Journal of Virological Methods 191 (2013) 48–54

Contents lists available at SciVerse ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

A novel indirect immunofluorescence test for the detection of IgG and IgA antibodies for diagnosis of Hepatitis E Virus infections

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Article history:
Received 15 October 2012
Received in revised form 7 March 2013
Accepted 14 March 2013
Available online 1 April 2013

Keywords:
Hepatitis E Virus
IgG
IgA
Immunofluorescence test
Recombinant ORF2
Serologic assay

A B S T R A C T

Hepatitis E Virus (HEV) causes epidemic infections in regions of poor hygiene in the developing world. Over the last years, however, increasing numbers of autochthonous infections in industrialized countries have been described, leading to new interest in this pathogen. Currently available serological test formats to detect IgG and IgM antibodies are mainly based on bacterially expressed ORF2 and ORF3 antigens and often give ambiguous results.

The objective of this study was the development of a different assay format for HEV diagnosis—a HEV immunofluorescence test (HEV-IFT) based on mammalian cells transiently expressing recombinant HEV ORF2 protein with a simple production and staining protocol and the investigation of its performance and methodical feasibility under diagnostic laboratory conditions. 31 sera of patients at different phases of HEV infection and 40 control sera from a non-endemic region were analyzed for anti-HEV IgG, IgM, and IgA antibodies.

The HEV-IFT detected successfully anti-HEV IgG and IgA, but not anti-HEV IgM antibodies. In the study group the HEV-IFT was able to confirm HEV infections and to support diagnosis when ambiguous results were obtained by commercial assays. Signal localization and staining patterns helped to gather additional information about reactive antibodies present in patient sera.

In conclusion the developed IFT for the detection of anti-HEV IgG and IgA antibodies can be used for diagnosis and for the serological confirmation of HEV infections.

1. Introduction

Hepatitis E is an acute, self-limiting disease that is spread by the fecal-oral route. Its causative agent, Hepatitis E Virus (HEV), is subdivided into four different genotypes found in various geographical regions (Schlauder and Mushahwar, 2001): genotype 1 (Asia), genotype 2 (Africa and Mexico), genotype 3 (Europe and Northern America) and genotype 4 (Asia). An increasing number of sporadic HEV infections in industrialized countries is assumed to be acquired as zoonosis from domestic livestock (Lewis et al., 2010). HEV is a positive-sense, single-stranded RNA virus. Its genome is approximately 7200 bases long and contains three open reading frames (ORFs) (Tam et al., 1991): ORF1 encodes a functional polyprotein, ORF2 the viral capsid which is partially overlapping ORF3, a protein of yet unknown function (Huang et al., 2007). During HEV infection viral RNA is observed by reverse transcriptase PCR (RT-PCR) from serum or stool samples. After a short phase of RNAemia virus-specific antibodies can be detected by Western blot, line immunoassay (LIA), enzyme linked immunosorbent assay (ELISA) or enzyme immunoassay formats (Mushahwar, 2008). The latter test formats utilize predominantly bacterially expressed ORF2 and 3 proteins, especially from the immunogenic C-termini of the respective proteins, to measure anti-HEV IgG and IgM (Ma et al., 2009). The most frequent formats of IgA detection are in-house ELISAs (Elkady et al., 2007; Gotanda et al., 2007; Mitsui et al., 2005; Takahashi et al., 2005, 2010; Zhang

Abbreviations: ANA, anti-nuclear antibody; EBV, Epstein Barr Virus; CMV, cytomegalovirus; DPBS, Dulbecco's phosphate-buffered saline; ELISA, enzyme linked immunosorbent assay; FITC, fluorescein isothiocyanate; HEV, Hepatitis E Virus; IFT, immunofluorescence test; LIA, line immune assay; NTR, non-translated region; ORF, open reading frame; O2C, open reading frame 2 C-terminus; RF, rheumatoid factor; RGS, arginine, glycin, serin; RT-PCR, reverse transcriptase PCR.

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http://dx.doi.org/10.1016/j.jviromet.2013.03.022

et al., 2009), modified commercial ELISAs (Tian et al., 2006) and LIAs (Herremans et al., 2007). Additional diagnostic options are based on the detection of HEV-specific T-cells (Wu et al., 2008) or viral antigen (Zhang et al., 2006) in patient blood samples.

