An erytropoietin measurement difficult to interpret

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ABSTRACT

Erythropoietin (EPO) regulates red blood cell production and is mainly produced in the peritubular cells of the adult kidney in response to a decrease in tissue oxygenation. EPO determination is requested in cases of reduced or increased erythrocyte production. Laboratory assays used to measure EPO in human serum are mainly based on enzyme-linked immunosorbent assays (ELISA). ELISA results, however, may be affected by the presence of heterophile antibodies. We report a case of analytical interference in EPO measurement.

Key word: *erythropoietin, analytical interference, heterophilic antibody, heterophile antibody*

CASE REPORT

In December 2023, a 77-year-old male patient, with a history of heart disease, diabetes, hypertension, and dyslipidemia, was addressed to the Blood Drawing Center of the Niguarda Hospital in Milan with a diagnosis of polycythemia. Blood tests revealed the presence of erythrocytosis and microcytosis, with a high value for hematocrit, while the other parameters of the complete blood count were all within the reference intervals; as was the lactate dehydrogenase value (Table 1). Erythropoietin (EPO), measured with the EPO ELISA kit (DRG Instruments GmbH, Germany) on the DSX Elisa System (DYNEX®, USA), returned a very elevated value, 384.7 mU/mL (r.i. 3.2-31.9) (Table 1). The EPO ELISA kit is a two-site ELISA immunoassay that uses two different mouse monoclonal antibodies againt well-defined domains of the human EPO molecule. One monoclonal antibody is biotinylated, while the other is labeled with horseradish peroxidase (HRP) for detection (Figure 1A). Absorbance readings are usually performed at 450 nm; however, when EPO concentrations are higher than that of the highest calibrator provided with the kit, a second reading is carried out at 405 nm, allowing for the extension of the calibration curve. Samples with EPO results above the highest calibrator value should be diluted with the Calibrator Zero also included in the kit (a buffered protein solution with no EPO content), re-assayed and the result multiplied by relative dilution factor, according to the manufacturer's instructions.

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Received: 31.05.2024 Revised: 25.06.2024 Accepted: 16.07.2024

Published on-line: 17.09.2024 DOI: 10.19186/BC_2024.040

The EPO concentration obtained for the patient was much higher than the value assigned to the highest calibrator (156 mU/mL); as a consequence, the sample was diluted 1:10; after dilution, EPO concentration was 35 mU/mL, and, on a second dilution, 32 mU/mL. Measurements were repeated on two other sample dilutions (1:5 and 1:2): EPO values were 21.5 mU/mL and 213.9 mU/mL, respectively. To be reliable, the values obtained with different dilutions of a single sample should be consistent and give approximately the same results once multiplied for the dilution.

In this case, a high variability of results was observed among the different dilutions analysed, suggesting the presence of an interferent. Actually, immunochemical methods, show high sensitivity and specificity, but can be affected by analytical interferences due to the presence in the sample of molecules that nonspecifically bind the antibody, or interfere with the formation of the antigen-antibody complex, or compete with the antibody for binding, as it happens when autoantibodies or heterophile antibodies are present in the sample (1).

To verify this hypothesis, a second blood sample was collected from the patient one month later; EPO was measured before and after sample treatment with Scantibodies® Heterophilic Blocking Tube (HBT, Scandibodies Laboratory, Inc., USA). The reagent blocks heterophile antibodies by means of specific binders, thus eliminating their interference. In the untreated sample, the measured EPO concentration was 279.8 mU/mL; after treatment with HBT, it was 7.6 mU/mL.

Table 1
Blood test results obtained in December 2023 and January 2024

First draw - December 2023			
Analyte	Result	Unit	Reference interval
Leucocytes	9.95	10^9/L	4.00 – 10.00
Erythrocytes	6.58	10^12/L	4.40 – 6.00
Hemoglobin	16.4	g/dL	14.0 – 18.0
Hematocrit	0.53	L/L	0.42 – 0.52
Mean corpuscolar volume	79.9	fL	82.0 – 97.0
Mean corpuscolar hemoglobin content	24.9	pg	27.0 – 33.0
Mean corpuscolar hemoglobin concentration	31.2	g/dL	32.0 – 36.0
Red cell distribution width	14.6	%	11.5 – 14.5
Platelets	157	10^9/L	140 – 440
Neutrophils	6.29	10^9/L	1.60 – 7.00
Lymphocyte	2.68	10^9/L	0.80 – 5.00
Monocytes	0.76	10^9/L	0.00 – 1.20
Eosinophils	0.18	10^9/L	0.00 – 0.45
Basophils	0.0	10^9/L	0.0 – 0.1
Lactate dehydrogenase	169	U/L	135 – 225
Erythropoietin	384.7	mU/mL	3.2 – 31.9
Second draw - January 2024	**Result**	**Unit**	**Reference interval**
Erythropoietin untrated sample	279.8	mU/mL	3.2 – 31.9
sample treated with Scantibodies Heterophilic Blocking Tube	7.6	mU/mL	3.2 – 31.9

