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Received: 17 April 2025 | Revised: 9 June 2025 | Accepted: 10 June 2025

DOI: 10.1111/vox.70069

SHORT REPORT

Vox Sanguinis ISBT International Society of Blood Transfusion

Unifying serological testing for cold agglutinins

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Funding information
Sanquin Blood Supply Foundation, Grant/Award Number: PPO-SHS-2022-22.13/L2687

Abstract

Background and Objectives: Cold agglutinins (CAs) are immunoglobulin M autoantibodies that optimally bind to red blood cells at low temperatures. The clinical significance of CAs is usually characterized by the CA titre and thermal amplitude. However, there is no consensus on the optimal testing strategy. In this study, we sought to unify CA testing.

Materials and Methods: A survey on CA evaluation was conducted in 17 Dutch transfusion laboratories. Additionally, samples from 16 patients with CAs were used to evaluate (pre)analytical variations in test outcomes.

Results: The survey on CA evaluation revealed heterogeneity in the indications for CA workup and in (pre)analytical conditions. Titration results between EDTA plasma and serum did not differ. However, titres were higher when 2% bovine serum albumin (BSA) was present in the titration medium. Routinely collected EDTA-blood kept at 37°C for 10 min before plasma isolation (*reheated*) showed equivalent results to plasma from blood kept at 37°C from collection through separation.

Conclusion: This study showed that different (pre)analytical conditions influence test outcomes in the characterization of CAs. Importantly, CA testing can effectively be performed with *reheated* blood samples, which will facilitate a more practical workflow for laboratories. International standardization of CA testing for diagnostic use is essential.

Keywords

autoimmune haemolytic anaemia, cold agglutinin disease, cold agglutinins

A list of authors and their affiliations appears in Appendix A.

Highlights

- Cold agglutinin (CA) testing is highly heterogeneous in clinical practice and is not well described in the literature.
- CA testing can be effectively performed with routinely collected blood samples that have subsequently been incubated and warmed at 37°C prior to serum/plasma separation from the cells.
- Standardization of the serological evaluation of CAs is essential to ensure accurate comparison of CA characteristics and treatment response in patients with CA-mediated autoimmune haemolytic anaemia.

INTRODUCTION

Cold agglutinins (CAs) are immunoglobulin M autoantibodies that bind to red blood cells (RBCs) with an optimal binding temperature of approximately 4°C. The I/IH/I antigens are most often targeted [1]. CA-mediated autoimmune haemolytic anaemia (cAIHA) occurs primarily when the CAs induce activation of the complement system. Additionally, CAs can lead to RBC agglutination in the acral circulation, leading to acrocyanosis.

Whether or not CAs can cause clinically significant haemolysis and/or RBC agglutination, depends on autoantibody characteristics, which are serologically reflected by the CA titre and/or thermal amplitude [2–4]. However, as we recently described, serological test approaches and possibly test outcomes differ between centres, making comparisons difficult [5]. Further research into the CA characteristics, and their correlation to severity and treatment response, would improve our understanding and management of cAIHA.

In the current study, we sought to investigate the effect of both the preanalytical and analytical conditions used for testing on the test outcome. We evaluated the routine serological testing procedures for CAs in Dutch clinical laboratories and assessed the impact of various blood collection methods and testing conditions on the serological outcomes.

MATERIALS AND METHODS

Survey on current serological workup CAs in the Netherlands

The current practice of the diagnostic workup of CAs in Dutch hospitals was surveyed using a questionnaire sent out via the professional association of transfusion laboratories. Questions focused on the indications for a CA laboratory workup and techniques used to characterize CAs.

Analysis of the influence of test variables on CA titre

Sample selection

The sample selection criteria included a complement-positive direct antiglobulin test (DAT), absence of bound immunoglobulin G (IgG)/immunoglobulin A autoantibodies and at least a grade 2+ macroscopic agglutination of test erythrocytes upon incubation of serum at 16°C in the CA screening test [6]. Blood samples from 16 patients were selected and comprised 12 cAIHA patients who, upon informed consent, were included in the Data Registry Autoimmune Hemolytic Anemia (DRAIHA) study (NCT04024202) and 4 patients that were sent to Sanquin. For two of these four patients, information on the autoimmune hemolytic anaemia (AIHA) diagnosis was lacking. Sample selection was based on blood sample availability sufficient for one or more comparisons. In order to establish reference values, residual blood samples from 100 healthy blood donors were used with an even distribution of blood group A, B, AB and O. All donors passed the donation health check and had normal haemoglobin values. Residual material from blood samples from both donors and patients was utilized in accordance with the institutional guidelines with informed consent.