Previous studies showed the usefulness of expressing recombinant HEV capsid proteins in various mammalian cell expression systems as diagnostic tools (e.g. ELISA, western blot) (Arankalle et al., 2007; He et al., 1993; Jimenez et al., 2009; Tsarev et al., 1993). Although expression products were detected by indirect immunofluorescence microscopy using acute phase sera and anti-IgG conjugate (Torresi et al., 1997), to our knowledge an indirect immunofluorescence test (IFT) for diagnosis of HEV infections has not been established yet. An obvious advantage of an IFT format based on a mammalian cell expression system compared to bacterial expression systems is the correct posttranslational modification of the presented antigen and higher preservation of specific antigenic epitopes. Although the function of posttranslational modification of the capsid protein is not understood completely, it was shown that glycosylation of recombinant ORF2 protein occurs during expression in mammalian cells (Graff et al., 2008; Jameel et al., 1996; Jimenez et al., 2012; Surjit et al., 2007; Torresi et al., 1999; Zafrullah et al., 1999).

IgM is known to be a sensitive marker for routine diagnosis of recent HEV infections (Tian et al., 2006; Zhang et al., 2009). However, discussion of the specificity of IgM assays is often found in literature (Takahashi et al., 2005; Tian et al., 2006). Therefore, there is still a need for new diagnostic markers in detecting recent HEV infections and some authors suggested using anti-HEV IgA antibodies for this purpose (Herremans et al., 2007; Tian et al., 2006). The occurrence of IgA within HEV-RNA positive patients is estimated to be up to 100% (Herremans et al., 2007; Takahashi et al., 2005; Tian et al., 2006). Various studies indicate that anti-HEV IgA detection is equally specific in diagnosing an acute hepatitis E compared to the detection of anti-HEV IgM (Herremans et al., 2007; Takahashi et al., 2005; Tian et al., 2006; Zhang et al., 2009). Overall most authors agree, that IgA assays are at least as specific as IgM assays and a combination of IgA and IgM testing would exhibit the highest sensitivity and specificity up to nearly 100% (Elkady et al., 2007; Herremans et al., 2007; Takahashi et al., 2005; Tian et al., 2006; Zhang et al., 2009). Takahashi et al. (2005) even suggest using IgA as the first-choice marker and diagnostic indicator for recent HEV infection.

This article reports the development of a novel indirect IFT based on recombinant HEV capsid protein expressed transiently in HEK293T cells, and the investigation of its suitability to confirm commercial anti-HEV assays by detecting anti-HEV IgG and IgA with a simple protocol under diagnostic laboratory conditions.

2. Materials and methods

2.1. Clinical specimens

23 sera (serum 1–23) from patients with laboratory confirmed acute Hepatitis E infections (20 of them (1–20) being HEV-RNA positive), eight sera (24–31) from patients with anti-HEV IgG prevalence, seven sera (32–38) from patients with unclear or negative HEV infection status, and 33 control sera (39–71) of patients with other clinically relevant conditions, such as other viral hepatitides (39–44), Epstein Barr Virus (EBV)-infections (45–55), cytomegalovirus (CMV)-infections (56, 57), rheumatoid factor positive disease (58–64) and pregnancy (63–71). Information about recent travel is known for five patients (5, 11, 20, 26, 27). These patients are indexed with "f" in Table 1.

2.2. Examination of the sera by serological and molecular biological methods

In total 71 sera were tested by either HEV recomBLOT, or HEV recomLINE (both: Mikrogen, Neuried, Germany) for the presence of anti-HEV IgG and IgM antibodies according to the manufacturer's instructions. 29 sera were tested by recomBLOT and 42 sera by recomLINE. This resulted from a change of the product line-up by the manufacturer (Mikrogen). Serum samples of acute phase patients were examined by in house nested PCR amplifying a fragment located in the 5′ NTR (data not published) or realtime RT-PCR adapted from Jothikumar et al. (2006) for the presence of HEV-RNA. All other virological parameters were determined by standard methods.

2.3. Generation of HEV ORF2 expression vector

Full length HEV ORF2 was PCR-amplified from an in vitro synthesized HEV genotype 1 genome fragment (Geneart, Regensburg, Germany) and cloned recombinatorially into a customized, pCR3-based, N-terminally His-tagged mammalian expression vector (Vizoso Pinto et al., 2009). The nucleotide sequence of the genotype 1 ORF2 is analogue to the sequence of the HEV reference sequence [genebank L08816] also used in our previous study (Osterman et al., 2012). The integrity of the construct pCR3-N-His-ORF2 was verified by restriction analysis and sequencing (AGOWA, Berlin, Germany). The expression vector was designed as Gateway® compatible vector to allow an easy and fast subcloning of respective ORF2 encoding cDNA from different HEV genotypes. Thus, users of the IFT can decide on their own which antigen genotype should be expressed in their respective laboratory.

