CHAPTER I

EPIDEMIOLOGICAL STUDIES ON ASF IN DOMESTIC PIGS AND ARGASID TICK ORNITHODOROS MOUBATA

INTRODUCTION

In 1972 pig farmers living in the area of the northern Transvaal subject to statutory control of ASF (Fig. 1) made representations for the relaxation of these measures since ASF had not occurred in the area during the previous 9 years. In answer to this request and to prove that the virus of ASF was still a real threat to domestic pigs within the endemic area, the Department of Agricultural Technical Services, undertook a virological survey based on the experience of the research workers in East Africa (Plowright, Parker & Pierce, 1969b). The object of the survey was to establish whether argasid ticks Ornithodoros moubata porcinus Walton, 1962, under South African conditions, were reservoirs of the virus of ASF and to acquire further information on their epidemiological significance. While the survey was under way in 1973 in the western region of the endemic area, the disease broke out in the domestic pigs of the eastern region.

MATERIALS AND METHODS

Field samples

Porcine specimens. Samples of porcine tissues received for virus isolation were tested immediately on arrival. Ten per cent (w/v)

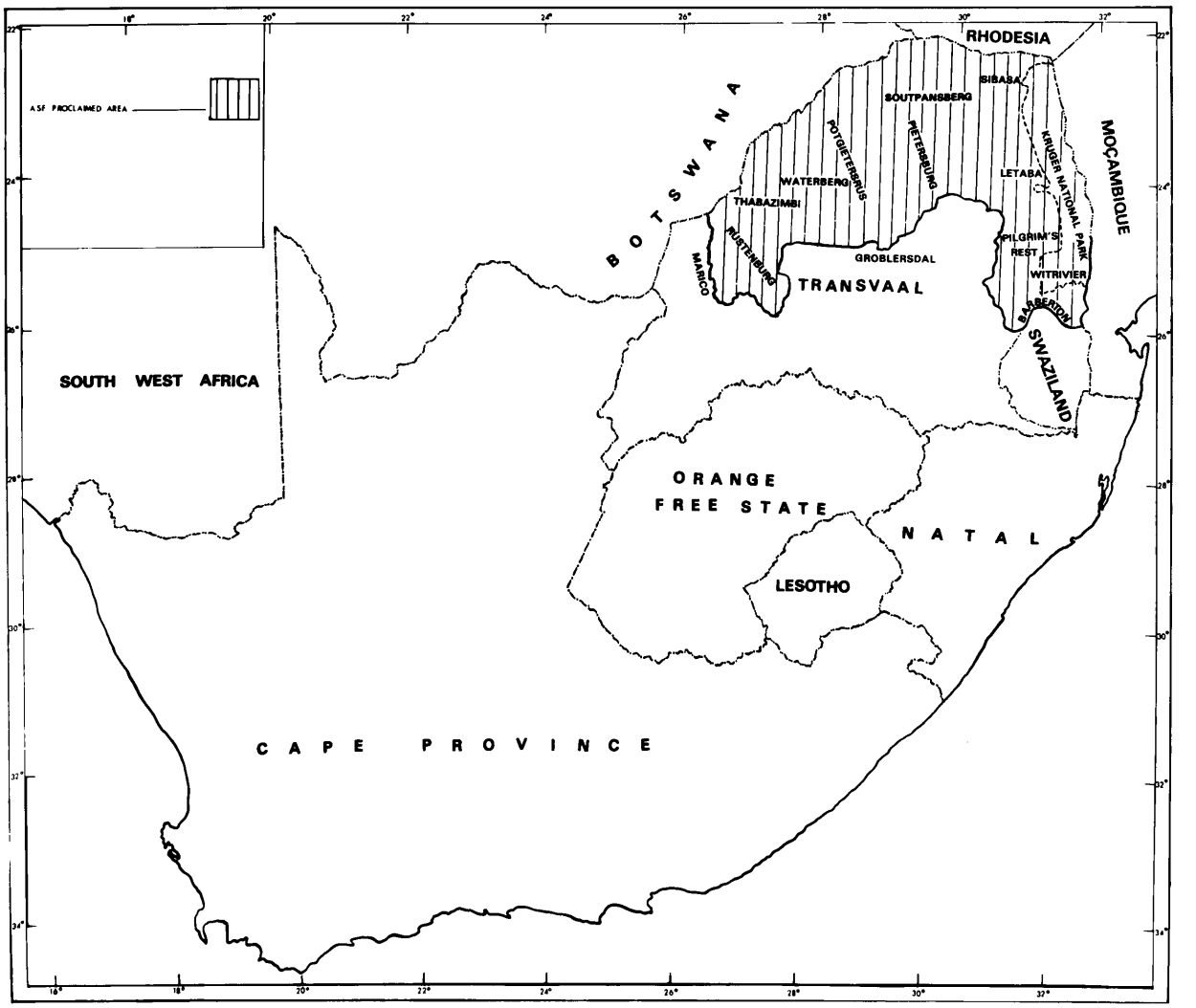


FIG. 1 Map of South Africa. The area in which ASF is endemic is indicated by shading.

suspensions were prepared in Eagle's medium (Macpherson & Stoker, 1962) containing 500 IU of penicillin, 500 ng of streptomycin and 250 ng of neomycin per ml. After 45 min incubation at 30°C, leukocyte cell cultures prepared in plastic tissue culture flasks were infected with 1 ml of the suspension. LLC-MK_2 cell cultures which preliminary experiments had shown to be susceptible to ASF virus were also used in this study.

If no evidence of haemadsorption and/or cytopathic effects could be demonstrated, the cultures were frozen and thawed three times and 1 ml transferred to a fresh culture. Three consecutive passages were carried out at weekly intervals before concluding that no virus was present in the material under examination.

Tick specimens. Ticks (Ornithodoros moubata) were collected from 246 burrows located on 170 farms in the districts of the Transvaal subject to statutory control of ASF. Twenty two farms located in the districts of Marico and Groblersdal (Fig. 1) outside the endemic area were also surveyed. Live ticks were collected and maintained by incubation at 30°C with 95% relative humidity according to the procedures described by Plowright et al. (1969a). Before maceration the ticks were identified (Walton, 1962) and the adults separated from nymphal forms. Unless otherwise stated 10 argasids were completely macerated in a Griffith's tissue grinder and suspended in 10 ml of Eagle's medium containing 500 IU of penicillin, 500 ng of streptomycin and 250 ng of neomycin per ml. The suspensions were centrifuged at 700 rpm (x g 82) for 3 min, after which the supernate was separated and leukocyte cell cultures infected. The cultures were observed for 10 days for the appearance of the haemadsorbing effect and/of cell lysis. Tick suspensions were considered negative if no virus could be isolated after three serial subcultures of the sample.

