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Feline blood genotyping versus phenotyping, and detection of non-AB blood type incompatibilities in UK cats

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OBJECTIVES: The aim of this study was to determine the agreement between AB blood phenotyping and genotyping and determine whether non-AB blood type incompatibilities exist in UK cats.

METHODS: Blood samples underwent phenotyping (A, B or AB) using microplate agglutination, and genotyping (AA, Ab or bb) using pyrosequencing of a fragment of the cytidine monophospho-N-acetyleneuraminic acid hydroxylase gene. Non-AB blood type incompatibilities were investigated by cross-matching against reference blood of the same phenotype.

RESULTS: Of 112 cats tested, 86 (77%) were blood phenotype A, 19 (17%) type B and 7 (6%) type AB. Genotype and initial phenotype agreed in 96% (107 of 112) of cats, but 5 were discordant; these were all B phenotype with either AA (n=2) or Ab (n=3) genotype. Two of the five cats that had repeat blood samples tested: one was reclassified as phenotype A; the other remained phenotype B. Two cats had incompatibilities on minor cross-match, but these were attributed to phenotyping errors.

CLINICAL SIGNIFICANCE: Unknown mutation(s) associated with phenotype B, resulting in false AA or Ab genotyping, were evident in a small number of cases in this study. No conclusive evidence for non-AB blood type incompatibilities was found.

INTRODUCTION

Blood groups arise due to the presence of genetically determined antigenic markers on the surface of erythrocytes (Knottenbelt 2002). Cats are different from other mammalian species in having only one major blood group system, the feline AB blood group system, which was first described in 1962 (Eyquem et al. 1962). Within this system cats are either blood type A, type B or type AB (Knottenbelt 2002). These different blood types arise due to differences in the neuraminic acid residues present on the erythrocyte surface; type A cats primarily have glycolyneuraminic acid (NeuGc) plus a small amount of acetyleneuraminic acid (NeuAc), whilst type B cats have only NeuAc (Andrews et al. 1992). The rare type AB cats have both NeuGc and NeuAc on the surface of their erythrocytes in roughly equal amounts (Andrews et al. 1992).

Recently the genetic basis of the AB blood group system has been investigated (Bighignoli et al. 2007). The enzyme encoded by the cytidine monophospho-N-acetyleneuraminic acid hydroxylase (CMAH) gene converts NeuAc (the only form present in type B cats) to NeuGc (the predominant form in type A cats) (Muchmore et al. 1989, Bighignoli et al. 2007). Mutations in CMAH in type B cats are believed to disrupt the enzyme's function, so that it can no longer convert NeuAc to NeuGc, and polymorphisms in CMAH have been used to define type A or type B cats (Bighignoli et al. 2007). The exact mechanism for the generation of type AB cats is unknown but Bighignoli et al. (2007) have speculated that the AB blood group system is designated by three alleles; the allele responsible for type A (A) is dominant to that associated with type B (b). The genetic basis for the type AB allele (a^b) has yet to be defined, but it is speculated to be a genetic locus dominant to b and recessive to A, i.e. A > a^b > b (Bighignoli et al. 2007).

As cats may possess naturally occurring alloantibodies against the blood type antigen they lack, incompatible first transfusions can result in significant reactions (Knottenbelt et al. 1999b). Potentially fatal haemolytic transfusion reactions can occur in type B cats given type A or AB blood, as type B cats invariably
have anti-A alloantibodies of high titre (Knottenbelt et al. 1999b, Klaser et al. 2005). Type A cats can have anti-B alloantibodies, but these are often of low titre. Although fatal transfusion reactions are unlikely in type A cats given type B or AB blood, the anti-B alloantibodies can mediate premature destruction of the transfused red blood cells (RBCs) (Knottenbelt et al. 1999b). Therefore accurate identification of blood types is important in feline practice to reduce the incidence of potentially fatal blood transfusion reactions. In addition, these naturally occurring anti-A alloantibodies in type B cats can cause neonatal isoerythrolysis (NI) and kidney death, when a type B queen gives birth to type A or AB kittens and transfers these alloantibodies to them via the colostrum (Knottenbelt 2002).

