Isolation, Cultivation and Characterization of a Poxvirus from some Horses in Kenya

By

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With 3 figures and 2 tables

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Viral diseases associated with skin lesions in the horse have been described by a number of investigators (1, 8, 12). The diseases described by McIntyre (12) and Hutchins (8) were caused by a virus which grew on the chorio-allantoic membrane of the developing chick embryo. It is assumed that the causal agent could be a pox virus. The causal agent of horse pox is reported to be a pox virus similar to that of vaccinia of cattle (1, 11). The available literature does not show whether studies of the different properties of the agents associated with these conditions have been made.

This paper reports results of work done with some isolates of a vaccinia-like pox virus obtained from naturally infected horses. The characterization of one of the virus isolates by electron microscopy has been reported (9).

Material and Methods

Tissue Culture

Monolayer tissue cultures of bovine calf kidney (CK), calf testis (CT) and bovine foetal skin (BFS) were prepared by trypsinization of fresh organs as described by Madin, Andriese and Darby (10). Chicken embryonic fibroblasts (CEF) were also prepared by the same technique. The cells were grown in Hank's balanced salt solution containing 15% unheated calf serum and 0.5% lactalbumin hydrolysate. When confluent sheets of cells had grown, they were washed three times with phosphate buffered saline (PBS) pH 7.2 and maintained in Eagle's minimum essential medium (MEM) containing 2% heat inactivated pig serum. Penicillin 100 i.u., streptomycin 100 µg. and 25 i.u. mycostatin per ml. were incorporated in both growth and maintenance media. Cell suspensions were seeded into 6 oz. medicine bottles and confluent monolayers were inoculated with 0.5 ml. of infective material.

Blind passages were carried out at 10 to 12 day intervals when no cytopathogenic change (CPE) was observed.
**Virus Strains**

The following strains were used to compare some of their properties with the virus isolated from horses.

Vaccinia (MRC) — Obtained as freeze-dried antigen from Dr. D. Metselaar, Medical Research Centre (M.R.C.), Nairobi. This is used for smallpox vaccination of man.

Cowpox (8911) — This strain was isolated from cattle by Dr. G. Davis, Veterinary Research Laboratory, Kabete. The virus was isolated from scabs of the same original case and passed 5 times in CK monolayers.

**Antiserum**

Immune serum against vaccinia (MRC) antigen was kindly supplied by Dr. D. Metselaar.

Antiserum for the horse strain was prepared by inoculating rabbits with infective serum — free tissue culture fluid containing $10^5$ TCID$_{50}$/0.1 ml. at 10 day intervals. The first four were intramuscular injections of 1.0 ml. virus suspension mixed with 0.5 ml. complete Freund's adjuvant and the subsequent two injections of 2.0 ml. virus suspension were given intravenously. Serum was obtained by bleeding from the marginal ear vein 10 days after the last injection. The clot was separated after 2 hours at room temperature and overnight at 4°C followed by centrifugation at 1000 x g. for 15 minutes. It was heat inactivated at 56°C for 30 minutes and stored at $-20$ °C.

**Inocula**

Samples from infected horses were ground in sterile chilled mortars, diluted to 20% (W/V) with maintenance medium containing 500 i.u. penicillin and 500 pg. streptomycin per ml. and centrifuged at 2,000 x g. for 15 minutes. The supernatant fluid was collected and kept at $-70$ °C.

Viral suspensions were prepared from infected monolayers when CPE was over 80%. The same procedure as that described for field samples was followed. The harvested fluid was dispensed into Bijou bottles in 1.0 ml. aliquots and kept at $-70$ °C until required.

**Titration of Virus**

Serial tenfold dilutions of the 7th passage of each virus strain were made in MEM and 0.1 ml. inoculated into each of four CK culture tubes. After an observation period of 10 days, the 50% TCID was calculated by the method of Reed and Muench (13).

**Serum Neutralization Test**

Amounts of 0.5 ml. of twofold serum dilutions made in MEM were mixed with equal volumes of virus suspension containing $10^2$ TCID$_{50}$/0.1 ml. The mixtures were kept at 37°C in a waterbath for 1 hour and 0.2 ml. amounts were added to each of four culture tubes and incubated at 37°C. They were examined daily and the final results recorded when control tubes showed the maximum CPE.