Serological methods

All serologic tests from these 16 patients were performed at Sanquin. The DAT was performed on fresh blood samples using column technique with monospecific reagents (Bio-Rad, Diamed GmbH, Switzerland) per manufacturer's instructions. CA titres were determined by twofold serial dilution prepared of thawed plasma or serum in glass tubes with phosphate-buffered saline (PBS) alone or with addition of bovine serum albumin (22% BSA, CE Immunodiagnostika GmbH) with a final concentration in the diluent of 2%.

Both the sample dilution and test erythrocytes (3% suspensions) were incubated separately for 10 min at the temperature at which the agglutination test was performed: 4, 16 or 30°C. Two drops of plasma/serum sample were added to one drop of test erythrocytes and incubated for 30 min at the given temperature. Thereafter, samples were centrifuged for 1 min at 200*g* at room temperature (RT) and agglutination was recorded. The reported titre is the inverse of the last dilution with macroscopic 1+ agglutination. In general, one dilution step difference is considered to fall within the normal intra-assay variation. The following preanalytical conditions were tested: "Standard" samples; plasma/serum sent in at RT, "Warm" samples; plasma/serum isolated from material that was collected and separated at 37°C, "Reheated" samples; EDTA-anticoagulated blood samples kept

at RT, incubated at 37°C for 10 min, followed by a centrifugation step at 37°C prior to plasma isolation.

RESULTS

Survey on serological workup CAs in the Netherlands

The survey results of 17 clinical transfusion laboratories are summarized in Table 1. The Dutch immunohaematology reference centre, Sanquin Diagnostics (Sanquin), all academic laboratories and 70% non-academic clinical transfusion laboratories where extended pre-transfusion testing is performed responded. Unexpected ABO blood grouping or antibody screening (14/17) and detection of complement deposition in the DAT (11/17) were the primary triggers for CA screening. CA screening (without titration) was performed at either 4°C, 16°C or both in 13 laboratories, with 1 laboratory using RT and 3 laboratories using a range of 4–37°C (Table 1a). Six laboratories performed CA titration upon physician request, following variable protocols (Table 1b). Almost all laboratories diluted the samples for titration with PBS without BSA. Five out of six laboratories defined the starting material in their standard operating procedures, using either serum ($n = 2$) or plasma ($n = 3$); one laboratory used either option based on availability. In three laboratories, blood samples were collected warm using prewarmed tubes and kept at 37°C before serum/plasma separation. In the other three, samples were kept at RT and prewarmed to 37°C before separation. Incubation temperatures varied between 4°C ($n = 2$) and 16°C ($n = 2$). Two academic laboratories determined CA titres at different temperatures between 4 and 30°C or 4 and 37°C, respectively.

All laboratories used tube testing for titration, with incubation times ranging from 1 to 20 h. Eight of the 17 laboratories determined CA thermal amplitude at various temperatures between 4 and 37°C (Table 1).

Comparison of different variables in serological workup to characterize CAs

Six *standard* EDTA plasma samples were titrated with either PBS or PBS/BSA (Figure 1a). Titration values ranged from 64 up to 32,000 in PBS and from 64 up to 256,000 with PBS/BSA medium. For two samples, there was no difference in CA titre. For four samples, higher titre results were obtained if PBS/BSA was used as a dilution medium. PBS/BSA was selected as the dilution buffer for subsequent analyses.

For 11 patients, the CA titre was determined at 16°C with either serum or EDTA plasma (Figure 1b). Titration values ranged from 64 up to 256,000 for both plasma and serum. For six samples CA titres were the same for serum and EDTA plasma, with two samples a one-step higher titre was obtained with serum, whereas for two samples plasma showed a higher titre (two titre steps). In one there was a five-step higher titre for the plasma sample.

TABLE 1 (a) Survey on serological cold agglutinin workup in 16 Dutch laboratories. (b) Conditions and procedures for performing the CA titre in 6/16 laboratories.

(a)

Indication for CA testing		CA screening temperature		CA titre performed		Thermal amplitude performed	
1. If DAT is positive for complement deposition	$n = 11$	4°C	$n = 3$[a]	No	$n = 11$	No	$n = 8$
2. Unexpected results during ABO blood grouping and antibody screening	$n = 14$	16°C	$n = 11$[a]	Upon request of the clinical physician	$n = 6$	Yes, at 16–30°C	$n = 3$
3. Upon request of a physician	$n = 10$	RT	$n = 1$			Yes, at 16, 20, 24 and 30°C.	$n = 1$
4. Suspicion of AIHA	$n = 7$	Range 4–30/37°C	$n = 3$			Yes, range up to 37°C	$n = 5$

(b)

Titration dilution		Blood sample type		Warm collected[b]		Incubation temperature		Incubation time	
PBS	$n = 5$	Plasma	$n = 3$	Yes	$n = 3$	4°C	$n = 2$	45–60 min	$n = 2$
Unbuffered saline	$n = 1$	Serum	$n = 2$	No	$n = 3$	16°C	$n = 2$	2 h	$n = 2$
		Both	$n = 1$			Range 4–30/37°C	$n = 2$	16–24 h	$n = 2$

Note: All laboratories work with tube testing.

