

ISOLATION IN MICE AND EMBRYONATED HEN'S EGGS OF A VIRUS ASSOCIATED WITH VAGINITIS OF CATTLE.

B. M. McINTOSH, D. A. HAIG and R. A. ALEXANDER,
Onderstepoort Laboratory.

In the Union of South Africa a disease of cattle which resembled that described as "epivag" or infectious epididymitis and vaginitis in Kenya by Daubney, Hudson and Anderson (1938) was first encountered in 1949 on a farm near Heidelberg in the Transvaal (van Rensburg 1949). A survey of the Union conducted by the Field Section of the Division of Veterinary Services revealed the presence of the condition on more than 400 farms in the Witwatersrand and the southern and north-western areas of the Transvaal. Only three infected farms were found in Natal and 19 in the Orange Free State, where it appeared to be confined to the north-eastern portion. No infection was found in the Cape Province (Diesel 1951).

Diagnosis had of necessity to be made on herd history and clinical examination and has been confined to those herds where frank cases of epididymitis could be detected. Since the incidence of clinical cases of epididymitis in some herds may be very low, the position on those farms where vaginitis only was encountered was uncertain.

From vaginal discharges collected from clinically affected animals in several different herds one or more agents were obtained which produced oedema and cellular proliferation on the chorio-allantoic membrane of hen eggs. Continued propagation for more than four serial passages was usually unsuccessful but in some instances passage up to 15 generations succeeded. Multiplication appeared to be confined to the membrane and embryo mortality was low. In no instance was it possible to maintain the virus more than a generation or two in suckling mice. Recently, however, a virus ("Rustenburg") has been isolated and maintained by serial passage in both mice and fertile eggs from a herd showing vaginitis which on clinical evidence is transmitted by bulls during coitus. None of the four bulls in this herd have developed epididymitis so that the relationship of this virus to epivag or possibly to a form of vaginitis of viral aetiology encountered by Blakemore (1952) in England where epivag is unknown is as yet uncertain.

Work done with this virus forms the basis of this report.

SOURCE OF MATERIAL.

Vaginal discharge was collected from cows in a herd in the Rustenburg district by a colleague. No infected bulls could be found on this farm but several of the cows showed vaginal discharge. A Friesland heifer in the laboratory was infected with this material by inserting a sterile swab, steeped in the pooled discharge from four cows into the vagina of the heifer for 18 hours after the method of Daubney (1939). When examined on the seventh day after infection,

the heifer showed marked reddening of the vaginal mucosa and a creamy yellowish mucoid discharge. These symptoms persisted until the eighteenth day when the heifer was slaughtered and vaginal mucosa was collected. This was mixed with an equal amount of saline, macerated in a Waring blender and lightly centrifuged. To the supernatant fluid penicillin and streptomycin were added in a final concentration of 500 units and 500 micrograms respectively. After standing one hour at room temperature it was used to infect mice and eggs.

A. Behaviour of the Strain in Mice.

Adaptation.

A family of nine one-day old Swiss albino mice was given intracerebrally 0.03 ml. of the vaginal mucous membrane extract. On the seventh day all the suckling mice appeared sick; the brains of four were harvested and used to infect another family of one-day old mice. The remaining mice were kept under observation for a further two weeks when they were discharged in good health.

All the mice in the second generation became sick within 48 hours at which time their brains were harvested, pooled, and used to infect a third family of suckling mice. These started to die on the second day after injection.

In all subsequent passages adult white mice were used. In those of the early generations the incubation period varied from five to seven days, but in later generations deaths occurred three to four days after injection. The mice usually appeared sick for about twelve hours before death. They were dull and weak and a few showed paralysis of the hind limbs but no excitability or hypersensitiveness was observed. From the sixth mouse generation there were no survivors.

Infectivity.

Serial ten-fold dilutions of four pooled brains from the 14th mouse generation were made in 10% horse serum saline. Four adult mice were injected intracerebrally with each dilution. The Ld 50 was about 10^{-4} . Similar titrations of viral activity in brains of suckling mice infected with material from the 25th generation gave an end-point of about 10^{-6} .

Gradocol Membrane Filtration.

Pooled brains from the fifth generation of the strain were injected intracerebrally into a family of one day old mice. When these mice were *in extremis* their brains were harvested and a 10% suspension made in 10% horse serum saline. After angle-head centrifugation at 2,000 r.p.m. for one hour, the supernatant fluid was passed through gradocol membranes of decreasing pore diameter. Suckling mice were injected intracerebrally with each filtrate. The results of this experiment are shown in table I.

Result.

From this table it is seen that activity could be demonstrated in filtrate from a 207 m μ A.P.D. membrane and possibly in the 106 m μ A.P.D. filtrate. The size of the virus particle was thus judged to be rather less than 100 m μ .

B. Behaviour in Developing Hen Eggs.

Fertile eggs were obtained from the pullorum-free flock of White Leghorns maintained at Onderstepoort. They were used after eight day preliminary incubation at 100° F.

