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Differences in capabilities of different enzyme immunoassays to detect anti-hepatitis E virus immunoglobulin G in pigs infected experimentally with hepatitis E virus genotype 3 or 4 and in pigs with unknown exposure

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A B S T R A C T

Hepatitis E virus (HEV), a major cause of acute viral hepatitis in humans in many developing countries, is highly prevalent in the pig population worldwide. The objective of this study was to assess the capability of three porcine prototypes of a human enzyme-linked immunosorbent assay (ELISA), an *in-house* ELISA and a line-immunoassay (LIA) to detect anti-HEV antibodies in pigs infected experimentally with HEV ($n = 57$), known to be negative for HEV infection ($n = 27$), or with unknown exposure to HEV infection (field samples, $n = 90$). All 27 samples from non-infected pigs were negative with all five assays. The earliest detection of anti-HEV antibodies occurred at 14 days post-inoculation (dpi) with four of five assays. From 42 dpi, all samples from infected pigs were detected correctly as anti-HEV positive. Kappa analysis demonstrated substantial agreement among tests (0.62–1.00) at 14 dpi and complete agreement (1.00) at 56 dpi. The overall area under the curve for all quantitative tests as determined by receiver operator characteristic analysis ranged from 0.794 to 0.831 indicating moderate accuracy. The results showed that all five assays can detect anti-HEV IgG antibodies accurately in pigs infected experimentally with HEV. In field samples, a higher prevalence of anti-HEV IgG was found in breeding herds than in growing pigs (100% versus 66.7–93.9%). These serological assays should be very useful in veterinary diagnostic labs for HEV diagnosis in swine.

1. Introduction

Hepatitis E virus (HEV) is a fecal-orally transmitted virus infecting humans and strains of HEV have also been genetically identified from numerous other animal species including domestic and wild pigs, deer, rats, mongoose, rabbits, chickens, and fish (Meng et al., 1997; Haqshenas et al., 2002; Emerson and Purcell, 2003; Fukai et al., 2006; Nakamura et al., 2006; Jiménez de Oya et al., 2007; Zhao et al., 2009; Johne et al., 2010; Boadella et al., 2010; Meng, 2010a,b; Batts et al., 2011; Takahashi et al., 2011). In humans, both epidemic and sporadic forms of acute hepatitis E have been recognized causing an acute, self-limiting hepatic disease, although chronic HEV infections have also been reported in organ transplant recipients and HIV patients (Kamar et al., 2008; Dalton et al., 2009; Legrand-Abravanel et al., 2010; Meng, 2010b). HEV infection has recently been associated with neurological diseases as well (Kamar et al., 2010, 2011). In pregnant women infected with HEV during the third trimester, mortality rates can reach up to 28% in some developing countries (Kumar et al., 2004; Adjei et al., 2009).

HEV is classified into the genus *Hepevirus* of the family *Hepeviridae* (Meng et al., 2011). It is a non-enveloped RNA virus with an icosahedral capsid that has a diameter of 32–34 nm. The genome is a single-stranded, positive-sense, RNA molecule that is approximately 7.2 kb in size and contains three open reading frames (ORFs) (Emerson and Purcell, 2003). ORF1 encodes nonstructural proteins, ORF2 encodes the capsid protein (Tam et al., 1991) and is composed of 660 amino acid residues (Mori and Matsuura, 2011) and ORF3 encodes a small multifunctional protein (Zafrullah et al., 1997). Several immunoreactive domains have been identified in ORF2 and ORF3 proteins (Yarbough et al., 1991; Kaur et al., 1992; Khudyakov et al., 1993). When peptides of HEV ORF1, ORF2 and ORF3 were investigated for immunogenicity, three of the seven peptides were found to be immunogenic (Qi et al., 1995). A recombinant protein designated as pB166, which consisted of overlapping peptides

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from ORF2, was found to be a neutralization epitope (Meng et al., 2001).

