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A STUDY OF THE AGGLUTINATION METHOD IN THE DIAGNOSIS OF GLANDERS.

by

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1012

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I. Introduction.

1. Glanders.

a. History.

b. Morbid Anatomy.

II. Diagnosis.

1. Mallein.

a. Preparation.

b. Method of Diagnosis.

2. Agglutination.

a. Theory.

b. Preparation of Test Fluid.

c. Technique of Applying Test.

1. Apparatus.

2. Securing Serum.

3. Dilutions.

4. Interpretation of results.

III. Experimental Data.

IV. Conclusions.

A STUDY OF THE AGGLUTINATION METHOD IN THE DIAGNOSIS OF GLANDERS.

Glanders is one of the most important infectious diseases of horses, asses and mules, and when transmitted to man one of the most fatal diseases of the human species. It runs an acute or chronic course attacking the lymphatic system, more especially in the upper air passages, lungs and skin. The disease is characterized by a strong tendency to the formation of small neoplasms or nodules which are likely to degenerate into ulcers from which exude a peculiar sticky discharge. In very acute cases a considerable rise of temperature and general debility may accompany the formation of the lesions. Glanders of the skin is known as farcy. By direct inoculation it may be transmitted to goats, rabbits, sheep, guinea pigs, field mice and several of the wild animals especially those of the cat tribe. Cattle, white mice, rats and domestic fowls are immune.

HISTORY:- Glanders appears to have prevailed in asses in Greece as noted by Aristotle. Its contagious prevalence in horses is recorded by Absyrtus in the time of Constantine and again by Vegetius Renatus in 381 A.D. In the central countries of Europe where the equine population is greatest and where there is the most extensive trade and movement among horses it secures the greatest relative number of victims. War, with its constant opportunities for infection, in crowded cavelry and artillery stables and the successive changes of place, tend greatly to enhance its ravages.

Thus in the German army it arose in 966 to 2058 per one hundred thousand per annum in the year of the Franco-German war; in Spain it was practically unknown until the Napoleonic war in the peninsula, but after this it proved a vertiable scourge; in Hindustan it was hardly known until the Sepoy rebellion yet its ravages greatly hampered the army movement in Afghanistan in 1879; from the United States it entered Mexico in army horses in1847. It became very prevelant in the armies during the Civil War of 1861-4, and was widely scattered over the whole country as a result of the sale of army horses and mules. Since that time as before, it has been most prevalent in the car stables of our great cities, though it has also gained a wild extension in many great horse breeding establishments in the Rocky Mountain regions, where however it proved much less destructive than in the east.

MORBID ANATOMY:- In chronic glanders, the most frequent location of the lesions are on the respiratory mucuos membranes, in the lungs, lymph glands and skin. The disease occurs in two forms, 1st., as circumscribed nodules with the formation of ulcers and cicatrices; and 2nd., as difuse or infiltrated lesions.

The most frequent location of the nodular form of glanders is situated upon the upper portion of the nasal septum and in the cavities of the turbinated bone. The nodules vary in size from a grain of sand to a millet seed, are more or less translucent, of a roundish or oval shape, and of a dirty gray or grayish color. The nodules may attain the maximum size of a pea and project somewhat above the surface of the mucuos membrane, surrounded by a reddish ring. Microscopically they consist of a large number of lymphoid cells, which disintegrate in the center of the nodule.

In consequence of the central fatty and purulent disintegration, the nodules become yellow in color, form ulcers and discharge. These ulcers are sometimes superficial, sometimes deeply lenticular or crateriform, surrounded by a hard indurated edge, and frequently becoming confluent with irregular and serated edges. The ulcers may increase in area or in deph and may even involve the underlying cartilage or bone, causing perforation of the septum nasi, and distensions of the maxillary or exostosis of the turbinated bones. The shallow lenticular ulcers may leave without leaving any visible changes; but the deeper ones after granulating leave a radiating star-shaped cicatrix which is either smooth or horny, and which, according to the shape of the ulcer, may be irregular oblong form. The nasal septum is frequently covered with these scars. They may also occur in the maxillary and frontal sinuses, in the gutteral pouches, in the eustachian tubes. They may also occur in the larynx, in the trachea, and even in the bronchi, particularly on the anterior surface, numerous long, oval ulcers or long pointed serrated scars are frequently found.