**Haemagglutination (HA) and Haemagglutination-Inhibition (HAI) Test**

Haemagglutination titrations were carried out in W.H.O. plastic trays. Dilutions of infective tissue culture (TC) fluid and fluids prepared from chorio-allantoic membrane (CAM) were made in calcium-magnesium saline con-
taining 1% normal rabbit serum (7) to inhibit lipid agglutination (15). Vaccinia-susceptible fowl erythrocytes at a concentration of 0.5% were used in 0.5 ml. volumes of virus-cell mixture. The trays were shaken and incubated for 40 minutes at 37 °C in a moist chamber.

To determine the anti-haemagglutinin activity of the antisera, serial two-fold dilutions of them were prepared in 0.5 ml. volumes of 0.5% vaccinia-susceptible fowl erythrocytes in calcium-magnesium saline. Equal volumes of antigen containing 4 HA units were added to each well. The trays were shaken and incubated for 40 minutes and thereafter the test read. In both the HA and HAI tests partial agglutination was taken as the end point.

Vaccinia (MRC) and cowpox (8911) virus antigens were used for screening fowl erythrocytes for their sensitivity to haemagglutination before such cells were used for HA and HAI tests.

**Agar-gel Diffusion Test**

One percent ion agar in 0.025 M sodium veronal buffer pH 8.3 was poured into petri dishes. Gel thickness of 6 mm. was employed with the wells 6 mm. in diameter and the centres 13 mm. apart. The plates were incubated at 37 °C for 1 hour and then transferred to a moist chamber at the same temperature. The test was read after 72 to 96 hours. Vaccinia (MRC) antigen was used at a 1/10 dilution in physiological saline. The other antigens and antisera were used undiluted.

**Sensitivity to Lipid Solvents**

Tissue culture suspensions of virus were mixed with diethyl ether B.P.* 20% (V/V) and the mixture held at 4 °C overnight. The ether was removed by pouring the liquid into plastic petri dishes (2) and the virus titrated in CK cells.

0.05 ml. of chloroform B. P.* was added to 1.0 ml. of TC virus suspension and shaken for 10 minutes at 4 °C. The reagent was removed by the same method as for ether and the virus titration done in CK monolayers.

**Experimental Animals**

Chick embryos. Ten to twelve day old chick embryos were inoculated with 0.5 ml. of infective fluid by the CAM route. They were incubated at 37 °C and candled daily. The embryonated eggs were examined 4 to 6 days post inoculation (p. i.). Blind passages were performed by grinding the harvested CAM in a chilled mortar with PBS as diluent and the supernatant fluid obtained by light centrifugation was used for further passage. Membranes inoculated with control diluent were similarly treated.

Baby chicks. Three-day old chicks were inoculated intrafollicularly in the wing by plucking out a few feathers and instilling 0.2 ml. of infective fluid into the feather follicles. Chicks of the same age inoculated with non-infective TC fluid on similar sites with the same technique were kept as controls. They were examined daily for the development of lesions.

Baby mice. Baby mice between the ages of 1 and 10 days were injected intraperitoneally either with 0.1 ml. of infective TC fluid or with 0.1 ml. virus suspensions harvested from CAM-adapted antigens. Controls were inoculated with non-infective TC fluid or supernate from non-infected CAM.

Calves, adult rabbits, guinea pigs, hamsters and mice were inoculated intradermally with TC infective fluid and observed daily for lesions.

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* May and Baker Ltd., Dagenham, England.
Results

All attempts to isolate virus from field samples in CT, BFS and CEF were unsuccessful. Five blind passages were carried out with each system. Virus was isolated in CK monolayers and CPE appeared in this cell system 18 hours p.i. The highest titre was obtained between 36 and 48 hours, by which time over 80% of the cells were completely degenerated and detached from the glass surface. CK propagated virus was adapted to grow in CT, BFS and CEF and produced CPE after one to two blind passages.

