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Application of ELISA *recom*Well HEV IgG (Human) for Detection of Virus-Specific Antibodies in Sera of Slaughtered Rabbits

Ewelina Bigoraj[1] · Artur Rzeżutka[1]

Abstract
HEV infections in animals are mainly diagnosed based on the presence of anti-HEV antibodies in sera using enzyme-linked immunosorbent assays (ELISA). In fact, commercial HEV ELISA kits are only available for selected animal species excluding rabbits. The demonstration of cross-reactivity of rabbit anti-HEV antibodies with human HEV strains makes possible diagnostic use of (human) ELISA kits for detection of specific anti-HEV antibodies in rabbit sera. The aim of the study was the application of ELISA *recom*Well HEV IgG (human) for identification of HEV seropositive rabbits at the time of slaughter. Native recombinant protein A was used as broadly reactive conjugate characterized by cross-species reactivity with IgG antibodies of human and other animal species. Several dilutions of protein A were employed and subsequently tested against the panel of control sera (positive, negative, and borderline) to determine its optimal concentration. Statistical analysis of OD values and their corresponding antibody titers obtained for each group of tested sera and protein A showed the most consistent performances at 1:40,000 dilution when compared to anti-human IgG (control) conjugate. Finally, the correct performance of the optimized test was verified using rabbit serum samples containing (or not) specific anti-HEV antibodies. As demonstrated here, HEV IgG (human) ELISA employing ORF2 antigens of human gt1 and gt3 HEV strains after modification and subsequent optimization can be used for the detection of anti-HEV antibodies in rabbit sera.

Keywords Hepatitis E virus · Rabbit · ELISA · Detection

Introduction

Hepatitis E virus (HEV) infects humans and many species of farm and wildlife animals (Johne et al. 2014). HEV infections in animals have an asymptomatic course with no significant impact on animal health. Such infections in humans and in particular in immunocompromised persons and pregnant women can cause serious health consequences (Goumba et al. 2010; Nelson et al. 2018). Although rabbit HEV strains belong to the rabbit HEV genotype cluster, they are genetically and antigenically closely related to pig and wild boar HEV strains which are known to infect humans (Yugo and Meng 2013; Johne et al. 2014; Doceul et al. 2016). The virus has been found in farmed, wild, and pet rabbits (Caruso et al.

2015). HEV infections in rabbits are asymptomatic and therefore tissues of asymptomatically infected animals can easily enter the food chain. Currently, there is a lack of data on cases of HEV infection in humans related to the consumption of rabbit meat but a risk of zoonotic infection with rabbit HEV potentially exists.

Rabbit meat is very popular among consumers in Europe, and its consumption in some countries reaches nearly 6 kg per capita. Due to a significant (21%) content of protein with good digestibility, mineral and vitamin B group content, and low levels of fat and cholesterol, it is recommended especially for allergic persons, pregnant women, and children (Szkucik and Pyz-Łukasik 2009; Dalle Zotte and Szendro 2011). In 2014, world production of rabbit meat amounted to 1.6 million tons and 25% of rabbit meat was produced in the EU, in countries such as France, Italy, and Spain (Dorning and Harris 2017), which also have the highest rate of rabbit meat consumption (FAO 2014). Poland supplies 10% of the world's production of rabbit meat while itself having a low consumption rate (Anonymous 2014). Similarly, to meat of other food animals intended for human consumption, rabbit meat should be free from any microbiological hazards. Different types of bacterial

✉ Artur Rzeżutka
arzez@piwet.pulawy.pl

[1] Department of Food and Environmental Virology, National Veterinary Research Institute, Al. Partyzantów 57, 24-100 Puławy, Poland

pathogens are nevertheless sporadically detected in raw rabbit meat (Khalafalla 1993; Pyz-Łukasik and Szkucik 2005).

