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Validation of HAV biomarker 2A for differential diagnostic of hepatitis A infected and vaccinated individuals using multiplex serology

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ABSTRACT

Background: Worldwide about 1.5 million clinical cases of hepatitis A virus (HAV) infections occur every year and increasingly countries are introducing HAV vaccination into the childhood immunization schedule with a single dose instead of the originally licenced two dose regimen. Diagnosis of acute HAV infection is determined serologically by anti-HAV-IgM detection using ELISA. Additionally anti-HAV-IgG can become positive during the early phase of symptoms, but remains detectable after infection and also after vaccination against HAV. Currently no serological marker allows the differentiation of HAV vaccinated individuals and those with a past infection with HAV. Such differentiation would greatly improve evaluation of vaccination campaigns and risk assessment of HAV outbreaks. Here we tested the HAV non-structural protein 2A, important for the capsid assembly, as a biomarker for the differentiation of the immune status in previously infected and vaccinated individuals.

Methods: HAV antigens were recombinantly expressed as glutathione-S-transferase (GST) fusion proteins. Using glutathione tagged, magnetic fluorescent beads (Luminex®), the proteins were affinity purified and used in a multiplex serological assay. The multiplex HAV assay was validated using 381 reference sera in which the immune status HAV negative, vaccinated or infected was established using the Abbott ARCHITECT® HAVAb-IgM or IgG, the commercial HAV ELISA from Abnova and documentation in vaccination cards.

Results: HAV multiplex serology showed a sensitivity of 99% and specificity of 95% to detect anti-HAV IgG/IgM positive individuals. HAV biomarker 2A allowed the differentiation between previously infected and vaccinated individuals. HAV vaccinated individuals and previously infected individuals could be identified with 92% accuracy.

Conclusion: HAV biomarker 2A can be used to differentiate between previously HAV-vaccinated and naturally infected individuals. Within a multiplex serological approach this assay can provide valuable novel information in the context of outbreak investigations, longitudinal population based studies and evaluations of immunization campaigns.

1. Introduction

Infection with hepatitis A virus (HAV) is a major public health problem all over the world, in the worst case resulting in an acute inflammation of the liver [1].

HAV belongs to the family of *Picornaviridae*, genus *Hepatovirus* and has a single stranded, positive orientated 7.5 kb RNA genome with one open reading frame (ORF) [2–6].

So far seven different genotypes have been identified from the known human isolates, however, only one serotype has been described until 2011 [7–9]. This allowed the development of a universally applied vaccine based on an inactivated, attenuated hepatitis A virus, which was licensed in 1995 [10–13]. An assortment of inactivated monovalent hepatitis A vaccines from various companies are available. For example Havrix, Vaqta and Avaxim are inactivated viral vaccines; Epaxal and HAVpur are virosomal

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vaccines [14,15]. Recommendations of hepatitis A vaccination in countries with low endemicity comprise high-risk groups, persons at occupational risk and travellers [16]. Countries with high or transitional endemicity and high burden of disease have implemented routine childhood immunization schedules against HAV for children between the ages of 12 and 24 month (e.g. Argentina, Greece) [17].

Vaccination with an inactivated vaccine can induce a different antibody response than infection with wild-type virus. Stewart et al. described the production of antibodies against the non-structural protein 3C exclusively in serum of acute infected patients but not in immunized subjects [18]. But still, the distinction between natural infection with HAV and an induced immunization by vaccination in serum samples remains difficult. Approaches have been made combining a commercially available HAV ELISA and a self-developed ELISA using recombinant 3C proteinase as antigen [18,19]. However, the necessity to combine assays is time-consuming and requires multiple amounts of serum.

For epidemiological studies the differentiation between infected and vaccinated individuals is of major interest, i.e. for serosurveys to assess the epidemiological situation during outbreaks or after HAV vaccination campaigns. So far studies involving the assessment of the vaccination status have to rely on the availability, completeness and legibility of vaccination cards which however may often not be the case [20]. Another challenge in assessing vaccine effectiveness is that HAV infection is often asymptomatic or subclinical, which raises the demand for a biomarker identifying natural HAV infections not confounded with HAV vaccinations. For countries that recently introduced a HAV vaccination strategy, the differentiation of HAV vaccinated and infected people via serum analysis would allow reliable assessments of the vaccination coverage and vaccine effectiveness and would thus provide greatly desired evidence for evaluation and potential adjustment of vaccination strategies [21,22].

For these reasons we developed a HAV multiplex serological assay with the aim to serologically differentiate between individuals naturally infected with HAV and those who received vaccination against HAV.