At least four major genotypes (1–4) of mammalian HEV belonging to a single serotype have been identified (Schlauder and Mushahwar, 2001). More recently, a novel strain of HEV was identified in rabbits and is a distant member of HEV genotype 3 (Zhao et al., 2009). The rat HEV, which was discovered recently, may belong to a new genotype (Johne et al., 2010). At least three genotypes of avian HEV have also been identified in North America, Australia, China and Europe (Haqshenas et al., 2001; Bilic et al., 2009; Peralta et al., 2009a). A genetically divergent strain of HEV was recently identified from cutthroat trout, and may belong to a separate genus (Batts et al., 2011). Swine HEV was the first animal strain of HEV identified (Meng et al., 1997). It is now known that HEV genotypes 1 and 2 are restricted to humans whereas genotypes 3 and 4 can infect humans, pigs, rabbits, deer and mongoose (Meng, 2010a), although a novel strain of HEV distinct from genotypes 3 and 4 has been identified recently from wild boars in Japan (Takahashi et al., 2011). Domestic and wild pigs and perhaps other species such as deer or rabbits are considered reservoir hosts (Meng, 2010a). Genotypes 1 and 2 HEV strains are endemic in human populations in many developing countries worldwide, whereas genotype 3 and 4 HEV strains are sporadic worldwide in both developing and industrialized countries (Meng, 2010b).

A number of methods have been used to detect HEV infection such as enzyme immunoassays (Engle et al., 2002; Arankalle et al., 2007; Hu et al., 2008; Jiménez de Oya et al., 2009; Ma et al., 2009), *in situ* hybridization (ISH) (Choi et al., 2004; Lee et al., 2009), immunohistochemistry (IHC) (Ha and Chae, 2004; Lee et al., 2009) and several nucleic acid amplification techniques (Erker et al., 1999; Smith, 2001; Ahn et al., 2006; El-Sayed et al., 2006; Enouf et al., 2006; Fernández-Barredo et al., 2006; Inoue et al., 2006; Jothikumar et al., 2006; Zhao et al., 2007; Ward et al., 2009). However, most of the above methods are performed in only a few research-focused laboratories. Many commercial and *in-house* enzyme immunoassay methods have been developed by incorporating recombinant antigens of ORF2 or ORF3 proteins in assays to detect anti-HEV immunoglobulin M (IgM) or G (IgG) in humans or other mammals (Arankalle et al., 2002; Engle et al., 2002; Blacksell et al., 2007; de Deus et al., 2008; Hu et al., 2008; Casas et al., 2009b; Jiménez de Oya et al., 2009; Ma et al., 2009; Peralta et al., 2009b; Rose et al., 2010).

Infectious HEV has been identified in manure slurry from commercial swine farms (Kasorndorkbua et al., 2005), not only potentially posing a risk for incoming HEV naïve pigs, but also pig handlers and farm workers (Drobeniuc et al., 2001; Meng et al., 2002). Retail pork livers have also been shown to contain infectious HEV (Feagins et al., 2007). The HEV RNA detected in wild boar livers was closely related to sequences recovered from humans in the same area (Kaba et al., 2010). Consumption of raw pig liver figatellu sausage, produced in France, has been linked to recent, acute HEV infection in humans (Colson et al., 2010). Cluster cases of acute hepatitis E have also been linked to consumption of raw or undercooked pork in Japan (Yazaki et al., 2003; Li et al., 2005). The zoonotic potential of HEV and its high prevalence in the commercial swine population warrants the assessment of available enzyme immunoassays that could be used to screen and maintain negative pig populations that are used for xenotransplanation (Meng, 2003) and export to countries where the consumption of raw pork products is common, such as in certain Asian countries including Japan (Mizuo et al., 2005). Therefore, the objective of the current study was to assess the diagnostic performance of four prototype and one *in-house* enzyme immunoassays on experimental serum samples obtained from negative control pigs, pigs infected with HEV genotype 3 (human or swine origin) or HEV genotype 4 (human origin) and field serum samples with unknown HEV exposure collected from pigs in commercial operations of the United States.

Table 1
Number of serum samples collected at each day post inoculation from the two sources of pigs (gnotobiotic and conventional pigs) organized by known exposure to HEV.