Several methods, which all require the use of blood samples, exist to determine feline AB blood phenotype. These include microplate agglutination, gel-based agglutination, card-based agglutination and immunochromatographic cartridges (Knottenbelt et al. 1999a, Stieger et al. 2005, Seth et al. 2011). The recent discovery of polymorphisms in cytidine monophospho-N-acetylneuraminic acid hydroxylase (CMAH) that are linked to blood type B (Bighignoli et al. 2007) have allowed the development of commercially available polymerase chain reaction (PCR)-based and sequencing-based blood genotype tests to identify the AA, Ab and bb genotypes [e.g. by targeting the G139A single nucleotide polymorphism (SNP) of CMAH, which is associated with the b allele]. However, Bighignoli et al. (2007) did not identify the mutation(s) responsible for blood type AB (a^b), and phenotype AB blood samples had genotypes indistinguishable (i.e. AA or Ab) from phenotype A samples. More recently, preliminary unpublished data from the authors’ laboratory, and personal communication with Prof. L. Lyons (University of Missouri, USA), have documented the existence of additional, rare polymorphisms in CMAH, particularly a C136T SNP in Exon 2, which explained some previously discordant blood typing results (phenotype B cats erroneously reported as genotype Ab) based on the G139A mutation. Further sequencing of regions of the CMAH gene is therefore required in order to determine the full set of polymorphisms associated with different feline blood types so that specific and more accurate PCR-based genotyping tests for blood type identification can be developed.

Although the AB blood group system is the main blood system that exists in cats, recent work has suggested the existence of a non-AB blood group system. Weinstein et al. (2007) in the USA reported the absence of a novel feline erythrocyte antigen named Mik in 4 of 66 cats tested, in association with the presence of anti-Mik alloantibodies, which mediated a clinically significant transfusion reaction despite the blood donor and recipient cat being AB-matched (Weinstein et al. 2007). Prospective investigations into the existence of non-AB blood type incompatibilities outside of the USA are required in order to determine whether cross-matching should be recommended in addition to conventional AB blood typing before feline transfusions.

The aim of this study was to prospectively analyse whole blood samples from UK cats in order to determine their AB blood phenotype (A, B or AB), using standard microplate agglutination, and their AB blood group genotype (AA, Ab or bb), using pyrosequencing of a fragment of CMAH containing previously reported mutations, and to determine the agreement between these methods. In addition, all samples were cross-matched against blood of the same AB blood phenotype, to determine whether non-AB blood group incompatibilities existed in this population.

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**MATERIALS AND METHODS**

**Sample collection**

Excess ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood available from cats presented to the Feline Centre, Langford Veterinary Services, University of Bristol for clinical evaluation for ill health or as potential blood donors from January to October 2012 was used for this prospective study. Full owner consent was obtained for inclusion in the study, and the study was approved by the University of Bristol Ethics Committee. Cats were excluded from analysis if insufficient blood was available or if they had received blood products prior to blood sampling. If subsequent blood type phenotyping and genotyping yielded discordant results, owners were offered repeat testing on submission of a new blood sample.

**Blood phenotyping**

Blood samples were phenotyped for the AB blood group system (i.e. type A, type B or type AB) using standard microplate agglutination, as previously described (Knottenbelt et al. 1999a) within 2 days of collection.

**Cross-matching**

Cross-matching of each sample with a reference blood sample from a cat of the same AB phenotype was performed within 2 days of collection from the test cat. Reference samples were kindly provided by a blood banking service (Animal Blood Resources International), and shipped on ice to the UK on a monthly basis; on receipt the samples were stored at 4°C until use (up to a maximum of 28 days).