2.4. Verification of HEV ORF2 antigen expression by pCR3-N-His-ORF2

In order to verify the capability of pCR3-N-His-ORF2 to express seroreactive His-tagged HEV ORF2 antigen in HEK293T cells (ATCC: CRL-11268) immunofluorescence microscopy was performed as described previously (Baiker et al., 2004). For staining cells were incubated with a 1:500 dilution of serum of a HEV positive patient (Table 1, 1) and a 1:2000 dilution of monoclonal mouse anti-RGS-His antibody (Qiagen, Hilden, Germany) in DPBS (Dulbecco's phosphate-buffered saline, Invitrogen, Basel, Switzerland). The secondary antibody solution consisted of 1:400 diluted Alexa Fluor 488 conjugated goat anti-human IgG/M (H+L) and Alexa Fluor 567 conjugated goat anti-mouse IgG/M (H+L) (both Invitrogen, Basel, Switzerland) supplemented with 4′,6-diamidino-2-phenylindole (1:10,000) for counterstaining (Fig. 1, upper panel).

2.5. HEV-IFT slide production and staining protocol

For the production of laboratory qualified HEV-IFT slides at a reasonable number, single scale transfection of HEK293T cells within individual Lab-Tek chambers with pCR3-N-His-ORF2 was replaced by batch transfection of a larger number of cells within 10 cm tissue culture dishes. Forty-eight hours post-transfection HEK293T cells were trypsinized, washed three times and resuspended thoroughly in DPBS. Cell number was adjusted to 750,000 cells ml^{-1}. Resuspended cells were applied to standard 10-field glass slides (one 20 μl drop of approximately 15,000 cells per field), air dried and encircled with a pap pen for immunohistochemistry (DAKO, Glostrup, Denmark). Slides were treated for 10 min in fixation solution, for 5 min in permeabilization solution and for 5 min in washing solution (all reagents: CINAkit, Argene, Verniolle, France). For long-term storage slides were kept at −80 °C. Transfection efficiencies (i.e. the amount of HEK293T cells expressing

Table 1
Anti-HEV IgG, IgM and IgA seroreactivity data of defined sera from patients with HEV infections.

Characterization	Serum no.	HEV RNA (PCR)[c]	Anti-HEV (Mikrogen)[a]		Anti-HEV (IFT)[b]	
			IgG	IgM	IgG	IgA
Acute hepatitis E	1	+	+	+	1024	1024
	2	+	+	+	1024	512
	3	+	+	+	1024	128
	4	+	+	+	1024	128
	5[f]	+	+	+	1024	128
	6	+	+	+	1024	64
	7	+	+	+	1024	32
	8	+	+	+	1024	32
	9	+	+	+	1024	16
	10	+	+	+	512	32
	11[f]	+	+	+	512	8
	12	+	+	+	512	8
	13	+	+	+	256	64
	14	+	+	+	128	64
	15	+	–	+	32	16
	16	+	+	+	1024	16
	17	+	+	–	1024	64
	18	+	+	+	512	–
	19	+	+	+	512	–[d]
	20[f]	+	+	+	256	–
Anti-HEV IgG prevalence	21	–	+[e]	+	–	?
	22	–	+	+	?	–[d]
	23	–	+	+	bg	bg
	24	nd	+	?	256	–
	25	nd	+	?	8	–
	26[f]	nd	+	?	8	–
	27[f]	–	+	?	8	–
	28	nd	+	–	256	–
	29	nd	+	–	128	–
	30	nd	+[e]	–	–	–
	31	nd	+[e]	–	–[d]	–[d]
Unclear/negative anti-HEV serostatus	32	–	?	+	256	?[d]
	33	nd	?	–	16	–
	34	nd	?	–	–[d]	–[d]
	35	nd	?	+	–	–
	36	–	?	+	–[d]	–[d]
	37	nd	–	–	–	–
	38	–	–	–	–	–

bg: very high background; nd: not done; +: positive; –: negative; ?: borderline.
[a] HEV recomBLOT/recomLINE, Mikrogen, Germany.
[b] Hepatitis E Virus immunofluorescence test (HEV-IFT): highest serum dilution [1:*x*] with still detectable specific signal.
[c] In-house nested reverse transcriptase PCR.
[d] Nuclear staining pattern.
[e] Isolated open reading frame 2 C-terminus band.
[f] Recent travel to endemic region.