Bacterial contamination or cytotoxic effects were only occasionally observed in infected cultures. To eliminate the latter, it was often sufficient merely to double the dilution of the standard tick suspension.

To find the ratio of infected to non-infected ticks the Ornithodoros moubata from known infected burrows were subdivided into groups of two and suspensions prepared by maceration of each pair in 2 ml of Eagle's medium. After centrifugation the supernate was separated and stored at 4°C. Pools were prepared by mixing 0,5 ml of each of 5 samples and then used for screening purposes. When a pool was found

to be infected, the suspensions used to form it were tested individually and those found positive were eventually titrated.

Cell culture preparation

Leukocyte cell cultures. Leukocyte cell cultures from porcine buffy coat (BC) were prepared according to the method described by Tubiash (1963) using donor pigs of 30 to 60 kg in weight. Blood was drawn from the jugular vein by negative pressure into an Erlenmeyer's flask containing 10 IU of sodium heparin, 100 IU of penicillin and 100 ng of streptomycin per ml; after gentle shaking the blood was rapidly transferred into a sufficient number of 100 or 50 ml volumetric flasks. After incubation at $30^{o}C$ for periods varying between 35 and 60 min, to allow for sedimentation of the red cells, the supernatant plasma was separated and the number of leukocytes counted in a haemacytometer. Finally the plasma was diluted in Eagle's medium supplemented with 40% heterologous pig serum to give a final concentration of 1×10^{6} leukocytes per ml. According to the requirements, Leighton tissue culture tubes or 25 cm^{2} plastic tissue culture flasks were seeded with 2 or 8 ml of the above preparation respectively, incubated at $37^{o}C$ and used 48 h later. A sufficient number of residual erythrocytes was always present in this type of culture for demonstrating the haemadsorbing effect.

$LLC\text{-}MK_{2}$ cell culture. A culture of $LLC\text{-}MK_{2}$-derivative (MK) cells was obtained from the American Type Culture Collection, Rockville, Maryland, U.S.A.

On arrival the cells underwent 4 serial passages and a frozen stock was prepared by suspending them in Eagle's medium containing 20% bovine serum and 10% dimethyl sulphoxide. Storage was carried out in a liquid nitrogen cabinet. For routine use, cells were sub-cultured 40 to 50 times, then discarded and a new batch was initiated from the frozen stock. Dispersion of cells was carried out by treatment with citrate buffered saline pH 7,4 containing 0,02% sodium versenate and 0,25% trypsin (Difco). The dispersed cells, counted in a haemacytometer were resuspended in a growth medium consisting of Eagle's medium containing 10% Difco-tryptose-phosphate broth and

supplemented with 10% bovine serum. The following antibiotics were used: Penicillin 100 IU, Streptomycin 100 ng and Tylan (Elanco) 5 ng per ml. Standard tissue cultures or Leighton tubes were seeded with 1 x 10^5 cells, whereas 25 cm^2 plastic tissue culture flasks were seeded with 1 x 10^6 cells. Monolayers were used when 80-90% confluent usually after 48 h incubation at 37°C.

Unless otherwise stated maintenance medium used after infection of the cultures consisted of Eagle's medium supplemented with 2% bovine serum. The medium was changed once on the 6th day of incubation.

Infection of cell cultures and infectivity titrations

Cell monolayers. Unless otherwise stated cell monolayers were rinsed in 3 changes of phosphate buffered saline pH 7,4 (PBS). Tissue culture tubes were infected with a 0,2 ml inoculum while in plastic culture flasks an inoculum of 1 ml was used. Adsorption was carried out at 37°C for 120 min during which time tube cultures were rotated, while flasks were kept stationary. Thereafter the inoculum was removed and 1,5 ml or 8 ml maintenance medium was added to the tubes or flasks respectively.

In order to determine the infectivity, serial tenfold dilutions of viral suspensions were prepared in Eagle's medium and four roller tube cultures were infected with each virus dilution.

Unless otherwise stated cell cultures were examined daily under the microscope for a period of 12 days for specific cytopathic effects (Cpe).

Leukocyte cell cultures. When the BC cell cultures were used, the appropriate virus preparation was inoculated into the original culture medium. Leighton tissue culture tubes received an inoculum of 0,2 ml whereas in the flasks an inoculum of 1 ml was used. Cultures were kept stationary and observed for 10 days for the appearance of haemadsorption (HAd) and/or cell lysis.

Infective titres were calculated according to the method of Reed & Muench (1938) and the results were expressed as the $\log_{10}$ 50% tissue

culture infective dose ($TCID_{50}$) or 50% buffy coat haemadsorbing dose ($BCHAd_{50}$) or 50% buffy coat infective dose ($BCID_{50}$).

Immunofluorescence technique

Direct method. The anti-ASF IgG fluorescein isothyiocyanate conjugate, prepared according to the methods described by Bool, Ordas & Botija (1970), was obtained from Dr C. Sanchez Botija, Patronato Biologia Animal, Madrid, Spain.

Infected as well as uninfected BC or MK cell cultures cultivated on coverslips were removed from the incubator when required. After rinsing in 0,01M PBS, cells were air-dried and fixed in cold acetone for 10 min. After a further rinsing they were overlaid with the conjugate diluted in 0,01M PBS and held for 45 min at 37°C in a humidified chamber. Cells were rinsed again in 3 changes of PBS and the coverslips were mounted in 50% phosphate buffered glycerol. Observations were carried out under a Leitz Orthoplan Microscope provided with a fluorescent vertical illuminator equipped with a HBO-200 W high pressure mercury vapour lamp. The following filter combination was used: 4 mm BG 38, KP 500, K 515 and S 525.

Indirect method. In the technique for detecting antibody to ASF virus, infected as well as uninfected cell cultures prepared and treated as above were overlaid with serial twofold dilutions of serum in 0,01M PBS. After 45 min at 37°C, cells were rinsed according to the standard procedure and overlaid for 45 min with a commercial rabbit-anti-porcine IgG fraction conjugated with fluorescein isothiocyanate (Miles-Seravac) diluted in 0,01M PBS. After further rinsing, coverslips were mounted and observed as described above. A positive reference control ASF immune serum was kindly supplied by Dr C. Sanchez Botija. A serum was considered negative when antibody to ASF virus could not be detected at a dilution of 1/2.

