A serological immunoassay for Hepatitis E virus (HEV) diagnosis based on genotype 3 Open Reading frame-2 (ORF-2) recombinant proteins produced in *Trichoplusia ni* larvae.

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Hepatitis E virus (HEV) is a major cause of acute hepatitis in humans and strains of genotypes 1 and 2 are endemic in many regions with sub-optimal sanitary conditions. In many industrialized countries, HEV strains of genotype 3 are highly endemic in swine and an increased number of autochthonous HEV infections by genotype 3 strains have been reported lately. Serological studies of HEV infection are often conducted with commercial assays based on peptides and recombinants proteins of HEV genotype 1 and 2 strains. In some patients with proven HEV genotype 3 infections, these assays failed to detect specific antibodies and they are not applicable or validated for detection of anti-HEV antibodies in swine. To elucidate thru hepatitis E incidence in regions where HEV genotype 3 infections can be expected, and to study HEV seroprevalence in swine, new tools with broad specificity to all genotypes of hepatitis E are needed. We present the expression and partial characterization of recombinant HEV genotype 3 ORF-2 proteins and their usefulness as diagnostic antigens in detecting anti-HEV antibodies in humans and swine with proven HEV genotype 3 infections. The recombinant antigens were produced at a relatively high yield and low cost upon infection of *Trichoplusia ni* larvae with recombinant baculoviruses expressing recombinant HEV genotype 3 ORF-2 proteins. The ELISA based on the recombinant proteins showed a good specificity and sensitivity for anti-HEV genotype 3 IgG detection in human and swine sera. These recombinant HEV genotype 3 ORF-2 proteins might be added to diagnostic kits containing HEV genotype 1 and 2 antigens to develop a broadly sensitive new diagnostic tool to diagnose hepatitis E.
Hepatitis E virus (HEV) causes an acute liver disease that is endemic in humans mainly in regions of Asia and Africa where sanitary conditions and water supplies are inadequate (8, 24, 32). The majority of HEV epidemics in Asia and Africa have been caused by genotype 1 strains. Genotype 2 has caused epidemics in Mexico and some regions of Africa. In China, epidemics were caused mainly by genotype 1, but recently genotype 4 has become the dominant cause of sporadic hepatitis E. Genotype 3 is widely distributed and is the cause of sporadic cases worldwide.

HEV is a spherical, non-enveloped virus of around 32-34 nm in diameter, whose genome is a single-stranded RNA molecule of positive polarity of approximately 7.2 Kb in length, containing 3 overlapping open reading frames (ORF) and a 3’ poly (A) tail (8, 24, 32). ORF-2, which encodes the viral capsid protein and harbors immunoreactive epitopes, is synthesized as a large glycoprotein precursor that is cleaved into the mature protein. HEV infecting mammals have been classified into four different genotypes, 1, 2, 3 and 4 (8, 20, 24, 32). HEV strains characterized from regions where only sporadic cases of hepatitis E were reported represent a diverse cluster of sequences largely grouped into genotype 3 (gt3).

Since in many industrialized countries an increased number of autochthonous HEV infections by gt3 strains have been reported lately (4, 6, 11, 12, 17, 23, 25, 31), HEV gt3 is now considered an emerging pathogen. Genotype 3 strains detected in humans and swine from the same geographical areas are genetically closely related (20, 23, 30, 31). This fact, together with the description of cases of acute hepatitis in people who ate uncooked deer meat or liver from pork or wild boar (19, 28, 33), and the recent detection of HEV-RNA and infectious virus in commercial pig livers sold in local
Since viremia is limited to the acute phase of the infection, the diagnosis of the disease is mainly dependent on serology, especially when only convalescent sera are available. The lack of an efficient cell culture system has hampered the use of natural viral antigens for serological diagnosis; therefore, different synthetic peptides and recombinant polypeptides expressed in various heterologous systems have been assayed for specific antibody detection (8, 24, 32). Currently, commercially available kits are designed to detect anti-HEV in human sera, or plasma, and include short fragments of ORF-2 and ORF-3 of gt1 and gt2, but not of gt3, the most prevalent in industrialized countries in swine and humans (20, 24, 32). Various reports indicate that commercial assays sometimes failed to detect specific antibodies in sera from patients with proven HEV gt3 infections (7, 10, 35). Therefore, the number of autochtonous HEV infections in industrialized regions may have been underestimated (18). These suggest the need for sensitive, specific, reproducible, standardized and validated assays to detect serological markers of HEV gt3 infection.

We describe a relatively simple, high yield and low cost production of genotype 3 HEV-ORF2 recombinant proteins in Trichoplusia ni (cabbage looper) larvae. The expressed proteins are recognized by human and swine sera and might be suitable diagnostic reagents for detection of HEV antibodies.

MATERIALS AND METHODS.