Infected fluids prepared from field specimens did not produce lesions on CAM after five blind passages. However, TC infective fluid produced lesions after the second blind passage. Pock lesions varying in size from 1.3 mm. to 4.0 mm. in diameter, which were opaque with a haemorrhagic periphery, were observed (Fig. 2). Subsequent passages produced similar pock lesions. No
pock lesions were observed on CAM inoculated with control non-infective TC fluids.

Cowpox (8911) virus inoculated in CT, BFS and CEF produced CPE 18 hours later, on first inoculation. The virus produced pock lesions on CAM also on first inoculation.

The new isolates were completely neutralized by antisera against vaccinia (MRC) and cowpox (8911) antigens. Neutralization by the former antiserum was 2 logs higher than that by the latter. The antigens were not neutralized by the antiserum prepared against the horse virus.

When TC virus suspensions obtained from the horse virus strains at different stages of passage in CT, BFS, CEF and CK cells were tested against vaccinia-sensitive fowl erythrocytes, there was no haemagglutination. Following the adaptation of TC propagated virus onto CAM, the supernatant fluid prepared from it agglutinated the erythrocytes. Haemagglutination was inhibited by vaccinia (MRC) and cowpox (8911) antisera. Antiserum prepared from TC propagated virus did not inhibit HA by CAM-adapted virus antigens.

Precipitation lines were formed between the vaccinia (MRC) antiserum and the antigens tested against it (Fig. 1).

Table 1

<table>
<thead>
<tr>
<th>Animal species</th>
<th>No. of animals inoculated</th>
<th>Age</th>
<th>Route of inoculation</th>
<th>Amount inoculated</th>
<th>Reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf</td>
<td>2</td>
<td>3 months</td>
<td>i / d</td>
<td>5.0 ml.</td>
<td>Localized pox</td>
</tr>
<tr>
<td>Rabbit</td>
<td>4</td>
<td>Adult</td>
<td>i</td>
<td>2.0 ml.</td>
<td>-</td>
</tr>
<tr>
<td>Guinea Pig</td>
<td>2</td>
<td>i</td>
<td>i</td>
<td>2.0 ml.</td>
<td>-</td>
</tr>
<tr>
<td>Hamster</td>
<td>4</td>
<td>i</td>
<td>i</td>
<td>1.0 ml.</td>
<td>-</td>
</tr>
<tr>
<td>Mice</td>
<td>10</td>
<td>i / p</td>
<td>i / d</td>
<td>0.3 ml.</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>i / p</td>
<td>i / p</td>
<td>0.5 ml.</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>1-4 day old</td>
<td>i / p</td>
<td>0.2 ml.</td>
<td>+ b</td>
</tr>
<tr>
<td>Chicks</td>
<td>6</td>
<td>3 days old</td>
<td>i / f</td>
<td>0.2 ml.</td>
<td>mild follicular reaction</td>
</tr>
</tbody>
</table>

*) Key
+ = positive reaction; - = no reaction; i/d = intradermally; i/p = intraperitoneally; i/f = intrafollicularly; b = positive reaction following adaptation onto CAM

Table 2

A summary of results from various tests with the horse virus,

<table>
<thead>
<tr>
<th>Virus used</th>
<th>Tests</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Animal pathogenicity</td>
</tr>
<tr>
<td></td>
<td>Bovine</td>
</tr>
<tr>
<td>Horse virus</td>
<td>+</td>
</tr>
<tr>
<td>Cowpox (8911)</td>
<td>+</td>
</tr>
<tr>
<td>Vaccinia (MRC)</td>
<td>0</td>
</tr>
</tbody>
</table>

*) Key:
i/d = intradermally; i/p = intraperitoneally; i/f = intrafollicularly; CAM = chorioallantoic membrane; CK = calf kidney; CT = calf testis; BFS = chicken embryo fibroblasts; HA = haemagglutination; HAI = haemagglutination-inhibition, antigens were tested against vaccinia (MRC) antiserum; Agar-gel diffusion test, antigens were tested against
Experiments on sensitivity to lipid solvents showed that the horse virus was resistant to ether but was chloroform labile.