Identification of virus

In all instances where it was considered necessary, the presence or absence of ASF virus in cell cultures was confirmed by immuno-fluorescence and/or negative or positive stained preparations examined

by electron microscopy (Els & Pini, 1977).

The haemadsorbing strains of virus were designated HAd^+ whereas those devoid of this characteristic were designated HAd^-.

RESULTS

ASF in domestic pigs

The 21 outbreaks of the disease in domestic pigs that occurred between May 1973 and December 1975 were grouped into 8 primary foci of infection as shown in Table 1. The first of these was reported in the eastern part of the Letaba district on a farm called Lillie-148.

TABLE 1 Occurrence of ASF in domestic pigs in the endemic area of the northern Transvaal between May 1973 and December 1975

Focus	No. of outbreaks	Month/Year	District	Virus isolated
1	1	5/1973	Letaba	HAd-
2	1	6/1973	Letaba	HAd+
3	4	6-8/1973	Pietersburg North	HAd+
4	7	9-11/1973	Pietersburg East/Letaba	HAd+
5	5	12/1973-1/1974	Witrivier	HAd+
6	1	3/1974	Thabazimbi	HAd+
7	1	7/1975	Potgietersrus	HAd+
8	1	11/1975	Pietersburg	HAd-

The origin of infection was traced to a warthog killed on the property and from which the meat was used for human consumption. The second focus occurred approximately a month later in the same district on a farm 35 km away from the previous one. Although the origin of the infection was not established, it is known that wild pigs were present in the area. The 3rd focus was recorded in the Pietersburg

district between June and August 1973. Three of the 4 piggeries affected were on adjoining farms but the source of infection could not be traced. In the 4th piggery approximately 60 km away the animals were fed meat scraps obtained from a local butcher who had purchased meat from one of the 3 farms primarily affected. The 4th focus of infection occurred in the eastern part of the Pietersburg district and adjoining farms in the Letaba district. Between the end of September and the end of November, 1973, seven outbreaks were confirmed. They probably all had a common origin and the disease spread through the movement of infected pork products. The 5th focus was recorded in the Witrivier district between December 1973 and January 1974. All 5 farms affected adjoin one another and wild pigs are found in the area. The 6th and 7th foci of disease occurred in the Thabazimbi and Potgietersrus districts in March 1974 and July 1975 respectively. Only one farm in each district was affected. It is known from the results of a survey described elsewhere that ticks Ornithodoros moubata infected with the virus of ASV were present in the warthog burrows in both areas. The last outbreak identified as 24823 was confirmed in November 1975 in the Pietersburg district from specimens taken from a dead pig from a farm where all swine had died over an unspecified period of time.

In 19 of the 21 outbreaks the disease had a peracute or acute course and diagnosis was confirmed by the appearance of the haemadsorbing effect in BC cell cultures infected with spleen or lymphnode tissue suspensions. HAd was observed in all instances within 24 h after infection of the cell system. In all cases the virological diagnosis was supported by histological examination of organ material. From the remaining 2 specimens, Lillie-148 and 24823, HAd^- strains of ASF were isolated (Pini & Wagenaar, 1974; Pini, 1976).

At the onset of the disease on farm Lillie-148, mortality was high and approximately 120 pigs out of 150 died within the first 2 weeks. During the following 7 weeks mortality continued but at a much reduced rate and a further 23 animals were lost. During the 8th week the remaining 7 apparently healthy animals were destroyed. Clinical observations on the affected animals were recorded on four different occasions. Temperature reactions in affected pigs varied

between 40 and 40,5°C and was accompanied by symptoms such as ataxia, hyperaemia of the skin of the abdominal area, diarrhoea and dyspnoea. At autopsy, moderate splenomegaly, generalized enteritis, congestion of the renal cortex and a marbled appearance of the lymphnodes was observed. In the seven apparently healthy animals killed during the 8th week no gross pathological changes were observed.

The results of histological studies carried out on organs taken from dead pigs were inconsistent. In the tissues of some animals, diffuse karyorrhexis of the nuclei of the endothelial cells could be seen, whereas in others no pathological changes of diagnostic significance were present.

Spleen, liver, kidney and lymphnode specimens were received on 3 different occasions at weekly intervals for virus isolation. In BC cell cultures infected with spleen or lymphnode suspensions cell lysis without HAd was observed between the 6th and 8th day of incubation, while with the other tissues the same changes were seen between the 9th and 12th day. When undiluted virus preparations were subinoculated on three consecutive occasions into freshly prepared BC cell cultures, lysis without HAd was seen after 4 to 5 days of incubation. HAd⁻ASF virus was isolated from the same organ suspensions in MK cell cultures, where Cpe were observed after a mean incubation time of 3 days.

During the outbreak identified as 24823 the only symptom reported was ataxia; no other reliable information was available. Specimens were not suitable for histological examination because of the advanced state of putrefaction. Spleen and liver suspensions were used to infect BC and MK cell cultures. On the 6th day post-inoculatation cell lysis without HAd was observed in the former cell culture and Cpe in the latter. The absence of haemadsorbing characteristics was confirmed by passaging the virus suspension on three consecutive occasions in BC cell cultures with similar results.

ASF virus in argasid ticks Ornithodoros moubata

The results of the survey summarized in Table 2 shows that 27.6% of the farms and 25% of the burrows were found to harbour ticks infected with the virus of ASF. A total of 15 597 ticks were examined, 22% were in the adult stage and 78% in one of the five nymphal stages.

TABLE 2 Distribution of farms and burrows infected with the virus of ASF

District	No. farms Tested	Infected	%	No. burrows Tested	Infected	%
Marico*	16	2	12,5	16	3	12,5
Rustenburg	25	9	36,0	56	14	25,0
Thabazimbi	86	23	26,7	114	33	28,9
Waterberg	21	6	28,5	33	8	24,2
Potgietersrus	26	7	26,9	31	4	12,0
Pietersburg	1	0		1	0	
Groblersdal*	6	0		6	0	
Letaba	7	4	57,4	7	4	57,4
Pilgrim's Rest	4	2	50,0	4	2	50,0
TOTALS	192	53	27,6	268	67	25

*Outside the controlled area

Virus was isolated from 15% of the suspensions prepared from adult ticks and 12% of those prepared from nymphal stages. A mean number of 57 ticks per collection was counted but this represented only a very small number of the tick population present in the burrows. The ratio between the number of ticks tested and the number of burrows found infected, is shown in Table 3. The percentage of burrows found to be inhabited by infected ticks, increased from 20,1 to 66,6% when the number of argasids tested increased from 10 to 200 or more per burrow.