Pig source	HEV exposure	Days post inoculation								
		0	7	14	21	28	35	42	49	56
Gnotobiotic	Negative	3	–	–	–	–	–	–	–	–
Gnotobiotic	HEV-3	–	3	3	2	1	–	–	–	–
Conventional	Negative	8	2	2	2	2	2	2	2	2
Conventional	HEV-3	–	3	3	3	3	3	3	3	3
Conventional	HEV-4	–	3	3	3	3	3	3	3	3

2. Materials and methods

2.1. Experimental design

A total of 84 experimental serum samples from pigs of known HEV exposure (12 obtained from gnotobiotic pigs and 72 obtained from conventional pigs) were tested for the presence of anti-HEV antibodies by four different enzyme-linked immunosorbent assays (ELISA-1 to ELISA-4) and a line-immunoassay (LIA). The experimental protocols for the animal studies were approved by the National Animal Disease Center and Iowa State University Institutional Animal Care and Use Committees (IACUC) and the Institutional Biosafety Committee (IBC), and international standards for animal welfare were followed. The experimental samples were divided into HEV-positive samples (total $n = 57$; HEV-3 = 33; HEV-4 = 24) and negative control samples ($n = 27$; Table 1). In addition, 90 field samples from pigs of unknown HEV exposure (U.S. field samples from 18 different commercial swine farms) were tested by three of the five assays (ELISA-1, ELISA-2 and ELISA-4). All experimental samples were tested in triplicate on three different plates for the porcine prototype ELISAs (ELISA-1, ELISA-2 and ELISA-3) to establish inter-assay variability. For ELISA-4 and the LIA, the experimental samples were tested in duplicate. ELISA-4 assay is an established *in-house* ELISA that has been used in previous studies (Meng et al., 1997, 1998a,b) and the LIA represented another prototype ELISA modeled after a human-based assay that is available commercially. Each of the samples with unknown HEV infection were tested in a single well format for each of the three assays used (ELISA-1, ELISA-2 and ELISA 4). The results were analyzed for diagnostic performance using the manufacturers' recommended cut-off and agreement among the assays was evaluated using kappa statistics (experimental and field samples). In addition, receiver operator characteristic (ROC) analysis was used to calculate the overall area under the curve (AUC) for all quantitative assays (experimental samples only).

2.2. Experimental samples from pigs with known HEV exposure

2.2.1. Pigs, viruses used, and inoculation

2.2.1.1. Gnotobiotic pigs. The pigs were acquired from a crossbred sow via surgery and immediately placed in one compartment of a sterile stainless steel isolator as described (Miniatis and Jol, 1978). Gnotobiotic pigs had no access to colostrum. At 1 week of age, the gnotobiotic pigs were intravenously inoculated with 2.5 ml of $1 \times 10^{3.8}$ 50% pig infectious dose (PID_{50}) of swine HEV genotype 3. Successful HEV inoculation was confirmed by quantitative HEV real-time RT-PCR on serum and fecal samples (data not shown).

2.2.1.2. Conventional pigs. The pigs were derived from a conventional HEV-positive U.S. swine herd by selecting seronegative

Table 2
HEV antigen coating differences of the five assays used in this study.

Assay	Open reading frame (ORF) 2						ORF3	
	N-terminal region		C-terminal region		Middle region			
	HEV-1[a]	HEV-3[b]	HEV-1	HEV-3	HEV-1	HEV-3	HEV-1	HEV-3
ELISA-1	+		+				+	
ELISA-2	+		+	+			+	
ELISA-3		+		+				+
ELISA-4					+			
LIA	+	+	+	+	+		+	+

[a] HEV genotype 1.
[b] HEV genotype 3.

piglets at 1 week of age and segregating and weaning those pigs at 2 weeks of age and transporting them to a biosafety level 2 (BSL-2) facility. All the conventional pigs had access to colostrum. The detailed experimental inoculation procedure for the conventional pig experiment has been described previously (Feagins et al., 2008). Allocation of pigs to treatment groups was random and treatment groups were housed in separate rooms and pens on a solid concrete floor. The conventional pigs were confirmed negative before intravenous inoculation at 3 weeks of age with 1 ml of 10^3 50% monkey infectious dose (MID_{50}) of human HEV genotype 3, 1 ml of 10^3 MID_{50} of human HEV genotype 4 or with 1 ml sterile phosphate buffered saline as negative controls.

