PATENT SPECIFICATION (11) 1 570 732

1 570 732

(21) Application No. 16400/77 (22) Filed 20 Apr 1977

(31) Convention Application No. 679840 (32) Filed 23 Apr 1976 in

(33) United States of America (US)

(44) Complete Specification Published 9 Jul 1980

(51) INT. CL.[3] A61K 39/27

(52) Index at Acceptance A5B 120 132 136 AA AC

(54) EQUINE RHINOPNEUMONITIS VACCINE

(71) We, PHILIPS ROXANE INC. residing at 2621 North Belt Highway, St. Joseph, Missouri 64502, United States of America, a Corporation organised and existing under the laws of the State of Missouri, United Stated of America, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

The invention relates to a vaccine for the protection of Equidae against the effects of a virulent strain of Equine Rhinopneumonitis virus, hereinafter referred to as ERP virus, to a method of preparation of a vaccine therefrom, and to a method of protection of Equidae.

The invention also relates to a process for the production of an immunogenically active virus which comprises the steps of attenuating the virulent strain of ERP virus by passage through a tissue culture, for example a Vero Cell Line at a reduced temperature for a number of serial passages sufficient to reduce the virulence of the virus without affecting its immunogenic character.

Rhinopneumonitis is an acute viral infection believed to be caused by type I Equine Herpes Virus, and is characterized by fever, leukopenia, and catarrhal inflammation of the respiratory tract. The viral disease predisposes to secondary bacterial infection which affects various parts of the respiratory tract. Abortion is normally a sequel when pregnant mares are infected.

Rhinopneumonitis occurs on farms in areas of concentrated horse breeding. A relatively uniform pattern is observed in central Kentucky, where both respiratory disease and abortion have been studied. Respiratory disease is observed almost exclusively in young horses, either on farms or when they are assembled for sale or training. Most farms in the area have outbreaks of rhinopneumonitis in the fall (Autumn) or early winter, especially in October, November and December. The outbreaks in young horses often are associated with weaning and assembling in winter quarters. Mares on the farms have no overt signs of disease at this time, but infection may be demonstrated by serologic tests. The disease spreads rapidly within a stable or training area, with all horses becoming infected within a few days or weeks.

Abortions have been observed in every month except July and August. They are most prevalent in mid-winter and early spring. Of 700 abortions recorded in Kentucky, 15% occurred in January, 24% in February, 29% in March, and 18% in April. Abortion may occur from the 5th month of gestation to full term. Some foals infected prenatally are born alive at full term. Data on 623 abortions show 11% occurring in the 8th month, 30% in the 9th month, 36% in the 10th month, and 19% in the 11th month. The data indicate a relation between age of the fetus and occurrence of abortion, but the breeding period of mares and the enzootic pattern in young horses have an important influence on the seasonal incidence and age of the fetus at the time of abortion.

The incubation time between nasal inoculation and abortion varies from 3 weeks to 4 months, establishing that the infection which results in abortion is coincident with the earlier enzootic infection of young horses on the farm. The virus spreads readily by direct contact, fomites and aerosolized secretions. Virus may be spread from one mare that aborts to others but in most outbreaks the evidence indicates that nearly all mares on the premises were infected 1 to 4 months before the first abortions occur.

The disease occurs annually in young horses on many farms that have not had new horses introduced, suggesting the presence of carriers among the adult horses. The regularity of outbreaks when young susceptible horses are assembled also indicated a carrier state. Outbreaks are frequent when susceptible

horses are assembled for sales, in yards, in training areas, at race meets and in military establishements.

The incubation period of ERP varies from 2 to 10 days. Primary infection of fully susceptible horses is manifested by fever and a serous discharge from the nostrils. Temperatures may reach 106 F, and fever persists for 1 to 7 days in uncomplicated cases. Afternoon temperatures usually are higher than in the morning. Leukopenia occurs in parrallel with fever. Both neutrophils and lymphocytes are depressed during the first 2 days of fever. Lymphocytes return to normal levels in 2 to 4 days, and neutrophils regain normal levels in 5 to 9 days. Feed and water consumption may be reduced. Mild congestion of the nasal mucous membranes is observed, and there may be palpable oedema of the mandibular lymph nodes. Enteritis and diarrhoea, oedema of the legs, and tendovaginitis are infrequent in uncomplicated cases. General depression is slight in horses kept at rest. All signs are exacerbated by forced exercise or work. Recovery is complete in 1 to 2 weeks unless complications develop.

Reinfection may occur at intervals of 4 to 5 months or longer. These subsequent infections are usually asymptomatic, afebrile, and do not result in complications, especially in adult horses on breeding farms. The effect of reinfection on horses in racing or subjected to hard work is not known.

According to the present invention there is provided a process for the production of an immunogenically active attenuated live equine rhinopneumonitis herpes virus which comprises the steps of

a. introducing a live virulent equine rhinopneumonitis virus into a tissue cell culture,

b. incubating the culture until the cellular cytopathology becomes evident,

c. harvesting a portion of the virus so produced and reintroducing the harvested virus into fresh tissue cultures,

d. repeating serial passage of the virus in tissue culture up to 300 times at an incubation temperature of 33°C or less, the number of passages and the temperatures being sufficient to produce an immunogenically active live avirulent virus; and

e. recovering said virus from said tissue culture medium.

The tissue cell culture may be a Vero Cell culture, and the incubation temperature for the serial passages may be from 23° to 33°C. At least 30 serial passages may be carried out and preferably at least 50 passages.

The vaccine of the present invention is an effective quantity of an immunogenically active avirulent live equine herpes I virus produced by the aforesaid process and a stabilizing solution, said virus being prepared from a virulent strain of Equine Rhinopneumonitis (ERP) Virus which was passed for example, for fifty (50) passages through the Vero Cell line (monkey kidney) at 26°C.