TABLE 3 Ratio between number of ticks tested and burrows found infected with ASF virus

No. of ticks per collection	No. of burrows Tested	Infected	%
10 - 49	154	31	20,1
50 - 99	87	22	25,2
100 - 199	20	8	40,0
200 or >	9	6	66,6

Estimation of viral contents. Collections of ticks which were found infected with the virus of ASF in the screening tests were subsequently titrated. During the interim, the preparations had been stored at $4^{o}C$ for periods not exceeding 12 days. It was assumed that no significant loss of infectivity would have occurred during the above intervals because of the stability of the virus. The infective titres of the suspensions are given in Table 4. Those of the adult ticks varied between $10^{4,2}$ and $10^{6,2}$ $BCHAd_{50}$ per suspension with a mean value of $10^{5,2}$ whereas those of the nymphal stages varied between $10^{3,7}$ and $10^{5,2}$ $BCHAd_{50}$ with a mean value of $10^{4,5}$.

Number of infected ticks. Adults and nymphal ticks from known infected burrows were subdivided into groups of two and suspensions prepared as previously described.

The results are reported in Table 5. It appeared that out of 520 adult ticks and 460 nymphae the mean percentage of infected argasids was 3,45 and 1,62 respectively. The results of the infectivity titrations of the positive suspensions gave results comparable to those obtained in the previous experiment.

TABLE 4 Infective titres of tick suspensions

	Tick suspension No.	$BCHAd_{50}$/suspension
Marico	190 A*	5,2***
	190 N**	4,2
Potgietersrus	237 A	5,2
	238 A	5,2
	238 N	5,2
Waterberg	233 A	5,7
	233 N	4,7
Thabazimbi	231 A	6,2
	231 N	5,2
	219 A	5,2
	219 N	4,7
	212 A	4,7
	212 N	4,7
Letaba	304 A	4,2
	304 N	3,7
	302 A	5,2
	302 N	4,2
	299 A	6,2
Pilgrim's Rest	298 A	4,7
	298 N	4,7

* Adult ** Nymphae *** Log 10

TABLE 5 Estimated number of infected ticks and their infective titre

	Adults	Nymphae
No. ticks	520	460
No. pools* infected / tested	9 / 52	4 / 46
No. suspensions** infected / tested	12 / 260	5 / 230
No. infected ticks %	12 - 24 2,3 - 4,6	5 - 10 1,08 - 2,16
Mean infectivity	5,4***	4,3

*Each pool = 5 suspensions

**Each suspension = 2 ticks

***Log 10 $BCHAd_{50}$/suspension

Appearance of the haemadsorbing effect in BC cell cultures infected with ASF virus from ticks. During the course of the survey it was observed that infected BC cell cultures were showing HAd of variable intensity at various intervals post-inoculation.

While with 72,7% of the pools the effect appeared after a mean incubation time of 3 days, with 12,5% it appeared between the 5th or 7th day of incubation. The remaining 14,8% of the preparations showed HAd on the 2nd serial passage only. From the results of the infectivity titrations it became evident that the delay and paucity of HAd could not always be correlated with a low viral content.

Segregation of a HAd^- and HAd^+ strain of ASF virus from a tick suspension. Adult tick suspension TS237 which had been found to have an infective titre of $10^{5,2}$ $BCHAd_{50}$ (Table 4) was used for this trial. BC cell cultures infected with the suspension showed a weak HAd effect

involving only a few cells followed by a rapid and total cell lysis occurring between the 6th and 7th day post-infection. Other tick suspensions with similar titres nevertheless showed strong, generalized HAd within the first 3 days of incubation.

The virus suspension at the 1st BC passage was diluted 10^{-1} and subinoculated into freshly prepared cell cultures. Weak and delayed HAd was again observed but the 3rd subculture showed typical HAd on the 2nd day post-infection, which became generalized by the 3rd day. For the 4th passage, decimal dilutions ranging from 10^{-1} to 10^{-6} were made and used to infect BC and MK cell cultures. The results of the 2 titrations are summarized in Table 6. The titre in BC was obtained on the 7th day post-infection, whereas in MK cells it was determined on the 10th day. In BC cell cultures inoculated with the 10^{-1} and 10^{-2} virus dilution HAd was observed on the 2nd day of incubation and was generalized by the following day. However, in the cultures infected with the 10^{-3} virus dilution HAd never involved more than a few cells whereas at the 10^{-4} dilution HAd was not observed at all. By the 7th day all the cells in the cultures infected with the 10^{-3} and 10^{-4} virus dilutions were lysed. The results of the titration in MK cells as detected by direct immunofluorescence indicated that virus was present in the 10^{-4} and 10^{-5} dilutions and suggested that the cell lysis observed in BC cell cultures was specifically caused by ASF virus.

TABLE 6 Infectivity titrations in BC and MK cell monolayers of strain TS237 of ASF virus at the 3rd passage in BC cultures

	BC cell cultures		MK cell cultures	
	HAd	Lysis	Cpe	FAT*
10^{-1}	+	+	+	+
10^{-2}	+	+	+	+
10^{-3}	+	+	+	+
10^{-4}	-	+	-	+
10^{-5}	-	-	-	+
10^{-6}	-	-	-	-

*Immunofluorescence

Passages in BC cultures were continued by the method of limiting virus dilutions as shown in Table 7 using those cultures in which only lysis of the cells was recorded and the presence of the virus confirmed by direct immunofluorescence. At the 5th passage, cell lysis only was seen in the cultures which received the 10^{-5} virus dilution whereas those infected with the lower dilutions also showed HAd. The infective titres recorded at the 6th, 7th, 8th and 9th passages were lower than expected. HAd was observed in the cultures infected with the 10^{-1} and 10^{-2} or 10^{-1}, 10^{-2} and 10^{-3} dilutions of virus whereas lysis was seen in the cultures infected with the 10^{-3} or 10^{-4} virus dilution respectively. At the 9th passage level only cell cultures which received the 10^{-1} virus dilution showed HAd which occurred within the first 24 h of incubation. In the cultures infected with the 10^{-2} and 10^{-3} dilutions only lysis was seen. When the limiting virus dilution was titrated in what represented the 10th serial passage, HAd was not recorded in any of the infected cell cultures although infectivity estimated by cell lysis on the 7th day of incubation was $10^{5,5}$ $BCID_{50}$ per 0,2 ml inoculum.