DISCUSSION

EPO is the hormone that regulates red blood cell production (2). Its molecule contains 165 aminoacids forming a heavily glycosylated protein with a molecular weight of approximately 30 kDa. EPO is mainly produced in the peritubular cells of the adult kidney, and, to a minor extent, also in the liver. EPO is released in response to a decrease in tissue oxygenation; the molecule binds to specific receptors on the erythroide progenitors cells in the bone marrow, promoting their differentiation into red blood cells (3). When blood oxygen levels normalize, the release of EPO decreases. In case of severe renal damage hindering the production of adequate amounts of EPO, the release of new red blood cells into circulation is decreased, resulting in the onset of anemia due to reduction of the red blood cell production (4). Anemia may occur also when EPO is present but the bone marrow does not respond to its action. These conditions may be observed in the course of bone marrow disorders or chronic diseases, such as rheumatoid arthritis. EPO is measured in case of anemia, in people with no manifest signs of iron or vitamin deficiencies; in case of very high red blood cell count (polycytheamia), or to investigate chronic kidney diseases or myeloproliferative disorders. Finally, the measurement of serum EPO may be useful to detect the recombinant molecule misuse in sport.

EPO can be quantified in human serum by different methods (5); one of them is ELISA. An antibody to the target protein is immobilized on the surface of microplate wells; the antibody is incubated first with the target protein, which is in the human blood, and then with another antibody, which is labeled with an enzyme that reacts on a substrate that is detected colorimetrically. Chemiluminescence immunoessays, which determine the antigen-antibody cross reactivity by measuring the intensity of the light emitted by a chemical and biological reaction, are an alternative to ELISAs.

EPO serum values, as measured in a reference human population, depend on the laboratory assay used. The expected EPO levels with the EPO ELISA kit method range from 3.22 to 31.9 mIU/mL (2.5° – 97.5° percentile).

Heterophile antibodies are endogenous antibodies against poorly defined antigens; they may be produced naturally, without a known cause, or may be observed after vaccination, infection, contact with animals, blood transfusion, autoimmune diseases, maternal transfer and dialysis (6). Heterophile antibodies can reagent immunoglobulins and other components used in immunoassays, therefore affecting test results indiscriminately and leading to false elevation or (rarely) false decreasing of measured values (7), as shown in figure 1B. Heterophile antibodies can also interfere with drug dosing, influencing clinical decisions regarding patient therapy. Such interference was observed in the case of a patient who showed high concentrations of tacrolimus, due to heterophile interference, even 14 days after the last drug administration. The interference was removed by treating the sample properly (8). The frequency of this kind of analytical interference is not clear in the literature, where discordant data can be found (9). When the interference of heterophile antibodies is suspected, firstly the sample should be re-analyzed with the same assay to exclude analytical errors due to pipetting inaccuracies, inefficient wash, tracer aggregates or other contaminants (10); then the sample should be tested using a different immunoassay, or it could be diluted and re-analyzed (11). Another method to remove heterophile antibodies is based on precipitation: the polymer polyethylene glycol (PEG) is very commonly used to separate the analytes bound to the antibodies from the free analytes, though the value obtained could be underestimated due to aspecific precipitation (12).

This report describes a case of heterophile antibody interference causing, in an ELISA method, false EPO increased values. The interference was highlighted

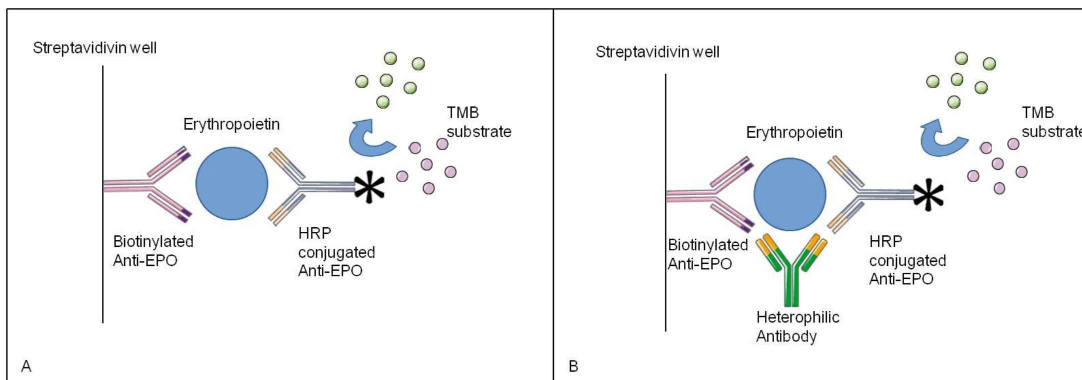

Figure 1
Normal sandwich immunoassay: any antigen found in the sample will bind to the capture antibody already coating the plate. Detection antibody labelled with an enzyme, usually horse radish peroxidase (HRP), is added and binds to any target antigen already bound to the plate. Finally, a substrate is added to the plate. ELISA assays are usually chromogenic using a reaction that converts the substrate (e.g. 3,3',5,5'-Tetramethylbenzidine,TMB) into a colored product which can be measured using a plate reader.
A sandwich assay where there is heterophilic antibody interference. The cross-binding of capture and reporter antibodies used in ELISAs results in a falsely exaggerated signal.

and confirmed by sample dilution and by removing the antibodies effect, which allowed to assess the “true” EPO level. The case suggests that, to increase patient safety and outcome, laboratory staff ought to be confident with the analytical methods in use and thoroughly investigate results that fall outside the assay specifications and performance. On the other hand, clinicians should interpret laboratory values with caution and should contact the laboratory professionals when the clinical picture contradicts laboratory results.

CONFLICT OF INTEREST

None

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