THE DIAGNOSIS OF DOURINE BY COMPLEMENT FIXATION

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INTRODUCTION

Dourine is a specific infectious disease affecting under natural conditions only the horse and the ass, transmitted from animal to animal by the act of copulation, and due to a single-celled animal parasite or protozoan, the *Trypanosoma equiperdum*. It is characterized by an irregular incubation period, the confinement of the first symptoms to the genital tract, the chronic course which it runs, and by finally producing complete paralysis of the posterior extremities, with a fatal termination, as a rule, in from six months to two years.

HISTORY OF DOURINE IN THE UNITED STATES

In the United States the disease was first suspected in 1885 and recognized in 1886 by Dr. W. L. Williams, who was then a veterinary practitioner at Bloomington, Ill. Officials of the State of Illinois took hold of the outbreak, and as a result of rigid prophylactic measures the disease was eradicated from the State in 1888, but not before an affected stallion had been shipped to Gordon, Nebr., thereby starting up a new center of infection in that locality.

In 1892 dourine was again brought into public notice by an outbreak among the breeding horses of northwestern Nebraska, the history of which suggested that it originated with this Gordon stallion. After an expenditure of about $5,500 by the Bureau of Animal Industry the disease was considered to have been eradicated from that section of the country. Five years later the infection again made its appearance in the same part of Nebraska, and early in 1899 the Bureau again began the work of eradication. Many inspections were made, and those animals which were found diseased were purchased and killed. Many obstacles were encountered, and the disease evidently kept smoldering during 1900.

In 1901 the infection reappeared with increased vigor, this time in the Pine Ridge and Rosebud Indian Reservations in South Dakota, in addition to northern Nebraska, and more stringent measures were immediately inaugurated to control the spread of the disease. However, eradication in this region was extremely difficult, owing to the wildness
of the country as well as of the horses and the fact that many horse owners would try to conceal from the inspectors animals which they knew to be affected with the disease. In 1906 the last suspicious cases of dourine were destroyed in South Dakota.

In the meantime, during the year 1903, dourine was reported in Van Buren County, Iowa, and successful steps were immediately taken to stamp it out. No connection could be established between this outbreak and that in Nebraska, but it was quite definitely determined that an imported Percheron stallion purchased by a company of farmers was responsible for its appearance.

Another outbreak of dourine was discovered in Taylor County, Iowa, in 1911. The diseased animals, together with all exposed stallions and mares, were immediately quarantined by the State. Those showing lesions of the disease and those exposed horses that reacted to the complement-fixation test were purchased by the Government and destroyed. It is now believed that the infection is entirely eradicated from Iowa. The source from which this center of infection was derived is only a matter of conjecture, but there is apparently no connection between this and any of the previous outbreaks. No authentic information as to the origin of the outbreak was discovered, but all cases lead back to a Percheron stallion which was imported in 1909 and brought direct to Lenox, Iowa.

Early in July, 1912, the State Veterinarian of Montana reported several suspicious cases of dourine in eastern Montana and forwarded blood sera from the suspected animals for the complement-fixation test. All but one sample gave positive results, thus establishing a new center of infection of dourine. From present indications this outbreak appears to be more extensive than any of the previous outbreaks, involving also two Indian reservations in North Dakota and South Dakota; but a force of 12 Federal veterinarians assisted by State representatives is at work on the disease, and the infection is well under control.

SEARCH FOR A METHOD OF DIAGNOSIS

The difficulty of diagnosing chronic and latent forms of dourine is generally recognized, and owing to this fact the control and eradication of this disease in horses has been of slow progress and sometimes ineffective. In such outbreaks it has been the custom to trace the disease as far as possible to its origin and then to keep under observation all mares and stallions which directly or indirectly have been exposed to the disease. At the same time animals which show clinical evidences of the affection are destroyed without delay. By this means several of the outbreaks which have occurred in the United States have been checked and eradicated.

The attempt to make a microscopical demonstration of the Trypanosoma equiperdum in affected horses is very frequently unsuccessful,
although our more recent experience proves that the organism may occasionally be found in the serous exudate of the plaques and also in the fluid of the edematous swellings of the genital organs in the stallions as well as in the mares.

Of course, this procedure of diagnosis can be attempted only when the disease occurs in farming localities where the animals can be readily observed and examined as desired. On the other hand, in the present outbreak in Montana and adjoining States the conditions make the diagnosis by the demonstration of trypanosomes impossible, and, likewise, animal inoculations can not be satisfactorily utilized for this purpose. Horses in that locality are bred under range conditions; they run wild and a round-up takes place only once a year. The difficulty of an examination, even clinically, of such animals is obvious, since they have not been broken to the halter and are troublesome to handle.