EDTA-anticoagulated whole blood was used for each test cat and whole blood in acid citrate dextrose anticoagulant was used for the reference cats. For all samples, plasma was separated from erythrocytes by centrifugation. The test and reference cat packed RBCs were washed twice in cold phosphate buffered saline (PBS) (pH 7.4, 0.01M) and a 2.5% suspension of washed RBCs was prepared. Cross-matching was performed in a microtitration system. Major (undiluted test cat plasma with reference cat RBCs) and minor (undiluted reference cat plasma with test cat RBCs) cross-matches were performed in addition to controls (undiluted test cat plasma with test cat RBCs, undiluted reference cat plasma with reference cat RBCs, test and reference cat RBCs in PBS). Each reaction was replicated in triplicate wells of a round bottomed 96 well microtitration plate. Plates were prepared and incubated at 37°C until control reactions formed a “button” at the base of the respective wells. Major and minor cross-match reactions were assessed by examining each well for evidence of haemagglutination (visible diffuse spread of RBCs over the base of the microtitration plate well) or haemolysis (visible diffuse red
colouration of plasma in the well). Haemagglutination and haemolysis was recorded as either present or absent.

**Blood genotyping**

Within 2 days of blood collection, 200 µL of EDTA-anticoagulated whole blood was removed from each sample and stored at −20°C for subsequent genotyping, which was performed in batches. DNA was extracted from 100 µL of EDTA blood and eluted into 100 µL of elution buffer using a commercial kit (Macherey-Nagel NucleoSpin Blood Kit, Fisher).

The PCR primers used for genotyping were designed using a fragment of feline *CMAH* obtained from the cat genome project (GenBank accession AANG01353910) to target the G139A and C136T mutations. Possible primer–dimer formation, self-complementarity and annealing temperatures for both the PCR and pyrosequencing reaction were optimised using Primer3 (Rozen & Skaletsky 2000), Mfold (Zuker 2003) and PyroMark assay design software (Qiagen Inc.) and resulted in PCR forward (5´-Biotin-GAAGACC GGCAAATTTTCACT-3´) and reverse (5´-CTCCTTGATGCTTGACAC-3´) primers being selected to amplify a region of 80 bp of *CMAH* (exon 2) (Fig 1). The pyrosequencing primer was 5´-ACACGT TTCTTGACAGCCCTCA-3´ (Fig 1).

The PCR comprised 12.5 µL 2x GoTaq mastermix (Promega), 0.5 µL 10 µM each forward and reverse primers, 10 µL water and 2 µL gDNA. Amplification was performed in a MJ PTC200 (Bio-Rad Labs. Ltd.) for 2 minutes at 95°C followed by 38 cycles of 95°C for 15 seconds and 58°C for 30 seconds.

Biotinylated PCR products were immobilised on streptavidin-coated Sepharose beads (GE Healthcare UK Ltd.), purified and annealed with the sequencing primer as described in the PyroMark Gold Q24 reagents kit instruction manual (Qiagen Inc.). Pyrosequencing was performed in a PyroMark Q24 (Qiagen Inc.) automated 24-well pyrosequencer according to the manufacturer’s instructions with a nucleotide dispensation order of GCTGTCGATCT. Pyrosequencing data were evaluated using PyroMark Q24 v2.0.6 software (Qiagen Inc.). Genotyping interpretation is summarised in Table 1. Samples found to have both the G139A and C136T SNPs were then subjected to targeted pyrosequencing of individual alleles to determine whether the SNPs were present on the same or different (i.e. compound heterozygotes) alleles.

**Screening for polymorphisms**

PCR amplification and DNA sequencing of segments of exons 1, 1a, 2, 3 and 10 of *CMAH* were also performed on samples discordant for phenotype and genotype to try and identify polymorphisms to explain phenotype. Four reference samples (two genotype AA and two genotype bb) also underwent this sequencing as controls.

**Statistics**

A minimum sample size of 104 was chosen as it predicted (http://sampsize.sourceforge.net/iface/) with 95% probability that at least three non-AB blood group incompatibilities would be found based on the prevalence reported in a previous study of 66 cats in the USA (Weinstein et al. 2007).