2.2.2. Collection, storage and the numbers of the experimental serum samples used

Blood was collected in 8.5 ml serum separator tubes (Fisher Scientific, Pittsburgh, PA), immediately centrifuged at $2000 \times g$ for 10 min at 4 °C and stored at −80 °C until use. Of the 84 samples tested in this study, 12 serum samples were collected from three gnotobiotic pigs inoculated experimentally with a swine HEV genotype 3 (Table 1). In addition, serum samples were also collected from eight conventional pigs (Table 1) inoculated experimentally with human HEV genotype 3 ($n = 24$ serum samples), or human HEV genotype 4 ($n = 24$ serum samples) or phosphate buffered saline ($n = 24$ serum samples). Serum samples collected on the day of inoculation were considered negative control samples.

2.3. Field samples from pigs with unknown HEV exposure

A total of 90 field serum samples were collected from pigs in 18 farms representing five U.S. states (Iowa, Illinois, Michigan, Minnesota and Wisconsin). The samples were taken from a variety of different stages of production: In total 45 samples were collected from nursery pigs (3–10 weeks of age), 21 from finisher pigs (10–15 weeks of age) and 24 from breeding age pigs (greater than 6 months of age). All serum samples were stored at −20 °C until testing.

2.4. Serology assays

The positive control for ELISA-1 through ELISA-3 was derived from serum collected from a highly positive pig from a Bavarian slaughterhouse. ELISA-1, ELISA-2 and ELISA-3 were validated by comparing the results to an ELISA (Axiom HEV Ab ELISA, Axiom GmbH, Bürstadt, Germany), which is available commercially, and the cut-off for each of these three ELISAs was determined by testing more than 500 serum samples and their corresponding meat juice samples (data not shown). The conjugate used for these three ELISAs was an anti-swine IgG conjugate (Anti-Swine IgG conjugate, Mikrogen, Neuried, Germany).

2.4.1. ELISA-1

This porcine-based prototype of a human HEV-based ELISA, which is available commercially, was validated by the manufacturer (*Recom*Well HEV IgG, Mikrogen, Neuried, Germany). The wells in this ELISA were coated with ORF2 (N-terminal and C-terminal region) and ORF3 proteins based on HEV genotype 1 (Table 2). The test was performed according to the manufacturers' instructions. For validation purposes the following conditions were met: Positive control optical density (OD) values were between 0.5 and 1.5, the negative control OD value was less than 0.15 and the cutoff OD value was between 0.15 and 0.4. To determine whether samples were positive or negative, the cutoff control OD was used. For samples to be considered negative, the sample OD value had to be less than the cutoff OD value. If the sample OD value was above the cutoff OD value × 1.2 it was considered positive. If a sample OD value was between the cutoff OD value and the cutoff OD value × 1.2 it was considered suspect.

2.4.2. ELISA-2

This ELISA was a modification of ELISA-1 and was produced under commercial settings for research purposes. In addition to the C-terminal ORF2 antigen from HEV genotype 1, a homologous protein of HEV genotype 3 was added (Table 2). The assay conditions were the same as described for ELISA-1. In order for the test to be considered valid, the following conditions were met: positive control OD values were between 0.8 and 1.95, negative control OD values were lower than 0.15 and cutoff OD value between 0.2 and 0.55. The test methods, positive and negative control evaluations and sample evaluations were the same as described for ELISA-1.

2.4.3. ELISA-3

ELISA-3 represents a variation of ELISA-1 and was produced under commercial settings for research purposes. The plates for ELISA-3 were coated with ORF2 (N-terminal and C-terminal region) and ORF3 proteins based on HEV genotype 3 only (Table 2). For validation purposes, the following conditions were met: positive control OD values were between 0.3 and 0.7, negative control OD levels were less than 0.12 and cutoff OD values were between 0.11 and 0.25. The test methods, positive and negative control evaluations and sample evaluations were the same as described for ELISA-1.

