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Cryoglobulins are immunoglobulins that precipitate in the serum upon cooling to below core body temperature and re-dissolve at higher temperatures. Cryoglobulinaemia may be life-threatening. The three types of cryoglobulinaemia are associated with a wide spectrum of haematological, autoimmune, and chronic infectious diseases, especially hepatitis C infection. Our laboratory has received 378 requests for cryoglobulin testing over the past 5 years, with a detection rate of 4.8% in the 271 patients involved. Twelve per cent of the specimens were not processed due to being at an inappropriate temperature on arrival at the laboratory. Clinicians should be aware of temperature requirements when requesting cryoglobulin testing in suspected cases, and for all relevant protein tests in patients with cryoglobulinaemia. Handling specimens at inappropriate temperatures in the pre-analytical and analytical phases of the investigation might lead to cryoprecipitation and therefore false-negative results. The potential pitfalls encountered with specimen handling, analysis, and result interpretation are discussed in detail.

## Introduction

Cryoglobulins were first described by Wintrobe and Buell in 1933, when found in a blood specimen from a patient with multiple myeloma, as precipitates that appeared in sera stored at a low temperature then disappeared when incubated at 37°C. Lerner and Watson first used the word "cryoglobulin" in 1947 to describe this type of serum protein. The exact mechanism of precipitation or gelling of these immunoglobulins (Ig) upon a decrease in temperature remains obscure, but it has been proposed that changes in the degree or site of glycosylation or sialylation, or structural modification of the variable portion of the heavy and light chains of the Ig may lead to this phenomenon.<sup>1-4</sup>

Cryoglobulinaemia is classified into three types according to Brouet et al.<sup>5</sup> Type I consists of a monoclonal Ig, usually IgM, and is associated with underlying lymphoproliferative disorders, though monoclonal gammopathy of undetermined significance may account for some cases of type I disease. Type II is composed of a mixture of immune complexes with a monoclonal component (usually IgM) and polyclonal IgG. The monoclonal Ig possess rheumatoid factor activity. Type II cryoglobulinaemia is frequently associated with lymphoproliferative disorders, autoimmune diseases, cold agglutinin disease and chronic infections, especially hepatitis C and, less commonly, hepatitis B. Type III consists of immune complexes formed by polyclonal Ig. The disease association is similar to type II, though underlying lymphoproliferative disorders are rare. The term "essential mixed cryoglobulinaemia" has been used to describe idiopathic type II and type III diseases and it was not until the early 1990s that many of these cases were found to be associated with hepatitis C virus (HCV) infections.<sup>6-8</sup>

Cryoglobulinaemia leads to systemic vasculitis with a clinical triad of purpura, arthralgia, and aesthenia. The disease mainly affects the small- to medium-sized arteries and veins due to depositions of immune complexes on the vessel walls which activate the complement system, or due to direct occlusion of these vessels.<sup>9,10</sup> Vasculitic manifestations are especially common in type II disease with a low cryoglobulin concentration, an unexplained phenomenon.<sup>11</sup> The kidney and the peripheral nervous system are particularly vulnerable.<sup>12</sup> Systemic manifestations may signify a grave prognosis.<sup>13</sup>

The prevalence of cryoglobulinaemia is strongly linked to the prevalence of HCV infection in a population. It has been reported in various populations that more than 40% of patients with HCV-related liver diseases had cryoglobulinaemia,<sup>14-16</sup> and HCV RNA was found to be highly concentrated in cryoglobulin.<sup>7</sup> In fact, the term "cryoribonucleic acid" has been proposed by a local group.<sup>17</sup> The prevalence of HCV infection in the general population in Hong Kong is low, with a prevalence among blood donors of 0.035 to 0.099% over the last decade.<sup>18</sup> In a recent report, 1.7% of the patients screened in a local tertiary institute were found to be positive for cryoglobulins over a 10-year study period, with 38%

