STRAINS OF AFRICAN SWINE FEVER VIRUS
ISOLATED FROM DOMESTIC PIGS
AND FROM
THE TICK ORNITHODOROS MOUBATA
IN SOUTH AFRICA

BY

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SUMMARY

Between 1973 and 1975, 21 outbreaks of ASF were confirmed
in the endemic area of the northern Transvaal after an interval
of 10 years in which the disease was silent. The new series of
outbreaks coincided with the isolation, for the first time in
South Africa, of two HAd^- strains of ASF virus. The first of
these virus isolates, Lillie-148, was obtained from swine which,
judging from circumstantial evidence, had been infected by a
warthog carrier of virus. The pigs on the farm were affected by
a form of disease with a lower pathogenicity than that observed
in previous epidemics. The second HAd^- strain 24823 was obtained
from a case from which neither clinical nor pathological obser-
vations were available. From the results of the biological tests
carried out at the laboratory, however, it was deduced that the
disease in the field may have had a chronic course.

When the carrier status of populations of the argasid tick
Ornithodoros moubata collected from warthog burrows was in-
vestigated, it was found that the situation in South Africa is
analogous to that in East Africa. Twenty five per cent of burrows
were found to be infected; the mean infective titres of the tick
suspensions varied between $10^{4,5}$ and $10^{5,2}$ $BCHAd_{50}$ and the mean
percentage of infected argasids varied between 1,62 and 3,45.
Infected ticks were also found in the Marico district, which is
adjacent to the endemic area, but ASF has never been recorded there.
From tick suspension TS237, showing both delayed and reduced haemad-
sorbing effect in buffy coat cell cultures, a HAd^- population of
ASF virus was segregated.

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From these observations it was inferred that ASF virus may mutate from the HAd^+ to the HAd^- form in the primary virus reservoir Furthermore, the virus appears to be evolving towards less pathogenic forms irrespective of prior adaptation of the infectious agent to domestic stock under the epidemiological conditions prevailing in South Africa.

During this investigation it was found that $LLC-MK_2$ cell cultures were susceptible to ASF virus. Cytopathic effects were observed in primary isolation and peak infectivity coincided with complete destruction of the cell monolayers, attained after three to four serial passages. The sensitivity of $LLC-MK_2$ cells for estimating the virus content of porcine tissues was in two instances comparable to that of buffy coat cells, but in another three cases it was 100 to 1000 times lower. It was concluded that $LLC-MK_2$ cells were a suitable complement to buffy coat cultures for the cultivation of ASF virus, particularly for HAd^- isolates.

After 35 to 45 serial passages in $LLC-MK_2$ cells the HAd^+ strains of ASF virus lost their haemadsorbing characteristics. A similar mutation, but more gradual, was also observed in buffy coat cell cultures.

The feasibility of plaque production was studied in $LLC-MK_2$ cell monolayers. Plaques were obtained with all the strains studied, irrespective of their adaptation to $LLC-MK_2$ or buffy coat cells when 0,4% Agarose was used as a solidifying agent. The diameter of plaques ranged from 0,3 to 3,0 mm and this characteristic was unrelated to the haemadsorbing properties of the strains used. Plaque technique was successfully used to detect the presence of HAd^- virus particles in HAd^+ populations by subculturing selected virus-plaques into buffy coat cultures.

The results of biological tests suggested that HAd^- strains have a reduced virulence which can vary within broad limits. The experience with strain Lillie-148 and 24823 showed that either acute or chronic or subclinical disease can follow infection of pigs with these isolates of virus. The results obtained with the two virus populations of strain TS237 emphasized the different degree of pathogenicity between HAd^+ and HAd^- virus. While the former was responsible

for a peracute or acute form of disease, the latter produced
chronic or subclinical infections. In pigs mild forms of ASF
also developed following the inoculation of HAd$^+$ strains obtained
after serial passages in cell cultures. It was concluded that
haemadsorption and pathogenicity are two characteristics that are
not linked and can be modified independantly.

OPSOMMING

Gedurende die tydperk 1973-1975 het Afrikaanse varkpes (AVP),
na 'n afwesigheid van 10 jaar, weer sy verskyning gemaak in die
endemiese gebied van Noord Transvaal en altesaam 21 bevestigde
gevalle is aangemeld. Die nuwe reeks uitbrake het saamgeval met
die eerste isolasie in Suid-Afrika van twee $\overline{\text{HAd}}$ stamme van AVP.
Die eerste virusstam wat geïsoleer is, was Lillie-148. Hierdie
virusstam is geïsoleer van 'n vark wat volgens omstandigheids-
getuienis deur 'n vlakvark besmet is. Die virus waarmee die varke
op die plaas besmet is, het 'n laer patogenisiteit gehad as virusse
van vorige uitbrake. Die tweede $\overline{\text{HAd}}$ stam nl. 24823 is verkry van
'n geval waar geen kliniese of patologiese waarnemings beskikbaar
was nie. Uit die resultate van laboratoriumtoetse is die gevolg-
trekking gemaak dat die siekte wel moontlik 'n kroniese verloop kon
gehad het.

Uit ondersoeke na die vektorstatus van populasies van die sagte
bosluis Ornithodoros moubata, wat verkry is uit vlakvarkgate, is ge-
vind dat die situasie in Suid-Afrika soortgelyk is aan dié in
Oos-Afrika. Daar is bevind dat 25 persent vlakvarkgate besmet is;
dat die gemiddelde virus konsentrasies van bosluissuspensies varieer
tussen $10^{4,5}$ en $10^{5,2}$ BCHAd$_{50}$ en dat die gemiddelde persentasie van
besmette bosluise wissel tussen 1,62 en 3,45. Besmette bosluise
is ook aangetref in die Marico-distrik wat aangrensend is aan die
ensoötiese gebied en waar AVP nog nooit voorgekom het nie. 'n $\overline{\text{HAd}}$
populasie van AVP virus is geïsoleer van 'n bosluissuspensie, TS237,
wat in wit selkulture 'n vertraagde en verminderde heem-adsorberende
effek getoon het.

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Uit hierdie waarnemings is die gevolgtrekking gemaak dat AVP virus in die aanvanklike virus reservoir instaat is om van HAd$^+$ na HAd$^-$ te muteer. Hieruit blyk dit dat onder die huidige epidemiese toestande, wat tans in Suid-Afrika heers, die virus skynbaar verander na 'n vorm van laer patogenisiteit. Dit geskied ongeag vroeëre aanpassing van die infektiewe agens by die plaaslike varkpopulasie onder heersende epidemiologiese toestande in Suid-Afrika.

Gedurende hierdie ondersoek is dit aangetoon dat LLC-MK$_2$ selkulture vatbaar is vir AVP virus. Primêre virus isolasies toon sitopatogeniese effekte. Infektiwiteit bereik 'n piek na drie tot vier agtereenvolgende oorspuitings met algehele vernietiging van sellae. Die gevoeligheid van LLC-MK$_2$ selle vir die bepaling van die virus inhoud van varkweefsel was in twee gevalle vergelykbaar met dié van wit selle. In drie ander gevalle was dit 100 tot 1000 keer laer. Die gevolgtrekking is gemaak dat LLC-MK$_2$ selle 'n geskikte aanvulling is vir wit selkulture vir die kweek van AVP virus, veral vir HAd$^-$ isolate.

Die heem-adsorberende eienskappe van die HAd$^+$ stam van AVP virus het verlore gegaan na 35 - 45 agtereenvolgende oorspuitings in LLC-MK$_2$ selle. In wit selkulture is 'n soortgelyke mutasie waargeneem, hoewel dit meer geleidelik plaasgevind het.

Die moontlikheid van plaket vorming in LLC-MK$_2$ sellae is ondersoek. Wanneer 0,4% agarose as stollingsagens gebruik is, het alle stamme wat ondersoek is plakette opgelewer ongeag of hulle aangepas was vir LLC-MK$_2$ selle of wit selle. Plakette se deursnee het gewissel tussen 0,3 en 3,0 mm. Hierdie eienskap is egter nie gekorreleer met die betrokke stamme se heem-adsorberende eienskappe nie. Die teenwoordigheid van HAd$^-$ virus partikels in HAd$^+$ populasies is aangetoon deur subkulture van geselekteerde plakette in wit selkulture te maak.

Uit die resultate van biologiese toetse is die gevolgtrekking gemaak dat die HAd$^-$ stamme 'n verlaagde virulensie het wat kan wissel tussen wye grense. Die ondervinding met stam Lillie-148 en stam

24823 het aangetoon dat varke wat met hierdie virus stamme besmet raak akute, kroniese of subkliniese siekte toestande ontwikkel. Die graad van verskil tussen die patogenisiteit tussen HAd^+ en HAd^- virus is beklemtoon deur die resultate wat verkry is met die twee virus populasies van stam TS237. Die HAd^+ stam veroorsaak perakute of akute vorms van die siekte terwyl HAd^- stam kroniese of subkliniese infeksie tot gevolg het. Matige vorme van AVP is ook verkry nadat varke geïnokuleer is met 'n HAd^+ stam wat 'n aantal oorspuitings in selkulture ondergaan het. Die afleiding is gemaak dat heem- adsorpsie en patogenisiteit twee eienskappe is wat nie verbonde is nie en dus onafhanklik van mekaar gemodifiseer kan word.

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I N D E X

INTRODUCTION

African swine fever (ASF) is an infectious disease of domestic pigs caused by a DNA virus (Haag & Larenaudie, 1965; Plowright, Brown & Parker, 1966; Enjuanes, Carascosa, Moreno & Vinuela, 1976b) that has been provisionally included in the family Iridoviridae (Fenner, 1976). The electron microscopic studies reported by Breese & De Boer (1966), Almeida, Waterson & Plowright (1967), Moura Nunes, Vigàrio & Terrinha (1975) all confirm its icosahedral symmetry. The virion appears to have at least two capsid layers surrounded by an outer envelope and an average diameter of 204 nm (Els & Pini, 1977). The number of capsomeres has not been counted, but it has been estimated to be in excess of 812 (Almeida et al., 1967)

Leukocyte cell cultures are the only system where the virus of ASF replicates without requiring previous adaptation (Malmquist & Hay, 1960). The erythrocytes that are added or are present in this system become adsorbed to the infected cells and this phenomenon has been used for some years as a convenient diagnostic test.

Infectivity of the virus is destroyed by treatment with ether, chloroform and heating at 60°C for 20 min but is not affected by variations of pH within the range of 4 to 13,4 (DeTray, 1963; Plowright & Parker, 1967).

The virus of ASF is indigenous to the African continent south of the Sahara. In this area warthogs (Phacochoerus aethiopicus), bushpigs (Potamochoerus porcus), giant forest hogs (Hylochoerus meinertzhageni) and argasid ticks (Ornithodoros moubata) may act as viral reservoirs (Steyn, 1932; De Kock, Robinson & Keppel, 1940; DeTray, 1963; Heuschele & Coggins, 1965; Plowright, Parker & Pierce, 1969a).

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The disease made its appearance with devastating effects, at the beginning of the century when the balance between the natural host and the infectious agent was altered by the introduction of domestic pigs into Africa (Montgomery, 1921). The virus has now escaped from this continent and has become established in the Iberian penisula (Manso Ribeiro, Azevedo, Teixeira, Braco Forte, Ribeiro, Oliveira, Noronha, Pereira & Vigàrio, 1958; Manso Ribeiro & Azevedo, 1961; Anonymous, 1961). The disease has also occurred in France (Larenaudie, Haag & Lacaze, 1964), in Italy (Mazaracchio, 1968) and in Cuba (Anonymous 1971).