2.4.4. ELISA-4

This *in-house* assay developed for detection of IgG anti-HEV in pigs was performed as previously described (Meng et al., 1997, 1998a), except that the plate was coated with a HEV ORF2 recombinant protein (GenWay Biotech, Inc, San Diego, CA, cat# 10-733-320087) (Table 2). Samples were considered positive in ELISA-4 if the OD value was equal to, or greater than 0.3. Confidence intervals of 99% based upon the frequency distribution of the absorbance values of normal sera were used to determine the cutoff for this ELISA as described previously (Lee et al., 2009).

Table 3
Detection rate by day post exposure in true positive samples ($n = 57$) from pigs inoculated experimentally with HEV genotypes 3 and 4. Data presented as number of positive samples by each assay/total number of samples considered true positive for that particular day.

Assay	7	14	21	28	35	42	49	56
HEV genotype 3								
ELISA-1	0/6	1/6	3/5	3/4	3/3	3/3	3/3	3/3
ELISA-2	0/6	1/6	3/5	3/4	3/3	3/3	3/3	3/3
ELISA-3	0/6	0/6	3/5	3/4	2/3	3/3	3/3	3/3
ELISA-4	0/6	1/6	3/5	3/4	3/3	3/3	3/3	3/3
LIA	0/6	1/6	3/5	3/4	3/3	3/3	3/3	3/3
HEV genotype 4								
ELISA-1	0/3	0/3	2/3	2/3	3/3	3/3	3/3	3/3
ELISA-2	0/3	0/3	2/3	2/3	3/3	3/3	3/3	3/3
ELISA-3	0/3	0/3	1/3	2/3	3/3	3/3	3/3	3/3
ELISA-4	0/3	1/3	2/3	2/3	3/3	3/3	3/3	3/3
LIA	0/3	0/3	2/3	2/3	3/3	3/3	3/3	3/3

2.4.5. Line-immunoassay

A porcine-based prototype of a commercially available kit for diagnosis of HEV in humans (*Recom*Line HEV IgG/IgM; Mikrogen, Neuried, Germany) was used. The test strips were coated with recombinant proteins originating from the N- and C-terminal parts as well as the middle region of the ORF2 and ORF3 proteins obtained from HEV genotypes 1 and 3 (Table 2). The test was conducted following manufacturers' instructions and the results were read both by human eye and digital-interpretation (*Recom*Scan software, Mikrogen, Neuried, Germany, connected to a flatbed scanner, Plustek OpticPro S28).

2.5. Analysis

2.5.1. Kappa statistics

A kappa statistic was calculated for paired tests using dichotomized data. For tests in which results were identified as positive, negative or suspect (ELISA-1, ELISA-2, and ELISA-3), suspect samples were considered positive. Values for kappa range from −1 to 1 where −1 indicates agreement worse than expected by chance, 0 equals agreement no better than expected by chance and 1 equals complete agreement (Sim and Wright, 2005). The following arbitrary standards for the strength of agreement as described by Landis and Koch were used: ≤0 = poor, 0.01–0.2 = slight, 0.21–0.4 = fair, 0.41–0.60 = moderate, 0.61–0.80 = substantial and 0.81–1 = almost complete (Landis and Koch, 1977). Statistical analysis was performed using the JMP® 8.0.1 software (SAS Institute Inc., Cary, North Carolina, USA).

2.5.2. Receiver operator characteristic curve analysis

The ability of the tests to discriminate between positive and negative samples (sensitivity and specificity) was evaluated using ROC curve analysis. The analysis was performed on animals with known HEV exposure (inoculated or non-inoculated) and independently of the manufacturers' recommended cut-off values. ROC curves were generated by plotting test sensitivity (*Y* axis) against 1-specificity (*X* axis) for all possible combinations. Performance of the tests was determined from the AUC generated from the plotted *X*, *Y* pairs. As the AUC approaches one (1.0), the higher the likelihood that the test will discriminate between infected and uninfected individuals. An arbitrary guideline for evaluation of the AUC values was used as follows: non-informative (AUC = 0.5), less accurate ($0.5 < AUC \leq 0.7$), moderately accurate ($0.7 < AUC \leq 0.9$), highly accurate ($0.9 < AUC < 1$) and perfect tests (AUC = 1) (Greiner et al., 2000). ROC curves and performance estimates were generated using MedCalc® Version 10.4.0.0 (MedCalc® Software, Mariakerke, Belgium) for each of the four quantitative ELISAs using the cumulative data from all days post inoculation (dpi) and the data from each of the individual dpi (7, 14, 21, 28, 35, 42, 49, and 56).