Diffuse glanders manifests itself as a diffused catarrh of the mucous membranes of the nasal and neighboring cavities with superficial ulceration, thrombosis of the veins, inflammatory infiltration of the submucosa, considerable thickening of the mucous membrane and the formation of peculiar radiating cicatrix. Both the nodular and infiltrated forms are found in the lungs. In the nodular form the lungs contain nodules varying in size from a millet seed to that of a pea. They are gray by transmitted light, glassy and pearl gray by reflected light, and are surrouned by a congested or hemorrhagic ring. The center of the nodules show a

pale yellow point in consequence of caseation and disintegration of the inner most cells. The nodules are of different sizes, of varying numbers, and of different ages. The formation of a capsule by a connective tissue membrane is induced by a reactive imflammation in the tissue surrounding the nodules.

Infiltrated glanders of the lungs form tumors of the size of a walnut to that of a child's head, consisting of disfuse glanderous infiltration of the alveoli and of the interstitial connective tissue. Frequently on section the infiltrated parts of the lungs resemble very closely a soft sarcoma. They are of a grayish dirty white color and are of a gelatinous consistency. In nodular and infiltrated glanders of the lungs and bronchial glands, and frequently the medistinal glands become enlarged indurated and studded with small foci of cell infiltration.

In glanders of the skin or farcy the nodules are found in the papillary layer, in the cutis and in the subcutaneous and superficial intermuscular tissue. They superate rapidly and form small ulcers. They change into large abscesses and discharge to the external surface. In the region of the nodules the lymphatic vessels are inflamed, swollen and frequently resemble a rosary or knotted cord. The neighboring lymph glands are swollen at first and soft, but later they become indurated by the growth of connective tissue and studded with dirty white nodules about as large as a pin head or with yellow foci of caseation.

Of the abdominal organs the spleen is the most frequently attacked. It then contains enbolic nodules which vary in size and either superate or become calcareous. Similar nodules occur though

not so often, in the liver, kidneys, testicles, the brain, muscles, heart and bones. Ulcers are very rare on the mucous membrane of the eyes, stomach and vagina.

ETIOLOGY:- This disease is due to the presence of a specific microorganism, the Bacterium mallei. Christot and Kiener claimed to have found the organism in the lesions of glanders in 1868. In 1881 bacilli were found by Bouchard in a glander abscess in man and these were cultivated and inoculated in a number of animals by Capitan and Charrin in 1882. Independently in the same year Löffler and Schütz discovered the bacterium. The organism is rod shaped, 2 to 5 microns long, by 0.5 to 1.4 broad. It is a non-motile facultative anaerobic and grows readily in a variety of culture media at a temperature of 37° C. On glycerin agar with milk it forms in 48 hours a milk-white layer changing to a yellowish brown. On potato it forms long slender filaments, in yellow, viscious, glistening colonies, changing to a fawn and darker. It grows best at 37° to 39° C. and growth ceases at 35° C. and above 42° C. It stains tardily in aniline colors but not at all by Gram's method. The bacterium often appears granular, and unequally stained in its different parts; frequently, when stained in tissues, it shows polar granules. It may be difficult to discover in old standing lesions of horses, but comes out quickly in recent lesions of experimental cases in Guinea pigs and donkeys. The bacterium has only a limited power of resistence, to destructive physical and chemical agents. It is killed in ten minutes at 55° C, and in two minutes at 100° C.,by a mercuric chloride solution 1 to 5000, by a phenol 5 to 100 or by a permanganate of potash 1 to 100. In

stables it may remain virulent for three or four months. Bacterium mallei does not grow in infusions of hay, straw or horse manure and it is doubtful if it can maintain a saphrophytic existance.

THE DIAGNOSIS OF THE DISEASE:- The accurate diagnosis of glanders in obscure cases and in horses that have been exposed but which fail to exhibit symptoms of the disease is one of the difficult problems in comparative medicine. The rapid strides that have been made in comparative pathology and bacteriology during the past few years and the diligent efforts among scientific investigators to bring to the front better methods of diagnosis which are comprehensive, accurate and rapid have been fruitful in supplying the wants of the practical scientific man or practitioner. Probaby the most value to the clinician of these recent methods of diagnosis has been the perfection of the agglutination test for glanders in such a degree that its successful practical application is assured, and it is now used in Austria and Prussia. Schütz and Meisser in Germany have been among the leaders in perfecting this method of diagnosing glanders.