His tagged HEV ORF2), was determined by incubation with 1:2000 diluted mouse anti-RGS-His primary antibody and 1:400 diluted Alexa Fluor 488 conjugated goat anti-mouse IgG/M (H+L) secondary antibody (Invitrogen, Renfrew, UK).

For diagnostic use, HEV-IFT slides were thawed in CINAkit washing solution. Eight fields were incubated with 40 μl patient's serum diluted with DPBS (1:8 to 1:1024) each. Additionally one field per slide was incubated with 40 μl diluted negative control serum (1:8 in DPBS) and one field with 40 μl DPBS (Fig. 2). First incubation was performed for 60 min at 37 °C in a humidified dark chamber. Slides were then rinsed briefly with DPBS, washed in fresh DPBS for 5 min at room temperature and incubated with one of the following secondary antibodies for 60 min at 37 °C in a humidified dark chamber: 30 μl fluorescein isothiocyanate (FITC) labelled rabbit anti-human IgG antibody (1:60), 30 μl rabbit anti-human IgA/FITC antibody (1:60), or 30 μl rabbit anti-human IgM/FITC antibody (1:30). Respective secondary antibodies (all DAKO, Glostrup, Denmark) were diluted in DPBS supplemented with 0.01% Evans blue (Sigma–Aldrich, Saint Louis, USA) for counterstaining. After secondary antibody incubation, slides were washed in DPBS, mounted and analyzed by immunofluorescence microscopy on a fluorescence microscope with a filter for FITC (Leica DMLB, Microsystems, Wetzlar, Germany) at 20× magnification.

3. Results

Transfection of HEK293T cells with pCR3-N-His-ORF2 resulted in the transient expression of His-tagged HEV ORF2 protein. The staining pattern of anti-RGS-His antibody (Fig. 1B) and acute hepatitis E patient serum (Fig. 1C) co-stained in the cytoplasm of all transfected cells analyzed (Fig. 1D). Morphology of HEK293T cells appeared differently on HEV-IFT slides as compared to Lab-Tek chamber slides: due to (re-)suspension and different fixation protocols, cells on HEV-IFT slides exhibited a roundish shape and appeared about 30% smaller (Fig. 1E–H) than cells grown on Lab-Tek chamber slides (Fig. 1A–D). As a result, the specific cytoplasmic immunostaining pattern of the His-tagged HEV ORF2 antigen changed from a spindle-shaped presentation on Lab-Tek chamber slides (Fig. 1B–D) to a ring-shaped presentation on the HEV-IFT slides (Fig. 1E and F). Unspecific reactivity of patient sera could be identified by different staining patterns of virtually all cells on the HEV-IFT slide (Fig. 1G and H). In contrast HEV positive serum

