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Blood Compatibility Testing in Asian Elephants Using an Indirect Antiglobulin Technique to Improve Captive Breeding Success

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ABSTRACT

Feto-maternal blood incompatibility causes hematological defects that can result in abortion and neonatal anemia and may lead to calf loss, including in elephants. The aim of this study was to examine blood compatibility in Asian elephants using the indirect antiglobulin technique, for use as a breeding management tool to reduce the risk of elephant fetal loss. Forty-four blood samples collected from 22 female and 22 male elephants were subjected to standard tube cross-matching tests and examined for macroscopic and microscopic agglutination reactions. The percentages of positive reactions across three female groups based on reproductive history (unmated, mated but not pregnant, parous) were 43.94, 53.03 and 45.80, respectively and did not differ ($p>0.05$). Moreover, all female sample showed positive reaction results at ambient and body temperatures. These results suggest blood incompatibility occurs in elephants. Thus, it could be beneficial to avoid mating between reactive blood groups.

Key words: Blood disorders, hematological defects, tube agglutination, cross-matching test, Asian elephant

INTRODUCTION

Blood group incompatibility is a disorder that occurs when the blood type of the mother conflicts with that of the fetus. During gestation, small amounts of fetal blood may enter the mother's circulation and stimulate an immune response if antigens on the fetal red cells, those inherited from the father, are perceived as foreign. The mother produces antibodies that pass through the placenta to the fetal circulation and can cause red blood cell hemolysis during the fetal and/or neonatal period (Kenner and Lott, 2013; Leifer, 2013). In humans, Hemolytic Disease of the Newborn (HDN) used to be a major cause of fetal loss and death among newborn babies. A major cause of HDN is an ABO incompatibility, arising when a mother with blood type O

becomes pregnant with a fetus of a different blood type (type A, B, or AB). The mother's serum contains naturally occurring anti-A and anti-B antibodies, which can cross the placenta and hemolyze fetal red blood cells. Although most cases of severe hemolytic disease result from Rh incompatibility, which is triggered by the D antigen, although other Rh antigens also can be involved. A sensitized Rh D-negative mother produces anti-Rh IgG antibodies against an Rh D-positive fetus. Clinical presentation varies from mild jaundice and anemia to hydrops fetalis, a serious condition marked by fluid accumulation in two or more fetal compartments that can result in heart failure and death. Approximately 15% of live births are at risk, but manifestation of disease develop in 0.3-2.2%. (Crowley, 2012; Kenner and Lott, 2013; Kliegman *et al*., 2015). They can also occur in other primate species that have a comparable placental structure to humans (Cotter, 2001).

In other species, blood incompatibility pathogenesis differs if the placental structure does not allow passage of maternal antibodies. In those cases, antibodies are secreted into colostrum and absorbed through the intestine by the newborn (Cotter, 2001). For example, strong IgG haemolysins can develop in dogs sensitized by a first incompatible transfusion or following pregnancy (Day, 2011). Cats are unique in that blood type B cats have naturally occurring anti-A antibodies without prior exposure and kittens that are type A develop hemolysis after nursing, which leads to severe illness or death (Harvey, 2012). In horses, Neonatal Isoerythrolysis (NI) is an important cause of anemia in newborn foals and can be life-threatening. The prevalence of NI varies among different breeds but has been reported to be 1% in thorough breds and 2% in standard bred horses. Sensitization to incompatible red blood cell antigens between foal and dam can occur after leakage of blood across the placenta during pregnancy or at delivery (Weiss and Wardrop, 2011). At birth, the foal ingests colostrum containing the alloantibodies, which bind to the red blood cells of the foal, resulting in agglutination, lysis, or both. The most common antigens involved in NI are Qa and Aa; therefore, mares without Qa and Aa factors are at an increased risk of producing NI-causing antibodies (Wilson, 2012).

Blood incompatibility may occur in elephants, although it has not yet been documented. Elephants exhibit characteristics of endotheliochorial placentation, which is common in carnivore species and associated with modest maternal to fetal transplacental antibody transfer (Nofs *et al*., 2013). Additionally, passage of a heavy fetus through the vagina can cause the placenta to detach from the endometrium by severing the narrow maternal placental hilus, which then leads to intrauterine hemorrhage (Allen *et al*., 2003). Still births, especially late term and neonatal death are significant problems of captive elephants in western zoos (Saragusty *et al*., 2009) and have also been observed in Thailand (Thongtip *et al*., 2009; unpublished data). It is not known, if reactions to blood incompatibility between the fetus and elephant mother are causes of these problems, but given the non sustainability of most captive elephant populations, it is imperative to understand causes of poor reproductive success.