In South Africa where stringent control measures have been systematically implemented, the virus has on the whole been success-fully contained in the natural reservoirs inhabiting the endemic area of the northern Transvaal and there is no indication, at present, that domestic pigs are playing a role in the epidemiology of the disease (Pini & Hurter, 1975). The lesions observed under field conditions are those of the acute form consisting of degenerative changes of the lymphoid tissue and vascular system leading to oedema, infarction, necrosis and extensive haemorrhages (Hess, 1971).

In Portugal, Spain and other countries where the virus has become established in the domestic swine population, ASF may be regarded as an evolving disease characterized by slow progression and mild patho-genicity (Hess, 1971; Coggins, 1974). The number of animals which survive the infection is increasing and chronic, subclinical and inapparent forms of disease have been reported (Manso Ribeiro, Nunes Petisca, Frarao & Sobral, 1963; Nunes Petisca, 1965; Sanchez Botija, 1965; Vigàrio, Terrinha & Moura Nunes, 1974). The lesions seen in swine affected by these types of infection include pericarditis, pneumonia, lymphadenitis, hepatitis and meningoencepha-litis (Moulton & Coggins, 1968).

Since pigs which recover from the infection normally withstand challenge with a homologous but not with a heterologous strain, it has been concluded that several immunological types exist (Walker, 1933; Henning, 1956; Malmquist, 1963). Systematic investigations into this aspect have been hampered, however, by the absence of demonstrable neutralizing antibodies in sera of pigs surviving the natural or experimental infection (Hess, 1971). These sera have an inhibitory

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effect on the haemadsorption of the erythrocytes to the infected
leukocytes but do not inhibit subsequent cell lysis (Malmquist, 1963;
Carnero, Larenaudie, Ruiz Consalvo & Haag, 1967; Coggins, 1968a).
This inhibition of haemadsorbtion appears to be isolate specific
(Hess, 1971). By this technique Vigàrio et al. (1974) have grouped
nine strains of virus into three serological groups while five
further isolates could not be classified antigenically as they were
devoid of haemadsorbing characteristics.

In South Africa the first recorded outbreaks of ASF occurred in
the Potgietersrus district of the northern Transvaal in 1926
(Steyn, 1928).   The control measures that were applied consisted of
slaughtering both affected and in contact swine, destruction of
manure, a prohibition on the restocking of farms within a period of
3 months and control over the movements of live pigs and pork products.
In spite of these measures, outbreaks of disease occurred in the
Cape Province between 1933 and 1939. The infection, which had apparently
entered the region through the movement of pigs from the Transvaal, was
eradicated by 1939, because the control measures which were implemented
did not allow the virus to become established in the domestic swine
population or in any other reservoir (Pini & Hurter, 1975). On the
contrary, in the endemic area of the Transvaal, where the eradication
of ASF would require the elimination of the natural virus reservoirs,
the disease still persists and between 1926 and 1972, it exhibited an
apparent cyclic occurrence. Two cycles representing active disease,
the first lasting 13 years from 1926 to 1938 and the second 12 years from
1951 to 1962, have been observed. In the intervening periods between
1939 and 1950 and between 1963 and 1972 the virus was apparently inactive.

The opportunity to undertake this investigation was provided by
the re-occurrence of the disease in 1973, 1974 and 1975 and by a
virological survey conducted to determine whether argasid ticks
Ornithodoros moubata play a role in the epidemiology of ASF.

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
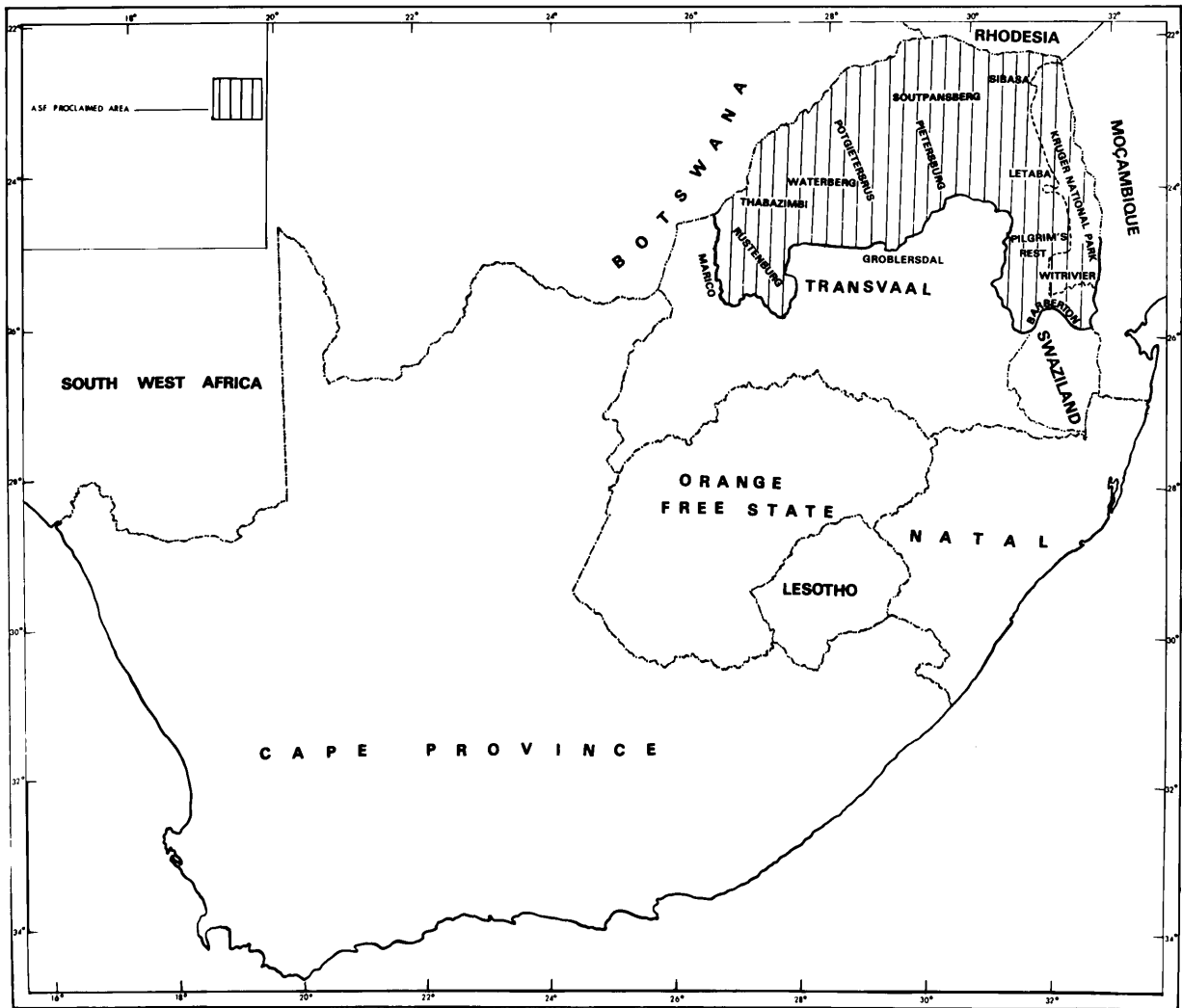isolation were tested immediately on arrival. Ten per cent (w/v)

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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

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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°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°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-MK$_2$ cell culture. A culture of LLC-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 1×10^5 cells, whereas 25 cm^2 plastic tissue culture flasks were seeded with 1×10^6 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 $37^{o}C$ 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 $37^{o}C$, 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

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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 haemad-sorbing 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 <u>Ornithodoros</u> <u>moubata</u>

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			No. burrows		
	Tested	Infected	%	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°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 suspen-
sions 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 in-
fectivity 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* $\frac{\text{infected}}{\text{tested}}$	$\frac{9}{52}$	$\frac{4}{46}$
No. suspensions** $\frac{\text{infected}}{\text{tested}}$	$\frac{12}{260}$	$\frac{5}{230}$
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

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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

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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 <u>Ornithodoros moubata</u> 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 <u>Ornithodoros moubata</u> 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 experi-
mental 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

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(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 $-70^{o}C$ 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 $-70^{o}C$.

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

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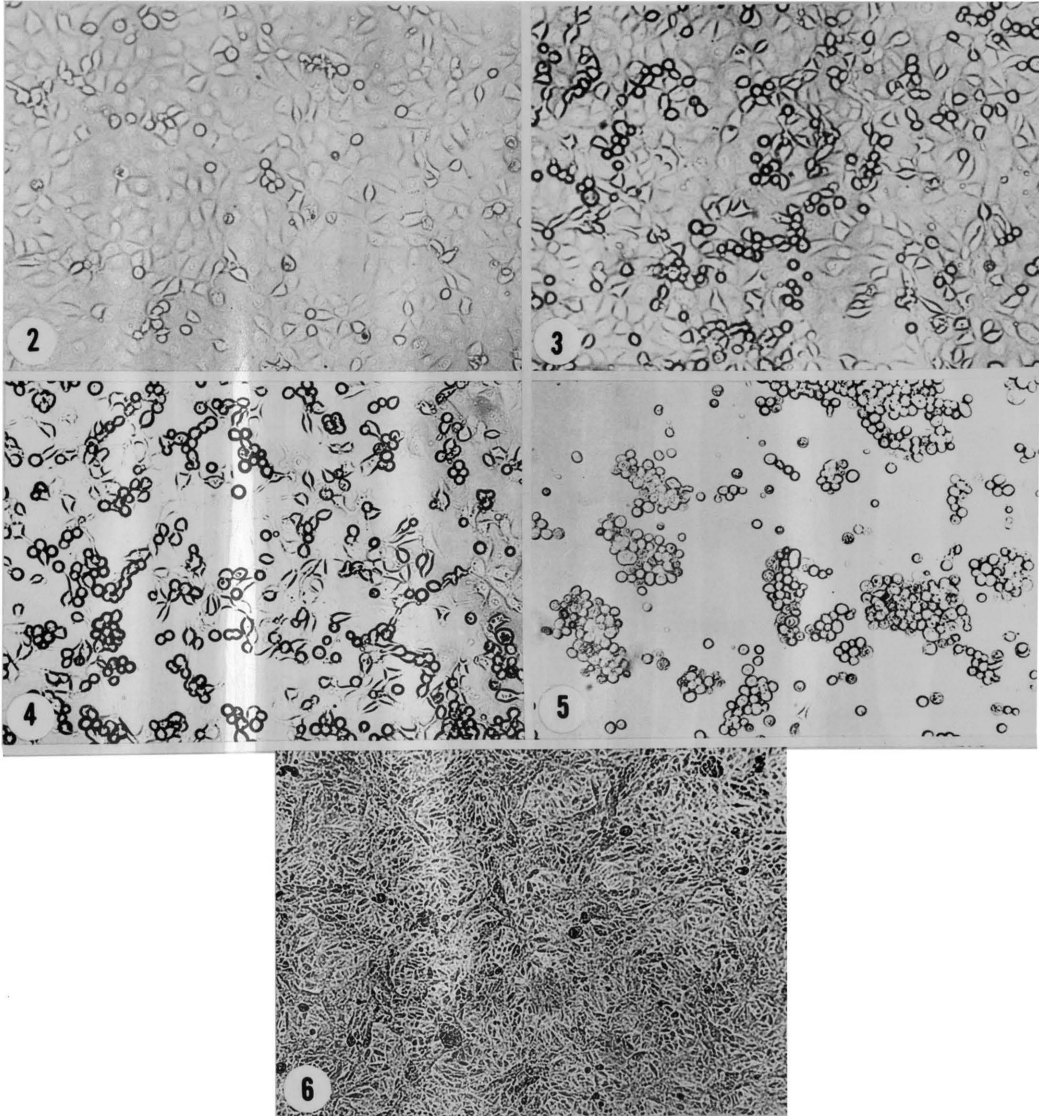
usually appeared as rounded conglomerates, sometimes composed of
2 to 3 smaller aggregates pushing the nucleus to an eccentric
position.