Our experience with the disease in Montana showed that only a limited number of animals were clinically affected. Nevertheless, the association of all the animals without any restriction in the breeding periods indicated that a larger number of animals would be found infected, which, as a matter of fact, has been proved by subsequent tests, as hereinafter shown.

Owing to the fact that until the last few years the eradication of dourine in this country was supposed to have been complete, the disease has received only slight attention as compared with other menacing diseases of our domesticated animals. It was not until the outbreak in the State of Iowa in 1911 that the necessity for devising a method of diagnosing this infection began to be fully realized. The value of being able to detect the latent and to verify the clinical cases became apparent. Otherwise, the necessity existed of maintaining a long-continued quarantine in those sections of the country where cases have been discovered. While little difficulty has been experienced in recognizing the advanced cases, a clinical examination alone naturally permitted many infected animals to escape detection, only to facilitate the further spread of the disease until the appearance of symptoms made the diagnosis unquestionable.

Inasmuch as the complement-fixation method of diagnosis has been employed with gratifying results in connection with numerous other diseases, the possibility of applying this method to dourine naturally suggested itself, and steps were therefore taken to determine the feasibility of its application to this disease.

It was very early discovered that the problem of preparing a satisfactory antigen would offer considerable difficulty. Efforts were primarily directed toward utilizing for this purpose the different organs of those horses that had succumbed to the disease. Several of the clinical cases were shipped from Iowa to the Bethesda Experiment Station during the outbreak referred to, in order that a more complete observa-
tion might be made of the development of the disease and that material might at the same time be available for the preparation of an antigen. From time to time, as these animals died, certain tissues were obtained which it was suspected might furnish the desired results, but although shake extracts of the spleens, livers, kidneys, and bone marrow, as well as alcoholic and acetone preparations, were employed under various conditions, the results were rather discouraging.

Subsequent to this time there came under our observation publications by numerous investigators who had given this subject consideration. It will suffice to mention the publications of Landsteiner, Müller and Pötzl, Levaditi and Yamanouchi, Hartoch and Yakimoff, Citron, Weber, Manteufel, Manteufel and Woithe, Zwick and Fischer, and Schilling, Claus, and Hösslin. The results in these instances appeared to have been unsatisfactory, which was also the case in the extensive work on the diagnosis of dourine by the Wassermann method by Trajan Pavlosévici, as he concluded that while antibodies can be demonstrated by this method in laboratory animals infected with trypanosomes, the method can not be utilized in stallions affected with dourine.

Later, Winkler and Wyschelessky, Mohler, and also Watson in their work on complement fixation as an aid in the recognition of trypanosomiasis indicated the good results obtained in the diagnosis of dourine. Likewise, Mattes in his work on the agglutination of trypanosomes obtained gratifying results, while Braun also concludes that complement fixation can be utilized for the diagnosis of trypanosome affections.

In the recorded publications it was observed that the more promising results were obtained by those who employed suspensions of pure trypanosomes. The organ extracts and other preparations of antigens generally used for this purpose proved unreliable. The procedure as recommended by various workers in obtaining an antigen from pure trypanosomes and using such a suspension as the antigen has also been tried by the writers with uniformly good results. The practical application of this procedure, however, would be very laborious and require a great deal of time, especially in cases where a large number of horses have to be tested by this method. Accordingly it was deemed advisable to devise a means by which an antigen could be prepared which would give similarly good results but would not require such delicate and laborious technique. In place of the specific trypanosome of dourine being utilized, the writers selected the surra organism, as it had been previously ascertained by several investigators that the reaction obtained was not absolutely specific for any one trypanosome infection but was rather of a group nature. As dourine is the only known trypanosome affection of horses existing in this country, the value of even a group reaction was immediately appreciated, and attention was directed to the carrying out of this idea in our diagnostic work.
In place of preparing suspensions of the trypanosomes, however, an antigen was made of the blood and macerated spleens of rats killed at the height of surra infection. This material was placed in a bottle containing glass beads and shaken for six hours, filtered through gauze, and carbolized. The results from this antigen proved satisfactory, and it was used repeatedly on the blood of the horses affected with dourine that were left of the Iowa shipment.

The smallest quantity of the serum which gave a positive reaction with the antigen was 0.05 c. c.; however, the various comparative tests indicated that fixation in tubes containing 0.2 c. c. of serum is sufficient for diagnostic purposes. Sera from normal animals, also those affected with various other diseases, failed to give a reaction. This antigen proved active on 10 consecutive days, but failed to produce fixation of complement on subsequent tests. Later attempts by the same procedure also resulted less satisfactorily, and it was therefore deemed advisable to try other methods in order to procure an antigen of more uniform action.