The vaccine can be prepared by a process which comprises the steps of inoculating a tissue cell culture with a virulent strain of ERP virus so that reproduction of the virus will occur, serially passing the virus so propagated in additional tissue cell cultures for a sufficient number of passes and under conditions such that the virus becomes attenuated to an avirulent condition without losing its immunogenic character.

According to a further aspect of the invention there is provided a process for the production of immunogenically active attenuated live equine rhinopneumonitis virus which comprises

a. introducing a live virulent equine rhinopneumonitis virus into a mono layer of a Vero permanent cell line,

b. incubating the culture from 3 to 5 days at a temperature of 37° C until the cytopathology of the cells is substantially complete,

c. harvesting a portion of the virus so produced and introducing the harvested virus into a freshly prepared stable cell mono layer of Vero cells,

d. incubating said culture at a temperature of 26°C for a period of time sufficient to result in cytopathology of at least a portion of the tissue cells,

e. serially passing a portion of the virus so produced in stable Vero cells at least 45 times at an incubation temperature of 26°C to produce an immunogenically active live avirulent virus; and

f. recovering said virus from said last serial passage.

Attenuation, or modification of the virulent or pathogenic character of bacteria or a virus to an avirulent or non-pathogenic state is well known in the art.

Modification or attenuation of morphology and/or pathogenicity has been brought about by many techniques. Sometimes the repeated serial passage in the host tissue attenuates the organism; or its serial passage in a tissue which is different from the host tissue sometimes leads to attenuation; or chemical shock to the organism, radiation treatment, low temperature passage, and other techniques have been used by the bacteriologist and the virologist to produce pathogenically inert organisms which retain their ability to cause their hosts to form antibodies or cell mediated immunity capable of effectively neutralizing a pathogen. The attenuation without loss of immunogenicity of a living organism is completely non-predictable and only empirical methods may be used to determine efficacy of any given technique. For example, a given technique that has been found applicable to one type of virus may not always be

applicable to another. Prior art techniques for attenuation of a virulent strain of ERP virus has been reported. For example, serial passage of a virulent ERP virus in hamster tissue with some adaptation has been reported by E.R. Doll in Vet. Bull. 32, 1493. U.S. Patent No. 3,725,542 described the attenuation of a virulent strain of ERP virus by cultivating and multiplying rhinopneumonitis viruses, deprived from a source selected from infected horses, infected foals, and aborted horse fetuses, in susceptible tissues selected from susceptible organs (such as monkey kidney) susceptible test animals, and susceptible permanent cell lines suitable for the cultivation and multiplication of said viruses, said multiplying involving from three to 10 passages; completely attenuating the viruses until they have lost pathogenicity for the horse by serial passage through the primary cell cultures obtained from sheep, pigs and piglets and from stable permanent cell lines derived from these primary cell cultures.

It is postulated that immunogenic protection against subsequent challenge by a virulent ERP virus does not occur unless the avirulent virus reproduces itself in the host animal and can be recovered and identified after vaccination occurs. To date, none of the attenuated virus strains reported possess this characteristic and do not furnish the desired degree of protection.

If the stable cell line from the African Green Monkey, *Cercopitheous aethiops,* the Vero cell line, is inoculated with a virulent ERP virus and serially passed in such a cell line at reduced temperatures, say 23°C to 33°C, the virus becomes attenuated and non-virulent but still retains its immunogenic character, that is, when introduced into an equine host, will trigger its immunological mechanism to produce viral antibodies and cell mediated immunity. The animal then becomes immune to subsequent infection, and the avirulent virus can be recovered from the vaccinated animal and identified.

Using ordinary serial passage techniques which are standard in the art, it has been found that the desired avirulent character has been developed without loss of immunogenicity after 30-60 passes at a temperature within the range of from 23° to 33°C. However, up to 250 or 300 passages or more may be used.

An attenuated live equine herpes I virus vaccine contains an effective quantity of an immunogenically active avirulent live equine herpes I virus produced by the inventive process as described above added to a stabilizing solution to form the vaccine therefrom.

The ERP vaccine of this invention may be administered to any species of the family Equidae by either extra - or interparenteral techniques. The vaccine, in liquid form, may be given intra-nasally, intra-orgally, intra-ocularally, intra-muscularly, intra-venously, and intraperitoneally. In a dry powdered form it is suitable for extra-parenteral administration. As is usual with vaccines of this type, from 3,000 to 100,000 $TCID_{50}$ units are administered in a single dose.

The steps of the inventive process may be more clearly described as follows:

1. *Reproduction of Virus*

Tissue cell cultures were prepared using Vero stable cell line derived from African Green Monkey at a passage level of 129. A standard glass culture vessel was seeded with a trypsinized suspension of Vero cells containing sufficient media to cover the cells to a depth of about ½ inch. Eagles Minimum Essential Medium (MEM), as defined in Handbook of Cell and Organ Culture by Merchant, et. al., Burgess Publishing Co., 1964, containing 10% foetal calf serum for growth medium (5% in maintenance medium) was used.

However, any standard growth medium may be used, such as, Lactalbumen Hydrolysate Medium in Hanks Balanced Salt Solution, Medium 199. The culture vessels were incubated at 37°C, for from 1-3 days after which time a monolayer of Vero cells was formed.

The vessel was the inoculated with *Equine Herpes I,* identified as an A183 isolate obtained from the University of California, using standard aseptic techniques and after 24-72 hours incubation at 37°C, the cytopathology of the cells was substantially complete indicating that the virus has reproduced itself. The virus was passaged 13 times in Vero cells at 37°C as above and then subjected to the attenuation procedure as detailed below.