2. Materials and methods

2.1. Generation of recombinant HAV proteins

The nucleotide sequence NC_001489.1 (NCBI Reference Sequence) for HAV strain HM175 was used. The genome belongs to the isolate HM175, genotype IB (isolate HM175, Human/Australia/HM175/1976). Full length coding sequences of structural HAV proteins VP1 (bp 2208–3107), VP2 (bp804-1469), VP3 (bp 1470–2207), VP4 (bp 741–803) and non-structural HAV proteins 2A (bp 3108–3674), 2B (bp 3675–3995), 2C (bp3996-5000), 3A (bp 5001–5222), 3B (bp 5223–5291), 3C (bp 5292–5948) and 3D (bp 5949–7415) were commercially synthesized (Eurofins Genomics, Ebersberg, Germany) after codon-optimisation for *Escherichia coli (E. coli)*. The HAV antigens were expressed as N-terminal GST fusion proteins using a modified pGEX4T3 vector as described by Sehr et al. [23]. All clones were verified by sequence analysis. Fusion proteins were expressed in *E. coli* BL21 in Terrific Broth medium at 20 °C over night and lysed in a high-pressure homogenizer (HTU-DIGI-Press, G. Heinemann). Successful full-length antigen expression was verified by Coomassie staining, Western Blot and GST-capture ELISA, to estimate concentrations of the specific antigens as previously described [23,24]. A concentration of 70 μg/ml total lysate protein or less was shown to be sufficient to reach antigen saturation and beads were loaded with lysates diluted to 1 mg/ml.

2.2. Multiplex serology

2.2.1. Covalent coupling of glutathione-casein and whole HAV to Luminex microspheres

Glutathione-Casein (GC) was produced as described previously [23] and coupled to spectrally distinct carboxylated, fluorescence labelled magnetic beads (MagPlex®; Luminex®) following the description by Waterboer et al. [25]. Deviating from the described protocol, a magnetic separator (Dyna Mag™-2, Life technologies) was used for the washing steps.

Whole formalin inactivated HAV (Aviva Systems Biology, Cat # OPMA04543) was coupled to magnetic beads using AMG™ Activation Kit for Multiplex Microspheres according to the manufacturer's protocol (Anteo Technologies, Cat # A-LMPAKMM). The activated magnetic beads (200 μl) were incubated with 20 μg/ml formalin inactivated HAV for 1 hour at room temperature.

2.2.2. Multiplex serology

All 11 HAV antigens were expressed as single GST-tagged proteins in *E. coli*, however only VP1, VP2, VP3, 2A, 2C and 3C could be produced in sufficient quantity and quality to be used for multiplex serology [25]. The complete bead set consisted of beads presenting these antigens, loaded and affinity-purified on GC coupled beads and beads directly coupled with whole inactivated HAV. Serum (2 μl) was used in a final dilution of 1:100.

2.3. Serum samples

2.3.1. Validation samples

For the validation of the assay 361 reference sera were collected from different German and international cooperation partners. We received 120 HAV negative samples from the National Reference Centre for HAV and HEV, Regensburg. This institute provided also HAV positive samples (10 samples), as did the Governmental Institute of Public Health of Lower Saxony (NLGA), Hanover (30 samples), the University Hospital Ulm (35 samples) and the Competence Network Hepatitis (HepNet) of the German Liver Foundation, Hanover (20 samples).

We received additional samples with infected and vaccinated status from the Ludwig-Maximilians-Universität München (LMU) (11 samples) as well as serum from vaccinated and infected children (age 0–14 years) from the National and Kapodistrian University of Athens, Greece (69 samples).

The serum status of all these samples had been determined by the provider with Abbott ARCHITECT® HAVAb-IgG/IgM and IgG, respectively in the case of the serum samples from Greece. The vaccination status had been confirmed using vaccination cards.

Additionally, we collected 35 samples from HAV vaccinated individuals in a survey at the Helmholtz Centre for Infection Research (HZI), (ethical approval, #2198–2014 MHH Hanover) and 31 samples during Pretest II of the German National Cohort (ethical approval Ethics Committee of the State Boards of Physicians of the German Federal State of Lower Saxony). The vaccination status was confirmed with the Hepatitis A virus Ab ELISA Kit (KA0284, Abnova) and vaccination cards.

2.3.2. Control samples

Three control serum samples were used in each run to monitor the quality of the run: Human standard IgG (Privigen 100 μg/ml infusion solution; CSL Behring), an anti-HAV IgG+ control from a previously, clinically diagnosed HAV infected individual and a pool

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