and unaffected farms (AF/CF)

Variable studied	Proportion of study group positive for variable (%)		Odds ratio	Statistical significance*
	AF	CF		
1. The ratio of other domestic livestock on the premises Cattle:dogs:cats:chickens	3:2·6:2·2:1	1:2·2:2·6:3	—	0.0001
2. The recent occurrence of illness in domestic animals other than horses	19.0	9.5	0.45	NS
3. The addition of horses to the farm over the last 12 months	72.7	85.7	0.44	NS
4. On farms where horses added, the addition of 5 or more horses to the farm over the last 12 months	44.0	61.1	0.49	NS
5. The temporary removal of horses from the farm over the last 12 months	83.4	90.5	0.67	NS
6. The presence of a neighbour with horses within 0.4 km	52.3	71.4	0.44	NS
7. The known presence of PHF on a neighbour's farm within 0.4 km	17.6	29.4	0.51	NS
8. The keeping of horses full-time at pasture (never stabled)	66.7	57.9	1.45	NS
9. The presence of excellent and lush pasture	33.3	5.5	8.5	0.03
10. The presence of weed, seed or insect damage in concentrate horse feed used	13.1	10.2	1.28	NS
11. Farm frequented by wild animals	72.2	82.4	0.56	NS
12. The recognized presence of rodents in the barn	71.4	65.0	1.34	NS
13. The recognized presence of rodents on the pasture	78.9	50.0	3.75	0.05
14. The presence of unexplained illness in people living or working on the premises	11.1	9.5	1.19	NS

*NS = not significant at $< P = 0.05$.

the same pasture, and 53% of unaffected horses on affected farms (UA) had one or more PHF case on the same pasture.

In the barn, 67% of the affected horses (AA) were the only case in the barn. Of affected premises, 58% had no PHF cases on the barn during previous years. Of affected horses, 9% had been affected with the disease previously.

In the AA/UA comparisons, all but one of the variables analyzed showed no significant risk associated with the presence of disease. A significant association was seen with the occurrence in previous years of PHF in the currently used barn. In the AA/UC comparisons, significant risk associations with the presence of the disease were found with four variables studied.

When the distributions of age, sex and horse-use within the AA group were compared to those of all the horses on the 41 premises studied, no significant difference could be identified. The results of this comparison are shown in Tables IV, V and VI.

TABLE IV

Comparison of sex distribution of AA horses with all horses on 41 survey farms

	Stallion	Mare	Gelding
Total horses on surveyed farms (%)*	7	43	50
Affected (AA) horses (%)	8	43	49

*No significant difference between the two distributions.

TABLE V

Comparison of age distribution of AA horses with all horses on 41 survey farms

	⩽ 3	4—6	7—10	11—15	16—20	> 20
Total horses on surveyed farms (%)*	18	15	27	22	13	5
Affected (AA) horses (%)	13	16	35	21	13	2

*No significant differences between the two distributions.

TABLE VI

Comparison of horse use distribution of AA horses with that of all horses on 41 survey farms

	Pleasure	Show	Event	Race	Polo	Breeding	Young
Total horses on surveyed farms (%)*	49	13	0	3	3	25	7
Affected (AA) horses (%)	34	37	6	1	1	14	7

*No significant differences between the distributions.

Faecal microbiology

Microbiology assay of faecal samples did not reveal any significant isolations. *Salmonella* sp. were isolated from only two horses in the UC group. No *Salmonella* sp. were isolated from affected horses.

Retrospective serology

All UC horses were seronegative to the IFAT for the causative *Ehrlichia.*

Discriminant analysis

The canonical discriminant analysis on the variables in Table VII gave a canonical R^2 value (analogous to the R^2 of multiple determination in regression) of 0.24 for the AA/UA grouping and 0.19 for the AA/U grouping. In terms of placement accuracy in discriminant analysis, the AA/U grouping placed affected and unaffected horses with 63 and 78% success, respectively, whereas the AA/UA grouping had 74 and 70% success, respectively. A summary of the stepwise analyses in Table VIII indicates that variables 1, 2, 9, 10 and 12 are among the best discriminators for both groupings. Further trials with variable subsets, using the five mentioned above as a core, led to the model using variables 1, 2, 5, 9 and 10. This model provided a canonical R^2 of 0.21 for the AA/UA grouping.

TABLE VII

Discriminant analysis: variables studies

Variable number and title	12 variable model	5 variable model
1. Size of current pasture	X	X
2. Number of other horses at pasture	X	X
3. The use of current pasture by other livestock	X	
4. The presence of abundant forage on current pasture	X	
5. Occurrence of PHF on currently used pasture or adjacent pasture in previous years		X
6. Number of other horses in barn	X	
7. The occurrence of PHF in barn in previous years	X	
8. The use of sawdust bedding	X	
9. Previous occurrence of PHF in horse studied	X	X
10. Number of horses introduced since January 1983	X	X
11. Number of horses introduced during last 30 days	X	
12. Number of horses introduced during last 10 days	X	

As a final exploratory procedure, a frequency histogram was constructed using only AA and UA horses. The groupings were based on the canonical variable score from CANDISC. Frequencies of horses in each class were then printed for each group of scores. Horses with extreme scores were analyzed using DISCRIM. Perfect placement of these horses was obtained. The low sample size resulting from this selective procedure limits the interpretation of this section of the analysis.

TABLE VIII

Discriminant analysis: stepwise analysis summary

Stage	AA/U			AA/UA		
	Variable no. entered	Partial R^2	$P < F$	Variable no. entered	Partial R^2	$P > F$
1	10	0.1015	0.0001	10	0.0792	0.0028
2	3	0.1018	0.0001	12	0.0320	0.0614
3	8	0.0817	0.0004	1	0.0336	0.0563
4	9	0.0473	0.0124	2	0.0463	0.0253
5	12	0.0516	0.0085			
6	1	0.0320	0.0545			
7	2	0.0379	0.0321			

DISCUSSION

Case-control studies have not been widely used as field investigation techniques in veterinary medicine, despite their extensive use in human medicine, although their frequency has certainly increased in recent years. Their most frequent use has been for the retrospective analysis of hospital clinical data (e.g. Willeberg, 1975) and of slaughterhouse data (Aalund et al., 1976; Willeberg, 1979). Recently the use has expanded to identify relationships between diseases (Dohoo and Martin, 1983) and in the study of complex multifactorial diseases (Pritchard et al., 1983).

In this study, the technique was chosen due to the low attack rate in the resident horse population (estimated at 19 per 1000), the lack of available data on the characteristics of that population, and the unknown aetiology of the disease at the time. The control subjects were selected from the horse population at large, and not from a limited group of hospital admissions, which is an advantage over the more conventional case-control studies which rely on hospital or slaughterhouse data.

The decision to select two control groups (UA and UC) was made for two reasons. Firstly and most important, in the absence of a definitive diagnostic test at the time, controls could not easily be defined. Secondly, the selection of unaffected premises ruled out the calculation of the odds

ratio by comparing with UC horses for certain parameters relating to the presence or previous presence of the disease, as groups had been selected on that basis. In these instances, AA/UA comparisons alone were made.

The problem of the identification of controls was of course extended to the identification of cases. It was decided to use the clinical classification of the five practices, based on the presence of fever, anorexia, leucopaenia, typical borborygmal sounds and diarrhoea, which was supplemented by the failure to isolate *Salmonella* sp. In retrospect, the clinical criteria proved very accurate, and subsequent serological confirmation of case and control classifications was made.

A potential disadvantage of the technique was the use of a questionnaire format, which inevitably limited the depth of information obtained. However, this method was the only one available whereby the information required could be obtained, and its accuracy was enhanced by ensuring that data were obtained by conducting personal interviews on the farm.

The results clearly indicate a lack of association with most of the variables so far analyzed, at least at the depth permitted by the questionnaire. These findings were particularly important in eliminating some of the various aetiologies which had been suggested at the time, especially in relation to common source ingested agents or toxins. With hindsight knowledge of the ehrlichial causation of the disease, the absence of such associations are upheld.

Significant positive risk associations at the horse comparison level were found between the presence of the disease and its previous presence in the barn, the presence of other livestock on the pasture, the presence of abundant forage on the pasture, the use of sawdust as bedding and the birth of a horse within the Potomac area. In the comparison of affected and unaffected farms, there was a significantly greater proportion of dogs and cattle on affected premises than unaffected premises. Significant risk associations were also found with the present of excellent and lush pasture, and with the recognized presence of rodents on the pasture. Current knowledge of the disease offers some explanation of these associations.

It has recently been shown that horses may remain carriers of the organism for at least 8 months following infection (B.D. Perry and Y. Rikihisa, unpublished data, 1985), potentially allowing a continuous source of infection on a farm following its first case. The role of other livestock as carriers is currently being investigated, but this phenomenon has been recorded for other rickettsia. Other ehrlichias for which vectors have been identified have been shown to be tick-borne (Ristic and Huxsoll, 1984), and the epidemiological characteristics of PHF indicate that it is probably arthropod-borne. Pastures with abundant forage are potentially more likely to support significant tick populations. Tick infestations of horses in the region are exclusively *Dermacentor variabilis* (M. Fletcher, J.W. Hansen, B.D. Perry and E.C. Turner, unpublished data, 1984; E.T. Schmidtmann, personal communication, 1984). The larval and nymphal stages of this tick feed on small

rodents (Sonenshine et al., 1966), offering a potential explanation of the significant association with rodents on pasture. The possible infection of small rodents with the causative *Ehrlichia* is currently under investigation.

The odds ratio of 2.9 associated with the use of sawdust bedding is more difficult to explain, as is the apparent association with birth within the Potomac area.

The discriminant analysis was performed to eliminate the effect of confounding variables. However, using variables with a significant odds ratio and others with potential as predictor variables, poor discrimination between affected and unaffected horses was obtained, unless the number of variables was reduced to five, and the comparison run only between AA and UC horses. In this instance, however, the sample size was reduced to 26, and all the control horses represented came from 3 farms.

All of the discriminant analyses require that the variables follow a multivariate normal distribution, at least approximately. The present data might not support that assumption. Furthermore, the data used to build the discriminant models were used to validate the models as well. Obviously this resulted in some upward bias in any measures of model adequacy, such as R^2 and percent of successful placement.

The results thus indicate that the disease is not contagious, that a 'point-source' infection related to ingestion is unlikely, and therefore that faecal-oral transmission is unlikely in the field. Recent experimental studies have confirmed the lack of infectivity of faeces (J.E. Palmer, unpublished data, 1985). The results do suggest that the disease is infectious, consistent with the involvement of an arthropod vector. The study thus provided a good indication of the nature of the condition even before the causative agent was isolated. Subsequent transmission studies using whole blood (A.L. Jenny, unpublished data, 1984; Whitlock et al., 1984) and agent isolation and identification (Rikihisa and Perry, 1984, 1985; Holland et al., 1984, 1985; C.E. Benson and J.E. Palmer, unpublished data, 1985) confirm the infectious nature of the disease.

The sporadic nature of the disease also suggests that the disease is of a low level of infectivity, or, if arthropod-borne, has a low challenge rate, or that there is a high level of population immunity. Recent studies (unpublished data; Whitlock et al., 1984) indicate a level of infectivity and clinical disease in experimental infections of almost 100%. This suggests the likely existence of a low challenge rate in the field, possibly the result of very low arthropod infection rates. The equal susceptibility of all age groups of horses is not consistent with the presence of any population immunity.

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Acute diarrhea in horses of the Potomac River area: Examination for clostridial toxins

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SUMMARY

Fecal specimens from horses in Montgomery County, Md, and in Fairfax and Loudoun counties, Va, were examined for *Clostridium perfringens* type A enterotoxin and for *C difficile* cytotoxin (92 and 108 specimens, respectively). The toxins were found in feces from horses that had experienced an acute diarrhea syndrome and from clinically normal horses. The toxins did not appear to be primary determinants of the diarrhea syndrome, although they may have contributed to the spectrum of clinical entities observed.

OVER THE PAST 4 YEARS, a disease syndrome characterized by acute diarrhea and high mortality has been observed with apparent increasing incidence in horses of an area adjacent to the Potomac River in Montgomery County, Md, and in Fairfax and Loudoun counties, Va. The number of clinically recognized and reported cases of this acute equine diarrhea syndrome reached 116 in this region during 1983.[1] More than one third of these cases proved fatal. Although a similar syndrome has been reported from other parts of the United States, the dramatic increase in incidence and focal nature of this disease has led to speculation that it is a new disease entity.

Clinical signs of this disease include prodromal fever accompanied by depression and anorexia, and usually followed by severe diarrhea.[1] There appears to be a wide spectrum of clinical manifestations during the acute phase, of which rapidly developing dehydration has been a constant feature. Laminitis is often found. The disease appears to be seasonal, with the highest incidence between the months of June and September. The causative agent(s) is unknown.

Several agents are documented as causes of acute diarrhea in the horse, including viruses, bacteria and their toxins, and toxic chemicals.[2] Of the bacterial toxins, those produced by certain species of clostridia are of particular interest. For example, large numbers of *Clostridium perfringens* type A were detected in the feces of horses with an acute diarrhea syndrome in Sweden[3] and in the feces of 2 of 23 horses with gastrointestinal disease in Illinois.[4] The enterotoxin produced by this bacterium has been demonstrated to cause hemorrhagic colitis in Shetland Ponies.[5]

The toxins produced by *C difficile* also deserve consideration, as they are recognized as the important etiologic agents in antibiotic-associated diarrheas of man and laboratory animals.[6-9] They also have been reported to cause colitis in a Kodiak bear[10] and diarrhea in pigs.[11] These extracellular proteins cause diarrhea and colitis by an unknown mechanism; clinical signs may appear during or following therapy with nearly all antimicrobial agents, parenteral aminoglycosides being the exception.[6]

In the study reported here, fecal specimens from horses in Maryland and Virginia were examined for toxins produced by *C perfringens* type A and *C difficile*.

Materials and Methods

Fecal specimens were obtained from 108 horses in Montgomery County, Md, and Loudoun and Fairfax counties, Va. A total of 51 specimens were from horses with acute diarrhea, and 57 were from clinically normal horses on affected and unaffected premises, and matched with respect to age, breed, and sex. Some horses were matched with more than 1 control. Fecal specimens (92>10 g; 16<10 g) were collected from the rectum of horses and

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The authors thank the Maryland State Department of Agriculture and the Maryland State Veterinary Diagnostic Laboratory for providing fecal specimens from horses with diarrhea. Technical assistance was provided by Linda Sparks Correll, Sarah Chalgren, and Sue Stevens.

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placed in plastic bags, and excess air was removed. Specimens were stored frozen until day of assay, when they were thawed slowly at 4 C, and a 5-g portion was centrifuged at 10,000 × g for 10 minutes for each of the toxin assays. The supernatant fluid was sterilized by passing it through a 0.22-μm membrane filter.[a]

Fecal supernatants were examined for *C perfringens* type A enterotoxin by measuring fluid accumulation greater than 130% of control values at 40 minutes after duplicate samples were inoculated into ligated intestinal loops of fasted, adult male mice (24 to 30 g).[12] Presence of enterotoxin was confirmed by loss of capability for fluid accumulation when fecal specimens preincubated with *C perfringens* type A antitoxin[b] were inoculated into the loop. Supernatant from a broth culture of *C perfringens* type A[c] was used as a positive control.[13] Negative controls were intestinal loops inoculated with phosphate-buffered saline, *C perfringens* antitoxin, and *C perfringens* toxin preincubated with antitoxin.

