PRELIMINARY CHARACTERIZATION OF VIRUSES ISOLATED FROM CASES OF EPIDIDYMITIS AND VAGINITIS IN CATTLE

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ABSTRACT

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Twelve slow-growing virus isolates propagated in tissue culture from cattle with epididymitis and vaginitis were identified as herpes viruses by virtue of their biological, physicochemical and morphological characteristics. None of the 12 virus strains proved to be serologically related to the infectious pustular vulvo-vaginitis-infectious bovine rhinotracheitis (IPV-IBR) virus, but a further 3 strains included in this study were identical with IPV-IBR virus.

Three of the slow-growing virus strains caused a mild anterior vaginitis when deposited in the vagina of heifers. A pool of 10 slow-growing viruses plus a strain of IPV-IBR virus produced anterior and posterior vaginitis in a heifer. the results are discussed in the light of the original description of vaginitis in the "epivag" syndrome.

Résumé

CARACTÉRISATION PRÉLIMINAIRE DE VIRUS ISOLÉS DE CAS D'ÉPIDIDYMITE ET DE VAGINITE DU BÉTAIL

Douze isolats de virus à croissance lente, propagés en culture de tissu à partir de bovins atteints d'épididymite et de vaginite, ont été identifiés comme virus herpes grâce à leurs caractéristiques biologiques, physicochimiques et morphologiques. On n'a trouvé aucune relation sérologique entre ces 12 souches virales et le virus IPV-IBR ("infectious pustular vulvovaginitis—infectious bovine rhinotracheitis"), mais 3 autres souches incluses dans cette étude étaient identiques au virus IPV-IBR.

Trois des souches virales à croissance lente ont provoqué une vaginite antérieure bénigne par dépôt dans des vagins de génisses. Une combinaison de 10 virus à croissance lente avec une souche de virus IPV-IBR a causé chez une génisse une vaginite antérieure et postérieure. On discute les résultats à la lumière de la description originale de la vaginite dans le syndrome "epivag".

INTRODUCTION

A herd disease syndrome manifesting itself as vaginitis and epididymitis has long been associated with infertility in cattle in Africa. Daubney, Hudson & Anderson (1938) recognized the condition in Kenya while Van Rensburg (1949) recorded the syndrome in the Transvaal and Orange Free State in South Africa.

Although vibriosis, brucellosis and trichomoniasis are under control on many farms, vaginitis and infertility in cattle remain a problem in South Africa and require further investigation. Various authors have investigated the potential aetiological role of infectious agents. McIntosh, Haig & Alexander (1954) succeeded in isolating a virus from cows with vaginitis in embryonated hen's eggs, but cattle infected experimentally with this virus developed a mild vaginitis only. Unfortunately this isolate was not available for inclusion in this study.

Viruses subsequently isolated by various workers at this Institute from cattle with epididymitis, orchitis or vaginitis were available, however, for studies which included biological physicochemical, morphological and serological characterization of the viruses, as well as a preliminary study of their pathogenicity in cattle.

MATERIALS AND METHODS

Virus strains

Fifteen virus strains were available for this study but for the sake of expedience they were not all included in every experiment. Table 1 summarizes the relevant history of each isolate. Originally, the primary isolation of all these viruses was undertaken either in primary foetal calf kidney (CFK) or foetal calf testis (VFT) cell cultures.

The strains were identified by the letter H, since they all proved to be herpes viruses, by a random number from 1–15 and by the plaque number selected for the tests. The strain isolated by Weiss (unpublished results, 1957), for example, was designated H 4/4 (Table 1). During the plaque purification processing of virus FH335 (Maré & Van Rensburg, 1961), 2 distinct viruses were obtained which were respectively designated H11/4 and H11/6, (results; Table 1). Some strains, such as H2 and H8, were used in biological tests prior to plaque purification.

Hellig (1965) isolated a number of viruses which were antigenically related to the virus of infectious pustular vulvo-vaginitis-infectious bovine rhinotracheitis (IPV-IBR). When one of the isolates was subcultured in 1969, a virus antigenically different from IPV-IBR was found and designated H7/2.

Viruses H14 and H15 were isolated by Maré (unpublished results, 1961) from the semen of bulls which showed epididymitis, but he did not attempt to identify them.

Prior to plaque purification, all viruses were subcultured in CFK line cells. The new stock antigens were frozen and thawed twice, centrifuged at $1\,500 \times g$ for 10 min and lyophilized in 0,5 mℓ volumes in glass ampoules after a solution of bovine albumin had been added to reach a concentration of 0,5%. The ampoules were sealed and stored at —20 °C.

Cell cultures

A locally developed foetal calf kidney (CFK) cell line at the 200th and 210th subculture was used for plaque purification of the viruses and other tests, including the preparation of stock antigens, haematoxylin-eosin staining (Paul, 1973) and neutralization tests. BHK-21 clone 13, African green monkey kidney (Vero) and a line of bovine kidney (MDBK)* cells were also used for comparison.

Primary foetal calf kidney and testis cells, used for the primary isolation of the viruses, were prepared by trypsinizing the fragmented kidneys in the cold (Paul, 1973). Stock line cell cultures were carried in Roux flasks and transferred to tubes or Petri dishes as required for each test.