Diagnosis of HEV infections in rabbits is based on molecular detection of the viral genetic material or detection of specific anti-HEV antibodies in the blood of tested animals (Burt et al. 2016; Di Bartolo et al. 2016). The use of molecular methods is not always possible as virus presence in animal blood is associated with viremia, which is transient and disappears when seroneutralising antibodies appear (Geng et al. 2011). Nevertheless, these methods are irreplaceable in determination of virus genotypes or identification of the sources of infection. In epidemiological studies on the prevalence of HEV infections in animals, mainly enzyme-linked immunosorbent assays (ELISA) are used. However, HEV ELISAs are only commercially available for selected animal species such as pigs, wild boars, and ruminants, but not for rabbits (Eiden et al. 2011; Costanzo et al. 2015). Demonstration of cross-reactivity of rabbit anti-HEV antibodies with human HEV strains (Izopet et al. 2012) makes possible diagnostic use of human ELISA kits for detection of specific anti-HEV antibodies in rabbits. The aim of the study was an adaptation of the ELISA recomWell HEV IgG kit (human) for identification of HEV seropositive rabbits at the time of slaughter.

Materials and Methods

ELISA

For the detection of the presence of anti-HEV antibodies in rabbit sera, a recomWell HEV IgG ELISA kit (Mikrogen Diagnostik) was used. This assay utilized a recombinant O2C fragment within HEV-ORF2 genotypes 1 and 3 (gt1 and gt3) as virus antigens.

Antibody-Binding Protein A (Conjugate) and Preparation of Control Rabbit Sera

A native recombinant protein A (Protein A-Peroxidase, Sigma-Aldrich) was used as a conjugate for the detection of rabbit anti-HEV antibodies using recomWell HEV IgG. This protein is characterized by cross-species reactivity with IgG antibodies of human, pig, rodents (rat and mouse), ruminants (cow, goat, and sheep), and companion animals (dog, cat, and rabbit). A panel of 28 positive and negative rabbit serum samples containing (or not) specific anti-HEV antibodies was prepared to verify the correct performance of the optimized test. Sera samples obtained from virus positive animals with (presence of HEV RNA in the liver) were considered "conditionally" positive sera, while other sera samples were labeled "conditionally" negative until their status was confirmed by in-house modified ELISA. The presence or absence of

asymptomatic HEV infections in rabbits of which sera were included in the panel of control negative and positive serum samples was checked by the detection of viral RNA in a pair of samples consisting of liver and serum taken from the same animal. Blood and liver samples were collected from each animal at slaughter. To obtain sera, after coagulation, blood was centrifuged at $1000 \times g$ for 10 min at 4 °C and subsequently frozen at -20 °C until use. Liver samples (2 g) were homogenized on ice with the addition of 1 ml PBS and then centrifuged at 2–8 °C for 5 min at $14000 \times g$. Isolation of viral RNA from liver homogenate and serum was performed using a QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. Detection of HEV RNA was carried out with a single-step real-time RT-PCR using an UltraSense One-Step Quantitative RT-PCR kit (Invitrogen) with primers and probe designed by Jothikumar et al. (2006). To control inhibition of the real-time RT-PCR, an IAC was included in each PCR reaction mix (Maunula et al. 2013). Additionally, to the IAC, the appropriate positive (HEV RNA standard) and negative (all reagents with distilled water instead of the template) controls were set up during molecular analyses (Maunula et al. 2013).

Adaptation and Optimization of ELISA

The recomWell HEV IgG assay was subjected to a modification in which the control conjugate (IgG anti-human antibodies) supplied with the assay was replaced with protein A (Protein A-Peroxidase, Sigma-Aldrich). This protein is reactive with human IgG and with antibodies of several animal species including rabbits. Dilutions of 1:20,000, 1:40,000, 1:60,000, and 1:80,000 of new conjugate in the sample dilution buffer were prepared.

In order to determine optimal concentration of protein A, each its dilution was tested 10 times against the panel of control sera (positive, negative, and borderline) included in the kit. In parallel, each control serum was also tested using a conjugate supplied with the kit. The analysis was carried out according to the manufacturer's instructions. The cut-off value was the arithmetic mean of the optical density (OD) values obtained for two replicates of doubtful serum sample. The quotient of serum OD and the cut-off value for doubtful serum sample gave the antibody titer, which was expressed in units (U) per ml. The serum was considered positive when its cut-off value exceeded the borderline seropositivity of 24 U/ml. When the value ranged from 20 to 24 U/ml, the serum was considered doubtful. A value below 20 U/ml indicated a negative result. The correct performance of the modified assay was verified by testing of positive and negative panel of rabbit sera with the use of the optimal dilution of protein A as the conjugate.