ATTENUATION

Using standard tissue culture flasks and the MEM growth medium (described above) monolayers of Vero cerlls were prepared. About 100 $TCID_{50}$/ml of the 13th passage virus was introduced into the flasks and the inoculated flasks were incubated at a temperature of 26°C. After about 8-10 days a small area of cell degeneration was noted and a second serial passage was made with some of the fluid medium. Incubation at 26°C continued and cellular cytopathology increased. After each serial passage, cytopathology increased and after 30-60 serial passages, cytopathology was found to be complete after 3-5 days incubation at 26°C. This attenuated virus was identified as A183 V26 P50 ERP vaccine.

The following *in vivo* experiments were carried out with the 50th or the 55th passage of the cold adapted virus. All ERP vaccine virus was administered deep I.M.

q A virulent ERP challenge was produced by serially passing the unattenuated A183 virus in nasal washes and whole blood through four horses. The equine passaged virus induced classical rhinopneumonitis symptoms of ERP as well as abortions in pregnant animals. The challenge ERP virus pool was used repeatedly for challenge of experimental horses with excellent results in non-immune animals. All challenge virus was administered intranasally.

Serum neutralization tests were used to

measure all serological responses to the ERP vaccine or challenge virus.

The purity of ERP vaccine virus produced as described above is determined by neutralizing the cytopathogenic effect (CPE) produced in tissue culture with known equine Herpes I typing serum.

The vaccine virus produces large syncytium in RK_{13} cells and Vero cells at 37°C. The syncytium can be used as a marker for the vaccine virus as the virulent ERP challenge virus produces individual cell rounding type CPE, in the above cell lines at 37°C.

The vaccine virus kills newborn hamsters and the vaccine virus was recovered from the dead hamsters producing the same type of syncytium marker. No clinical symptoms of equine viral diseases were induced in vaccinated horses or foals in varying age groups.

The vaccine virus can be isolated from equine peripheral leukocytes from 2 to 11 days following ERP vaccination. Other virus agents are not isolated following vaccination, except the indigenous Herpes II group of viruses. Also, the vaccine virus recovered from the horses has the vaccine marker (syncytium).

The experimental vaccine virus A183 V26 P50 was checked for the following adventitious viral agents by known techniques, and none was found to be present:
Equine infectious Anemia virus, Equipe Herpes II group of viruses, Equine Adeno virus, Equine Encephalomyelitis virus group and Equine Influenza group (A/Equi/1; A/Equi/2).

PREPARATION OF VACCINE

The attenuated virus prepared as described above, was grown in large quantites using the same growth medium described above in standard monolayer Vero cells flasks. The flasks were incubated until cytopathology was completed, usually in from 3-4 days. The growth medium was then harvested and admixed with a stabilizing solution and divided into unit doses of approximately 1,000 to 10,000,000 $TCID_{50}$/ml each in the usual vaccine vials. The material was then freeze-dried in the vials and sealed for use.

Experiment I

The following experiment was designed to determine if the ERP vaccine developed was safe and to see if the vaccine virus transmitted to contact horses. In addition, the efficaciousness of the vaccine in vivo was to be tested against the challenge virus.

A group of 6 mixed breed horses of both sexes were housed under the same roof in a group pen. Feed consisted of oats and grassy hay and self-feeding waterers were present. The group received the attenuated ERP vaccine. All vaccinations were given deep I.M. The first vaccination was followed by a similar vaccination in 28 days. All horses were challenged with virulent ERP virus 56 days following the first vaccination. The following parameters were monitored; all horses were checked daily for clinical signs of ERP and temperatures and serums were monitored throughout the test. Three horses were selected to monitor virus isolations from the buffy coat cells and nasal swabs. Virus isolations were started on two contact controls at the time of challenge. Complete blood counts were frequently made on the horses being monitored for virus isolations.

Bacterial isolations and identification were frequently made on horses exhibiting nasal drainage. Principals were numbers 104, 77, 52, 53 and 54. Contact control was number 79. Results are shown in Experiment I tables below:

EXPERIMENT I
Serum Neutralizations

	Vaccinated Twice I.M.						Challenge				
	1st Vac	+12 days	+21 days	2nd vac. +28 days	+12 days 2nd vac.	+20 days 2nd vac.	+28 days 2nd Vac.	+11 days Challenge	+21 days Challenge	+29 days Challenge	+66 days Challenge
P52	16	128	512	256	128	512	256	512	512	1024	
P53	8	256	512	1024	128	512	128	256	128	256	
P54	4	64	512	256	256	256	128	512	128	256	
P77	0	N.D.	2048	512	256	256	128	1024	256	256	
P104	0	0	64	8	64	128	128	256	256	128	
C79		0	0	0	0	0	0	512	2048	2048	

P = Principal
C = Contact Control
N.D. = Not Done

None of the principals in this experiment had any clinical signs of ERP

EXPERIMENT I
Equine Herpes Virus Isolation

	1st Vaccination 0 day	+3	+4	+5	+6	+7	+10	+12	+14	2nd Vaccination +28	+3	+4	+5	+6	+7	+8	+12	+14	+28	Challenge +1	+2	+3	+4	+7
P77 BS			I	I		I	+							II		II			I	+				
NS							II	II									II	II	II	+				
P104BC				I		I													II	II				
NS							II	II		II	II			II	II		II	II	II	II	I	I	II	+
C79*BC																							I	I
NS										NOT DONE										I	I	I	I	

(continuation)

	+8	+9	+10	+11	+14	+16	+18	+21
P77 BC								
NS				II	II	II	II	II
P104 BC								
NS		II	II	II	II	II	II	II
C79*BC			I					I
NS	I	I	I	II	II	II	II	II

* Abortion 15 days following challenge
Herpes I Virus recovered from fetus.