<u>The assessment of the susceptibility of MK cell cultures to</u>
<u>strains of ASF virus</u>

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 immuno-fluorescence.

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.

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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).
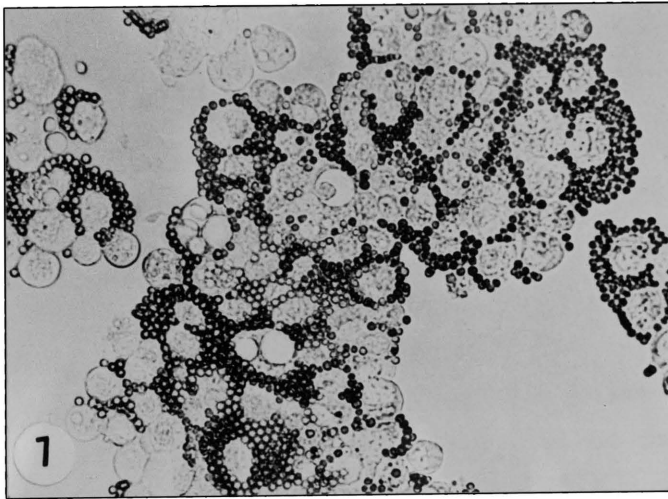
28

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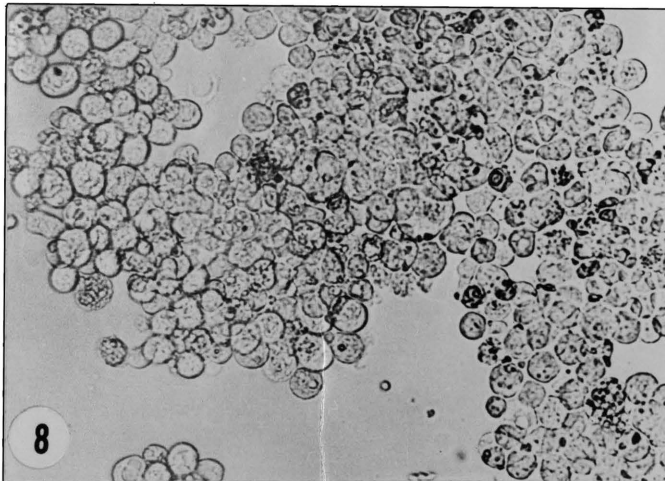

FIG. 8   MK cell culture infected with
         strain Lillie-148 HAd$^-$ of ASF
         virus with negative haem-
         adsorption.   x 150

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.

CHAPTER   III

REPLICATION   OF   ASF   VIRUS   IN
LLC MK$_2$ CELL CULTURES

INTRODUCTION

Studies on the replication of ASF virus in cell culture
lines are scarce and probably this is because of the difficulties
encountered in adapting this virus to various cell systems.

Plowright et al., (1966) reported on the growth curve of
ASF virus in pig kidney cell lines and on the inhibitory effects
of 5-iodo and 5-bromo-2' deoxyvridine and Actinomycin-D.
Coggins (1966) studied the growth of three isolates in PK-2a
and BC cell cultures. Cpe were observed on about the 5th day
post-inoculation when the infective titres were in excess of
10^5 TCID$_{50}$.   Virus release was slower in pig kidney than in
BC cells and the level of cell associated virus exceeded that
of the free virus through out the replicative cycle. Pig
kidney cells were less sensitive than BC cells in determing
infective titres. This author also established that ASF virus was
unaffected by trypsin, freezing and thawing and ultrasonic
treatments.

Because the results of the previous experiments indicated
that MK cells could be used as a complementary system to the
BC cell cultures, it was decided to study the replication and
the maximum yield of harvested virus in MK cell monolayers.

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MATERIALS AND METHODS

Virus

Two strains of virus, Lillie-148 HAd$^-$and 2055 HAd$^+$, both
at the 12th passage in MK cells, were used. The arabic number
after the type of cell culture, indicates the passage level at
which the strain was used.

Viral adsorption

Replicate titrations were carried out by infecting MK cell
monolayers with serial dilutions of both virus preparations ranging
from 10^{-1} to 10^{-7}. Adsorption was allowed to take place for fixed
intervals at 37°C in a rotating apparatus. At the end of each interval,
one set of titrations was rapidly removed from the incubator, the inoculum
discarded and each culture tube washed in 4 changes of PBS. After the
addition of maintenance medium the cultures were returned to the incubator.

Assessment of the amount of virus adsorbed to cells after 120 min incubation

MK cell monolayers in tissue culture tubes were infected with 0,2ml
of the two undiluted virus preparations and rotated while incubating at
37°C for 120 min. A control aliquot of the virus suspension was maintained
at 37°C for 2 h. After completion of the incubation period the inoculum was
removed and the monolayers washed in 4 changes of PBS; the cells were then
scraped and harvested in Eagle's medium and centrifuged. Cells recovered
from five tubes were considered equivalent to 1ml of inoculum. They
were disrupted by freezing and thawing and titrated together with the
control aliquot.

Growth curves

Monolayers in culture tubes were infected as for the previous
experiment. At the end of the 120 min adsorption period they were
washed in 4 changes of PBS and the maintenance medium added.

Five culture tubes were taken at random, immediately after the addition of the medium and at intervals of 24 h. They were subsequently frozen and thawed on 3 successive occasions, pooled and titrated.

Virus cell-association and the effect of freezing and thawing

The 10^{-1} dilutions of the two virus preparations were used to infect flasks containing monolayers of MK cells.

Three flasks were removed from the incubator at intervals corresponding to the four stages of the Cpe. The cells were scraped with the aid of a rubber policeman after which the contents of the three bottles were pooled, homogenized and an aliquot taken. The remaining culture fluid was centrifuged at 3 200 r p m (x g 1717) for 30 min, the supernate was decanted and stored at 4°C. The sedimented cells were washed three times in PBS. After the last centrifugation the cells were resuspended in a volume of maintenance medium equal to that of the original. Infectivity titrations were carried out with each preparation obtained.

Samples of medium and cell mixtures, obtained after generalization of Cpe from the experiment above, were frozen and thawed three times. Titrations were carried out before and after centrifugation at 3 200 r p m for 30 min.

RESULTS

Each result reported is the mean of three experiments conducted under identical conditions.

Viral adsorption

The results of the infectivity titrations conducted to establish the rate of adsorption with the two strains of ASF

virus are given in Fig. 9. It was concluded that adsorption was complete after an incubation period of 120 min.

Assessment of the amount of virus adsorbed to cells after 120 min incubation

While the mean infectivity of the inoculum of strain Lillie-148 before adsorption was $10^{5,35}$ that of cell adsorbed virus was $10^{4,1}$ $TCID_{50}$. With strain 2055 the viral titres were $10^{5,5}$ and $10^{4,5}$ $TCID_{50}$ respectively. This suggested that with both preparations approximately 90% of the virus was adsorbed within the 120 min period.

Growth curves

Results are given in Fig. 10. Using a mean virus input of $10^{5,3}$ $TCID_{50}$, peak virus titres were obtained for both preparations, on the 6th day post-inoculation and coincided with the complete destruction of the monolayers. Peak infective titres persisted for at least 24 h before thermal inactivation occurred.

Virus cell-association and the effect of freezing and thawing

The results of the experiments given in Table 11 indicate that ASF virus is not readily separated from infected cells. Freezing and thawing of the virus preparations did not have any detrimental effect on infectivity, however, when it is followed by centrifugation, the infectivity of virus stocks is lowered.

FIG. 9   Adsorption of ASF virus in MK cells
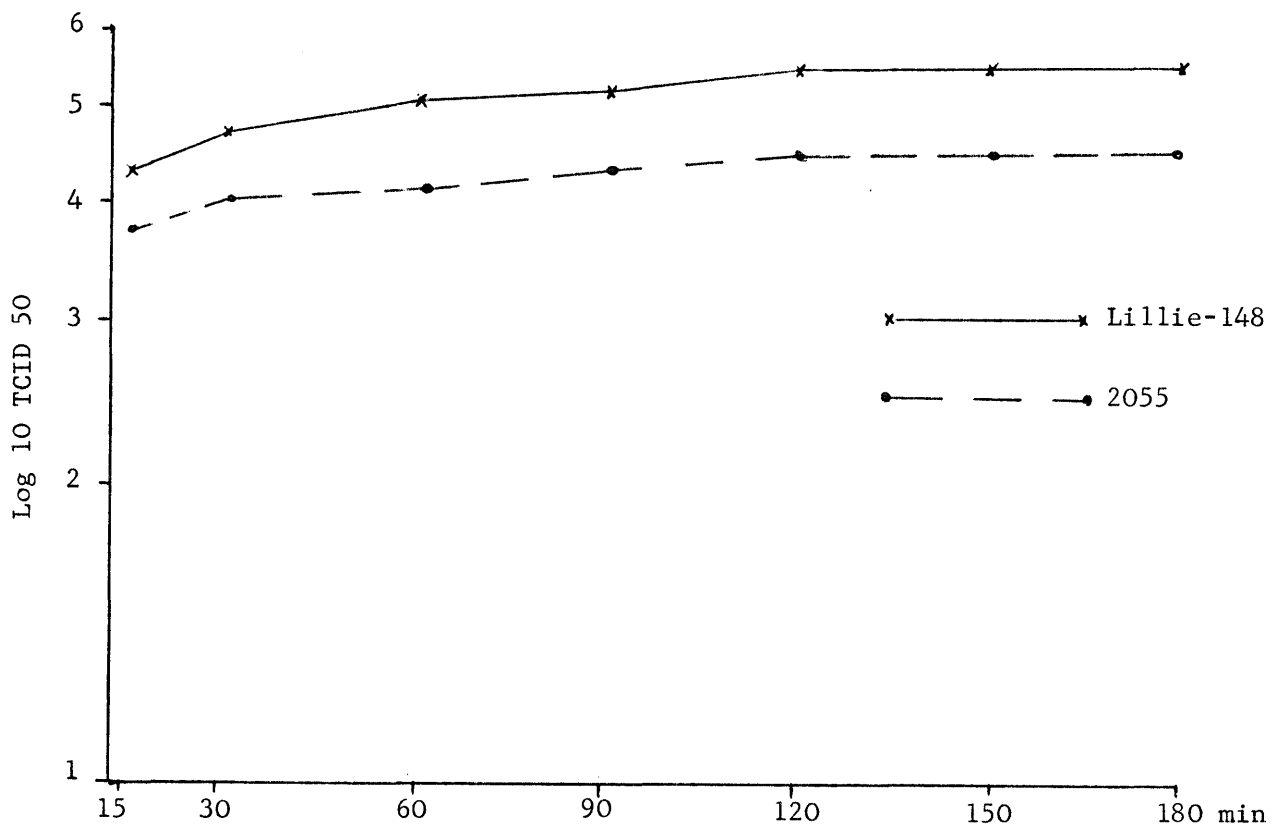
FIG. 10   Growth curve of ASF virus in MK cells
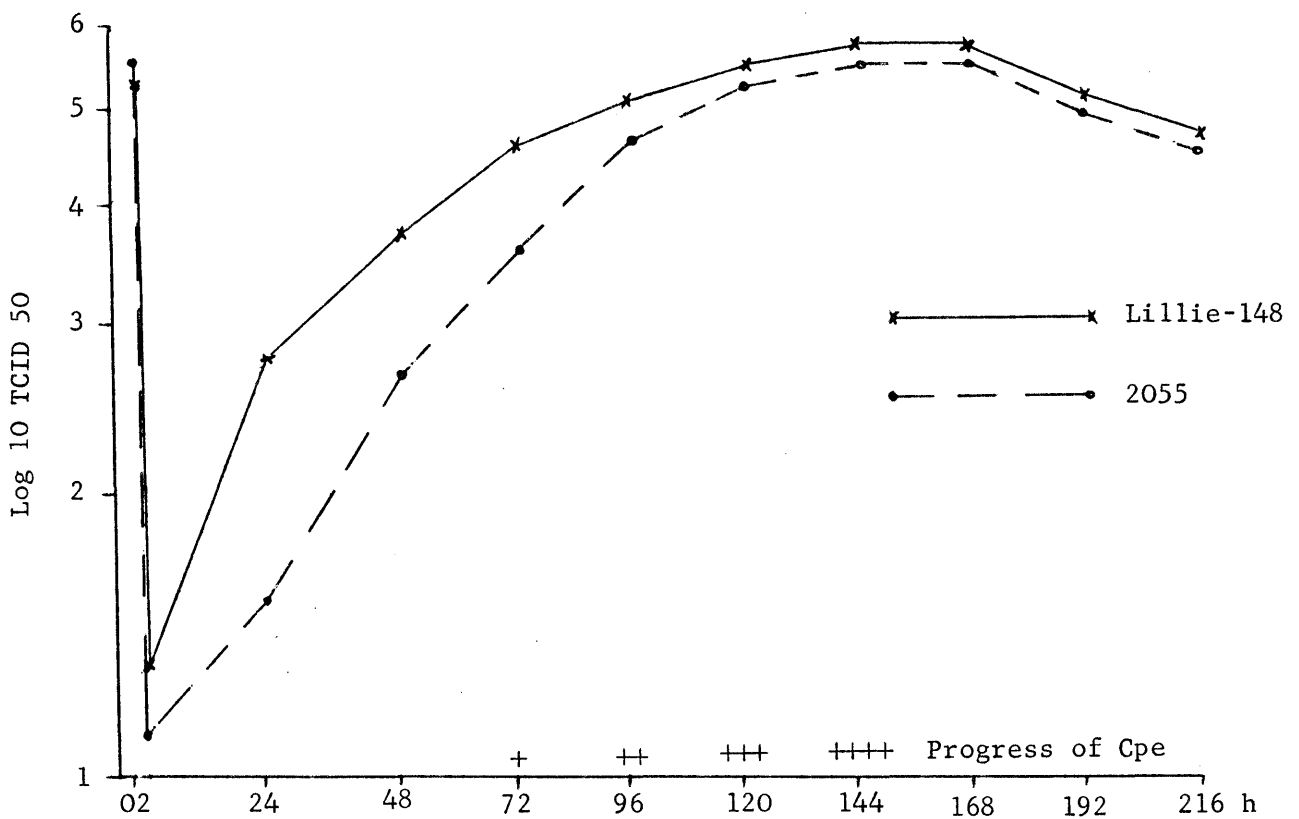
TABLE   11   Infectivity of free and cell-associated virus
during replication of strains Lillie-148 and
2055 MK12

