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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

of 16 different IgG+ serum samples from the reference sample pool of IgG+ infected individuals.

2.4. Assay validation and statistical analyses

The assay validation was based on a publication by Shankar et al., using three validation panels of reference sera [26].

Validation panel 1, consisting of 50 HAV IgG negative sera, was used for the first validation step, determining the antigen specific cutoff, defining the threshold above which a serum is considered positive for the particular antigen. The panel was measured twice each by two analysts. Normal distribution for all antigens was assessed after transformation into natural logarithm (ln) scale and exclusion of outliers (±1.5 interquartile range (IQR)). The assay run means and variances for each antigen were compared using ANOVA. The antigen cutoff can either be a fixed cutoff, resulting in the same value in all future HAV multiplex assays, or a floating cutoff, calculated anew for each run using normalisation sera. According to Shankar et al. a fixed cutoff (mean + 1.645 standard deviation) is determined when mean and variance are the same for all runs; else the floating cutoff is assigned. In the first validation step the normalisation factor (fixed cutoff – mean of HAV negative normalisation sera) was determined, using ten of the 50 negative sera from panel 1 as normalisation sera. Each subsequent run contained these normalisation sera and the floating cutoff was calculated as mean of the normalisation sera + normalisation factor, thus adjusting the cutoff for a lower or higher run specific mean fluorescence intensity (MFI). An example is shown in the supplementary Table 1 for panel 2.

Validation panel 2 was used to define which antigens are necessary to identify and distinguish HAV negative, infected and vaccinated individuals. This assay cutoff defines for which or how many HAV antigens a serum must be positive to be considered HAV infected or vaccinated. Validation panel 3 was used to assess assay accuracy. A detailed description of the panels can be found in Table 1.

For standardization, the individual antigen reactions in the three control serum samples were categorized: low MFI (20–199), medium MFI (200–1999), high MFI (2000–8000) and very high MFI (>8000), allowing the detection of errors in assay performance in case of systematic deviations from the expected category.

All statistical tests were performed two-sided, and P values below 0.05 were considered significant. Box plots depict ln-transformed MFI values. The line inside the boxes represents the median, the diamond the mean. The boxes are delimited by the first and third quartile and whiskers extend to the 1.5 × IQR, respectively. Outliers are presented as circles.

For statistical analysis and graphical presentations of the results SAS 9.2 was used.

3. Results

Three panels with reference sera were used for the validation of HAV multiplex serology, listed in Table 1. Validation panels 1 and 2, used to determine the specific antigen cutoff and assay cutoff, contained adult sera; panel 3, used to determine assay accuracy, additionally contained sera from vaccinated and infected children. Furthermore, validation panel 2 had a higher share of HAV RNA or IgM positive samples while panel 3 contained more prevalent IgG positive samples.

Due to differences in the mean for each of the four measurements of validation panel 1, a floating cutoff had to be determined as antigen cutoff for future assay applications. Supplementary Table 1 shows the normalisation factor calculated across all four runs of panel 1 and its application to calculate the floating cutoff for the run of validation panel 2.

Fig. 1 shows the reaction of the three HAV serum sample groups (negative (0), infected (1) and vaccinated (2)) towards the different antigens used in the assay. While HAV negative serum samples (0) were below the antigen cutoff for all the antigens (HAV – 3C) HAV vaccinated serum samples (2) reacted only against the whole virion (HAV) but not against the recombinantly expressed single antigens VP1 – 3C. HAV infected serum samples (1) showed reactivity against HAV and the recombinant antigens. Thus, the sensitivity and specificity per antigen was calculated separately for HAV infected and vaccinated samples (Table 2). Differentiating between HAV negative and infected individuals, antigen 2A exhibited the highest sensitivity (88%[$CI_{95\%}$: 77–99%]) and specificity (98% [$CI_{95\%}$: 95–102%]) among the recombinant antigens, while the HAV virion could be used to differentiate between HAV negative and vaccinated individuals with a sensitivity of 100% and specificity of 97%[$CI_{95\%}$: 91–103%]) Thus, a combination of the whole HAV virion and 2A could be used as assay cutoff to determine the HAV immune status. Positivity against whole HAV virion and 2A indicated a previously HAV infected state while antibody reactivity against only whole HAV virion determined an HAV vaccinated immune state (Table 3). Based on these findings for panel 2 the sensitivity of the HAV multiplex serology was 99%[$CI_{95\%}$: 96–101%] and the specificity 100%. The accuracy for detecting vaccinated and infected individuals was 97%[$CI_{95\%}$: 91–103%] and 88% [$CI_{95\%}$: 77–99%], respectively. For confirmation of the assay accuracy, validation panel 3 was analysed (Table 4). The sensitivity and specificity to distinguish HAV positive (anti-HAV IgG/IgA/IgM +) from HAV negative sera was 99%[$CI_{95\%}$: 98–101%] and 95% [$CI_{95\%}$: 85–105%], respectively. Serum samples from vaccinated individuals could be identified with 91%[$CI_{95\%}$: 85–97%] accuracy and HAV infected samples with 78%[$CI_{95\%}$: 69–87%] accuracy. The remaining 22% were assigned a vaccinated immune status.