The following procedure was next employed:

After successive examinations of the blood of a dog infected with surra, about 200 c. c. of blood were drawn from the jugular vein when the microscopic examination revealed an extreme infestation with the parasite. The blood was drawn into a 1 per cent potassium-citrate solution in large centrifuge tubes of 100 c. c. capacity. A quantity of potassium-citrate solution was used equal to the amount of blood drawn into each tube, and 0.5 gram of saponin was added to each tube in order to dissolve the red blood corpuscles. After a thorough shaking and after complete hemolysis had taken place, the tube was centrifuged for 30 minutes at 2,500 revolutions, and the supernatant fluid was siphoned off. The residue, which was of an opaque color and consisted principally of trypanosomes, was then thoroughly mixed and shaken up with salt solution, when it was again placed in the centrifuge; this washing was repeated three times. After the last washing the thrown-down opaque mass was emulsified with 50 c. c. of salt solution and titered as to its merits as an antigen for dourine tests. The results were highly satisfactory, and the titer was established at 0.5 c. c. of this emulsion per tube. However, the disadvantages of this method—namely, the difficulty in the preparation of this antigen and also the small quantity which was obtainable from a single bleeding of a dog—were soon apparent.

In July, 1912, the outbreak of dourine in Montana was discovered, as already mentioned. Several samples of blood sera from clinical cases were forwarded by the State authorities to the Bureau of Animal Industry for verification. Positive reactions were obtained in numerous instances with antigens thus prepared, establishing conclusively the presence of the disease in that State, as well as suggesting the possibilities of the test as a means of its eradication. It was not long before dis-
covery was made that the disease was quite widely spread in Montana owing to the previous failure to recognize it. In an endeavor to comply with the request of the State authorities to make diagnoses in a large number of animals, it was soon apparent that a different method would necessarily have to be devised in order to make the desired progress.

PREPARATION OF ANTIGEN

Various organs from rats just dead from surra were tried out in both fresh and preserved states, and the results which were obtained from the fresh suspension of the macerated spleen of a rat just dead from surra were the most promising. In order to establish whether such an antigen would constantly, or at least in most instances, give the results desired, it was repeatedly tested on positive sera of horses affected with dourine, as well as on horse serum known to be free from immune bodies of dourine. After repeated tests on horses clinically affected with dourine had shown the antigen to be uniformly constant in its action, the procedure of diagnosing dourine by this method was definitely adopted. It was at this time that our present method of preparing antigen was first employed, which is as follows:

Gray or white rats are infected with surra by the injection of 0.2 c. c. of blood from a rabbit infected with that disease. Since tests have to be made every day to keep up with the large number of cases submitted and as the antigen proves effective only when prepared fresh, it was arranged that at least two rats should die daily with the disease. When the rats appeared to be at the point of death late in the afternoon it was found that placing such rats in the ice chest until they died furnished a better antigen than when they have died in the cage during the night and have to be used the following morning.

The spleens of the rats are removed, placed in a mortar, and ground up with a small amount of salt solution to a pulpy mass. From time to time more of the salt solution is added, and the suspension thus obtained is filtered twice through a double layer of gauze into a test tube. The quantity of the suspension from each spleen is made up to 40 c. c. by dilution with salt solution.

This suspension constitutes the antigen for the tests of the suspected dourine sera. Dr. Jacob Traum, who was temporarily assigned to this work, found that when the suspension was titered against sera in graduated quantities from a known positive and a known negative case the best results were obtained, and this method has since been adopted. The quantity of antigen employed is double the amount necessary to produce complete fixation with positive serum. The following table gives the method practiced in titrating the antigen:
Table showing method of titration of antigen for the complement-fixation test in dourine.

| Tube No. | NaCl solution, 1 | Serum | Antigen, 1 | Complement, 3 | Hemo-
|          | C. c. | C. c. | C. c. | C. c. | lytic
|          | O. 15 | O. 05 | I    | I    | serum, 4
|          | I     | I     | I    | I    | Blood
corpuses, 6
|          | I     | I     | I    | I    | For 1 hour in incubator.

Positive serum.

| Tube No. | NaCl solution, 1 | Serum | Antigen, 1 | Complement, 3 | Hemo-
|          | C. c. | C. c. | C. c. | C. c. | lytic
|          | O. 15 | O. 05 | I    | I    | serum, 4
|          | I     | I     | I    | I    | Blood
corpuses, 6
|          | I     | I     | I    | I    | For 1 hour in incubator.