### Key words

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## 冷凝蛋白血症：臨床和化驗室的研究

冷凝球蛋白是免疫球蛋白先冷卻至核心體溫之下沉澱在血清裏，再升溫溶解而生成。冷凝蛋白血症可以致命，共分為三種，常見於多種疾病，包括血液系統出血性疾病、自身免疫性疾病、各種慢性感染性疾病，特別與丙型肝炎有關。本化驗室過去五年共接378宗冷凝球蛋白測試，在271位病人中檢出率為4.8%。12%樣本由於送達化驗室時溫度不適當而無法進行測試。因此，凡是對懷疑個案做冷凝球蛋白測試、以及對冷凝蛋白血症患者要做所有相關的蛋白質測試，臨床醫護人員在處理樣本時必須留意測試對溫度的要求。分析前和進行分析時，在不適當的溫度處理樣本可能導致冷沉澱，造成假陰性的結果。本報告仔細討論樣本處理、分析，結果詮釋過程中可能出錯的各種陷阱。

Initial laboratory investigations showed normal renal and liver functions except a reversed albumin-globulin ratio (serum total protein 82, reference range [RR]: 62-81 g/L; albumin 36, RR: 34-48 g/L; globulin 46 g/L). The haemoglobin level was 114 (RR: 134-171) g/L. In view of the reversed albumin-globulin ratio, specific protein tests were ordered and the Ig pattern (immunoturbidimetric, Modular Analytics, Roche Diagnostics, Mannheim, Germany) showed a lowish IgA level (0.65, RR: 0.70-4.00 g/L), a depressed IgM level (0.17, RR: 0.40-2.30 g/L), and a normal IgG level (10.20, RR: 7.00-16.00 g/L). The C3 level was normal (1.02, RR: 0.88-2.01 g/L) but the C4 level was borderline low (0.15, RR: 0.16-0.47 g/L). Rheumatoid factor was undetectable. Serology testing showed that the patient was a hepatitis B carrier.

Blood was also sent for cryoglobulin testing; 10 mL of clotted blood was received at 37°C and centrifuged in warm conditions at 3500 revolutions per minute (rpm) for 7 minutes. Two aliquots were made from the serum obtained. One of them was kept at 4°C while another aliquot was kept at 37°C. Both aliquots were inspected daily for precipitate over 3 consecutive days. Cryoprecipitate was detected on day 3 in the aliquot kept at 4°C while the aliquot kept at 37°C remained clear. Total protein concentration (colorimetric, Modular Analytics) was measured in the serum kept at 37°C and also in the supernatant of the serum kept at 4°C after centrifugation. The difference in total protein concentration between the two specimens gave an approximated concentration of the cryoprecipitate measuring 16.6 g/L. The cryoprecipitate was washed with cold normal saline thrice. All the cryoprecipitate re-dissolved upon warming to 37°C, indicating a positive cryoglobulin result. Under warm conditions, serum protein electrophoresis (PE) and immunofixation (IF; Paragon Electrophoresis Systems, Beckman Coulter, Fullerton, CA, US) were performed on the serum kept at 37°C, the supernatant serum kept at 4°C and the re-dissolved cryoprecipitate. An IgG lambda paraprotein, quantified as 18.1 g/L by densitometric scanning, was detected in the gamma region in the warm serum. Features of immune paresis were also present. Similar findings were obtained in the PE and IF of the supernatant and the re-dissolved cryoprecipitate (Fig 1), suggesting that not all the paraproteins were cryoproteins. Free lambda light chains were detected in the patient's urine by PE and IF (not shown). The Ig pattern of the warm serum showed a depressed IgA level (0.5 g/L) and IgM level (0.09 g/L), and an elevated IgG level (23.4 g/L), consistent with the serum PE and IF findings. A bone marrow aspiration and trephine biopsy (BMAT) revealed 6% mature-looking plasma cells. A skeletal survey showed no osteolytic lesions. There was no lymphadenopathy. Findings from a peripheral blood smear and a biopsy of the skin on the lower limb were compatible with cryoglobulinaemia.