I = Equine Herpes I Virus
II = Equine Herpes II Virus

+ = Unidentified Virus
P = Principal
C = Contract Control
BC = Buffy Coat
NS = Nasal Swab

An examination of the data reported in the TABLES of Experiment I above plus the absence of clinical signs indicate the following:

1. The vaccine did not induce disease symptoms following either vaccination.

2. The vaccine virus did not transmit to the contact control animals over a 56 day period of being held together, prior to challenge. There were numberous isolations of *Equine Herpes II* virus throughout this experiment.

Although the control, C79, demonstrated the normal clinical symptoms of Equine Rhinopneumonitis, that is inflammation of the respiratory tract, anorexia and general malaise, a temperature increase to several degrees above normal within two days after challenge with virulent virus, and a severe depression of the white blood cell count, the vaccinated principals were protected against the virulent challenge virus as measured by lack of clinical symptoms, normal body temperatures and normal white blood cell counts.

Experiment 2

The following experiment was designed to determine if the attenuated ERP vaccine was safe in pregnant mares (no clinical symptoms or abortions) and to determine the efficacy of the vaccine in pregnant animals against a virulent ERP challenge, to obtain information concerning ERP passive antibody transferred to the foals, the safety of the vaccine at different time intervals of pregnancy, and to determine if the vaccine virus would transmit to contact controls which were non-vaccinated pregnant animals.

Experiment 2 was divided into four groups of pregnant horses. Two groups were vaccinated once and challenged intranasally 40 days later with virulent ERP virus. One of the aforementioned groups received the 50th passage level of the cold adapted vaccine virus. The remaining two groups of pregnant horses were vaccinated twice, 20 days apart in a similar manner as above with the same passaged levels of cold adapted vaccine virus. The latter two groups were challenged intranasally 50 days following the first vaccination. A fifth group was used as challenge control horses and foals.

The following parameters were measured: temperature, virus isolations and identification, CBC, serum neutralization titers and clinical symptoms. The above parameters were measured in both the mares and foals. The foals were monitored for virus from blood leukocytes and nasal turbinates immediately upon being found and two or three days after birth. Also, serums were collected at the time of foaling of the newborn colts and weekly thereafter. In some cases, burlap barriers were placed over the mammary gland to prevent suckling after parturition, in order to assure some precolostrum serum samples. ERP colostrum neutralizing antibody was measured in some mares. Also, many paired serum samples were collected from colts and mares at birth or later.

Many of the foals born during this experiment were vaccinated with the attenuated ERP vaccine virus at 10 to 21 days of age, and this data will be reported from a safety point of view in Experiment 3. Data obtained from this Experiment are set out in the Experiment

2 TABLES below.

EXPERIMENT 2
ERP Serum Neutralizations and Pertinent Data

Group 1 - Pregnant Mares Vaccinated Twice with A183-V26-P50 Virus

I.D. No.	8-2-74 1st Vac 0 day	+14	1-3-74 2nd vac +21	+5	+14	2-4-74 Chall. +32	+6	+14	+21	Live Del.	Abort	Death after Del.
P65	16	512	1024	512	512	Colt born 1 day pre chall.				27-3-74	-removed	
P70	16	512	512	256	512	256	256	256	256	4-5-74		
P150	0	128	256	256	256	Colt born 3 days pre chall.				30-3-74	-removed	
P155	4	256	512	512	1024	256	256	256	512	20-5-74		
P157	8	256	256	512	512	512	256	256	64	21-4-74		
C140*	>32	16	>16	16	16	16	32	1024	1024	Male		
C74	Preg. Horse added at Chall.					>16	32	512	1024	7-4-74		

Group 2 - Pregnant Mares Vaccinated Twice with A183-V26-P55 Virus

P151	4	512	1024	512	256	256	128	512	256	13-4-74		
P152	8	64	128	128	128	128	128	256	128	22-4-74		
P153	2	128	128	128	128	Colt born 6 day pre chall.				27-3-74	-removed	
P154 **	2	128	256	256	512	512	512	512	512	28-4-74		30-4-74
P156	4	512	256	512	2048	512	>1024	512	512	Not Preg		
C148	8	8	8	8	8	8	16	512	1024	Male		
C159	Preg. Horse added at Chall.					>2	4	256	1024	NA	19-4-74	

Group 3 - Pregnant Mares Vaccinated Once with A183-V26-P50 Virus

	20-2-74 0 day	+7	+14	+21	Chall. +40	1-4-74 +7	+14	+21	+35		
P160	2	64	512	256	256	256	256	128	128	5-4-74	
P161	16	16	1024	1024	1024	256	512	256	256	23-2-74	23-2-74(1)
P162	2	64	512	512	1024	512	256	256	128	22-4-74	
P164	16	64	1024	1024	512	1024	512	512	1024	24-6-74	
P165	4	16	256	512	256	256	256	256	256	15-5-74	
C163	8	32	>16	32	64	32	512	512	256	16-4-74	
C158	Preg. Horse added at Chall.				>32	64	1024	1024	1024	9-4-74	10-4-74

Days Post Vac. Birth Occurred	Days Post Chall. Birth Occurred	Virus Recovery from foal of fetus	Foral Neutralizing Ad Titers 3-14 Days of Age
47	NA	–	256
85	32	–	1024
50	NA	–	128
101	48	–	512
72	19	–	512
NA	NA	NA	NA
58	5	–	4
94	41	–	128
73	20	–	16
47	NA	–	128
79	26	–	NA
NA	NA	NA	NA
NA	NA	NA	NA
NA	17	I	NA
44	4		128

* C140 and C148 animals were male contact control

** Mare 154 did not care for her foal

(1) Died during delivery

(2) Aborted 1 day before vaccination

P = Prinicipal

C = Control

CC = Challenge Colt

CM = Challenge Mare

Continuation Experiment 2
(continuation)

