

ATTENTION

Radial Immunodiffusion Test
For Quantitation Of Equine IgM
In Serum Or Plasma

Plate must be stored upside down. Avoid near freezing temperatures. Accuracy can only be assured with proper storage

1. Intended Use:

The quantification of equine IgM in horses aids in the diagnosis of combined immunodeficiency disease (CID). IgM levels increase in newborn foals from passive transfer of colostrum.

2. Summary

Single radial immunodiffusion tests have evolved from the work of Fahey and McKelvey¹ and Mancini et al². They are specific for the various proteins in serum or other fluids and depend on the reaction of each protein with its specific antibody.

When the wells in antibody containing gels are completely filled with the antigen, the precipitin rings that develop after 10-20 hours at room temperature are measured. The diameter of the ring and the logarithm (base 10) of the protein concentration are related in a linear fashion. Using appropriate reference standards, the concentration of unknown samples may be measured.

Immunoglobulin M (IgM) is the largest of the immunoglobulin molecules and because of its large size it is restricted almost entirely to the intravascular space. These macromolecules are highly efficient agglutinators of particulate antigens such as bacteria and red blood cells. When a foreign antigen is introduced into a host for the first time the synthesis of IgG and IgM antibodies begins almost simultaneously, however, the level of IgM antibodies peaks within a few days and then declines more rapidly than the level of IgG antibodies. Combined Immunodeficiency Disease: An autosomal recessive type of this disease has been identified in Arab foals. Affected animals are frequently asymptomatic during the first several months of life, but become progressively more susceptible to microbial infections as maternal antibody wanes. Arab foals with the disorder frequently succumb to adenovirus pneumonia or other infections at around 2 months of age. The foals are persistently lymphopenic. Precolostral serum samples have no detectable IgM antibody. Immunoglobulin levels will be normal following nursing, but will progressively decrease after that time compared to normal foals. Affected foals at ages 28 to 70 days were reported to lack IgM in all cases. Heterozygous individuals show no signs. The incidence of CID in Arabian foals is approximately 2 to 3%, suggesting a prevalence of about 25% for the carrier state.^{3,4,5}

3. Principle

Radial immunodiffusion is based on the diffusion of antigen from a circular well radial into a homogeneous gel containing specific antiserum for each particular antigen. A circle of precipitated antigen and antibody forms, and continues to grow until equilibrium is reached. The diameter of the rings is a function of antigen concentration. After overnight incubation, the zone diameters of reference sera are plotted against the logarithm (base 10) of the antigen concentration. If equilibrium is reached, the reference sera zone diameters are squared and plotted against their concentration (linear). At intervals in between, a linear relationship does not occur. Unknown concentrations are measured by reference to the standard curve.

4. Reagents

A. Radial immunodiffusion plates contain specific antiserum in agarose gel, 0.1M phosphate buffer pH 7.0, 0.1% sodium azide as bacteriostatic agent, 1 ug/ml amphotericin B as an antifungal agent. Plates also contain 0.002M ethylenediaminetetraacetic acid. Store at refrigerator temperatures (2 to 8 C).

B. Equine Reference sera - (Pooled equine serum at three levels*). Contains sodium azide (0.1%) as bacteriostatic agent. Store at refrigerator temperature.

5. Specimen Preparation and Handling

A. Collect whole blood without anticoagulant and allow to clot at room temperature.

B. Separate serum by centrifugation at about 200 rcf within 2-3 hours after collection.

C. Plasma may be used, but non-specific precipitation of fibrin may obscure precipitation rings. In addition, liquid anticoagulants such as ACD fluid will dilute the specimen.

6. Procedure**A. Materials Provided**

1. One Radial Immunodiffusion plate.
2. Reference Sera: 4x0.25 mls.
3. Directions for use.

B. Materials Required

1. Blood collection tubes
2. Centrifuge (200rcf)
3. Microliter dispenser (5 microliters)
4. Normal control sera (optional) - available separately
5. Measuring device - calibrated in 0.1mm increments
6. Two cycle semi-logarithmic graph paper and/or linear graph paper.

