APPLICATION OF THE COMPLEMENT-FIXATION TEST TO THE DEMONSTRATION OF RINDERPEST VIRUS IN THE TISSUE OF INFECTED CATTLE USING RABBIT ANTISERUM. I. RESULT WITH THE AKABETE AND PENDIK STRAINS OF VIRUS (1)

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The cattle population of our country is undoubtedly highly susceptible to rinderpest, having had no previous contact with this infection. In the case of an outbreak a quick and reliable method of diagnosis would be very useful to prevent the rapid spread of the disease.

In a previous report (1) it was demonstrated that after careful extraction of infected rabbit spleen with acetone-ether, it is possible to obtain rinderpest antigen free of nonspecific complement-fixing activities. With properly-prepared rinderpest rabbit antisera this extracted material gives excellent fixation of complement. The present paper will give the results of experiments using complement-fixation methods, in which the presence of viral antigen was detected at the various stages of experimental infection with the Kabete and Pendik strains of rinderpest virus. The technique adopted will be described in detail.

LITERATURE REVIEW

The application of the complement-fixation test to the diagnosis of various viral infections of cattle has led to unexpected findings. Whereas anti-bacterial cattle sera such as that containing *Brucella abortus* antibodies (2,3) fix complement readily with homologous bacterial antigens, anti-viral cattle sera are general exceptions to this rule in that they only rarely fix complement with homologous viral antigen. This peculiarity of cattle viral antisera was recognized by Minett in 1927 and by Cuica in 1929 as has been mentioned by Brooksby (4) in his review of the earlier serological studies of foot-and-mouth disease. Later, Traub and Mohlmann (5) and Brooksby (4) made similar observations but pointed out that guinea-pig hyperimmune serum was complement-fixing and could be used in the detection of virus in infected bovine tissue. During the Canadian outbreak of foot-and-mouth disease this method of detecting the virus was also utilized in our laboratory It was further determined by Rice *et al* (6,7) that non-complement fixing antibodies in bovine sera could be demonstrated by an indirect form of the complement fixation test previously developed for the detection of antibodies in non complement-fixing avian antisera (8). Working with rinderpest, Pellegrini *et al* (9) 1952 reported on the non fixability of complement by bovine serum antibodies. They claimed that good positive results to be

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obtained by inactivating the complement in bovine serum with 1.5 per cent sodium chloride instead of by heat. In other laboratories, including our own, the salt inactivation method has proved unsatisfactory (7).

As pointed out in a previous paper (1) laboratory methods for the diagnosis of rinderpest should then be oriented mainly towards the detection of the virus in infected tissue rather than towards the demonstration of antibodies in convalescent serum since in this highly fatal disease these hardly have time to develop before death occurs. The complement-fixation test has been widely used in the diagnosis of various viral diseases for the detection of the agent as well as for the demonstration of antibodies. For the former a good complement-fixing antiserum prepared in a suitable species of animal must first be made available. Nakamura (10) in 1936 found that sera of hyperimmune rabbits did not react with extracts of lymphatic glands of cattle infected with rinderpest virus. In 1940, the same author with Wagatuma (11), not having recognized the atypical behaviour of cattle viral sera, tried but failed to obtain fixation with the sera of cattle convalescent from rinderpest. They claimed, however, that sera of cattle hyperimmunized with infected lymphatic glands gave good fixation with a boiled extract of such infected glands. This fixation, they considered to be "specific" and not related to the presence of normal tissue isoantibodies. So far as we know this work has not been confirmed by other workers. More recently Nakamura (12) reported that sera of rabbits previously infected with the lapinized strain of rinderpest virus do not react with a heated antigen prepared from lymphatic glands of infected calves. The same sera when tested with raw antigen gave nonspecific complement-fixation. To avoid this nonspecific reaction with antigens prepared from raw lymphatic gland extract, this author heated the rabbit sera to 60°C. and reduced the fixation period in the test to four hours at 4° to 7°C. When he tried this technique with immunized cattle sera, he failed to detect any fixation. Nevertheless, he claimed that reactions could be obtained with non-inactivated sera from immunized cattle when these were tested with desiccated lymphatic glands as antigen. In his initial studies, 94.9 per cent of the vaccinated cattle were found to react in the test, whereas in the last trials only 19.4 per cent reacted. In our hands non-inactivated sera have given generally unsatisfactory results because of their anticomplementary properties and tendency to give nonspecific fixation with various antigens (7). Hence we try to avoid their use if possible.

