Are we getting our hormones right?
ASVCP Veterinary Laboratory Professionals Session I

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Background. Although, for many decades, the measurement of hormones has been based on immunoasssays, in recent years there has been increasing availability of endocrine measurement systems on different platforms.

Objectives.

- To summarise the key functional elements of immunoassays**.**
- To highlight the impact of the choice of measurement methods on hormone results and potentially clinical decisions.
- To highlight that differences occur between methods or between labs even for conserved hormone structures.
- To highlight the importance of method validation or verification and an appropriate animals based quality control system.

Examples from abbreviated method comparison studies, EQA schemes and interference studies combined with theoretical understanding support the objectives.

Results will vary between methods and between labs using the same method. The degree of variation complicates the interpretative guidance published in textbooks and clinical guidance.

Clinical Pathologists and Clinicians need to understand the limitations of the analytical methods upon clinical decisions as based so as to minimize mis-diagnosis. Limitations are best understood through a combination of appropriate method validation/verification, QC systems and EQA programs.

TO CROSSMATCH OR NOT TO CROSSMATCH?

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To crossmatch or not to crossmatch? A still controversial question! This presentation will discuss best practices in terms of blood compatibility in dogs and cats with an emphasis on crossmatch testing. The advantages and disadvantages of the different crossmatch techniques will be discussed, as well as possible pitfalls. The presentation will be punctuated by clinical cases, their crossmatch interpretation and resulting clinical decisions...to transfuse or not to transfuse?!

Introduction:

The demand for transfusions has increase dramatically to support anemic and bleeding canine and feline patients requiring more and more sophisticated care. Multi blood transfusions recipients are more common leading to blood compatibility challenges in some cases. Although systematic blood typing for DEA 1 in dogs and AB blood groups in cats has become common practice to ensure effective and safe transfusions, what about assessing blood compatibility beyond these systems?

1. Blood Compatibility in Dogs:

1.1 The clinical importance of the Dog Erythrocyte Antigen (DEA) 1 system

DEA 1 is considered the most important canine blood group system, both because of its well-documented immunogenicity and of its overall prevalence. A DEA 1+ blood transfusion in a DEA 1- dog will invariably elicits a strong alloantibody production, and lead to hemolytic transfusion reaction if subsequent DEA 1+ blood is transfused. The risk of sensitization, and thereafter transfusion reactions, is high if untyped blood transfusions are used as roughly half of the canine population is DEA 1+ (47-65%, with geographical and breed variation), while the other half is DEA 1-. Thus, DEA 1 blood typing has been recommended before any blood transfusion in dogs for decades, which is facilitated nowadays by standardized typing kits using monoclonal antibodies based on agglutination reactions (DMS/RapidVet-H) or on chromatographic techniques (Alvedia). Weakly positive DEA 1+ canine blood donors should be classified as DEA 1+. Since it remains unclear if a weakly DEA 1+ dog could mount an immune response if transfused with strongly DEA 1+ RBC, it is advisable to use DEA 1 - blood in weakly DEA 1+ dogs.

1.2 The significance of other DEAs and novel blood canine blood types

Other DEAs have been described but are considered clinically less important than DEA 1 either because of a less immunogenic nature (ex: DEA 3, 5 and probably 7) or because of their overall prevalence. For instance, DEA 4 is a high frequency antigen with a prevalence of more than 97% in most tested populations. Its immunogenicity has been documented with a well-described post-transfusion acute haemolytic reaction in a DEA 4-negative canine patient previously sensitized in a clinical context. However, given its high prevalence, the clinical scenario rarely occurs. That said, the rare DEA 4-negative dogs are at high risk of transfusion incompatibility and hemolytic reaction if they must receive multiple RBC transfusions given the rarity of DEA 4-negative blood.

Similarly, the Dal-positive phenotype was found to be extremely common in dogs, i.e. 98% of canine population is Dal-positive. Since its discovery, the uncommon Dal-negative phenotype has been described with an increased prevalence in some specific breeds, including Doberman Pinschers (42.4%, n: 432), Dalmatians (11.7%, n: 128), and

Shih Tzus (57.1%, n: 21). The high prevalence of Dal-positive dogs puts Dal-negative dogs at high risk of sensitization following blood transfusion, and compatible Dal-negative blood may be extremely difficult to find. Moreover, the Dal antigen appears to be highly immunogenic, with anti-Dal antibodies detected as early as 4 days post-transfusions and still observable > 2 years posttransfusion, putting sensitized Dal-negative dogs at lifelong risk of reacting to a second transfusion.

