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Technical note

# Assay interference caused by antibodies reacting with rat kappa light-chain in human sera

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ABSTRACT

The enzyme-linked immunosorbent assay (ELISA) and its derivatives are powerful tools used in research, in the clinic, and in many other analytical and quality control settings. In general, ELISAs are robust, reproducible and reliable. However, a number of pitfalls of ELISAs have been described over the years. The issue of rheumatoid factor (RF), autoantibodies against the Fc portion of IgG, is well recognized (yet often forgotten), as are problems arising from heterophilic antibodies induced by external antigens that cross-react with self-antigens. A few years ago focus was on human anti-mouse antibodies (HAMA) concomitant with the increased use of mouse monoclonal antibody therapy, a problem that is now diminishing due to development of humanized antibodies. Issues pertaining to food antigens or environmentally encountered antigens are less recognized. We report a recently encountered example of the latter resulting in interference in a solid-phase sandwich assay. Due to the set-up employing a monoclonal rat IgG for capture and a monoclonal rat IgM for development the interference had to be human antibodies reacting with rat light-chain. Out of 102 Danish Caucasian blood donors we found a prevalence of anti-rat kappa light chain antibodies of close to 40% (39/102, defined as at least 2-fold elevated measurements), with around 6% (6/102) having very high levels (defined as at least 4-fold elevated measurements), yielding significantly higher measurements in the assay designed to measure the complement component MAp19 in serum samples. The interference could be blocked by the addition of rat immunoglobulin to the sample buffer. An individual, who had been followed over time, demonstrated a periodic increase of interfering antibodies, highlighting that it is an independently varying parameter and thereby a variable interference in assays. Our results highlight a major pitfall of potential relevance to many sandwich-type assays, as well as an approach to rectify such problems.



## 1. Introduction

Ever since the first description of the enzyme-linked immunosorbent assay (ELISA) (Engvall and Perlmann, 1971; Van Weemen and Schuurs, 1971), there has been a steady increase in the number of variations and applications of this technique. For the first decade of its existence (1971–1980) 965 hits are recorded in PubMed on the search term "ELISA", for the second decade (1981–1990) 22,332, the third (1991–2000) 52,210 and fourth 84,969 (2001–2010). The usefulness and importance of this technique and its derivatives are self-evident.

One important variation of the ELISA is the sandwich ELISA, which is used for many important clinical assays. The method relies on coating microtiter wells with a capture antibody, application of a sample containing the analyte allowing this to be captured specifically in the well, followed by detection with a second antibody using various detection systems. This is a powerful method employing the affinity and specificity of antibodies in two steps.

*Abbreviations:* ELISA, enzyme-linked immunoassay; TRIFMA, time-resolved immunofluorometric assay; RF, rheumatoid factor; MAp19, mannan-binding lectin-associated protein of 19 kDa.

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A commonly encountered problem in solid-phase sandwich assays when analyzing clinical samples is the presence of rheumatoid factor (RF), i.e., autoantibody directed towards the Fc portion of IgG (Rose et al., 1948; Waaler, 2007). Whereas significant levels of RF are found in 80% of rheumatoid arthritis patients and 70% of Sjögren's syndrome patients, low levels of RF is present in virtually all individuals. Indeed, the level of RF increases in general threefold when we are infected or vaccinated (Welch et al., 1983). As the Fc portion of IgG is highly conserved through the mammalian genus, presence of RF in samples can lead to a false-positive signal in most sandwich assays, independently of the source of the antibodies employed, by directly cross-linking the coated capture antibody and developing antibody, a problem which is exacerbated in more recent multiplexed assays (Naot et al., 1981; Todd et al., 2011). A common remedy is the addition of aggregated human immunoglobulin to patient samples in order to quench RF.

