Journal of Virological Methods 193 (2013) 278–283

Contents lists available at ScienceDirect

Journal of Virological Methods

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

Development of a fluorescent microbead-based immunoassay for the detection of hepatitis E virus IgG antibodies in pigs and comparison to an enzyme-linked immunoassay

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Article history:
Received 28 March 2013
Received in revised form 31 May 2013
Accepted 6 June 2013
Available online 14 June 2013

Keywords:
Hepatitis E virus (HEV)
Swine
Serology
Enzyme-linked immunosorbent assay (ELISA)
Fluorescence microbead-based immunoassay

A B S T R A C T

Swine hepatitis E virus (HEV) is a zoonotic virus and pigs are considered as an important reservoir. Swine HEV infection is widespread and most pig herds are infected. Humans can be infected with swine HEV via consumption of undercooked pork or through direct contact with infected pigs. To minimize the risk of zoonotic transmission, sensitive tools to assess the HEV infection status of pigs and pork products are needed. The objective of this study was to develop a fluorescent microbead-based immunoassay (FMIA) for the detection of IgG antibodies against swine HEV and compare it to an *in-house* enzyme-linked immunoassay (ELISA). Three sets of samples were utilized: (A) samples from pigs infected experimentally with different strains of HEV (positive controls, $n = 72$), (B) samples from known HEV-negative pigs (negative controls, $n = 62$) and (C) samples from pigs of unknown HEV infection status ($n = 182$). All samples were tested by both ELISA and FMIA. The results on the experimental samples with known HEV exposure indicate that both assays have a specificity of 100% while the sensitivity ranges from 84.6% (ELISA) to 92.3% (FMIA). The overall prevalence of HEV IgG antibodies in field samples from pigs with unknown HEV exposure was 21.9% (40/182) for the ELISA and 21.4% (39/182) for the FMIA. The two assays had an almost perfect overall agreement (Kappa = 0.92).

1. Introduction

Hepatitis E virus (HEV) is the causative agent of hepatitis E, a viral infection of the liver which can range from a mild, acute, self-limiting hepatitis to fulminant hepatitis with mortality rates ranging from 0.2 to 4.0% in humans (Kamar et al., 2012). The mortality rate in pregnant women infected with HEV can reach up to 30% (Kumar et al., 2013). Outbreaks of human hepatitis E are often associated with consumption of contaminated water or water supplies via the fecal-oral route. Other routes of transmission include foodborne transmission through the consumption of contaminated shellfish, animal meats and meat products, infection via infected blood products or organs (Kamar, 2011; Matsubayashi et al., 2008), and vertical transmission. The importance of foodborne transmission was emphasized in recent years, as more and more sporadic cases of acute hepatitis E have been detected in industrialized nations in patients with and without a history of traveling to endemic areas.

Hepatitis E virus is the only member of the genus *Hepevirus* belonging to the family *Hepeviridae* (Meng, 2011). HEV is a non-enveloped, single-stranded, positive sense RNA virus with a diameter of 27–34 nm and a genome size of approximately 7.2 kb (Emerson and Purcell, 2003). The virus contains both 5′ and 3′ untranslated regions and three discontinuous open reading frames (ORF). ORF2 overlaps ORF3 but neither overlaps ORF1 (Huang

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http://dx.doi.org/10.1016/j.jviromet.2013.06.010

et al., 2007). HEV ORF1 encodes non-structural proteins like the RNA-dependent RNA polymerase, methyltransferase, RNA helicase and cysteine protease (Karpe and Meng, 2012; Koonin et al., 1992). ORF2 encodes the capsid protein, while ORF3 encodes a multifunctional small phosphoprotein (Chandra et al., 2011; Kenney et al., 2012; Tam et al., 1991; Zafrullah et al., 1997).

At least four known major genotypes and two putative genotypes of mammalian HEV have been identified so far. HEV genotypes 1 and 2 have only been identified in humans so far and are frequently associated with human epidemics in Asia, Africa and Mexico. Genotypes 3 and 4 circulate in the human and other animal species including pigs. In addition to humans and pigs, genotype 3 HEV has also been identified from deer, mongoose, rabbit and rat (Cossaboom et al., 2011; Meng, 2010). While HEV genotype 3 has a demonstrated global prevalence (Dalton et al., 2008; Meng et al., 1997), genotype 4 HEV has been reported in sporadic human cases in Asia and Europe (Garbuglia et al., 2013; Hakze-van der Honing et al., 2011; Nishizawa et al., 2003; Tesse et al., 2012). Novel strains of HEV belonging to putative new genotypes have recently been identified from rats in Germany and the United States (Johne et al., 2010; Purcell et al., 2011), and from wild boars in Japan (Sato et al., 2011). Currently, swine HEV infection is widespread in pork producing countries and most pig herds are positive globally (Chandler et al., 1999; Rose et al., 2011; Takahashi et al., 2005).

