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TECHNICAL INFORMATION

ImmunO™

Catalog Number: 646371
Equine Anti-Globulin Test

Description: Anti-Globulin reagent has been widely used for the diagnosis of autoimmune hemolytic anemia (AIHA) in a variety of species. AIHA is an autoimmune disease characterized by antibody coated red blood cells that either lyse in the presence of complement, or are subject to phagocytosis by the liver and spleen, resulting in a severe anemia. Although not common AIHA has been reported in the horse[1-4].
MP Immunobiologicals equine anti-globulin reagent is also useful in the neonatal isoerythrolysis test.
The anti-globulin procedure has also been used in equine blood typing[5-8].
Reagents: Antibodies are produced in rabbits by immunization with equine immunoglobulins and formulated with selected b globulins including the C3 component of complement. The resulting antiserum is heated at 56°C for 30 minutes to inactivate rabbit complement. Heteroagglutinins are absorbed with pooled normal equine red blood cells. The reagent is tested with normal and antibody-coated equine red blood cells to assure its specificity.
Equine anti-globulin reagent is presented in the lyophilized form. One milliliter of the reconstituted antiserum is adequate for 10 determinations by the tube test method or 50 by the microliter procedure.
Reconstitution: Reconstitute with 2.0 ml of sterile distilled water.
Specimen Collection and Preparation:

A.Collect approximately 5 ml of blood in a serum tube or in EDTA. Clotted bloods transport best and are recommended for specimens not drawn in your laboratory.
B.EDTA Bloods: Centrifuge to separate RBCs and decant plasma for those collected in EDTA.
C.Clotted Bloods: Pour off serum from clotted specimens. Add 2 ml of physiological saline (0.9 g/dl) and gently break the clot, suspending the RBCs. Centrifuge to separate and decant the saline.
D.Pipette 0.1 ml of packed cells into a 12 x 75 mm test tube and resuspend in 4.9 ml of saline. Centrifuge, decant and resuspend four times with the same volume of physiological saline. Following the last wash the specimen should be a 4% RBC suspension (5 ml total) of 4X washed cells. (0.2 ml packed cells to 4.8 ml of normal saline).
E.Test specimens immediately after washing and as soon after collection as possible.

Procedure:

A.Materials Required:

1.Equine anti-globulin reagent.
2.Physiological saline (0.9 g/dl)
3.12 x 75 mm test tubes
4.0.1 ml pipettes

B.Negative control cells should be selected from a previously tested, negative animal. RBCs are prepared in the same manner as patient specimens.

C.Preparation of Anti-Globulin Dilutions:

1.Select three 12 x 75 mm test tubes per specimen and label each set with specimen and label each set with specimen identity and dilution 1:2, 1:4 and 1:8.
2.Pipette 0.1 ml of anti-globulin reagent per specimen or control into tube 1:2. (e.g. for 2 specimens and a control pipette 0.3 ml total.
3.Pipette 0.1 ml of physiological saline per specimen or control into tubes 1:2, 1:4 and 1:8.
4.Vigorously mix tube 1:2 and transfer 0.1 ml per specimen or control of the mixture to tube 1:4. Vigorously mix tube 1:4 and transfer 0.1 ml per specimen or control of the mixture to tube 1:8. Vigorously mix tube 1:8. These are serial, twofold dilutions of anti-globulin reagent at titers of 1:2, 1:4 and 1:8 respectively.

D.Testing:

1.Label four 12 x 75 mm test tubes for each specimen or control to be tested. Label each set CS, C1:2, C1:4, Cl:8, PS, P1:2, P1:4 and P1:8 etc.
2.Pipette 0.1 ml washed negative controls cells into tubes labeled CS, C1:2, C1:4 and C1:8.
3.Pipette 0.1 ml washed patient cells into tubes labeled PS, P1:2, P1:4 and P1:8.
4.Pipette 0.1 ml physiological saline into tubes CS and PS.
5.Pipette 0.1 ml of anti-globulin reagent dilution 1:2, 1:4 and 1:8 into tubes C1:2, C1:4 and C1:8 respectively. Repeat for tubes P1:2, P1:4 and P1:8.
6.Mix all tubes gently and incubate at 37°C for 15 minutes (incubation time is critical to this test).
7.Following incubation observe the tubes for agglutination. Tubes in which agglutination is not readily apparent should be checked microscopically, since a positive may occur without signs of agglutination detectable by the unaided eye.