Table 1 shows results from animal inoculations. Localized lesions developed in calves and 3-day old chicks. Baby mice up to 4 days old were very susceptible to CAM adapted virus but refractory to TC propagated virus. The mice developed generalized pock lesions which were observed on the skin areas over the back, flanks and the abdomen (Fig. 3 A, 3 B). Some baby mice died before visible lesions developed but virus was detected by HA reaction using tissue and organ-ground extracts. Extracts from control animals did not haemagglutinate erythrocytes. Inoculations in rabbits, guinea pigs, hamsters and adult mice were unsuccessful (Table 1).

![Fig. 3. Extensive lesions on the back of baby mice after intraperitoneal inoculation with CAM-adapted horse virus](image)

<table>
<thead>
<tr>
<th>Tests</th>
<th>serology</th>
<th>other tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>pathogenicity</td>
<td>tissue cultures</td>
<td></td>
</tr>
<tr>
<td>cowpox (8911)</td>
<td>+</td>
<td>+ a</td>
</tr>
<tr>
<td>vaccinia (MRC)</td>
<td>+</td>
<td>+ b</td>
</tr>
<tr>
<td>cowpox (8911)</td>
<td>+ a</td>
<td>+ b</td>
</tr>
<tr>
<td>vaccinia (MRC)</td>
<td>+ a</td>
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</tr>
<tr>
<td>vaccinia (MRC)</td>
<td>+ a</td>
<td>+ b</td>
</tr>
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<td>cowpox (8911)</td>
<td>+ a</td>
<td>+ b</td>
</tr>
<tr>
<td>vaccinia (MRC)</td>
<td>+ a</td>
<td>+ b</td>
</tr>
<tr>
<td>cowpox (8911)</td>
<td>+ a</td>
<td>+ b</td>
</tr>
<tr>
<td>vaccinia (MRC)</td>
<td>+ a</td>
<td>+ b</td>
</tr>
</tbody>
</table>
Table 2 summarizes the results of the various tests which were done with the horse virus, cowpox (8911) and vaccinia (MRC).

**Discussion**

Observations made in the course of this work on the immunological properties of the horse virus indicate that it is related to vaccinia virus. The difference lies in its weak antigenic properties and infectivity.

Antiserum from rabbits inoculated with the horse antigens did not react with vaccinia (MRC) and cowpox (8911) antigens but antisera against vaccinia (MRC) and cowpox (8911) antigens neutralized, inhibited haemagglutination and formed precipitation lines with the horse virus. Serum from the affected horses did not react in any way either with the homologous or the heterologous antigens despite the fact that one of the horses has had the disease for over 2 years. Inoculation of one of the animals with an autogenous vaccine prepared from scabs and biopsies followed by tissue culture virus suspensions did not produce encouraging results (unpublished data).

Calves inoculated with the virus did not develop lesions on re-exposure and serum from these animals neutralized the antigens. It was also observed that TC virus was not pathogenic for baby mice, whereas after CAM adaptation it became pathogenic for these animals. The virus harvested from the CAM acquired a haemagglutinating property which was inhibited by vaccinia (MRC) and cowpox (8911) antisera. Apart from CK cells, it was difficult to adapt the virus to other tissue culture systems, to some animals and to the CAM, unlike cowpox (8911) and some vaccinia virus strains described by other investigators (3, 4, 5, 6, 7).

Some properties of this virus are similar to those described for vaccinia viruses by other workers (4, 7, 11). It is well established that there is a great variability in the characters of vaccinia strains (7). The only property which is probably relatively constant is the antigenic structure as determined by neutralization and complement-fixation tests (14). It would appear that the horse virus is serologically closely related to vaccinia virus.

There are reports that horse pox is a variant of cowpox (1, 11). In Kenya, horse pox has not hitherto been reported and the disease seen in these animals differs from the descriptions of the disease in the literature (1). Although the clinical picture of the disease resembles that described as viral papular dermatitis by some authors (8, 12), the difference is that while viral papular dermatitis runs a clinical course of between 2 and 6 weeks and is highly contagious (8, 12), this disease has a prolonged clinical course and appears to be much less contagious (unpublished observations).