TABLE 7 Serial passages in BC cell cultures of limiting virus dilutions of strain TS237

Virus dilution Log 10	Passage No.											
	5		6		7		8		9		10	
	HAd	Lysis	HAd	Lysis	HAd	Lysis	HAd	Lysis	HAd	Lysis	HAd	Lysis
-1	+	+	+	+	+	+	+	+	+	+	-	+
-2	+	+	+	+	+	+	+	+	-	+	-	+
-3	+	+	+	+	-	+*	+	+	-	+*	-	+
-4	+	+	-	+*	-	-	-	+*	-	-	-	+
-5	-	+*	-	-	-	-	-	-	-	-	-	+
-6	-	-	-	-	-	-	-	-	-	-	-	-

*Chosen for following subculture

From the 10th passage onwards, subcultures were carried out twice a week by inoculating BC cells with a 10^{-1} dilution of virus. Only cell lysis withou HAd was observed at each serial passage up to the 25th, at which stage subculturing was temporarily stopped and the identity of the virus stock prepared was confirmed once more by immunofluorescence.

In a parallel series the same virus suspension at the 3rd passage level in BC cell cultures was serially subcultured 25 times in the same cell system, by using the 10^{-1} dilution of virus instead of the limiting dilution. HAd increased in intensity and by the 4th passage appeared within the first 24 h of incubation and affected all the cells by the 2nd day.

Infectivity titrations carried out at the 5th, 15th and 25th passages gave a mean titre of $10^{6,0}$ $BCHAd_{50}$ per 0,2 ml. Cell lysis was never observed in the absence of HAd.

By means of the above mentioned procedures strain TS237 was separated into two sub-strains one of which was HAd^{+} and the other HAd .

CONCLUSIONS

From the 21 outbreaks of ASF that occurred between 1973 and 1975 in domestic pigs inhabiting the endemic area of the Transvaal, 19 HAd^{+} and 2 HAd virus isolates were obtained. With the HAd^{+} strains of virus the haemadsorbing effect was observed in BC cell cultures in the first 24 h post-infection. For the isolation of the HAd strains, BC cell cultures were not considered entirely satisfactory because cell lysis did not appear before the 6th day post-infection and its specificity had to be confirmed by supplementary tests.

In the outbreaks from where HAd^{+} strains of virus were isolated the disease had an acute course. On the contrary, pigs on the farm Lillie-148 from where the first HAd^{-} strain of ASF was recovered were affected by a less virulent form of disease. The outbreak 24823 occurred in an isolated area where all pigs had died over an

unspecified period of time and clinical observations were not recorded by the owner. An HAd strain of ASF virus was isolated from the tissues taken from a carcass in an advanced state of putrefaction, that apparently represented the last pig on the property.

The reservoir state of Ornithodoros moubata was demonstrated in the collections obtained in the endemic area from warthog burrows.

Infected ticks were however, also detected in three of 16 burrows in the Marico district that adjoin the endemic area but where ASF in domestic pigs has never been recorded.

The infective titres of suspensions prepared from adult ticks were higher than titres of suspensions prepared from ticks in the nymphal stages, the mean infectivity was $10^{5,2}$ and $10^{4,5}$ $BCHAd_{50}$ per suspension respectively. The success rate of isolating ASF virus increased with the number of ticks tested and from the results of the experiment it was anticipated that only a low percentage of Ornithodoros moubata were in fact infected. This was later confirmed by testing pairs of 520 adult ticks and 460 nymphae. It was found that the mean percentage values for the two groups were 3,45 and 1,62 respectively.

During the course of this investigation it was observed that leukocytes infected with tick suspension TS237 showed weak HAd, while cell lysis was a predominant feature. This phenomenon was apparently due to the isolation of a virus population having both HAd^{+} and HAd^{-} components.

CHAPTER II

SUSCEPTIBILITY OF CELL CULTURE LINES TO ASF VIRUS

INTRODUCTION

The confirmation of the diagnosis of ASF by the isolation of the causative agent has presented certain difficulties since the virus may be considered fastidious when cultivated in experimental systems. Malmquist & Hay (1960) were the first to succeed in propagating ASF virus in cell cultures using leukocytes from porcine blood or bone marrow. In this system the virus replicates readily producing haemadsorption of swine erythrocytes followed by cell lysis. The leukocyte system, however, has drawbacks which include techniques of preparation and maintenance, fragility during handling and variables in the haemadsorbing characteristics of the virus. This situation has prompted investigators to look for alternative cell culture systems.

Plowright & Ferris (1956-1957) were unsuccessful in adapting strains of virus to primary pig kidney and testis cells. Greig, Boulanger & Bannister (1967) propagated six strains of virus in primary pig kidney cells, but only five of them produced Cpe after incubation periods varying between 30 and 100 days, depending upon the strain studied.

Pig kidney cell lines were also tested for their susceptibility to ASF virus. Malmquist (1962) observed Cpe, of variable intensity, after prolonged incubation of strains of virus in the PK-2a cell line. This author concluded that the cytopathic characteristics were not firmly established because after one passage in pigs the re-isolated virus did not reproduce the same Cpe. Sanchez Botija

(1963a) encountered difficulties in identifying cellular changes in pig kidney cell cultures using five different strains of virus. Only two of them produced cytopahtic changes after 90 and 180 days of incubation respectively.

Cell lines from tissues of other animal species have also been investigated. A strain of ASF virus, serially passaged in BC cell cultures, was cultivated in BHK cells (Stone & Hess, 1967), but no details in respect of any Cpe were published. Vigàrio, Relvas, Ferraz, Ribeiro & Pereira (1967) failed to induce the formation of infective virus in a line of African green monkey kidney cells. Hess (1971) was able to adapt two strains of ASF virus to the Vero cell line and Cpe were observed after two consecutive passages carried out at weekly intervals but no further details were published. More recently Enjuanes, Carascosa, Moreno & Vinuela (1976a) using ASF virus which had undergone serial passages in porcine leukocytes demonstrated early Cpe in Vero cells after eight consecutive sub-cultures carried out at weekly intervals.