Negative serum.

| Tube No. | NaCl solution, 1 | Serum | Antigen, 1 | Complement, 3 | Hemo-
|          | C. c. | C. c. | C. c. | C. c. | lytic
|          | O. 15 | O. 05 | I    | I    | serum, 4
|          | I     | I     | I    | I    | Blood
corpuses, 6
|          | I     | I     | I    | I    | For 1 hour in incubator.

1 0.85 per cent NaCl solution.
2 Suspension of macerated spleen from rat.
3 The determined smallest quantity established by titration.
4 Sensitized rabbit serum.
5 5 per cent suspension of red blood corpuscles of sheep.

Half the quantity of antigen which in the negative serum does not inhibit hemolysis, provided this quantity is at least double the amount necessary to produce complete fixation with the positive serum, indicates the titer of the antigen. For instance, if tubes Nos. 1, 2, 3, and 4 of negative serum show complete hemolysis and Nos. 5 and 6 slight inhibition, and at the same time tubes Nos. 6, 5, 4, 3, and 2 of positive serum show complete fixation and No. 1 partial fixation, the quantity of antigen for the test proper would be 0.2 c. c. of the antigen.

Occasionally the antigen does not prove satisfactory for the test and has to be discarded. In these cases the fixation in all tubes is apparently due to the excessive amount of proteids from the spleen. Experience has shown that the excessively large spleens contribute such an antigen. This, of course, is indicated by the titration undertaken prior to the regular test. At other times it was found that the antigen proved satisfactory the following day, after it was allowed to stand in the test tube overnight and the supernatant fluid drawn off for the antigen. This is then retitred and the titer established in accordance with the results of the test.

THE COMPLEMENT-FIXATION TEST

The test proper for the diagnosis of dourine is carried out in a manner similar to that practiced for the diagnosis of glanders. 1

1 A more detailed description of the technique of this method as applied to glanders is given by Mohler and Eichhorn in Bulletin 136, Bureau of Animal Industry, entitled "The diagnosis of glanders by complement fixation."
The hemolytic system consists of sensitized rabbit serum, serum from a guinea pig, and a 5 per cent suspension of washed sheep corpuscles.

The serum to be tested is, of course, inactivated for one-half hour at 56°C and is used in the tests in quantities of 0.15 c. c., since it has been found that fixation in this quantity is obtained only with sera of horses affected with dourine. Tests to determine the smallest quantity of serum of horses having dourine which will give a fixation showed that in several instances even 0.02 c. c. of serum was sufficient to give a complete fixation.

The complement from the guinea pig is always titered previous to the test, as it is absolutely necessary to use the exact amount of the complement to obtain the best results, since a deficiency or an excess of the complement would interfere greatly with the reaction.

In the numerous cases which have been tested the results were almost invariably definite, and only on a very few occasions was it found necessary to make retests on cases which appeared atypical. The reaction is always very marked, and in our work only a complement fixation with the quantity of serum mentioned is recognized as a positive reaction. It is only proper that in the tests the usual number of checks should be employed in order to insure reliable results.

Since the testing has been undertaken by the method described, 8,657 samples have been examined from Montana and the Cheyenne and Standing Rock Indian Reservations in North Dakota and South Dakota. Of these, 1,076 gave positive reactions, which appears to be a very large proportion, but when it is remembered that these animals were kept under range conditions without sanitary or veterinary control and also that before the disease was recognized as dourine it had been diagnosed for a long period as some other affection, it will be apparent that the opportunity for the spread of the disease was ideal.

With the present system of diagnosis, by which even the latent cases can be determined, it is hoped to eradicate the disease quickly. All the horses in the infected localities will be submitted to the complement-fixation test, and by cooperation with the State authorities means will be devised to dispose of the affected animals in such a way as to make the further spread of the disease impossible. The animals which were destroyed as a result of the disease in the above-named localities and which were diagnosed by the complement-fixation test showed in most instances some lesions indicative of the disease. In some of the cases there were no indications of a progressive paralysis, but the lesions existing in the genital organs of either the male or female were sufficient for confirmation of the diagnosis by the complement-fixation test.

It is therefore evident that the diagnosis of trypanosome infections of both man and animal by the complement-fixation test is of very great importance, especially in countries where only one of these protozoan
diseases exists. By this means it is possible to determine all infected persons or animals within a short time and adopt such hygienic measures as would be best suited for the control of the infection. Furthermore, the introduction of a disease like dourine into any country could also be guarded against by a compulsory requirement of this test on all horses imported from countries in which dourine is present.

**BIBLIOGRAPHY**