MATERIALS AND METHODS

Preparation of rabbit typing antisera

The rabbit typing antisera were prepared according to the technique described in one of our earlier reports (1). Rabbits were injected with 3 ml. of an approximately 1:25 saline suspension of rabbit spleen infected with the Japanese strain of lapinized rinderpest virus. Bleedings were taken prior to infection and served as normal controls. Fifteen days after infection the rabbits were bled out and each individual serum tested by the com-

plement-fixation test using as antigen extracts of known-infected rabbit or cattle spleens that had been chemically extracted. Rabbit antisera which in a 1:20 or 1:40 dilution fixed three 50% haemolytic units of guinea-pig complement were pooled and served as "standard" typing sera. They were kept in either a frozen or desiccated form.

Preparation of Extracts of Infected Cattle Spleen

Six calves were artificially infected at the Grosse Ile Experimental Station, three with the Pendik, the other three with the Kabete strain of rinderpest virus. The calves were approximately 5 months old and each one received subcutaneously 2 ml. of a 1:10 dilution of infected spleen emulsion. Temperatures were taken twice daily until the animals were killed or died. All but one of the calves were bled, killed thereafter and the spleen removed at once; one calf died before this could be done.

Immediately after collection of the spleen the capsule was removed carefully, and the remaining material weighed and extracted with acetone-ether according to a modification of the method of Casals *et al* (13) as described in one of our previous reports (14). This consisted of grinding 100 grams of the splenic material in a Waring blendor or in a mortar with approximately 500 ml. of chilled acetone. After 20 minutes shaking in a mechanical shaker placed in the refrigerator at a temperature of approximately 9°C., the ground tissue was centrifuged at 2,000 r.p.m. for 30 minutes and re-suspended in the same volume of chilled acetone. This procedure was repeated once with acetone, then with a mixture of acetone and anhydrous ether, then twice with anhydrous ether only. All extractions were made, as much as possible, in the cold room. The last ether extraction was for a period of 24 hr., chloroform to a concentration of 1 per cent being added to the extract to inactivate the virus. The last ether extraction could be reduced to 20 minutes without materially changing the results obtained in the test.

After the final extraction, the material was centrifuged, the supernatant discarded and the residual ether evaporated under vacuum for a minimum of two hours or until the sticky splenic material appeared as a dry powder. The dry powder was re-suspended in one volume (100 ml.) of physiological saline and placed overnight at approximately 9°C., in a mechanical shaker. The following morning, the suspended tissue was centrifuged for 15 min. at 2,000 r.p.m. The sediment was discarded and the supernatant fluid frozen. Before freezing, an aliquot of the fluid, equivalent to 1 gram of the original spleen material, was injected into a calf to detect any infectivity. This infectivity test was performed for additional safety measure but could be omitted in case of urgency. Before being tested for antigenicity, the frozen material was thawed and centrifuged in an angle centrifuge for one hour at approximately 10,000 r.p.m. The supernatant fluid was then frozen in small amounts until ready to serve as antigen.

Complement-Fixation Technique

The methods used in the titration of complement and standardization of amboceptor are those described in the Standard Methods of the Division of Laboratories and Research of the New York State Department of Health (15). Preliminary titrations of all antigens were made by a single two-fold antigen dilution method, using 0.1 ml. of antigen dilutions, 0.1 ml. of three 50 per cent haemolytic units of guinea pig complement, and 0.1 ml. of 1:5 diluted rabbit antiserum. The period of fixation was 18 hours at approximately 9°C. followed by 30 minutes at 37°C., after the addition of 0.2 ml. of a 2.5% suspension of maximally-sensitized sheep red cells the density of which was adjusted with the aid of a Klett-Summerson photo-electric colorimeter.