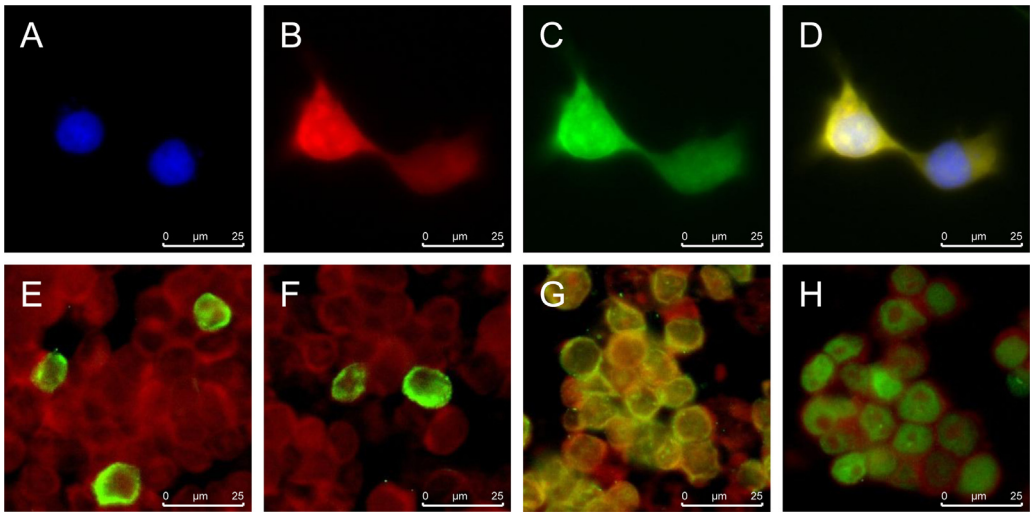

Fig. 1. Co-staining study and staining features of the Hepatitis E Virus immunofluorescence test (HEV-IFT). The upper panel shows the results of the co-staining study using Lab-Tek chamber slides. The red staining pattern of anti-RGS-His antibody (B) and green staining of anti-HEV IgG antibodies prevalent in a serum of a HEV-RNA positive patient (C) co-stained in the cytoplasm of all transfected cells analyzed (D; merged with blue nuclear 4′,6-diamidino-2-phenylindole counterstain, A). The lower panel shows close-up views of HEV-IFT slides. Typical transfection efficiency of 5–10% was determined by green anti-RGS-His antibody staining (E). In contrast to HEV-RNA positive patient serum (F), unspecific reactivity of sera could be identified by a homogenous cytoplasmic staining (G) or by a homogenous nuclear staining (H) of virtually all cells. Fluorescein isothiocyanate labelled rabbit anti-human IgG antibody were used for green staining in, F–H. In the lower panel Evans blue was used for red counterstaining. Magnification is 63-fold.

stained only 5–10% of all cells (Fig. 1F). This correlates well with the transfection efficiency of 5–10% determined by anti-RGS-His antibody (Fig. 1E).

Fig. 2 depicts results of serum 10 (Table 1) using the easy to handle HEV-IFT slides. Anti-HEV IgM antibodies could neither be detected within serum 10 (Fig. 2, middle panel), nor in the sera of other hepatitis E patients.

The reactivity of HEV ORF2 antigen with different patient sera is shown in Tables 1 and 2. Results regarding the HEV IFT reactivity can be briefly summarized as follows: the HEV-IFT was able to detect IgG in 20 PCR-positive sera and IgA in 17 PCR-positive sera. Using Mikrogen assays, only one IgG and one IgM test did not react with PCR-positive sera. Neither IgG nor IgA antibodies were detected by HEV-IFT in three sera tested positive for IgG and IgM antibodies with the HEV Mikrogen tests. In the group of eight sera, which were HEV IgG positive, the HEV-IFT also detected IgG antibodies in six of them. Five sera with borderline IgG titers were tested positive for IgM in three cases in the commercial assays but only two of them were positive for IgG using the HEV-IFT. IgA reactivity was negative in all five cases. Two negative control sera remained negative throughout testing.

From six sera of patients with known viral hepatitis A, B or C, two were positive for IgG and one for IgM using Mikrogen assays (Table 2). Using the HEV-IFT only one serum showed the combination of positive IgG and IgA. In the group of 11 EBV positive sera, six were positive for IgG and/or IgM in commercial testing. Five of them showed positive reactivity in the HEV-IFT test and none reacted for IgA. In two patients with CMV infection no anti-HEV antibody was detected at all. From five rheumatoid factor positive patients four had detectable IgG antibodies in Mikrogen tests and two in IFT tests. No reactivity for IgM or IgA could be detected within the group of rheumatoid factor positive patients. These results are similar for the reactivity of sera from nine pregnant patients. In the latter group, IgG was detected twice using commercial assays and three times using the HEV-IFT.

