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Comparison of Methods for Determining ABO Blood Type in Cynomolgus Macaques (*Macaca fascicularis*)

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Thorough examination of ABO blood type in cynomolgus monkeys is an essential experimental step to prevent humoral rejection during transplantation research. In the present study, we evaluated current methods of ABO blood-antigen typing in cynomolgus monkeys by comparing the outcomes obtained by reverse hemagglutination, single-nucleotide polymorphism (SNP) analysis, and buccal mucosal immunohistochemistry. Among 21 animals, 5 were type A regardless of the method. However, of 8 serologically type B animals, 3 had a heterozygous type AB SNP profile, among which 2 failed to express A antigen, as shown by immunohistochemical analysis. Among 8 serologically type AB animals, 2 appeared to be type A by SNP analysis and immunohistochemistry. None of the methods identified any type O subjects. We conclude that the expression of ABO blood-group antigens is regulated by an incompletely understood process and that using both SNP and immunohistochemistry might minimize the risk of incorrect results obtained from the conventional hemagglutination assay.

Abbreviation: SNP, single nucleotide polymorphism.

Cynomolgus macaques (*Macaca fascicularis*) have been recognized as an appropriate model for transplantation research because of their similarity to humans in immunologic, genetic, and physiologic aspects.[8,9] Identification of ABO blood-group antigens is a prerequisite not only for donor–recipient matching to prevent rejection but also for transfusion during emergency animal care. In humans, the forward hemagglutination test, in which blood type is determined by using the antigen–antibody reaction between antigens on RBC and antisera, is recognized as the 'gold standard' for ABO blood-antigen determination. However, such a system is infeasible in macaques because they lack ABO antigens on their RBC.[7] Therefore, hemagglutination assays using surrogate human blood cells and the antisera of the test animal is used.

Although the hemagglutination assay is simple, cost-effective, and reliable on many occasions, its results are prone to subjectivity, and it has several technical limitations. The largest drawback of the agglutination assay arises from age-associated differences in the levels of serum antibody secreted in each animal.[20] The gel test method, in which hemagglutination is examined by assessing the differential movement of nonagglutinated and agglutinated RBC in dextran acrylamide gel after centrifugation, was introduced as an alternative to conventional hemagglutination in cynomolgus monkeys.[4] However, interpreting the grade (0 to +4) can be subjective; therefore other validation data, obtained by using methods, such as DNA analysis and immunohistochemistry, are required to determine the blood type of cynomolgus monkeys.

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It is well established in humans that the A and B allele encode distinct glycosyltransferases that convert the H antigen to the respective A and B antigens, whereas an O-type person expresses an unmodified H antigen.[22] Although a specific single-nucleotide polymorphism (SNP) in the ABO locus controls this activity, the molecular mechanism of blood-group antigen expression in cynomolgus macaques is not fully delineated. A proposed new method for ABO typing in cynomolgus macaques is based on the results of sequence comparison between various nonhuman primates and uses quantitative real-time PCR coupled with the TaqMan probe assay.[16,17] Such genetic analysis can clearly show the antigenic characteristics on the DNA level with the strength of high-throughput analysis of multiple samples.[6] Despite the tremendous potential of that approach in immunohematologic applications, the SNP that govern ABO antigen expression in cynomolgus monkeys remain unclear. For example, the null allele (O) in cynomolgus monkeys lacks the stop codon mutation found in the human ABO locus,[16,22] prompting questions regarding whether O-type animals exist and how the A- or B-antigen moiety is generated from H antigen in cynomolgus monkeys. Therefore, discrepant outcomes between hemagglutination and modern DNA analysis methods are still possible.

ABO antigens in Old World monkeys are expressed in a variety of tissues, including the endothelium of the vasculature of most organs, the epithelium of the gastrointestinal tract, saliva, and exocrine secretions.[15,19] If appropriately set up, immunohistochemistry provides unequivocal results regarding blood-group antigen expression.[2] However, the level of antigen expression on the epithelium differs among subjects,[20] and the quality of cells collected from epithelial tissues (for example, buccal mucosa) can vary widely, depending on the salivation status and general health conditions (for example, hydration) of the tested animals.