3. Results

3.1. Experimental samples from pigs with known HEV exposure

Based on recommended cut-off criteria, all 27 negative control samples (11 independent samples collected right before inoculation and 16 dependent samples collected from 2 animals over time) were negative with all four ELISAs as well as with the LIA. The results on the samples collected from the pigs at different days post experimental HEV infection are summarized in Table 3. Suspect samples were not identified with any of the assays. The results for the LIA were similar regardless of interpretation method (eye versus digital; data not shown). Seroconversion was first detected at 14 dpi in 11.1% (1/9; ELISA-1, ELISA-2 and the LIA) to 22.2% (2/9; ELISA-4) of the samples. At 21 dpi all assays detected correctly 3/5 genotype 3 samples. At this dpi, ELISA-1, ELISA-2, ELISA-4 and the LIA identified 2/3 correctly whereas ELISA-3 detected only 1/3 genotype 4 samples as being positive. Overall, 50% (4/8; ELISA-3) or 62.5% (5/8; ELISA-1, ELISA-2, ELISA-4 and the LIA) of the samples were detected correctly. Identical results for all assays were observed at 28 dpi with 71.4% of the samples (5/7) detected as positive: 3/4 genotype 3 and 2/3 genotype 4 samples were positive. At 35 dpi all three genotype 3 samples were identified correctly by all assays except ELISA-3, which detected only 2/3 of the genotype 3 samples as being positive. By 42 dpi all samples from pigs inoculated experimentally with HEV were found to be positive with all assays (Table 3). All enzyme immunoassays had substantial agreement (0.62 ± 0.33 to 1.00 ± 0.00) by 14 dpi and complete agreement by 56 dpi (1.00 ± 0.00).

The overall AUCs and the sensitivity and specificity on each dpi based on the overall ROC optimized cut-off of the four quantitative assays are displayed in Table 4. All four assays exhibited similar AUCs and performance characteristics with values ranging from 0.794 to 0.842 which indicates moderate accuracy. By 28 dpi, using the ROC calculated optimized overall cut-offs, all four assays had a sensitivity ≥62.5% and a specificity ≥98.8%. By 42 dpi, all four assays exhibited a sensitivity of 100% and a specificity ≥97.6%.

Table 4
ROC optimized overall cut-off and corresponding sensitivity and specificity of each of four enzyme-linked immunosorbent assays (ELISA) by days post inoculation and cumulative AUC.

Assay	Optimized cutoff	Day post inoculation								AUC
		7	14	21	28	35	42	49	56	
ELISA-1	0.16	3.3, 98.8	33.3, 98.8	59.3, 98.8	79.2, 98.8	100.0, 98.8	100.0, 98.8	100.0, 98.8	100.0, 98.8	0.831
ELISA-2	0.15	6.0, 99.3	34.0, 99.1	62.2, 99.3	87.5, 99.3	100.0, 99.3	100.0, 99.3	100.0, 99.3	100.0, 99.3	0.842
ELISA-3	0.09	93.3, 2.4	23.3, 97.6	59.3, 97.6	62.5, 97.6	100.0, 97.6	100.0, 97.6	100.0, 97.6	100.0, 97.6	0.794
ELISA-4	0.26	20.0, 100.0	30.0, 100.0	55.6, 100.0	87.5, 100.0	100.0, 100.0	100.0, 100.0	100.0, 100.0	100.0, 100.0	0.822

Table 5
Detection rate of positive samples from pigs with unknown HEV exposure collected in 18 farms from three production stages.