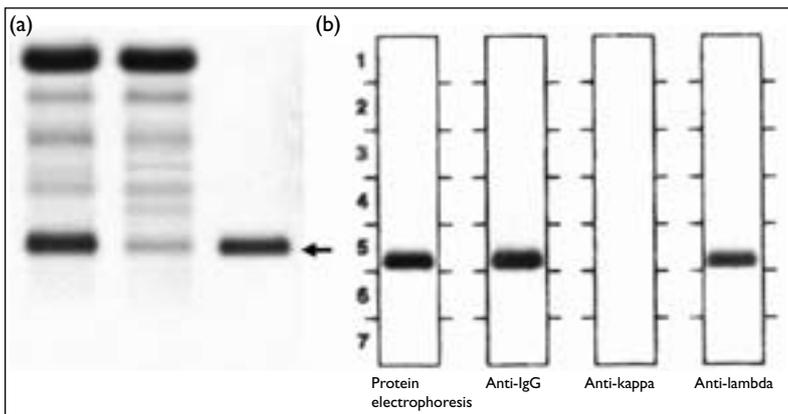


FIG 1. (a) Serum protein electrophoresis of the serum kept at 37°C (left), the supernatant serum kept at 4°C (middle) and the re-dissolved cryoprecipitate (right). Paraproteins were observed in the gamma region (arrow) of all the three specimens. (b) Immunofixation of the re-dissolved cryoprecipitate. It was characterised as an immunoglobulin G (IgG) lambda paraprotein

of these positive cases being HCV carriers.<sup>19</sup> Here, we report a case with HCV-negative cryoglobulinaemia. Precautions needed when carrying out this special protein investigation and the pitfalls encountered with test analyses and result interpretations will be discussed.

### Case report

A 61-year-old man with known hypertension presented to a private practitioner in 2001 with a 2-month history of intermittent but increasing bilateral calf and ankle pain. He was diagnosed as having gout. The pain persisted despite treatment with colchicine and analgesics so he attended the emergency department in April 2001. Physical examination revealed bilateral necrotic scabs covering his lower limbs to mid-calf level. There was also gangrenous change on the toes and the feet were cold and tender. Peripheral pulses were strong with preservation of muscle power. The examination of the cardiovascular, respiratory, and abdominal systems was unremarkable.



TABLE 2. Clinical information provided on the request forms when requesting cryoglobulin testing

Clinical information	%
Vasculitis/skin rash/gangrene	20.6
Lymphoproliferative disorders	11.4
Autoimmune diseases	8.2
Liver problems	5.5
Arthritis	4.0
Anaemia	3.4
Others	30.2
No information given	16.7

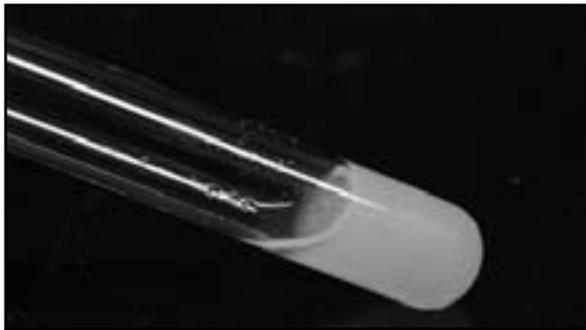


FIG 3. Gelling of the serum specimen of patient 4 after standing at 4°C for 24 hours

reported in the literature,<sup>11,21</sup> probably due to the small number of positive cases in our series. The highest concentration of cryoglobulin was found in a patient with Waldenstrom's macroglobulinaemia (Fig 3). Patients with type I cryoglobulinaemia tended to have higher concentrations of cryoglobulins than those with type II and type III, a pattern consistent with the literature.<sup>22</sup> Four patients were hepatitis B carriers and one patient was anti-HCV positive. One of these 13 patients was a carrier of both hepatitis viruses.

In all the 378 requests, the most commonly provided clinical information was cutaneous manifestations, followed by lymphoproliferative disorders and autoimmune diseases (Table 2). Forty-five requests were not processed because the specimens were at a suboptimal temperature on arrival at the laboratory. Strict temperature control is a prerequisite for the investigation of cryoglobulins because of the temperature-dependent nature of these serum proteins. Over-heating of the blood specimens leads to protein denaturation while cooling of the specimens to below body temperature risks cryoprecipitation before laboratory processing. Prompt delivery to the laboratory via a specially arranged courier after blood taking using a pre-warmed collection tube is essential. The specimen should be kept at 37°C using a vacuum flask or other suitable devices during transportation. Laboratories

providing this service should monitor the pre-analytical quality of the specimens.