Table 1. ABO blood typing of individual macaques by using various methods

Macaque no.	Country of origin	AB typing method		
		Hemagglutination	SNP	Immunohistochemistry
AI-27	Cambodia	A	A	A
XI-03	Cambodia		A	A
11-8	Cambodia		A	A
IM-8	China		A	A
IM-9	China		A	A
AI-10	China	B	B	B
AI-14	Cambodia		B	B
IM-4	China		B	B
IM-5	China		B	B
AI-18	Cambodia		B	B
AI-08	China		AB	B
AI-17	Cambodia		AB	B
AI-21	Cambodia		AB	AB
AI-16	Cambodia	AB	AB	AB
10-5	Cambodia		AB	AB
IM-2	China		AB	AB
IM-3	China		AB	AB
IM-7	China		AB	AB
15-12	Cambodia		AB	AB
5-5	China		A	A
10-1	Cambodia		A	A

Given those viewpoints, we evaluated current ABO typing methods for cynomolgus macaques by comparing the results from hemagglutination assays with those of SNP analysis and immunohistochemistry.

Materials and Methods

Animals, sedation and sera. Four female (age, 3.9 ± 1.8 y; weight, 2.9 ± 0.4 kg) and 17 male (age, 6.0 ± 1.3 y; weight, 4.81 ± 1.19kg) cynomolgus monkeys were used in this study. All animals originated from either Cambodia or China. All procedures were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals*[10] and the Animal Welfare Act[1] and in the animal facility of the Primate Organ Transplantation Research Center at Genia (Sung-nam City, Korea). All experiments were approved by the IACUC at Genia. Animals were individually housed indoors on a 12:12-h light:dark cycle and were fed standard macaque biscuits (Harlan Laboratories, Seoul, Korea) and fresh fruit twice daily. Animal rooms were maintained at 23 to 25 °C and 40% to 60% relative humidity with 15 changes of conditioned air hourly. Chlorinated and filtered fresh water was provided ad libitum. To collect blood samples, macaques were fasted overnight and sedated with intramuscular ketamine hydrochloride (10 mg/kg, Yuhan, Seoul, Korea). Once animals were sedated (approximated 10 min after injection), approximately 1 mL of blood was collected from a femoral vein and dispensed equally into a serum-separator microtainer (Becton Dickinson, Franklin Lakes, NJ) and an EDTA-containing microtainer (Becton Dickinson).

All macaques were seronegative for simian retrovirus type D, SIV, simian T cell lymphotropic leukemia virus, measles virus, and herpes B virus, as determined by ELISA (VRL Laboratories, Suzhou City, Jiangsu, China). Tuberculosis testing was completed every 6 mo, and negative results were obtained with an immunochromatographic test kit (SDBioline TB Ag MPT64RAPID, Standard Diagnostics, Yongin-si, South Korea). Routine husbandry and sample collection were conducted by the same caretakers.

Hemagglutination assay. The hemagglutination assay was conducted as previously described[2] with minor modification. Briefly, whole-blood cells from A, B, and O blood donated from healthy volunteers were diluted 1:5 with PBS. The diluted whole blood cells were centrifuged at 900 × *g* for 3 min at room temperature, and the supernatant was removed. We repeated the washing twice and resuspended the cell pellet in 5 mL PBS. To minimize nonspecific reactions, we centrifuged 500 µL of prepared human O-type whole-blood cells at 900 × *g* for 3 min, removed the supernatant, gently resuspended the pellet in 200 µL serum from the test animals, and incubated the mixture for 30 min at room temperature. We then centrifuged these preadsorbed serum samples at 900 × g for 5 min and transferred the supernatant to a new tube. We then used a pipette tip to mix 25 µL of the preadsorbed serum with 25 µL of prepared human A- or B-type whole-blood cells on a white acryl plate, and the agglutination reaction was determined within 30 s of its onset. When an O-type macaque was found, the assay was repeated with serum that had been preadsorbed with human O-type whole blood cells for additional 2 rounds (total, 3 preadsorption reactions).