Blood compatibility testing or cross-matching determines the *in vitro* compatibility of blood between two subjects and whether antibodies are present in the blood of one subject that will react against the other's red cells. For example, reactions between blood from a male and female conducted at room temperature and 4°C are used for detection of IgM antibodies that may readily agglutinate red blood cells, whereas reactions at 37°C and the indirect antiglobulin test are examined for detection of IgG antibodies. Agglutination or hemolysis at any stage is indicative of incompatibility (Kawthalkar, 2012). Anti-IgG is an antiglobulin reagent used for the indirect antiglobulin technique to demonstrate the in-vitro coating of red blood cells with antibody

molecules. Most clinically significant antibodies in red blood cell serology are of the IgG class. The IgG may coat red blood cells but not cause detectable agglutination without the addition of the antiglobulin reagent, which effects agglutination by crosslinking the IgG molecules on red blood cell (Erhabor and Adias, 2013).

Therefore, the objective of this study was to determine if blood incompatibility occurs between male and female Asian elephants, which could prove to be a valuable test to enhance reproductive outcomes and reduce the risk of fetal loss.

MATERIALS AND METHODS

Animals: Forty-four Asian elephants (22 males, 22 females), aged 3-60 years, from elephant camps in Chiang Mai and Lampang provinces, Thailand were included in the study. Females were classified into three groups by reproductive status: unmated (N = 6), mated but not pregnant (N = 3) and parous (N = 13).

Blood sample collection and preparation: Venous blood (10 mL) was collected from a marginal ear vein. For females, blood was allowed to clot and then centrifuged at 2,000 g for 15 min to obtain serum. For males, whole blood was washed three times with physiological saline (0.85% sodium chloride in distilled water) by centrifugation at 300 g for 1 min to remove plasma and a 4% red cell suspension was prepared. Samples were stored at 4°C until cross-matching procedures were performed. The refrigerated samples were viable up to 7 days of storage.

Cross-matching procedures: One drop of 4% male red blood cell suspension was added to a tube containing of two drops of female serum. Combinations of serum reactions were conducted between all 22 male and 22 female elephant samples; thus, a total of 484 reactions were conducted. Each set of samples was examined after four successive phases involving different temperatures. In Phase 1, after mixing of red cells and serum, each tube was incubated at 25°C for 5 min, centrifuged at 3,000 g for 15 sec and agglutination was initially scored visually by placing the tube on a white background. For Phase 2, the same tube was incubated at 4°C for 5 min, centrifuged and examined visually again and in Phase 3 tubes were examined again after incubation at 37°C for 30 min and centrifugation. Phase 4, also known as an indirect antiglobulin test for detection of IgG-mediated agglutination, the red cells in the tube were washed three times in saline by centrifugation at 300 g for 1 min followed by addition of one drop of 14 mg mL^{-1} rabbit anti-elephant IgG (Vongchan, 2013), centrifugation and visual examination.

Scoring cross-matching reactions for agglutination: All reactions were examined macroscopically and microscopically and the scoring methods are presented in Table 1.

Table 1: Grading of blood compatibility agglutination reactions

Macroscopic	Microscopic	Grading
Solid clump, clear background	No free cells	4+
Several large clumps, clear background	Several large agglutinates	3+
Several small clumps, clear background	Many medium-sized agglutinates (>20 cells) among free cells	2+
Several small clumps, cloudy background	Medium to small-sized agglutinates (6-20 cells) among free cells	1+
Tiny aggregates, cloudy background	Small-sized agglutinates (3-5 cells), many free cells	Weak
No agglutination, very cloudy background	No agglutination, all cells separate	Negative

Adapted from Blaney and Howard (2013)

Macroscopic grading was performed after each phase of treatment by gently dislodging the cellular pellet and scoring each tube based on the degree of agglutination (4+, 3+, 2+, 1+ and weak). Bright field microscopy with 10X magnification was used to confirm negative reactions.

Statistical analysis: A Chi square test (R program version 3.1.1; R Core Team, 2014) was used to examine differences in agglutination reactions. Statistical difference in the agglutination reaction between phases was compared. The difference between female groups in the agglutination reaction after the indirect antiglobulin technique was analyzed.

RESULTS

Numbers and percentages of agglutination reactions across the four phases of treatment are shown in Table 2. There were differences in reaction rates between phases ($p<0.001$), with few reactions for Phase 1 and higher reactions for Phase 2 ($p<0.05$), the latter of which was similar to Phase 3 ($p>0.05$). For Phase 4, after the addition of rabbit anti-elephant IgG, the percentage of positive reactions increased markedly compared to Phase 3 ($p<0.001$). Macroscopically, clumps of agglutinates were apparent in positive reactions, whereas no agglutination with a cloudy background was observed in negative reactions, as shown in Fig. 1a and b. Microscopic evaluations confirmed visual assessments; agglutinates were apparent in positive, but not negative reactions Fig. 2a and b.