Shedding of the virus occurs through feces and has been demonstrated for up to eight weeks (Feagins et al., 2008) while viremia is transient and ranges from one to six weeks in infected pigs under experimental conditions (Bouwknegt et al., 2009; Feagins et al., 2008). Anti-HEV IgG antibodies develop approximately two to four weeks after HEV infection (Bouwknegt et al., 2009; Feagins et al., 2008). During natural HEV infections, anti-HEV IgM antibodies are typically first detected around 12 weeks of age (3 weeks after the viremia onset) and are present for five to seven weeks (de Deus et al., 2008). In a recent study, 7% (36/516) of meat juice samples collected from pigs at slaughter were positive for IgM antibodies against HEV (Wacheck et al., 2012). Anti-HEV IgG antibodies are first detected approximately 3 weeks after the IgM rises and pigs remain seropositive until slaughter (de Deus et al., 2008) at approximately 6 months of age. Compared to detection of viral RNA by conventional reverse transcription polymerase chain reaction (RT-PCR) or real-time RT-PCR, serology is rather inexpensive and less technical and therefore ideal for screening of large numbers of samples and for diagnostic application in developing countries. Another advantage of diagnosis by serology rather than by PCR detection of viral RNA is that the length of HEV viremia in pigs is transient, thus the window for detecting HEV nucleic acids in blood is very short.

The fluorescent-microbead immunoassay (FMIA) is an evolving novel diagnostic tool. The FMIA utilizes microspheres that are identified by a specific spectral signature. The ability to uniquely identify various groups of microspheres creates the capability to couple a unique microsphere group with a specific antigenic target and to test for multiple antigenic targets in one reaction. The number of antigenic targets that can therefore be placed in a single reaction is limited only by the number of unique spectral target groups available. Indications are that FMIA tests can improve sensitivity compared to conventional assays such as ELISAs (van Gageldonk et al., 2008). Because the assay also offers the ability to detect more than one pathogen in a single reaction, the savings in cost of time, labor and reagents could be substantial. The development of a sensitive and specific FMIA for the detection of swine HEV IgG antibodies is described and compared to an *in-house* anti-HEV IgG ELISA using samples from pigs infected experimentally with HEV and field samples from pigs with unknown HEV status.

2. Materials and methods

2.1. Experimental design and biological specimens utilized

2.1.1. Experimental design

Serum samples from pigs infected experimentally with HEV and pigs with unknown HEV status were evaluated for presence of anti-HEV IgG antibodies by both ELISA and FMIA. Sera collected prior to the HEV experimental infection and sera from the sham-inoculated group were used as negative control. The *in-house* ELISA used is a modification of an ELISA described previously (Meng et al., 1997, 1998; Zhang et al., 2011), while the development and evaluation of the FMIA is described here. Both detection assays are based on the same HEV immunodominant ORF2 antigen as described in Sections 2.2 and 2.3. All samples utilized in this study were tested in duplicate. The experimental designs for the animal studies conducted previously were approved by the Iowa State University Institutional Animal Care and Use Committee and by the Iowa State University Institutional Biosafety Committee.

2.1.2. Experimental samples

One-hundred-and-thirty-four serum samples from 36 specific-pathogen-free (SPF) pigs from two separate HEV studies (Feagins et al., 2008; Sanford et al., 2012) were used in the present study (Table 1). In the first study, 2 pigs were intravenously inoculated with 1 ml sterile phosphate buffer solution (PBS) and served as negative controls, 2 pigs were inoculated with 1 ml of a genotype 3 human HEV strain (strain US-2), and 2 pigs received 1 ml of a genotype 4 human HEV strain (strain TW6196E). Blood samples were collected two days before inoculation and weekly thereafter for eight weeks (Feagins et al., 2008). To verify successful challenge in the pigs infected experimentally, seroconversion was determined by using an ELISA described previously (Meng et al., 1998). Specifically, seroconversion started at day post-inoculation (dpi) 14 and at dpi 28 all inoculated pigs had seroconverted to IgG anti-HEV (Feagins et al., 2008). As expected, all pigs in the control group inoculated with PBS remained seronegative throughout the study (Feagins et al., 2008). A total of 22 negative control samples (all samples collected on dpi 0 and samples from the sham-inoculated negative control group from dpi 0 to 56) and 32 samples (dpi 7–56) from pigs infected experimentally were tested (Table 1).

Serum samples utilized from the second study included 6 negative control pigs (vaccinated with PBS at dpi −28 and −14 and challenged with PBS at dpi 0), 6 positive control pigs (vaccinated with PBS at dpi −28 and −14 and challenged with HEV at dpi 0), 16 vaccinated and challenged pigs [vaccinated with swine HEV capsid antigen ($n=5$), rat HEV capsid antigen ($n=5$), or avian capsid antigen ($n=6$) at dpi −28 and −14 and challenged with HEV at dpi 0] (Sanford et al., 2012). The HEV challenge was conducted intravenously with a genotype 3 swine HEV (strain Meng). Blood samples were collected 28 days before challenge and weekly thereafter for eight weeks (Sanford et al., 2012). Successful infection by evidence of seroconversion was determined by an ELISA described previously (Meng et al., 1998). At dpi 21, all 6 positive control pigs had seroconverted to IgG anti-HEV and all vaccinated pigs had seroconverted at dpi 0 and remained seropositive through the end of the study (Sanford et al., 2012). As expected, all pigs in the control group inoculated with PBS remained seronegative throughout the study (Sanford et al., 2012). A total of 40 negative control samples from 28 pigs and 40 samples from the positive control group ($n=24$; dpi 7–28) and vaccinated and challenged groups ($n=16$; dpi 28) were tested (Table 1).

2.1.3. Field serum samples

A total of 182 field serum samples were arbitrarily selected from routine submissions to the Iowa State University Veterinary