During the preliminary stages of the virological survey designed to establish the role of ticks Ornithodoros moubata in the maintenance of ASF virus in the endemic area, it was anticipated that it would have been desirable, for the reasons discussed, to have had available a cell system for use as an alternative or complement to the BC cell cultures.

MATERIALS AND METHODS

Virus

For the preliminary investigations strain CV of ASF virus which had been isolated in 1961 from an outbreak of disease in domestic pigs and stored at -70°C was used. The 5th passage of the virus in BC cell cultures was subcultured and a fresh stock was prepared and stored in 1,0 ml aliquots at -70°C.

The other 10 strains 2055, 6367, 9746, 9192, 8061, 10615, 11018, 14918, Lillie-148 and 24823 represented field material and were obtained during the outbreaks of ASF which occurred in the northern Transvaal between 1973 and 1975. They were stored in 1,0 ml aliquots at -70°C as 10% (w/v) spleen suspensions prepared in Eagle's medium. Eight of these isolates were HAd^+ while two, Lillie-148 and 24823, were HAd^-. Their identity had been previously confirmed by direct immunofluorescence and/or electron microscopy.

Cell cultures

The origin and maintenance of MK cell cultures have been described. Vero, MDBK and Hela cell lines were obtained from the American Type Culture Collection. Pig kidney Stice cell cultures were received through the courtesy of Dr Derbyshire from the Institute for Research on Animal Disease, Compton, England and MS cells from Dr Ozawa while working at the Razi Institute, Teheran, Iran.

All the cell lines metioned above were maintained and propagated by a procedure similar to that described for MK cell cultures.

Infection of cell cultures

For screening purposes or serial passages, monolayers prepared in plastic flasks were infected with 1,0 ml of undiluted virus suspension, unless otherwise indicated.

To assess the susceptibility of the cell lines, 3 consecutive passages were carried out at weekly intervals. On the 7th day of incubation the infected monolayers were frozen and thawed three times at -20°C and the medium and cell mixtures were used to infect freshly prepared cell cultures. At the completion of the 3rd serial passage the cultures were examined by direct immunofluorescence and subinoculated into BC cells to assess whether ASF virus was present in the absence of microscopic cellular changes.

Staining of cell cultures with haematoxylin and eosin

In order to detect inclusion bodies, MK cell cultures cultivated on 20 x 8 mm coverslips were rinsed in PBS, fixed in Bouin's fluid and stained with haematoxylin and eosin according to standard procedure.

Preparation of porcine erythrocytes for the detection of HAd

Packed porcine erythrocytes, washed in three changes of PBS, were resuspended in Eagle's medium and added to cell cultures at a final concentration of 1/500.

RESULTS

Preliminary assessment of the susceptibility of cell lines to ASF virus

When strain CV of ASF virus was used to test the susceptibility of different cell lines, no evidence of viral replication or viral survival was observed at the 3rd serial passage in MDBK and Hela cells. Pig kidney Stice and MS cell cultures did not show evidence of Cpe, but the presence of ASF virus was confirmed by immunofluorescence and subinoculation into BC cells.

Moderate Cpe were observed in Vero cells within 48 h after primary infection. The effects consisted mainly of focal rounding of the cells which progressed slightly during the ensuing incubation period. In the 2nd and 3rd serial passage Cpe remained focal in nature. The presence of ASF virus was confirmed by the auxiliary tests previously indicated.

MK cell monolayers showed evidence of Cpe in the first passage 48 h after infection and this became more pronounced during the ensuing incubation period with the result that on the 6th day the monolayers were completely destroyed. On 2nd and 3rd passage, similar

Cpe with complete destruction of the cells were again observed. Immunofluorescence and subinoculation in BC cell cultures confirmed the presence of ASF virus. These results appeared to indicate that MK cell cultures were the most suitable system for the detection and cultivation of ASF virus.

In order to exclude the presence of contaminating agents as a possible cause of Cpe, uninfected as well as infected cultures of MK cells were examined on 3 successive occasions by standard bacteriological techniques and by electron microscopy, using positive and negative staining methods. Other than ASF virus in the infected cultures, no evidence of contamination could be found.

The Cpe in MK cell cultures

The Cpe observed in MK monolayers infected with the CV strain of ASF virus could be differentiated into four stages over a period of 6 days.

The early effects (scored +) involved the rounding of cells in small foci of approximately 10 to 20 cells in contrast to scattered round cells sometimes observed in normal non-infected monolayers. Twenty-four to 48 h later the foci became larger and increased in number (scored ++) until the majority of the cells were affected and started to detach from the flask's surface (scored +++). The last stage (scored ++++) was characterized by complete rounding and massive detachment of cells. The development of the Cpe is illustrated in Fig. 2 to 6.

In preparations stained with haematoxylin and eosin the uninfected cells had an expanded polygonal cytoplasm, whereas those infected had a reduced and rounded cytoplasmic mass with an apparent increased affinity for basophilic stains. The nucleus was in an eccentric position and often showed an indentation which seemed to be caused by a large intracytoplasmic eosinophilic inclusion body structurally resembling a ball of thread and with the nucleoli fragmented. These findings were similar to those observed by immunofluorescence where fluorescent inclusion bodies

FIG. 2-5 Development of Cpe in MK cell cultures infected with strain CV of ASF virus. x 75