SNP analysis. We extracted genomic DNA from blood by using a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA). For amplification of exon 7 of the ABO locus, PCR was conducted by using the following primer pair, as previously reported:[16] 5′ CCT GCC TTG CAG ATA CGT G 3′ (forward) and 5′ CAG CTG ATC ACG GGT TCC 3′ (reverse). We used the following PCR protocol: 94 °C for 5 min for initial denaturation; 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 40 s; and final extension at

72 °C for 5 min. All PCR reagents including Top-Taq polymerase, dNTP, and buffer were purchased from Davinch K (Seoul, Korea). We prepared the reaction cocktail in 100-µL quantities as follows: 300 to 500 ng of template DNA, 0.4 µM each of the forward and reverse primer, 8 µL 10mM dNTP, and 0.5 µL *Taq* polymerase. After amplification was confirmed by 1% agarose gel electrophoresis, we purified the PCR product by using silica-based membrane columns (MEGAquick-spin Total Fragment DNA Purification Kit, Intron Biotechnology, Daejeon, Korea) and sequenced it by using the forward primer. We analyzed the chromatogram image to determine SNP by visually confirming the nucleotides of the SNP locus with FinchTV software (Geospiza, Seattle, WA).

Immunohistochemistry. Buccal swab immunohistochemistry was performed as described previously.[2] Briefly, we collected mucosal epithelial cells by swabbing the inner surface of each macaque's mouth with a cotton swab and then applying the swab to a microscope slide. After air-drying, the slides were submerged in ice-cold acetone for 10 min for fixation. The slides were air-dried again and then subjected to immunohistochemistry. We used antiA (1:100 in PBS; Z2A, Santa Cruz Biotechnology, Dallas, TX) or antiB (1:50 in PBS; Z5H-2, Santa Cruz Biotechnology) antibodies for 1 h, washed the slides with PBS 3 times, and then incubated them with goat antimouse IgM-FITC (1:100 in PBS, Santa Cruz Biotechnology). After washing, the slides were mounted using the VectaShield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). We obtained digital images under an inverted microscope (BX53, Olympus, Tokyo, Japan) equipped with a power supply for a mercury burner (U-RFL-T, Olympus) and a digital camera (DP73, Olympus).

Statistical analysis. To examine whether the number of macaques with each blood type is deviated from the expected value, the immunohistochemistry results of animals from each country of origin were tested for Hardy–Weinberg equilibrium at a probability of 0.05 by calculating a χ^2 value with 2 degrees of freedom and a Yates correction factor of 0.5.[3,17] Under our assumption that no O-type macaque was present, the expected ratios of A, B, AB types were calculated as $0.25(p^2)$, $0.25(q^2)$, and $0.50(2pq)$ for the number of A-, B-, and AB-type macaques, respectively.

Results

ABO blood typing by hemagglutination. The hemagglutination assay showed the presence of A, B and AB types, in the cynomolgus macaques we tested (type A, 5; type B, 8; type AB, 8). Positive hemagglutination was readily visible within 30 s, and negative reactions were deemed to be type AB. Agglutination did not occur from reaction with O cells in any of the cases. During initial screening, we found a single O-type animal (no. AI-18, Table 1); therefore hemagglutination was retested by using serum that had been preadsorbed for an additional 2 rounds. Consequently, we could clearly demonstrate that this animal was type B. We then cross-checked the hemagglutination results by using SNP analysis and immunohistochemistry.

ABO typing by SNP analysis. Using standard PCR amplification, we easily obtained a 710-bp fragment of the ABO locus that contained 4 SNP (Figure 1 A) and then sequenced the products. The entire chromatogram was scanned, and polymorphisms among types A, B, and AB were detected at 4 positions within the fragment (Figure 1 B and C).[16,17] In type A macaques, the results obtained by hemagglutination and SNP typing were completely consistent with each other. In contrast, 3 of the 8 macaques determined to be type B by hemagglutination were type AB according to SNP genotype. The results also indicated