MALLEIN:- The method of diagnosis in most general use in the United States to-day is the Mallien test. Mallien is the sterilized and concentrated toxic product obtained from a pure culture of bacterium mallei in a peptonized glycerin bouillon. When injected hypodermically in a small physiological dose this has no effect upon a sound horse but in one affected with glanders it developes in several hours an extended swelling in the seat of innoculation, hot, tense and painful, which continues to enlarge from 24 to 36 hours and does not subside for four or five days. From the margin of the local lesion, swollen lymphatics may often be

traced running toward the adjacent lymphatic glands. There is also a decided dullness, prostration, inappetence, staring coat and tremors. The temperature rises 1.5° to 2.5° and upward after the eighth hour after innoculation, attaining its maximum from the tenth to the eithteenth hour and slowly subsiding from the forty-eighth to the sixtieth hour.

The animal to be tested should be in its customary environment and should not be subjected to any conditions which would cause excitment. Reaction sometimes fails in advanced cases of glanders but in such a case other symptoms are usually diagnostic so that mallien is superfluous and should not be misleading. The greatest care should be taken to prevent infection from the syringe, nozzle, skin, hands, etc., as other infections may give rise to local swellings and hyperthermia. If the first test leaves the matter in doubt, the animal should be secluded and tested again in a month.

AGGLUTINATION:- The successive acquistion of definite knowledge of the nature of the lesions of this disease, the life history of its specific bacterium and the effects of mallien have each tended to minimize error in diagnosis, yet the last and most apparently successful method is that based upon the power of the blood serum of an infected animal to agglutinate in high dilutions, the infecting bacterium. For an intelligent understanding of the phenomenon of the agglutination, a brief statement of its history, the properties of agglutinins and the character of agglutination is here introduced.

In 1889 it was found that Pseudomonas pyocaneus cultivated in the serum of an animal immunized against the organism did

not produce a diffused turbidity of the serum such as appears it is grown in a serum of a non-immunized animal. Instead of being scattered through the culture medium as usual, the organisms were joined in little clumps which settled in the bottom of the tubes. This was the first recognition of the phenomenon of agglutination, but its application to diagnosis was not made until 1896. Since 1896 the application of the agglutination method in diagnosis has been more or less successfully made in typhoid fever, hog cholera, Asiatic cholera, pneumonia, bubonic plague, tuberculosis and other infections. The power of the blod of glandered animals to agglutinate Bacterium mallei exceeds this power in other diseases to such a degree that the differential diagnostic significance of agglutination in glanders cannot be denied.

The suspension fluid used in this test is prepared by cultivating the third generation of Bacterium mallei for a period of forty-eight to seventy-two hours on an acid glycerin agar culture, nothing but the third generation should be used. It has been found that all cultures of the Bacterium mallei do not agglutinate satisfactorily. It has also been shown by experiment that a suitable culture when obtained is liable at unexpected intervals to lose its responsiveness to the agglutinin. This can be forestalled by passing the organism through a Guinea pig at leat once in three weeks. In order to have a suitable culture on hand, subcultures should be made every few days. A culture more than 72 hours old should not be used. The growth from the agar culture should be washed by the aid of a sterile loop, into distilled water containing 0.85 per cent sodium chloride and 0.5 per cent carbolic acid crystals. This suspension is then placed in a ther-

mostat at 60° C. for two hours, which kills the bacteria. A temperature higher than 65° C and lower than 60° C. should be avoided. After heating, the suspension is thoroughly triturated and filtered through sterile cotton. The filtrate thus prepared is diluted with the carbolized salt solution until it is of a faintly cloudy appearance.