Days Post Vac. Birth Occurred	Days Post Chall. Birth Occurred	Virus Recovery from foal or fetus	Floral Neutralizing Ad Titers 3-14 Days of Age
3	NA	–	NA
61	21	–	64
123	83	–	
84	44	–	64
55	15	–	512
NA	8	1	NA

EXPERIMENT 2 (continuation)
ERP Serum Neutralization and Pertinent Data

I.D.	20-2-74 1st Vac 0 day	+7	+14	+21	4-1-74 Chall. +40	+7	+14	+21	+35	Live Del.	Abort.	Death after Del.
Group 4 - Pregnant Mares Vaccinated Once with A183-V126-P55 Virus												
P166	0	4	256	256	128	128	64	16	32	NA		
P167	2	64	1024	1024	512	512	512	512	512	Preg?	19-2-74	
P169	4	64	128	512	128	256	128	128	64	5-4-74		
P170	32	128	256	1024	1024	512	256	256	128	23-6-74		
P171	4	64	128	64	128	64	64	256	512	27-5-74		
C168	16	8	4	4	16	32	1024	1024	512	Preg?		

Group 5 - ERP Serum Neutralization titers on Challenge Control Horses and Foals

	Chall	+7	+14	+21
C119	128	64	512	
CM174	8	16	4	128
CC174	2	0	16	2
CM175	32	32	512	256
CC175	0	0	2	32

Days Post Vac. Birth Occurred	Days Post Chall. Birth Occurred	Virus Recovery from foal of fetus	Foal Neutralization Ab Titers 3-14 Days of Age
NA	NA	–	NA
44	4	–	8
122	82	–	
96	56	–	1024

* C140 and C148 animals were male contact controls
** Mare 154 did not care for her foal
(1) Dies during delivery
(2) Aborted 1 day before vaccination

P = Principal
C = Control
CC = Challenge Colt
CM = Challenge Mare

The table in Experiment 2 above shows the safety and the efficacy of the attenuated ERP vaccine of the present invention. The horses were vaccinated and challenged and the date of Groups 1 and 2 which were vaccinated twice and Groups 3 and 4 which were vaccinated once, show both the safety and the efficacy of the attenuated ERP vaccine in the pregnant mares during and after pregnancy and no positive equine Herpes I vaccine virus was isolated from the newborn foals.

VIRUS ISOLATION TABLES

The number of ERP virus isolations following first and second ERP vaccinations are compared in the Virus Isolation Data Tables below. The number of ERP virus isolations made following vaccination and challenge are compared between vaccinates and control animals.

TABLE IV

Equipe Herpes Virus Isolations

Group 1 (A183-V26-P50) - Vaccinated Twice I.M.

I.D		1st Vac 0 day	+3	+5	+7	+10	+12	+14	2nd Vac 0 day	+3	+5	+7	+10	+12	+14
P65	BC			I	I					I					
	NS		+												
P70	BC				I	I	I								
	NS														
P150	BC			I	I	I									
	NS														
P155	BC			I	I	I	I	I	I	I					
	NS				I										
P157	BC			I	I	I	I								
	NS				I										
C140	BC										+	+	+		
	NS								+						
C74	BC														
	NS														

Group 1 (A183-V26-P50) - Vaccinated Twice I.M.

I.D.		Chall 0 day	+2	+4	+6	+7	+9	+11	+14	+17	+21	+23	+28	+48
P65	BC													
	NS													
P70	BC										I			
	NS													
P150	BC													
	NS													
P155	BC													I
	NS													
P157	BC													
	NS													
C140	BC			I	I	I	I							
	NS				I	I			I					
C74	BC			I	I	I	I		II					
	NS				I	I								

Group 2 (A183-V26-P55) - Vaccinated Twice I.M.

P151	BC			I	I	I	I	I							I
	NS														
P152	BC			I	I	I									
	NS						I								
P153	BC	II	I		I	II	I			II					
	NS														
P154	BC			I	I	I									
	NS														
P156	BC			I	I										
	NS														
P148	BC			II	+	+				II			II	II	II
	NS														
P159	BC														
	NS														

Equine Herpes Virus Isolations
(continuation)

I.D.		Chall 0 day	+2	+4	+6	+7	+9	+11	+14	+17	+21	+23	+28	+48
Group 2 (A183-V26-P55) - Vaccinated Twice I.M.														
P151	BC													
	NS													
P152	BC													
	NS													
P153	BC													
	NS													
P154	BC			I	I	I								
	NS													
P156	BC													
	NS													
P148	BC		II	I	I	I		I	I			II		
	NS													
P159	BC				I	I	I	I	I	I	I			
	NS			I	I	I	I	I	I	I				

* Herpes I Isolation Made at Parturition
P = Principal
C = Control
BC = Buffy Coat
NS = Nasal Swab
+ = Unidentified Virus
I = Equine Herpes I Virus
II = Equine Herpes II Virus

I.D.		Vac.	+5	+7	+14	Chall 0 day	+2	+4	+6	+7	+9	+11	+14	+17	+21	+28	+48
Group 3 (A183-V26-P50) - Vaccinated one I.M.																	
P160	BC		I	I													
	NS																
P161	BC		I	I													
	NS			I													
P162	BC			I													
	NS																
P164	BC		I	I													
	NS																
P165	BC				+												
	NS																
C163	BC							I		I	I						
	NS																
C158	BC							I		I	I	I	I				
	NS									I	I	I	I				
Group 4 (A183-V26-P55) - Vaccinated Once I.M.																	
P166	BC			I													
	NS																
P167	BC		I	I													
	NS																
P169	BC		I	I													
	NS																
P170	BC																
	NS				+												
P171	BC																
	NS																
C168	BC						I	I		I	I	I					
	NS									I	I						

Equine Herpes Virus Isolations
(continuation)

P = Principals
C = Controls
BC = Buffy Coat
NS = Nasal Swab
I = Equine Herpes I Virus
+ = Unidentified Virus