*Maden Darby bovine kidney, supplied by American Type Culture Collection

TABLE 1 Origin of virus isolates used in this study

Virus strain	Original designation	Type of sample	Clinical signs of original case	Virus growth-rate in tissue culture	Isolated by
H 1/2....	Epiv. 1/67...	Vaginal swab.....	Vaginitis..............	Fast.......	A. Theodoridis (1967).
H 2/2....	Epiv. 1/70...	Tissue suspension of epididymis + testes	Epididymitis + orchitis	Slow......	A. Theodoridis (1970).
H 3/4....	G 26........	Vaginal swab.....	Anterior vaginitis and cervicitis	Slow......	J. Maré & S. J. van Rensburg (1961).
H 4/4....	Epiv. 2/57...	Vaginal discharge	Vaginitis..............	Slow......	K. E. Weiss (1957).
H 5/1....	C 375.......	Vaginal swab.....	Vaginitis..............	Slow......	J. Maré & S. J. van Rensburg (1961).
H 7/2....	C 2864......	Vaginal discharge	Posterior vaginitis.......	Slow......	H. Hellig (1965).
H 8/1....	Cotyl. herpes	Cotyledon tissue..	Abortion in 6th month...	Slow......	A. Theodoridis (1970).
H 9/1....	P 10........	Vaginal discharge	Anterior vaginitis.......	Slow......	J. Maré & S. J. van Rensburg (1961).
H10/2....	B 2.........	Semen..........	Epididymitis...........	Slow......	J. Maré & S. J. van Rensburg (1961).
H11/4....	FH 335......	Vaginal discharge	Vaginitis..............	Fast.......	J. Maré & S. J. van Rensburg (1961).
H11/6....	GH 335.....	Vaginal discharge	Vaginitis..............	Slow......	A. Theodoridis (1975).
H12/1....	Bantam......	Semen..........	Epididymitis...........	Slow......	J. Maré & S. J. van Rensburg (1961).
H13/1....	B 78........	Viginal swab.....	Posterior vaginitis.......	Fast.......	J. Maré & S. J. van Rensburg (1961).
H14......	Epiv. 1/61...	Semen..........	Epididymitis...........	Slow......	J. Maré (1961).
H15......	Epiv. 2/61...	Semen..........	Epididymitis...........	Slow......	J. Maré (1961).

All cells were cultured in Eagle's medium (Macpherson & Stoker, 1962), containing 10% bovine serum, 200 IU penicillin/mℓ, 200 μg streptomycin/mℓ, 200 IU colistin sulfate/mℓ and 2,5 μg amphotericin B/mℓ.

Plaque assay

A modification of the method used by Howell (1969) was followed for plaque assaying. Monolayers of CFK line cells were cultured in 60 × 15 mm Petri dishes* at 37 °C in 5% CO$_2$. The confluent monolayers were washed twice with Hanks's balanced salt solution, inoculated with appropriate dilutions of the virus concerned and 4 mℓ of agar overlay added to each dish. The agar overlay was prepared as follows: (a) Earle's salt solution plus sodium bicarbonate (required volume of a 3% sodium bicarbonate solution to give 1,4 mg/mℓ) 66%; (b) Eagle's medium 34%; (c) agarose** 0,5%.

The cultures were observed daily for the development of cytopathogenic effects (CPE), under an inverted light microscope and the shapes and sizes of the plaques were recorded.

The plaques were selected under the inverted microscope and removed by suction through a pipette with a rubber tube attached. Each plaque was suspended in 2 mℓ Hanks's salt solution and tubes with CFK line cells were each inoculated with 0,2 mℓ of the plaque suspension. The cultures of the 2nd subinoculation were harvested and lyophilized after addition of a solution of 0,5% bovine albumin. This stock antigen was used for all subsequent tests.

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**Miles Seravac, Cape Town

Preparation of specific immune serum

Rabbits. Roller tube cultures of primary CFK cells were infected with 0,2 mℓ of a 10^{-1} dilution of each virus. When the CPE involved more than 50% of the cells, they were harvested, frozen and thawed twice in ethanol and solid CO$_2$ and centrifuged at 1 500 × g for 10 min. The supernatant fluid was aspirated and stored at 4 °C for injecting the rabbits. Virus suspensions with a titre of at least 10^6 TCID$_{50}$/mℓ were used to inject the rabbits and each rabbit received 3 mℓ intramuscularly (im) and 3 mℓ intraperitoneally (ip). The injections were repeated 3 weeks later and 10 days thereafter blood was collected from the heart and the serum separated. Two weeks after this the rabbits received 1 mℓ of an emulsion consisting of equal volumes of virus and Freund's complete adjuvant at 5 different sites intradermally. Serum was again collected 15 days later and stored at —20 °C.

Cattle. Antiserum against virus strain H8, cultivated in primary CFK cells and not plaque-purified, was produced in 2 serologically negative cows. Each animal received 5 mℓ of the virus suspension im and 3 mℓ intravaginally. Both animals were observed for clinical signs and serum was collected 6 weeks after inoculation.

Serum was also obtained from the animals used in the pathogenicity studies on some of the viruses (see below).

Known antisera to classical IPV-IBR and bovine ulcerative mammillitis virus produced in cattle were included in the test for the purpose of comparison.

Virus assay and serological tests

Virus titrations and neutralization tests were carried out in roller tube cultures of CFK cells. Titrations

were performed by standard procedures using tenfold dilutions of the virus concerned. Cytopathic changes were recorded for 10 days and end-points were calculated by the method of Reed & Muench (1938).

In the neutralization tests following, tenfold dilutions of virus were mixed with equal volumes of the rabbit antisera diluted 1:5 in phosphate buffer. After the serum-virus mixtures were incubated for 3h at 37 °C in a water-bath, 1,0 ml of each serum-virus mixture was transferred to at least 2 roller tube cultures. The cultures were then incubated at 37 °C. The microscopic development of CPE was recorded and on the 10th day the titre and neutralization indices were calculated by the method of Reed & Muench (1938).