**Summary**

Two strains of pox virus isolated from naturally infected horses were adapted to the chorioallantoic membrane (CAM) of developing chick embryos and to tissue cultures of chicken fibroblasts and bovine calf kidney, bovine calf testis and bovine foetal skin.

Isolation of the virus was achieved in calf kidney monolayers. After that, it was adapted to grow in the other tissue cultures and the CAM. The virus acquired a haemagglutinating property after adaptation onto CAM and also became pathogenic for baby mice by the intraperitoneal route. Calves became infected by intradermal inoculation and chicks by the intrafollicular route.

The virus is sensitive to chloroform but resistant to ether. Both isolates show weak antigenic properties. Immune serum prepared against vaccinia and
cowpox viruses respectively, neutralized the horse virus. The antisera also inhibited haemagglutination when this property was acquired by the virus after CAM adaptation. Precipitation lines were formed between vaccinia immune serum and the horse antigen.

These results suggest an immunological relationship between the horse virus and vaccinia and cowpox viruses.

The disease condition is discussed in comparison with horse pox and viral papular dermatitis.

Acknowledgements

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Zusammenfassung

Isolierung, Züchtung und Charakterisierung eines Pockenvirus von Pferden in Kenya

Zwei Pockenvirusstämme, isoliert von natürlich infizierten Pferden, wurden an die Chorioallantrismembran (CAM) von Hühnerembryonen und an Zellkulturen aus Hühnerembryofibroblasten, Kälbernieren, Kälberhoden und fetaler Rinderhaut adaptiert.


Résumé

Isolement, reproduction et caractérisation d'un virus de la variole équine au Kenya

Deux souches de variole ont été isolées chez des chevaux infectés naturellement et adaptées à la membrane chorioallantoïque (CAM) d'embryons de poulets et à des cultures cellulaires de fibroblastes d'embryons de poulets, de reins de veaux, de testicules de veaux et de peau de foetus bovins.

L'isolement du virus a réussi dans des cultures de reins de veaux. La souche s'adapta par la suite aux autres cultures cellulaires et à la «CAM». Après son adaptation à la «CAM», ce virus développait des propriétés
hémagglutinantes et devint pathogène pour des souriceaux par voie intra-péritonéale.

Le virus est sensible au chloroforme, mais résistant à l'éther. Les deux isolements étaient faiblement antigènes. Les immunosérums contre le virus de la vaccinie et de la variole bovine neutralisèrent le virus équin. Les antisérums inhibèrent également l'hémagglutination du virus « CAM ». Il y eut des lignes de précipitations entre un immunosérum de vaccinie et un antigène de variole équine. Les résultats laissent supposer qu'il existe un rapport entre le virus équin, le virus de la vaccinie et le virus de la variole bovine.

On compare et discute les signes caractéristiques de la maladie avec la variole équine et la dermatite vierale.

Resumen

Aislamiento, cultivo y caracterización de un virus variólico equino en Kenia

Dos estirpes de virus variólico, aisladas de caballos infectados naturalmente, se adaptaron a la membrana corioalantoidea (MCA) de embriones de pollo y a cultivos celulares de fibroblastos de embriones de pollo, riñones de ternera, testículos de ternero y piel de feto bovino.

El aislamiento del virus se logró en cultivos renales de ternera. Después se pudo adaptar y multiplicar en otros cultivos celulares y en la MCA. Tras su adaptación a la MCA, este virus desarrolló propiedades hemoaglutinantes y se tornó patógeno para ratones lactantes por vía intraperitoneal.

El virus es sensible frente al cloroforme, aunque étterresistente. Ambos productos aislados solo poseían una antigeneidad débil. Los sueros inmunes frente a virus Vaccinia resp. de la viruela vacuna neutralizaban el virus equino. Los antisueros inhibían también la hemoaglutinación del virus MCA. Entre el suero inmune Vaccinia y el antígeno viral equino se formaban líneas de precipitación. Los resultados autorizan la sospecha de que existe cierta relación inmunológica entre el virus equino y el virus Vaccinia y el de la viruela vacuna.

Las características nosológicas se discuten comparativamente con las de la viruela equina y dermatitis papular vírica.

References


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