Supplementary Information 4: Recommendations for Laboratory Performance and Interpretation of the Direct Antiglobulin Test and Flow Cytometry for Erythrocyte-Bound Antibodies

Direct antiglobulin test (DAT)

- Laboratories should establish the effects of sample handling and storage.
- Owing to considerable variation between reagents, individual laboratories should determine the titer that defines a positive result.[1,2]
- Comparison of microtiter, capillary or gel tube methods shows that all are acceptable for initial testing.[2] However, confirming negative gel tube results by microtiter has been recommended.[3] Microtiter testing is preferred to the tube method because it allows a greater range of dilutions and minimizes sample volume requirements.[4] Enzyme-linked immunosorbent assays, which use optical density to detect anti-erythrocyte antibodies, are considered less specific than DAT assays that evaluate samples for agglutination.[5]
- For microtiter testing, to avoid false negative results due to high concentrations of anti-erythrocyte antibodies (i.e. the prozone effect), serial dilutions are recommended.[1-3,6] Diluting beyond the range recommended by the DAT reagent manufacturer may be required to overcome the prozone effect in some cases.[1]
- Evidence is mixed regarding the optimum temperature(s) for the DAT, with some studies suggesting that testing at 37^{o}C only is adequate[2,7] and others finding that additional IMHA cases were detected when tested at both 4^{o}C and 37^{o}C.[3,6,8-11] A recent study has suggested that although it is more difficult to interpret than microtiter testing at 37^{o}C,

room temperature incubation may also be acceptable.[2] This variation between studies may reflect differences in optimal binding temperatures for DAT reagents, so it is recommended that individual laboratories determine optimal temperature conditions. For patients with clinical signs consistent with cold agglutinin disease, a DAT at 4°C and 37°C is recommended. Some studies have reported increased false positive DATs [9,12] or spontaneous agglutination of erythrocytes from normal animals[13] at 4°C. Based on recommendations from human medicine, when the DAT is positive or spontaneous agglutination is present at 4°C but not at 37°C, consideration of titer and thermal amplitude is recommended to determine the likely clinical significance.[14]

- A polyvalent reagent reactive against IgG, IgM and C3 is considered acceptable for initial diagnosis.[1] However, when available, some monovalent reagents may increase sensitivity.[3,8] Positivity for IgA in the DAT or other methods is rare in cats[9] and dogs[15]; therefore, inclusion of anti-IgA antibodies in the routine DAT is not required. However, based on reported IgA-induced AIHA in humans[16] in rare DAT-negative cases where IMHA is strongly suspected clinically, additional testing for IgA may be helpful.

Flow cytometry

- Laboratories should provide guidance regarding pre-analytical handling, including acceptable delay between sample collection and analysis.
- Owing to variability in reagents and flow cytometers, individual laboratories should optimize assay conditions and determine their own objective criteria for defining positive results.[17] In the absence of veterinary guidelines, laboratories should adopt quality control procedures from recommendations for clinical flow cytometry on human specimens.[18]

- Antibodies detected for IMHA cases vary between published reports. Some studies fail to detect additional IMHA cases[19] or report a decrease in specificity[20] when anti-IgM and/or anti-C3 antibodies are included in addition to anti-IgG antibodies. In contrast, the largest series of IMHA cases reported 8/54 IMHA cases were positive only for IgM and 1/54 was positive for only IgA.[15] As this may in part reflect variability in reagents and assay design, we suggest that individual laboratories design test panels based on information about the sensitivity and specificity of each of the antibodies used in confirmed IMHA cases and in sick patients with a diagnosis other than primary or secondary IMHA.

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