The complement fixation test (CF) on the bovine sera was conducted according to the method described by Cunningham (1973). Untreated tissue culture antigens of virus strains H1/2, H2/2 and H5/1 were used in the test.

Physicochemical properties

Chloroform sensitivity.—Virus suspensions harvested from CFK cell cultures with advanced CPE were centrifuged at $4\,000 \times g$ for 90 min and the supernatant fluid was removed for the test. Chloroform sensitivity was determined according to the method described by Bögel & Mayr (1961). Both the supernatant fluid and sediment of treated as well as untreated control cultures were assayed for virus content in CFK cell cultures.

As an additional control a strain of enteric cytopathic bovine orphan (ECBO) virus was included in the tests as a known, chloroform-stable virus (Oellermann, Els & Verwoerd, 1967).

Nucleic acid.—The type of viral nucleic acid was determined by means of 5-iodo-2-deoxy uridine (IUDR) according to the method of Hahnefeld & Hahnefeld (1964). Cell cultures were treated with 30 μg/ml IUDR in Eagle's medium for 24 h prior to infection and, after virus adsorption, medium containing the same concentration of IUDR was again added to the cultures after each had been seeded with 0,2 ml of 1:10 diluted virus suspension and adsorption had been allowed to take place at 37°C for 1 hr. Untreated control cultures were infected with the same virus concentration. When the CPE involved 50% or more of the cells in the untreated cultures, the virus yield in both treated and untreated cell cultures was assayed.

A preparation of ECBO virus, representing a known RNA virus, was included as a control in the experiments.

Electron microscopy

Cultures of CFK cells infected with the virus strains H8/1, H2/2 and H5/1 were examined by electron microscopy. The cells from culture tubes showing advanced CPE were detached from the glass with a long needle and the resulting suspensions were centrifuged at $1\,000 \times g$ for 5 min. The pelleted cells were pre-fixed in 3% glutaraldehyde for 1 h and post-fixed in 1% osmium tetroxide as described by Lecatsas & Weiss (1968).

All the viruses included in this study were also examined by the negative contrast staining method of Almeida & Howatson (1963). In this case pelleted cells were disrupted with distilled water and stained with 3% phosphotungstic acid at pH 6. A drop of this mixture was transferred to a formvar carbon-coated grid and the excessive fluid drained with blotting paper. After air-drying, the grid was examined in a Siemens Elmiskop 1A electron microscope.

Pathogenicity

Bull.—A serologically negative bull was infected with virus H2 (not plaque-purified) at the 5th CFK passage level. Ten ml of the virus suspension with a titre of $10^{6,5}$ TCID$_{50}$/ml was rubbed onto the mucous membrane of the prepuce with a sterile gauze swab. The animal was examined clinically on several occasions. Sera collected before and 6 weeks after infection were tested for neutralizing antibodies to H2 virus.

Cows and calves.—Two serologically negative cows (6221 & 6219) were exposed to infection with the 3rd CFK passage of virus H8 (not plague-purified) by depositing 5 ml of virus suspension in the anterior vagina. The titre of this virus was $10^{6,0}$ TCID$_{50}$/ml. Five ml of the same virus suspension was injected intravenously into each of 3 cows (7589, 7577 & 7574) and intranasally into 2 calves (7976 & 7935). The animals were examined clinically every 2nd day during the first 3 weeks following infection. Sera collected before and again 5 weeks after inoculation were tested for neutralizing antibodies to H8 virus.

Heifers.—Three sexually mature heifers (780, 9403 and 779) were infected with plaque-purified viruses H1/2, H2/2 and H5/1 respectively. In each case the inoculum consisted of 5 ml of virus suspension with a titre of $10^{6,5}$ TCID$_{50}$/ml deposited in the anterior portion of the vagina as well as 5 ml administered intravenously.

In order to determine the combined effect of multiple virus strains on the clinical and serological response in a heifer, virus H1/2 and a pool of 10 virus strains (H1/2, H2/2, H3/4, H4/4, H5/1, H7/2, H8/1, H9/1, H10/2, H11/6) serologically unrelated to classical IBV/IBR virus were used to infect a 6-month-old heifer calf (584). Each virus strain had a titre of at least $10^{6,0}$ TCID$_{50}$/ml. The calf was injected intravenously with 5 ml of the pooled virus suspension and an equal volume was deposited in the anterior vagina.

Samples of citrated blood and vaginal swabs were collected daily from the above-mentioned heifers for 14 days and assayed for virus content. The animals were examined clinically by means of a speculum at weekly intervals for a period of 8 weeks following infection except in the case of heifer calf 584 which was examined for a period of 10 months. Serum was collected from these animals before and again 8 weeks after infection and assayed for antibodies.

RESULTS

Studies in cell cultures

The cytopathic effects of the plaque-purified viruses were studied in CFK cells.