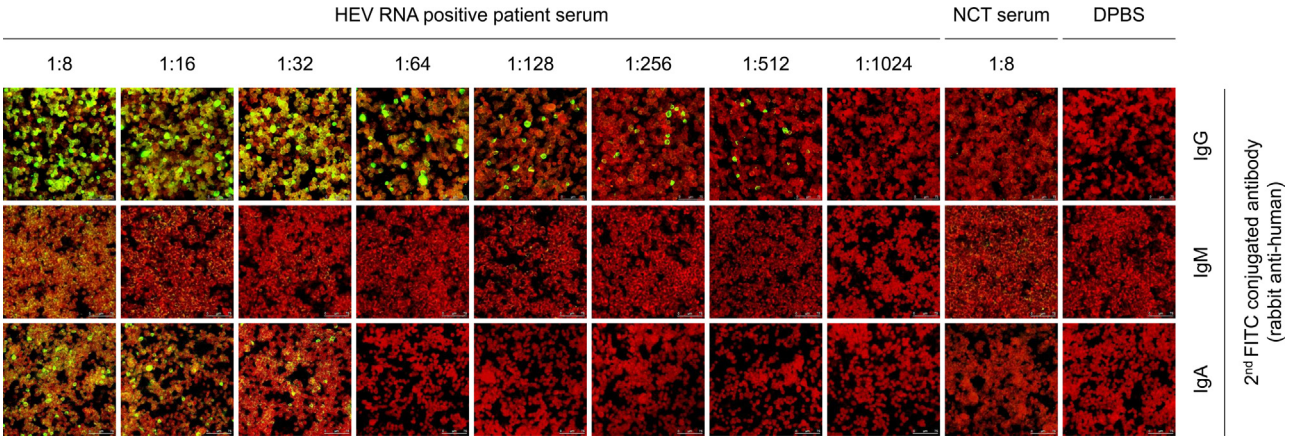

Fig. 2. HEV-IFT slide sample—IgG, IgM and IgA signal detection in a HEV-RNA positive serum. This figure depicts the HEV-IFT results of serum 10 (Table 1). Eight fields of the HEV-IFT slide were incubated with diluted patient's serum (1:8 to 1:1024 in Dulbecco's phosphate-buffered saline (DPBS)). Additionally one field per slide was incubated with diluted negative control serum (1:8 in DPBS) and one field with DPBS. The titers of anti-HEV IgG (upper panel) and IgA (lower panel) antibodies could be detected as 1:512 and 1:32, respectively. Anti-HEV IgM antibodies could not be detected within this patient serum (middle panel). Magnification is 20-fold.

Table 2
Anti-HEV IgG, IgM and IgA seroreacitvity data of defined sera from patients with other clinically relevant conditions.

Laboratory confirmed diagnosis	Serum no.	Anti-HEV (Mikrogen)[a]		Anti-HEV (IFT)[b]	
		IgG	IgM	IgG	IgA
Hepatitis A	39	+[d]	–	–	–
	40	–	–	–	–
Chronic hepatitis B	41	+	?	1024	16
	42	–	–	–	–
Hepatitis C	43	–	+	–	–
	44	–	–	–	–
EBV infection	45	+	+	128	–
	46	+	+	64	–
	47	–	–	256	–
	48	–	–	128	–
	49	–	–	128	–
	50	+[d]	–	–	–
	51	+[d]	–	–	–
	52	–	+	–[c]	–
	53	–	+	–[c]	–
	54	–	–	–	–
	55	–	–	–	–
CMV infection	56	–	–	–	–
	57	–	–	–	–
RF positive disease	58	+	–	256	–
	59	+	–	128[d]	–
	60	+[d]	–	–	–
	61	+[d]	–	–[c]	–[c]
	62	–	–	–	–[c]
Pregnancy	63	+	–	1024	–
	64	+	–	256	–
	65	–	–	256	–
	66	–	–	–	–
	67	–	–	–	–
	68	–	–	–	–
	69	–	–	–	–
	70	–	–	–	–
	71	–	–	–	–

EBV: Epstein Barr Virus; CMV: cytomegalovirus; RF: rheumatoid factor; +: positive; –: negative; ?: borderline.
[a] HEV recomBLOT/recomLINE, Mikrogen, Germany.
[b] Hepatitis E Virus immunofluorescence test (HEV-IFT): specific signal detectable up to serum dilution 1:*x*.
[c] Nuclear staining pattern.
[d] Isolated open reading frame 2 C-terminus band.

4. Discussion

The confirmation of the co-staining of immunofluorescence signals of anti-RGS-His antibodies and patients IgG/M in HEV-ORF2 expressing HEK293T cells indicated the feasibility of an IFT for HEV diagnosis. The developed HEV-IFT slide is an economical method for diagnostic laboratories with few work-steps and short hands-on time. Comparative imaging of differently produced IFT slides showed only minor differences in cell morphology with similar staining properties.