Assay	Nursery pigs	Finisher pigs	Breeding age pigs
	3–10 weeks of age	10–15 weeks of age	Older than 6 months
ELISA-1	27/45	17/21	24/24
ELISA-2	27/45	19/21	24/24
ELISA-4	42/45	20/21	24/24

3.2. Field samples from pigs with unknown HEV exposure

Based on the recommended cutoff criteria and dependent on the assay used, 60.0–93.3.4% of the nursery pigs were seropositive for HEV (Table 5). In finisher pigs the prevalence of HEV seropositive pigs ranged from 81.0% to 95.2% depending on the assay. In breeding herds 100% of the animals tested were positive for anti-HEV antibodies by all three assays (Table 5). ELISA-1 and ELISA-2 had substantial agreement (0.63 ± 10) and agreement between ELISA-1 and ELISA-4 (0.19 ± 0.11) and ELISA-2 and ELISA-4 (0.17 ± 0.10) were slight.

4. Discussion

The results indicate that four of the five assays evaluated in this study (ELISA-1, ELISA-2, ELISA-4 and the LIA) could discriminate reliably between the absence and presence of anti-HEV antibodies with adequate sensitivity and specificity suggesting that they are effective for detecting anti-HEV antibodies in pigs known to be exposed to HEV. The samples used in this study were from pigs infected experimentally with HEV genotype 3 of human origin, HEV genotype 4 of human origin, or HEV genotype 3 of swine origin. In addition, field samples from pigs with unknown HEV infection were tested. Pigs with no HEV infection were also included (gnotobiotic and conventional pigs) in order to ensure that a portion of the samples were true negative for assay validation.

The results on the experimental samples showed that all assays detected antibodies against HEV with high specificity and varying sensitivity. This was related to the dpi analyzed. In humans it has been determined that anti-HEV IgM antibodies appear in the early phase of clinical illness and last 4–5 months whereas the IgG response develops shortly thereafter and can remain high for up to 4.5 years after the acute phase of the disease (Dawson et al., 1992). In previous experimental trials using pigs, it was determined that a detectable humoral immune response against swine HEV is present 2–3 weeks after experimental infection (Kasorndorkbua et al., 2003; Bouwknegt et al., 2009; Casas et al., 2009a). As expected, samples that were collected from pigs infected with HEV experimentally were negative with all assays at 7 dpi. Detection of anti-HEV antibodies started at 14 dpi with a sensitivity of 0% (ELISA-3), 11.1% (ELISA-1, ELISA-2, LIA), and 22.2% (ELISA-4). In the later stages of infection, detection of positive animals was very similar between the assays and there was no difference in identifying positive pigs infected with HEV genotype 3 or HEV genotype 4. These findings are not surprising, as a porcine or human origin based recombinant ORF2 antigen ELISA was found capable of detecting seroconversion to HEV genotypes 1–4 following experimental infection of primates (Engle et al., 2002). Moreover, it was found that the antigens were interchangeable with respect to their ability to detect human and swine anti-HEV antibodies (Engle et al., 2002). Similarly, when an ELISA based on a human HEV genotype 1 ORF2 antigen and an ELISA based on a swine HEV genotype 4 ORF2 antigen were used and compared, identical results were obtained (Arankalle et al., 2007). An ELISA based on human HEV genotype 3 ORF2 antigen expressed by the *Trichoplusia ni* larvae was found to detect anti-HEV IgG in human and swine sera with good specificity and sensitivity (Jiménez de Oya et al., 2009).

Variable numbers of antibodies are formed against selected antigens and the selection of the ideal coating antigen is very important. For example, it has been shown that antibodies directed against putative neutralizing epitopes were detected inconsistently in HEV seropositive humans and rhesus monkeys (Zhou et al., 2004). ELISA-1 and ELISA-2 were coated with similar coating antigens (HEV genotype 1 based with the addition of a HEV genotype 3 ORF2 C-terminal based antigen for ELISA-2) and had identical detection rates. While assays that generate continuous data are not comparable directly with assays that generate dichotomized test results, the LIA, ELISA-1 and ELISA-2 had similar temporal results. Interestingly, ELISA-3, which was coated solely with HEV genotype 3 antigens detected fewer positive samples especially at early time points, 14, 21 and 35 days, after HEV infection compared to the other assays. The four major mammalian HEV genotypes 1–4 shared >85% amino acid sequence identity in the capsid protein; however, the majority of the amino acid divergences were found in the N-terminals 111 amino acid residues (Mori and Matsuura, 2011). This may explain the differences seen between ELISA-1 and ELISA-2 (based on HEV genotype 1) compared to ELISA-3 (based on HEV genotype 3). A comparison of the exact locations and amino acid compositions of the coating antigens used in the present study was not possible as the antigen information from the assays that were produced commercially was considered proprietary.

