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Discordant diagnostic results due to a hepatitis B virus T123A HBsAg mutant

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ABSTRACT

HBsAg immunoassay results are occasionally discordant among primary and confirmatory assays or with respect to other markers of HBV infection. Such discordance has been observed repeatedly in Canada with samples having a mutation at HBsAg codon 123 (sT123A). Detection of recombinant expressed HBsAg protein having either sT123 or sA123 was evaluated with one manual and six automated HBsAg immunoassays. The recombinant mutant HBsAg was non-reactive by Abbott AxSYM, while the Abbott ARCHITECT Quantitative and Qualitative II, ADVIA Centaur, and VITROS ECi detection signal was reduced compared with the wild-type protein, approaching the assay cut-off for certain assays, dependent upon the level of protein. The Roche Elecsys and manual immunoassays detected both wild-type and mutant proteins comparatively. The sT123A mutation leads to loss of detection by immunoassays commonly used in Canadian diagnostic laboratories, which may produce misleading results and diagnoses.

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1. Introduction

Loss of HBsAg detection due to mutations within the immunodominant antigenic ("*a*") determinant is an important consideration when resolving discrepancies between screening and confirmatory immunoassays or among the HBV laboratory marker profile. HBsAg antigenicity depends on the epitope conformation formed by disulfide bridges among cysteine residues that result in several putative 'loops' within the "*a*" determinant (Alavian et al., 2013). The competency of commercially available immunoassays to detect various mutations located within and outside the "*a*" determinant has been well investigated (Lou et al., 2011; Louisirirotchanakul et al., 2010; Ly et al., 2006; Mühlbacher et al., 2008; Servant-Delmas et al., 2012), and it is established that assay performance may be affected by these mutants such that HBsAg detection is abolished or significantly decreased (Alavian et al., 2013; Servant-Delmas et al., 2012). Such mutations may therefore lead to 'false' occult hepatitis B infection (Samal et al., 2012).

Detailed conformational analysis of the "*a*" determinant predicts that the residues between amino acids 121 and 124 form a mini-loop due to clustering of amino acids in the vicinity of cysteine residues as well as possible intermolecular disulfide bonding (Salisse and Sureau, 2009; van Hemert et al., 2008). Residues within this mini-loop are critical for proper detection by many HBsAg immunoassays (Ly et al., 2006; Mühlbacher et al., 2008; Servant-Delmas et al., 2012) due to the essential role of this epitope in HBsAg antigenicity (Tian et al., 2007b). In particular, a substitution at amino acid 123 from threonine to asparagine (sT123N) was found to reduce virion formation and secretion, in part due to the N-glycosylation generated by the asparagine substitution (Li et al., 2015; Wu et al., 2010). The sT123N mutation often results in decreased (Tian et al., 2007b) or abolished (Servant-Delmas et al., 2012) detection of HBsAg, depending on the immunoassay platform; however, there are discrepancies among studies [i.e., ADVIA Centaur results within Ly et al., (Ly et al., 2006) and Mühlbacher et al. (Mühlbacher et al., 2008)] which may be due to differences in recombinant protein expression levels (Li et al., 2015; Martin et al., 2012) or in the context of multiple mutations (Servant-Delmas et al., 2012).

The Canadian National Microbiology Laboratory investigated two cases of diagnostic marker profile discrepancy, such that the specimens had consistently low levels of HBsAg but moderate to high levels of HBV DNA. Upon sequence analysis, the samples, which were genotype B, had a threonine to alanine substitution at amino acid 123 (sT123A), either alone or within a background of other non-genotypic surface substitutions. Whereas the sT123N mutation has been well described (Servant-Delmas et al., 2012; Tian et al., 2007b; Wu et al., 2010), the sT123A mutation in isolation has not been well characterized with

Abbreviations: sT123A/N, threonine to alanine/asparagine substitution at amino acid 123 of the HBV surface antigen (HBsAg); "*a*", immunodominant antigenic determinant; EIA, enzyme immunoassay; TBS, tris-buffered saline; TBS-T, tris-buffered saline - tween; S/N, signal/noise; S/Co, signal/cut-off; NGS, next generation sequencing.

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immunoassay platforms other than the Abbott Architect (Lou et al., 2011; Verheyen et al., 2012); thus, the aim of this study was to evaluate the detection of the sT123A mutant by various serological assays to investigate its role in diagnostic escape.

2. Materials and methods

2.1. Samples

Serum samples were referred for HBsAg mutational analysis based on a description of discrepant diagnostic markers ("HBsAg result close to cut-off; signal/noise (S/N) = 7.83" with either "HBV DNA positivity" or "moderate to high HBV DNA levels") from a 30-year-old female and 54-year-old male. HBsAg testing for both samples was initially performed by the AxSYM assay (Abbott Diagnostics, Mississauga, ON, Canada).

2.2. HBsAg assays

The serological assays evaluated included 6 automated platform assays and one manual enzyme immunoassay (EIA). The AxSYM HBsAg and ARCHITECT HBsAg Quantitative and Qualitative II assays (list numbers 6C36 and 2G22, respectively; Abbott Diagnostics), ADVIA Centaur HBsAg assay (Siemens Healthcare Diagnostics, Deerfield, IL), VITROS HBsAg assay (Ortho Clinical Diagnostics, Markham, ON), Elecsys HBsAg II Quantitative assay (Roche Diagnostics, Laval, QC) and the manual third generation HBsAg One EIA (International Immuno-Diagnostics, Foster City, CA) were performed according to the manufacturer's instructions.