Duplicate, 10-fold dilutions of the fecal supernatants were prepared in phosphate buffer (0.05 *M*, pH 6.9) and analyzed for *C difficile* toxins by cytotoxicity to Chinese hamster ovary cells (CHO-K1), using previously described procedures.[14] Cytotoxic substances were detected when 20 μl of fecal supernatant, at dilutions >10:1, caused rounding of cells in Microtiter wells after preincubation with buffer or nonspecific antitoxin. The presence of *C difficile* toxin was confirmed by lack of cytotoxicosis when toxic supernatant was preincubated with specific antitoxin.[15] Toxin of *C difficile* (VPI strain 10463) produced in broth culture was used as control. Presumptive identification of *C difficile* was accomplished by staining and microscopic examination, as well as by observing colonies that appeared after inoculation of fecal material on egg yolk agar plates containing cycloserine, cefoxitin, and fructose.[16,17,d]

Results

Clostridium perfringens type A enterotoxin was found in 23 of the 92 specimens tested. This toxin increased the weight/length ratios of mouse-ligated intestinal loops except when preincubated with antitoxin to *C perfringens* type A. A higher proportion (33%, or 14 positive of 42 tested) of the specimens were from horses with diarrhea, as compared with specimens from controls (18%, or 9 positive of 50 tested) although chi-square analysis did not indicate a statistically significant difference. For the fecal specimens from clinically normal horses, 5 of 18 from affected premises contained the enterotoxin, compared with 5 of 32 from unaffected premises. Three fecal specimens from clinically normal horses increased fluid volume in ligated intestinal loops, but this effect was not abolished by preincubation with *C perfringens* type A antitoxin. The causative factor for fluid accumulation in these specimens was not determined.

Also, a cytotoxin that could be neutralized with *C difficile* antitoxin was detected in 3 of the 108 fecal specimens tested, including 2/57 control and 1/51 diarrheal specimens. The bacterium *C difficile* was presumptively identified in 11 of 25 samples tested, including the 3 that were positive for cytotoxin. Of the 12 diarrheal samples tested, 4 contained bacteria presumptively identified as *C difficile*. As the fecal specimens had been frozen, bacterial counting was not attempted.

Discussion

Compared with what has been reported in human medicine (0%),[18] a relatively high proportion (25%) of the equine fecal specimens tested contained an enterotoxin that could be neutralized with *C perfringens* type A antitoxin. The quantity of enterotoxin in these samples approximated that of a single mouse LD_{50} per 100 mg of feces.[12,19] However, not all of the horses with enterotoxigenic feces had acute diarrhea. Thus, this enterotoxin cannot be implicated as the sole causative agent of the acute equine diarrheal syndrome, although it was found more frequently in clinically affected horses than in clinically normal horses. According to our findings, the latter horses were more likely to be from farms on which other horses had suffered from diarrhea. These observations and the reports implicating *C perfringens* enterotoxin in other diarrheas[3–5] suggest that the toxin, in combination with other, unidentified factors, may contribute to the severity of the clinical manifestations recognized in the field.

Some fecal specimens did contain *C difficile* cytotoxin. Previous antibiotic administration was not a common feature of the medical history for horses with diarrhea; perhaps that is why cytotoxin was not detected with the same frequency as it is in certain types of diarrhea in man. In adult human beings, *C difficile* cytotoxin is found with highest frequency in patients given antibiotics (17% to 25% and 97% to 100% of those with antibiotic-associated diarrhea without and with pseudomembranous colitis, respectively).[6] *Clostridium difficile* has been identified in feces from other healthy Equidae.[20] As in human infants,[21] the presence of this bacterium in equine species does not necessarily appear to be associated with the presence of cytotoxin or with diarrhea.

Although the identity of the causative agent(s) of the acute diarrhea syndrome affecting horses in the area adjacent to the Potomac River is not known, the present study indicates that the clostridial toxins under investigation may not be primary determinants of this disease syndrome, and that attention should be paid to other microbiologic and toxicologic agents of interest.

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[b]Burroughs Wellcome, Animal Health Division, Kansas City, Mo.
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Letters

Rickettsial link with acute equine diarrhoea

From Dr Y. Rikihisa and others

SIR, — We wish to report the demonstration by transmission electron microscopy of a rickettsial organism in the large colon of a pony experimentally infected with Potomac horse fever (acute equine diarrhoea syndrome).

The disease, which is characterised by fever, anorexia, leucopenia and diarrhoea, has occurred with apparently increasing incidence during the summer months of the last six years, in the counties adjacent to the Potomac river in Maryland and Virginia, USA (Knowles and others 1984). Reports of the disease have also been received from other areas in the United States.

Examinations for salmonella have proved negative and clostridial toxins do not appear to play a role in the aetiology (Ehrich and others 1984). A recent epidemiological study concluded that the disease was infectious but not contagious (Perry and others 1984).

The isolation of a coronavirus-like agent from affected horses was reported by Huang and others (1983), but this agent did not cause disease when inoculated into ponies (Knowles and others 1984). Earlier this year, it was reported that recovered horses developed serum antibodies to *Ehrlichia sennetsu*, as detected by the fluorescent antibody technique (Jenny 1984).

Following that, we experimentally infected a pony by the intravenous transfusion of 350 ml whole blood from early untreated field cases of the disease. After an incubation period of nine days, the pony exhibited a fever of 38·6°C and a lymphopenia (774/μl) accompanied by depression and anorexia. This was followed by progressive accumulation of fluid in the large intestine. Clinical signs were consistent with those seen in early field cases, and were reproduced in subsequent transmissions. The pony was killed four days after the onset of clinical signs, at which time diarrhoea was not present.

At post mortem examination, the contents of the stomach, small intestine, caecum, large and small colon were of watery consistency, but the faeces in the terminal part of the small colon were normal. Gross lesions included erosions in the fundus of the stomach with few in the sternal flexure of the large colon. Haematoxylin and eosin-stained sections revealed degenerative changes in areas of the deep mucous epithelia in the large colon. The mucous membrane was infiltrated by a mixed population of inflammatory cells including lymphocytes, plasma cells, macrophages, neutrophils and eosinophils.

Fresh specimens of the gross lesion of the large colon were prepared for transmission electron microscopy as previously described (Rikihisa and Ito 1979). There were focal necrotic areas in the submucosa, and many cells were disrupted or had pyknotic nuclei with numerous membrane vesicles in the cytoplasm.

Close to the base of the intestinal gland, macrophage-like cells embedded in the collagen matrix and cellular debris were found containing microorganisms with the morphological characteristics of rickettsia in the cytoplasm (Fig 1). The organisms were round and 0·5 to 0·7 μm in diameter. They contained fine strands of DNA and ribosomes. The organisms were surrounded by a double bileaflet membrane. The thickness of the inner membrane was 5 to 6 nm and that of the outer membrane was 8 nm, with an intermembrane space of 12 to 15 nm. The ultrastructure clearly resembled that of rickettsial organisms.

However, neither the outer nor the inner leaflet of either membrane showed thickening as in the genus *Rickettsia* (Silverman and Wisseman 1978). All the microorganisms were found in loose membrane vacuoles which occasionally contained small membrane vesicles of about 50 to 100 nm in diameter (Fig 2). It was rare to find more than one or two organisms in a thin sectioned profile.

The round shape of the microorganism and its location in vacuoles of macrophage-like cells suggest that it belongs to the genus *Ehrlichia* in the Family Rickettsiaceae, supporting the serological findings of Jenny (1984) of seroconversion of infected horses to the antigen *Ehrlichia sennetsu*.

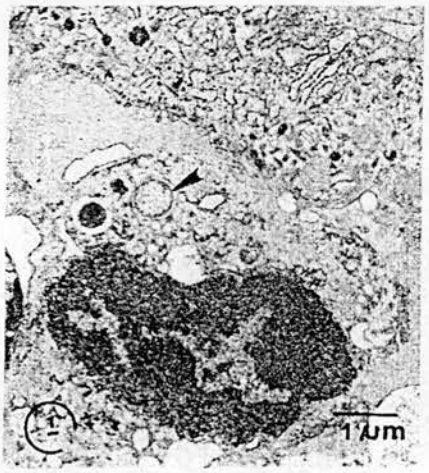

FIG 1: Rickettsial organism (arrowhead) in the cytoplasm of a macrophage-like cell in the connective tissue of the large colon of an infected horse

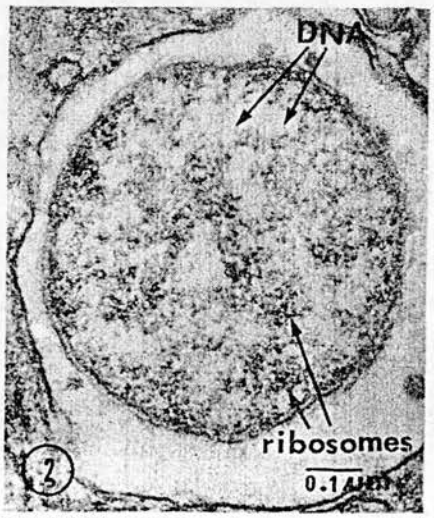

FIG 2: Rickettsial organism in the vacuole of the cytoplasm of a host-cell

We acknowledge the support of the Morris Animal Foundation in this study.

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Letters

Causative agent of Potomac horse fever

From Dr Y. Rikihisa and Dr B. D. Perry

SIR, — Further to our recent letter (*VR*, October 13, p390), we wish to report on the successful culture in human histiocytes of rickettsial organisms consistent with the genus Ehrlichia from the leucocyte fraction of ponies experimentally infected with Potomac horse fever (acute equine diarrhoea syndrome).

Leucocyte fractions were prepared from blood (350 ml volume) obtained from ponies in the early febrile stage of the disease. Leucocytes were aseptically separated using Histopaque 1077 (Sigma). Horse leucocytes were cultured in square flasks with RPMI medium containing 10 per cent calf serum at 37° C in a humidified atmosphere of 5 per cent carbon dioxide/95 per cent air. Two days later, when a monocyte monolayer was formed, a suspension culture of human histiocytes was added. Aliquots of cell suspensions were taken from each flask on days 5, 7, 10, 13 and 22 after histiocyte inoculation.

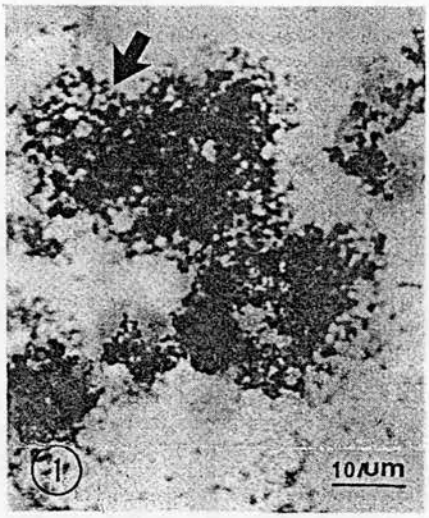

FIG 1: Clusters of organisms appearing as black dots in human histiocyte cultures at day 10 of infection. Many organisms are outside the disrupted cells (arrow). (Giemsa)

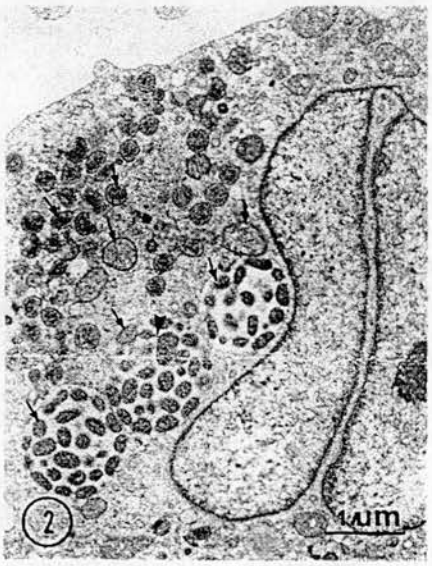

FIG 2: Transmission electron micrograph of ehrlichial organisms (arrows) in cytoplasmic vacuoles of human histiocyte culture at day 10 of infection. Smaller organisms, including some organisms dividing by binary fission (arrowhead), were in loose vacuoles. Large organisms were found individually enveloped in a tight membrane

The presence and degree of infection with rickettsial organisms were determined on smears of cultured cells by modified Giemsa and fluorescent-antibody staining techniques as previously described (Rikihisa and others 1979). For the immunofluorescent-antibody technique, cultured cells were fixed in acetone and reacted with horse serum from a recovered clinical case of the disease. (Serum provided by Dr A. L. Jenny, National Veterinary Service Laboratory, Ames, Iowa). Cells so treated were then incubated with fluorescein isothiocyanate-labelled goat anti-horse IgG. Final confirmation of infection was conducted by preparing cultured cells for transmission electron microscopy as previously described (Rikihisa and others 1979). From day 10 of culture, Giemsa stain and immunofluorescent-antibody labelled preparations revealed distinct positive staining for intracytoplasmic ehrlichial organisms (Fig 1). Transmission electron microscopy clearly revealed ehrlichial organisms with an ultrastructure and intracytoplasmic location identical to those we demonstrated in the large colon of infected ponies (Fig 2).

This opens the possibility of specific serodiagnosis and rapid agent isolation from blood leucocytes of animals infected with Potomac horse fever, which we now prefer to call equine ehrlichial colitis.

We wish to acknowledge the support of the Morris Animal Foundation in this study.

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From Ms C. J. Holland and others

SIR, — The purpose of this letter is to communicate some early information on the discovery of the causative agent of the disease known as Potomac horse fever. The disease, characterised by fever, anorexia, leucopenia and occasional diarrhoea, with a fatal outcome in approximately 30 per cent of the cases, was first reported in the vicinity of the Potomac River in the State of Maryland in 1979. Since then, the disease has become well established in the eastern part of the United States and is being observed

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Ultrastructural Study of Ehrlichial Organisms in the Large Colons of Ponies Infected with Potomac Horse Fever

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Potomac horse fever is characterized by fever, anorexia, leukopenia, profuse watery diarrhea, dehydration, and high mortality. An ultrastructural investigation was made to search for any unusual microorganisms in the digestive system, lymphatic organs, and blood cells of ponies that had developed clinical signs after transfusion with whole blood from horses naturally infected with Potomac horse fever. A consistent finding was the presence of rickettsial organisms in the wall of the intestinal tract of these ponies. The organisms were found mostly in the wall of the large colon, but fewer organisms were found in the small colon, jejunum, and cecum. The organisms were also detected in cultured blood monocytes. In the intestinal wall, many microorganisms were intracytoplasmic in deep glandular epithelial cells and mast cells. Microorganisms were also found in macrophages migrating between glandular epithelial cells in the lamina propria and submucosa. The microorganisms were round, very pleomorphic, and surrounded by a host membrane. They contained fine strands of DNA and ribosomes and were surrounded by double bileaflet membranes. Their ultrastructure was very similar to that of the genus *Ehrlichia*, a member of the family *Rickettsiaceae*. The high frequency of detection of the organism in the wall of the intestinal tract, especially in the large colon, indicates the presence of organotrophism in this organism. Infected blood monocytes may be the vehicle for transmission between organs and between animals. The characteristic severe diarrhea may be induced by the organism directly by impairing epithelial cell functions or indirectly by perturbing infected macrophages and mast cells in the intestinal wall or by both.

Potomac horse fever (PHF) has been reported with apparently increasing frequency during the summer months of the last 7 years in the counties adjacent to the Potomac River in Maryland and Virginia (9). Reports of the disease have recently been received from other areas in the United States (J. E. Palmer, personal communication). The clinical signs of the disease include fever, anorexia, leukopenia, watery diarrhea, and dehydration (9). In 1983, 42 of 116 Maryland horses affected with PHF died or were euthanatized. Among the 32 cases reported in Virginia, there were 10 deaths. In Pennsylvania, there were 4 deaths in 25 cases, and in 1984 18 of 108 horses in the same region died.