C. General

1. Do not overfill or underfill wells. An improperly filled well yields erroneous results and the same specimen should be placed in another well. Overfilling with a 5 microliter sample indicates that some gel shrinkage has occurred.

2. Reference serum zone diameters should be measured at the same time as test sera. If a delay in measurement is anticipated allow sufficient intervals between filling wells.

3. The time of filling each plate should be marked on the cover and if more than one plate is filled, they should be read in order of filling.

4. Excess moisture is required to prevent drying. Replace each plate in its plastic bag and reseal carefully before incubation.

5. Shrinkage of gel or oval shaped wells indicates drying and the plate should not be used.

6. If temperature fluctuations are anticipated, the plates in their bags may be incubated in an insulated container. Fluctuations in temperature may result in multiple precipitin ring formation.

7. Unused sections may be run at a later day if the plate has been stored at 2 to 8 C between incubations in its plastic bag. Check carefully for evidence of drying.

8. *Rough granulation of the gel indicates freezing, plates should be discarded.*

D. Performance of Test

1. Remove plates from refrigerator to room temperature approximately 30 minutes before filling wells. Do not open bag until ready for use.

2. If excess moisture is present, remove plate from bag and remove cover until evaporation has dried the surface and wells. Replace cover until used.

3. For best results, four wells should be filled with reference sera for each plate. Location of each should be noted. Mix each vial of reference serum thoroughly.

4. Deliver specimen to well by placing the pipette tip at the bottom of the well. Allow the well to fill to the top of the agar surface. Avoid bubbles to ensure proper volume and diffusion of sample. Visualization may be aided by placing the plate on dark background. If practice is required, a used plate may be utilized.

5. More consistent results may be obtained when wells are fill with a 5 microliter pipette.

6. Mark time of completion on plate cover and replace cover.

7. Replace plate in bag and reseal carefully.

8. Incubate plate upright on a flat surface at room temperature (20^o 24^o C) for 6-20 hours for Overnight readings and over 24 hours for End Point readings. See C6 above.

E. Calibration

1. Using the reference sera provided in kits determine their ring diameters to the nearest 0.1mm.

2. Using 2 or 3 cycle semi-logarithmic graph paper, plot the concentration on the Y-axis and the zone diameters on the linear or X-axis for each protein for Overnight readings.

3. Using regular graph paper, plot the concentration on the X-axis and the zone diameters squared on the Y-axis for each protein for End Point readings.

4. Draw a straight line of "best fit" between the three points. A curved line usually indicates that the incubation time and/or temperature should be reduced for overnight values. For valid results, a smooth curve should be fitted to the points and control sera included for additional verification.

F. Quality Control

For consistent results and a comparison of lot to lot, day to day, and week to week variations, a "normal" and abnormal serum should be included each day. The diameters and concentrations obtained can be charted to determine means and standard deviations. For the same specimen, an appropriate series of wells on the same plate should yield diameters within 0.2mm of one another. Control sera should be freshly thawed or reconstituted.

G. Reference Sera

All reference sera supplied have been calibrated from two Standard sera. The Standard Sera were calibrated against the appropriate purified proteins.

7. Results

Determine the concentration of each unknown of specimen protein by reading its zone diameter on the reference curve and the corresponding concentration from the X-axis. Zone diameter must be squared for End Point calibration.

8. Interpretation of Results and Limitation of the Procedure

A. When an unknown diameter exceeds that of the top standard, the specimen should be diluted with saline and rerun.

B. When an unknown diameter is smaller than that of the lowest standard, its concentration should be reported as "less than" the concentration of the reference serum. If available, "low level" radial immunodiffusion plates may be utilized.