**RESULTS**

**Samples**

One hundred and twelve blood samples were available. Most were from ill cats (n=67), some were from potential blood donors (n=42) and a few did not have the reason for blood sampling recorded (n=3). The samples were from 82 non-pedigree cats (74 domestic shorthair; 8 domestic longhair) and 30 pedigree cats [Devon rex (n=5); Siberian (n=5); ragdoll (n=4); Persian (n=2) or Persian-cross (n=1); British shorthair (n=3); Burmese (n=2); Siamese (n=2); Birman (n=1); LaPerm (n=1); Maine coon (n=1); snowshoe (n=1); Somali (n=1); tiffanie (n=1)].

**Blood phenotyping**

Initial phenotyping revealed 86 (77%) type A cats, 19 (17%) type B cats and seven (6%) type AB cats. Of the 86 type A cats 24% [n=21; ragdoll (n=4), Siberian (n=3), Burmese (n=2), Siamese (n=2), British shorthair (n=2), Persian or Persian-cross (n=2),...
and one of each of Devon rex, LaPerm, Maine coon, snowshoe, Somali and tiffanie) were pedigree cats. Of the 19 type B cats 47% [n=9; Devon rex (n=4), Siberian (n=2), Birman (n=1), British shorthair (n=1) and Persian (n=1)] were pedigree cats. All type AB cats were non-pedigree cats.

**Cross-matching**
Cross-matching yielded an incompatibility reaction on the minor cross-match (test RBCs with reference plasma) in two cats (cats #68 and #116) of phenotype B. The incompatibility reaction took the form of haemagglutination (with no haemolysis) in each case.

**Blood genotyping**
Pyrosequencing (Table 1) identified 59 homozygous AA and 39 heterozygous Ab (38 with G139A and one with C136T). These 98 cats were genotyped as A or AB based on the genotyping results. The remaining 14 cats were bb: 10 were homozygous bb (G139A) whilst 4 were compound heterozygotes (G139A and C136T). No cats had both G139A and C136T SNPs on the same allele or were homozygous C136T.

These genotyping results include five cats that would have been incorrectly genotyped (one Ab cat would have been incorrectly typed as AA, and four bb cats would have been incorrectly typed as Ab) had C136T not been included in the genotyping assay.

**Discordant results**
Initially, five genotyping results were discordant with the initial phenotype results (all non-pedigree cats). All five were initially a B phenotype with either homozygous AA (n=2, including cat #116 whose RBCs showed a minor cross-matching reaction with reference type B plasma) or heterozygous Ab (n=3) [two with G139A (including cats #18 and #68; cat #68 had also had a minor cross-matching reaction between its RBCs and reference type B plasma) and one with C136T] genotype. Cats #18 and #68 had repeat blood samples tested: cat #18 remained phenotype B, whilst cat #68 was reclassified as phenotype A and repeat cross-matching for cat #68 with reference phenotype A blood was compatible, suggesting cat #68 was truly phenotype A. Cat #116 could not be retested, but its original B phenotype is suspected to be erroneous as its minor cross-match incompatibility reaction with reference type B plasma is consistent with it being truly phenotype A or AB (in agreement with its AA genotype) rather than phenotype B.

**Screening for polymorphisms**
PCR amplification and DNA sequencing of exons 1, 1a, 2, 3 and 10 of CMAH on the five discordant samples failed to identify any consistent polymorphisms that explained the phenotype (data not shown). The four reference samples yielded polymorphisms appropriate to their phenotype (data not shown).

**DISCUSSION**
The initial phenotyping results generated in this study agreed with genotyping in 107 of the 112 (96%) cats. The genotyping assay (Fig 1) included C136T, as well as G139A, as a target for the pyrosequencing assay. Omission of C136T in the genotyping assay would have resulted in five genotyping errors; incorrectly genotyping bb cats as Ab is a particularly worrying consequence of an assay omitting C136T, as this would lead to an incorrect phenotype prediction for those cats. Although 96% represents very good agreement of phenotyping and genotyping in this study, two of the five discordant cats were actually believed to have had initial phenotyping errors that had incorrectly classified them as being phenotype B. Both these cats had genotypes consistent with phenotype A or AB (Ab for cat #68 and AA for cat #116), and cross-matching results consistent with being phenotype A or AB, as they generated incompatibilities on the minor cross-match between their RBCs and the reference phenotype B plasma believed to contain anti-A alloantibodies. Repeat blood phenotyping and cross-matching confirmed that cat #68 was phenotype A. Cat #116 could not be retested to confirm its phenotype as A or AB.