One clear advantage of the HEV-IFT over other serological methods is that the investigator can specifically assess the localization of the antibody staining pattern by microscopy. Thus, unspecific reactivity of sera could be identified by a homogenous cytoplasmic staining (Fig. 1G) or by a homogenous nuclear staining (Fig. 1H) of virtually all presented cells. The latter could be observed frequently in sera derived from patients with anti-nuclear antibodies (ANAs) as for example patient serum 31.

The results of probing 71 sera by the HEV-IFT are discussed in the following: among twenty-three sera of patients with laboratory confirmed acute hepatitis E only minimal differences in sensitivity of IgG detection between HEV-IFT and commercial tests could be detected (Table 1, 1–23). Sera 15 and 16 (Table 1) were drawn from the same patient during early and late phase of RNAemia, respectively. Positive anti-IgG titers during early phase were only detected by the IFT and a rise in IgG titers during course of infection gives a hint on the semi-quantitative properties of the HEV-IFT. Among 48 sera of patients who were not diagnosed with an acute hepatitis E (24–71), 13 showed positive anti-HEV IgG titers in both, commercial and HEV-IFT assays, seven only within the commercial and six only within the HEV-IFT assay. The fact, that all seven anti-HEV IgG positive sera detected exclusively by the commercial recomBLOT/LINE exhibited an isolated ORF2 C-terminus (O2C) band, can be interpreted in two ways: either anti-O2C IgG represents a highly sensitive and long persisting marker for past HEV infections as suggested by Mohn et al. (2009), or O2C antigen exhibits a potential of unspecific IgG crossreactivity. Results from a recent study (Wenzel et al., 2013) support the hypothesis that the IgG positive results determined with the recomLINE are true positives, and further support the seemingly high rate of positive anti-HEV IgG results, which are in line with data published for Germany (Faber et al., 2012).

Because of the frequent false positive rate in serological tests during pregnancy (Zacharias et al., 2004), nine sera of healthy pregnant women (Table 2, 63–71) were analyzed for the presence of anti-HEV antibodies. No serum was reactive for IgM or IgA in any assay, but two (63, 64) exhibited a positive IgG titer within commercial, and three (63–65) within HEV-IFT testing. These findings suggest that a high false positive rate of IgA results seems not to be crucial in interpreting HEV-IFT results in pregnant patients.

Reviewing recent literature, false positive IgM results seem to be often caused by IgM rheumatoid factors (Takahashi et al., 2005; Tian et al., 2006; Zhang et al., 2009). Therefore, five sera of rheumatoid factor positive patients (58–62) were analyzed for the presence of anti-HEV antibodies in both assays. In all five sera, neither positive IgM, nor IgA antibodies could be detected. The high rate of anti-HEV IgG positive sera in the recomLINE assay (four out of five) – two of which could be confirmed by HEV-IFT – suggested that rheumatoid factors might not only play a role in false positive IgM, but also in IgG results. An influence of rheumatoid factor on IgA specificity was not detected.

In total, 23 sera were tested positive for the presence of anti-HEV IgG and IgM antibodies using commercial tests. Five of these sera were negative for detectable IgA antibodies when assayed by HEV-IFT. This antibody pattern can be accounted for hepatitis E patients with false negative IgA, as well as for any disease unrelated to hepatitis E exhibiting false positive IgM. The latter could be observed for two sera of patients diagnosed with a recent EBV infection (Table 2, 52, 53). The overall high prevalence of anti-HEV IgG and/or IgM antibodies within the control group of sera of patients with EBV infection (Table 2, 45–55) shows that serologic HEV diagnosis during EBV infection needs to be handled with care (Fogeda et al., 2009) and detection of viral RNA might be the only reliable marker for HEV infection in these cases. Other subpopulations of patients reported to exhibit a higher rate of false positive anti-HEV IgM results are Hepatitis A Virus or CMV infected patients and patients with chronic hepatitides or auto-immune disorders (Elkady et al., 2007; Fogeda et al., 2009; Takahashi et al., 2005; Tian et al., 2006). In one serum of a patient with a chronic Hepatitis B Virus infection (Table 2, 41) the HEV-IFT showed positive results for both, IgG and IgA assuring the diagnosis of an existing HEV co-infection (Wu et al., 2009) over an unspecific increase of antibody levels caused by a hepatitis B "flare".

According to literature persistence of anti-HEV IgA is described with up to five months, which is longer than anti-HEV IgM or HEV-RNA in the diagnosis of an acute hepatitis E (Elkady et al., 2007; Takahashi et al., 2005; Tian et al., 2006; Zhang et al., 2009).