Strain	Progression of Cpe	Medium	Cells	Cells + Medium
Lillie-148	+	3,3*	4,5	4,8
	++	4,5	5,0	5,2
	+++	5,3	5,5	5,8
	++++	5,6	5,6	5,8**
	Freezing & thawing			5,8
	& centrifugation			5,6
2055	+	2,5	3,6	3,6
	++	3,5	4,5	4,6
	+++	5,0	5,5	5,5
	++++	5,2	5,5	5,6**
	Freezing & thawing			5,6
	& centrifugation			5,0

*Log 10 $TCID_{50}$/0,2 ml. Mean of 3 infectivity titrations

**Preparation used to study effect of freezing & thawing
followed by centrifugation.

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CONCLUSIONS

These results suggest that ASF virus replicating in MK cell monolayers has an optimal adsorption time of 120 min, during which approximately 90% of the virus population is adsorbed on to the cells. With the two preparations used, Cpe were observed when the viral concentration had reached mean values of $10^{4,0}$ $TCID_{50}$. The peak infective titres coincided with the complete destruction of the monolayers and thermal inactivation started 24 h later. At least 50% of the virus remains cell associated when Cpe is generalized and three cycles of freezing and thawing did not seem sufficient to break up this association.

CHAPTER IV

INFLUENCE OF SERIAL PASSAGE IN CELL CULTURES
ON THE HAEMADSORBING CHARACTERISTICS OF ASF VIRUS

INTRODUCTION

Adsorption of erythrocytes is an effect which has been observed
with cell cultures infected with a variety of viruses and it is
apparently due to the incorporation of a virus protein into the plasma
membranes of the infected cells (Shelokov, Vogel & Chy, 1958;
Laurenaudie, Toulier, Santucci & Carnero, 1967).

Cell cultures infected with ASF virus haemadsorb porcine erythro-
cytes (Malmquist & Hay, 1960; Malmquist, 1963; Greig et al., 1967).
The mechanism of the reaction has been studied by Breese & Hess (1966)
and Larenaudie et al. (1967) with inconclusive results, though the
effect apparently occurs at the level of the intact cell membrane.
Haemadsorbing characteristics are not essential for infectivity
(C. Sanchez Botija & R. Sanchez Botija, 1965; Coggins, 1968b;
Pini & Wagenaar, 1974 and Pini 1976) and according to Vigàrio et al.
(1974) the envelope of the virus may be of importance in haemadsorption
since non-enveloped virions in his laboratory failed to produce this
effect.

To assess whether maintenance in cell cultures had any in-
fluence on the haemadsorbing characteristics of the isolates of ASF
virus, serial passages were carried out in parallel in MK and BC
cells.

36.

MATERIALS AND METHODS

Virus

Strains of ASF virus 2055 HAd$^+$, CV HAd$^+$, Lillie-148 HAd$^-$ and TS 237 were used. From the results of previous experiments the latter strain was an heterogeneous population of HAd$^+$ and HAd$^-$ virus. The serial passages of strains 2055 and Lillie-148 were initiated from the original spleen suspensions stored in the interim at -70°C whereas for strains CV and TS 237 they were initiated from the BC stocks at the 6th and 3rd passage respectively. Identity of viruses was confirmed at regular intervals by immunofluoresence.

Assay method

Cell monolayers in plastic tissue culture flasks were infected with 0,2 ml of the 10^{-1} virus dilution. When Cpe or HAd or cell lysis had generalized, cultures were frozen and thawed three times and used for the next passage. Infectivity titrations were carried out at intervals for each series in MK and BC cell cultures simultaneously, according to the methods described.

RESULTS

An average of two subcultures per week were made with the various strains of virus in the BC series while an average of 1,5 passages per week were undertaken in the MK series. No significant differences were observed with regard to the duration of incubation at the various passage levels. Unless otherwise stated, no significant abnormalities were seen, in the appearance and intensity of HAd in BC cells whenever this effect was present.

Strain 2055 HAd$^+$

MK series. Forty six serial passages were carried out in MK cell cultures. When comparative titrations were carried out in MK and BC cell cultures at the 7th, 15th, 26th, 41st and 46th subculture, the infective titres were found to be comparable in both systems. As shown in Table 12 strain 2055 lost its haemadsorbing characteristic between

the 41st and 46th MK passage and the infective titre in BC cells at
the 46th subculture was estimated on the presence of cell lysis.

TABLE   12   Assessment by infectivity titrations in BC cells of
              the haemadsorbing characteristics of strain 2055 of
              ASF virus following serial passages in BC or MK cell
              cultures

Serial passages Strain 2055	Infectivity titres in BC cell cultures		
	HAd and/or	Lysis	Difference
MK 7	4,0*	4,0	0
15	3,5	3,5	0
26	6,5	6,5	0
41	5,5	5,5	0
46	0,0	6,5	6,5
BC 11	4,5	4,5	0
21	4,5	4,5	0
41	5,5	5,5	0
70	4,5	4,5	0
75	4,5	5,5	1,0
76	3,5	6,5	3,0

*Log 10 $BCHAd_{50}$ or $BCID_{50}$ / 0,2 ml

BC series.   Seventy six serial passages were carried out.
Comparative titrations in the MK and BC cell cultures at the 11th,
21st, 41st, 70th, 75th and 76th passages showed that MK cells were
less sensitive in establishing the infectivity of strain 2055 adapted
to BC cell cultures.   The mean virus titres in the former system were
1000 times lower than in the latter.   From the results given in
Table 12 the presence of an HAd^{-} virus population became demonstrable
in the limiting virus dilutions between the 70th and 75th passage.

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Strain CV HAd$^+$

MK series. Forty one passages were carried out. Comparative titrations in MK and BC cell cultures at the 10th, 19th, 37th and 41st passage showed that both systems were equally sensitive for estimating virus infectivity. The results are given in Table 13 Strain CV lost its haemadsorbing characteristic between passage 37 and 41.

TABLE 13   Assessment by infectivity titrations in BC cells of the haemadsorbing characteristics of strain CV of ASF virus following serial passages in BC or MK cell cultures

Serial passages Strain CV	Infectivity titres in BC cell cultures		
	HAd and/or	Lysis	Difference
MK 10	4,5*	4,5	0
19	5,5	5,5	0
37	4,5	4,5	0
41	0,0	5,0	5,0
BC 9	4,0	4,0	0
18	4,5	4,5	0
43	6,0	6,0	0
56	5,5	5,5	0
61	5,5	6,5	1,0
75	4,5	6,5	2,0
76	3,5	6,5	3,0

*Log 10   BCHAd$_{50}$ of BCID$_{50}$ / 0,2 ml

BC series.   Seventy six passages were made.   Comparative titrations carried out in MK and BC cell cultures at the 9th, 18th, 43rd, 56th, 61st and 75th and 76th passage confirmed that the former system was 1000 times less sensitive in estimating the infectivity of the strain adapted to BC cells. As shown in Table 13 a HAd$^-$ virus population became demonstrable at the terminal virus dilution by the 61st BC passage.

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MK series. Only 10 subcultures were made. The sensitivity of BC
and MK cell cultures was found comparable when infectivity titrations
were made in both systems at the 2nd, 5th, 7th and 10th passage.
As shown in Table 14 the HAd⁻ virus population was already predominant
at the 2nd passage and haemadsorbing virus could not be detected by
the 7th passage.

TABLE 14   Assessment by infectivity titrations in BC cells of the
             haemadsorbing characteristics of strain TS 237 of ASF
             virus following serial passages in BC or MK cell
             cultures

Serial passages Strain TS 237	Infectivity titres in BC cell cultures		
	HAd and/or	Lysis	Difference
MK 2	4,5*	5,5	1,0
5	2,5*	5,5	3,0
7	0,0	5,5	5,5
10	0,0	5,5	5,5
BC 5	7,5	7,5	0,0
15	5,5	5,5	0,0
25	5,0	5,0	0,0
37	6,5	6,5	0,0
45	4,5	5,5	1,0
50	5,5	6,5	1,0
55	5,0	5,0	0,0
60	6,0	6,0	0,0

*Log 10 $BCHAd_{50}$ or $BCID_{50}$ / 0,2 ml

BC series. Sixty subcultures were made and comparative titrations in
BC and MK cell cultures carried out at passage 5, 15, 25, 37, 45, 50, 55
and 60. The BC system was again 1000 times more sensitive than the
MK system for estimating infectivity.

CHAPTER V

PLAQUE PRODUCTION BY ASF VIRUS IN LLC-MK$_2$ CELL CULTURES

INTRODUCTION

The results of the previous experiments showed that heterogeneous populations of ASF virus may be obtained in primary isolation and that the virus has a tendency to mutate from the HAd$^+$ to the HAd$^-$ form after serial passages in BC or MK cells. The methods of passaging limiting virus dilutions in BC cultures to detect and segregate heterogeneous populations is obviously cumbersome. Coggins (1968b) concluding his report on the segregation of HAd$^-$ strains of virus of ASF stated that since haemadsorbtion continued to reappear after inoculation of HAd$^-$ virus into pigs, "a better method of separation of the virus particles had to be developed before excluding reversion of HAd$^-$ to HAd$^+$ forms."