Statistical Analysis

A two-way analysis of variance (ANOVA) with interactions was used to assess significant differences between OD obtained for each group of tested control serum samples (positive, negative, and borderline sera) and control anti-human IgG conjugate including serial dilutions (1:20,000, 1:40,000, 1:60,000, and 1:80,000) of protein A—a tested conjugate. Furthermore, differences between the OD values and the antibody titers (U/ml) obtained for each dilution of protein A in comparison to the control conjugate within each group of sera were analyzed using a one-way analysis of variance.

All calculations were performed with a Statgraphics Centurion v. XV (Statpoint Technologies, Warrenton, USA).

Results

The OD values obtained for each group of tested sera (positive, negative, and borderline) with protein A dilutions and control conjugate (IgG anti-human antibodies) differ significantly ($P < 0.001$) (Table 1). The one-way analysis of variance performed on the basis of OD values for positive sera indicated the presence of statistically significant differences ($P < 0.001$) between control conjugate and protein A dilutions. Only the OD values obtained for positive sera when tested with protein A at 1:40,000 dilution did not differ significantly from the OD values of the control conjugate. It is noteworthy that solely OD values obtained at 1:20,000 dilution of the tested conjugate differed significantly from the control (Fig. 1a). In the case of negative sera, the values were not significantly different ($P = 0.604$). A significant difference ($P < 0.001$) between the tested dilutions of protein A and the control conjugate was shown for borderline sera. In fact, the OD values gained for tested sera samples using both 1:40,000 and 1:60,000 dilutions of the conjugate did not differ significantly compared to the control, although they differed significantly from each other (Fig. 1b). Analyzing the antibodies

titers that define the tested serum as positive, negative, or borderline, there were not significant differences present ($P = 0.008$) between the group of positive sera tested with protein A (1:40,000 dilution) and the control conjugate. Similarly, the titer of negative sera obtained for the conjugate did not differ either at 1:40,000 or 1:60,000 dilution from the sera titers obtained with the control conjugate.

The correct performance of the optimized ELISA for the detection of anti-HEV IgG antibodies in rabbits was evaluated using a panel of rabbit sera samples. All "conditionally" positive sera originated from virus-positive animals as confirmed by molecular testing showed the level of anti-HEV IgG in a range from 56.7 to 183.7 U/ml (Table 2). In the case of sera that were considered "conditionally" negative based on molecular testing, ELISA also confirmed their seronegative status.

Discussion

ELISA-based assays are commonly used as screening tests in diagnostics of human and animal viral infectious diseases (Khudyakov and Kamili 2011; Abravanel et al. 2013). However, they are not always available for a wide variety of viruses infecting different animal species. Nowadays, dedicated ELISA assays are available for detection of HEV antibodies in human and pig serum. Although HEV has several genotypes, it has only one serotype. Therefore, expressed antigens from one virus genotype can theoretically react with antibodies directed against all four virus genotypes (Zhou et al. 2004; Zhao and Wang 2016; Wang et al. 2017). Additionally, it has previously been shown that different mammalian HEV genotypes have cross-immunoreactive epitopes (Zhao and Wang 2016). Taking this into account and considering the facts that rabbit HEV strains are closely related to genotype 3 HEV strains and recombinant capsid protein of rabbit HEV cross-reacted with antibodies raised against avian, rat, swine, and human HEV (Cossaboom et al. 2012), an attempt was

Table 1 Results of determination of the optimal concentration of protein A in ELISA

Control sera	OD for control conjugate		OD for protein A							
			1:20,000		1:40,000		1:60,000		1:80,000	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Positive	1.215*	0.097	1.981*	0.254	1.508	0.296	0.899*	0.181	0.671*	0.187
Negative	0.061	0.003	0.059	0.013	0.058	0.011	0.058	0.008	0.053	0.004
Borderline 1	0.346*	0.030	0.544*	0.089	0.410	0.091	0.275	0.058	0.219*	0.048
Borderline 2	0.360*	0.023	0.557*	0.128	0.406	0.136	0.256	0.042	0.217	0.040