Of the three remaining discordant cats, all phenotype B, one (cat #18) was tested again and remained phenotype B despite the Ab genotype, while the other two cats could not be retested (cats #43, genotype AA, and #77, genotype Ab). None of these three cats showed cross-matching incompatibilities with reference phenotype B blood, suggesting that their B phenotype was genuine. Therefore, these cats are likely to have an additional unknown novel mutation(s) in CMAH that affects their A allele(s) resulting in a true bb genotype and lack of CMAH expression or enzymatic activity. However, extensive sequencing of CMAH exons failed to identify any new polymorphisms that could explain their B phenotype.

Despite the relatively high agreement reported, the use of genotyping to predict phenotype ahead of blood transfusions has limitations as it cannot differentiate between phenotypes A and AB. However, unlike phenotyping, genotyping can be used by breeders to identify cats carrying the b allele to direct breeding programmes to prevent or predict NI. In this study genotyping was carried out on DNA extracted from blood samples, but mouth swabs provide an alternative source of DNA for genotyping and are easily collected by breeders. It is important that genotyping is as accurate as possible to enable breeders to predict breeding with confidence, but it should be highlighted to breeders that, using the known mutations, genotyping is currently approximately 96% accurate.

Ideally, in the discordant cats identified in this study, cross-matching with the other reference blood phenotypes available should have been performed to help characterise their phenotype. Indeed some have recommended that all cats phenotyped as B or AB are confirmed by back-typing of their plasma/serum with known phenotype A and B RBCs (Proverbio et al. 2011). However, the volume of reference phenotype B and AB blood available in this study was limited, and additionally genotyping was performed on samples in batches, meaning that genotyping results were not available at the time of phenotyping to highlight discordant cats. However, the phenotyping errors reported here with a reference method, and those highlighted by others in previous studies using in-house blood phenotyping kits.
(Proverbio et al. 2011, Seth et al. 2011), suggest that ideally all phenotype B (and AB) cats are confirmed by repeat pheno-
typing or back-typing. The phenotyping errors in this study
can only be attributed to technical errors in performance of
what were complex experiments involving testing of multiple
samples in multiple microtitration plates on single occasions. In
a routine diagnostic setting, it is believed that such errors would
be less likely to occur when fewer clinical case samples are being
evaluated at a time.

No conclusive evidence for non-AB blood type incompatibil-
ties was found in this study. The only apparent incompatibilities
on cross-matching were in cats that were believed to have had
phenotyping errors; indeed, repeat cross-matching of one cat
with reference blood of its revised phenotype was compatible.
However, because of the limited number of cats providing refer-
ence blood samples (one per blood type per month) for cross-
matching in this study, there may be a limited ability to detect
non-AB blood type incompatibilities. As the prevalence of non-
AB blood type incompatibilities is unclear at this time, a larger
population of reference animals may provide a more representa-
tive sample of the general population of cats. In addition, the
reference samples used had to be obtained from the USA, as no
blood banking services exist in the UK, and the geographical dif-
ference between the source of reference blood and test blood may
have influenced the results obtained.

In conclusion, genotyping appears to be accurate in predicting
phenotype as either A or AB, or B, in the majority of cats and can
be used by breeders to help control NI by the identification of
phenotype as either A or AB, or B, in the majority of cats and can
have influenced the results obtained.

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Conflicts of interest

None of the authors of this article has a financial or personal
relationship with other people or organisations that could inap-
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ENB and ST collected the samples for analysis. MJD per-
formed the blood typing and cross-matching. CRH designed
the pyrosequencing assay, performed the DNA extraction, PCR,
pyrosequencing and sequencing. ST, MJD and CRH conceived
the study and participated in its design. ST coordinated the study
and was principal investigator. ST and ENB drafted the manu-
script. All authors read and approved the final manuscript.

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