Fig. 2 shows the immune response towards the two biomarkers needed to define the immune status classified into the six groups:

Table 1
371 HAV serum samples were used in the validation process according to infection or vaccination status and their distribution among the three validation panels (number of samples (n)). The serum samples were assigned to six groups: negative adults (A), infected (B) and vaccinated (C) children, vaccinated (D) adults and infected (E) and acute (F) infected adults.

HAV status	Group	n total	n panel 1	n panel 2	n panel 3
Purpose of panel			Antigen cutoff	Assay cutoff	Assay accuracy
HAV IgG negative	Adults (A)	126	50	56	20
HAV IgG positive/infected	Children (B)	22	–	–	22
HAV IgG positive/vaccinated	Children (C)	47	–	–	47
HAV IgG positive/vaccinated	Adults (D)	67	–	33	34
HAV IgG positive/infected	Adults (E)	44	–	–	44
HAV RNA or IgM positive	Adults (F)	55	10	34	11
Total		361	60	123	178

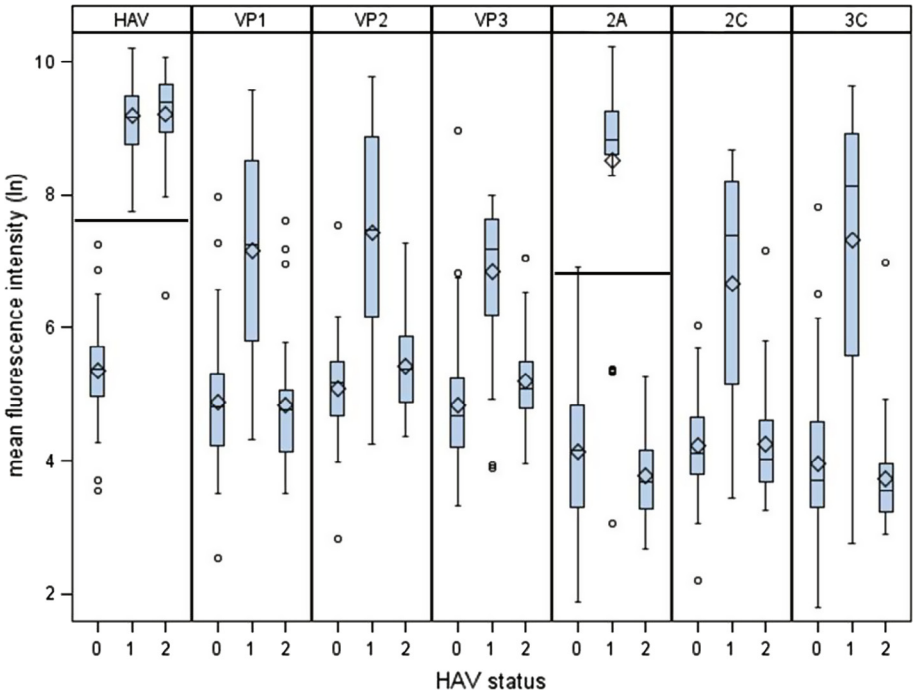

Fig. 1. Validation of multiplex serology for the different HAV antigens with negative (0; n = 56), infected (1; n = 34) and vaccinated (2; n = 33) serum samples in serum panel 2. Lines across indicate the floating cutoffs for HAV virion (HAV) (7.3) and 2A (6.6).

Table 2
Depicted are the sensitivity and specificity with which the different serum groups, HAV vaccinated (VAC) and infected (INF), from validation panel 2 reacted towards the used antigens. While the HAV vaccinated serum samples only responded against the whole viral particle coupled directly to the beads (HAV virion), antibodies in HAV infected samples bound also to the recombinant HAV antigens.

	HAV virion		Recombinant HAV antigens					
			VP1	VP2	VP3	2A	2C	3C
Sensitivity (%)	100	100	68	68	88	88	82	85
Specificity (%)	97	100	91	98	80	98	82	85
Serum status	VAC	INF	INF	INF	INF	INF	INF	INF

Table 3
Distribution of serum samples in validation panel 2 according to the reference immune status and the determined status in HAV multiplex serology (HAV multiplex). Definition of HAV virion positive samples as vaccinated and HAV virion and 2A positive samples as HAV infected would result in a sensitivity of 99% and a specificity of 100%. 88% of infected samples and 97% of vaccinated samples would be correctly identified.

