# 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.

# 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 x $10^6$ 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.

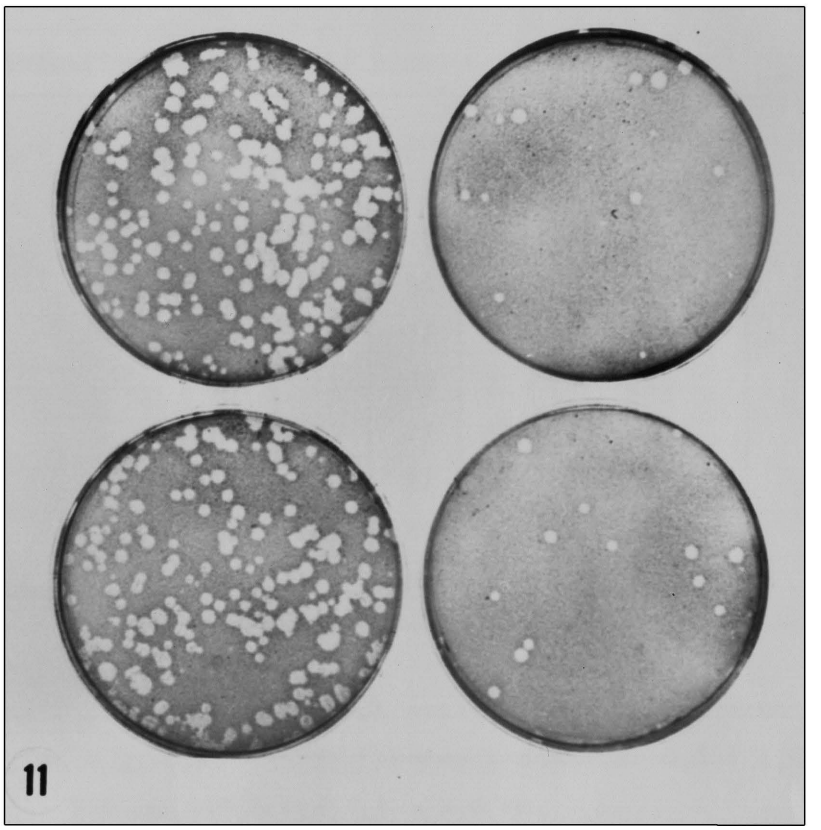


FIG. 11 Plaques formed by strain Lillie-148 of ASF virus at the 20th passage in Mk cell cultures.

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 subdivided in 3 groups. Each group was overlaid with a medium containing 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 monolayers 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.

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 $NaHCO_3$.

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 overlaid 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 $40^{\circ}C$ 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.

## RESULTS

### 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°C. The results are summarized in Table 28. In pig 384 a temperature of 40,2°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 | Pig No. 384 |
|---|---|---|
| Temperature reaction Range °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°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°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 broncho-pneumonia, 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°C on the 5th and 41,1°C on the 6th day. On the following day the temperature dropped to 37,9°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 °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 | Pig No 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°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 °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°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 macroscopic 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°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 instances.

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.

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

| Type of response | Pig No. 588 | Pig No. 589 |
|---|---|---|
| Temperature reaction | | |
| Range °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 $^oC$ | 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 - $BCHAd_{50}$

$41^oC$ on the 4th day post-inoculation. In pig 594 temperature reactions of 40 and $40,8^oC$ were recorded on day 4 and 14 respectively. No other clinical signs were observed. Viraemia was detected in both animals whenever they were bled. Mean infective 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 examination of both animals showed interstitial pneumonia and lymphadenitis 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 increased. 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 demonstrating 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 concentrations 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 correlation 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 subclinical 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

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

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 subcultured 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 (Vigário 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

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

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

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; Vigário 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,

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.