Group 5 - ERP Challenge Control Horses and Foals

I.D.		Chall. 0 day	+3	+4	+5	+7	+9	+12	+14	+21	+28
C119	BC	II			I	I	I				
	NS					I					
CM174	BC				I	I	I		I	I	
	NS										
CC174	BC		I			+					
	NS		I		I		+	I	I		
CM175	BC				I	I	I			I	
	NS										
CC175	BC		I		I	I	I		I		
	NS				I	I	I	I	I		

CM = Challenged Mare
CC = Challenged Colt
BC = Buffy Coat
NS = Nasal Swab
I = Equine Herpes I Virus
II = Equine Herpes II Virus
+= Unidentified Virus

Examination of the data reported in Groups 1 to 5 of Table IV above indicate the following:

1. The ERP vaccine was safe when used in pregnant animals in varying stages of pregnancy.

2. The pregnant mare vaccinates were protected from abortion and ERP symptoms following challenge. Three principal progeny died. One abortion occurred one day prior to vaccination and the start of the experiment. One foal died two days following birth from a neglectful mother and one foal died during delivery from a first foal mother. *Equine Herpes I* virus was not recovered from these three progenies. The two deaths which occurred while on test were attributed to chance and in no way connected with ERP, no *Equine Herpes I* isolates were recovered from the blood leukocytes or nasal turbinates from these foals.

3. The 12 challenge controls exhibited typical ERP symptoms - depression, febrile response, anorexia and leukopenia. Five suspected pregnant control mares presented one abortion and a dead foal at one day of age. Challenge virus was recovered from both of the these progenies. Two mares presented live normal foals; one fifteen days and the other, five days following challenge. No challenge virus was recovered from these two foals. It is questionable whether the remaining challenge mare was pregnant.

4. Substantial ERP passive immunity was passed in the colostrum to the progenies providing there was adequate time between challenge and parturition.

5. Based on non-rising ERP neutralizing serologies and negative virus isolation, the vaccine did not transfer to the two contact control mares or to the two stallions kept in confinement.

Experiment 3

The following experiment was designed to give information concerning (a) safety of the vaccine in foals, as exhibited by signs and clinical pathology examinations; (b) determine when a foal may be vaccinated in the face of maternal antibody; (c) what type of virus isolation pattern results in a two week old vaccinated foal; (d) efficaciousness of the ERP passive antibody.

The following parameters were measured in Experiment 3, Groups 1 through 4: body temperature, CBC, virus isolations from the nasal turbinates and blood leukocytes, ERP neutralizing serologies and daily observations for clinical signs of disease.

Group 1 - Colt ERP Vaccination Response:

This group of eight colts came from mares with a well documented history concerning their ERP status. The foals were I.M. vaccinated with a single freeze dried 1 ml dose of A183 V26 P55 ERP vaccine containing $10^{5.8}$ logs of virus/2 ml which was constituted with 2 ml of sterile water prior to use.

Group 2 - Colt ERP Vaccination Response:

a. Colt number 6 was an orphan colt. No ERP history was available on the orphan's mother.

b. Colt number 74 came from a challenge control mare which delivered a foal five days after an intranasal challenge of virulent ERP virus. The challenge virus was isolated from the mare, however, no ERP virus was isolated from the foal after parturition. It

was apparent that no increase in ERP colostrum antibody occurred in the short interval prior to birth, after challenge.

Group 3 - Colts Challenged in with Virulent ERP Virus:

This group of colts consisted of four which were challenged intranasally by catheter with 10 ml of virulent ERP virus which contained $10^{2.7}$ logs of virus/2 ml. The ERP history of the mares involved was well documented.

Group 4:

This group consisted of two colts coming from mothers with unknown ERP histories. The colts were challenged in a similar manner as the colts in group 3.

The results of this experiment are set out in the TABLES below.

EXPERIMENT 3
Colt and Mare Data Sheet

Group 1: Colt Vaccinates (from Mares Vaccinated and/or Challenge Prior to Foaling) Receiving one I.M. Dose of A-183 V26 P55 Virus

		Status of Mare at Parturition		Colt's ERP Neutralizing Antibody Titer			
Mare & Colt No.	Group Designation	Days Post First Vacc.	Days Post Challenge	ERP Neut. Titer	Hours or Days Post Parturition 0 to 16 hrs	3 to 10 days	Age and Neutralizing Ab Titer at Time of Vaccination and/or Challenge
48	1x Vac., no Chal.	59	NA	512	0	256	11 days -
65	2x Vac., no Chal.	47	NA	512	1024	256	23 days - 512
151	2x Vac., Chal.	94	41	256	0	128	9 days - 128
152	2x Vac., Chal.	73	20	128	4	16	10 days - 16
162	1x Vac., Chal.	61	21	256	32	64	10 days - 64
163	1x Vac., Chal.	55	15	512	4	512	22 days - 64
165	1x Vac., Chal.	84	44	128	32	64	7 days - 64
171	1x Vac., Chal.	96	56	256	512	1024	9 days - 1024

Group 2: Vaccinated Colts
Sub Group A (no History or mare)

6	No vac., no chal.	NA	NA	Info not available	Info not available	128	22 days - 64

Sub Group B (time inadequate for transfer of passive Ab from mare to foal)

74	No vac., chal.	NA	5	16	0	4	31 days - 4

Group 3: Challenge Colts (from Mares Vaccinate and/or Challenged Prior to Foaling) Receiving Wild-Type A-183 Virus I.N.

68	Chal., 1x Vac	78	483	512	512	1024	13 days - 256
70	2x Vac., Chal.	85	32	0	1024	1024	11 days - 512
155	2x Vac., Chal.	101	48	512	0	32	16 days - 64
157	2x Vac., Chal.	72	19	128	32	512	11 days - 512

Group 4: Challenge Control Colts (from Non-Vaccinated & Non-Challenged Mares) Receiving Wild Type A183 Virus I.N.