TABLE I.

Gradocol Membrane Filtration of the Mouse-adapted Strain of Virus.

A.P.D.	Days after Injection.							
	1.	2.	3.	4.	5.	6.	7.	8.
406 m μ	0/4*	0/4	4/0	—	—	—	—	—
317 m μ	0/5	1/4	4/0	—	—	—	—	—
207 m μ	0/3	0/3	0/3	1/2	0/2	0/2	1/1	0/1
106 m μ	0/4	0/4	0/4	0/4†	0/4	0/4	0/4	0/4

* Numerator denotes mice dead.
Denominator denotes mice alive.

† Mice appeared sick.

The same mucous membrane extract that was used to initiate infection in day old mice was used for inoculation onto the chorio-allantoic membrane of twelve eggs.

After six days incubation in a forced draught incubator at 35° C. all the embryos were alive but on opening the eggs the membranes were found to be thick and oedematous with a marked opacity at the site of injection. These membranes were macerated and after light centrifugation the supernatant fluid was used for injection onto the membranes of other eggs. In this way serial passages were made at intervals of from four to six days.

Before the fourth generation only an occasional embryo died, but on further passage the percentage mortality increased.

The lesions produced became progressively more marked and by the twelfth egg generation infected membranes showed marked oedema with a number of pock marks which tended to coalesce in the centre of the lesion.

Embryos of the twelfth C.A.M. passage found dead on candling on the sixth day were harvested. A 10% emulsion was made in broth and the supernatant fluid after light centrifugation was used to infect other eggs by the yolk sac route, the dose being 0.1 ml. Passage was continued by subinoculating embryos harvested on the day when the majority died, usually on the fourth day at 35° C. No difficulty was experienced in maintaining this embryo-yolk sac passage for seven generations by which time embryo deaths commenced on the third day and 100% mortality occurred by the sixth day. The harvested embryos were slightly oedematous and were covered with numerous haemorrhages.

A family of day-old suckling mice was given macerated chorio-allantoic membranes from the twelfth egg generation intracerebrally. Five of seven mice died on the fourth day.

A gradocol membrane filtration was made with chorio-allantoic membranes from the fourth generation. After maceration the membranes were centrifuged at 2,000 r.p.m. for one hour. The supernatant fluid was then diluted 1/10 in broth and further clarified by passage through asbestos pulp. The clarified fluid was then passed through a gradocol membrane of 610 m μ A.P.D. and used to infect eggs by the C.A.M. route.

ISOLATION OF A VIRUS ASSOCIATED WITH VAGINITIS OF CATTLE.

When examined six days later well developed lesions were found in the chorio-allantoic membranes resembling those in eggs injected with unfiltered infected material.

INFECTION OF CATTLE WITH MOUSE AND EGG ADAPTED STRAINS.

Two Friesland cows and two Hereford heifers were infected with material containing the mouse and egg adapted strains of virus by the method of Daubney. The animals were examined at intervals with the aid of a vaginal speculum.

A. *Mouse adapted strain.*

Friesland cow 5456.

This cow was infected with a 10% suspension of four brains from the fourth mouse generation.

When examined four days later she showed some reddening of the vaginal mucosa and about 20 ml. of an opaque odourless discharge which contained numerous white floccules. The tail and perineal region became soiled with a sticky substance. On the tenth day there was about 50 ml. of an opaque yellowish mucoid material.

The condition then improved rapidly and by the twelfth day after infection only a small amount of clear mucus was present. However, on the 18th day there was again a small amount of opaque mucous material present.

Hereford heifer 5539.

This heifer was infected in the same way with material from the sixth mouse generation.

On the third day after infection she showed about 25 ml. of opaque mucous material in the anterior portion of the vagina. By the fifth day the tail region was soiled and a large amount of slightly opaque material was found in the vagina; the mucous membrane was reddened and bled easily on manipulation.

On the seventh day after infection very little abnormality was to be seen but when this heifer was examined on the 13th day a moderate amount of opaque mucous material was found again.

B. *Egg adapted strain.*

Friesland cow 5454.

This cow was infected with macerated chorio-allantoic membranes from the third generation.

On the fourth day after infection this cow showed about 10 ml. of opaque mucous material; the mucous membrane was reddened and bled easily. The tail became soiled and long strings of slightly opaque material were frequently seen hanging from the vulva over a period of two weeks. When examined on the seventh day and again on the fifteenth day after infection only a small amount of opaque material was found.

Hereford Heifer 5541.

This heifer was infected with material from the fifth egg generation.

On the third day after infection a small amount of yellowish opaque material was found on the floor of the vagina; the mucosa of the anterior part of the

vagina was reddened. On the following day the amount of discharge had increased considerably and it contained numerous floccules; the mucosa was now quite red and bled easily. By the sixth day, however, the condition had improved considerably. On the 13th day, however, again a small amount of opaque mucoid material was present.

Conclusion.