Abbreviations: AIHA, autoimmune hemolytic anaemia; CA, cold agglutinin; DAT, direct antiglobulin test; PBS, phosphate-buffered saline; RT, room temperature.

[a]One laboratory performs screening at 4 and 16°C.

[b]Blood samples are collected warm using prewarmed tubes and kept at 37°C until serum/plasma is separated from the cells. Other samples are kept at room temperature and then prewarmed to 37°C before separation.

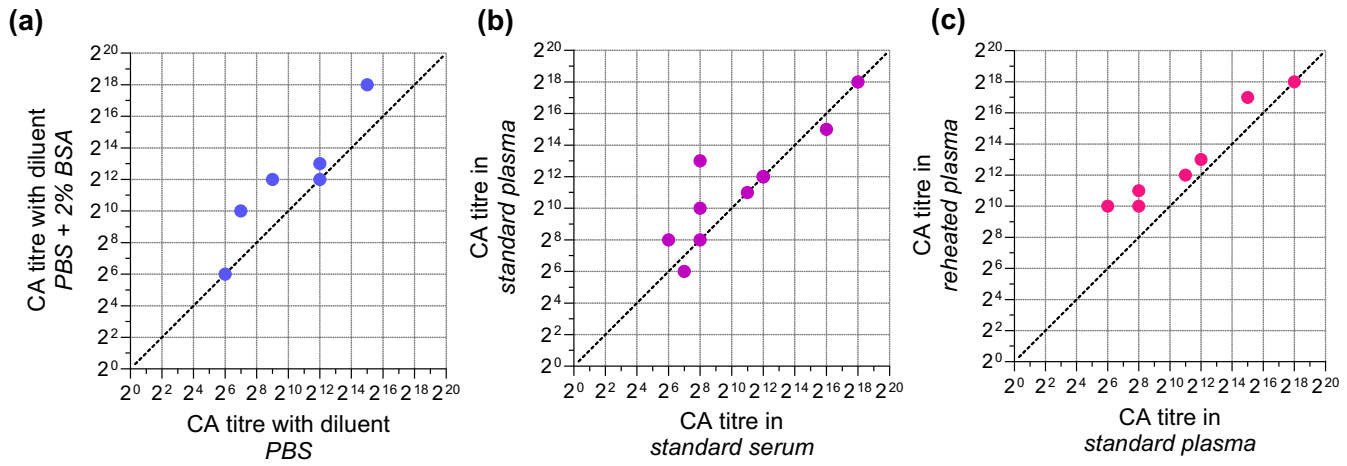

FIGURE 1 Comparison of test variables in cold agglutinin (CA) titration at 16°C. (a) EDTA-anticoagulated plasma titrated in either phosphate-buffered saline (PBS) or PBS with 2% bovine serum albumin (BSA). (b) Serum and EDTA plasma (in PBS with 2% BSA). (c) *Standard* EDTA plasma versus *reheated* EDTA plasma (in PBS with 2% BSA). Titres were expressed as 2^X, where X represents the number of twofold serial dilutions (1:64 = 2^6, 1:16,000 = 2^{14}).

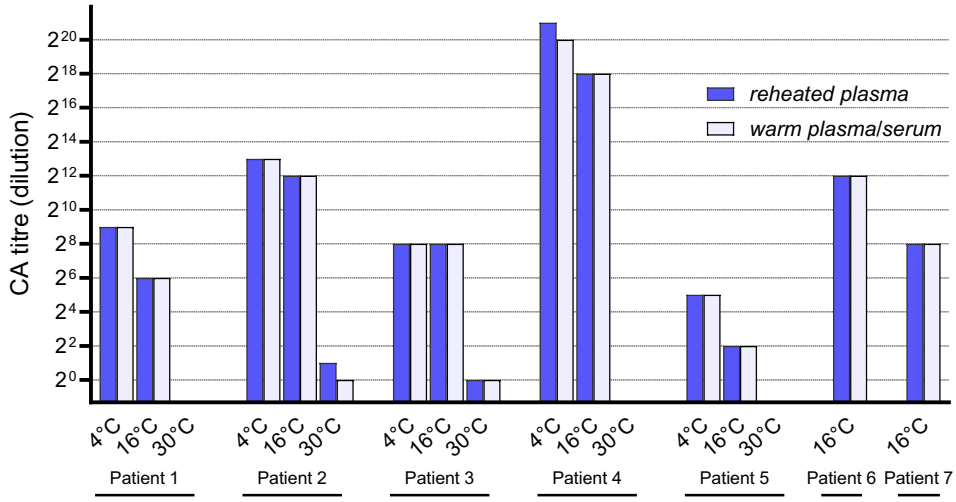

FIGURE 2 Cold agglutinin (CA) titration in *warm* blood samples were compared with CA titres in *reheated* EDTA-anticoagulated plasma of the same patients.