The viruses, according to the rate of development of CPE in cultures infected with 10^3 TCID$_{50}$/ml, could be divided into a fast-growing group (the IPV-IBR related viruses) consisting of H1/2, H11/4 and H13/1 and a slow-growing group (not related to IPV-IBR—see neutralization tests) consisting of H2/2, H3/4, H4/4, H5/1, H7/2, H8/1, H9/1, H10/2, H12/1, H14 and H15 (Table 1; Fig. 1)

In the case of the slow-growing group (Fig. 1), CPE were first noticeable on the 3rd day in the cells at the periphery of the culture and they slowly extended

to involve the entire culture. Complete destruction of all the cells invariably took up to 10 days (Fig. 1). The cells appeared rounded, shrunken and less translucent and formed irregularly-shaped groups. The cells did not become detached immediately and no empty spaces were visible during the early stages despite cell destruction at the initial foci. During the later stages the culture medium became progressively cloudy as cells became detached from the monolayers. The plaques of the slow-growing viruses remained small, being approximately 2 mm in diameter. They were granular and compact, well demarcated and round.
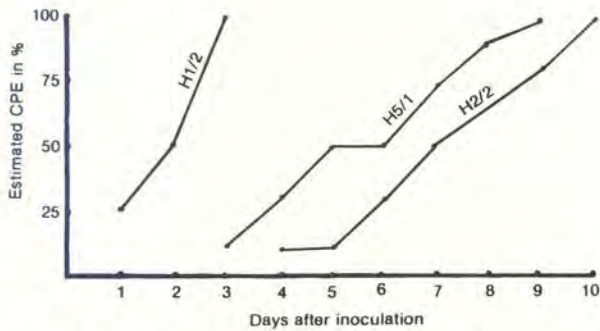

FIG. 1 Rate of development of CPE of fast and slow-growing viruses in CFK cells

The fast-growing viruses showed the typical progression of CPE of IPV-IBR virus. Foci of rounded cells occurred all over the culture on the 2nd day and empty spaces soon appeared in the centre of the foci. The cells of the entire monolayer were affected after the 3rd or 4th day. The plaques of the IPV-IBR group of viruses were large (5 mm) and rounded, translucent cells could be seen on the edges.

With the exception of virus H8/1, which failed to produce CPE in BHK21 cells, all the viruses produced CPE in CFK, BHK21, MDBK and Vero cell cultures. The nature of the CPE and rate of development varied according to the cell type.

Virus H8/1 did not produce CPE in BHK21 cells, but infected cultures harvested 8 days after inoculation had a virus titre of $10^{6,0}$ TCID$_{50}$/mℓ in CFK cells.

Heamatoxylin-eosin stained cultures of all 15 virus strains revealed the presence of large eosinophilic intranuclear Cowdry type A inclusions. The nuclei and cytoplasm of many infected cells were enlarged (giant cells) and an early cellular destruction was visible in the centre of the foci which appeared multi-layered.

Neutralization tests with immune sera

The pre- and post-immunization sera of the rabbits injected with each of the slow-growing strains of virus, including the serum samples collected after administration of the virus strains in Freund's complete adjuvant, were negative for neutralizing antibodies to the homologous and heterologous virus strains (2/2, H3/4, H4/4, H5/1, H7/2, H8/1, H9/1, H10/2, H12/1, H14 and H15). Only the IPV-IBR-related strains, H1/2, H11/4 and H13/1, induced a strong immune response in rabbits and showed cross-neutralization to one another. There was no cross-neutralization between the slow-growing viruses and the IPV-IBR related viruses, whereas complete cross-neutzalization occurred between the latter and a known IPV- IBR strain included as control.

An examination of the paired serum samples (pre- and post-infection sera) of the heifers, cows, calves and the bull showed that the slow-growing viruses induced neither neutralizing nor CF antibodies. The IPV-IBR-related virus H1/2 induced strong neutralizing and CF antibodies in Heifer 780.

No antigenic relationship between bovine ulcerative mammillitis virus and the viruses under investigation was found in the neutralization tests.

Physicochemical properties

Chloroform completely inactivated all the viruses under study whereas IUDR treatment reduced the titres of the 13 virus strains tested by at least 90% (Table 2).

Electron microscopy

The electron microscopy of cells infected with virus strains H8/1, H2/2 and H5/1, as representatives of the slow-growing group, revealed a typical herpes virus morphology. This is illustrated in Fig. 3, 4 & 5. Unenveloped icosahedral particles, ± 80 nm in size, could be seen in the nuclei of the cells and other

TABLE 2 Effect of chloroform and IUDR on virus strains

Virus strain	Treatment with chloroform			Treatment with IUDR		
	TCID$_{50}$/mℓ		Logs of inhibition	TCID$_{50}$/mℓ		Logs of inhibition
	Untreated	Treated		Untreated	Treated	
H 1/2..........	$10^{6,5}$	$10^{0,5}$	6,5	$10^{6,5}$	$10^{4,5}$	2,0
H 2/2..........	$10^{6,0}$	$10^{0,5}$	6,0	$10^{7,5}$	$10^{1,0}$	6,5
H 3/4..........	$10^{6,5}$	$10^{0,5}$	6,5	$10^{6,5}$	$10^{1,0}$	5,5
H 4/4..........	$10^{6,5}$	$10^{0,5}$	6,5	$10^{5,5}$	$10^{0,5}$	5,5
H 5/1..........	$10^{6,5}$	$10^{0,5}$	6,5	$10^{5,5}$	$10^{0,5}$	5,5
H 7/2..........	$10^{7,0}$	$10^{0,5}$	7,0	$10^{5,5}$	$10^{0,5}$	5,5
H 8/1..........	$10^{6,0}$	$10^{0,5}$	6,0	$10^{7,5}$	$10^{5,0}$	2,5
H 9/1..........	$10^{6,5}$	$10^{0,5}$	6,5	$10^{6,5}$	$10^{1,0}$	5,5
H10/2..........	$10^{6,5}$	$10^{0,5}$	6,0	$10^{6,0}$	$10^{3,5}$	2,5
H11/4..........	$10^{6,5}$	$10^{0,5}$	6,5	$10^{6,5}$	$10^{3,5}$	3,0
H11/6..........	$10^{6,5}$	$10^{0,5}$	6,5	$10^{6,0}$	$10^{3,5}$	2,5
H12/1..........	$10^{6,5}$	$10^{0,5}$	6,5	$10^{7,5}$	$10^{0,5}$	7,5
H13/1..........	$10^{7,0}$	$10^{0,5}$	7,0	$10^{6,5}$	$10^{5,0}$	1,5
ECBO..........	$10^{6,5}$	$10^{6,5}$	$10^{0,5}$	$10^{6,5}$	$10^{6,5}$	0,5

developmental stages, such as unenveloped empty capsids, some with rod-shaped cores and others with partly formed or dense large cores, are illustrated (Fig. 3 & 5). Enveloped particles, ±120 nm in size, were found intra- (Fig. 5) and extra-cytoplasmatically (Fig. 4).