In contrast to other canine erythrocyte antigens described which were discovered following experimental or accidental sensitization of dogs by blood transfusions, Kai 1 and Kai 2 blood types were recently identified following the production of monoclonal antibodies. Presumed anti-Kai alloantibodies were documented in 4 dogs 21 days post-transfusion (Kai 1+ receiving Kai 2+ positive blood, and vice-versa). Although the antigenic nature of Kai 1 and Kai 2 is likely, their clinical relevance is currently unknown. Given its reported prevalence in North America (94% of dogs were Kai 1+, 1% Kai 2+ and 5% Kai negative), sensitization of the rare Kai 2+ and Kai negative dogs is likely if they were to be transfused.

1.3 Naturally occurring alloantibodies (NoAb) in dogs

First-time transfusions to dogs are considered safe without prior blood crossmatching, as dogs do not possess clinically significant NoAb. Similarly, pregnancy does not sensitize dogs to RBC antigens. Although mild immunologic incompatibilities have been documented in first-time transfusion recipients their clinical importance needs further investigation. Indeed, despite several recent publications documenting the presence of naturally occurring anti-DEA 7 antibodies in up to 50% of all DEA 7-negative dogs, the clinically significance of such antibodies has not been documented.

1.4 Blood crossmatching in dogs: the "when and why"?

As multiple blood transfusions recipients are more common, blood incompatibility scenarios are more likely. Transfusion reactions may occur after a sensitized dog receives blood that is mismatched for a RBC antigen other than DEA 1 and hemolytic transfusion reaction have been described against DEA 4, Dal, and another unspecified common RBC antigen. Given the lack of clinically significant NoAb, a blood crossmatching is recommended in any dogs that have received a transfusion $\geq$ 4 days previously or have an unknown transfusion history.

As mentioned above, the rarity of DEA 4-negative, Dal-negative, and Kai 2+/negative phenotypes pauses particular clinical challenge as compatible blood may be difficult to find. Except for DEA 1 and Kai 1/2 canine blood typing relies on polyclonal reagents produced following the sensitization of negative dogs. Nowadays, there is a complete lack of accessibility to DEA 6 and 8 reagents, and clinical access to Kai blood typing is not available. Recent commercialisation of DEA 4, 5 and Dal bedside typing cards and recent production of a monoclonal murine anti-Dal antibody may eventually facilitate finding compatible blood for previously sensitized patients, but a blood crossmatch will remain the first step to assess the serologic compatibility between the anemic recipient and potential donor.

2. <u>Blood Compatibility in Cats:</u>

2.1 The clinical importance of the AB blood group system

The AB blood group system characterized by Auer and Bell is the predominant blood group system in cats. Three phenotypes occur: type A, type B, and type AB. The

types are defined by NoAb against the antigen they lack, which have been documented to be produced in kittens as young as 6-8 weeks of age.
Of up-most clinical significance is the presence of highly potent anti-A agglutinins and hemolysins in virtually all type B cats, which can result in severe acute hemolytic transfusion reaction, and even death, if type A blood is administered to a type B cat. Similarly, the strong anti-A NoAb present in the colostrum of type B cat can also be responsible for neonatal isoerythrolysis in type A or type AB kittens born to a type B queen. In contrast, only a third of type A cats have alloantibodies in their plasma capable of weak macroscopic agglutination of type-B RBC, and type AB cats do not possess alloantibodies against either A or B antigens.

Type A is the most common blood type. Type B is less common, and type AB is very rare. The percentage distribution of types A and B in domestic shorthair (DSH) and longhair cats (DLH) can vary markedly with geographic location worldwide. Type B domestic cats are uncommon in most countries, however, in other countries such as Australia, Greece and Turkey, their prevalence can reach up to 36% of the non-pedigree feline population. Similarly, the variation per breed is significant, ranging from 0% up to 60% of Type B among different purebred cats. Breeds such as Turkish Van and Angora cats are reported to have a prevalence of type B cats of 60% and 46.4%, respectively. Type AB is exceedingly rare and only in breeds in which type B is detected. Compared to all other cat breeds, Ragdolls are commonly type AB with reported frequency in Italy ranging of 18 to 24%.