Examinations for salmonellae have proved negative, and clostridial toxins do not appear to play a role in the etiology of the disease (2). From a recent epidemiological survey it was concluded that the disease is infectious, but not contagious (B. D. Perry, J. E. Palmer, J. B. Birch, R. A. Magnusson, D. Morris, and H. F. Troutt, Proc. Soc. Vet. Epidemiol. Prev. Med., 1984, p. 148–153. The isolation of a coronavirus-like agent from affected horses was reported (7), but this agent did not cause disease when it was inoculated into ponies (9). After reports that disease could be transmitted by the inoculation of whole blood from horses with early untreated PHF into susceptible horses (R. H. Whitlock, J. E. Palmer, C. E. Benson, H. M. Acland, A. L. Jenny, and M. Ristic, Proc. 27th Annu. Meet. Am. Assoc. Vet. Lab. Diagnosticians, 1984, p. 103–124), it was reported that recovered horses developed serum antibodies to *Ehrlichia sennetsu*, as detected by the fluorescent antibody technique (A. L. Jenny, Am. Assoc. Vet. Pract. Newsl. no. 2, p. 64–65, 1984). There was no seroconversion to *Ehrlichia equi*, but some slight seroconversion to *Ehrlichia canis* (the etiological agent of canine tropical pancytopenia) occurred (6). The clinical signs of PHF differ from equine ehrlichiosis caused by *E. equi* (4). Equine ehrlichiosis has a lower mortality rate and is not associated with diarrhea.

We had previously reported briefly on the first demonstration of microorganisms with an ultrastructure similar to that of the genus *Ehrlichia* in macrophages of the large colon of a pony infected by transfusion of whole blood from a horse with early untreated natural PHF (Y. Rikihisa, B. D. Perry,

TABLE 1. Identification of ehrlichiae in organs by electron microscopy

Organ	Detection of ehrlichiae in pony no.[a]:						
	1	2	3	5	7	8	13
Duodenum	/	/	/	/	/	−	−
Jejunum	+	+	+	/	/	/	/
Ileum	/	/	/	/	+	−	/
Cecum	−	/	−	−	++	++	+
Large colon	+++	+++	+++	++	++	++	+
Small colon	/	++	++	/	+	−	+
Stomach	−	−	−	/	−	−	−
Liver	−	−	−	/	/	/	/
Spleen	−	−	−	/	−	−	−
Lymph nodes							
Mesenteric	−	−	−	/	/	/	−
Ileal	/	/	/	/	/	−	/
Cecocolic	/	/	/	/	+	/	/
Cerebrum	−	/	−	/	/	/	/
Cerebellum	−	/	−	/	/	/	/

[a] +, Ehrlichiae detected; −, ehrlichiae not detected; /, Specimens not examined.

* Corresponding author.

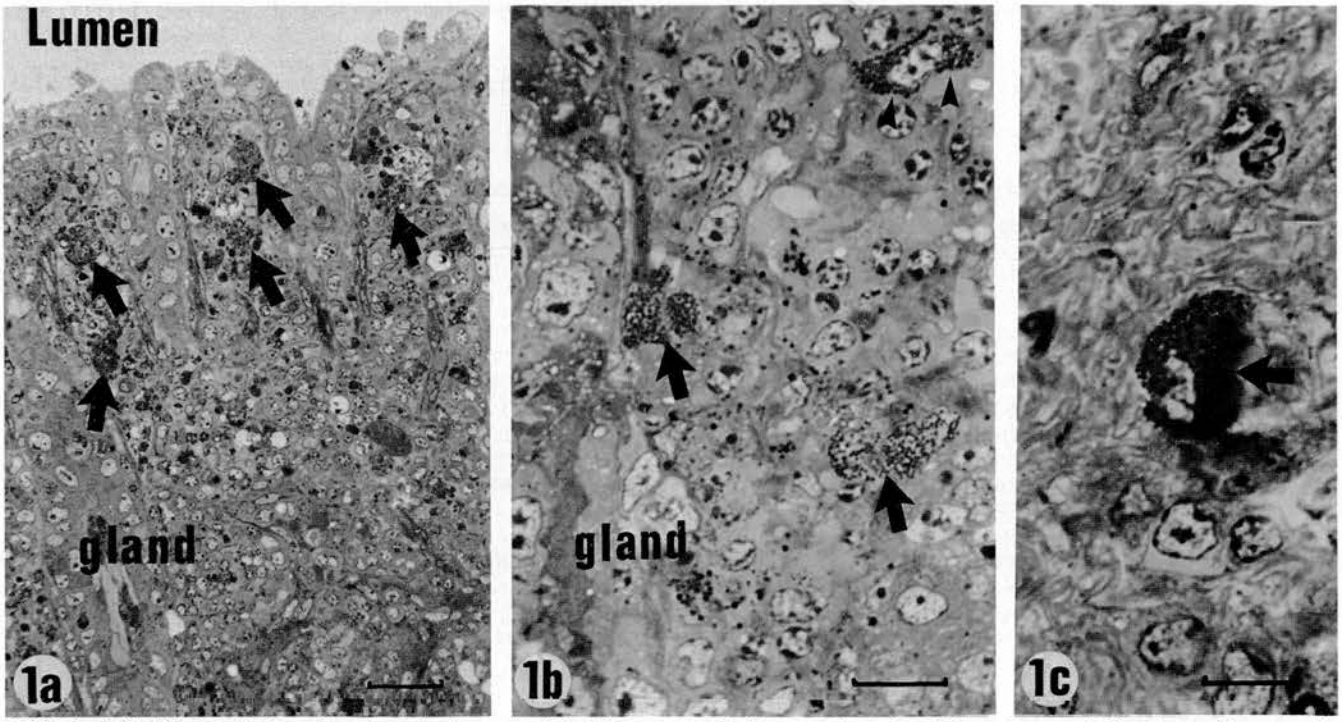

FIG. 1. (a) Thick vertical section through the wall of the Epon-embedded large colon of an infected pony. Infiltrations of mast cells, eosinophils, lymphocytes, and plasma cells are seen in the lamina propria. Note the focal necrotic regions (arrows) in the lamina propria. Toluidine blue stain. Bar, 20 μm. (b) Higher magnification of the deeper portion of the lamina propria of the large colon showing cells containing numerous dark stained microorganisms (arrows). Note most of the mast cell granules are larger (arrow heads) than the microorganisms. Toluidine blue stain. Bar, 10 μm. (c) Thick section of Epon-embedded large colon of an infected pony. A macrophage in the submucosa contains numerous intracytoplasmic microorganisms (arrow). Toluidine blue stain. Bar, 10 μm.

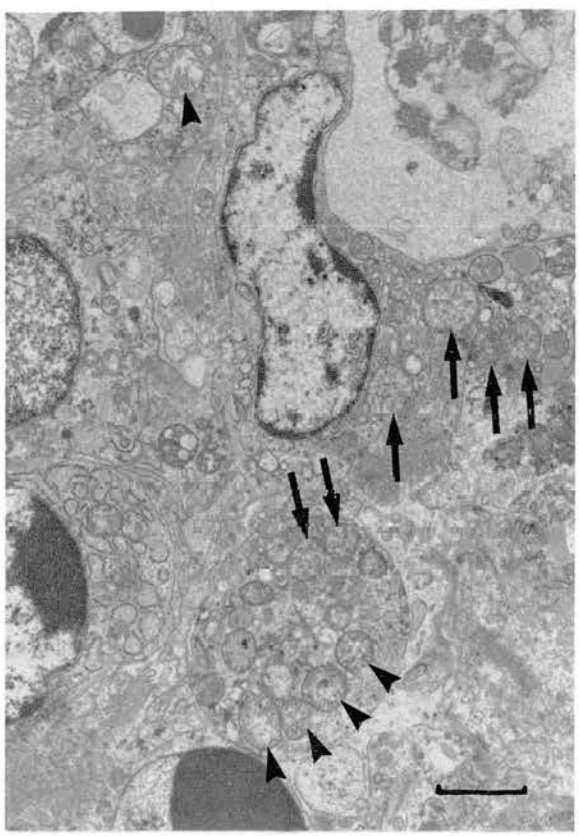

FIG. 2. Transmission electron micrograph of macrophages in the lamina propria of the large colon of an infected pony. Note numerous ehrlichial organisms (arrows) in the cytoplasm of macrophages. The connective tissue contains collagen bundles as well as much debris from disrupted cells. Also note swollen mitochondria with sparse cristae in the cytoplasm of macrophages and other cells (arrow heads). Bar, 1 μm.

and D. O. Cordes, Vet. Rec. Lett. **115**:390, 1984). This is the full report on this investigation. Later we isolated this organism in a human histiocyte cell line from PHF-infected pony leukocytes (Y. Rikihisa and B. D. Perry, Vet. Rec. Lett. **115**:554, 1984); the organisms were ultrastructurally identical to the microorganisms found in the infected colons. Inoculation of this organism to ponies resulted in clinical manifestations that were similar to those seen in the natural disease, and the organisms were reisolated from blood of inoculated ponies (6, 15). Although the organism was identified as the causative agent of PHF, its localization and detailed ultrastructure in the infected horse have not been reported. In this article we focus on a detailed description of the pleomorphic ultrastructure of the ehrlichial organisms and their host tissue and cell associations in the experimentally infected ponies.

MATERIALS AND METHODS

Infection of ponies. Two female ponies were each experimentally infected by the intravenous transfusion into the jugular vein of 350 ml of whole blood obtained from two

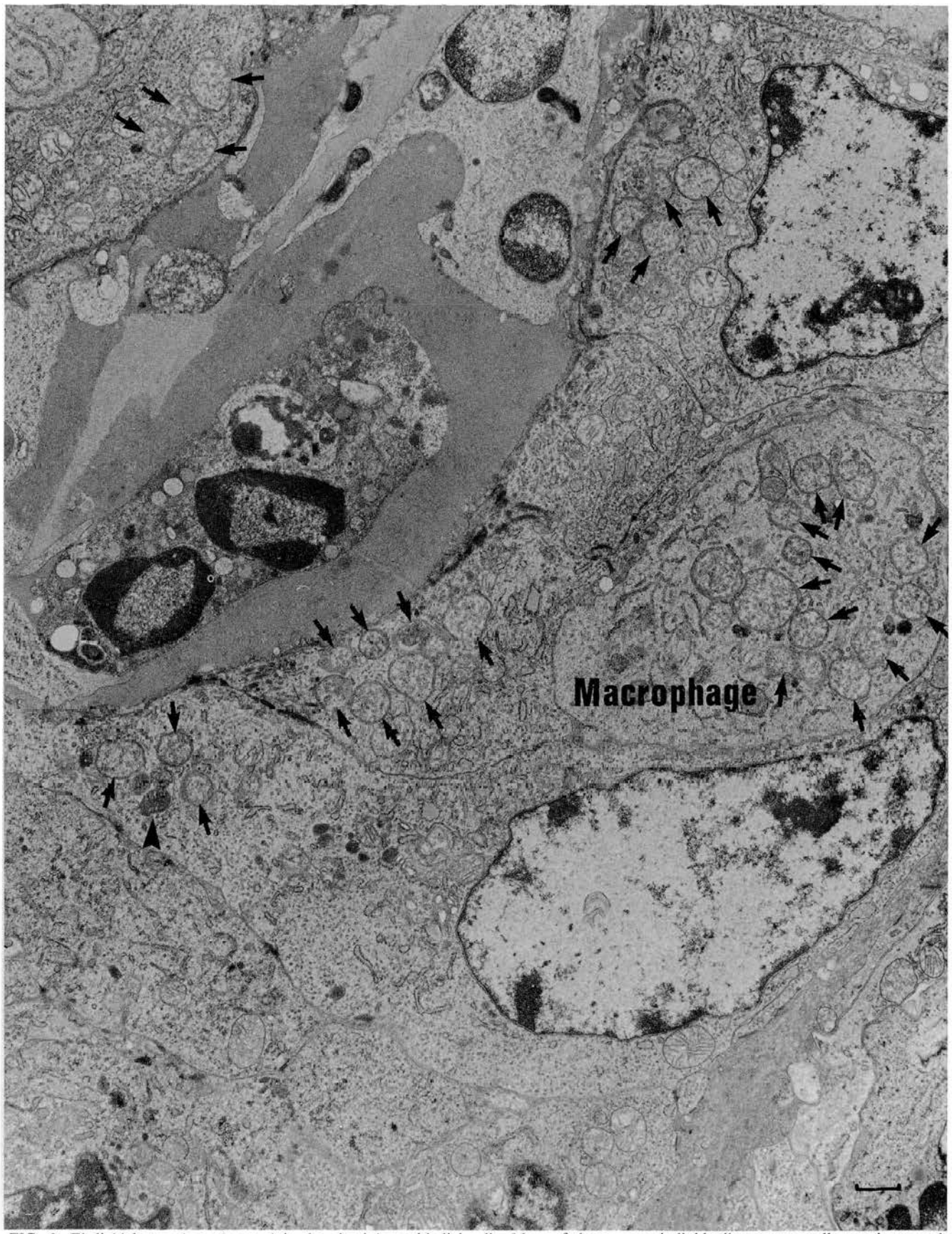

FIG. 3. Ehrlichial organisms (arrows) in the glandular epithelial cells. Most of them occur individually; some small organisms made clusters in a vacuole (arrow head). Note a migrating macrophage with numerous intracytoplasmic microorganisms. Bar, 1 μm.

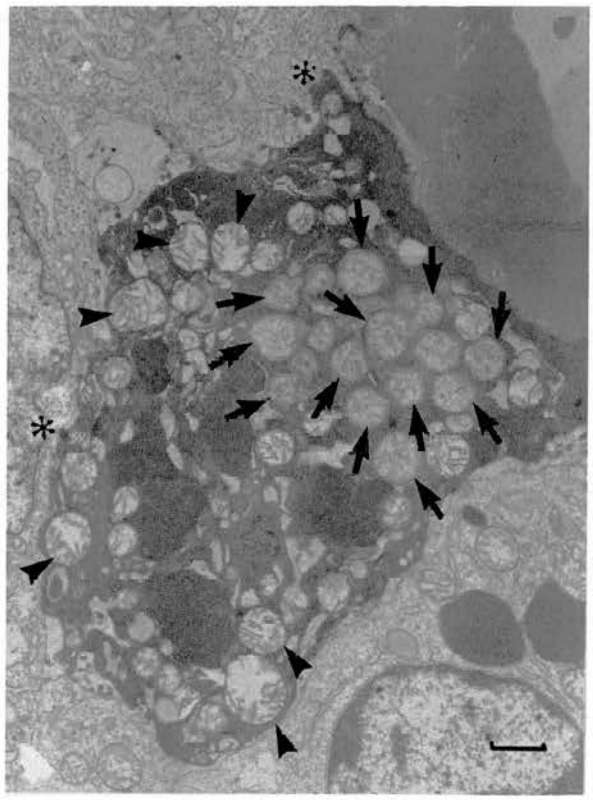

FIG. 4. Dying epithelial cell still maintaining its intercellular junction (*) contained numerous ehrlichiae (arrows). Note swollen mitochondria (arrow heads) Bar, 1 μm.

early untreated field cases of the disease in Maryland. After an incubation period of 9 to 14 days, the ponies were febrile (38 to 40°C) and had a lymphopenia (772 to 1,188 lymphocytes per μl), accompanied by depression and anorexia. Another group of 14 ponies was subsequently transfused from these two ponies. All ponies were seronegative to the antigens of *Babesia caballi*, *Babesia equi*, and equine infectious anemia virus (A. L. Jenny, personal communication). In all the ponies except one, clinical signs typical of PHF developed. Diarrhea developed in 88% of the experimentally infected ponies. The ponies were euthanatized by intravenous injection of T-61 (American Hoechst Corp., Somerville, N.J.) for necropsy 1 to 5 days after the onset of clinical signs. Three noninfected ponies were also euthanatized to examine any common microorganisms or viruses in their tissue and normal structure of their organs.

Blood leukocyte culture. Leukocyte fractions were prepared from blood (350-ml volume) obtained from ponies in the early febrile stage of the disease. Leukocytes were aseptically separated with Histopaque 1077 (Sigma Chemical Co., St. Louis, Mo.). Pony leukocytes were cultured in square flasks (25-cm^2 culture area) with RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) containing 10% calf serum at 37°C in a humidified atmosphere of 5% CO_2 95% air. Seven days later, the monocyte monolayer was fixed for electron microscopic observation.