All 15 plaque-purified virus strains examined by the negative contrast staining method revealed particles with the typical morphology of the family *Herpetoviridae* (Fig. 2).

Pathogenicity

Virus of the slow-growing group

Bull.—The bull infected with virus H2 (not plaque-purified) via the prepuce failed to develop symptoms or lesions during the observation period of 6 weeks. No abnormalities could be detected in either the preputial mucous membrane, testes or epididymis. Reisolation of virus was not attempted. Paired serum samples were negative for neutralizing antibodies to this virus.
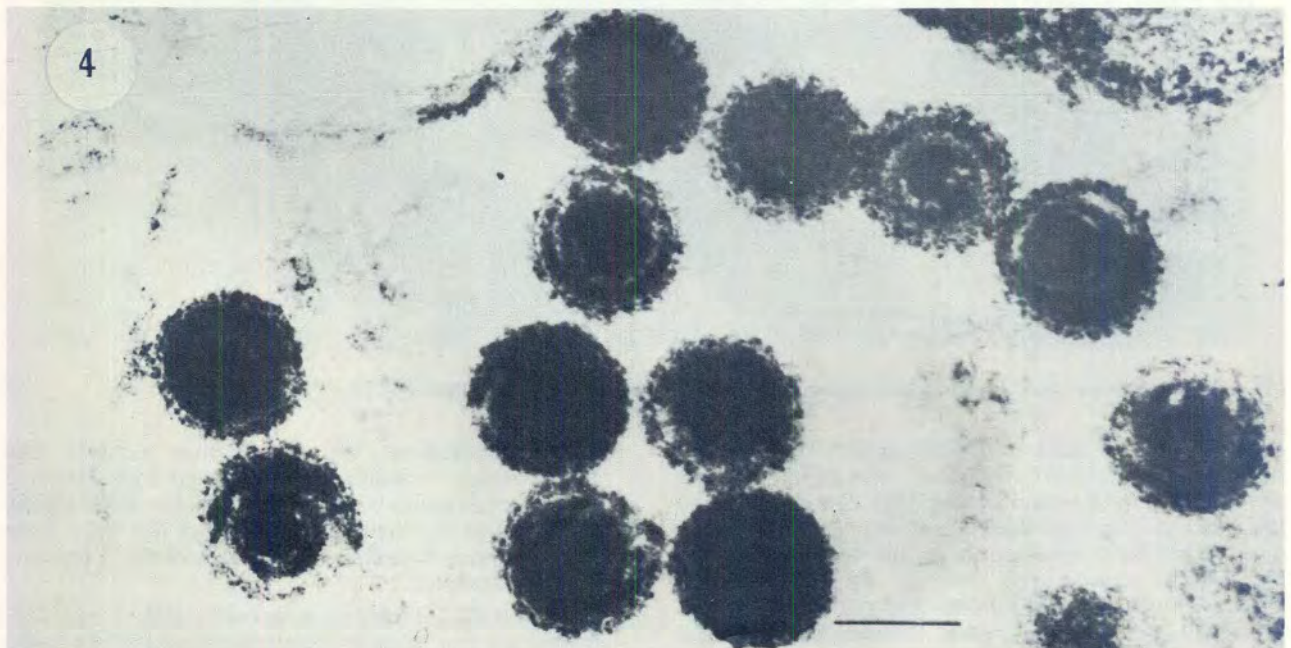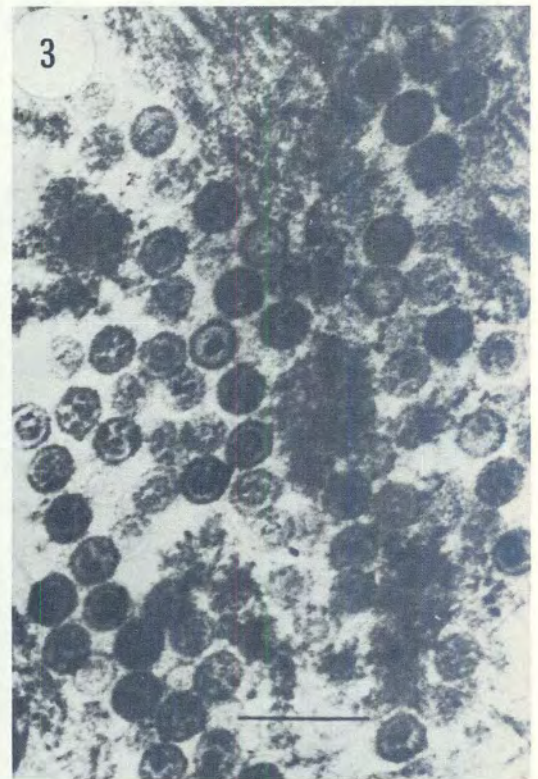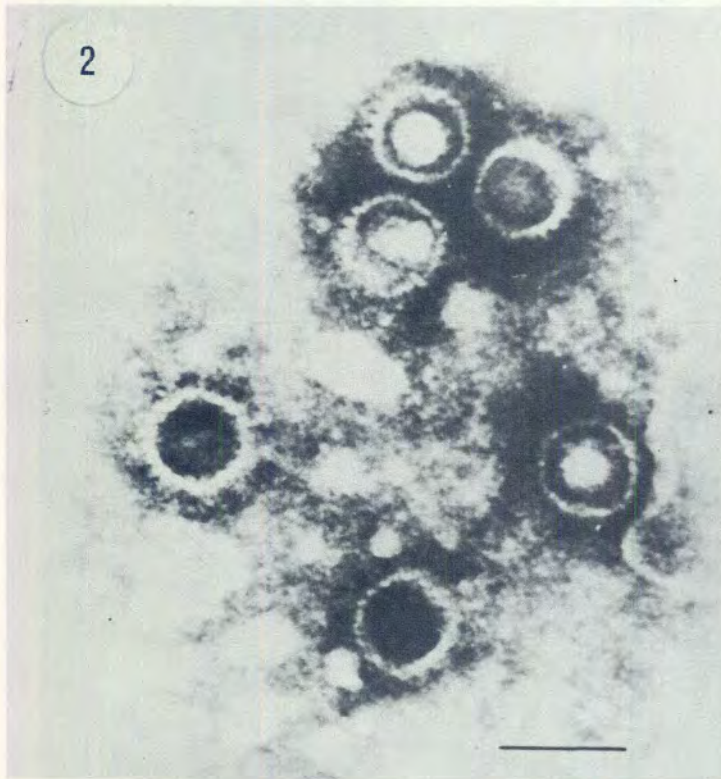