In making the test at least ten c.c. of blood is drawn from the jugular vein under aseptic precautions. As soon as the clot forms the supernatent serum is drawn off and diluted with water or normal salt solution, using one part serum to forty parts of dilutent. For the purpose of testing the reaction of this diluted serum in our experimental work in the bacteriological laboratory at the Kansas State Agricultural College, the Parke, Davis & Company agglutometer was used. This consists of a case containing five sets of glass tubes of four each, each tube having a capacity of about six c.c. One set of four tubes is used in making a single test. The first tube is labeled 200, the second, 500; the third, 800; and the fourth 1200. Into each is poured three c.c. of the "Test Fluid" or suspension. Into the tube of suspension labeled 200 is introduced 0.6 c.c. of the 1 to 40 dilution making a dilution of one part of serum to 200 parts of the suspension fluid, into the tube labeled 500 is introduced 0.24 c.c. of the diluted serum thus making the dilution 1 to 500; in the tube labeled 800 is introduced 0.15 of the diluted serum making a dilution of 1 to 800; and into the tube labeled 1200 is put 0.105 c.c. of the diluted serum making a dilution of 1 to 1200.

This is then placed in an incubator or other warm place having a temperature of about 37° C. or (98 " F.). The agglutina-

tion may begin in six or eight hours and may be complete at the end of twenty-four hours. Unless all reaction are complete at the end of twenty-four hours the tubes should remain undisturbed and should be examined at the end of forty-eight hours, as reactions may occur after twenty-four hours, the reaction occasionly not becoming complete until the end of 72 hours. No further reaction will occur after 72 hours. The reaction shows a layer of agglutinated and precipitated bacteria covering the entire convexity at the bottom of the tube. This film-like sediment may become so dense that it rolls in at the periphery. The test fluid becomes clear in the lower dilutions and in cases of a strong reaction serum it may become clear in all the tubes. If a clearly visible, film-like, flocculent layer of bacteria covers the bottom of the tube, the reaction is to be regarded as positive whether the supernatent fluid has become completely clear or not. The tubes should not be shaken at any time while the reaction is taking place as this might interfere with a complete reaction. When a negative reaction is present there appears a small, round, concentrated spot composed of organisms, which have settled in the center of the convexity of the tube while the degree of cloudiness of the test fluid remains apparently unchanged. This usually occurs in dilutions above the 1 to 200 tube. In making the examination of the tubes, a well lighted window should be faced and the tubes turned toward the window at such an angle that the greator part of the bottom is visible. A black pasteboard should be placed against the bottom tube, between it and the window thus forming a black background. Turn the tubes slowly around and the film-like flocculent mass of agglutinated bacteria in the bottom of the tube will be clearly apparent when

a positive reaction is present.

Diagnosis can be made in the following manner: 1. If negative results occur in all four tubes glanders is not present. 2. With positive results in the tube labeled 200 and negative results in the other three tubes, a positive diagnosis of glanders is not to be made. 3. With a reaction in the tubes labeled 200 and 500 and the absense of symptoms of glanders in the animal and no signs of a positive reaction in the tubes labeled 800 and 1200, the case would be considered possibly suspicious and the animal should be retested about three weeks later. If the second test gives the same results as the first the animal should be regarded as free from glanders. 4. If a positive reaction occurs in the tubes labeled 200, 500 amd 800 the case should be regarded as very suspicious. A similar reaction as the first upon retesting indicates that the animal is free from glanders. 5. If a positive reaction occurs in all four tubes even in the absense of symptoms a positive diagnosis of glanders should be given at once. 6. Positive reactions in any of the tubes higher than the tube labeled 200 when symptoms of glanders are present indicate glanders and the animal should be condemned. 7. The degree of infection may possibly be revealed by the reaction being either tardy or prompt, weak or strong.

EXPERIMENTAL DATA:- Our work in the laboratory consisted chiefly in testing serum from both normal and infected horses and the inoculation of Guinea pigs to confirm the results; the maintainance of the virulence of various cultures of Bacterium mallei and the preparation of suspension fluid.

About three hundred and fifty centimeters of suspension

fluid was made from a third generation growth on artifical media, on a Parke, Davis culture of Bacterium mallei according to the directions above given. The efficiency of this suspension fluid compared favorably with suspensions procured from Parke, Davis & Company and Dr. V. A. Moore of the New York State Veterinary College.

The following results were obtained from cases tested, with Parke, Davis suspension fluid.

CASE No. 1.- Black Percheron mare, age eight, weight 1650, one posterior limb much enlarged and covered with farcy buds of two years standing. Positive reaction in 200 and 500. Confirmed by Mallein test with a rise of $3\frac{1}{2}°$ but a Guinea pig innoculated with pus from a farcy bud on the animal, showed no signs of orchitis or subcutaneous swelling.