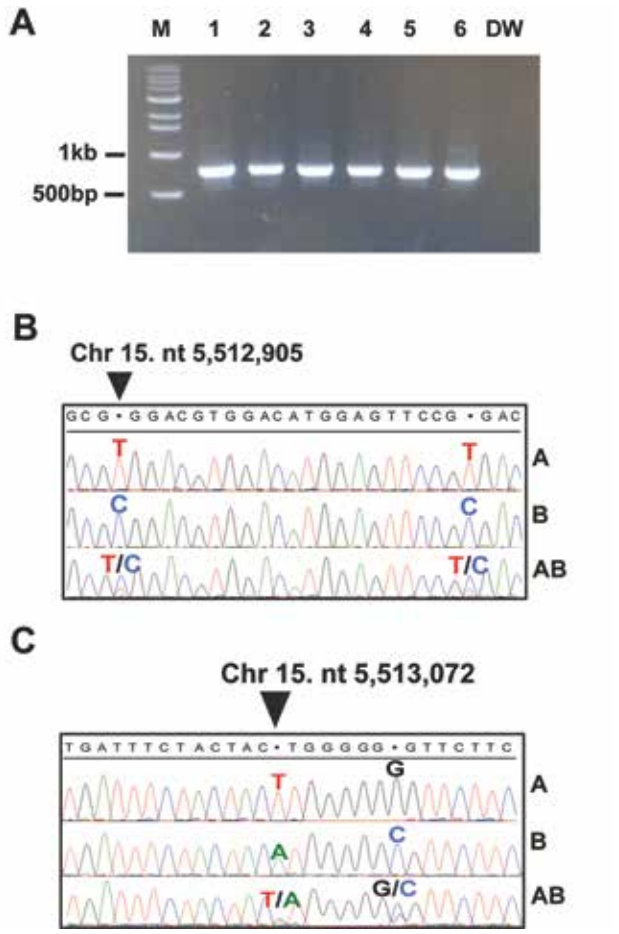

Figure 1. Amplification of the AB locus for SNP typing. (A) Agarose gel electrophoresis revealed a 710-bp fragment that was detected by PCR. Lanes 1 through 6, 6 different DNA templates; DW, distilled water. The agarose concentration was 1.2%. (B, C) Chromatogram illustrations showing all 4 SNPs. Nucleotide positions were numbered based on the NCBI reference sequence (AF052078.1).

that among the 8 serologically type AB animals, 6 had matching SNP serology results, and the remaining 2 had a type A allele (Table 1).

ABO typing by immunohistochemistry. To confirm the blood type of each macaque, we performed immunohistochemistry of buccal mucosal tissue by using antiA or antiB antibodies (Figure 2). We found complete consistency among the 3 methods in type A animals. Among the 3 macaques with AB genotypes among the 8 animals that initially had been labeled as type B according to hemagglutination results, one expressed both A and B antigens (as its AB allele indicated), and the other 2 expressed only the B antigen despite the presence of both the A and B alleles. Two A-allele macaques among the 8 initially defined as type AB expressed only the A antigen, showing consistency between their SNP and antigen expression results. The overall results are summarized in Table 1.

Statistical analysis. Regardless of the country of origin, the distribution of the blood types analyzed by immunohistochemistry did not differ from the values expected according to Hardy–Weinberg equilibrium at a probability of 0.05 and analyzed by the χ^2 test (Table 2).

Discussion

The purpose of this study was to evaluate the reliability of hemagglutination assay for determining the ABO blood type