SD, standard deviation obtained for 10 replicates

*Denotes a statistically significant difference compared to control conjugate ($P < 0.001$)
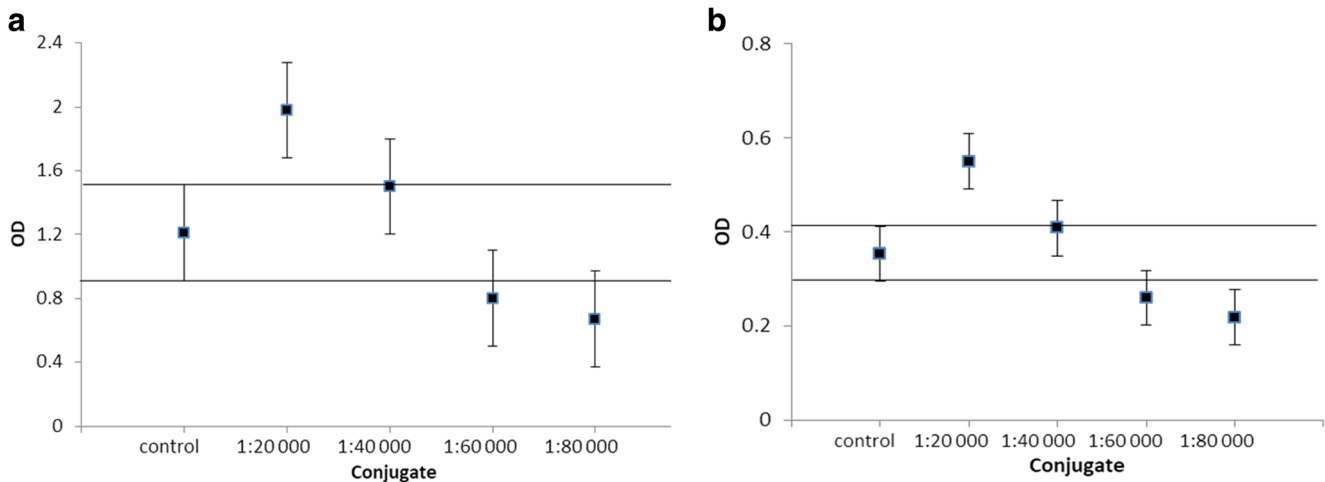
Fig. 1 OD values of positive (a) and borderline (b) sera obtained when analyzed with tested dilutions of the conjugate. Values outside the confidence interval indicated conjugate dilutions which differ significantly from the mean. The horizontal lines shows the ranges of confidence intervals (lower and upper limit) for the average OD value

undertaken to apply a commercially available ELISA containing antigens derived from human gt1 and 3 HEV strains to the detection of anti-HEV antibodies in rabbit sera.

The HEV genome contains three open reading frames (ORFs) that encode structural proteins (ORF2) capable of inducing an immunological response and non-structural proteins (ORF1 and ORF3) essential for virus replication in cells (Emerson and Purcell 2003; Johne et al. 2014). Currently, different synthetic peptides and recombinant antigens of ORF2 and 3 of genotypes 1, 2, and 3 are used in serological diagnostics of human and animal HEV infections (Osterman