The number of positive agglutination reactions in Phase 4 occurred from each male are shown in Table 3. All males showed a positive reaction to some of female serum with one male that showed positive reactions to all female serum. Number of positive agglutination reactions in Phase 4 occurred from each female are shown in Table 4. All females in this study showed a positive reaction to the red blood cells of males. The number and percentage of positive agglutination reactions using the indirect antiglobulin method (i.e., Phase 4) across female reproductive categories are shown in Table 5. Mated but not pregnant elephants yielded highest positive results (53.03%), as compared to parous (45.80%) and unmated (43.94%) females, but these were not

Table 2: Number and percentage of positive and negative responses in each phase of the agglutination reaction

	No. of reactions			
	Positive		Negative	
Phases	No.	%	No.	%
Phase 1	3	0.62	481	99.38
Phase 2	13	2.69	471	97.31
Phase 3	10	2.06	474	97.94
Phase 4	224	46.28	260	53.72

Chi square test, between four phases ($p<0.001$), between phase 1 and 2 ($p<0.05$) and between Phase 3 and 4 ($p<0.001$)

Table 3: Number of positive agglutination reactions in Phase 4 for each bull elephant

Male ID	No. of positive reactions	Male ID	No. of positive reactions
1	9	12	9
2	8	13	12
3	5	14	10
4	5	15	11
5	7	16	9
6	11	17	7
7	11	18	10
8	22	19	15
9	10	20	13
10	6	21	11
11	10	22	13

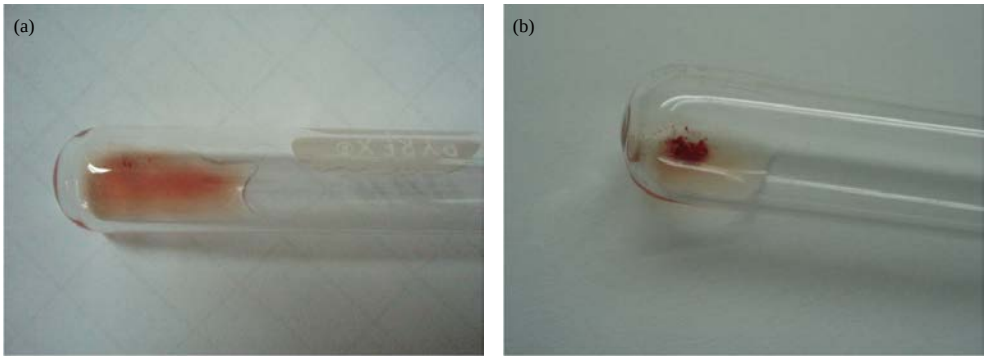

Fig. 1(a-b): (a) Negative and (b) Positive agglutination reactions based on macroscopic observation

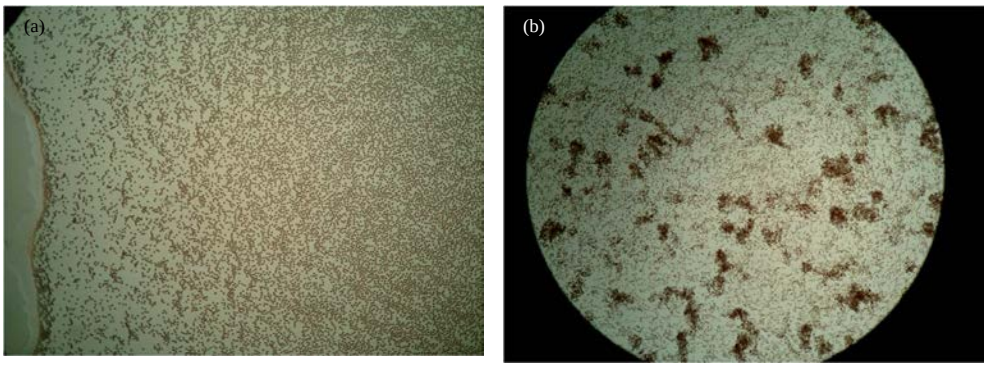

Fig. 2(a-b): (a) Negative and (b) Positive agglutination reactions based on microscopic evaluations

Table 4: Number of positive agglutination reactions in Phase 4 for each female elephant

Female ID	No. of positive reactions	Female ID	No. of positive reactions
1	4	12	6
2	4	13	4
3	18	14	10
4	14	15	14
5	10	16	11
6	8	17	14
7	3	18	18
8	16	19	12
9	16	20	9
10	4	21	14
11	5	22	10