For seven patients *standard* EDTA plasma was compared with *reheated* EDTA samples (Figure 1c). For six of these blood samples, the CA titre was higher if *reheated* plasma was tested. In one sample with a very high titre (1:256,000) there was no difference in CA titre between the two types of materials.

Subsequently, CA titres were determined ($n = 7$) with *reheated* plasma versus *warm* plasma or serum (Figure 2). CA titres were determined at 4, 16 and 30°C. There was no difference in titre as all samples obtained similar CA titres between the two material sources or within one titre step difference. In all five samples for which a temperature amplitude was performed, the titre decreased with increasing temperature, consistent with cold antibody characteristics. Three samples showed no reactivity at 30°C (Patients 1, 4 and 5), of which one showed a titre of 2,000,000 at 4°C.

Blood samples from 100 healthy blood donors (53 men and 47 women) were used to investigate how often CAs may be present in a healthy population. In this series, no complement deposition was observed in the DAT. At 4°C, CAs were found in 30/100 *reheated* plasma samples with a maximum titre of 4. Six CA titres were positive at 16°C, but only undiluted (1:1). In none of the samples CAs were found at 30°C. Neither the sex of the donor nor the ABO blood group was correlated with having a detectable CA titre at 4°C (data not shown).

DISCUSSION

Most literature defines clinically significant CAs as the presence of haemolysis in combination with a monospecific DAT showing strong complement (C3d) deposition together with negative or weak IgG deposition, and a CA titre of 64 or greater at 4°C, although clinical disease with low-titre CAs has been reported [7–9]. However, test

procedures used in studies applying this cut-off value and the influence of different analytic variables on the titre are not well described. We evaluated the current CA serological testing practices in the Netherlands and found them to vary widely. Therefore, we set out a series of experiments to explore the impact of these varying protocols.

Based on our data, and in line with earlier findings by Garratty et al., it seems that adding BSA to the titration medium enhances the sensitivity of titration results [2], most likely by preventing loss of CAs due to adherence to the test tube.

Using this titration method, we did not observe a significant difference in test results between plasma and serum as a sample source. Apart from one unexplained outlier, test results were within the normal variation of one titration step.

For optimal CA testing, it is often recommended to keep the blood specimen at 37°C from sampling until the serum/plasma is separated from the cells [3]. Our results show that CA testing can be effectively performed with routinely collected blood samples that are subsequently incubated and warmed at 37°C prior to serum/plasma separation. Given the logistical difficulties associated with obtaining blood samples taken and kept at 37°C, this observation can facilitate a more practical workflow for laboratories to perform reliable titration of CAs.

The presence of low-titre (<64) CAs, reactive only at 4°C or to a lesser extent at 16°C, is a well-known phenomenon in healthy individuals. These CAs do not indicate the presence of cAIHA but may be related to a recent infectious episode [10]. Low-titre CAs were detected at 4°C in 30 out of 100 healthy blood donors, however, no CAs were detectable at 30°C. CA titre evaluation in *reheated* EDTA plasma of healthy blood donors did not lead to unexpectedly high CA titres, supporting our conclusion that this type of material can be used for testing in patients with CAs.

A limitation of this study is the low number of samples tested, which is related to the rarity of cAIHA. However, considering the consistent results, we are confident that these data can be used to implement *reheated* plasma as an equal alternative for CA titre determination.

In conclusion, current practice on CA evaluation is highly heterogeneous. Our data provide practical insights to help unify the serological workup of CAs. Standardizing the serological workup is a crucial step toward establishing evidence-based cutoffs for clinically relevant CA titre and thermal amplitude in larger cohorts of patients.

ACKNOWLEDGEMENTS

The project was supported by Sanquin Blood Supply Foundation (PPO-SHS-2022-22.13/L2687).

M.J., F.V.M.M. and C.C.F. designed the research study and acquired and analysed the data, M.E.G. acquired and analysed the data, M.J. wrote the first draft of the manuscript, F.V.M.M. designed the figures, reviewed and edited the manuscript, J.M.I.V. and M.d.H. reviewed and edited the manuscript and C.C.F. supervised the research and reviewed and edited the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Jalink M, Mulder FVM, George ME, Vos JMI, de Haas M, Folman CC, et al. Unifying serological testing for cold agglutinins. Vox Sang. 2025;120:944–9.

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