It was thought that the plaque technique could provide this improved method and would permit the study of the composition of populations of ASF virus with regard to their haemadsorbing characteristics. It was also considered paramount to assess whether plaques could be obtained without prior adaptation of the virus to the cell system used.

Among the various isolates that were successfully adapted to cell cultures to date, at least three have been reported to produce plaques under agar overlay. For this purpose Parker & Plowright (1968) used pig kidney cell cultures while Enjuanes et al. (1976a) used Vero cells.

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MATERIALS AND METHODS

Virus

Strain Lillie-148 MK20 of ASF virus was used to study the influence of various environmental factors on the production of plaques. After the optimal conditions had been determined plaque production was investigated further with strains 2055 MK41 and 46, CV MK37 and 41, CV BC43 and 56, 24823 BC1 and with the HAd⁻ virus population of strain TS 237 BC26 which had been segregated according to the methods described.

Cell Cultures

Disposable 60 x 15 mm plastic tissue culture petri dishes were seeded with $1,2 \times 10^6$ cells. Cell monolayers were confluent after 24h incubation at 37°C in a humidified water jacket incubator gassed with 5% carbon dioxide in air.

Unless otherwise stated monolayers were washed in three changes of PBS and infected with the appropriate virus dilution of strain Lillie 148 MK20. Adsorption was carried out for 120 min at 37°C after which the inoculum was drained and the overlay added. The overlay was prepared from 0,8% Agarose (Miles Seravac) in Earle's salt solution without sodium bicarbonate which was melted and mixed with an equal volume of Eagle's medium supplemented with bovine serum at a final concentration of 2%. The concentration of sodium bicarbonate was adjusted to 1,5 mg per ml. The prepared overlay was held at 44°C prior to its addition to the monolayer.

Plaque Assay

When strain Lillie-148 was used, a virus stock was diluted to give a mean count of 60 plaques per petri dish.

To study plaque production under optimal conditions with the other strains of ASF virus, decimal dilutions from 10^{-1} to 10^{-7} were prepared and 0,2 ml was used to infect each of four or five petri dishes as well as four tissue culture tubes per dilution. In order to investigate the haemadsorbing characteristics of the virus populations

obtained under the agar overlay, plaques were selected according to standard procedures from monolayers showing one to three plaques per dish and used to infect BC cell cultures.

Monolayers were stained with neutral red at a final dilution of 1/30000 in maintenance overlay medium. Plaque counting was carried out 6 to 15h later.

Plaque size was estimated under a microscope provided with an ocular micrometer.

Results were statistically analysed for a completely randomized experiment or one way classification model. The analysis of variance showed if differences among results were significant or not. The comparison between mean results for the least significant differences were done by Tukey's method (Snedecor & Cochran, 1967).

RESULTS

From the results of the preliminary experiments it was established that MK cell monolayers could be maintained for 12 days under not less than 8 ml of the overlay medium with a depth of 4,8 mm. This incubation time was similar to that used for titrations carried out in monolayers prepared in rotating tissue culture tubes. Under our experimental conditions, increasing the volume of the overlay or the addition of a second overlay during the period of incubation did not appear to significantly prolong the viability of the cells.

Influence of incubation time on plaquing efficiency

Monolayers were overlaid and stained with neutral red from the 3rd to the 12th day post-infection. Results are shown in Table 16 and Fig. 11. Plaques became visible on the 3rd day of incubation and their mean number increased eightfold by the 9th day; thereafter they remained stationary and by the 11th day started to lose their definition and the count decreased. No significant differences were found between the counts made from the 7th to the 10th day post-inoculation.
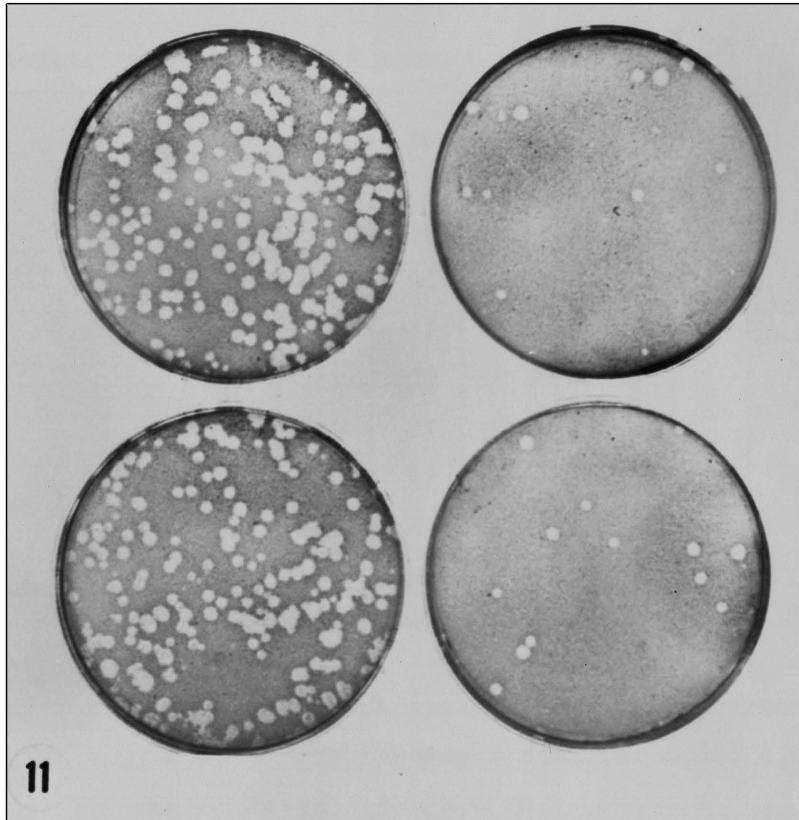
45

FIG. 11   Plaques formed by strain Lillie-148 of
           ASF virus at the 20th passage in Mk cell
           cultures.

TABLE 16   Plaque efficiency of strain Lillie-148 of ASF virus
            under 0,4% Agarose.

Day post-infection	No. of plaques				Total	Mean
3	11	9	10	10	40	10
4	14	16	13	16	59	15
5	30	27	26	28	111	28
6	49	48	54	49	200	50
7	64	72	76	68	280	70
8	69	71	79	72	291	73
9	83	86	71	83	323	81
10	86	75	72	84	317	79
11	57	69	69	61	256	64
12	61	59	69	68	257	64

On day 9 plaques could arbitrarily be subdivided according to
size into three groups: small, having a diameter 0,5 - 1,0 mm,
medium 1,1 - 2,0 mm and large 2,1 - 3,0 mm.

Influence of different concentrations of Agarose on plaquing
efficiency

Confluent monolayers were infected and petri dishes sub-
divided in 3 groups. Each group was overlaid with a medium con-
taining a final concentration of agarose of 0,6, 0,5 and 0,4%
respectively. On the 9th day post-infection neutral red was added
and thereafter the plaques were counted and measured.

The results are given in Table 17. When the concentration
of agarose was increased from 0,4 to 0,6% the number of plaques
was reduced by approximately 90%. The plaque size in the mono-
layers overlaid with 0,6 or 0,5% agarose varied between 0,4 -
2,0 mm, whereas with the lowest concentration, plaque size varied
between 0,5 - 3,0 mm.

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TABLE 17   Influence of different concentrations of Agarose on
             plaquing efficiency of strain Lillie-148 of ASF virus

Agarose %	No. of plaques					Total	Mean
0,6	4	8	4	6	2	24*	5
0,5	32	44	28	36	42	182*	36
0,4	58	60	68	66	70	322**	64

*Size 0,4 - 2,0 mm
**Size 0,5 - 3,0 mm

When 0,4% agarose was used, monolayers were stained satisfactorly within a period of 6 h. When however the concentration of agarose was increased to 0,6% the staining of cells was slower and plaques were more readily counted after an incubation of about 12 h.

Influence of neutral red on plaquing efficiency

During the course of the two experiments previously described it was observed that neutral red when used at a final concentration of 1/30 000 had an inhibitory effect on plaque formation. The number of plaques counted on the 4th or 6th day of incubation increased marginally when counting was repeated on the 9th day, while in monolayers stained on the 8th or 9th day plaque formation proceeded according to the results shown in Table 16.

Influence of different types of solidifying agents on plaquing efficiency

Cell monolayers infected with an identical virus preparation were overlaid with media containing different types of gels. Agarose at a concentration of 0,4%, Iono Agar No. 2 (Oxoid) at 0,9% and carboxy methyl cellulose (Koch Light Laboratories) at 1% were used. In the latter medium the concentration of sodium bicarbonate was increased to 2 mg/ml. The results of plaque counting are shown in Table 18. Iono agar and carboxy methyl

cellulose had an inhibitory effect on plaque formation by ASF
virus.

TABLE 18   Influence of different types of soldifying agents on
plaquing efficiency of strain Lillie-148 of ASF virus

	No. of plaques					Total	Mean
Agarose 0,4%	51	53	57	59	60	280	56
Iono Agar No. 2 0,9%	14	16	14	16	16	76	15
Carboxy Methyl Cellulose 1%	4	0	5	4	1	14	3

Influence of the $NaHCO_3$ concentration in the overlay medium

Media with concentrations of $NaHCO_3$ varying from 0 to 4,5 mg/ml
were used to overlay infected monolayers. The results of plaque
counts are given in Table 19. The absence of $NaHCO_3$ drastically
reduced the number of plaques, whereas with concentrations of
$NaHCO_3$ varying from 1,5 to 4,5 mg/ml the number of plaques did
not show significant variations. The plaque size with the 1,5 mg/ml
$NaHCO_3$ concentration varied within the limits of 0,5 - 3,0 mm,
while with the higher concentrations it appeared to be more uniform
with limits between 1 - 2,0 mm. The definition of the plaques was
optimal with the lower concentration of $NaHCO_3$.

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TABLE 19   Influence of different NaHCO$_3$ concentrations on
             plaquing efficiency of strain Lillie-148 of ASF
             virus.

NaHCO$_3$ mg/ml	No. of plaques					Total	Mean
0	4	7	0	5	2	18	4
1,5	36	48	32	42	44	202*	40
3,0	26	36	40	40	34	176**	35
4,5	32	24	36	34	32	158**	32

*Size   0,5 - 3,0 mm

**Size   1,0 - 2,0 mm

Influence of serum from different animal species in the overlay medium

Porcine, adult and foetal bovine serum at a final concentration
of 2% were used to supplement standard overlay medium. The results
are shown in Table 20. Plaque sizes were similar for all three
media, but statistical analysis of plaque counts indicated that
plaquing efficiency was enhanced by adult bovine or porcine serum.
Since the latter appeared to enhance plaque definition it was
adopted for the subsequent experiments.

TABLE 20   Influence of serum from different animal species on
              plaquing efficiency of strain Lillie-148 of ASF virus

Serum 2%	No. of plaques					Total	Mean
Adult Bovine	50	50	52	54	46	252	50
Foetal Bovine	42	34	44	40	44	204	41
Porcine	54	56	50	40	46	246*	49

*Best definition

Influence of adsorption time on plaquing efficiency

Monolayers were infected and incubated for set periods varying from 30 to 180 min. At the end of each interval the inoculum was drained; the monolayers washed in three changes of PBS and the overlay added. The results of the experiment are given in Table 21. Adsorption times of less than 120 min significantly lowered plaquing efficiency.