From the observations on these four animals it is evident that a definite, though mild vaginitis resulted from infection with material from both the mouse and egg adapted strains of the virus.

DISCUSSION.

The diagnosis of infectious epididymitis and vaginitis is at present most difficult and uncertain. It is based upon the clinical lesions in individual animals considered in relation to the herd history. Where cases of vaginitis with copious discharge of opaque odourless exudate are found in a number of cows and frank cases of epididymitis are present in the bulls, a positive diagnosis is justified. Unfortunately in many herds the typical picture is not found. This has led to considerable confusion.

It appears from observations in South Africa that bulls exposed to infection may take a considerable time to develop clinical epididymitis; it is possible that no epididymitis may develop after infection. Evidence of infection in females may clear up before clinical symptoms in the bulls are detectable. For example a farm in Natal was declared infected in August 1949 because the owner had introduced 170 cows a short time previously from a known infected farm in the Transvaal. At the same time he had purchased four young Friesland bulls from an apparently clean herd. At that time a number of the cows were showing typical vaginal discharge; the bulls appeared normal. When this herd was again examined in February 1951, i.e. 18 *months later*, the cows appeared to be normal and the bulls as yet had not developed clinical epididymitis.

Again in August 1949 another farm in Natal was declared possibly infected on the evidence of typical vaginal discharge in a number of the cows. It was not until the latter part of 1951 that the bull in this herd developed lesions and was found to have become sterile. This bull was slaughtered and the diagnosis of specific infectious sterility was confirmed histologically.

On this farm it was noticed that a number of heifers at the age of six to ten months showed vaginal discharge. This would indicate that the disease might be transmitted by means other than coitus. Similar observations have been made in other herds known to be infected with "evipag" (van Heerden 1952).

Because of the variety of forms shown by the disease in Kenya, it has been suggested (Daubney, Hudson and Anderson, 1938) that there are possibly two types of infectious vaginitis. One, usually a mild condition, occurs in heifers and even young calves before service as well as in the mature breeding females. The second type is more serious, appears to be spread only by coitus and, once introduced, leads to general infertility in the herd.

As the bulls in the herd from which the Rustenburg virus was obtained, did not show clinical epididymitis, it is considered that more information on this virus must be obtained before its relationship to evipag or possibly to the virus of Blakemore can be established.

ISOLATION OF A VIRUS ASSOCIATED WITH VAGINITIS OF CATTLE.

Lesions produced in heifers by both the mouse and egg adapted strains in females were mild. However, a definite vaginitis did result. The animals used in these experiments were kept housed in an insect-proof stable and it has been observed frequently that animals kept under these conditions react more mildly to infection than do those running under more natural conditions.

The results of neutralisation tests with this strain of virus by sera from animals on infected and clean herds and work on the relationship between this strain and those which have not proved fatal to mice or egg embryos are being investigated.

SUMMARY.

From infected material obtained from various herds virus has been isolated in several instances in developing hen's eggs, and in one instance also in mice.

The strain which could be propagated in both mice and eggs was taken 25 generations by intracerebral passage in mice. In early passages day-old mice were used but later passage was continued in adult mice. In these, mortality occurred regularly after three to four days. Gradocol membrane filtration indicated that the particle diameter was less than 100 m μ .

In developing hen's eggs this strain was propagated for twelve generations by chorio-allantoic membrane passage. Definite lesions on the membrane were observed and occasional embryos died. Subsequent passages were made by the injection of infected embryo material into the yolk sac. In this way the virus was readily maintained a further ten generations, at which stage it regularly killed all embryos.

Cows and heifers infected with material from both the mouse and egg propagated lines of this strain showed definite, though mild, symptoms of vaginitis.

The possibility of various forms of vaginitis occurring in bovines as well as the relation of this virus to these conditions is discussed.

REFERENCES.

- ALEXANDER, R. A. (1938). Studies on the neurotropic virus of horsesickness. VI. Propagation in the developing embryo. *Onderstepoort Jl.*, Vol. 2, No. 1, pp. 9-19.
- BLAKEMORE, F. (1952). Personal communication.
- DAUBNEY, R. (1939). Epididymitis in bulls associated with vaginitis in female stock. *Ann. Rep. Vet. Dept., Kenya Colony*, for 1938, pp. 77-78.
- DAUBNEY, R., HUDSON, J. R., AND ANDERSON, J. (1938). Preliminary description of a form of sterility in cattle associated with vaginitis in female stock, and with chronic changes in the epididymis and in the testicles of bulls. *East Afr. Agric. Jl.* Vol. 4, pp. 31-34.
- DIESEL, A. M. (1951). Personal communication.
- HENNING, M. W. (1949). *Animal Diseases in South Africa*. Central News Agency Ltd., South Africa.
- HUSTON, P. E. (1947). Cited by Henning (1949).
- VAN HEERDEN, J. S. (1952). Personal communication.
- VAN RENSBURG, S. W. J. (1949). Personal communication.