Electron microscopy. At necropsy, specimens (10 to 20 samples per organ per pony) of digestive and lymphatic organs were prepared for transmission electron microscopy as previously described (14). Briefly, specimens were cut into 3-mm cubes and fixed overnight at 4°C in a mixture of 2.5% paraformaldehyde, 5% glutaraldehyde, and 0.03%

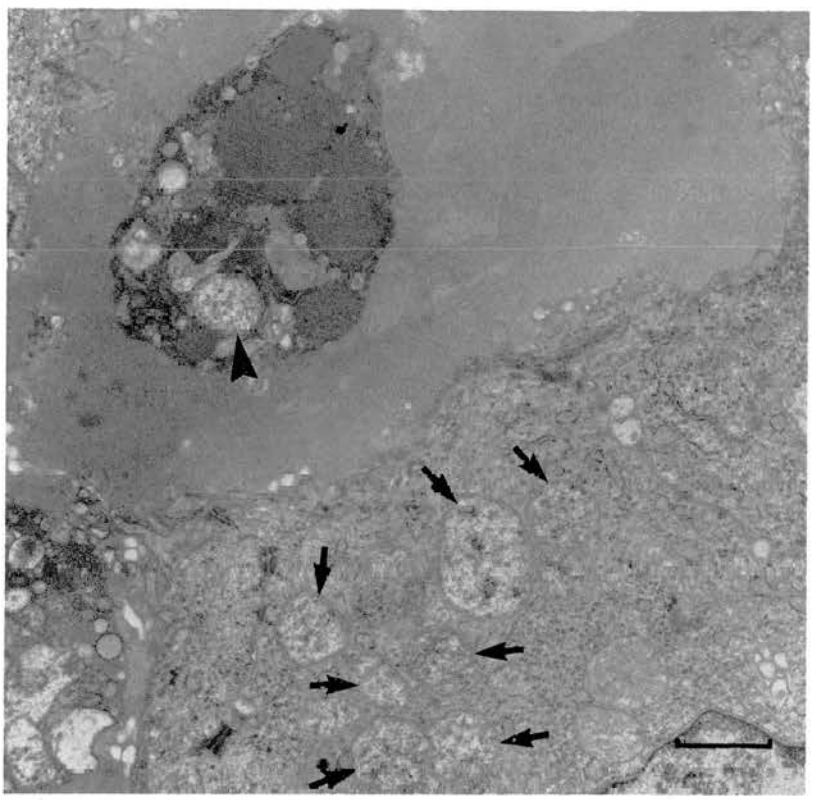

FIG. 5. Exfoliated cell in the glandular lumen carrying an ehrlichia (arrow head). Glandular epithelial cells also contain numerous ehrlichiae (arrows). Bar, 1 μm.

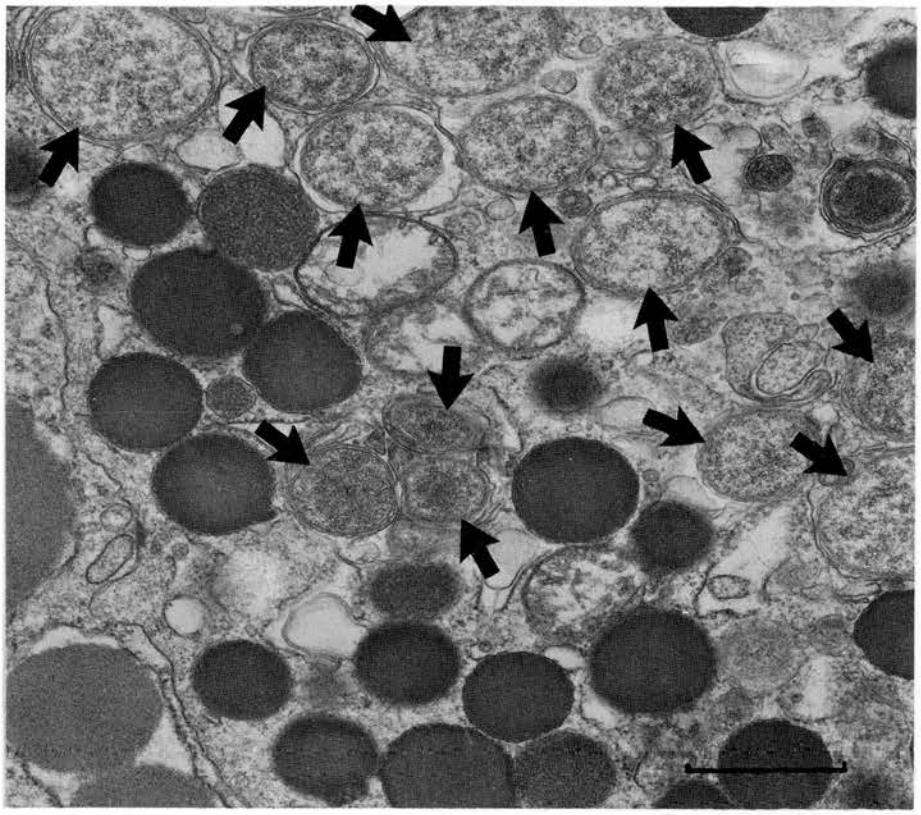

FIG. 6. Ehrlichial organisms (arrows) in the cytoplasm of a mast cell in the submucosa of the colon of an infected pony. All of the microorganisms were surrounded by the host cell membranes. Note that mast cell granules have not coalesced with ehrlichia-containing vacuoles. Bar, 1 μm.

trinitrophenol in 0.1 M cacodylate buffer (pH 7.4) and postfixed in 1% OsO_4 in 1.5% potassium ferrocyanide. After block staining in 1% uranyl acetate in maleate buffer (pH 5.2), tissues were dehydrated in a graded series of ethanols and propylene oxide and embedded in Poly/Bed 812 (Polysciences, Inc., Warrington, Pa.). Blood monocytes cultures were treated the same way. The cells were scraped off the bottom of the culture flask with a razor blade before being transferred to propylene oxide. Thin sections (60 to 90 nm) were cut, stained with uranyl acetate and lead citrate, and examined with a JEM 100 CXII electron microscope.

RESULTS

With electron microscopy, rickettsial microorganisms were consistently found in the wall of the large colon of the ponies that had developed clinical signs of PHF (Table 1), whereas they were absent in the intestinal organs of control ponies. They were also detected in the small colon, jejunum, ileum, cecum, and cecocolic lymph node. The rickettsial organisms were not detected in the walls of duodenums, gastric mucosa, or other organs examined (Table 1). The organisms were detected by electron microscopy in the small number of monocytes derived from pony blood only after they had been in culture for 7 days. No other microorganisms or viruses were found consistently in any organs examined.

Since the host cell association of the organism was identical throughout the intestinal tract and the largest numbers of organisms were detected in the walls of the large colons of the infected ponies, our present study illustrates the ultrastructure of this organism in that organ.

At low magnification, the intestinal wall where rickettsiae were identified by transmission electron microscopy was almost intact. The intestinal glands (crypts), lamina propria, submucosa, muscularis externa, and serosal layers were all present. In comparison to controls, infiltration of mast cells, eosinophils, lymphocytes, and plasma cells was more pronounced, and congestion of some small blood vessels in the lamina propria and submucosa was found.

With light microscopy it was very difficult to detect the rickettsial microorganisms in any of the tissues examined, unless the cells were heavily infected (Figs. 1b and c). The organisms were round and stained dark purple with toluidine blue and reddish-purple with Giemsa stain. They were generally smaller and less distinct than the mast cell granules. Eosinophils had easily distinguishable, much larger, refractile, greenish-blue granules with the toluidine blue stain.

Small, focal necrotic areas were observed in the lamina propria between the intestinal glands in some areas of the large colon of infected ponies (Fig. 1a). When these areas were observed by electron microscopy, macrophages containing numerous rickettsial organisms were found in the connective tissue containing much debris from disrupted cells (Fig. 2). The organisms were also found in the macrophages in lamina propria (Fig. 1b) and submucosa (Fig. 1c), which appeared intact. A large number of organisms were found in glandular epithelial cells (Fig. 3) and in the macrophages migrating among them (Fig. 3). Exfoliating epithelial cells, still maintaining some of their intercellular junctions, also contained numerous organisms (Fig. 4). There were some degenerating cells in the lumen of the intestinal glands

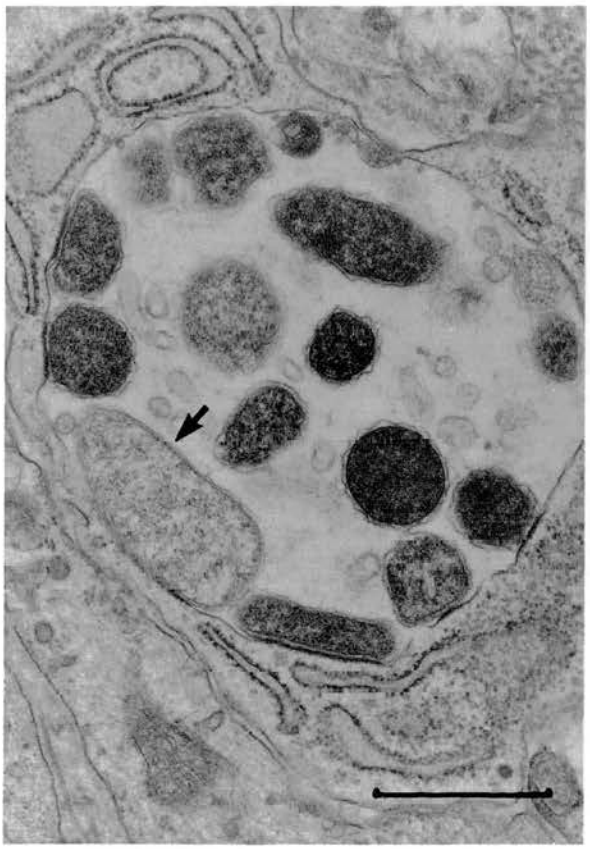

FIG. 7. Dense and smaller forms of organisms and a lighter and larger form are found in the same vacuole. The larger one (arrow) occurs peripherally and is apparently tightly associated with the host membrane. It would probably later pinch off from the rest of the vacuole, leaving remaining smaller and denser organisms. Bar, 1 μm.

that contained rickettsiae (Fig. 5), indicating that microorganisms were actually shed into the lumen. The same microorganisms were also found in the cytoplasm of mast cells in the lamina propria and submucosa (Fig. 6). The mast cell granules did not coalesce with organism-containing membrane vesicles (Fig. 6). The organism was rarely found in plasma cells. The organisms were limited to these four types of cells. Goblet cells and endocrine cells in the glandular epithelium endothelial cells, eosinophils, neutrophils, erythrocytes, smooth muscle cells, fibroblasts, and mesothelial cells were all negative for their presence.

The microorganisms were round and very pleomorphic, and at least two forms were discernible. There were also intermediate forms. Small (0.2 to 0.4 μm in diameter) electron-dense forms (Fig. 3, 7, and 8), including organisms undergoing binary fission (Fig. 9), were found in loose host membrane vacuoles (Fig. 3, 7, 8, and 11). Most of the larger (0.6 to 1.5 μm in diameter) and less electron-dense organisms were individually enveloped by very tight host membrane (Fig. 3, 4, 5, 6, and 10). These larger forms were difficult to identify even under the electron microscope because of their similar density to the host cell cytoplasm. Although the two forms were mostly in separate host vacuoles, occasionally smaller electron-dense organisms and peripherally located electron-lucent organisms were found in the same vacuole (Fig. 7), suggesting that the host membrane surrounding the larger forms was being pinched off from the main vacuole. Some vacuoles contained small, single membrane vesicles apparently derived from the outer membrane of the organism (Fig. 8). Some small, double membrane vesicles (0.05 to 0.1 μm in diameter) with electron-dense cytoplasm were also found (Fig. 11), suggesting the occurrence of unequal binary fission.

The organisms contained fine strands of DNA and ribosomes (Fig. 10) and were surrounded by double bileaflet membranes. Smaller, dark forms had an extensive fuzzy coating on the outer membrane (Fig. 11). The thickening of the outer or inner leaflet of either membrane, suggesting the presence of the peptidoglycan such as that reported in the genus *Rickettsia* (18), was not found in either form of the organism.

Despite the large number of microorganisms they contained, most host cells were morphologically intact, except for the presence of swollen mitochondria (Fig. 2 and 4). Similar swollen mitochondria were also seen in circulating monocytes of the infected ponies.

DISCUSSION

Identical rickettsial organisms were consistently found in the ponies transfused with blood from horses naturally infected with PHF, indicating that this organism was transmitted from horses to ponies via the blood. No other microorganisms or virus were consistently identified in these ponies.

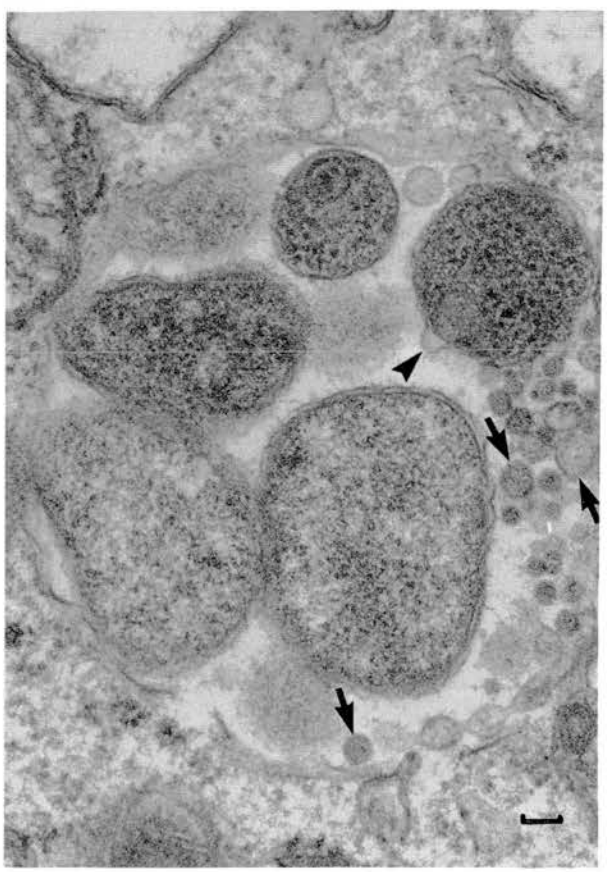

FIG. 8. Some vacuoles contain, in addition to intact ehrlichiae, many membrane vesicles (arrows); apparently the outer membrane vesicles pinched off (arrow head). Bar, 0.1 μm.

These rickettsial organisms were found throughout the intestinal tract and were highly concentrated in the wall of the large colon. Organisms were intracytoplasmic and not found outside of the host cell, supporting our tissue culture data indicating that these are obligate intracellular parasites (Rikihisa and Perry, Vet. Rec. Lett. **115**:554, 1984). The organisms were round and host membrane-bound, thus different from the genus *Rickettsia*. Although a small proportion of affected and unaffected horses in Maryland have been shown to be seropositive to *Rickettsia rickettsii* (Perry and Farhang-Azad, unpublished data), this was apparently independent of previous exposure to PHF. This result also suggests an absence of cross-protective immunity between *R. rickettsii* and the PHF agent. The organism was ultrastructurally rather similar to the genus *Ehrlichia*, thereby supporting the serological study of Jenny (Am. Assoc. Vet. Pract. Newsl. no. 2, p. 64–65, 1984.), in which infected horses were found to develop serum antibodies against *E. sennetsu*. With respect to host cell specificity, however, the PHF agent is different from any of the known ehrlichial organisms. *E. canis* (13) and *E. sennetsu* (5) are found in monocytes or macrophages, and *E. equi* is found in granulocytes (4). Other members of the tribe *Ehrlichieae*, such as organisms of the genus *Cowdria*, grow preferentially in endothelial cells (19), and organisms of the genus *Neorickettsia* grow in reticulocytes in the lymph nodes of dogs (1). In fact, none of the rickettsiae found so far prefers to grow in intestinal epithelial cells of mammals, although many of them grow in the gut of their blood-sucking arthropod vectors (8, 16, 17).