FIG. 2 H8/1 negatively stained with 3% phosphotungstic acid at pH 6. Bar=150 nm
FIG. 3 H8/1 virus particles at various developmental stages in the nucleus of sectioned CFK cells. Note typical herpes virus morphology. Bar=250 nm
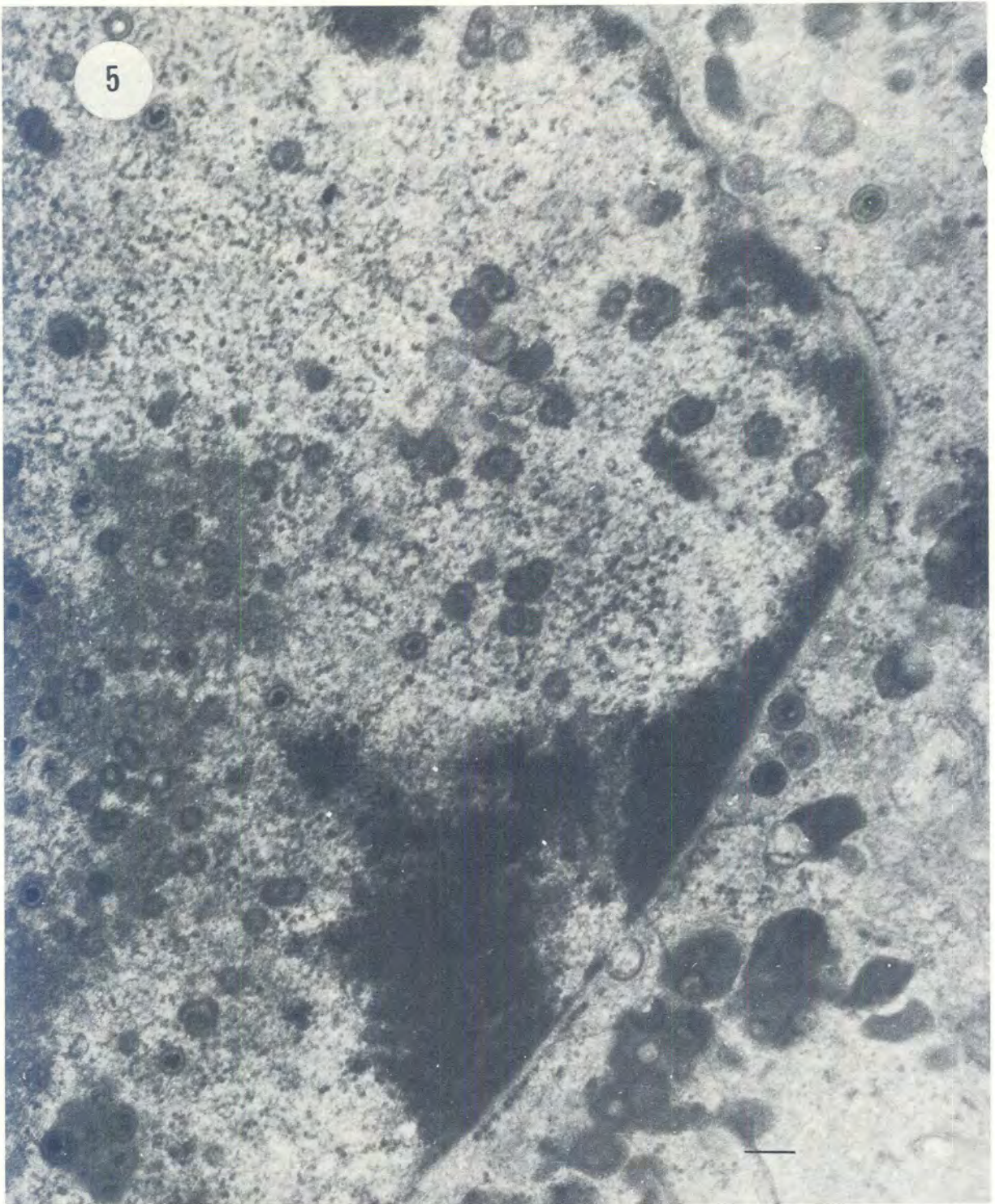FIG. 4 H8/1 virus, sectioned mature particles outside the cell. Bar=90 nm