ELISA-4 was coated with a recombinant truncated antigen of the human HEV genotype 1 isolate SAR-55 located in the middle region of ORF2 and spanning amino acids 452-617. Compared to ELISA-1 and ELISA-2 as well as the LIA, ELISA-4 showed a slightly higher detection rate at 14 dpi. These results are not surprising as it was found previously that the truncated ORF2 protein contains the most immunogenic site on ORF2 when compared to the C-terminus, N-terminus and varying ORF3 peptides (Zhou et al., 2005). Besides coating antigen properties, other factors may contribute to different outcomes and include coating density, incubation time, differences in reagents such as buffers, and others. Finally, the nature of the secondary detection antibody (anti-swine IgM versus anti-swine IgG) also contributes to differences in detection rates. In this study, all assays except the LIA utilized anti-swine IgG antibodies as secondary antibody. Interestingly, the LIA, which detected both anti-HEV IgG and IgM antibodies, was not found to be more sensitive on the experimental samples evaluated compared to any of the other assays.

For comparison of detection rates among field samples of unknown HEV infection, only three of the five assays were used. The LIA was not used as this test is more expensive and time consuming when testing high numbers of samples and would therefore not be an ideal assay for use in veterinary diagnostic labs. ELISA-3 was also excluded due to its poor performance on experimental samples compared to the remaining assays. Overall there was a higher prevalence of anti-HEV-antibodies in breeding herds compared to finisher and nursery pigs. This is in agreement with a longitudinal study of swine HEV in Spanish farrow-to-finish pig herds where an increase in prevalence of anti-HEV-IgM in pigs at slaughter age was observed which was in contrast to 13 week old pigs where anti-HEV-IgM were first detected (Casas et al., 2011). Furthermore, the seropositive rate was 82.5% in sows, 53.9% in finisher pigs, and 63.4% in nursery pigs in eastern China (Zhang et al., 2008).

ELISA-1 and ELISA-2 showed substantial agreement (kappa = 0.63) which was likely due to utilizing similar antigens. Agreement of ELISA-1 and ELISA-2 with ELISA-4 was only slight according to kappa values; however, this was not surprising as most of the differences in detection of HEV positive samples by these two assays occurred in the nursery stage of production.

At this particular production stage, anti-HEV antibodies in the piglets could have been acquired passively (IgG) or may have been due to an active HEV infection (mixture of IgM and IgG). All the assays used for this part of the study (ELISA-1, ELISA-2 and ELISA-4) utilized anti-swine IgG in the secondary detection step and therefore should have only detected anti-HEV IgG.

5. Conclusions

Four of the five enzyme immunoassays tested in this study could be used for the detection of anti-HEV antibodies in pigs. The four assays demonstrated similar diagnostic performance; however, in field samples with unknown HEV infection ELISA-4 was found to perform better in nursery pigs. Interestingly, modified versions of two of the assays (ELISA-1 and LIA) are available currently for detection of anti-HEV antibodies in humans and in this study, prototypical uses of these enzyme immunoassays with swine sera were utilized by replacing the anti-human IgG conjugate with anti-swine IgG. ELISA-2 with anti-swine IgG will soon be available commercially for use in veterinary diagnostic laboratories on serum and meat-juice samples. A species-independent ELISA produced commercially that is capable of detecting anti-HEV antibodies in human and several animal sera including pigs, has been described (Utsumi et al., 2011) and is available in some areas of the world; however, the adaptation of assays utilized in the current study, which were developed specifically for the use in pig samples, represents a potentially greater opportunity for veterinary diagnostic laboratories to integrate HEV serology into their laboratories and utilize serological data to better assess the epidemiology and importance of HEV in swine production systems.

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