C. Lack of a precipitin ring may be due to:

1. Sample not applied to well
2. A concentration too low to be detected by the method
3. A concentration too high, resulting in the formation of soluble complexes, which are not precipitated

D. For the determination of CID in a young Arabian foal it is important that blood from the foal is drawn before the foal suckles. A normal foal will have low circulating levels of IgM while a CID foal has no IgM. After suckling the IgM test will be meaningless, because both foals will absorb IgM from the colostrum and their blood levels will be equivocal. Maternal antibodies will have to clear from the foal ¹⁷before an RID test can accurately measure the foals IgM immunoglobulin.

E. Presuckle colostrum can be diluted 1 part to 3 parts in saline.

F. **These plates do not measure substitute colostrum sources of IgM from Goat, Sheep, or Cow.**

9. Expected Values

The half-life of IgM from colostrum is 20 to 23 days ^{6,7} therefore serum immunoglobulin levels are lowest between 1 to 2 months of age ^{8,9}.

Immunoglobulin levels appear to vary among breeds. It has been suggested that environmental or husbandry factors, rather than genetic breed differences, may be a primary cause ¹

AVERAGE IMMUNOGLOBULIN LEVELS ^{12,13}

FOAL AGE	IgM MG/DL
Presuckle	12
Postsuckle	17-30
3-9 months	39-61, ³
Adult	89-151
Colostrum	90-309

These values, determined by different testing methods, are intended as a guideline - each laboratory should establish it's own "normal" range. Values vary with age and should be separately established.

10. Performance Characteristics

A. For investigational use only. The performance characteristics of this product have not been established.

11. References

1. Fahey, J.L. and McKelvey, E.M. Quantitative determination of serum immunoglobulins in antibody agar plates. J. Immunol. 94,84, 1965.
2. Mancini, G., Carbonara, A.O. and Heremans, J.F. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry 2, 235, 1965.
3. McGuire, T.C., Poppie, M.J. and Banks, K.L. (1974), "Combined (B-andT-Lymphocyte) Immunodeficiency: A Fatal Genetic Disease in Arabians Foals", J.Amer. Vet Med Assoc., 174,70.
4. McGuire, T.C., Banks, K.L. and Poppie, M.J. (1975) "Combined Immunodeficiency in Horses: Characterization of the Lymphocyte Defect", Clin. Immunology and Immunopathology, 3,555.
5. Koterba, Drummond and Kosch "Equine Clinical Neonatology", pp.2750295. Lea and Febiger 1990.
6. Jeffcott, L.B.: Studies on passive immunity in the foal. J. Comp. Pathol., 84:93, 1974.
7. Jeffcott, L.B.: Passive immunity and its transfer with special reference to the horse. Biol. Rev., 47:439, 1972.
8. Perryman, L.E.: Immunological management of young foals. Comp. Cont. Educ. Pract. Vet. 3:S223, 1981.
9. Jeffcott, L.B.: Some practical aspects of the transfer of passive immunity to newborn foals. Equine Vet. J., 6:109,1974.
10. Allen, P.Z. and Dalton, E.J. (1975), "Studies on Equine Immunoglobulins", Immunology, 28, 187.
11. McGuire, T.C. and Crawford, T.B. (1973), "Passive Immunity in the Foal: Measurement of Immunoglobulin Classes and Specific Antibody", Amer. J. Vet. Res., 34, 1299.
12. Hopkins, Mel, M&M Veterinary Laboratory. 313-439-2698. Private communication.
13. Jorgensen, D. Triple J Farms.
14. Rouse, B.T. (1971), "The Immunoglobulins of Adult Equine and foal Sera: A Quantitative Study", Br. Vet. J. 127,45.
15. Makimura, S., Tomoda, I. And Usui, K. (1975), "Quantitative Studies on Immunoglobulins and Transferrin In Equine Serum", Jap. J. Vet. Sci., 37,187.
16. Perryman, L.E., McGuire T.C., Hilbert B.J. Selective Immunoglobulin M Deficiency in Foals. JAVMA 170:212-215, 1977.
17. Makimura, S., Tomoda, I. and Usui, K. (1975), "Quantitative Studies on Immunoglobulins and Trasferrin in Equine Serum", Jap. J. Vet. Sci., 37,187.

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