FIG. 5 H2/2 virus particles at various developmental stages in sectioned CFK cells. Bar=200 mm

Females.—Cows 6221 and 6219, which received H8 (not plaque-purified) by the intravaginal route, developed a mild posterior vaginitis. On the 2nd day the mucosa was hyperaemic and small vesicles filled with turbid fluid were visible on the ventral aspect of the vulva around the clitoris. A mucopurulent discharge was present from the 2nd until the 7th day. By the 12th day the vaginal mucosa had returned to normal. Reisolation of virus was not attempted.

Cows 7589, 7577 and 7574 infected intravenously and calves 7976 & 7935 infected intranasally with virus H8/1 (prior to plaque purification) remained normal throughout the observation period. The paired serum samples of the above-mentioned 5 experimental animals were negative for neutralizing antibodies to this virus. Reisolation of the virus from the blood, vagina and nasal passages of the 5 animals was not attempted.

Heifer 9403, infected intravaginally with virus H2/2, showed no rise in body temperature and virus could only be isolated from the vagina until the 5th day (Table 3). The vaginal mucosa remained normal until the 11th day when the anterior part became hyperaemic and showed strings of pus adhering to the wall.

TABLE 3 Body temperature and virus isolation from experimentally-infected heifers

Days after infection	Heifer 789			Heifer 9403			Heifer 779			Heifer 584		
	Rectal temperature in °C	Virus H1/2 isolated from		Temperature	Virus H2/2 isolated from		Temperature	Virus H5/1 isolated from		Temperature	Virus isolated from	
		Vaginal swab	Blood		Vaginal swab	Blood		Vaginal swab	Blood		Vaginal swab	Blood
1	38,9	Nt	−	38,4	Nt	−	38,4	Nt	+	38,6	Nt	−
2	39,2	+	−	38,5	+	−	38,7	+	+	38,9	+ S+F	+F
3	39,6	+	+	38,3	+	−	38,7	+	−	40,0	+ S+F	+F
4	39,6	+	−	38,5	+	−	38,8	+	−	40,2	+F	−
5	39,9	+	−	38,3	+	−	38,8	+	−	39,7	+F	−
6	38,7	+	−	38,8	−	−	38,9	−	−	39,5	+F	−
7	38,8	+	−	38,8	−	−	39,0	−	−	39,3	+F	−
8	38,1	Nt	−	38,3	Nt	−	38,9	Nt	−	39,3	Nt	−
9	38,9	+	Nt	38,3	−	Nt	38,3	−	Nt	39,1	+F	Nt
10	38,9	−	Nt	38,3	−	Nt	38,9	−	Nt	39,6	+F	Nt
11	39,2	+	Nt	38,5	−	Nt	39,2	−	Nt	39,9	+F	Nt
12	38,8	−	Nt	38,6	−	Nt	39,0	−	Nt	39,6	−	Nt
13	38,9	−	Nt	38,4	−	Nt	38,7	−	Nt	39,0	−	Nt
14	38,7	−	Nt	38,9	−	Nt	38,3	−	Nt	39,0	−	Nt

+ = virus isolated
− = virus not isolated
Nt = not tested
S = slow-growing virus
F = fast-growing virus

The yellow purulent discharge was noticeable until the 20th day. On the 30th day a white opaque discharge was observed which continued for 3 days. Small reddish nodules approximately 2 mm in diameter appeared on the mucosa of the posterior vagina on the 45th day after infection and persisted for 6 weeks.

Heifer 779 infected with virus H5/1 responded in a similar manner. There was no detectable febrile reaction and virus could also be isolated from the vagina until the 5th day post-infection (Table 3). The vaginal lesions were exactly similar to those found in Heifer 9403.

Virus of the fast-growing group

Heifer 780, infected intravaginally with virus H1/2, developed a vaginitis characteristic of that described for IPV-IBR virus. On the 2nd day the mucosa of the posterior vagina and vulva became hyperaemic and was covered with vesicles whose content soon turned yellowish. A purulent discharge was observed on the ventral surface of the vagina. The body temperature was slightly elevated on Days 2–5 and virus was isolated from the blood on the 3rd day and from the vagina up to the 11th day (Table 3). The vesicles gradually became confluent but no ulcers formed and the mucosa appeared normal by the 14th day.

Pool of slow- and fast-growing viruses

Heifer calf 584, infected intravaginally with a pool of viruses consisting of 10 slow-growing and 1 fast-growing strain, showed a definite febrile response which was most marked on the 3rd and 4th days and remained slightly elevated until the 12th day (Table 3).

Virus was isolated from the vagina until the 10th day post-infection and from the blood on the 2nd and 3rd days only. Slow-growing virus was isolated from the vagina on the 2nd and 3rd days only, while fast-growing virus identified as H1/2 was isolated regularly until the 10th day. Only fast-growing virus (H1/2) could be isolated from the blood samples.