Given its clinical importance, AB blood typing in cats has been recommended before a transfusion for decades, which is facilitated by point of care card agglutination and immunochromatographic typing kits. When unavailable in emergency situation, a blood crossmatch could be used to detect strong AB incompatibilities (i.e. if recipient was type B and donor type A).

2.2 The significance of the Mik antigen and newly described Feline Erythrocyte Antigen (FEA)

Based on the presence of a NoAb in three blood donor cats, the Mik blood group system was described in 2007. Incompatible crossmatch reactions with plasma from a transfusion-naïve blood donor cat (named Mike) resulted in a detailed survey of blood compatibilities within Penn Animal Blood Bank blood donors. Upon further investigation of 65 type A donors, a total of three unrelated cats (Mik-negative) were found incompatible with the same cats (Mik-positive), yet compatible to each other, indicating that they lacked the same RBC antigen (Mik antigen) and had natural anti-Mik alloantibodies. Three type B and one type AB cat also appeared to express the Mik antigen. The clinical relevance of anti-Mik alloantibodies was documented after an acute hemolytic transfusion reaction following inadvertent transfusion of Mik-positive blood to a thereafter confirmed Mik-negative renal transplant recipient upon its first blood transfusion. Unfortunately, anti-Mik antibodies are no longer available preventing future investigations.

Again, based on the presence of NoAb, our research group at the Université de Montréal has recently begin mapping the corresponding feline erythrocyte antigens (FEA) behind these incompatibilities and identified five different putative FEA. FEA 1, 4 and 5 were most frequent with a prevalence of 84%, 65% and 96%, respectively. Only FEA 1 was significantly associated with NOAb ($P = 0.005$), which were observed in 8 of 43 FEA 1-negative cats (19%). Because of its prevalence and association with NOAb, FEA 1 may correspond to the lost Mik antigen. Experimental transfusion of FEA-1 negative, transfusion-naive cats with FEA-1 blood results in production of post-transfusion anti-FEA 1 alloantibodies (titer 1-32) as early as 5 days post-transfusion (titer 1-32). This study, which included the presentation of a case report, confirmed the potential immunogenicity

of FEA 1, as some cats appear to have strong anti-FEA 1 NoAbs (titer 256) that can result in acute hemolytic reaction secondary to receiving blood from a FEA 1-positive donor. With similar experimental transfusion, we are currently assessing the immunogenicity of the other FEAs described, and in doing so, we have recently identified a novel antigen (FEA 6) following sensitization and highlighted a strong association between FEA 1 and 4 (i.e. cats are either positive for one or the other, but not both).

2.3 Naturally occurring alloantibodies in cats

In recent literature, there is some evidence for the presence of other naturally NOAb outside the AB blood group system, like anti-Mik and most recently anti-FEA 1, but their clinical importance has not been defined. As such, McClosky et al documented major crossmatch incompatibilities outside of the AB system in 23 of 154 transfusion-naive cats (14.9%). Similarly, Sylvane et al identified 10 of 52 major crossmacthes performed in transfusion-naïve cats to be incompatible (19%). However, the crossmatch screening of 112 cats in the United Kingdom failed to detect any non-AB incompatibilities. Similarly, the prevalence of non-AB RBC incompatibilities in previously transfused cats has been reported to be approximately 25-27%.

The clinical significance of NoAb and post-transfusion alloantibodies outside the AB system is poorly defined. In a prospective controlled study, 48 client owned transfusion-naïve anemic cats were randomized to receive either a type- and crossmatch-compatible packed RBCs transfusions or non-crossmatched blood type compatible pRBCs. Their findings did not detect any difference in the increase in PCV after transfusion or in the incidence of transfusion reactions between crossmatched and uncrossmatched groups. This contrast with a retrospective study conducted in 2014 which showed that the transfusion of type- and crossmatch-compatible RBCs to cats compared to typed non-crossmatched RBCs was associated with a significantly greater increase in post-transfusion PCV. In McClosky et al study, the administration of type- and crossmatch-compatible pRBCs was not associated with a greater increase in post-transfusion PCV, however, these recipients experimented significantly less febrile transfusion reactions compared to cats that received AB type-specific pRBCS without crossmatch. However, crossmatched-incompatible cats were not included (i.e. transfused) for obvious ethical reasons as controlled in any of these study, therefor lacking a true control group.