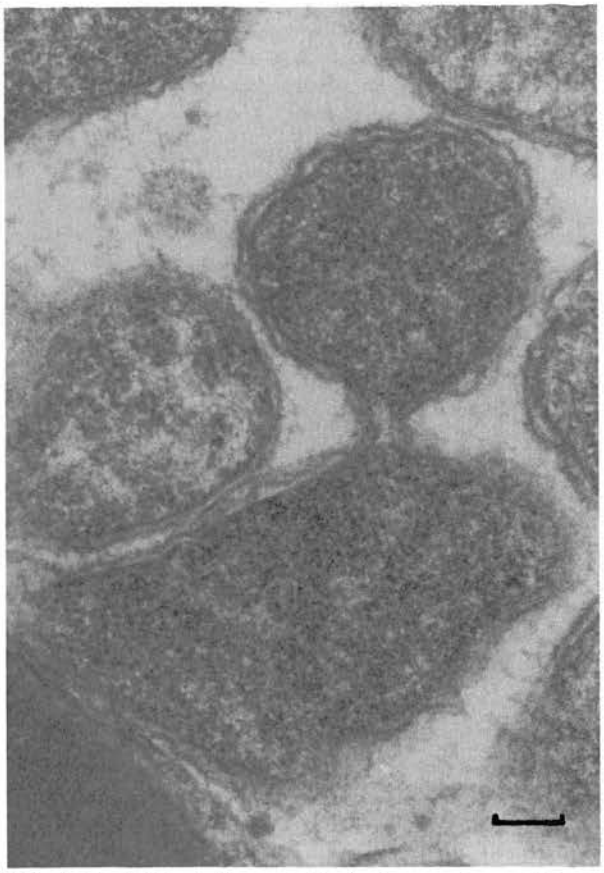

FIG. 9. Ehrlichiae in binary fission in the cytoplasm of glandular epithelial cells of the colon. Bar, 0.1 μm.

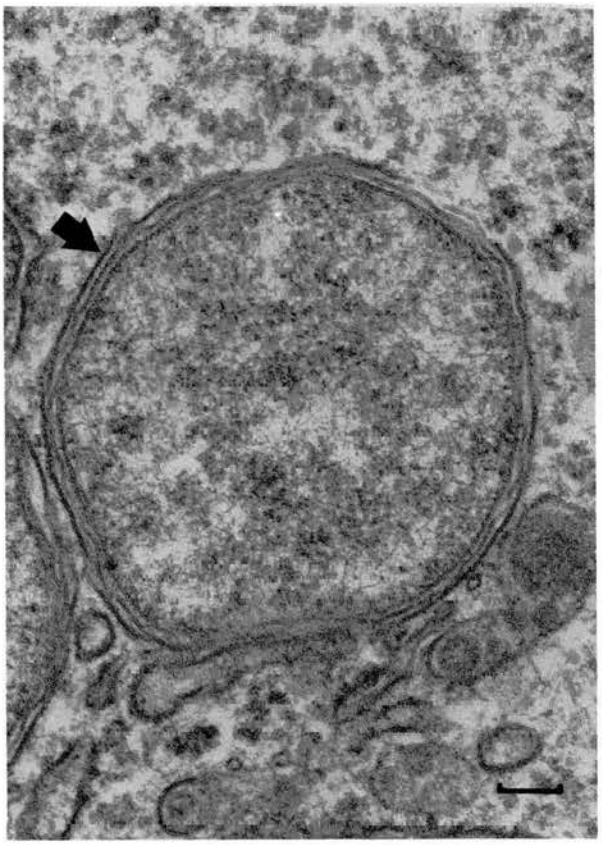

FIG. 10. Larger ehrlichial organism individually enveloped with a tight host membrane (arrow). Note its two unit membranes, clear DNA filaments, and ribosomes. Bar, 0.1 μm.

The PHF agent was very pleomorphic, suggesting a development from electron-dense forms to larger electron-lucent forms similar to *Chlamydia* spp. (12). However, unlike *Chlamydia* spp., binary fission was found in the electron-dense forms. The numerous aberrant forms, such as outer membrane vesicles or minicell-like structures (3), may not play a direct role in the life cycle of the PHF agent, but may have an effect on the host immune response once they are released extracellularly.

The most characteristic clinical sign of this disease is a progressive fluid accumulation in the large intestine, followed by a sometimes "explosive" diarrhea, possibly due to impaired fluid adsorption in the intestinal wall. In *Campylobacter jejuni* and *Shigella* spp. infections, mucosal damage and inflammation account for the ensuing diarrhea (10); however, such extensive damage was not a consistent finding in PHF. The mucosa was relatively intact as in cholera (11), suggesting that interference with epithelial cell function may be caused biochemically by the organisms multiplying in epithelial cells, macrophages, and mast cells.

In the present study, the sloughed epithelial cells containing ehrlichiae observed in the lumen of the intestinal glands suggest the possibility of transmission through contamination of grasses or soil with feces containing exfoliated epithelial cells bearing live organisms. Preliminary experiments involving oral administration of fecal material from affected horses (J. E. Palmer, personal communication) in-

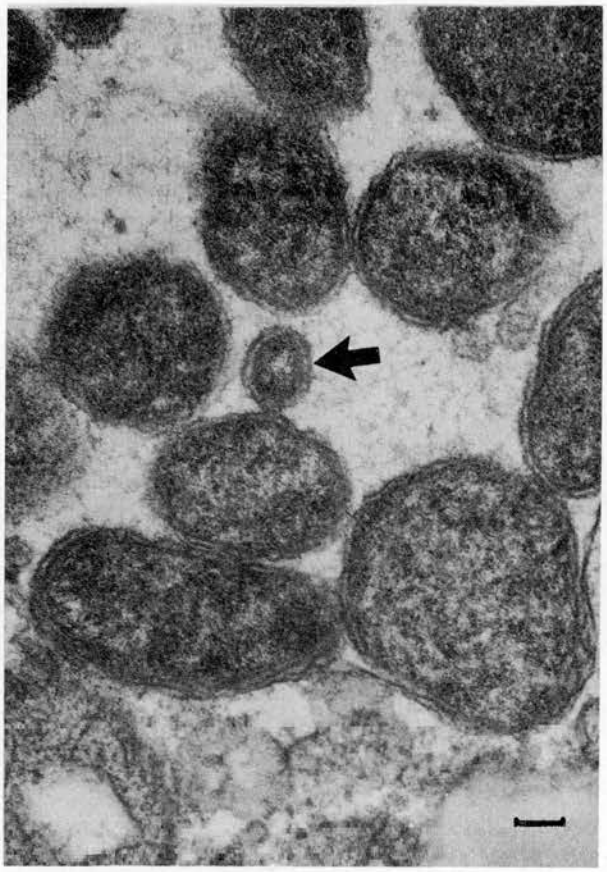

FIG. 11. Vacuole containing minicell-like structures (arrow) as well as ehrlichial organisms. Note extensive fuzzy coating extending from outer membrane. Bar, 0.1 μm.

dicate, however, that fecal material from clinically affected horses is not likely infective to susceptible horses. The seasonal nature of the disease and its restricted geographical distribution suggest that it is arthropod borne. This would imply that the multiplication of the organism in the epithelial cells in the intestinal wall plays a primary role in the pathogenesis of the disease, but is not of significance in its transmission. Although the organisms were fewer in number, circulating blood monocytes may be the vehicle for transmission of organisms between animals.

ACKNOWLEDGMENTS

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Causative Ehrlichial Organisms in Potomac Horse Fever

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An ehrlichia was consistently isolated from the peripheral blood leukocyte fraction of ponies that had been experimentally infected with Potomac horse fever by whole blood transfusion from naturally infected horses. The organism was propagated in a human histiocyte cell line for 3 to 5 weeks and then inoculated intravenously or intradermally into healthy adult ponies. Clinical signs of Potomac horse fever, which varied in the degree of severity, occurred 9 to 14 days post-inoculation in all of the ponies. One pony died 20 days post-inoculation. The ehrlichial organism was reisolated in the human histiocyte cell line from the blood leukocyte fraction of all of the experimental ponies on each day that samples were examined (days 9, 10, 11, 19, and 39). These organisms were identical to those originally detected in the wall of the intestine of ponies with clinically diagnosed Potomac horse fever when compared by light and electron microscopy and an immunofluorescence labeling technique. The immuofluorescent antibody titer became positive in a pony at 20 days postinjection. These results indicate that the ehrlichial organism is the causative agent of Potomac horse fever.

Potomac horse fever (PHF) (equine ehrlichial colitis) is characterized by fever, anorexia, leukopenia, diarrhea, and dehydration, and is associated with a case fatality rate of approximately 30% (2). The disease was recently identified by veterinary practitioners in the counties adjacent to the Potomac River in Virginia and Maryland. One of the landmarks in investigations into its cause is the transmission of the disease by the inoculation of whole blood from clinical cases into susceptible horses (R. H. Whitlock, J. E. Palmer, C. E. Benson, H. M. Acland, A. L. Jenny, and M. Ristic, Proc. 27th Am. Assoc. Vet. Lab. Diagnost. 1984, p. 103–124). Subsequently, it was reported that some recovered horses show seroconversion to *Ehrlichia* sp., as assayed by the fluorescent antibody technique (Jenny, Am. Assoc. Vet. Pract. Newsl. no. 2, p. 64–65, 1984). This seroconversion is to *Ehrlichia sennetsu*, with lower levels of conversion to *Ehrlichia canis*, and no seroconversion to *Ehrlichia equi*. *Ehrlichia* spp. are obligate intracellular parasites that are members of the family *Rickettsiaceae*.

After that report, Rikihisa et al. (Vet. Rec. Letter, **115**:390, 1984) briefly reported on the demonstration of ehrlichial organisms in the wall of the large colon of an experimentally infected pony. Later, systematic electron microscopic examination of various organs from 16 experimentally infected ponies revealed a consistent finding of the ehrlichial organisms in the wall of the intestinal tract, especially the large colon. No virus or other microorganisms are detected consistently in those ponies. Control, noninfected ponies do not have ehrlichial organisms (4). Large numbers of intracytoplasmic organisms are found in both macrophages and glandular epithelial cells and are observed to be reproducing at these sites (4). Furthermore, Rikihisa and Perry (Vet. Rec. Lett. **115**:554, 1984) briefly reported the isolation and identification of an *Ehrlichia* sp. from infected ponies in a human histiocyte cell line. Holland et al. (1; Vet. Rec. Lett. **115**:554, 1984) reported the culture of an ehrlichial agent in primary cultures of canine blood monocytes which produces clinical diseases consistent with PHF when inoculated into a pony.

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Our study was designed to experimentally reproduce PHF in ponies by inoculation of the ehrlichial organisms grown in a human histiocyte cell line, to reisolate them after the expression of clinical disease, and to monitor the development of antibodies in the pony after inoculation by immunofluorescent antibody assay.

MATERIALS AND METHODS

Culture of ehrlichiae. Blood samples of approximately 350 ml were collected into sterile collecting bags containing citrate phosphate dextrose solution, USP (Fenwall Laboratories, Deerfield, Ill.) from each of six ponies in the early febrile stage of the disease at 3 to 18 days after blood transfusion from naturally infected horses and centrifuged at 1,600 × *g* for 10 min. After removal of plasma, the buffy coat was aspirated and mixed 1:1 (vol/vol) with Hanks balanced salt solution. The mixture was layered on Histopaque 1077 (Sigma Chemical Co., St. Louis, Mo.) at a 5:3 (vol/vol) ratio and centrifuged at 800 × *g* for 15 min at room temperature. The interface containing leukocytes was collected and centrifuged at 1,000 × *g* for 5 min. The pellet was suspended in Hanks balanced salt solution and washed by centrifugation two further times. These horse leukocytes were cultured in square tissue culture flasks (25-cm^2 culture area) with RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) containing 10% calf serum (GIBCO) at 37°C in a humidified atmosphere of 5% carbon dioxide–95% air. After 1 to 2 days, when a monocyte monolayer was formed, floating lymphocytes were discarded, and monocyte monolayers were rinsed twice with the culture medium. A suspension culture of human monocyte-like, histiocytic lymphoma U-937 cell (CRL1593; American Type Culture Collection, Rockville, Md.) propagated in the same culture medium was added to the monolayer. The U-937 line was established by C. Sundstrom and K. Nilsson in 1974 from malignant cells obtained from the pleural effusion of a 37-year-old white man with diffuse histiocytic lymphoma (3). Samples of cell suspension were taken from each flask and examined by Diff-Quik stain (Harleco, Gibbstown, N.J.), transmission electron microscopy, and indirect immunofluorescent antibody labeling.

TABLE 1. Isolation of ehrlichial organisms from ponies infected by whole blood transfusion

Pony no.	Days after transfusion	Days in culture ehrlichiae detected[a]
8	13	10
13	11	6
15	17	12
24	18	7

[a] By Diff-Quik stain.

Ehrlichia-positive and contamination-negative cultures were continuously passed in normal histiocyte cultures.

Indirect immunofluorescent antibody labeling. Serum provided by A. L. Jenny (National Veterinary Service Laboratory, Ames, Iowa) and sera that we obtained from experimental infection studies both gave identical results. A sample of the cultured cells was fixed on a glass slide in acetone and reacted with horse serum from a recovered clinical case of the disease. Cells so treated were then incubated with fluorescein isothiocyanate-labeled goat anti-horse immunoglobulin G (IgG; Cooper Biomedical, Malvern, Pa.), washed with phosphate-buffered saline (pH 7.4) containing Evans blue counterstain (Sigma), and observed with a Nikon Optiphot epifluorescent microscope.

Electron microscopy. Infected histiocyte cultures were fixed in a mixture of 2.5% paraformaldehyde, 5% glutaraldehyde, and 0.03% trinitrophenol in 0.1 M cacodylate buffer (pH 7.4) and postfixed in 1% osmium tetroxide in 1.5% potassium ferrocyanide. After block staining in 1% uranyl acetate in maleate buffer (pH 5.2), cells were dehydrated in a graded series of ethanol and propylene oxide and embedded in Poly/Bed 812 (Polysciences, Inc., Warrington, Pa.). Thin sections (60 to 90 nm) were cut and stained with uranyl acetate and lead citrate and examined with a JEM 100 CXII electron microscope.

Ehrlichia infection. Mature female ponies were purchased locally in southwestern Virginia. Ponies were acclimatized for 1 to 4 weeks before inoculation, and were shown to be negative to equine infectious anemia (immunodiffusion test), *Babesia equi*, and *Babesia caballi* (complement fixation text) (A. L. Jenny, personal communication). Histiocyte cultures (1×10^7 to 4×10^7 cells in 3 to 6 ml of RPMI 1640 medium) heavily infected with ehrlichial organisms and free of any contamination by other microorganisms and virus (determined by Diff-Quik and Gram staining, blood agar culture, and electron microscopy) were harvested and held at 4°C for 1 to 20 h before inoculation. In two ponies organisms were inoculated intravenously. Two further ponies were inoculated intradermally with 1.0 ml of culture, at each of 5 body sites. Two ponies inoculated with noninfected histiocyte cultures served as uninfected controls. Initially, ponies were not subject to the serological test for the PHF agent, but the one recovered pony was examined serologically by the indirect immunofluorescent antibody test to the PHF agent on days 0, 4, 6, 11, 13, 18, 20, 25, and 31 post-inoculation.

Reisolation of ehrlichiae. From each pony, 350 ml of blood was obtained from the jugular vein in citrate phosphate dextrose solution after the onset of fever. This occurred 3 to 18 days after the inoculation of infected histiocyte culture. The samples were processed for culture of ehrlichiae as previously described.