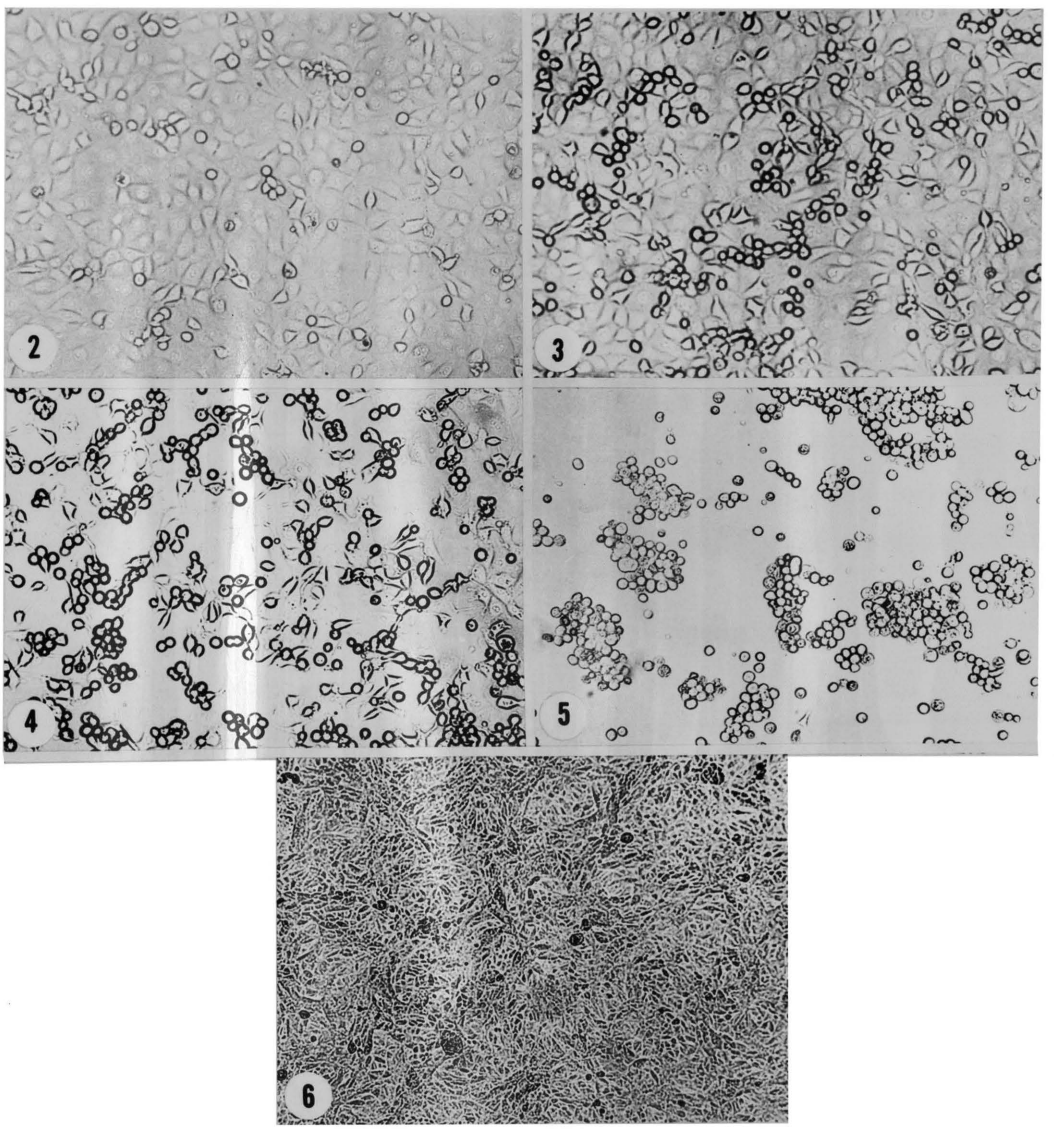
2 Stage 1 (+). Early Cpe

3 Stage 2 (++). Foci of Cpe enlarging.

4 Stage 3 (+++). Large number of cells affected, some detaching.

5 Stage 4 (++++). Complete destruction of monolayer.

FIG. 6 Uninfected control culture. x 30



usually appeared as rounded conglomerates, sometimes composed of 2 to 3 smaller aggregates pushing the nucleus to an eccentric position.

The assessment of the susceptibility of MK cell cultures to strains of ASF virus

To assess the susceptibility of MK cell cultures and to compare their sensitivity to that of the BC cell system the following experiments were conducted.

Experiment 1. Ten per cent (w/v) spleen suspensions were prepared from specimens 2055, 6367, 9746, 9192, 8061, 10615, 11018, 14918, Lillie-148 and 24823. They were used to infect MK cell monolayers prepared in plastic tissue culture flasks which were observed daily for a period of 7 days. The results are given in Table 8. The earliest Cpe were observed between the 1st and 4th day post-infection in all the specimens examined, but under the conditions of this experiment, Cpe did not progress beyond stage 2. The identity of the virus isolates was confirmed by immunofluorescence

TABLE 8 Isolation of ASF virus and progression of Cpe in MK cell cultures.

Specimen No.	Progression of Cpe						
	Days post-inoculation						
	1	2	3	4	5	6	7
2055 HAd^+	.	.	.	+	+	++	++
6367 HAd^+	.	.	+	+	++	++	++
9746 HAd^+	.	+	+	++	++	++	++
9192 HAd^+	.	+	+	+	++	++	++
8061 HAd^+	+	++	++	++	++	++	++
10615 HAd^+	.	.	+	+	++	++	++
11018 HAd^+	.	.	.	+	++	++	++
14918 HAd^+	.	.	+	++	++	++	++
Lillie-148 HAd^-	.	+	++	++	++	++	++
24823 HAd^-	.	.	.	.	+	++	++

Experiment 2. Five of the 10 specimens employed in the previous experiment were selected because of their ability to produce Cpe in MK cell cultures at various intervals of time after infection. Serial dilutions of the original spleen suspensions ranging from 10^{-1} to 10^{-6} were used to infect BC and MK cell cultures prepared in Leighton tissue culture tubes with and without coverslips. In the former system infective titres were estimated by the appearance of HAd and/or cell lysis and in the latter system by the appearance of Cpe and immunofluorescence.

The results of the infectivity titrations are given in Table 9. Early Cpe (+) in MK cell monolayers were observed between the 3rd and 6th day post-infection with a mean incubation time of 4,2 days. Virus titres between $10^{2,5}$ and $10^{5,0}$ $TCID_{50}$ per 0,2 ml were obtained, by the observation of Cpe, between the 6th and 12th day post-infection with a mean incubation time of 9 days.

TABLE 9 Infectivity titrations of spleen suspensions in MK and BC cell cultures

Specimen No.	Viral Titres			
	MK		BC	
	Cpe	FAT*	HAd	Lysis
8061	5,0**	5,5	5,5	
14918	2,5	3,5	4,5	
2055	2,5	3,5	5,5	
6367	2,5	3,0	5,5	
Lillie-148	2,5	3,5	None	2,5

*Direct immunofluorescence

**Log 10 ID_{50}/0,2 ml

Strong specific fluorescence was detectable within 24 h post-infection with the 10^{-1} virus dilutions and the infective titres for the haemadsorbing strains of virus were obtained by this technique between the 2nd and the 5th day post-inoculation with a mean incubation time of 3,5 days. The titre of the HAd^- Lillie-148 strain was obtained by immunofluorescence within the first 24 h.

