A Critical Appraisal of Current Practice in the Detection, Analysis, and Reporting of Cryoglobulins

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To assess current practice in the detection, analysis, and reporting of cryoglobulins, a questionnaire was sent to 140 laboratories. Only 36% of laboratories used standard procedures (tube preheating, transport in container, and sedimentation and/or centrifugation at 37 °C) to ensure that the temperature did not drop below 37 °C until after serum separation. Time periods allowed for cryoprecipitation at 4 °C varied from 12 h to 9 days, with 30% of laboratories allowing precipitation for <3 days. After cryoprecipitation, 81% of laboratories resolubilized the cryoprecipitate at 37 °C, and 77% further immunotyped the cryoprecipitate. After analysis, 5% referred the sample for confirmation, and 37% reported the cryoglobulin concentration in the cryoprecipitate as cryocrit, total protein concentration, and/or immunoglobulin concentration. Only 3 laboratories (2%) provided cryoprecipitate-specific reference values for total protein content, and none provided reference values for immunoglobulins. We believe standardization is needed for cryoglobulin detection to avoid missed diagnoses and improve the comparability of results. Laboratories should ensure that sample temperature does not drop below 37 °C until after serum separation. The serum should cryoprecipitate at 4 °C for at least 3 (preferably 7) days. The cryoprecipitate should be washed and resolubilized at 37 °C for further analysis.

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Cryoproteins are serum proteins that reversibly precipitate at temperatures below 37 °C. There are 2 types of cryoproteins, cryoglobulins and cryofibrinogens. Cryoglobulins are immunoglobulins that precipitate both from serum and plasma, whereas cryofibrinogens, which consist of fibrinogen-fibrin complexes, precipitate only from plasma (1).

Cryoglobulinemia was first described by Wintrobe and Buell in 1933 and has been associated with the Meltzer triad of palpable purpura, arthralgia, and asthenia. The clinical significance of cryoglobulinemia is the consequence of intravascular precipitation of immunoglobulins, which can produce (reversible) mechanical obstruction of small vessels leading to the Raynaud phenomenon and immune complex-mediated vasculitis, particularly in the skin, peripheral nerves, and kidneys (2).

There are 2 distinct types of cryoglobulins. Type I cryoglobulins are monoclonal immunoglobulins associated with immunoproliferative disorders. Type I cryoglobulins account for about 10% of cryoglobulins, usually precipitate within 24 h, and can produce symptoms of hyperviscosity and thrombosis (2). Mixed cryoglobulins, in contrast, are composed of immune complexes containing polyclonal immunoglobulins with (type II) or without (type III) a monoclonal component and can take up to 1 week to become apparent by precipitation. Mixed cryoglobulins account for approximately 90% of cryoglobulins and are associated with chronic inflammatory diseases and infection. Mixed cryoglobulinemia is characterized by immune complex-mediated vasculitis and multiple organ involvement (2).

Detection of cryoglobulinemia can aid in the diagnosis of certain syndromes and is part of the diagnostic work-up for other conditions (e.g., monoclonal gammopathies) (3). The Chapel Hill nomenclature for systemic vasculitis is the only disease classification system in which the presence of cryoglobulins is mentioned, i.e., for essential cryoglobulinemic vasculitis (4). Quantitation of cryoglobulins can be used to make therapeutic decisions (plasmapheresis) and to assess the efficacy of therapeutic interventions.

Although serum and plasma are necessary for detecting cryofibrinogenemia, cryoglobulin analysis is traditionally performed only on serum (1). Several authors have described different analytical approaches...
highlight the need for standardization and interpretation by clinicians. Reports in the literature exist among laboratories that search for cryoglobulins were included for further analysis (Fig. 1). The number of samples analyzed per year varied between 1 and 1500 (median 40). The 25th and 75th percentiles were 15 and 85, respectively. (The questionnaire is available in the online data supplement at http://www.clinchem.org/content/vol54/issue1.)

**CRITICAL APPRAISAL**

**TEMPERATURE CONTROL BEFORE SERUM SEPARATION**
It is well recognized to be crucial that blood should be collected, transported, and clotted at 37 °C, and most authors stress the need for warm centrifugation to avoid false-negative results (6,9,10). Temperature control is particularly important for type I cryoglobulins, which are often present at concentrations >5 g/L (1,11). High concentrations of monoclonal cryoglobulins tend to precipitate at higher temperatures, and it has been suggested that the higher the temperature at which cryoglobulins precipitate, the more dramatic the symptoms (1,11). There are also reports of missed diagnosis of type II cryoglobulins due to inadequate temperature control (7,8). For type III cryoglobulins, temperature control is not crucial because they slowly precipitate over a period of days (1,2).

In our survey, 48 of the 137 laboratories (35%) took all the standard precautions: preheating of tubes, transport at 37 °C, and sedimentation/centrifugation at 37 °C (Table 1). Insufficient temperature control is recognized as the single most important cause of missed diagnosis of cryoglobulinemia (1). Inadequate temperature control during blood collection and transport before the sample arrives in the laboratory is a common cause of concern for laboratories. Often nonpreheated tubes were used (40%) and samples were brought to the laboratory by hand-held transport (19%). When adequate temperature control cannot be ensured during sample collection and transport, then patients should be required to come to the laboratory for sample collection to avoid false-negative results. In many laboratories (47%) the sample was centrifuged without maintaining the temperature at 37 °C, most likely because of a lack of a heated centrifuge. When no preheated centrifuge is available, cells should be allowed to sediment at 37 °C, and serum should be separated at 37 °C without centrifugation.