2.4 Blood crossmatching in cats: the "when and why"?

In addition to AB blood typing, recent literature supports blood crossmatching in cats prior to a blood transfusion even in transfusion-naive cats because of the presence of NoAb. Though hemolytic transfusion reactions due to non-AB incompatibilities appear to be rare, and systematic crossmatching does involve additional financial cost and time, the author believe that prevention of unnecessary transfusion reactions (however rare) is in the best interest of the patient and conforms with current best practice transfusion medicine.

That said, in emergency situation, the time required to perform a blood crossmatch may not be realistic. However, because of the risk of post-transfusion alloantibody production which has been observed in up to 25% of anemic cats, a crossmatch should definitely be performed in previously transfused cats as early as 4 days post-transfusion.

On a side note, if xenotransfusion was to be considered for an anemic patient, pre-transfusion blood crossmatching should absolutely be performed.

3. Blood Crossmatching in Dogs and Cats: the "How"!

Many veterinary hospitals rely on a standardized tube crossmatching procedure which requires very little material, but some expertise notably in cats because of the tendency for rouleaux formation (see Table 1). Even with experience, the grading of the agglutination reactions (+/- hemolysis) is subjective.

Gel column techniques (DiaMed and Ortho Clinical) have been evaluated and found to be simple, sensitive, and standardized methods to crossmatch dogs, cats: the objectivity of the results makes it an ideal research tool. However, they require a special incubator and centrifuge.

Gel-based and immunochromatographic in-house crossmatching kits are now available, which facilitates the procedure and the interpretation of results, but have variable reported sensitivity and specificity (DMS Laboratories, Flemington, NJ and Alvedia, Limonest, France). Additional incompatibilities may be recognized by using addition species-specific antiglobulin-enhanced gel column tests (Alvedia, Limonest, France).

Regardless of the technique use, performing an auto-control is essential as the presence of auto-agglutination despite RBC washing process may render the blood crossmatch uninterpretable; unfortunately, most kits skip this important step, limiting the interpretation of blood incompatibilities. In case persistent autoantibodies are present, because the anemic patient will require a blood transfusion regardless, grading the incompatibility reactions may help the clinician choose the "least incompatible" blood units.

Obviously, a pre-transfusion compatible crossmatch does not prevent sensitization of the patient against donor cells within the following days and weeks. In the same regard, it does not give information about the blood type of the patient (although a type B cat may be strongly suspected). If DEA 1 and AB incompatibilities do not explain a blood incompatibility, extended blood typing is recommended and may facilitate identifying a compatible blood donor.

Table 1. Standard Tube Crossmatching Procedure (major and minor):

Step 1:

- Collect 1-2 mL of donor blood in an EDTA tube, or use an equivalent 1-2 mL of crossmatch segments from unit of blood being considered.
- Collect 1-2 mL of recipient blood in an EDTA tube.
- Centrifuge both donor and recipient blood in separate labeled tubes for 5 minutes (1000 *g*).
- Remove plasma using a pipette and save in separate labeled tubes for later use (use different pipettes for donor and recipient).
- Wash the remaining packed RBCs three times by filling tubes with 0.9% saline, gently resuspending, centrifuging (1000 *g*) for 5 minutes, and decanting off the saline (discard the saline).
- After a third wash, add 0.2 mL of washed packed RBCs from the donor red-top tube to 4.8 mL of 0.9% saline (in a separate tube), and gently mix to obtain a 3%-5% RBC solution (bright cherry red). Make a similar 3%-5% RBC solution with the washed recipient RBCs.

Step 2:

In a test tube:

- Mix 2 drops of recipient plasma and 1 drop of donor RBC suspension (major cross-match).
- Mix 2 drops of donor plasma and 1 drop of recipient RBC suspension (minor cross-match).
- Mix 2 drops of recipient plasma and 1 drop of recipient RBC suspension (auto-control).
- Incubate at 37°C (ideally, but room temperature is acceptable) for 15-20 minutes.
- Centrifuge for 30 seconds.
- Observe the plasma for hemolysis. Gently resuspend the RBC button by tapping the tube, and examine for agglutination clumps.
- Hemolysis or agglutination indicates incompatibility.

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