et al. 2012; Riddell et al. 2000; Pezzoni et al. 2014; Wang et al. 2017). This could, to some extent, be explained by the fact that major antigenic epitopes of HEV are located more in the ORF2 (Wang et al. 2001) and ORF3 (Zhao et al. 2009) regions than in the ORF1 coding region (Osterman et al. 2012). Further studies characterizing the immunogenic properties of the ORF2- and ORF3-based recombinant virus proteins showed that ORF2 polyprotein, and especially its C-terminus part (O2C or p166 protein), is characterized by the highest immunogenicity among all tested virus antigens so far (Liang et al. 2010; Osterman et al. 2012). It is noteworthy that differences in the antigenicity of the ORF3 proteins of human gt1 and 4 HEV strains were observed (Ma et al. 2009). As with ORF3 proteins but to a lesser extent, the genotype-specific seroreactivity of ORF2 antigens was also found (Osterman et al. 2012). In this study, the ELISA *recom*Well HEV IgG which allows the detection of anti-HEV antibodies in human sera was adapted and optimized for detection of virus-specific antibodies in rabbit serum samples. The assay used utilizes O2C protein (432–660 aa) as antigen-capturing rabbit anti-HEV antibodies. This protein showed cross-reactivity among different genotypes and subgenotypes of HEV strains isolated from humans, pigs, boars, and monkeys (Khudyakov et al. 1999; Li et al. 2000; Meng et al. 2001; Johne et al. 2014; Pérez-Gracia et al. 2014). As demonstrated in previous studies, the applicability of ORF2 recombinant proteins (ORF2 C-terminal portion; 394–660 aa and its truncated form at the N- and C-terminals) in serodiagnostics of HEV infections in pigs has been shown (Pezzoni et al. 2014). Another diagnostically relevant region within ORF2 virus sequence encodes protein at position of 452–617 aa (Obriadina et al. 2002). Recently, the performance of several commercial assays for detection of anti-HEV antibodies in human and animal sera was evaluated (Zhang et al. 2011; Wenzel et al. 2013; Shrestha et al. 2016). The results indicated significant

Table 2 Results of molecular and serological tests of rabbit liver and sera samples

No. of sample	Real-time RT-PCR		ELISA	
	Serum (Cq)	Liver (Cq)	OD	U/ml*
1	–	24.16	2.73	125.37
2	–	27.67	4.00	183.70
3	–	35.49	4.00	183.70
4	–	32.26	3.34	153.48
5	–	27.00	3.14	144.34
6	–	40.01	3.37	154.99
7	–	28.65	3.33	152.84
8	–	32.77	1.67	76.74
9	–	28.60	1.95	89.37
10	–	28.82	1.77	81.47
11	–	28.22	2.47	113.43
12	–	24.93	1.23	56.67
13	–	31.57	2.20	101.22
14	43.91	27.51	1.39	63.97
15–28	–	–	< 0.22	< 10.15

*Cut-off value 0.435

differences in the assays' diagnostic sensitivities. Additionally, assays based on proteins derived from ORF3 HEV may show poor sensitivities (Wang et al. 2001; Ma et al. 2009). In this study, a recombinant protein A reactive against the antigenic determinants of HEV strains was evaluated as a universal conjugate in ELISA. This native protein is able to bind with IgG antibodies present in sera of different mammals. It also permits use of the control sera supplied with the assay during its optimization. A panel of control rabbit sera was employed to gain better understanding about the correct test performance. They were selected according to the results obtained from molecular testing of pairs of samples, i.e., rabbit liver and serum collected from the same animal. Although a direct identification of the virus presence in rabbit sera was unsuccessful, the presence of HEV RNA in liver samples indicated animal seropositivity. Only one serum sample was positive for HEV RNA with a low level (63.97 U/ml) of anti-HEV antibodies determined by the use of the modified ELISA *recom*Well HEV IgG. Subsequent serological testing of other rabbit sera collected from HEV-positive animals also showed the presence of specific anti-HEV antibodies. Consequently, OD values obtained for control sera and anti-human IgG conjugate gave most consistent performances when an appropriate concentration of protein A was used.

In summary, the use of serological tests for screening of animals at the farm level or those entering the food chain for the presence of potentially zoonotic viral pathogens is an efficient and inexpensive way to determine animal HEV seropositivity. As demonstrated here, the antibodies against rabbit HEV cross-reacted with O2C capsid proteins derived from human gt3 HEV strains, confirming the suitability of the anti-HEV IgG (human) ELISA used for detection of anti-HEV antibodies in rabbits. The results have also shown that protein A can be employed as the universal conjugate in ELISA, although a determination of its broad reactivity with sera of different animal species requires further studies.

Compliance with Ethical Standards

Conflict of Interest Ewelina Bigoraj declares that she has no conflict of interest. Artur Rzeżutka declares that he has no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Informed Consent Not applicable.

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