**CRYOPRECIPITATION**
After serum separation, most laboratories (66%) allowed the sample to cryoprecipitate at different temperatures. Although most authors report performing cryoprecipitation only at 4 °C, some authors find that cryoprecipitation at different temperatures has merit (10). The duration of cryoprecipitation at 4 °C varied from 12 h to 9 days (median 3 days). Although monoclonal immunoglobulin cryoprecipitates (type I) usually appear within 24 h, mixed cryoglobulins may appear only after several days (1,9). A minimum incubation period of at least 3 days (9) to preferably 7 days (1,6,12) is required to avoid missing a diagnosis of mixed cryoglobulinemia.

All laboratories performed visual observation of the cryoprecipitate. Most laboratories (72%) compared the same sample at different temperatures and approximately 1 in 3 laboratories (32%) compared serum and plasma. After cryoprecipitation, 60% of laboratories washed the sample, 81% resolubilized the cryoprecipitate at 37 °C, and 19% concentrated the sample before further analysis. The number of washes ranged from 1 to 5, with a median of 3. Washing the cryoprecipitate and checking the resolubility at 37 °C is recommended to avoid contamination from precipitation of normal serum proteins (6).

Seven laboratories that refer positive samples for confirmation were excluded from further analysis (Fig. 1).

**CRYOGLOBULIN QUANTIFICATION AND IMMUNOTYPING**
There was wide variation in the type of results reported, ranging from a simple qualitative negative/positive result to a quantitative report including cryoglobulin type and total protein content and/or immunoglobulin concentration in the cryoprecipitate. Of the 127 laboratories included for the postidentification phase, 50 (39%) reported the cryocrit, the total protein content, and/or the immunoglobulin concentration in the cryoprecipitate (quantitative); 48 (38%) answered negative/positive only; and 29 (23%) provided additional information without reporting the cryoglobulin concentration in the cryoprecipitate (nonquantitative).

Forty-six of the 50 laboratories that provided a quantitative report and 55 of the 77 laboratories that provided a nonquantitative report performed im-
munofixation analysis on the cryoprecipitate. Of the 101 laboratories (57%) that further immunotyped the cryoprecipitate, 98 performed immunofixation or immunoelectrophoresis, and 3 used protein electrophoresis only.

For type I cryoglobulins, which are often present at concentrations >5 g/L, reporting the presence or absence of an important visual cryoprecipitate that can be resolubilized is acceptable. For mixed cryoglobulins, however, which constitute >90% of the total cryo-
globulins and are typically observed in chronic inflammatory conditions (6), further analysis and quantification is important to improve sensitivity and specificity, because visual inspection of mixed cryoglobulins at low concentrations lacks accuracy for detection and quantification. Diagnosis of low concentrations of mixed cryoglobulins is important because there is, in general, no relationship between the cryoglobulin concentration and the severity of the symptoms (2, 13).

**REFERENCE VALUES**

Of the 11 laboratories that provided reference values, only 3 (2%) applied cryoprecipitate-specific reference values, whereas the other 8 used reference values for serum proteins and/or immunoglobulins, which are not valid for the cryoprecipitate. Three laboratories that had cryoprecipitate-specific reference values provided different reference values using total protein content: 0–10 mg/L serum (no reference provided), 0–50 mg/L serum (no reference provided), and 0–80 mg/L (12). None of the participating laboratories provided cryoprecipitate-specific reference values as immunoglobulin concentrations.

The lack of formal cryoprecipitate-specific reference values hampers the direct quantification of cryoglobulins, because many healthy individuals also have detectable amounts of cryoglobulins (2, 6). Although several authors have looked at total protein content in the cryoprecipitate to establish reference values, (12, 14, 15), only 3 laboratories (2%) provided cryoprecipitate-specific reference values for total protein content. This result could be related to the lack of standardization of cryoglobulins detection, variation in the suggested reference values, methodological problems (e.g., young healthy controls instead of diseased controls), or the lack of hepatitis C (HCV) serology. There is an association between essential cryoglobulinemia and HCV infection (5).

Total protein content and cryocrit are only indirect measures of the cryoglobulin concentration because they also detect the presence of other proteins such as albumin and fibrinogen. When cryocrit is determined without washing and resolubilizing the precipitate, particular care has to be taken when interpreting the results (6). Direct quantification of immunoglobulins in the cryoprecipitate could overcome this problem, but no cryoprecipitate-specific reference values exist for immunoglobulins.

**Conclusion**

The results of our questionnaire underline the need for standardization of the detection, analysis, and reporting of cryoglobulins. To avoid missing a diagnosis of monoclonal cryoglobulins, laboratories should ensure that the sample temperature does not drop below 37 °C during the preanalytical phase. For the diagnosis of mixed cryoglobulins, serum should be allowed to cryoprecipitate at 4 °C for at least 7 days, the cryoprecipitate should be washed and resolubilized at 37 °C, and the cryoglobulin should be quantified to improve accuracy and allow comparison of results between laboratories. Because cryocrit and total protein are only indirect measures of the cryo-immunoglobulin concentration, further research is warranted to provide reference values for cryo-immunoglobulins.

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