The posterior vagina appeared hyperaemic and a few vesicles were present on the 2nd day. A muco-purulent vaginal discharge was visible on the 3rd day and numerous yellow vesicles, which first appeared on the dorsal part of the vagina, later spread all over the surface of the posterior vaginal mucous membrane. The vesicles became confluent, with strings of pus adhering to the vaginal wall. Mucopurulent eye and nasal discharges were also observed on the 3rd day. The mucopurulent vaginal discharge continued until the 21st day, although the vesicular lesions had disappeared by the 12th day. Vaginoscopy on the 10th day showed that the cervix and the mucosa of the anterior vagina were hyperaemic and moist, and bled easily. A tenacious, yellowish, odourless mucoid discharge was observed on the ventral surface of the anterior vagina. One month after infection a whitish vaginal discharge was observed. During the 6th week light-red nodules, which bled easily when touched, appeared on the mucosa of the posterior vagina and vulva and were still present 10 months after infection when the last examination was carried out. The tenacious, yellowish, mucoid discharge persisted around the cervix. After the 10th day virus could not be isolated from swabs taken from the anterior vagina or the mucoid discharge on several occasions during the 10-month observation period.

Discussion

The physico-chemical, morphological and certain biological characteristics of both the 12 slow-growing and the 3 IPV-IBR related virus strains employed in this study undoubtedly relate them to the family *Herpetoviridae*. However, the serological results showed that the slow-growing virus strains are not antigenically related to the 3 IPV-IBR strains. This confirms the work of Maré & Van Rensburg (1961), who also found 2 serologically distinct groups of herpes viruses associated with vaginitis in cows and epididymitis in bulls on farms where the so-called

"epivag" syndrome described by Daubney, Hudson & Anderson (1938) and Van Rensburg (1949) was prevalent. Furthermore, the results also showed that the 15 virus strains used in this study are not related serologically to the herpes virus of bovine ulcerative mammilitis.

What still needs to be determined is whether the 12 slow-growing virus strains are serologically related to other herpes viruses of cattle such as DN-599 (Mohanty, Hammond & Lillie, 1971), DDV-71 (Parks & Kendrick, 1973) and other herpes viruses not related to IPV-IBR virus. The 2 outstanding biological characteristics, namely, the slow-growth rate in tissue culture and the inability to produce neutralizing and CF antibodies, agree with those described for DN-599 (Mohanty et al., 1971). Potgieter & Maré (1974) showed, however, that it is possible to elicit the production of neutralizing antibodies to DN-599 virus by administering purified virus in complete Freund's adjuvant to rabbits.

Although definite conclusions cannot be drawn from the limited number of animals used for the pathogenicity studies, it would appear that the fast-growing IPV-IBR strains are more inclined to produce a pustular vulvo-vaginitis, indistinguishable from that described by Kendrick, Gillespie & McEntee (1958). However, Maré & Van Rensburg (1961) conclusively showed that the FH-335 strain of IPV-IBR virus which they used in their studies was able to produce primarily an anterior vaginitis accompanied by a copious muco-purulent discharge in heifers and a transient epididymitis, vesiculitis and balanoposthitis in bulls. On the other hand, experimental infection of cows and heifers with some of the 12 slow-growing virus strains individually produced a variable reaction, ranging from a mild pustular vulvo-vaginitis to a severe anterior vaginitis with a copious muco-purulent discharge, this subsequently followed by a granular vaginitis in the posterior vagina. No disease symptoms could be elicited in a single bull infected with one of the virus strains, nor was there any clinical response in cows and calves injected intravenously and intranasally, respectively, with another strain. However, since the susceptibility of the experimental animals to the slow-growing virus strains could not be determined serologically, the variation in response could be due to previous exposure to 1 or more of these viruses.

The fact that both slow-growing and IPV-IBR related virus strains had been isolated previously in the same herd from different animals showing the typical "epivag" syndrome (Maré & Van Rensburg, 1961) prompted the infection of the heifer calf 584 with a pool of 10 slow-growing and 1 IPV-IBR-related virus strain. It is interesting to note that this animal developed clinical signs which can be divided into 2 distinct phases. The initial phase was observed for the first 2 weeks after infection and involved the posterior vagina with lesions resembling those of IPV (Kendrick, Gillispie & McEntee, 1958). The 2nd phase was observed during the 3rd week after the abovementioned lesions had disappeared and mainly involved the anterior vagina, although the posterior part was also affected to a lesser degree. The lesions, which were characterized by an anterior cervicovaginitis accompanied by a profuse tenacious mucopurulent discharge, resemble the "epivag" syndrome in females described by Daubney et al. (1938), Van Rensburg (1949) and Maré & Van Rensburg (1961). In addition, the persistent nodular rash which was observed in the posterior vagina during the 2nd phase has been reported by Daubney et al. (1938) in unserved

heifers and has also been described previously as "Knötchenausschlag" (Isopani, 1887; cited by Erhardt 1896), "vaginitis verucosa" (Tromsdorf, 1894); "infectious catarrhal vaginitis" (Ostertag, 1901); "colpitis granularis" (Liebetanz, 1901) and "colpitis infectiosa" (Nieberle & Cohrs, 1931).

Although it has been shown that an anterior vaginitis resembling the description of the "epivag" syndrome in females can be produced by slow-growing herpes virus strains as well as certain IPV-IBR related virus strains, it has not yet been possible to reproduce the epididymitis characteristic of this syndrome in bulls. It is possible that "epivag" may be due to infection with more than one infectious agent, including viruses and other micro-organisms. Admittedly, the number of experimental animals was limited in the pathogenicity trials described in this paper and no attempts were made to determine the presence of other intercurrent infections. Therefore, much further work is indicated before conclusions can be made about the role of herpes viruses in the aetiology of "epivag".

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