Immunofluorescent antibody titration of pony sera. The infected histiocytes at 10^3 cells per well were fixed on a 14-well glass slide in acetone and reacted with serially diluted pony sera. The cells so treated were then incubated with fluorescein isothiocyanate-labeled goat anti-horse IgG as described above. The reliability of our immunofluorescent antibody titration technique was verified through A. L. Jenny. He had sent 10 unknown sera, including positive PHF sera, simultaneously to us and to M. Ristic at the University of Illinois. The results of the titrations were almost identical from the two laboratories.

RESULTS

Culture of ehrlichiae. From day 6 to 12 of culture, Diff-Quik-stained and immunofluorescent antibody-labeled preparations revealed the distinctive positive staining for intracytoplasmic microorganisms (Table 1). A similar preparation is shown in Fig. 1 and 2. Transmission electron microscopy clearly revealed numerous round microorganisms with fine DNA strands and ribosomes surrounded by two bileaflet membranes of their own and one host membrane. A similar preparation is shown in Fig. 3. The cultures were negative for any other microorganisms, virus, or unusual structures. Histiocytes cultured with normal pony leukocytes were negative for any microorganisms or virus. The organisms were more numerous in histiocyte cultures than in the cells of the infected pony intestine (4).

Reisolation of ehrlichiae from ponies. When 10^7 histiocytes heavily infected with the ehrlichial organisms (a mean count in excess of 10 organisms per infected histiocyte) were inoculated intravenously into two ponies, both animals developed clinical signs of the disease 9 days later. A biphasic fever was recorded on days 2 and 3 and on days 9 and 10 post-inoculation. Both animals were depressed and anorectic, and the intestinal sounds indicated the progressive accumulation of gas and fluids in the large intestine. Neither of the ponies developed diarrhea. Two ponies inoculated intradermally with 4×10^7 infected histiocytes developed the typical clinical signs of PHF, which included fever, depression, anorexia, and diarrhea. In one pony, death occurred 20 days post-inoculation (B. D. Perry, Y. Rikihisa, and G. Saunders, Vet. Rec. Lett. **116**:246, 1985). The inoculation of noninfected histiocyte cultures caused no change in control ponies when administered by the same routes.

All attempts to reisolate ehrlichial organisms from circulating blood leukocyte fractions were successful. Organisms were reisolated from intravenously inoculated ponies on days 9, 10, 11, 19, and 39 post-inoculation (Table 2). At the latter isolation the pony had made a complete clinical recovery. At day 19 post-inoculation when reisolation was attempted, the presence of ehrlichial organisms was confirmed in an intradermally inoculated pony.

Light micrographs (Fig. 1), immunofluorescent staining (Fig. 2), and electron micrographs (Fig. 3) of one of the reisolated cultures are shown. Their intracytoplasmic distribution, ultrastructure, and antigenicity were identical to those of the original isolate.

Titration of pony sera. Pony 19 recovered from a mild clinical response after the intravenous injection of pure cultured microorganisms, and its immune response was monitored. The sera were negative by immunofluorescent antibody titration to the PHF agent from day 0 to day 20 postinjection. From day 20, the titer exceeded 1:80 and gradually increased to reach a peak of 1:1,280 on day 24 postinjection.

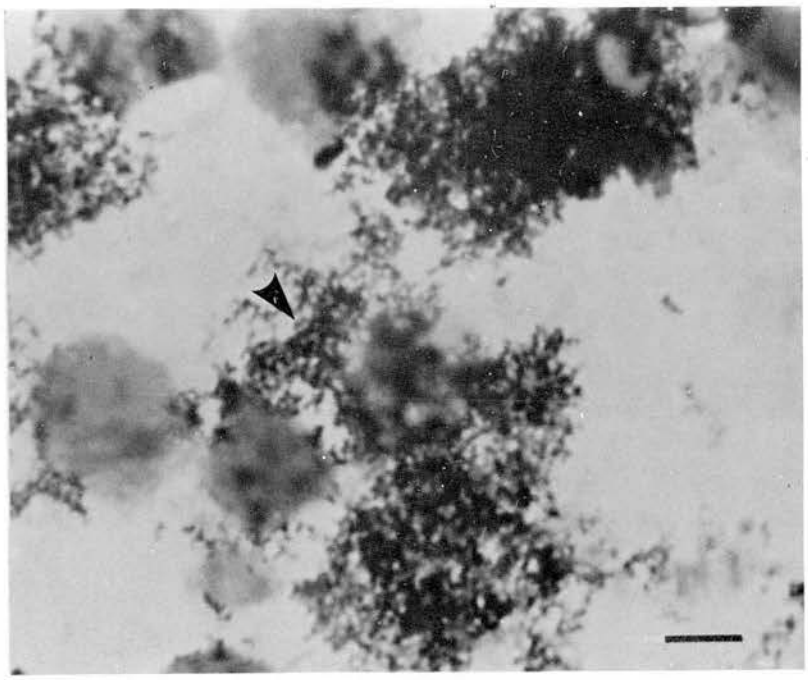

FIG. 1. Clusters of organisms appearing as black dots in human histiocyte cultures at day 9 of infection. Numerous organisms are also outside the disrupted cells (arrow). Diff-Quik stain. Bar, 10 μm.

DISCUSSION

In the accompanying paper we reported that the causative ehrlichial organisms are always found along the intestinal wall of the ponies experimentally infected by transfusion of whole blood from horses naturally infected with PHF (4). In this paper we show that the identical organisms were isolated in pure culture in human histiocyte cell lines, that the inoculation of this culture produced a similar disease in experimental ponies, and that the organism was recovered from these ponies. No other microorganisms or virus were consistently found or isolated in these or normal ponies, thus meeting the criteria of Koch's postulates to prove that the organism described is the causative agent of PHF. The apparent variation in clinical response and gross pathology between individual ponies is consistent with that seen fol-

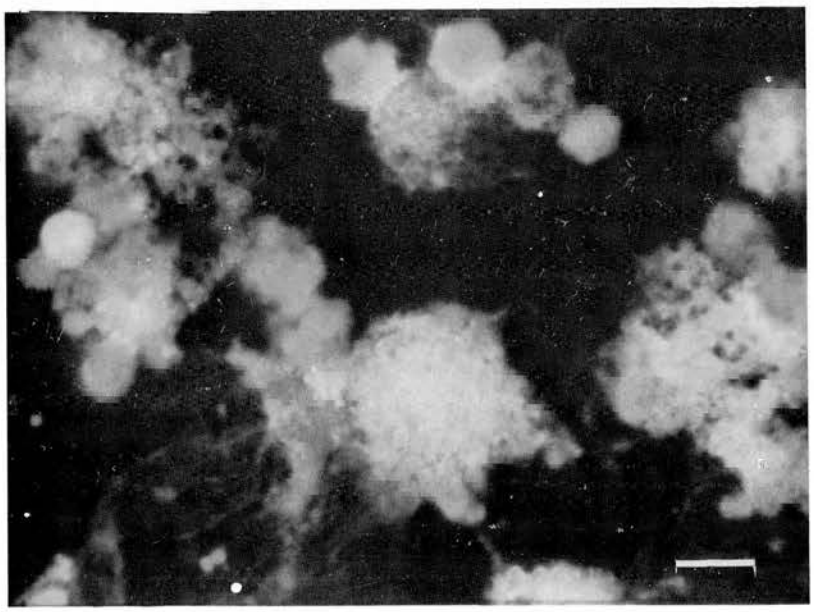

FIG. 2. Ehrlichial organisms in human histiocyte cultures labeled with PHF-convalescent pony sera and fluorescein-conjugated anti-horse IgG. The numerous white dots are the microorganisms. The histiocytes were counterstained with Evans blue. Bar, 10 μm.

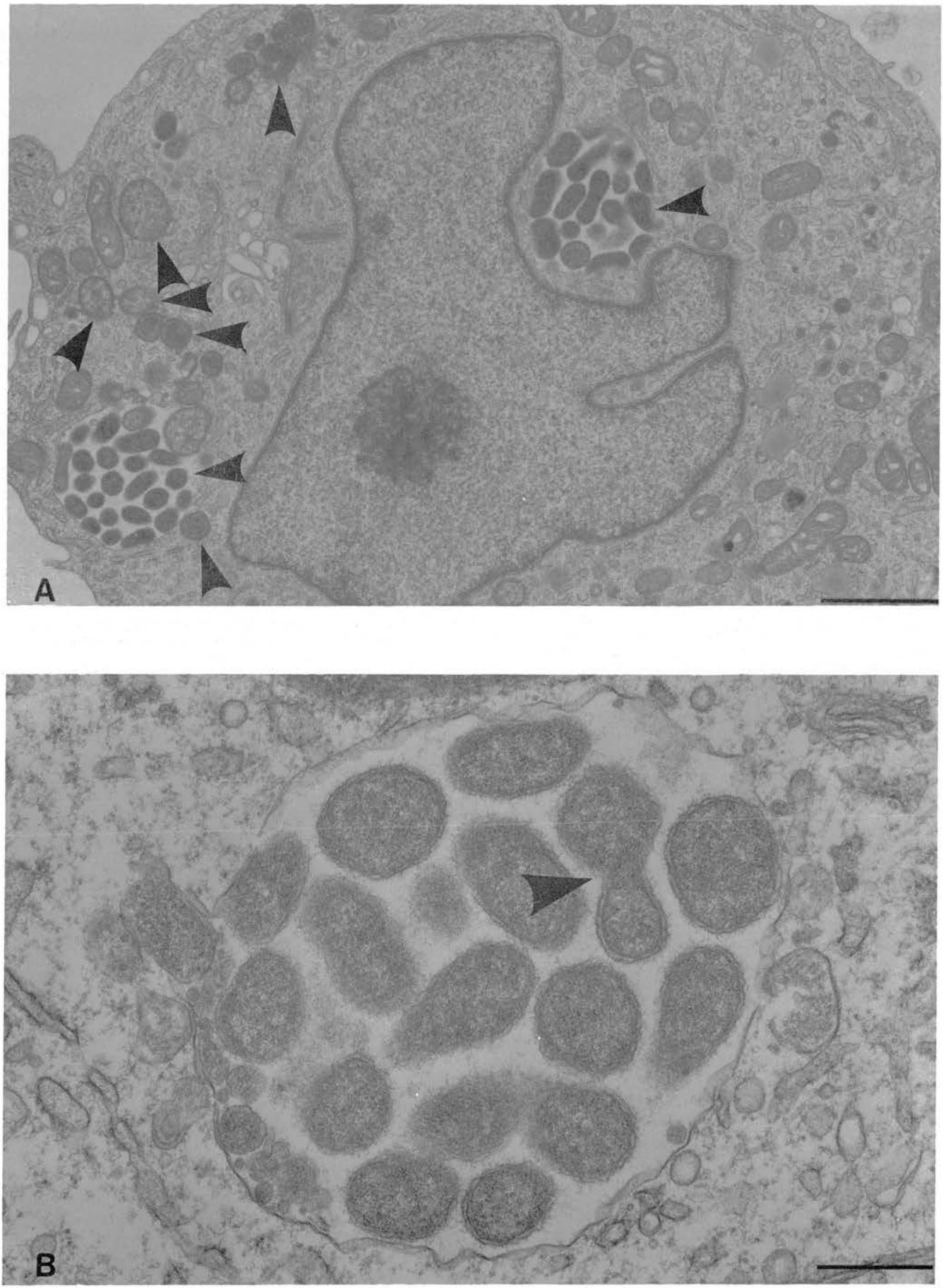

FIG. 3. (A) Transmission electron micrograph of ehrlichial organisms (arrowheads) in cytoplasmic vacuoles of human histiocyte cultures at day 9 of infection. Bar, 1μm. (B) At higher magnification, an organism dividing by binary fission (arrowhead) is evident. Note the fuzzy coating extending from outer membrane. Bar, 0.5 μm.

TABLE 2. Reisolation of ehrlichial organisms from ponies infected with cultured organisms

Pony no.	Days after inoculation	Days in culture ehrlichiae detected[a]
18	9	11
	10	11
	11	10
	13	8
19	9	11
	10	10
	11	8
	39	7
26	19	8

[a] By Diff-Quik stain.

lowing whole blood inoculation (B. D. Perry, Y. Rikihisa, and D. O. Cordes, unpublished data; A. L. Jenny, personal communication) and in field cases (2).

The histiocyte culture technique was very effective for isolating the ehrlichiae compared with the classical mouse inoculation method used for other ehrlichiae. The histiocytes multiply rapidly and are easy to maintain in suspension culture when compared with the primary monocyte cultures that have been used by other workers (1). Since human histiocytes are cultured in suspension and have a morphology distinct from that of horse monocytes, it is clear that we are not transferring or injecting infected horse monocytes. The infected human histiocyte is also useful as an antigen for immunofluorescent antibody assay because of less nonspecific binding of anti-horse IgG to the cells of human origin.

In the accompanying paper we demonstrated that the morphology of the organism both in infected ponies (4) and in cell culture was consistent with the genus *Ehrlichia*, but distinct from other genera of the family *Rickettsiaceae*. This distinction from other rickettsia has recently been confirmed serologically by other workers (1).

The persistence of ehrlichiae in apparently healthy, recovered ponies at 39 days or more post-inoculation suggests that recovered horses may serve as reservoirs and carriers of this disease. We are investigating the duration of persistence of organisms after inoculation and the duration and nature of immunity of ponies to homologous challenge.

Studies are in progress to correlate the successful isolation and clinical signs with the development of specific serum antibodies in infected ponies. Since the serum antibody titer becomes positive to this agent at 20 days post-inoculation, the use of the histiocyte culture technique should be explored as a means of diagnostic confirmation. The histiocyte culture technique and serological assay of horses will provide the means for further studying the epidemiology of PHF.

ACKNOWLEDGMENTS

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Enterocolitis Caused by *Ehrlichia* sp. in the Horse (Potomac Horse Fever)

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Abstract. Potomac horse fever was reproduced in 15 ponies by transfusion of whole blood originally from two natural cases and subsequently from ponies infected by the transfusions. Incubation periods varied from 9 to 15 days. Affected ponies developed varying degrees of fever, diarrhea, anorexia, depression, and leukopenia. Eleven affected ponies were killed, three died in the acute phase of the disease, and one did not show clinical signs. The most consistent post-mortem findings were fluid contents in the cecum and large colon, and areas of hyperemia (of inconstant degree and distribution) in mucosae of both small and large intestines. Multifocal areas of necrosis occurred in mucous membranes. Ehrlichial organisms were most common in the cytoplasm of epithelial cells, macrophages, and mast cells of the large colon.

A clinical disease characterized by fever, depression, diarrhea, and leukopenia has occurred each summer since the late 1970's in horses in Maryland and Virginia in the vicinity of the Potomac River. The disease is popularly known in the region as Potomac horse fever (PHF) or as acute equine diarrhea syndrome. Clinical features of the natural disease have been reported.[6,14] The disease occurs seasonally, usually between early June and late September each year. In the initial stages of the disease, fever ranges from 101 to 107 F (33.3 to 44.6 C), and is accompanied by depressed demeanor and decreased food intake. Fecal consistency changes, in most cases, first to a soft mass resembling bovine feces. In a proportion of affected horses, it then attains a watery consistency within 1 to 3 days. There is a temporary decline in the numbers of circulating leukocytes to less than 4,000/ml. Several days after the acute phase of the disease about 30% of affected animals exhibit other signs including colic, subcutaneous edema of the legs and ventral abdomen, and laminitis. Approximately 25% of clinically affected horses either die or are killed for humane reasons.