In contrast to IgM, false positive IgA results are reported frequently for patients with acute hepatitis A and B (Elkady et al., 2007; Tian et al., 2006). Noticeably, none of the 33 control sera used was found to be false positive for anti-HEV IgA, indicating a high specificity of the HEV-IFT.

Lacking detectable anti-HEV IgA antibodies during acute HEV infections may be accounted by several reasons. Variable patterns of negative serological IgA but also of IgG and IgM testings have already been discussed in the literature (Elkady et al., 2007; Gotanda et al., 2007; Herremans et al., 2007; Mitsui et al., 2005; Takahashi et al., 2005, 2010; Zhang et al., 2009). One possible reason for false negative IgM and IgA results is the competition of these types of antibodies with IgG for the limited numbers of antigen binding sites, especially when patient IgG titers are extraordinarily high (Takahashi et al., 2005; Tian et al., 2006; Zhang et al., 2009). A second explanation could be a primary immunodeficiency. Patients with IgA deficiency, the most common form of primary immunodeficiency with a prevalence of about 1:400 in Caucasian Europeans (Weber-Mzell et al., 2004), might lead to insufficient production of anti-HEV IgA antibodies in serum during acute HEV infections (Takahashi et al., 2005; Zhang et al., 2009). Reduced or absent anti-HEV IgA response was also observed in patients infected parenterally (e.g. blood transfusions), because viral replication is not able to stimulate an IgA response if the mucosal immune system is evaded (Herremans et al., 2007). Different pathogenic capabilities of the various HEV genotypes might play additionally a role in the stimulation of IgA immune responses. Herremans et al. (2007) speculated that HEV genotype 1 strains could lead to higher IgA-production during more-extensive mucosal infection than mild infections caused by the zoonotic genotype 3 strains (Herremans et al., 2007). Last but not least, the sensitivity of anti-HEV IgA assays might be dependent on the genotype of the presented HEV antigen (Elkady et al., 2007; Herremans et al., 2007; Osterman et al., 2012). Therefore, negative IgA results in this study may be explained by the fact that the HEV-IFT is based on a genotype 1 antigen whereas the most common form of autochtonous hepatitis E in Germany is caused by genotype 3 strains (Wichmann et al., 2008).

The HEV-IFT requires only a very small amount of serum compared to the commercial test used in diagnostic laboratories (four to 20 times less serum required). This is a substantial advantage in volume limited settings such as pediatric samples or specimen from small animals in veterinary medicine (Drexler et al., 2012).

This study is limited by the low number of available HEV-RNA positive patient samples and therefore it was not possible to perform a statistically validation of the HEV-IFT. The reason why this IFT can detect antibodies from class G and A, but not M remains unclear. Insufficient permeabilization of the cell membrane and therefore inhibited accessibility to the cytoplasmatic HEV ORF2 antigen for IgM pentameres were ruled out by the evaluation of different permeabilization protocols (data not shown).

5. Conclusions

In conclusion, the use of a portfolio of laboratory tests rather than one single test should be used for HEV diagnosis, especially in the case of ambiguous results. The results are in agreement with several published studies which showed the capability of anti-HEV IgA antibodies to support serological HEV diagnosis, but still claim the need to clarify substantially the clinical and epidemiological significance of IgA for HEV diagnosis (Mitsui et al., 2005; Takahashi et al., 2005; Tian et al., 2006).

This manuscript presents a novel semi quantitative indirect IFT based on recombinant HEV capsid protein expressed transiently in HEK293T cells. This assay is suitable to confirm commercial anti-HEV IgG assays and also to determine the anti-HEV IgA status of patients with putative hepatitis E infections using a simple and economical protocol feasible under diagnostic laboratory conditions.

Acknowledgements

Financial support by the FoeFoLe (Foerderprogramm fuer Forschung und Lehre) of the Ludwig-Maximilians-University of Munich to AO, AB and HN, by the Deutsche Forschungsgemeinschaft (BA 2035/3-1 and SPP1230 priority program "Mechanisms of gene vector entry and persistence") to AB, and the Bundesministerium fuer Bildung und Forschung (BMBF BioChancePLUS/FKZ: 0315182) to AB and HN is acknowledged gratefully. We thank gratefully Daniela Huzly for providing kindly sera 15 and 16 and Helga Mairhofer for performing PCR analysis on clinical specimens.

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