The HBsAg assay formats for the AxSYM and ARCHITECT involve a single monoclonal/polyclonal, dual monoclonal/single polyclonal (Quantitative) and dual monoclonal/ combination monoclonal/polyclonal (Qualitative II) capture/tracer arrangement, respectively. The format for the ADVIA Centaur HBsAg assay, VITROS HBsAg assay, and International Immuno-Diagnostics HBsAg EIA assay involves a single monoclonal/monoclonal capture/detection arrangement, while the Elecsys HBsAg quant assay format involves a dual monoclonal/ combination polyclonal capture/detection arrangement.

The limit of detection or cut-off value, indicating analytical sensitivity, of each assay as stated by the assay manufacturer based on the WHO 1st international HBsAg standard (code no. 80/549; AxSYM, VITROS, ADVIA Centaur) or 2nd international HBsAg standard (NIBSC code 00/588; both ARCHITECT assays, Elecsys, International Immuno-Diagnostics), is as follows: AxSYM, 0.03 IU/mL; ARCHITECT Quantitative, 0.05 IU/mL; ARCHITECT Qualitative II, 0.021 IU/mL; ADVIA Centaur, 0.0661 IU/mL; VITROS, 0.069 IU/mL; Elecsys, 0.05 IU/mL; and International Immuno-Diagnostics, 0.1–0.05 IU/mL.

2.3. PCR amplification and cloning

HBV DNA was extracted from 200 μl serum of one T123A clinical specimen by silica gel filtration (QIAamp DNA Blood Mini Kit, Qiagen, Inc., Mississauga, ON, Canada). Amplification of extracted DNA was performed using AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA) using HBV primers specific for an approximately 941 bp HBsAg-coding region product from nucleotides 55 to 995 of the HBV genome (Osiowy et al., 2011).

The sT123A containing amplicon was cloned using a TA-based ligation method (pDrive cloning vector; Qiagen, Inc.) and a single clone was used for further manipulation.

2.4. Site-directed mutagenesis

Site directed mutagenesis of nucleotide 367 of the mutant HBsAg coding region from guanine (*G*CC; Alanine) to adenine (*A*CC; Threonine) was performed by assembly PCR (Stemmer et al., 1995) to obtain a wild-type HBsAg sequence at amino acid 123, according to the methods described in Supplementary File 1. Selection of oligonucleotide primers (Integrated DNA Technologies, Toronto, ON) for assembly PCR (Supplementary Table 1) was based on the mutant clone sequence and facilitated by using the Gene2Oligo web-based tool [http://berry.engin.umich.edu/gene2oligo/; (Rouillard et al., 2004)].

2.5. Expression of HBsAg

Mutant (A123) and wild-type (T123) HBsAg sequences were subcloned into the mammalian expression vector pcDNA3.1+ (ThermoFisher Scientific, Burlington, ON). Two clones were selected based on their identical sequence to the original clones for transfection and expression of HBsAg in human hepatoma HepG2 cells, according to methods described in Supplementary File 1.

2.6. HBsAg quantification

Expressed HBsAg in culture supernatant was quantified by comparison to the WHO 2nd international standard for HBsAg (NIBSC code number 00/588). Duplicate dilutions of both the HBsAg standard (ranging from 0.026 IU/mL to 33 IU/mL) and the concentrated culture supernatant (undiluted, 1:10, and 1:100) were diluted and assayed in culture medium. Initial quantification was performed using the International Immuno-Diagnostics HBsAg EIA, ARCHITECT (Quantitative) and VITROS assays.

2.7. Western blot analysis and relative quantification of expressed HBsAg

Ten microliters of equally concentrated culture supernatant from each transfection was electrophoresed on a 10% NuPAGE® Bis-Tris polyacrylamide gel (ThermoFisher Scientific). Electrophoresed protein was transferred to PVDF membrane by the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Mississauga, ON) and the membrane was blocked for 1 hour with 50% Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) and 50% tris-buffered saline (TBS; 20 mM Tris–HCl, pH 7.5, 500 mM NaCl). Multiplex detection of HBsAg and vinculin, as a loading control, was performed following incubation with a goat anti-HBsAg polyclonal antibody (#70-HG15; Fitzgerald Industries International, Acton, MA) diluted 1/1000 and a mouse anti-vinculin monoclonal antibody (#ab18058; Abcam Inc, Toronto, ON) diluted 1/2000 in 50% Odyssey Blocking Buffer and 50% TBS-tween (TBS-T; 0.1% Tween-20) for 24 hours at 4°C. Primary antibodies were detected following incubation with secondary antibodies conjugated with infrared dye; an anti-goat IRDye 800 antibody (#926-32214, LI-COR Biosciences) for HBsAg detection and an anti-mouse IRDye 680 antibody (#926-68020, LI-COR Biosciences) for vinculin detection, diluted 1/15 000 and 1/20 000, respectively, in 50% Odyssey Blocking Buffer and 50% TBS-T plus 0.01% sodium dodecyl sulfate for 1 hour at room temperature. The membrane was washed 4 times for 5 minutes each with TBS-T between each step. A final 5 min wash in TBS was done to remove the Tween 20 before detection.

Infrared dye-conjugated antibodies were detected using the Odyssey Infrared Imaging System (LI-COR Biosciences) to capture both the 800 nm and 700 nm channels simultaneously at 169 μM resolution for detection of HBsAg and vinculin, respectively. Quantification was performed with the Li-Cor Odyssey system application software and Image Studio Lite V.5.0 analysis software (LI-COR Biosciences).

2.8. Statistical analysis

Comparison among wild-type and mutant IU/mL, index or signal/cut-off (S/Co) values was performed by Student's T test. A *P*-value less than 0.05 was considered significant.

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