Gross lesions reported from field cases included ulceration of the glandular and pyloric regions of the stomach, patchy congestion of the duodenum, focal areas of congestion in the cecum, anterior and midcolon, and watery contents in these organs. Vesicles are sometimes seen on the oral mucosa.[6]

An epidemiological study reported that the disease was infectious, but not contagious.[8] Subsequent clinical and field observations have confirmed this finding (unpublished data). An agent resembling a coronavirus was isolated from affected horses,[5] but it did not cause the disease when inoculated into ponies.[6] In another study, horses recovered from the disease were found to have serum antibodies to *Ehrlichia sennetsu.*[7] In 1983 the disease was transmitted by transfusions of whole blood from affected horses into ponies[14] (Dr. A. L. Jenny, personal communication). In 1984, whole blood was transfused from two field cases into two ponies. Potomac horse fever was reproduced and, by electron microscopy, rickettsial organisms with the morphology of the genus *Ehrlichia* were seen within cells in the wall of the large colon.[11,12] These organisms were subsequently cultured in human histiocytes using infected leukocytes harvested from horses in the early stage of the disease.[10] Another report[4] also described the isolation of the *Ehrlichia* sp. from an experimentally infected horse and its cultivation in equine and canine monocyte cultures. Subsequently, organisms derived from cell culture were inoculated into ponies and produced the disease.[4,9,11] Some ponies used in this study were the subjects of previous reports.[10–13] This report describes the pathology of the acute stage of the disease in 15 ponies infected using whole blood transfusions.

Materials and Methods

The 15 ponies were of mixed breed, sex, and age and originated from rural environments. Because it was the intention to study tissue changes in the acute phase of the disease to avoid as far as possible complicating change due to secondary effects, ponies were killed within the first 4 days of clinical disease.

Approximately 350 ml of whole blood was taken into sterile collecting bags containing citrate phosphate dextrose anticoagulant solution USP (Ferrwall Labs, Deerfield, IL) from each of two field cases (A and B) in the acute stage of Potomac horse fever in early August 1983 in Maryland. Case A died 3 days later, and case B made a clinical recovery.

To reproduce the clinical disease, ponies were used as re-

Table 1. Experimental transmission of *Ehrlichia* sp. causing colitis in ponies.

Donor Horse		Recipient Horse*				
Pony	Days Post-transfusion	Pony	Febrile[f]	Leucopenia[l]	Diarrhea[d]	Killed
Case A	—	1	15–16	—	(15–16), 17	17
Case B	—	2	5, 12–13	—	(7), 12–13	13
2	14	3	6–10	2–4	(5), (11)	11
1	16	4	13–23	16	(12), 13–14, 20–22	23 (died)
3	8	5	11–13	12–24	(12–14), 15–16, (17–18)	18
4	10	6	—	—	—	—
5	9	7	4–7, 13–15	4–10	(14), 15–16	16
7	13	8	3–6, 11–12	—	12	12
8	12	9	10–13	—	10	13 (died)
7	16	10	9–11	—	(11)	12
8	12	12	10–14	—	(12–13), 14	14
10	6	13	4–10	—	(13), 14	14
13	11	14	—	7	(11)	12
13	11	25	14–16	15	—	16 (died)

* Episodes of febrile reaction, diarrhea, leukopenia, and death are given in days post-transfusion (pt). [f]: a febrile reaction was a rectal temperature >38.5 C; [l]: leukopenia occurred when leukocytes <4,000/μl; [d]: figures in ( ) indicate days when feces resembled bovine feces; other figures represent liquid feces.

Cases A and B were horses with the natural disease. They were donors for ponies 1 and 2. In turn ponies 1 and 2 became donors for ponies 3 and 4, they in turn became donors for ponies 5 and 6, respectively, and so on.

cipients of whole blood from others in the acute phase of the disease. Clinically normal ponies of mixed breed, sex, and age were obtained from local sources in southwestern Virginia. They were acclimatized for 3 to 21 days, initially fed pasture and hay until transfused with infected blood, at which time they were accommodated individually in pens screened to exclude arthropods. Anthelmintic or antibiotic treatments were not administered prior to transfusion. The ponies were fed a daily diet of good quality hay when housed.

Whole blood obtained from field cases A and B was cooled with ice packs and transported 250 miles to an infectious disease transmission facility. Each sample was inoculated 24 to 26 hours after collection into two separate recipient ponies. Each received in excess of 250 ml of whole blood. Following the incubation period and expression of clinical disease, these recipient ponies became donors of infected blood for the subsequent recipient pony in the series. Blood for transfusion was collected when the subject exhibited early stages of depression, fever, anorexia, leukopenia, and diarrhea. Recipients were transfused immediately.

Observations were made at least once a day prior to and following transfusion. Records were kept each day of rectal temperature, heart rate, respiratory rate, abdominal sounds, fecal consistency, food consumption, and demeanor of the animal. Blood samples were collected daily using ethylenediamine tetraacetic acid as an anticoagulant for complete blood cell count, and a clotted blood sample was obtained for serology.

Ten ponies were killed with T61 euthanasia solution (American Hoechst Corp., Sommerville, NJ) within the first 4 days of acute clinical disease. Routine necropsy examination was made immediately after death. The stomach and intestines were examined first. Photographs were taken of the luminal surface of the intestines after removal of feces. Samples selected for light microscopy were fixed in 10% neutral buffered formalin, processed through standard paraffin-embedding methods, and routinely stained with hematoxylin and eosin. Samples of adjacent tissue were taken for electron microscopy. Samples of stomach, small intestine, large colon, cecum, small colon, and spleen were cut into 3 mm cubes and fixed overnight at 4 C in a mixture of 2.5% paraformaldehyde, 5% glutaraldehyde, and 0.03% trinitrophenol in 0.1 M cacodylate buffer (pH 7.4), and postfixed in 1% OsO_4 in 1.5% potassium ferrocyanide. After block staining in 1% uranyl acetate in malate buffer (pH 5.2), tissues were dehydrated in a graded series of ethanols and propylene oxide and embedded in Poly/Bed 812 (Polysciences, Inc., Warrington, PA). Thick sections (1 μm) of Epon-embedded large colon of the infected ponies were originally examined under a light microscope. Thin sections (60–90 nm) were cut from the areas which appeared to contain organisms, stained with uranyl acetate and lead citrate, and examined with a JEM 100 CXII electron microscope.

Results

Clinical signs

Of 15 ponies in this study (Table 1), three died and 11 were killed within 4 days of the onset of acute clinical phase of the disease. One pony (6) did not develop clinical disease, but subsequently developed serum antibodies against the causative *Ehrlichia* sp., and its blood remained infectious for other horses for more than 7 months (unpublished data).

Fever preceded or coincided with the onset of diar-

Table 2. Summary of gross lesions in the digestive tracks of 14 ponies with ehrlichial colitis.

Pony	Stomach (fundus)	Small Intestine	Cecum	Large Colon	Small Colon
1	Erosions <1 cm	—	Hyperemia: mild, apical, 6 cm	Ulcers: few 1–2 mm	—
2	Hyperemia: mild, Ulcers: <0.5 cm	Hyperemia: mild, Ulcers: 1–2 mm (jejunum)	—	Hyperemia: marked (rvc) 20 × 5 cm, Ulcers: <0.5 cm	—
3	Hyperemia, Erosions: moderate, extensive	Hyperemia: mild (jejunum)	Hyperemia: mild, Ulcers: few 1–2 mm	Hyperemia: extensive, moderate	—
4	Congestion: marked	Congestion: mild (jejunum)	Congestion: extensive, marked; Contents red-brown	Congestion: extensive, marked; Contents red-brown	Congestion: extensive, marked; Contents red-brown
5	Erosions: moderate	—	—	—	—
7	—	Hyperemia: mild (ileum)	Hyperemia: moderate, Petechiae: few, Ulcers: 1–2 mm	—	—
8	—	Hyperemia: patches (duodenum)	Hyperemia: mild, patchy	Hyperemia: marked 20 cm (rvc)	—
9	Fibrinous exudate	Eccymoses (duodenum) (jejunum); Contents brown	Congestion: marked; Postmortem discoloration; Contents dry	Congestion: marked; Postmortem discoloration; Contents dry	—
10	Congestion: marked fibrinous exudate	Hyperemia: mild, patchy (jejunum, ileum)	—	Hyperemia: mild 20 cm; Petechiae	—
12	Hyperemia: mild	—	—	—	—
13	Hyperemia: mild	—	—	Hyperemia: mild 10 cm (rdc), Ulcers: 1–2 mm	—
14	—	Hyperemia: mild 30 cm	Hyperemia: mild	—	—
25	—	—	Hyperemia: marked, diffuse	Hyperemia: marked, diffuse	—

Ponies 4, 9, 25 died with the disease; pony 6 survived; the other ten ponies were killed in the acute clinical phase of the disease.
rvc = right ventral colon; rdc = right dorsal colon; — = no gross lesion.

rhea (Table 1). Depressed demeanor and decreased food consumption also coincided with the onset of diarrhea and were the first clear evidence of clinical disease. The passing of soft feces resembling the consistency of normal bovine feces or liquid diarrhea occurred in 13 of 14 clinically affected ponies. Only nine of the 15 animals developed liquid feces. Details of the leukograms of these and other horses will be reported.[1]

These signs were usually followed by initial decreased intestinal sounds and an increase in the volume and frequency of sounds associated with fluid accumulation. There was also an approximate doubling of the heart rate and respiratory rate. Rapid breathing was frequently abdominal in type in terminal stages. Dehydration followed diarrhea and was evidenced by slowed capillary refill time and delayed retraction in skin-pinch test on the neck. There was no evidence of edema, colic, laminitis, or oral vesicles in these ponies.

Gross findings

The most consistent gross changes were in the large colon and cecum and to a lesser extent in the small intestine (Table 2).

The contents of the small intestine were watery or mucoid in most ponies and of a catarrhal nature in one. Gross changes in the wall of the small intestine were absent in four ponies and were confined to small

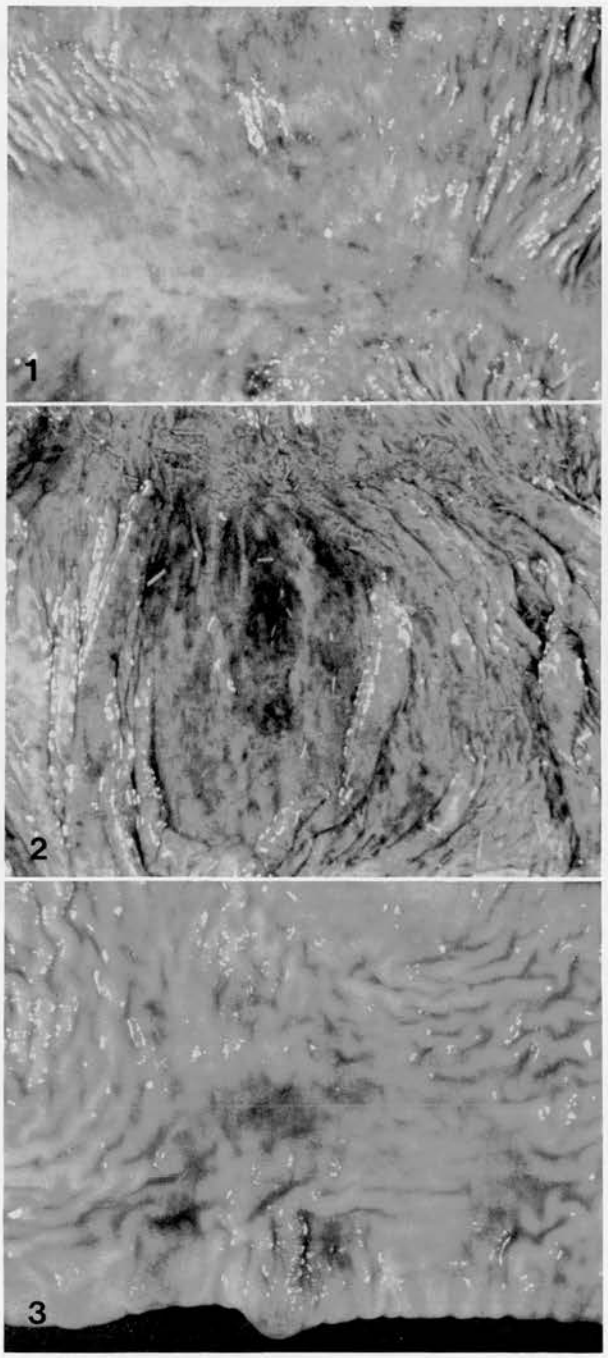

Fig. 1. Mild localized patches of hyperemia of mucous membrane of the jejunum. Pony 2.

Fig. 2. Patches of hyperemia, and petechiae (dark areas). Mucosa of the cecum. Pony 4.

Fig. 3. Patches of hyperemia (dark areas) of mucous membrane of the large colon. Pony 8.

patches of hyperemia 1 to 5 cm in diameter in eight others. Petechiae and ulcers of 1 to 2 mm diameter were in some areas of hyperemia. Ecchymoses were in the duodenum of one animal (Fig. 1). Changes in the small intestine were much less severe than those of the cecum and large colon.

The contents of the cecum (a mixture of water and poorly digested hay) were of approximately normal volume, but of very fluid consistency. In some animals the wall of the cecum was grossly normal, while in others there were regions of hyperemia 5 to 10 cm in diameter (Fig. 2), or small fields of ulcers and petechiae 1 to 2 mm in diameter. There was extensive hyperemia of the wall of the cecum in three ponies (4, 9, and 25) that died in the acute phase.

The contents of the large colon were of approximately normal volume, but very fluid. The contents of both the cecum and large colon were pale brown and had a fetid odor. In ponies that died in the acute phase, the contents were reddish brown. Gross lesions in the large colon resembled those in the cecum and consisted of hyperemic patches (Fig. 3) often with closely juxtaposed ulcers and petechiae. These areas were irregular in distribution and shape and were more common in the right dorsal colon. In half of the ponies, the wall of the cecum and large colon were grossly normal apart from ulcers caused by *Strongylus* sp. All ponies were parasitized by *Gasterophilus* sp. in the stomach and by low to moderate numbers of *Strongylus* sp. in the large intestine.

There was no gross change in the wall of the small colon of twelve ponies, but in two ponies there were irregular areas of hyperemia. In the ponies without diarrhea, the contents in the rectum were of normal consistency, but of an abnormal fluid consistency in the proximal part of the small colon. In ponies with diarrhea the contents were fluid throughout.

Histopathology

Changes seen by light microscopy were consistent with those seen at necropsy and were mostly confined to the cecum and large colon. Similar lesions were occasionally seen in the small intestine. In a control pony unaffected by ehrlichial colitis, the mucous membrane of the large colon and cecum was composed of the normal epithelium with crypts and had an intact layer of surface epithelial cells along the lumen of the organ. A mixed population of inflammatory cells dominated by eosinophils was present in deeper layers of the lamina propria and submucosa of this pony.