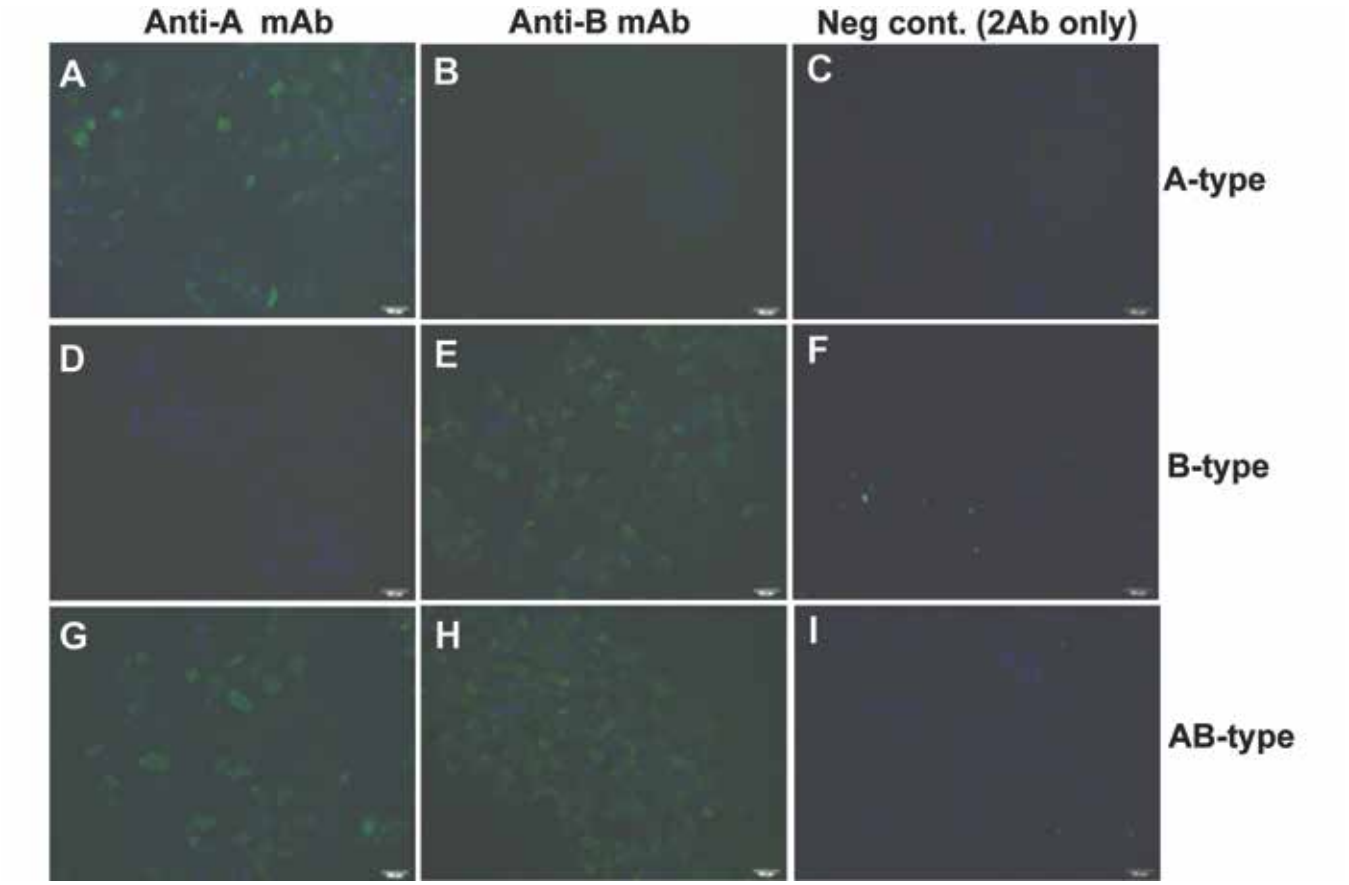

Figure 2. Immunohistochemical detection of AB histo-blood antigens in buccal mucosal tissues. Smears were fixed, hydrated, and bound with mouse monoclonal antibodies (mAb) against A or B antigens from human blood. After being stained with FITC-conjugated anti-mouse IgM, the tissues were mounted by using antifade medium containing DAPI. The blood type of each macaque was determined by staining the tissue with either antiA (A, A-type) or antiB antibody (E, B-type) or both (G and H, AB-type). Negative reactions were determined by the absence of stained cells (B, D). As a negative control, only the secondary antibody (2Ab) was used for analysis. The corresponding blood type is indicated at the right end of each row. Magnification, 100×.

Table 2. Origin and the number of macaques with each phenotype as determined by immunohistochemistry

	ABO type			
Country of origin	A	B	AB	χ^2 P^a
Cambodia	4	3	4	0.567
China	3	4	3	0.975
Total (observed frequency)	7 (0.33)	7 (0.33)	7 (0.33)	0.484

[a] $P < 0.05$ was regarded as significantly deviated from Hardy–Weinberg equilibrium.

in cynomolgus macaques by comparing the results obtained from SNP analysis and immunohistochemistry. The reverse hemagglutination assay has been used to determine the ABO blood antigens in cynomolgus macaques in light of the absence of ABO blood antigens on their RBC. Unfortunately, this technique does not always yield consistent results in macaques, which necessitates implementing other methods (i.e., DNA or immunohistochemical analyses) to confirm their blood types. In this context, one group developed a sequence-specific priming-based multiplex PCR technique to determine the ABO blood types of rhesus macaques.[16] A more convenient and sophisticated method, namely quantitative genotyping by real-time quantitative PCR using the TaqMan system, is now available for cynomolgus macaques.[17] Another powerful technique currently available for determining blood type is immunohistochemical detection of distinct A or B antigens present on buccal mucosal tissue. This method offers additional advantages over other techniques in that it is applicable to diverse animal species, including pigs, baboons, and macaques, by using same experimental protocol, making this technique an essential tool, especially for xenotransplantation research.[2]

We found that, for some macaques, the results differed among the methods (Table 1). One report of a human study demonstrated that the hemagglutination assay could produce discrepant results in consecutive tests in certain cases, suggesting the complexity of ABO allele expression and the presence of other unknown A or B subgroup alleles.[14] Therefore, confirming the contribution of weakly expressed antigens is tremendously important.