TABLE 21   Influence of duration of viral adsorption on plaquing efficiency of strain Lillie-148 of ASF virus

Adsorption time min	No. of plaques					Total	Mean
30	32	33	30	29	38	162	32
60	37	40	36	45	40	198	40
90	39	38	45	46		168	42
120	59	51	52	59	52	273	55
150	59	57	58	54	53	281	56
180	65	55	58	54	55	287	57

Influence of rinsing cell monolayers after adsorption

At the end of the 120 min adsorption time, the inoculum was removed and the infected monolayers were washed either once, twice or three times with PBS. Control cultures were over-laid immediately after the removal of the inoculum. The results of plaque counts shown in Table 22 indicate that the rinsing of infected monolayers did not have significant effect on plaque efficiency.

TABLE 22   Influence of washing cell monolayers after
             adsorption of strain Lillie-148 of ASF virus

No. of washing	No. of plaques				Total	Mean
0	85	76	72	70	303	76
1	77	78	81	79	315	79
2	72	73	76	70	291	73
3	73	69	77	79	298	74

Influence of age of cell monolayers

Confluent monolayers were infected with the identical virus
preparation when they were 1, 2 and 3-day-old. On the 9th day
post-infection plaques were counted and the results are given in
Table 23. No difference in plaque number was observed between
cell monolayers 1, 2 or 3-day-old. In the latter group plaques
were smaller and the majority of them appeared to be within
the range 0,4 - 1,0 mm.

TABLE 23   Influence of cell age on plaquing efficiency of
             strain Lillie-148 of ASF virus

Age of cell culture	No. of plaques					Total	Mean
1	50	52	46	40	46	234	47
2	53	50	45	39	47	234	47
3	49	50	48	43	40	230	46

Influence of volume of inoculum

Monolayers were infected with volumes varying from 0,1 to
0,75 ml. The results given in Table 24 indicate that volumes of
inoculum above 0,2 ml had a significant influence in lowering

plaque efficiency, in addition their distribution appeared to
be more even when a volume less than 0,5 ml was used.

TABLE 24   Influence of volume of inoculum on plaquing
             efficiency of strain Lillie-148 of ASF virus

Volume (ml)	No. of plaques					Total	Mean obtained/expected	
0,1	22	24	17	21	16	100	20	20
0,2	36	38	37	40	38	189	38	40
0,5	85	87	91	91	84	438	88	100
0,75	133	141	126	136	129	665	133	150

Influence of different diluents during adsorption

The following diluents: Eagle's medium, PBS, Tris saline,
Tris saline with 0,1% bovine albumin (Cohn fraction V) with a pH
of 7,1 were used. Monolayers were infected with the appropriate
virus dilution prepared in the same type of diluent used for
rinsing the monolayers. The results are shown in Table 25. No
significant differences in plaque counts and size were observed
with any of the diluents tested.

TABLE 25   Influence of different solutions for rinsing cell
             monolayers and preparing virus dilutions on plaquing
             efficiency of strain Lillie-148 of ASF virus

Solution	No. of plaques					Total	,Mean
Eagle	33	35	35	37	40	180	36
PBS	30	32	40	37	35	174	35
Tris saline	31	34	37	38	36	176	35
Tris saline 0,1% bovine albumin	33	32	34	34	35	168	34

Influence of different pH during viral adsorption

Four stock solutions of Tris saline with a pH of 6,5, 7,0, 7,5 and 8,0 were prepared by the addition of N HCl. They were used for preparing sets of virus dilutions and for the rinsing of monolayers. From the results shown in Table 26 it was concluded that the diluent with pH 7,0 was optimal for plaque production.

TABLE 26   Influence of different pH for rinsing cell monolayers and preparing virus dilutions on plaquing efficiency of strain Lillie-148 of ASF virus

Tris Saline pH	No. of plaques					Total	Mean
6,5	41	31	35	37	38	182	36
7,0	47	45	37	42	40	211	42
7,5	30	33	35	34	30	162	32
8,0	30	38	35	45	35	183	37

Plaque formation by various strains of ASF virus

The optimal conditions for plaque formation defined in the previous series of experiments with strain Lillie-148 MK20 were used for comparative studies on plaque production with other strains of ASF virus.

The results are shown in Table 27. When infectivity titrations were carried out in MK cell cultures by the methods of plaque counting and observation of Cpe in rotating cell monolayers, the former technique compared favourably with the latter.

TABLE 27   Infective titres of different strains of ASF
             virus and their haemadsorbing characteristics
             following plaque selection.

Strain	Cell culture & passage level	TCID$_{50}$*	PFU**	Size/mm	HAd after plaque selection
Lillie-148	MK 20	4,6	$3,0 \times 10^5$	0,5-3,0	-
2055	MK 41	5,0	$1,3 \times 10^5$	0,4-2,4	+
	46	5,5	$1,0 \times 10^5$	0,4-2,4	-
CV	MK 37	4,0	$1,3 \times 10^4$	0,3-1,6	+
	41	4,5	$3,0 \times 10^4$	0,3-1,6	-
	BC 43	3,0	$3,3 \times 10^3$	0,2-1,2	+ or -
	56	2,5	$3,0 \times 10^3$	0,2-1,2	-
24823	BC 1	6,0	$1,0 \times 10^6$	0,5-2,5	-
TS 237	BC 26	4,2	$4,0 \times 10^4$	0,4-1,5	-

*Log 10 TCID$_{50}$ / 0,2 ml

**Plaque forming units / 0,2 ml

The HAd$^-$ isolate 24823 gave rise to plaques varying in size
within the range 0,5 - 2,5 mm, with the medium and large plaques
being predominant.

Of the 2 HAd$^+$ isolates 2055 and CV the latter formed smaller
plaques.   The loss of haemadsorbing characteristics which
occurred during the course of serial passages in BC or MK cell
cultures did not have significant influence on plaque size whereas
adaptation to MK cell cultures seemed to favour the appearance of
larger plaques. The outline of plaques was regular and well defined
with plaques of 0,4 mm or larger but was irregular and poorly
defined in the case of smaller plaques. During the course of plaque
selection of strain CV BC 43 it was found that only half of the
plaques formed in petri dishes infected with limiting dilutions
were derived from HAd$^+$ virus while the other half originated from
HAd$^-$ virus. The chances of isolating HAd$^+$ virus were equal from
small or large plaques.

The CV strain adapted to BC cell cultures had the lowest plaque efficiency and infectivity in cell cultures tubes.

CONCLUSIONS

When the influence of the environmental factors on plaque efficiency was studied with strain Lillie-148 of ASF virus, it was found that the optimal incubation time was 9 days and optimal concentration of Agarose was 0,4%. The size of the plaques varried within the range of 0,5 and 3 mm. Iono Agar No.2 Oxoid and carboxy methyl cellulose had an inhibitory effect on plaque efficiency. The other optimal parameters for plaque production were : (a) cell monolayers at the time of infection 1 to 2-day old; (b) 0,2 ml volume of inoculum; (c) an adsorption time of 120 min; (d) the incorporation in the overlay of 1,5 mg of $NaHCO_3$ per ml of medium and 2% porcine serum.

When the above findings were applied to other strains of ASF virus, they all produced plaques, irrespective of their adaptation or not to the MK system. Plaque size was not influenced by the haemadsorbing characteristics of the strain studied and the technique was effective in assessing the haemadsorbing characteristics of a virus population and in segregating HAd⁻ populations.

CHAPTER VI

BIOLOGICAL TESTS IN DOMESTIC PIGS

INTRODUCTION

ASF is a disease in South Africa that is characterized by a short course with mortality close to 100% and with pronounced generalized haemorrhagic lesions. In the outbreak which occurred in 1973 on the farm Lillie-148 the pattern of the disease was different, as the course was protracted and pathological changes were inconsistent.

Where ASF has become established in domestic swine, the severity of the disease has decreased and the incidence of subacute and chronic forms has increased (Scott, 1965). Mild forms of the disease may be characterized by a moderate pyrexia only.

Moulton and Coggins (1968) and Coggins, Moulton and Colgrove (1968) studied the pathological changes in pigs inoculated with attenuated strains of ASF obtained by serial passages of the virus in BC cell cultures. Viraemia was readily demonstrated in the majority of animals, although the virus tended to disappear 35 days post-inoculation. The pathological changes most consistently observed were interstitial or necrotic pneumonia, pericarditis, arthritis, meningoencephalitis and hyperplasia of the lymphnodes.

Following the segregation of HAd⁻ strains of ASF virus Coggings (1968b) suggested that they were relatively avirulent. Vigàrio et al. (1974) reached a similar conclusion since their

HAd$^-$ isolates were obtained from swine affected with chronic lesions of pneumonia only.

To conclude this series of experiments, it was decided to study the pathogenicity of ASF in domestic pigs infected with some of the HAd$^-$ and HAd$^+$ strains of virus.

MATERIALS AND METHODS

Virus

The response of swine to inoculation with HAd$^+$ and HAd$^-$ ASF virus was studied with the following strains: Lillie-148 and 24823 HAd$^-$, segregates TS237 HAd$^-$ and HAd$^+$, CV and 2055 HAd$^+$. They were inoculated into pigs as original spleen suspensions or after serial passages in BC or MK cell cultures as indicated.

Experimental animals

Biological tests were carried out in susceptible Large White cross Landrace pigs weighing 30-40 kg, bred in isolation at the Laboratory. Before infection the serum of each animal was tested for absence of antibody to ASF virus by indirect immunofluorescence.

Assay method

Pigs were inoculated intramuscularly with 1 ml of the appropriate virus preparation. Temperatures were taken in the early mornings and only those of 40°C or above were considered to be febrile. For the detection of viraemia animals were bled every other day and 1 ml of whole blood was tested.

Spleen, liver, lung, kidney, tonsils, brain and mesenteric, hepatic, mediastinal, and mandibular lymphnodes taken after death or after the animals were sacrificed, were used to prepare 10% (w/v) suspensions in Eagle's medium and tested for the presence of virus. A sample was considered negative when no evidence of HAd and/or cell lysis and Cpe could be found after three consecutive

passages in BC and MK cell cultures. Infectivity was estimated in BC cell cultures and expressed per ml of blood or g of tissue.

Histopathology was considered consistent with the diagnosis of ASF when the lesions observed complied with the description given by De Kock, Robinson & Keppel (1940) and Moulton & Coggins (1968) for pigs infected with virulent strains of virus. In all other instances the nature of the histological changes were as recorded.

<div align="center">RESULTS</div>

Non-haemadsorbing strains of ASF virus

Strain Lillie-148. Two pigs were each inoculated with $10^{3,2}$ $BCID_{50}$ of virus contained in the original spleen suspension stored at $-20^{o}C$. The results are summarized in Table 28. In pig 384 a temperature of $40,2^{o}C$ was recorded on the 5th day post-inoculation, on the 8th day the animal developed a diarrhoea which was haemorrhagic on the following morning and death occurred on the 11th day.

TABLE 28 Results of biological tests in domestic pigs inoculated with 10% spleen suspension of specimen Lillie-148 HAd^{-}

Type of response	Pig No.	
	383	384
Temperature reaction Range ^{o}C	40- 41,4	40,2 - 41,2
Day p.i.	6 to 17	5 to 11
Viraemia	5,7*	5,7
Tissue infectivity	6,2	6,2
Macro- and microscopic lesions at death	Chronic ASF	Acute ASF
Type of virus	HAd^{-}	HAd^{-}

*Log_{10} / ml or g - $BCID_{50}$

During this period pig 384 showed a temperature warying between 40,2 and 41,2^{O}C, dyspnoea and anorexia. No other clinical symptoms were seen. At autopsy severe haemorrhagic lesions were recorded in the lymphnodes, lungs, kidney, bladder, stomach and large intestine.