Patches of hyperemia were seen by the light microscope as accumulations of erythrocytes in capillaries or within the tissues in the superficial zone of the lamina propria (Fig. 4). In some locations there was degeneration and coagulation of erythrocytes. Loss of surface epithelium, presence of strands of fibrin on the surface of mucous membranes, and small foci of necrosis sometimes accompanied these changes (Fig. 5). Debris from epithelial and inflammatory cells was present in small foci in all layers of the mucous membrane including depths of the crypts, but was most

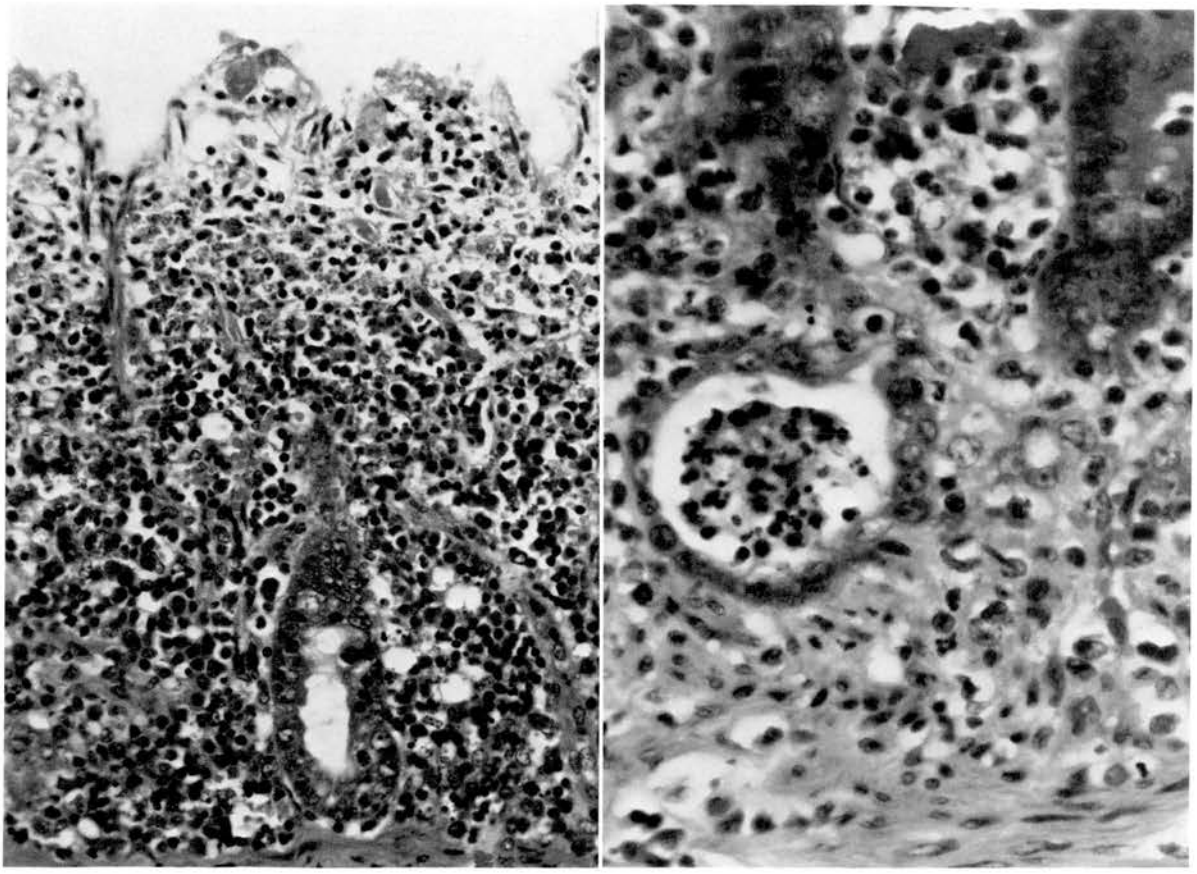

Fig. 4. Mucous membrane of large colon showing loss of superficial epithelium and fibrin deposits on the surface; congestion and infiltration by mononuclear cells. Pony 3. HE.

Fig. 5. Focus of necrotic cells in the base of a crypt in the mucous membrane. Pony 1. HE.

common in the superficial parts of the lamina propria. In the three ponies that died, superficial mucous membrane was absent, and deeper parts of the mucous membrane were congested.

A mixed population of moderate to large numbers of inflammatory cells was present in the lamina propria and in some cases in the submucosa. The population included eosinophils, plasma cells, lymphocytes, macrophages, mast cells, and neutrophils (all more numerous than in the control pony). There were hyaline changes in segments of the wall of small blood vessels, in the submucosa of the large colon, and there was mineralization of the intima of arterioles. There was also evidence of slight edema around these vessels.

Electron microscopy

Large numbers of ehrlichial organisms were present in the cytoplasm of deep glandular epithelial cells and in mast cells and macrophages in the lamina propria and submucosa of the large colon. They were particularly numerous in macrophages near large blood vessels. Fewer organisms were also found in the macrophages in the small colon and in other organs.

Two morphological forms of the organisms occurred. One was a small electron-dense structure that had undergone binary fission within vacuoles in the cell cytoplasm. The other form was larger, less electron-dense, and present as accumulations of organisms surrounded by a closely applied host membrane (Fig. 6). Occasionally both forms were seen in the same vacuole. The organisms were bounded by a thin double membrane with a fuzzy coating on the outer membrane.

Discussion

Ponies exhibited a varied expression of clinical signs during disease and the range was similar to those described previously.[6,14] Ponies killed in the acute phase of the disease did not exhibit colic, laminitis, or edema of the limbs as reported in natural field cases.

Gross lesions were confined to the small intestine, cecum, and colon. Three of the 15 ponies did not develop gross lesions, and five others exhibited only minor, localized lesions. The remaining seven ponies had more marked lesions in the mucous membrane of the cecum and large colon with minor localized changes

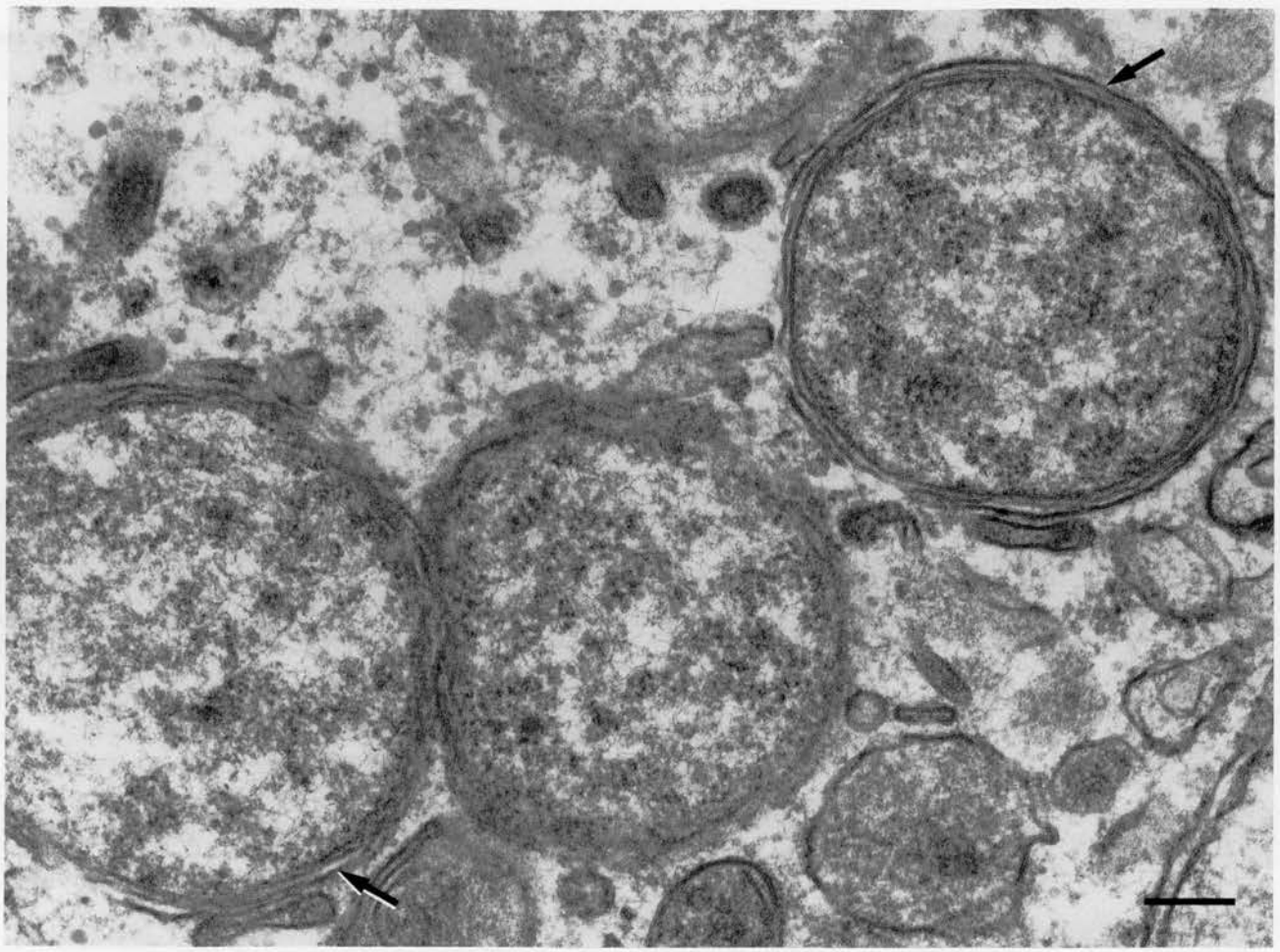

Fig. 6. Ehrlichiae surrounded by a closely applied host membrane (arrows) in a macrophage. Bar = 1.5 μm.

occasionally present in the small intestines. The most consistent change in all ponies was the very fluid nature of the approximately normal volume of the contents of the cecum and large colon, contrasting with the thicker, less fluid consistency of that of the unaffected animal. The redness of the contents of the cecum and large colon of one pony (4) that died in the acute phase was probably caused agonally by blood released from congested autolysed vessels immediately before or after death. Gross changes in the wall of the cecum and large colon were marked in some ponies, but distribution was variable. Hyperemia and petechiae, when present, were useful indicators of the disease, but their absence did not exclude the diagnosis.

Dehydration, seen in all ponies, indicated there had been interference with fluid absorption across the surface of the mucosa of the cecum and large colon independent of the presence of gross lesions in the mucous membrane. Light microscopy revealed an increase in the erythrocyte concentration in the superficial part of the lamina propria within capillaries and in the tissue, thus indicating necrosis of the capillary that resulted in petechiae and ecchymoses. This was followed by sloughing of the surface epithelium of the mucous membrane, focal necrosis of the glandular and crypt epithelium in the lamina propria at all levels, and a small amount of fibrinous exudate on the luminal surface.

Pathologic changes, together with the presence of ehrlichial organisms seen by electron microscopy in epithelial cells, mast cells, and macrophages in the lamina propria, suggest a general interference by the organism with epithelial cell function in the large colon and cecum, and/or indirectly through the perturbations of infected macrophages and mast cells in these organs. Tests made on feces have failed to show evidence of clostridial toxins[2] (R. J. Carman, personal communication).

Since no ehrlichial organisms were found in the stomach wall, gastric hyperemia, erosion, and ulcera-

tion were considered to be not directly related to the ehrlichial infection. They are common incidental findings in horses. While lesions occurred elsewhere, they were interpreted as being of minor incidental nature and not directly related to the ehrlichial infection.

Hyaline changes in segments of the wall of small blood vessels in the submucosa of the large colon and changes in the intima of arterioles were attributed to the effects of parasites, mostly *Strongylus* sp. There was evidence of slight edema around these vessels in horses with ehrlichial enterocolitis. Eosinophils and lymphocytes were attributed to infestation by parasites.

To determine precisely which tissue changes may be attributed to the ehrlichial organism and which were due to helminth parasites and other agents would require the use of recipient animals raised specific pathogen-free, and that has obvious practical difficulties.

Ehrlichial enterocolitis is apparently a different disease from equine ehrlichiosis.[3] The latter syndrome has not been reported with signs of diarrhea or intestinal involvement. The edema of the lower parts of the limbs and ventral abdomen may occur in horses with either disease. While edema was not seen in the 15 animals of this report, mild edema has been observed in the ventral abdominal area[14] of other animals with ehrlichial enterocolitis (unpublished data). We did not see petechiae or ecchymoses in the subcutaneous tissues as reported in equine ehrlichiosis, nor were our ponies jaundiced. Changes reported in blood vessels in equine ehrlichiosis[3] were not interpreted as part of the acute clinical phase of the disease in ehrlichial colitis of the horse.

Horses with Potomac horse fever develop serum antibodies against the causative *Ehrlichia* and can be assayed by an indirect fluorescent antibody (IFA) test[10,11] It has been shown that this organism is closely related to *E. sennetsu,*[4] but it appears to be serologically distinct from *E. equi,* the agent of equine ehrlichiosis.[3,4]

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The Veterinary Record, March 2, 1985

Intradermal transmission of Potomac horse fever

From Dr B. D. Perry and others

SIR, — The causative agent of Potomac horse fever (PHF), an ehrlichia, has recently been identified in infected horses (Rikihisa and others 1984) and grown in cell culture (Rikihisa and Perry 1984), and the disease successfully reproduced by the inoculation of this organism into susceptible horses (Holland and others 1985, Rikihisa and Perry 1985). The natural disease in horses is seasonal, occurring from May to October. Despite the infectious nature of the disease, it does not appear to be contagious (Perry and others 1984), suggesting that an arthropod vector is involved in its transmission, as occurs with other species of the genus *Ehrlichia*. This letter reports the successful transmission of the disease to a pony by the intradermal route.

The causative ehrlichia was grown on human histiocyte cell lines following primary isolation from a horse suffering from clinical PHF. Infected cells were harvested and infection was confirmed by examining Giemsa stained smears of cultured cells. Infected cells were counted using a haemocytometer, and 4×10^7 infected cells suspended in 5 ml RPMI 1640 medium were pipetted into a sterile centrifuge tube and sealed. One ml aliquots of the cell suspension were inoculated by the intradermal route into a susceptible pony. The five distinct inoculation sites were located on the neck, flank and rump. Whole blood in anticoagulant (350 ml) was taken on day 19 after inoculation and leucocytes were aseptically separated and cultured as previously described (Rikihisa and Perry 1984).

After nine days' incubation, the pony developed a fever of 38·3°C accompanied by progressive depression (Fig 1). Fifteen days after inoculation it stopped eating completely. Diarrhoea developed 18 days after inoculation and the pony died two days later.

At post mortem examination, lesions were consistent with those of PHF. The stomach had a few ulcerations, all less than 1 cm, near the margo plicatus. The small intestine contained only mucus and four focal areas of haemorrhage were observed, which were located in the duodenum (two), distal jejunum (one) and ileum (one). These were 2 to 5 cm in size and round, stellate or irregular in shape. A vague hyperaemia was evident throughout the mucosa of the small intestine.

The large colon, caecum and small colon contained watery brown fluid. No lesions were seen on the mucosa, except for two discrete ulcers in the caecum. These were oval, 1 cm long and 5 cm apart.

From day 7 of culture a heavy growth of the causative ehrlichia was observed and the identity of the organism was confirmed by immunofluorescent microscopy and electron microscopy as previously described (Rikihisa and Perry 1984).

The clinical and pathological pictures were consistent with that of PHF (Whitlock and others 1984, unpublished data) with an incubation period similar to that described previously. Isolation of the causative agent in pure culture from whole blood confirmed the diagnosis.

Transmission of the disease to horses under experimental conditions has previously been achieved by the intravenous transfusion of whole blood from an acutely sick horse (Jenny 1984, Whitlock and others 1984, Rikishisa and others 1984, Holland and others 1985) and by the intravenous inoculation of *E potomacensis* in cell culture (Holland and others 1985, Rikihisa and Perry 1985). In the field, it is likely that the disease is transmitted by a biting arthropod, and we are currently investigating the role of the American dog tick *Dermacentor variabilis*. The present study indicates that the intradermal route is extremely effective in the transmission of the disease and produces an incubation period apparently indistinguishable from that of intravenous transmission.

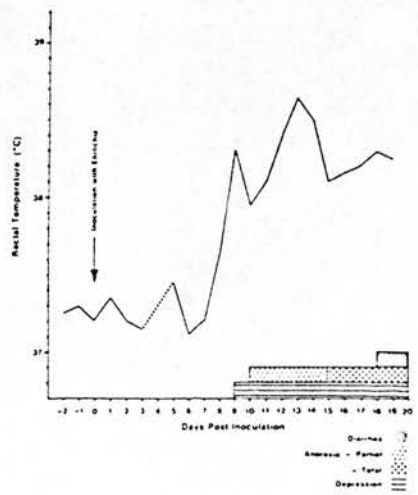

FIG 1: Clinical response to the intradermal transmission of Potomac horse fever

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