Test Panel Configuration and Composition:
Test Panel Configuration and Composition:

	Negative Control			
	CS	C1:2	C1:4	C1:8
Negative Control Cell	X	X	X	X
Patient Cells	--	X	--	--
Normal Saline	X	--	--	--
Anti-Globulin Reagent	--	1/2	1/4	1/8
	Patient No.			
	PS	P1:2	P1:4	P1:8
Negative Control Cell	--	--	--	--
Patient Cells	X	X	X	X
Normal Saline	X	X	--	--
Anti-Globulin	--	1/2	1/4	1/8

Procedural Adaptation to Microdilution Plates: Equine anti-globulin test is easily adapted to a variety of commercially available microdilution plates. Preparation of RBCs and diluted anti-globulin reagent is the same as in the macrotube procedure. Testing of patient and control cells is performed in the 0.2 ml wells of a microdilution plate ("U" or "V" bottom plates are recommended). Volumes for RBCs, saline and diluted anti-globulins are 0.05 ml each, compared to 0.1 ml in the macrotube method.
Addition of commercially available microdilution loops and droppers will further simplify the procedure, eliminating the separate steps for preparation of anti-globulin reagent dilutions. Each specimen or control requires four wells.

1.Drop 0.05 ml of physiological saline into four consecutive wells. Drop an additional row for each additional control or specimen.
2.Drop 0.05 ml of equinine anti-globulin reagent stock solution into the second well of each row.
3.Use a 0.05 ml microdilution loop to mix the reagent and saline in the second well, first row. Transfer 0.05 ml with the loop to the third well and mix. Transfer 0.05 ml from the third to the fourth well and mix. Rinse and blot (dry) the loop. Repeat for each row of wells.
4.Drop 0.05 ml of negative control cells into each well in row one.
5.Drop 0.05 ml of patient RBCs into each well of row two. Repeat, using a new row for each patient.
6.Gently shake the microdilution plate back and forth to mix the cells.
7.Incubate at 37°C for 30 minutes.
8.Following incubation, observe wells for agglutination by stirring samples (with a toothpick) and viewing wells for agglutination. Negative samples will remain in suspension (no clumping) whereas positive samples will show distinct cell clumping.

Note: You must gently stir the samples and read as recommended. Do not use patterns of settling to determine positives or negatives.
Interpretation of Results: A greater degree of agglutination in patient specimen tube(s) (wells) compared to negative control cell and patient cell/saline tubes indicates that the patients RBCs are coated with antibody and/or complement. For a valid positive test, the control tubes should show less or no agglutination when compared to the agglutinated patient specimen tube.
Precautions: Anti-globulin reagent should not be used undiluted. Follow dilution instructions. Hemolysis at any of the recommended dilutions is not considered a positive reaction. When significant hemolysis is observed, deterioration of patient cells should be considered as a possible cause. Some lots of anti-globulin reagent may show hemolysis when freshly

reconstituted. This pink to light red color is caused by RBCs used in absorption of the serum and will not interfere with the test results.
Neonatal Isoerythrolysis Description: Neonatal isoerythrolysis (NI) in the horse is a severe hemolytic condition in newborn foals involving immunological mediated lysis of red cells[9-11].
The condition is caused by red cell alloantibodies if previously exposed to genetically incompatible RBCs through pregnancy, transfusion or tissue vaccines.
If the foal inherits RBC antigens from its sire (to which the dam has been previously sensitized) occurrence of isoerythrolysis is possible. During pregnancy the presence of the problem RBC antigens from the foal serves as a "booster immunization" resulting in a high titer of alloantibodies in colostrum[11].
The isoerythrolysis test may be performed as a predictive or diagnostic test. The predictive test is performed using RBCs from the stallion and serum from the mare. The direct and indirect diagnostic tests may be performed after the foal has taken colostrum from the mare and shows clinical signs of NI. The direct test is performed first and requires only the foal's RBCs and serum or colostrum from the mare. (Mare serum is preferred).
Predictive NI Test:
Specimen Collection:

A.Collect approximately 5-10ml of clotted blood from the mare and stallion approximately 3-4 weeks prior to foaling date.
B.Centrifuge the samples and transfer the mare's serum to a separate tube.
C.Heat the mare's serum in a water bath at 56°C for 30 minutes to inactivate complement. Use immediately or store at 4°C.
D.Pour off the serum from the stallion's tube, then add 5 ml of physiological saline to the clot. Vigorously agitate the clot with a wooden stirrer. Transfer the saline with suspended RBCs to a separate tube.
E.Wash the RBCs from the stallion 3 times with PS. Resuspend cells at a ratio of 0.2 ml packed cells to 4.8 ml of PS to yield a 4% suspension.

Preparation of Absorbed Rabbit Complement:

1.Reconstituted rabbit complement is cooled to 0-4°C in a refrigerator overnight.
2.10 to 20 ml of horse blood is collected in ACD, Alsever's or heparin and the red cells washed 3x to 4x with cold saline.
3.One volume of washed red cells are added to 4 volumes of cold rabbit complement and the tube with red cells and rabbit complement incubated at refrigerator temperature 0-4°C for 2 hours. The red cells are removed by centrifugation at 4°C.
4.The rabbit complement is tested by adding 0.1 ml of solution to 0.1 ml of a 4% suspension of washed horse RBCs. The tube is incubated for 30 minutes at 37°C. If there is no lysis of the red cells the rabbit complement is ready for use. If lysis occurs, repeat steps 1 to 4. A second absorption should remove all remaining natural antibody reactivity from the rabbit complement.

Precaution: Keep reconstituted rabbit complement at refrigerator temperature. Avoid prolonged exposure to room temperature which can reduce complement activity. It is advisable to aliquot and freeze the unused rabbit complement.
Procedure:

A.Add one volume (0.1 - 0.5ml) of the 4% suspension of stallion RBCs to an equal volume of mare's serum (previously heat inactivated at 56°C for 30 minutes). Incubate for 15 minutes at room temperature.
B.Centrifuge the incubated stallion RBCs and wash one time with PS.
C.Resuspend the cells to a 4% suspension. Place 0.1 ml of the suspension in three separate tubes.
D.To the first tube add 0.1 ml of undiluted MP equine anti-globulin reagent. To the second tube add 0.1 ml of rabbit complement (pre-absorbed with normal equine RBCs). To the third tube add 0.1 ml PS. Gently mix all three tubes with a wooden stirrer and incubate for 15 minutes at room temperature.

Interpretation of Results: Agglutination in the tube containing anti-globulin reagent and/or lysis in the tube containing rabbit complement is considered a positive test. The third tube should not have agglutination or lysis. A positive test indicates that the foal is a risk to develop neonatal isoerythrolysis if permitted to obtain colostrum from the mare.
Isoerythrolysis - Direct Diagnostic Test: The direct diagnostic test should be performed on foals suspected of having NI. If this test is negative the indirect test should also be performed.
Specimen Collection:

A.Obtain a clotted blood or EDTA sample from the foal.
B.Centrifuge the clotted blood or EDTA sample and remove the serum or plasma.
C.Remove a 0.1 ml sample of packed RBCs and place in a tube. Wash the cells three times with PS.
D.After washing resuspend to a final concentration of 4% RBCs. (0.2 ml packed cells to 4.8 ml of PS).