The tests were then repeated with serial dilutions of complement ranging from 1, 2, 3, 4.5, 6, 9 and 12 — Fifty per-cent haemolytic units. From the percentage haemolysis recorded with the different complement dilutions, the amount of complement necessary to produce 50 per cent haemolysis with each of the different antigen dilutions was estimated by graphic methods.

RESULTS

Using the complement fixation techniques and the methods of preparing antisera and antigen described in a previous paper, (1) the optimum time for harvesting spleens from cattle experimentally infected with the Kabete or Pendik strains of rinderpest virus was determined. Various modifications of the procedures were tried with a view to improving the test for diagnostic purposes.

Antisera

Whereas hyperimmunization of rabbits is a general procedure in producing antisera of high titre, this has not proved satisfactory in rinderpest. On the contrary, it was found that rabbits with a good complement fixing titre at the 10th. to the 25th. day after infection, exhibited only a weak reaction after a second or third recall dose of the virus. For this reason in the present work, only a primary infective dose was administered and the rabbits were bled at the period when the serum titre seemed likely to be the highest, i.e. 15 days after infection. However, all rabbits did not produce a serum titre as high as 1:20 or 1:40. Sera of lower titre were discarded and also those showing marked anticomplementary activity.

Rabbit sera maintained in a frozen state for two years showed a slight decrease in titre and a little increase in anticomplementary activity. They were still usable when tested with the Pendik beef antigen prepared approximately 18 months earlier. It seemed possible that keeping the rabbit typing sera in a desiccated form would prevent change in fixability and anticomplementary activity. This possibility is now being tested. On reconstitution with saline 2 weeks after desiccation, the sera prepared for the present work did not show any change in activity.

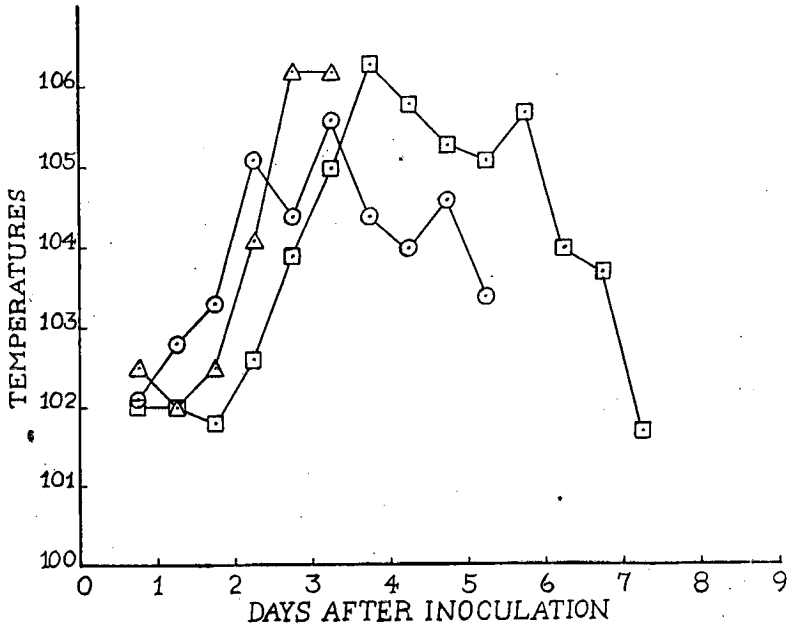
Antigen

As reported previously (14) attempts to concentrate the antigenic material in the spleen extract by centrifuging at 40,000 r.p.m. did not increase the potency of the antigen: the supernatant after centrifugation at 10,000 r.p.m. has proven to be a richer source of antigen than the re-suspended 40,000 r.p.m. sediment. This additional centrifugation at high speed had some advantages when dealing with a splenic extract highly contaminated with haemoglobin such as those derived from rabbit spleen, but it has no advantage with the beef spleen extracts which contain very little haemoglobin.