CASE No. 2.- Black percheron horse, mate to No. 1 for two years, gave negative reactions in all four dilutions. Serum from cases 1 and 2 in duplicate test gave same results.

CASE No. 3.- Bay mare four years old suffering from slight attack of tetanus. Negative results in all four dilutions.

CASE No. 4.- Gray livery horse 10 years old, apparently in good health, gave negative reactions in all dilutions.

CASE No. 5.- Bay mare in plethoric condition suffering with azotouria. Reactions in dilutions of 200 and 500.

CASE No. 6.- Black mare apparently normal. Negative results in all four dilutions.

The following cases were tested with our own preparation of suspension, Parke, Davis suspension and Dr. Moore's suspension for the purpose of obtaining the relative efficiency of the different preparations.

CASE No. 7. - Bay mare old and emanciated, heavy in foal. Positive reactions in dilutions of 200 and 500 in all three preparations but owing to the absence of symptoms the animal was not condemned.

CASE No. 8. - Gray horse 25 years old, greatly emaciated, no symptoms of glanders. Positive reaction in 200 dilution and slight reaction in dilution of 500. Animal not condemned.

CASE No. 9. - Old gray horse greatly emaciated otherwise apparently normal. Test gave negative results in all three suspensions.

CASE No. 10. - Brown horse suffering with extreme case of fistulous withers, much pus having been absorbed. Negative results in all three preparations of suspension fluid.

INTERPRETATION OF RESULTS:- Case No. 1 was condemned from the evidence furnished by clinical symptoms and positive reactions in dilutions of 200 and 500 and a positive reaction with the Mallien test. The fact that it did not agglutinate the higher solutions nor produce clinical symptoms in a Guinea pig is accounted for in that frequently in cases of long standing the organisms loose their virulence to a certain degree.

CASE No. 2. - Showed no clinical symptoms of the disease yet one would expect to find a well developed case of glanders after an exposure of two years as was the case with this animal.

CASE No. 3.- Was not condemned.

CASE No. 4. - Not condemned.

CASE No. 5.- Owing to lack of clinical symptoms was not condemned. It has been found that normal serum may sometimes agglutinate as high as 500. This is very rare, however.

The animal should be retested after a few weeks.

CASE No 7. - Similar to No. 5. The agglutination may have been partly due to the animal having beem in foal. Should be retested.

CASE No. 8. - Normal blood frequently agglutinates 200.

CONCLUSION:- From the observation and results of our work, we suggest the following conclusions.

1sr. That the diagnosis of glanders by the agglutination method is more rapid, practical and more efficient that any other yer devised. It has the advantage that it can be used in those cases where there is a rise in temperature and consequently where Mallein could not be used.

2nd. That its rapid application is a very important factor with the busy practitioner. By this method when everything is in readiness it only requires a few minutes to make a test and oftentimes results can be obtained in six or eight hours. Whereas when horses are tested with Mallein it requires from thirty-six to forty-eight hours before a thorough test is completed and most of this time is consumed in taking temperatures.

3rd. That it is practical as animals are not prevented from working during the test. The horses need not be taken out of the harness to draw anounce of blood.

4th. That it relieves the veterinarian of false accusiations. Many times owners of animals tested with Mallein will accuse the veterinarian of injecting something into the animal's system determental to its general health. In this method there is no systemic disturbances nor local swellings.

5th. That this method will serve admirably for the de-

tection of occult cases of glanders which are dangerous to the health of the other animals and may be the spreaders of the disease.

6th. That the maximum dilution of normal serum that is capable of producing agglutination is 1 to 500.

7th. That the highest agglutination dilution of the serum of diseased horses not glandered does not exceed that of normal serum.

8th. That the interpretation of the results where the maximum dilution is about 1 to 500 gives the greatest difficulty. All cases of this kind, unless there are unquestioned diagnostic symptoms or lesions, should be held for subsequent test.

9th. That it offers a more practical method for the complete extermination of glanders.

10th. That the labor and cost of its application is much less than any other method of diagnosis.

BIBILOGRAPHY.

1. Law, Veterinary Medicine, Vol. IV.

2. Moore, Pathology of Infectious Diseases.

3. American Veterinary Review. Oct. 1906, May 1907, Sept. 1907.