Procedure:

A.Place 0.1 ml of the 4% RBC suspension into each of three tubes.
B.To one of the tubes add 0.1 ml of undiluted MP equine anti-globulin reagent; to the second tube add 0.1 ml of rabbit complement (pre-absorbed with normal equine RBCs; to the third tube add 0.1 ml of PS.
C.Incubate all three tubes at 37°C for 15 minutes.

Interpretation of Results: Agglutination in the tube containing anti-globulin reagent and/or lysis in the tube containing rabbit complement is considered a positive test.
Note: If the RBCs in the saline control are lysed, the results of anti-globulin and rabbit complement tube cannot be interpreted. Red cells from foals with NI are often fragile and will lyse spontaneously. It is not possible to interpret the test if lysis occurs with the PS control. A new sample of blood should be obtained.
Isoerythrolysis - Indirect Diagnostic Test: - Specimen Collection:

A.Prepare a 4% suspension of RBCs from the foal and a serum sample from the mare (heat inactivated at 56°C for 30 minutes). Prepare the foal RBCs according to steps A-D under Specimen Collection in the Direct Diagnostic Test. Prepare the mare's

serum sample as in steps A-C under Specimen Collection in the Predictive NI Test.

Procedure:

A.Add one volume (0.1 - 0.5ml) of the 4% suspension of foal RBCs to an equal volume of heat inactivated mare serum. Incubate 15 minutes at room temperature.
B.Centrifuge the RBCs and wash once with PS.
C.Follow steps A-C of the Direct Diagnostic Test Procedure.

Interpretation of Results: The interpretation of the test is similar to that of the Direct Diagnostic Test. See precautions for limitations of the Predictive and Diagnostic Test for NI.
Precautions: Results obtained with this reagent in the Diagnostic Test should not serve as the sole criterion in a final diagnosis of NI.
While the predictive test can indicate that the foal is a risk to develop NI, it is possible for both false positives and negatives to occur. The tester should be aware the equine erythrocytes are prone to rouleux formation.
References:

– Anderson, I.J. Idiopathic autoimmune haemolytic anaemia in a horse, N.Z. Vet. J. 22, 102, 1974.
– Collins, J.D., Autoimmune haemolytic anaemia in the horse, Proc. 1st Int. Equine Symp. 343, 1975.
– Farrelly, B.T., Collins, J.D. and Collins, S.M., Autoimmune haemolytic anaemia (A.H.A.) in the horse, Irish Vet. J. 20, 42, 1966.
– Noda, H. and Watanabe, Y., Relationships between blood groups and hemolytic disease of newborn foal, Jap. J. Zootech, Sci. 46, 180, 1975.
– Stormont, C., Suzuki, Y. and Miller, W.J., Bacterially mediated false-positive reactions in lytic blood-typing test, Nature 186, 147, 1960.
– Stormont, C. and Suzuke, Y., Paternity test in horses, Cornell Vet. 55, 365, 1975.
– Stormont, C., Suzuke, Y. and Rendel, J., Application of blood typing and protein tests in horses. In Blood Groups of Animals IXth European Blood Groups Conf. Publishing House of the Czechoslovak Acad. of Sciences, Prague, 1965.
– Stormont, C., Suzuke, Y. and Rhode, E.A., Serology of horse blood groups, Cornell Vet. 54, 439, 1964.
– Stormont, C., Neonatal Isoerythrolysis in domestic animals: A comparative review. In advances in veterinary science and comparative medicine, edited by C.A. Brandly and C.E. Cornelius, Academic Press, New York, 1975.
– Suzuki, Y., Stormont, C. and Trommershausen-Smith, A., Alloantibodies: The blood groups they define, Proc. 1st Int. Equine Symp. 34, 1975.
– Trommershausen-Smith, A., Suzuki, Y. and Stormont, C., Alloantibodies: Their role in equine neonatal isoerythrolysis, Proc. 1 st Int. Equine Symp. 349, 1975.

Note: This product may contain a preservative such as sodium azide, thimerosal or proclin. Please see lot specific chemical credential for preservative information.