174	NA	NA	NA	Info not available	Info not available	Info not available	14 days - 0
175	NA	NA	NA	"	"	"	20 days - 0

EXPERIMENT 3 - PART B
FOAL VIRUS ISOLATION IDENTIFICATION AND/OR CHALLENGE

GROUP I – Vaccinates:

		+3	+5	+7	+9	+10	+12	+13	+14	+21	+27
48	BC			I	+						
	NS										
65	BC			I							
	NS										
151	BC			I	+						
	NS										
152	BC	I	I	I							
	NS		I	I	I						
162	BC		I	I							
	NS										
163	BC				I	I	I				
	NS										
165	BC		I	I	I						
	NS										
171	BC			I	I						
	NS										

GROUP 2 – Vaccinates:

6	BC		+	+							
	NS										
74	BC	I	I	I	II	II	II		II		
	NS								II	II	I

GROUP 3 – Foals with Passive ERP Ab - Challenged:

68	BC			I			I		I		
	NS										
70	BC		I	I					I		
	NS										
155	BC		I	I	I		I		I		
	NS		I	I	I		I				
157	BC		I								
	NS							I			

GROUP 4 – Challenge Control Foals

174	BC	I		+							
	NS	I	I		+		I		I		
175	BC	I	I	I	I				I		
	NS		I	I	I		I		I		

I = Equine Herpes I Virus
II = Equine Herpes II Virus
+ = Unidentified Virus

None of the vaccinated foals showed any adverse clinical signs. Occasionally, *Streptcoccus zooepidemicus* bacteria was isolated from nasal passages during infrequent nasal drainages which had no pattern in relationship to the ERP Vaccine.

None of the four challenged foals (Group 3) containing ERP colostrum antibody became sick or depressed like the challenged foals lacking ERP colostrum antibody.

It was apparent that the foals normal body temperature was higher than their adult counterparts. Also, environmental stress, such as handling the foals and laying in the hot sun appeared to have a greater influence on temperature fluctuations. A tendency toward a leukopenia appears to occur generally in the vaccinated foals between 3 to 7 days. The leukopenia is more severe in the challenged control foals than in the vaccinated foals.

A marked febrile response between 2 to 6 days after infection was noted in the challenged foals lacking ERP passive antibody. There appears to be no consistent temperature rise following ERP vaccination or ERP challenged foals with high levels of ERP passive antibody. One colt (155) exhibiting the lowest neutralizing ERP antibody (1:64) showed a temperature rise similar to control challenge colts, however, no depression or anorexis was noticed.

The following salient points may be extracted from experimental data obtained.

1. The Vero cell, cold-adapted (26°C) virus replicates *in vivo* when given I.M., producing a protective (humoral and cell mediated) immunity against virulent ERP challenge virus administered intranasally.

2. The vaccine virus is non-pathogenic when given to pregnant mares and to foals coming from various ERP immunological backgrounds.

3. The vaccine virus can be isolated from horses having had previous ERP exposure (as measured by ERP neutralizing antibody) or from animals with no neutralizing antibody. The virus may be frequently recovered from washed blood leukocytes or infrequently from nasal turbinates.

4. The ERP vaccine virus is not transmitted to contact control horses, based on sero non-conversion of contact control animals, and upon negative virus isolation data from control animals.

5. Neutralizing serologies from vaccinated animals compare favorably with those induced by virulent ERP challenge virus.

6. High titer colostral ERP antibody passively transferred to foals apparently protects (no visible symptoms) the foals against virulent challenge ERP virus.

7. Cell mediated immunity appears to play an important part in protecting horses against ERP abortions and clinical signs of ERP, however, no feasible way has yet been found to measure ERP cellular immunity other than direct challenge experiments.

The number of virus isolates recovered following vaccinations with a replicating ERP vaccine or ERP virulent virus exposure is dependent largely upon the type and degree of immunity that the host exhibits. Virus isolations from foals with high levels of colostral ERP neutralizing antibody are quite prevalent. These foals are considered to have humoral immunity, and which depending upon the degree, does ameliorate symptomatology when challenged with virulent virus. However, humoral immunity plays little, if any, part in shutting off a virus carrier state. In contrast, active immunity induced with virus infection, be it a replicating vaccine virus or virulent virus, induces both cell mediated and humoral immunity. It is apparent from the data that after re-exposure to replicating vaccine virus or virulent virus, the virus carrier state is practically eliminated; however, it is not absolute. This appears to exist in non-ERP sensitized animals, and in previously ERP sensitized animals, based on serologies. It is obvious that cellular immunity, like humoral immunity, wanes with time. The time interval involved with depletion of ERP cellular immunity is unknown at this time.

8. The ERP vaccine virus does not cross the placental barrier, based on negative netralizing pre-colostral serum samples from eight newborn foals. Also, no positive *Equine Herpes I* vaccine virus was isolated from 18 newborn foals, from vaccinated-challenged mares. It also appears that passively acquired ERP neutralizing antibody depletion curves on five foals in isolation are uninterrupted in their decline, indicating no in utero exposure of *Herpes I* virus as there appears to be no active induction of *Herpes I* antibody.

9. ERP vaccine virus isolations were more frequently recovered from blood leukocytes than from the nasal turbinates. This finding also applied to intranasally administered vaccine virus. Although only two principals out of four produced isolates, all were from blood leukocytes. Many of the nasal virus (vaccine) isolations recovered in the experiments may have originated from minute amounts of blood absorbed from capillaries damaged in the process of taking samples with six inch long nasal swabs from animals in motion. Of course, some nasal swab samples were lost due to bacteria and mould contamination.