In pig 383 a temperature reaction of 40°C was recorded on the 6th day post-infection, and for the following 11 days it varied between 40 and 41,4^{O}C. The animal fed normally until the last day and the other clinical signs observed were an arched back, recumbency and dyspnoea. The animal died on the 18th day post-infection. At autopsy a severe sero-fibrinous pericarditis with the pericardium adhering to the heart and the pleura was observed, while the pericardial sac and the thoracic cavity contained abundant serosanguineous fluid. There was bronchopneumonia, congestion of the intestine and the margins of the spleen showed infarction. Histological lesions in both animals were consistent with a diagnosis of ASF.

Virus was isolated from blood samples of both pigs when bled and from the organs taken at death. The viraemic titres varied between $10^{5,3}$ and $10^{6,1}$ BCID$_{50}$ with a mean value of $10^{5,7}$. The infective titres of the organs were between $10^{5,4}$ and $10^{6,7}$ BCID$_{50}$ with a mean value of $10^{6,2}$. All isolates were HAd$^-$.

Two more pigs were inoculated with 10^4 BCID$_{50}$ contained in a suspension prepared from the spleen of pig 383. The results are summarized in Table 29.

In animal 382 the temperature reaction was 41,3^{O}C on the 5th and 41,1^{O}C on the 6th day. On the following day the temperature dropped to 37,9^{O}C and the animal died on the morning of the 8th day without showing any evident clinical sign of disease. At autopsy lymphnodes were haemorrhagic, the spleen was enlarged, the kidneys and the bladder showed petechiation and the lungs were oedematous. Histopathology was consistent with a diagnosis of ASF.

TABLE   29   Results of biological tests in domestic pigs
               inoculated with 10% spleen suspension of pig 383
               from previous experiment

Type of response	Pig No.	
	381	382
Temperature reaction		
Range $^{\circ}$C	40,2 - 41,8	41,3 - 41,1
Days p.i.	12 to 16	5 to 6
Viraemia	5,8*	6,0
Tissue infectivity	6,0	6,3
Macro- and microscopic lesions at death	chronic ASF	acute ASF
Type of virus	HAd$^-$	HAd$^-$

*Log_{10} / ml or g - $BCID_{50}$

In pig 381 a temperature reaction of 40,2°C appeared on
the 12th day post-inoculation and varied between 40,2 and 41,8°C
during the following 4 days. The animal died on day 17. The
macroscopic lesions observed at autopsy were similar to those
observed in pig 383 and histopathology suported a diagnosis of
ASF.

Virus was isolated from all the blood samples of both pigs
and from the organs taken after death. Infective titres were
within the same range indicated in the previous experiment.
All isolates were HAd$^-$.

Strain 24823.   Since the original specimens were putrefied
and contained only traces of the infective agent, it was decided
to use a virus preparation obtained from the spleen, after one
passage in BC cell cultures. Two pigs were each inoculated
with $10^{6,3}$ $BCID_{50}$ and the results are summarized in Table 30.

TABLE 30   Results of biological tests in domestic pigs
inoculated with strain 24823 BC1 HAd‾

| Type of response | Pig No | |
	537	538
Temperature reaction	None	None
Viraemia	N D*	N D
Tissue infectivity	N D	N D
Macroscopic lesions at autopsy	gastritis**	pneumonia***
Histopathology	interstitial pneumonia & lymphadenitis	
Serum titre	$10^{-1,3}$**	$10^{-2,5}$***

*Not Demonstrable

**at 21 days p.i.

***at 62 days p.i.

Pyrexia was not detected in any of the 2 animals during the entire observation period and temperature varied between 38,5 and 39,9^{o}C. Both pigs showed diarrhoea on the 6th day post-inoculation only and they were listless between the 9th and 10th day. Viraemia could not be demonstrated in BC and MK cell cultures on the days of bleeding. Pig 537 was killed on the 21st day; the carcass was in a good condition and with the exception of an ulcerative gastritis no other macroscopic lesions were observed. Serum from the blood sample taken on the last day of observation showed an antibody response to ASF virus at a dilution of 1/20. On histological examination interstitial pneumonia and lymphadenitis with some haemorrhages were found.

Pig 538 was sacrificed on the 62nd day post-inoculation. At autopsy the carcass was in a good condition; the left lung however, showed a subacute lobular pneumonia. On histological

examination interstitial pneumonia and lymphadenitis were found.
The antibody titre to ASF virus at the 62nd day was 1/320.

No virus was isolated in BC and MK cell cultures from any
of the organs taken from both pigs.

Strain TS237. Two pigs 553 and 554 were each inoculated
with $10^{6,5}$ $BCID_{50}$ of the HAd^- segregated strain of TS237, serially
subcultured for 26 times in BC cell cultures, according to the
method described. The results are summarized in Table 31.

TABLE 31   Results of biological tests in domestic pigs
inoculated with strain TS237   BC26 HAd^-

Type of response	Pig No.	
	553	554
Temperature reaction		
Range ^{o}C	40,1 - 40,6	None
Day p.i.	7 to 22	
Viraemia	*4,0 to traces	N D**
Tissue infectivity	5,8	N D
Macroscopic lesions at autopsy	pneumonia	None
Histopathology	interstitial pneumonia & lymphadenitis	
Serum titre	$10^{-2,8}$	$10^{-2,5}$
Type of virus	HAd^-	None

*Log_{10} / ml or g - $BCID_{50}$
**Not Demonstrable

Pig 554 did not develop pyrexia and temperatures ranged
between 38,3 and $39,7^{o}C$ over the entire observation period of
60 days. No other clinical signs of disease were observed.
Viraemia could not be demonstrated at the times of bleeding. On

the 21st day post-inoculation the antibody response to ASF detected by immunofluorescence was 1/160 and had risen to 1/320 by the end of the observation period. When the animal was killed on the 60th day the carcass was in a good condition and no macro-scopic changes were seen. The histological lesions were those of interstitial pneumonia and lymphadenitis. The tissue samples tested for the presence of virus were found to be negative.

Pig 553 showed a temperature reaction between the 7th day and 22nd day post-inoculation which ranged between 40,1 and $40,6^{o}$C. On day 13 the animal was listless and on day 21 it developed an oedema on the right lower flank which persisted until day 35. Viraemia was detected between day 7 and day 31 post-inoculation. Between day 7 and 15 the infective titres varied between $10^{3,5}$ and $10^{4,0}$ $BCID_{50}$, thereafter they started to decline and by day 31 viraemia could be detected only on second passage in cell cultures. HAd^{-} virus was isolated in all in-stances.

On day 21 an antibody titre of 1/160 was detected. When the animal was killed on day 60 the antibody titre had risen to 1/640; the carcass was in a good condition but the lungs showed oedema and a severe penumonia with histological lesions of broncopneumonia, probably of bacterial origin. The mediastinal lymphnodes were enlarged and the histological examination showed lymphadenitis. HAd^{-} ASF virus was isolated from liver, lung, kidney, spleen, mediastinal lymphnodes and tonsils, whereas the other organs tested were negative. The infective titres ranged between $10^{5,6}$ and $10^{6,0}$ $BCID_{50}$ with a mean value of $10^{5,8}$.

Haemadsorbing strains of ASF virus

Strain TS237. Two pigs, 588 and 589, were each inoculated with $10^{6,2}$ $BCHAD_{50}$ of strain TS237 HAd^{+} which in conformity with the HAd^{-} counterpart had undergone 26 serial passages in BC cell cultures as previously described. The results are given in Table 32.

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TABLE 32 Results of biological tests in domestic pigs inoculated with strain TS237 BC26 HAd[+]

| Type of response | Pig No. | |
	588	589
Temperature reaction		
Range $^\circ$C	40,2 - 41,2	40,2 - 41,3
Days p.i.	3 to 9	2 to 5
Viraemia	6,6*	6,5
Tissue infectivity	7,2	7,0
Macro- and microscopic lesions at death	acute ASF	peracute ASF
Type of virus	HAd[+]	HAd[+]

*Log_{10} / ml or g - $BCHAd_{50}$

Pig 589 had pyrexia from the 2nd to the 5th day post-inoculation that ranged between 40,2 and 41,3°C. On the 6th day the temperature dropped to 39,7°C and the animal died. Other clinical signs were prostration, dehydratation, dyspnoea and cyanosis of the auricula. The macroscopic and histological lesions were all suggestive of a peracute form of ASF. HAd[+] virus was isolated from each blood sample and from all the organs tested. Mean infective titres were $10^{5,6}$ and $10^{7,0}$ $BCHAD_{50}$ respectively.

Pig 588 reacted in a similar fashion and was killed in extremis on the 10th day post-inoculation. The results of autopsy, histopathology and isolation of virus were as described for pig 589.

Strain CV BC 43. Two pigs, 590 and 594 were each inoculated with $10^{6,1}$ $BCHAD_{50}$ of strain CV serially subcultured 43 times in BC cell cultures according to the method described. Results are summarized in Table 33. Pig 590 had a temperature reaction of

TABLE   33   Results of biological tests in domestic pigs
inoculated with strain CV BC43 HAd$^+$

Type of response	Pig No.	
	590	594
Temperature reaction		
Range $^\circ$C	41	40 - 40,8
Day p.i.	4	4 and 14
Viraemia	4,5 - 1,5*	4,5 - 1,5
Tissue infectivity	3,0	None
Macroscopic lesions at autopsy	haemorrhagic lymphnodes	pneumonia
Histopathology	interstitial pneumonia & lymphadenitis	
Type of virus	HAd$^+$	HAd$^+$

*Log$_{10}$ / ml or g - BCHAd$_{50}$

41°C on the 4th day post-inoculation. In pig 594 temperature
reactions of 40 and $40,8^\circ$C were recorded on day 4 and 14 respec-
tively. No other clinical signs were observed. Viraemia was
detected in both animals whenever they were bled. Mean in-
fective titres declined from their peak levels of $10^{4,5}$ BCHAD$_{50}$
on day 4 to $10^{1,5}$ on day 27, when the animals were sacrificed.
At autopsy haemorrhages were observed in the lymphnodes of the
cephalic area and thoracic cavity of both swine. In pig 590
however, splenic lymphnodes also had cortical haemorrhages
whereas in pig 594 an interstitial diffuse pneumonia with focal
haemorrhages was seen. The results of the histological exami-
nation of both animals showed interstitial pneumonia and lymph-
adenitis with some haemorrhages. When organ suspensions were
tested, 10^3 BCHAD$_{50}$ of virus was only detected in the spleen of
pig 590.

Strain 2055 MK41. Two pigs, 608 and 609, were each inoculated with $10^{5,1}$ BCHAD$_{50}$ of strain 2055 serially sub-cultured 41 times in MK cell cultures according to the methods described. Results are summarized in Table 34. No clinical

TABLE 34   Results of biological tests in domestic pigs inoculated with strain 2055 MK 41 HAd^{+}

Type of response	Pig No	
	608	609
Temperature reaction	None	None
Viraemia	N D*	N D
Tissue infectivity	N D	N D
Macroscopic lesions at autopsy	None	None
Histopathology	interstitial pneumonia & lymphadenitis	
Serum titre	$10^{-1,6}$	$10^{-1,6}$

*Not Demonstrable

signs were observed and viraemia was not detected during the 30-day observation period. At autopsy macroscopic lesions were absent and virus was not detected in any of the organs tested. On histological examination however, interstitial pneumonia and lymphadenitis were found in both swine. An antibody titre of 1/40 was demonstrated in the sera taken 30 days post-inoculation.

CONCLUSIONS

From the results of these experiments it appeared that the HAd^{-} strains of ASF virus were characterized by a pathogenicity which varied within broad limits. The inoculation of strain Lillie-148 into domestic pigs was followed by either an acute or

chronic form of disease while subclinical forms of disease
followed infection with strains TS237 and 24823. The results of
the biological tests with the latter isolate raised doubts
regarding the immediate cause of death of the pigs on the farm.
From the biological tests it was difficult, however, to assess
how the disease would have evolved after a longer observation
period and under less favourable environmental conditions.