		Reference immune status			
		Negative	Infected	Vaccinated	Total
HAV multiplex	HAV neg.	56	0	1	57
	HAV/2A pos.	0	30	0	30
	HAV pos./2A neg.	0	4	32	36
	Total	56	34	33	123

Table 4
Distribution of serum samples in validation panel 3 according to the reference immune status and the determined status in HAV multiplex serology (HAV multiplex). The sensitivity is 99%, specificity 95% and the accuracy to detect infected individuals is 78% and for vaccinated individuals 91%. Values in brackets show the share of IgM positive.

		Reference immune status			
		Negative	Infected	Vaccinated	Total
HAV multiplex	Negative	19	0	1	20
	Infected	0	60 (10)	6	66
	Vaccinated	1	17 (1)	74	92
	Total	20	77 (11)	81	178

negative adults (A), infected-IgG+ (B) and vaccinated (C) children, vaccinated (D), infected-IgG+ (E) and acute infected-IgM+ (F) adults. While the immune response could still be differentiated into anti-HAV negative and anti-HAV positive, according to the antigenicity towards whole HAV virion, infected children (B) showed a 20–30% reduced response towards the 2A antigen com-

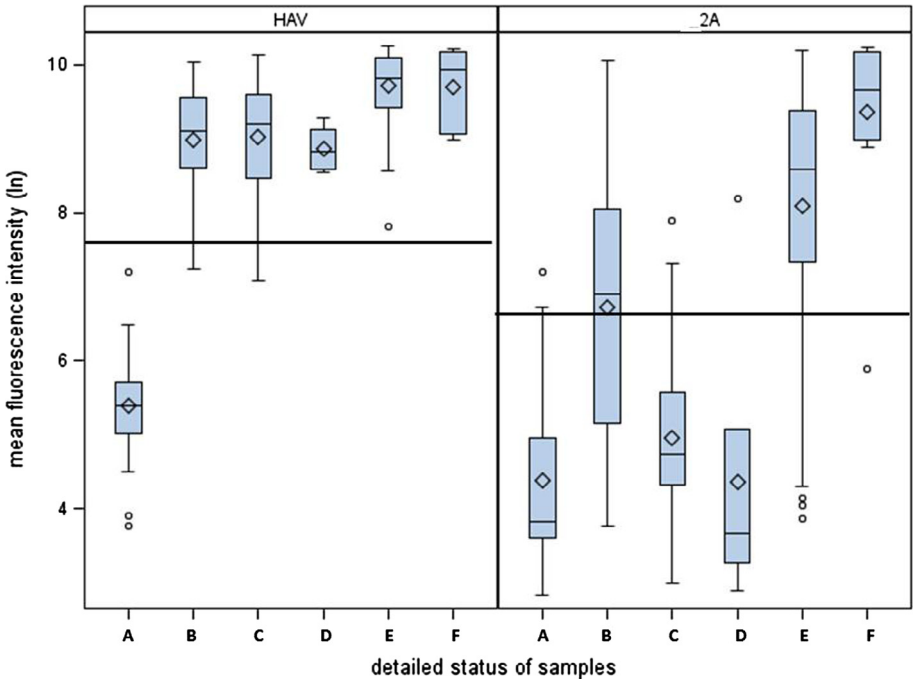

Fig. 2. Distribution of serum samples in panel 3 according to age groups and HAV immune status: negative adults (A; n = 20), infected children (B; n = 22), vaccinated children (C; n = 47), vaccinated adults (D; n = 34), infected adults (E; n = 44), acute infected adults (F; n = 11). The lines across indicate the floating cutoffs for the virion (HAV) (6.7) and 2A (6.7).

pared to adults (E, F). Furthermore, antigenicity against 2A was reduced about 10% in HAV prevalent-IgG+ sera (E) compared to IgM positive serum samples (F).

Of the serum samples, defined by the provider as HAV infected serostatus due to the source and IgG+ status, 12 samples were consistently (≥4 measurements) misclassified as HAV vaccinated. They were 2A negative and showed a low response towards the remaining HAV fusion antigens.

4. Discussion

To fulfil the need to differentiate between the immune responses of previously HAV infected individuals and HAV vaccinated ones, we developed and clinically validated a HAV multiplex serological assay which requires minimal amounts of serum and allows high-throughput application.

To date, only experimental ELISA assays have been developed to distinguish HAV infected from vaccinated individuals based on the exclusive expression of non-structural proteins during HAV infection. The assays were tested using experimentally infected and vaccinated chimpanzees [18,27]. None of the assays has progressed to an applicable state for human samples.