Table 5: Number and percentage of positive and negative agglutination reactions after the indirect antiglobulin technique, classified according to female group

	No. of reactions					
	Positive		Negative		No. of females/total	
Groups	No.	%	No.	%	Positive	Negative
Unmated	58	43.94	74	56.06	6/6	0
Mated but not pregnant	35	53.03	31	46.97	3/3	0
Parous	131	45.80	155	54.20	13/13	0
Total	224	46.28	260	53.72	22/22	0

significantly different ($p>0.05$). In two cases, there were positive reactions between blood samples from breeding pairs that resulted in the abortion of two fetuses at 17 months of gestation without evidence of infectious disease or poor management (1 calf from Thongtip *et al*., 2009; 1 calf from unpublished data).

DISCUSSION

This is the first study to assess blood compatibility between male and female Asian elephants and found positive agglutination reactions in about 46.28% of the potential breeding pairs. These results suggest that blood incompatibility could compromise reproductive success in this species and that cross-matching procedures should be conducted before doing mate introductions. Hemolytic anemia is the result of immune incompatibility between donors and recipients, or between mother and offspring. Antibodies involved can be IgM or IgG with intravascular and/or extravascular hemolysis. Testing blood compatibility at different temperatures (4, 25 and 37°C) allowed us to examine differential immunoglobulin reactions. The IgM, naturally, is the first antibody produced in response to an antigenic stimulus. The IgM usually does not react at temperatures above 30°C, but reacts best at 4°C (Graeter *et al*., 2014). The positive reactions at these temperatures suggests that the serum of these elephants contained IgM, but because much of it is in pentameric form, it is incapable of crossing the placental barrier (Kayser *et al*., 2011). The IgG is the major antibody produced in the secondary immune response and usually requires temperature at 37°C to exhibit optimal reactivity (Graeter *et al*., 2014). The positive results at this stage suggests that the serum of the respective elephant contained the alloantibody of the type IgG, which occurs as a monomer and can cross the placenta. While some agglutination was observed at this temperature, the reaction was significantly stronger after the addition of anti-elephant IgG (indirect antiglobulin test). This suggests that the antiglobulin reagent formed cross-links between antibody molecules that have bound to the surface of red blood cells. This promoted the formation of agglutination and allowed for visual observation of an antigen-antibody reaction. The IgG passed through the placenta can provide the newborn with a passive form of protection against pathogens, but in certain rare circumstances, these antibodies may also harm the fetus or neonate; for instance, when they are directed against epitopes expressed by tissues that the mother has reacted against immunologically (i.e., blood incompatibility) (Kayser *et al*., 2011). Our results showed that female elephants exhibited positive reactions to all phases of cross-matching conditions, so the serum likely contained both IgM and IgG. The difference in degree of reaction between antigen and antibody is affected by a variety of factors including particle charge, electrolyte concentration and viscosity, antibody type, antigen-to-antibody ratio, antigenic determinants and physical conditions (e.g., pH, temperature, duration of incubation) (Turgeon, 2013). One male that showed positive reactions to all female serum may due to a high antigenicity of the red cells. Regardless, the finding of significant positive reactions, especially using the indirect antiglobulin method, suggests that blood incompatibility could be a problem for breeding elephants.

To further characterize the responses, results for the indirect antiglobulin tests were divided according to three reproductive categories. While we anticipated agglutination reactions might occur in some elephants that had produced one or more calves, we were surprised to see responses in mated but not pregnant females as well and to approximately the same degree. They had been in a breeding program, so they may have become pregnant and aborted at an early stage, a condition that could not be detected externally or from a change in behavior. So, these elephants may have been exposed to foreign antigens from an aborted fetus. This possibility is supported by

a study that found early fetal loss through evaluations of transrectal ultrasound examinations and serum progesterone analyses occurs in Asian elephants (Lueders *et al*., 2010). The positive results observed in unmated females could be due to the mother's passive immunity. Previous reports of mammals with endotheliochorial placenta, such as dogs, cats and elephants, found around 5-10% of antibody transfer from the mother to the offspring through the placenta (Chucri *et al*., 2010). Because every female elephant tested had a reaction that yielded a positive result when using the indirect anitglobulin technique, it can be inferred that the serum of every female elephant contained IgG. Of particular interest was the blood incompatibility between bulls and two cows that was suspected to be a cause of the abortions.

CONCLUSION

This study found some male/female elephant pairs have incompatible blood types, which could cause problems with fetal loss. We recommend that blood compatibility testing be conducted to avoid breeding of incompatible pairs, especially those with a high grade of reaction, to protect against this risk for blood disorders. In addition, these results suggest areas for future study regarding elephant blood groups and feasibility of blood transfusion.

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