The stage of the disease process at which the spleen is harvested is the most important factor in obtaining tissue rich in virus. A good criterion for judging the stage of the disease process and the optimum time for harvesting of the tissue is the temperature of the animal. Figure I gives the temperatures of three calves infected with the Kabete strain of virus and figure II for three calves infected with the Pendik strain. It will be observed on comparing these two figures that the animals infected with the Kabete virus gave a fluctuating type of temperature curves whereas plateau shape curves lasting three days were given by the animals infected with the Pendik virus. In both cases the first animal was killed on the third day after infection which was a little less than 24 hours after the rise in

Figure I

Temperatures of the cattle infected with the Kabete rinderpest virus, taken twice daily from the day of infection to the collection of the spleens.



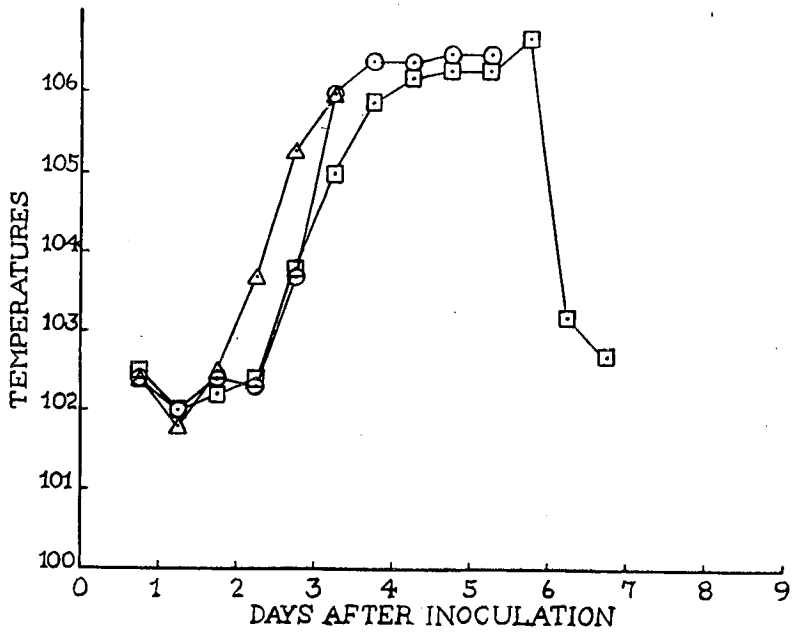
temperature. The second animal in both cases was killed on the fifth day after infection which was approximately 72 hours after the rise in temperature. The temperature of the animal infected with the Kabete virus was at this time beginning to decrease whereas the one infected with the Pendik virus still had a temperature around the maximal point. The spleen from the remaining animal in each group was harvested on the seventh day after infection. The temperature of both animals had been in the normal range for approximately 10 hours before death. The Kabete animal was still alive but in a moribund stage when sacrificed whereas the Pendik animal had been dead for one or two hours before the spleen was harvested.

The amount of complement fixed with rinderpest antiserum by the antigens prepared from these spleens collected at different intervals after infection varied greatly according to the period of harvesting and the strain of virus.

The spleen collected from the calf killed 3 days after infection with the Kabete strain did not show any appreciable fixation of complement with homologous antiserum. (Figure III). The haemolytic curves obtained by plotting the amount of complement required for 50% haemolysis against the amount of antigen used were essentially the same in tests with normal and rinderpest sera. The antigen prepared from spleen collected on the 5th. day after infection with Kabete virus fixed 8.3 units of complement in a 1:4

Figure II

Temperatures of the cattle infected with the Pendik rinderpest virus, taken twice daily from the day of infection to the collection of the spleen.