   With the peracute, acute and chronic forms of disease
the demonstration of the infective agent in the blood stream
or organs did not offer difficulties and high concentrations of
virus were found. With the subclinical forms the demonstration
of the infective agent in the tissues of the host failed when cell
cultures were used as indicator system. By using domestic swine
and increasing the number of observations to monitor the presence
of the virus, the probability of demonstrating viraemia and
persistence of the infective agent in organs could have been in-
creased. This, however, does not detract from the conclusion
that the pathogenesis of the disease has two distinct patterns.

   In subclinical forms, infection was confirmed by demonstra-
ting a rising antibody response to ASF virus. Histopathology
appeared to become of greater importance since interstitial
pneumonia and lymphadenitis, as described by Moulton & Coggins
(1968), were consistent changes of swine undergoing this type of
infection. This suports the hypothesis that minimal concentra-
tions of virus may have been present in the infected animals.

   The results obtained with the HAd^+ and HAd^- virus populations
of isolate TS237 on the other hand, seem to point to a corre-
lation between haemadsorbing characteristics and pathogenicity of
the strains. While the HAd^+ virus was responsible for a peracute
or acute course of disease, the HAd^- counterpart caused a sub-
clinical infection. Maintenance of virus in the cell system, in
this instance, had no influence on the pathogenicity, since both
strains underwent an identical number of passages in BC cells.

The results obtained with strains 2055 MK41 and CV BC43 however, showed that HAd$^+$ strains were also responsible for sub-clinical or mild forms of disease. This seems to discount the importance of the haemadsorbing property on the pathogenicity of the virus and seems to indicate that the two characteristics are not linked and can be modified independantly.

DISCUSSION

The cyclic re-occurrence of ASF in domestic pigs in the
endemic area of the northern Transvaal is a phenomenon to be
elucidated and requiring further observations. Whether this
situation can be related to an immune status of the warthog and
bushpig populations or of an unknown virus reservoir still
remains to be proved. In Spain, under different epidemiological
conditions, between 1960 and 1973, ASF has occurred in epidemic
waves. In 1962, 1965, 1969 and 1972 the number of outbreaks
were at their lowest level and initially, the remission of the
disease was attributed to the control measures implemented
(Sanchez Botja, 1965; Sanchez Botija and Sanchez Botija, 1965;
Sanchez Botija, 1974 personal communication).

In South Africa the widespread and sudden re-occurrence
of the disease after an interval of 10 years together with the
results of the survey carried out to obtain information on the
epidemiological significance of ticks Ornithodoros moubata
confirmed the necessity of maintaining the strict statutory
control of ASF.

The first of the outbreaks reported here occurred in
early winter and the incidence of the disease during this season
was comparable with that of spring and summer. Therefore it
appears that the observation reported by Scott (1965) that in
East Africa, ASF is prevalent in spring, during the farrowing
season of the warthogs, cannot be confirmed under the conditions

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prevailing in South Africa.

The new series of outbreaks coincided with the isolation, for the first time in South Africa, of two strains of virus devoid of haemadsorbing characteristics. The first of these strains, Lillie-148, was obtained from pigs affected by what appeared to be a protracted and less virulent form of disease characterized by inconsistent histopathological findings.

The second HAd^- strain, 24823, was isolated from an outbreak from which epidemiological, clinical or pathological observations were not available. However the results of the biological tests carried out at the laboratory seem to indicate that, contrary to any previous experience, the disease in the field may have had an extended course.

When the carrier status of Ornithodoros moubata was investigated it was found that the situation in southern Africa is analogous to that in East Africa (Plowright et al., 1969a & b). In this country mean infective titres were $10^{5,2}$ per adult tick suspension and $10^{4,5}$ BCHAd$_{50}$ per suspension prepared from nymphae; and are comparable with the results obtained by Plowright and his collaborators (1969a). The mean percentage of adult and nymphal ticks infected, was calculated to be 3,45 and 1,62 respectively, whereas in East Africa the ratio of infection for adult ticks varied within the limits of 1,7 and 3,8% and that of nymphae was 3 to 16 times lower (Plowright et al., 1969b).

The overall percentage of infected burrows was however lower in the Transvaal. The detection of infected ticks in the Marico district was interesting since ASF has never been reported in this area. The district was included in the endemic area, once the results of this investigation had become available.

Plowright et al. (1969a & b) did not describe abnormalities in the haemadsorbing characteristics of the virus isolated from ticks. Under the experimental conditions described here, however, the time of detection, the intensity and the progress of HAd, on certain occasions, varied for reasons unrelated to the infectivity of the tick suspensions. In one instance, with sample TS237, it was demonstrated that the delay and paucity of the

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haemadsorbing effect which was accompanied by pronounced lysis, was caused by the isolation of a virus population formed of both HAd^+ and HAd^- components.

HAd^- strains of ASF virus were obtained in primary isolation as early as 1963; however it was stated that after two to three passages in BC cell cultures or inoculation into pigs the HAd^- isolates regained their typical haemadsorbing characteristics (Sanchez Botija, 1963b; Sanchez Botija & Ordas, 1970). On account of the results obtained with strains TS237, it appears that the above statements should be reassessed. In fact when low dilutions of virus suspensions of strain TS237 were serially sub-cultured in BC cells the HAd^- component, originally present in the virus population was apparently lost, whereas by passaging limiting virus dilutions the HAd^- component was successfully segregated.

The strains devoid of haemadsorbing characteristics obtained in primary isolation in Spain, were probably heterogeneous populations which lost the HAd^- component by subcultures in BC cells or inoculation into animals.

More recently the isolation of strains of ASF virus with apparently stable HAd^- characteristics has also been reported in Portugal (Vigàrio et al. 1974). They were obtained from the lungs of pigs showing chronic lesions of pneumonia. In the Iberian peninsula the appearance of HAd^- isolates seems to coincide with the more frequent observation of subacute, chronic and inapparent forms of disease. It would appear that the relative low incidence of isolation of HAd^- strains of virus does not correlate with the higher incidence of mild cases of ASF and it may therefore be assumed that the virus mutates towards a less virulent form, without loosing its haemadsorbing characteristics.

The isolation of strain Lillie-148, from an outbreak of disease which, from circumstantial evidence, was caused by a

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warthog virus carrier and the observation made with isolate TS237, seems to support the hypothesis that this type of mutation may occur through an unknown mechanism, in the original virus reservoirs. There would appear to be a resemblance between the evolution of the infectious agent in the Iberian peninsula and that taking place in South Africa.

When discussing the epidemiology of ASF, Scott (1965) distinguished between an old cycle, occurring in regions such as East and South Africa, and a new cycle occurring in Spain and Portugal. Where the latter occurs, the presence of domestic pig carriers of ASF virus may cause noticeably less virulent forms of disease, because the infectious agent "in tending toward a more satisfactory accommodation to a newly acquired host" (Hess, 1971). In East and South Africa the outbreaks of disease exhibit the characteristics of primary epizootics because the virus has been on the whole successfully contained in the indigenous reservoirs and the "accommodation" has not apparently taken place. The recent demonstration of HAd⁻ forms of virus in South Africa seems to be the first indication of a spontaneous tendency of ASF virus to mutate towards a lower virulence, irrespective of prior adaptation to the domestic stock. If confirmed, the difference between the old and new epidemiological cycle discussed by Scott (1965) will loose significance and the control of the disease may turn into a challenge requiring a new approach.

During the course of this investigation, it was found that MK cell cultures appeared to be the most sensitive amongst the cell monolayers so far tested. Cpe could be observed in primary isolation between the 1st and 4th day post-inoculation, although the sensitivity in estimating virus content of porcine tissues were only comparable in two instances to that of BC cells, whereas in another three it was 100 to 1000 times lower. When immunofluorescence was applied for diagnostic purposes to infected MK cell monolayers, using a technique similar to that described by Heuschele and Hess (1973) with PK 15 cell cultures, infectivity

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could be estimated after a mean incubation time not exceeding 3,5 days.

The full adaptation of the virus to MK cells for maximum progression of Cpe and yield of infectivity could be obtained after three to six serial passages. It was concluded that MK cells were a suitable complement to BC cell cultures for the replication of ASF virus particularly in the presence of HAd^- isolates. Strains of virus adapted to MK cell cultures had an optimal adsorption time of 120 min during which 90% of the virus had become absorbed to the cells. Peak infectivity coincided with complete destruction of the monolayer, however, at this stage 50% of the infectious agent remained cell associated. Inclusion bodies were seen and following the addition of porcine erythrocytes HAd was observed in cell cultures infected with HAd^+ virus.

During the course of serial passages in MK and BC cell cultures of various strains, it was observed that HAd^+ virus maintained in BC cells, when subcultured into MK monolayers, required a period of adaptation similar to that necessary for the field strains. On the contrary HAd^- isolates and strains of virus adapted to MK monolayers grew readily when subcultured in BC cells. The two HAd^+ porcine virus isolates used in the experiment, lost their haemadsorbing characteristics between the 35th and 45th MK passages. Using the heterogeneous population of isolate TS237, the selection of the HAd^- mutant population occurred as early as the 7th MK passage, whereas in BC cell cultures the HAd^+ population was initially selected. Serial passages of the porcine HAd^- isolates in MK or BC cells did not reveal any change in this respect. A similar type of mutation was observed when the two HAd^+ porcine strains of ASF virus were serially passaged in BC cells. Mutation did not however, appear before the 61st passage and occurred as a gradual phenomenon.

It appears that an analogous mutation may have taken place in PK-2a cell monolayers used by Malmquist (1962) for

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subculturing the Hinde strain of ASF virus. He stated that it became increasingly more difficult to carry out titrations in BC cells because of delayed reactions that did not appear before the cultures deteriorated.

From the field and laboratory observations reported here it appears that HAd$^-$ populations of ASF virus exist in the natural reservoirs but the mutation can also be induced by serial passages in cell cultures. By implication it is reasonable to assume that HAd$^-$ virus may have been present in experimental vaccines used in field trials in the Iberian peninsula.

The feasibility of plaque production in MK cell cultures was also investigated. Plaques were obtained with all strains studied, irrespective of their adaptation to MK or BC cells. It appeared that the choice and concentration of the solidifying agents were critical parameters whereas the other environmental factors had only minimal influence. Plaques varied between 0,2 and 3,0 mm in diameter and on the whole were larger than those observed by Parker & Plowright (1968) and Enjuanes et al. (1976a), under different experimental conditions. Variations in plaque size could not be related to the haemoadsorbing characteristics of the virus; however, the technique was successfully used to detect the presence of HAd$^-$ virus particles in HAd$^+$ populations by subculturing selected virus-plaques into BC cell cultures.

From the results of the field observations and biological tests in swine it was concluded that in the presence of HAd$^-$ strains of ASF virus, the observance of infected animals must be in excess of the 2 months used in the course of this work. It was also confirmed that the HAd$^-$ strains have a reduced virulence (Coggins, 1968b; Vigàrio et al., 1974) which, can vary within broad limits as the experience with strains Lillie-148 and 24823 indicates. The results obtained with the two virus populations of isolate TS237 emphasized the different degree of pathogenicity between HAd$^+$ and HAd$^-$ virus. While the former was responsible for peracute or acute forms of disease,

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the latter was responsible for chronic or subclinical infection. Since mild forms of ASF were also obtained following inoculation into swine of HAd$^+$ strains, after serial passages in cell cultures, it seems appropriate to conclude that haemadsorption and pathogenicity are two characteristics that are not linked and can be modified independantly.

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