The BC cultures proved to be 3 to 1 000 times more sensitive than MK cells in estimating infectivity of the haemadsorbing field strains of virus and the titres were obtained following a mean incubation time of 5,5 days. With the HAd^- strain of virus, lysis of the cells at the 10^{-1} virus dilution could be observed only on the 7th day post-infection and a titre of $10^{2,5}$ $BCID_{50}$ per 0,2 ml was obtained by the 10th day.

The adaptation of ASF virus to MK cell cultures

Although the results of Experiments 1 and 2 indicated that field strains of ASF virus replicate in MK cell cultures with Cpe, it appeared that a period of adaptation was required to enhance multiplication of the virus and progress of Cpe. To determine the limits of the adaptation period, 3 strains of ASF virus, Lillie-148, 2055 and 14918, isolated in MK cell cultures were serially subcultured using the 10^{-1} dilution of virus. Infectivity titrations were carried out at the 3rd, 6th and 9th passages using viral suspensions harvested when the Cpe appeared to have reached maximum intensity.

The results of this experiment are given in Table 10. Between passage 1 and 3 only the first and second stages of the Cpe were observed, but the infectivity at the 3rd subculture was higher than that obtained in primary isolation. Complete adaptation of the 3 strains of virus occurred between the 3rd and 6th passage, at which stage complete Cpe and maximum viral infectivity was obtained. However, the mean incubation time to reach the end point of the titrations remained fixed at 9 days. There was no difference in the viral titres established by immunofluorescence and observation of Cpe at the 6th and 9th passage levels.

TABLE 10 Infective titres of 3 strains of ASF virus following serial passages in MK cell culture

Passage No.	Titre estimated by	Virus strain		
		Lillie	14918	2055
3	Cpe	4,5*	4,0	4,0
6	Cpe	6,0	6,0	6,5
	FAT**	6,0	6,0	6,5
9	Cpe	6,2	5,5	6,0
	FAT	6,2	5,5	6,0

*Log 10 $TCID_{50}$/0,2 ml **Immunofluorescence

The haemadsorbing effect in MK cell cultures infected with ASF virus

Monolayers of MK cells were infected with the following strains of ASF virus at their 12th passage in MK cells: $CVHAd^{+}$, 2055 HAd^{+} and Lillie-148 HAd^{-}. Pig erythrocytes were added at various intervals to infected as well as to uninfected cell cultures.

In the cultures infected with strains CV and 2055, HAd followed the appearance of the Cpe and could be observed within 4 to 6 h after the addition of the erythrocytes. The degree of HAd was proportional to that of the Cpe. In the initial stage of Cpe, rosettes of erythrocytes were at first seen only around individual cells but when Cpe were advanced the majority of cells were haemadsorbing and cluster formations were common (Fig. 7). Some of the clusters could be seen floating in the microscopic field. Removal of the infected medium and rinsing of the monolayers before the addition of erythrocytes did not appear to have any influence on HAd. This effect was never observed in uninfected cell cultures or in those infected with the HAd^{-} Lillie-148 strain (Fig. 8).

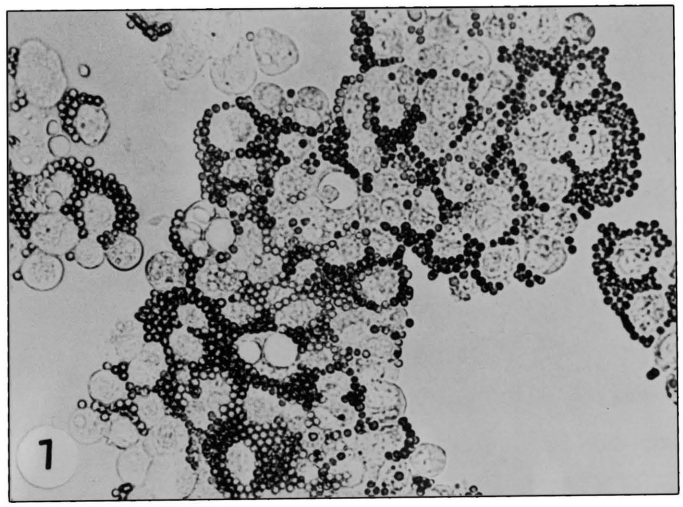


FIG. 7 MK cell culture infected with strain 2055 HAd^{+} of ASF virus showing haemadsorbed pig erythrocytes. x 200

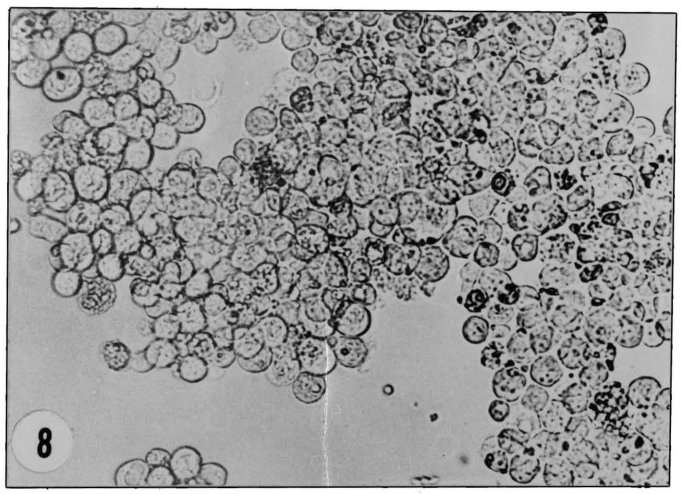


FIG. 8 MK cell culture infected with strain Lillie-148 HAd^{-} of ASF virus with negative haem-adsorption. x 150

CONCLUSIONS

From these results it appears that the selected strains of ASF virus replicate in MK cell cultures producing Cpe on primary isolation. Cpe were observed any time between the 1st and 5th day post-infection, with variations apparently dependant upon the strain and concentration of virus in the inoculum. The infective titres of 2 strains, 8061 HAd^+ and Lillie-148 HAd^-, were comparable when simultaneously assessed in BC and MK cell cultures. With the other three virus isolates the mean infective titre in BC cell cultures was $10^{2,6}$ times higher than in MK cell monolayers. To obtain the full progression of the Cpe and peak infective titres, the strains had to be serially subcultured in MK cells four to six times. MK cell monolayers, showing Cpe produced by HAd^+ strains of virus, haemadsorbed porcine erythrocytes.