Once cryoglobulinaemia has been diagnosed, all the blood specimens sent for protein testing, including total protein (as part of the liver function test), Ig patterns, serum PE, cryoglobulins, and rheumatoid factor must be sent using temperature precautions to avoid falsely low results due to cryoprecipitation. In the case reported above, the first IgG result was 10.2 g/L when the specimen was handled at room temperature before the diagnosis of cryoglobulinaemia had been made. When the IgG test was repeated 2 weeks later, in a specimen sent at 37°C, the level was 23.4 g/L. It is very likely that cryoprecipitation occurred in the first specimen, leading to an underestimation of the IgG concentration. Clinicians should be aware of this potential pitfall when requesting protein tests for patients with cryoglobulinaemia. On the other hand, because Ig patterns and serum PE may not be performed daily in some laboratories, proper preservation of the specimens at 37°C before and during analysis is mandatory. Centrifugation of all the blood specimens must be performed at 37°C. Our laboratory information system is capable of generating an alert whenever blood specimens from a patient known to have cryoglobulinaemia are received. This helps to avoid generating erroneous protein results from specimens delivered and handled without taking temperature precautions.

The cut-off level for a quantitatively positive cryoglobulin test is variable and no consensus is available. Some report 'positive' as being greater than 0.001 mg/mL while others define the cut-off as above 0.02 mg/mL.<sup>22</sup> Some laboratories compare patients' specimens with control sera, and this is the approach adopted by our laboratory. In our series of patients, the concentration of cryoglobulins ranged from less than 1 g/L to over 40 g/L. For cases like patient 4 (Table 1, Fig 3), the presence of cryoglobulin would not be missed because of the very high concentration of cryoglobulins forming a gel upon cooling to below core body temperature. In cases where the cryoglobulin concentrations are low, the cryoglobulins may not be easy to identify or the result may even be misinterpreted as negative. Adequate specimen volume is essential to ensure reasonable test sensitivity. In our practice, we require at least 9 mL of clotted blood, ie two 5 mL-filled plain blood tubes, for the investigation. A larger volume of blood is needed for those positive cases where the quantity of cryoglobulin is too low for characterisation procedures. It is essential that the specimens be kept warm during the analysis to prevent cryoprecipitation at the origin of application of serum for PE and IF. Correct characterisation is an important component of cryoglobulin investigation because it has a direct impact on the line of investigation, like testing

for hepatitis serology, complement, autoimmune markers, and on patient management. Laboratories providing cryoglobulin testing should perform characterisation of the cryoglobulins before issuing the final report.

Turbid specimens and fibrins are other nuisances when interpreting cryoglobulin results. Turbid specimens usually result from hypertriglyceridaemia and can be avoided by using fasting specimens. Blood from patients with coagulation disorders, or those on anti-coagulant therapy and specimens contaminated with heparin or other anti-coagulants may show delayed clotting in the blood tubes. Upon standing, fibrinogen in the plasma converts to fibrin with the formation of serum. The appearance of fibrin may resemble that of cryoglobulin and may be misinterpreted as such. In addition, fibrinogen and fibrin may possess cryoprecipitable properties.<sup>23</sup> To get rid of the problems with fibrinogen and fibrin, serum instead of plasma should be used when investigating cryoglobulinaemia, and a prolonged clotting time should be allowed for specimens from

susceptible patients. Re-dissolving the precipitate at the end of the investigation is helpful for differentiating between cryoglobulins and fibrinogen or fibrin. Cryoglobulin dissolves upon warming to 37°C, sometimes with the aid of a drop of 0.1 M acetic acid but fibrin in serum does not behave the same way. Characterisation with the use of serum IF also helps clarify the Ig nature of the cryoglobulins.

In conclusion, the investigation and diagnosis of cryoglobulinaemia, as for many other medical conditions, require multidisciplinary inputs. Clinicians have to be aware of the specimen requirements for investigating cryoglobulinaemia, ie adequate specimen volume and appropriate temperature precautions. The same should be applied to all relevant protein tests in patients known to have cryoglobulinaemia. Laboratory technicians have to make a judgement about the integrity of, and to properly handle, the specimens. Pathologists should be aware of the potential pitfalls in order to avoid misdiagnosing this uncommon but potentially debilitating disease.

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