Regardless of the methods examined, identical results were obtained only in type A animals. At present whether such finding is biologically meaningful is unknown, in light of the small population tested. The observed incongruity may be associated with the relatively high level of antiB antibodies in the macaque serum, although additional tests for verifications (for example, serum dilution) are needed. Among the 7 serologically type B animals in our study, sequence analysis showed 3 to be type AB. Such conflicting results strongly suggest the limitations of the conventional reverse hemagglutination assay. Moreover, 2 among the 3 SNP-determined type AB animals lacked A antigen expression. Epigenetic regulation, that is, silencing of the A or B allele by methylation of CpG islands in the promoter region, could yield such discrepancy.[11]

Table 3. Comparison of the various ABO typing methods.

	Hemagglutination	SNP analysis	Immunohistochemistry
Relative technical level	simple	high	intermediate
Speed	quick (few seconds)	prolonged (more than 24 h)	intermediate (3 h)
Applicability in the field	high	poor	poor
Error rate of the procedure	low	Intermediate (PCR failure, sequencing error)	Intermediate (collection of too few cells, defective specimen preparation, nonspecific antigen–antibody binding)
Need for supporting methods	high	intermediate	intermediate
Subjectivity	high	low	low

Although immunohistochemistry of mucosal tissue can be used to obtain the most definitive results at the antigen level, several things should be considered. First, the expression level of ABO antigens on the buccal epithelium of cynomolgus monkeys likely is affected by its differentiation status, as occurs in human oral epithelium;[5] therefore, buccal swab tissue may not always provide appropriate material for immunohistochemistry. Second, because the histo-blood group antigen is found in saliva also, additional experimental steps to remove saliva or purify buccal cells before fixation would provide clearer data. Using SNP genotyping so that the antigenic profile can be determined at the DNA level would be ideal. Last, examining the Lewis system antigens will further strengthen the validity of the immunohistochemistry results,[18] given that Lewis antigens are known to regulate the secretor status of ABO blood group antigens.[21] In rhesus macaques, the prevalence of secretor animals, that is, Le(a– b+) type, is higher than that of nonsecretors, that is, Le(a + b–) type,[18] but studies on Lewis antigen distribution in cynomolgus macaques are few.[13] Regardless of the species, it would be the best to compare the immunohistochemistry results with those from other experimental tools (for example, SNP typing) to determine ABO blood types, especially in weak secretors (Le[a+b+]) and nonsecretors (Le[a+ b–]).

It is still unclear whether type O macaques exist. In the present study, we identified a single O-type animal in initial hemagglutination screening, which included serum that had been preadsorbed by using human O cells. To fully remove the heteroagglutinins that may still present in the serum, we increased the preadsorption reaction and finally obtained clear B-type agglutination results, which were supported by SNP typing and immunohistochemistry. One study reported that 28.0% of 729 cynomolgus macaques were type O, as determined by agglutination between undiluted human cells and diluted animal serum.[12] In contrast, other colleagues[2] showed that 1 of 8 macaques (12.5%) was type O, as supported by both buccal mucosal immunohistochemistry and serum hemagglutination with human cells.[2] Given that this latter study was supported by positive staining of the H antigen on buccal mucosal tissue with antiH antibody, it is highly indicative that type O cynomolgus monkeys exist. However, we cannot exclude the possibility of positive binding reaction between anti-H antibody and tissues of A- or B- type individuals, considering that H antigens are also the precursors of A and B antigens.[21]

We conclude that the hemagglutination assay is not sufficient for determining ABO blood type in cynomolgus monkeys unless supported by SNP analysis and immunohistochemistry of epithelial tissues. Finding novel SNP in other exons or regulatory regions will provide critical information on the molecular basis of O allele expression.

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