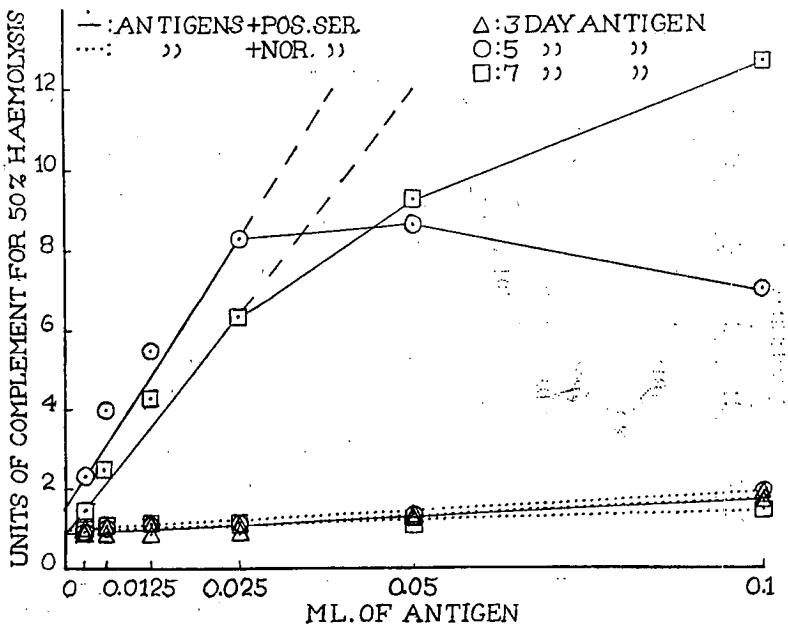


dilution which is equivalent to a 0.025 ml. of antigen in 0.1 ml. With large amounts of antigen, 0.05 ml. and 0.01 ml., 8.7 and 7 units of complement were fixed respectively. The antigen prepared with the spleen collected at the seventh day period from the animal infected with the same virus, fixed 6.4 units of complement when 0.025 ml. amount of antigen was used in the test, 9.3 units with 0.05 ml. of antigen, and 12.7 units of complement with 0.01 ml. of antigen. These three amounts are obviously equivalent to 0.1 ml. of 1:4, 1:2 and undiluted antigen. Both with the fifth and the seventh day Kabete antigens an approximately straight haemolytic curve was obtained when antigen amounts smaller than 0.025 ml. were used in the test. With the two largest quantities of antigen used, the amount of complement fixed did not increase in proportion to the amount of viral antigen incorporated to the test. An insufficient quantity of antiserum or the presence of pro-complementary substances in the antigen may have been responsible. However this deviation from linearity did not confuse the interpretation of the results.

In figure IV are given the results of the tests performed with the antigens prepared from the spleen collected from the Pendik infected animals. The antigen prepared from spleen removed on the 3rd day after infection fixed 3.5 units of complement when used in an 0.025 ml. amount. The 0.05

Figure III

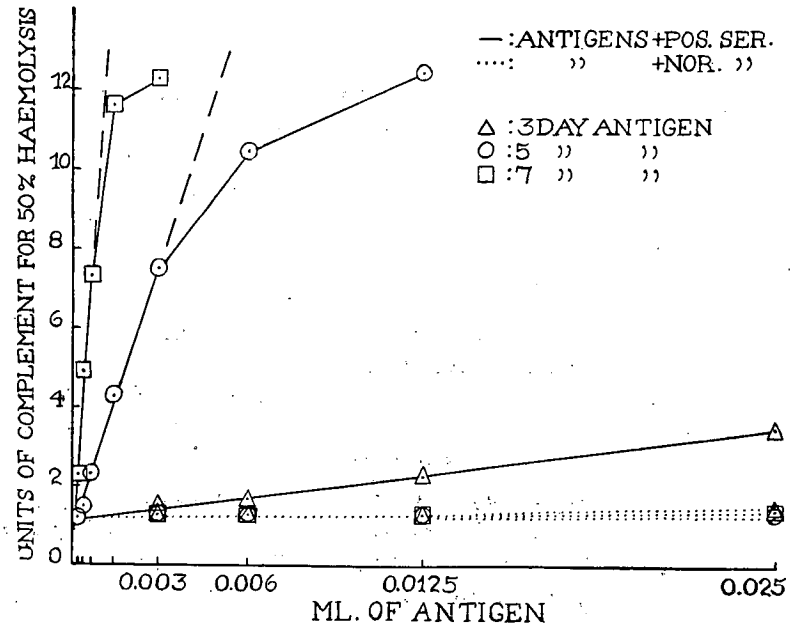
Amount of complement fixed by antigens, prepared from cattle spleens collected at various intervals during infection with the Kabete rinderpest virus, when tested with immune and corresponding normal rabbit sera.