Unlike in the veterinarian context, where vaccines are designed with deletions in non-structural proteins to allow easy differentiation between infected and vaccinated animals (DIVA) [28–30], the human HAV vaccines are based on full virus particles [10–13]. Therefore assays detecting antibodies against a single antigen only, were insufficient for our approach. Using the multiplex platform from Luminex® allowed us to combine promising recombinant HAV antigens for the targeted differentiation of HAV vaccinated and infected individuals. The non-structural protein 2A, allowed us to differentiate between vaccinated and infected individuals [31]. However, serum samples from vaccinated individuals generally showed a poor response against recombinant antigens produced in *E. coli*, including the structural surface proteins. HAV antigens are produced by mammalian cells upon infection. Their expression in *E. coli* can result in incorrect folding or posttranslational modification. Furthermore, whole virus presents conformational epitopes consisting of amino acid stretches overlapping VP1 and VP3, thus generating neutralizing epitopes not present on the individual antigen [32,33]. Our recombinant antigens have most likely not been folded correctly, thereby probably presenting only linear epitopes. This may have reduced the number of presented epitopes, thus reducing the signal strength of reactive antibodies present in vaccinated serum samples. Antibodies produced through HAV vaccination are only targeting the three dimensional virion and not the potential linear epitopes, presented to the immune system only during the replication and assembly process. Serum from vaccinated individuals therefore does not contain antibodies reactive to our recombinant HAV antigens, but is exclusively reactive against the whole HAV virion.

Our assay achieved a sensitivity of 99% and specificity of 95%. More weight is placed on false positive sera in panel 3 due to a comparatively low number of negative sera. A combination of results from panel 2 and panel 3 would lead to a specificity of 99%. The assay accuracy for the distinction of HAV infected and vaccinated individuals with 78% and 91% respectively, leaves room for improvement. This may be attributed to the mixed composition of the HAV infected serum pool, consisting in panel 3 of mainly IgG + serum samples and only to a smaller percentage of IgM+ samples. The status 'HAV infected' was assigned by the provider due to source (age of sample donor >70, lack of vaccination information) and IgG+ status. From the samples concerned, 12 (71%) were continuously measured as samples from HAV vaccinated individuals in HAV multiplex serology. Nine of the relevant samples were collected from children in a study where hepatitis A vaccination history was retrieved from hospital records [34]. Most likely, the relevant children's immune status as 'vaccinated' was not documented in the vaccination cards and therefore misdiagnosed as infected in the respective study. Infection with HAV leads to a lifelong immunity and usually takes place during early childhood in

populations endemic for HAV [35]. The low reactivity of these nine samples against antigen 2A can therefore not be explained by a developing immune system in children in general, since adults, infected during childhood, show a strong response against 2A even years later.

The remaining three IgG+ samples were from adults from a sample set, of which the infection status was assumed due to the age (>70) of the subject. The HAV vaccination was implemented in 1995 in Germany thus the assumption was that older sample donors were infected during childhood in case of anti-HAV IgG positivity. It is conceivable however that a small proportion of these sample donors was not infected during childhood but received the vaccination at a later stage. In a sensitivity analysis hypothetically reassigning these 12 samples to the status of HAV vaccinated would result in an accuracy of 92% to identify vaccinated and (previously) HAV infected individuals from a serum pool.

To our knowledge, our assay is the first to allow distinction between HAV vaccinated and previously infected status thus allowing the investigation of the serological status in human serum samples independently from the documented vaccination status, to observe vaccination coverage rate in populations and to examine effectiveness and effectivity of vaccination campaigns.

This is of particular relevance in countries where, in contrast to the originally licensed vaccination scheme single dose regimens are being implemented such as in Argentina in 2005 [36].

Furthermore, the ability to serologically distinguish HAV infected and vaccinated serum samples can provide improved risk assessments for epidemics in populations where a relevant proportion of HAV vaccinated individuals is to be expected as was the case in Germany 2012 or in Italy 2013 [37,38]. Furthermore this approach may complement existing biomarkers for clinical vaccine trials and licensing procedures. In the field of occupational medicine it may serve to confirm infection despite prior vaccination.

In conclusion, our high-throughput HAV multiplex serology allows the differentiation of HAV infected and vaccinated individuals with high sensitivity and specificity and thereby introduces a novel diagnostic tool for vaccine trials, seroepidemiologic surveys and individual risk ascertainment in occupational medicine and outbreak investigations.

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Authors' contribution

Katrin Bohm designed and performed the experiments and wrote the paper; Angela Filomena and Nicole Schneiderhan-Marra provided advice in the coupling of the HAV virion to the beads and reviewed the paper; Gérard Krause developed the project idea, contributed to the concept and reviewed the paper; Claudia Sievers contributed to experimental planning, analysed the data and reviewed/edited the paper. All authors approved the final version of the submitted manuscript.

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Conflict of interest

KB, GK and CS declare that a patent application has been filed.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2017.08.089.

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