10. Three overt cases of clinical strangles occurred, and many isolations of *Streptococcus zooepidemicus, Strep. equisimilis,* strep-Group E. *Staph Aureus* and one foal was found to have *Streptocossus equi.* These bacteria were periodically isolated from on-test animals. All of these bateria have the capability of producing clinical signs of fever and/or nasal drainage, therefore, they were frequently monitored to help to determine the cause of any clinical abnormality which might tend to confuse the results obtained from on-test animals. In most

instances, the bacterial flare-ups were quite obvious when the horse temperature and total leukocyte charts are reviewed.

Streptococcus zooepidemicus is frequently a companion agent with the Equine Rhinopneumonitis virus which is not surprising in view of its ubiquitous nature in the equine population. It would seem that any severe stress of the horse may incite *Strep. zooepidemicus* to flare up.

In one embodiment a virulent A183 isolate of an Equine I Herpes virus is introduced into a monolayer of a Vero permanent cell line which is incubated 3 to 5 days at 37°C and cytopathology of the cells was completed. A freshly prepared stable cell monolayer of Vero cells was inoculated with a portion of the produced harvested virus. The culture was again incubated to produce cytopathology of at least a portion of the tissue cells. Thereafter, a portion of the virus produced was serially passed at least 30 times at an incubation temperature of 26°C and the immunologically active line avirulent virus was recovered.

WHAT WE CLAIM IS:—

1. A process for the production of an immunogenically active attenuated live equine rhinopneumonitis herpes virus which comprises the steps of:

a. introducing a live virulent equine rhinopneumonitis virus into a tissue cell culture;

b. incubating the culture until the cellular cytopathology becomes evident;

c. harvesting a portion of the virus so produced and reintroducing the harvested virus into fresh tissue cultures;

d. repeating serial passage of the virus in tissue culture up to 300 times at an incubation temperature of 33°C or less, the number of passages and the temperature being sufficient to produce an immunogenically active live avirulent virus; and

e. recovering said virus from said tissue culture medium.

2. A process according to Claim 1 wherein said tissue cell culture is Vero cell culture.

3. A process according to Claim 1 wherein the incubation temperature of the serial passages is maintained at one within the range of from 23° to 33°C.

4. A process according to Claim 1 wherein the incubation temperature of each serial passage is about 26°C.

5. A process according to Claim 1 wherein the serial passage is carried out at least thirty times.

6. A process according to Claim 1 wherein at least 50 serial passages are carried out at incubation of from 23°C to 33°C.

7. A process for the production of immunogenically active attenuated live equine rhinopneumonitis virus which comprises the steps of:

a. introducing a live virulent equine rhinopneumonitis virus into a monolayer of a Vero permanent cell line;

b. incubating the culture from 3 to 5 days at a temperature of 37°C until cytopathology of the cells is substantially complete;

c. harvesting a portion of the virus so produced and introducing the harvested virus into a freshly prepared stable cell monolayer of Vero cells;

d. incubating said culture at a temperature of 26°C for a period of time sufficient to result in cytopathology of at least a portion of the tissue cells;

e. serially passing a portion of the virus so produced in stable vero cells at least 45 times at an incubation temperature of 26°C to produce an immunogenically active live avirulent virus; and

f. recovering said virus from said last serial passage.

8. An attenuated live equine herpes I virus vaccine containing an effective quantity of immunogenically active avirulent live equine herpes I virus produced by the process according to any one of Claims 1 to 7 adding said recovered virus to a stabilizing solution to form a vaccine therefrom.

9. A vaccine according to Claim 8 wherein said tissue cell culture is Vero cell culture.

10. A vaccine according to Claim 8 wherein the incubation temperature of the serial passages is maintained at one within the range of from 23° to 33°C.

11. A vaccine according to Claim 8 wherein the incubation temperature of each serial passage is about 26°C.

12. A vaccine according to Claim 8 wherein the serial passage is carried out at least thirty times.

13. A vaccine according to Claim 8 wherein at least 50 serial passages are carried out at incubation temperature of from 23° to 33°C.

14. An attenuated live equine rhinopneumonitis virus produced according to Claim 1 or Claim 7 and adding said recovered virus to a stabilizing solution to form the virus vaccine therefrom.

15. The method of protecting members of the family Equidae from equine rhinopneumonitis virus infection which comprises administering to said Equidae an effective dose of a live attenuated ERP virus vaccine when prepared by a process according to any one of Claims 1 to 6 or Claims 8 to 13.

16. A method according to Claim 15 wherein said tissue cell culture is Vero cell culture.

17. A method according to Claim 16 wherein the incubation temperature of the serial passages is maintained at one within the range of from 23° to 33°C.

18. A method according to Claim 16 wherein the incubation temperature of each serial passage is 26°C.

19. A method according to Claim 16 wherein the serial passage is carried out at

least thirty times.

20. A method according to Claim 16 wherein at least 50 serial passages are carried out at incubation temperatures of from 23° to 33°C.

21. The method of protecting equines from ERP virus infection which comprises administering to said equines an effective dose of a live attenuated ERP virus vaccine when prepared by a process according to Claim 14.

22. A process according to Claim 1 or Claim 7 for the production of an immunogenically active attenuated live equine rhinopneumonitis herpes virus substantially as hereinbefore described.

23. An attenuated live equine herpes I virus vaccine according to Claim 8 or Claim 14 substantially as hereinbefore described.

24. A method of protecting equidae from Equine Rhinopneumonitis virus infection according to Claim 21 substantially as hereinbefore described.

R.J. BOXALL
Chartered Patent Agent
Berkshire House,
High Holborn,
London WC1V 7AQ
Agent for the Applicants

Printed for Her Majesty's Stationery Office by MULTIPLEX techniques ltd., St. Mary Cray, Kent. 1980. Published at the Patent Office, 25 Southampton Buildings, London WC2 1AY, from which copies may be obtained.