and 0.1 ml. amounts fixed 5.7 and 9 units of complement respectively. The increased quantity of complement fixed by such an antigen in the presence of the rinderpest antiserum may thus be of definite diagnostic value since the haemolytic curve obtained was definitely steeper than that for tests of the same preparation with normal rabbit serum. With the antigen prepared from the Pendik spleen collected 5 days after infection, the haemolytic curve could not be evaluated with the greater quantities of antigen used because complete fixation was obtained throughout the tests. This antigen was of such high complement fixing titre with the rinderpest antiserum that it had to be used in 0.003 ml. or smaller amounts before a curve could be obtained, that is in a 1:32 or higher dilution. With 0.003 ml. of antigen, 7.5 units of complement were fixed and with 0.006 and 0.0124 ml. of antigen 10.5 and 12.5 units respectively. With the 7th day splenic antigen even more complement was fixed than with the 5th day material so the former antigen had to be used in a 1:128 or higher dilution before a haemolytic curve could be obtained. The amount of complement fixed in the test was 7.3 units with 0.0007 ml. of antigen and 11.6 and 12.5 units respectively with 0.0015 and 0.003 ml. of antigen. The Pendik antigen derived from the spleen collected seven days after the infection was therefore approximately 50 times more potent than the corresponding one collected

Figure IV

Amount of complement fixed by antigens, prepared from cattle spleens collected at various intervals during infection with the Pendik rinderpest virus, when tested with immune and corresponding normal rabbit sera.



three days after infection and about 35 times more potent than the Kabete antigen also collected 7 days after infection. The prozone effects noted with the two larger amounts of the 5th and 7th Kabete antigens were likewise apparent with the corresponding Pendik antigen.

DISCUSSION

From these experimental data it would be surmised that an uncertain result from the diagnostic standpoint might be expected if the spleens from cattle suspected of having rinderpest were to be collected very early in the disease processes. Should the animals be infected with a strain of virus resembling the Kabete in its behaviour, the results might be negative on the third day after infection. However, if the infection were with a rapidly developing strain similar to the Pendik, the test might be positive even at this early stage of infection. However, on the fifth day after infection when the disease process would be well established, both the Kabete and Pendik infections could be easily diagnosed by the complement-fixation test.

The fact that the extract of the spleen of an animal infected for three days with the Kabete strain of virus may not react in the complement-fixation test is not likely, however, to create a practical difficulty in the diagnosis of this infection since at this early stage the symptoms are so relatively minor that the attention of the cattle owner, or even of an expert, would seldom if ever be alerted. It would be only when the febrile period has existed for a few days that symptoms such as stupor, loss of appetite and so on would attract the attention of the attendant. If spleens were harvested at this stage, the diagnosis could easily be made within 3 days or less by the complement-fixation test described above.

SUMMARY

A complement-fixation test, in which rabbit serum collected 15 days after infection with the Japanese strain of lapinized rinderpest virus was employed as a source of typing antibodies, readily detected the presence of rinderpest virus in acetone-ether extracted cattle spleens as early as three days after artificial infection with the Pendik strain of the virus. With the Kabete strain of virus the test gave positive results from the fifth day after infection up to the death of the animal.

The complement-fixation test, because of its specificity and possibility of providing a reliable result within three days or less after receiving the suspicious material, promises to be a useful and rapid method of diagnosing rinderpest of cattle.

RESUME

Une épreuve de fixation du complément, dans laquelle du sérum de lapin, prélevé quinze jours après l'infection avec une souche japonaise de virus de la peste bovine adaptée au lapin, a été employée comme source d'anti-corps pour typage, détecte la présence du virus de la peste bovine dans des extraits

acétono-éthériques de rates bovines, aussi tôt que trois jours après infection artificielle avec la souche Pendik du virus. Avec la souche Kabete, l'épreuve donne des résultats positifs à partir du cinquième jour après infection jusqu'à la mort de l'animal.

L'épreuve de fixation du complément promet d'être une méthode utile et rapide pour le diagnostic de la peste bovine, par suite de sa spécificité et de la possibilité de fournir des résultats probants en moins de trois jours après réception de matérial suspect.

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