A related problem is the prevalence of antibodies to food antigens (Husby et al., 1985). Thus in one study the prevalence of anti-bovine IgG (presumably mainly encountered in dietary cow's milk and meat) was 95% (Andersen et al., 2004). Most hybridomas used for production of monoclonal antibodies to be used in solid-phase assays are grown in medium containing fetal calf serum (FCS). Although FCS is supposed to be low in immunoglobulin it still contains IgG and IgM to a varying degree depending on the manufacturer. Whereas the IgG and IgM levels of adult serum have been estimated at 18.9 mg/ml and 2.6 mg/ml, respectively, those of fetal calf serum (5 months to birth) were estimated at 40–160 μg/ml and 10–120 μg/ml, respectively (Butler, 1971; Sawyer et al., 1973; Williams et al., 1975). Despite this marked difference between the adult and the fetal levels, the fetal Ig level is significant when one considers that it co-purifies on Protein A and Protein G columns. Some researchers have even estimated that at the 10% FCS commonly employed in hybridoma cultures, only 5% (about 50 μg out of 1 mg total Ig/ml) of immunoglobulin in culture supernatants is the monoclonal antibody of interest, with the remaining 95% being of bovine origin (Harlow and Lane, 1988). This may cause false-positive reactions due to cross-linking of contaminating bovine IgG present in both the coating and the developing antibody preparations used in sandwich immunoassays. The problem can be circumvented by the specific depletion of bovine antibodies from hybridoma culture supernatants or the purified antibodies, or by the addition of bovine immunoglobulin to the sample dilution buffer to quench anti-bovine Ig present in serum samples. While these issues are known, we still encounter commercial kits where such artifacts produce false results.

A third related issue is that of heterophilic antibodies, induced by external antigens, and which cross-react with self-antigens. A subset of this was able to cross-link immunoglobulins from two different species (Bjerner et al., 2005a). Some of these are generated in response to food antigens as mentioned above, whereas another subset is generated in response to monoclonal antibody therapy (Koshida et al., 2010). This problem has been particularly pronounced in connection with mouse monoclonal antibody therapy, but is diminishing due to transition to humanized antibodies or human monoclonals. Heterophilic antibodies arising in response to animal husbandry have also been reported, e.g., in a case of an assay relying on rabbit monoclonals (Park et al., 2003). Some researchers distinguish between heterophilic antibodies, broadly cross-reactive and of low affinity, and human anti-animal antibodies (HAAA) induced by the immunoglobulin in question, and which are species specific and of high affinity (Kaplan and Levinson, 1999).

We recently encountered another variant of interference in a solid-phase assay employing a rat monoclonal IgG for coating and a rat monoclonal IgM for development. The assay was constructed to measure the MBL-associated protein of 19 kDa (MAp19). MAp19, a polypeptide of 19 kDa, consists of the first two domains of MASP-2 plus an additional 4 C-terminal residues. Through the two domains shared with MASP-2, MAp19 is able to associate with MBL and ficolins, pattern-recognition molecules of the lectin pathway of complement, albeit with a lower binding affinity than that of MASP-2. By virtue of this competitive binding, MAp19 has been suggested to control complement activations, since MASP-2 is the serine protease responsible for lectin pathway activation. The 4 C-terminal residues of MAp19 is the only feature distinguishing it from MASP-2, and an antibody directed towards a peptide comprising this motif is employed in the assay. During the initial tests of the assay, some individuals were unusually high in MAp19, raising suspicion of assay interference. Running the assay on a mock coat of non-specific rat IgG revealed that these individuals harbored significant levels of anti-rat Ig antibodies able to cross-link the antibodies used for capture and development, thus giving rise to high false-positive signals. The interference was ablated by the inclusion of an excess of rat IgG in the sample dilution buffer leading to the successful construction of a reliable assay (Degn et al., in press). Here we characterize the interference and discuss the potential impact of such interferences in similar assays.

## 2. Materials and methods

### 2.1. Two versions of an assay for MAp19

#### 2.1.1. First version

Initially, a sandwich assay was developed, involving capture with mAb 6G12 (binding site in the N-terminal domains shared by MASP-2 and MAp19) and detection of bound MAp19 with biotinylated anti-MAp19 antibody, 4D12, directed against the C-terminal of MAp19, followed by $Eu^{3+}$-labeled streptavidin. Wells were coated with 4 μg 6G12 per ml PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM $KH_2PO_4$, 8.1 mM $Na_2HPO_4$, pH 7.4), o.n. at 4 °C. Residual binding sites in the wells were then blocked with HSA, 1 mg/ml TBS (10 mM Tris–HCl, 140 mM NaCl, 15 mM $NaN_3$, pH 7.4), followed by wash 3× with TBS/Tw (TBS containing 0.05% v/v Tween-20), and incubation with serum samples diluted 20-fold in MAp19 buffer (TBS/Tw, 1 M total NaCl, 10 mM EDTA, containing 100 μg ΔnhIg (heat-aggregated human immunoglobulin (ΔnhIg) (nhIg, Beriglobin, ZLB Behring GmbH; incubated 30 min at 63 °C and centrifuged 10 min at 3000 g to remove insoluble complexes)) per ml) o.n. at RT. The ΔnhIg was added as a safeguard against RF. After washing the wells 3× again, biotinylated 4D12 was added at