Protein expression and analysis of recombinant ORF-2 proteins in insect larvae
Construction of recombinant baculoviruses, Bac1-ORF2r and Bac1-Δ-ORF2r, expressing the entire gt3 histidine-tagged ORF-2 and a truncated form of it lacking the 111 first aa at the N-termini, respectively, and their production upon infection of Sf21 insect cells has been reported by us recently (13). To obtain both recombinant proteins at a larger scale in an easy and economic way, Trichoplusia ni (T. ni) larvae were reared under level 2 safety conditions as previously described (2, 22). Briefly, larvae were sedated by incubation on ice for 15 min, injected with different doses (1 to 3x10⁶ pfu/larvae) of recombinant baculoviruses (Bac1-ORF2r or Bac1-Δ-ORF2r) near the proleg (forward along the body cavity), kept in growth chambers at 28º C and, then, harvested at different times post-inoculation and frozen immediately at ~20º C until processed as described (22).

Protein analyses were carried out by Western blot. Briefly, processed protein larvae extracts were resolved in 10% SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Richmond, CA, USA), which were then blocked for 1 hour with PBS-0.05% Tween-20 (PBST) containing 5% skim milk. The membranes were subsequently incubated at 4º C overnight with a commercial mouse anti-histidine monoclonal antibody (Clontech Laboratories, Inc., Mountain View, CA, USA) and then with a goat anti-mouse horse-radish-peroxidase (HRP) conjugated secondary antibody (Sigma-Aldrich, St. Louis, MO, USA), or with either positive and negative human, or swine, sera before the addition of goat anti-pig, or goat anti-human, IgG-specific HRP conjugated immunoglobulin (MorphoSys AG, Martinsried-Plangg, Germany) as secondary antibodies. Finally, bands were detected by addition of 2.5 mM luminol-0.4 mM p-cumaric acid-100 mM Tris-HCl pH 8.5-0.018 % H₂O₂. Larvae extracts obtained upon inoculation with an irrelevant baculovirus, and similarly processed, were used as control.
Partial purification of recombinant ∆-ORF2r protein.

Recombinant HEV ∆-ORF2r protein from larvae extracts was partially purified in native form with Imidazol (Merck, Mollet del Vallés, Spain) by means of their histidine tails using His-GraviTrap columns (GE Healthcare, Alcobendas, Spain) following manufacturer’s instructions. A 10, 20 and 500 mM Imidazol concentration was used in the binding, washing and elution buffers. Protein concentration was determined by gel densitometry using known amounts of purified bovine serum albumin (BSA) as control. Integrity of partially purified proteins was analyzed by Western blot as described above.

∆-ORF-2-based ELISA.

Partially purified ∆-ORF2r protein was tested as ELISA antigen. Microplates (Polysorp, Nunc, Roskilde, Denmark) were coated with 50 µl/well of serial dilutions of purified protein (2.5-80 ng/well) in 50 mM carbonate/bicarbonate buffer, pH 9.6, and incubated overnight at 4° C. All following incubations were carried out for 1 h at 37° C under constant shaking. After washing thrice with PBST, plates were incubated with 100 µl/well of blocking solution (PBST-3% skim milk); then, serum diluted in blocking buffer was added in duplicate wells. Plates were washed again with PBST before addition of 50 µl/well of goat horse-radish peroxidase conjugated anti swine, or anti human, IgG secondary antibody diluted 1:30.000 (0.03 µg/ml) and 1:10.000 (0.06 µg/ml), respectively (MorphoSys, Martinsried-Plangg, Germany). After the incubation with the secondary antibody, plate wells were washed three times before 50 µl/well of substrate solution (OPD-0.056% H2O2) was added. Plates were incubated (dark, room temp, 10 min) and then the reaction was stopped by addition of 50 µl/well of 3N H2SO4.
and read at 495 nm in an ELISA microplate reader (Tecan Genios Ag., Vienna, VA, USA). Previously characterized negative and positive swine and human (5, 27) sera were included in the assay as controls.

In addition, for comparison purposes, a commercial kit (Genlabs Diagnostics Inc, CA, USA) for specific detection of anti-HEV IgG antibodies in human sera was also used following manufacturer’s instructions. The procedure was modified when swine sera were tested, so that, a conjugated anti-swine IgG was used as secondary antibody as describe above. This ELISA is based on recombinant proteins from ORF-2 and ORF-3 genes from gt1 and gt2 HEV strains expressed in E. coli. The cut-off for the commercial assay was described by the manufacturer and Absorbance values were expressed as P/N (Absorbance value of the test sample/Absorbance value of the negative control + 0.5). A P/N value > 1 was considered positive.

A battery of previously characterized randomly selected 40 human and 66 swine sera were used for comparison purposes. Swine sera were kindly provided by Dr. M. Martín and had been previously tested using a SAR55 (gt1) based ELISA (27). In all cases in which HEV-RNA could be detected in these samples, it was a gt3 strain (27). Most of the human sera tested were kindly provided by Dr. M. Buti, and have been assayed previously with the same commercial test used here. They are from a non-endemic area (Catalonia, Spain), and the infecting genotype is not known (5). Six human sera (10) were also from a non-endemic area (the Netherlands) and had been previously tested with two commercial kits, the one used here and the IgG/IgM Recomblot (Mikrogen GmbH, Neuried, Germany). In five cases the patients were infected by a gt3 strain and in one case by a gt1 stain (10).
RESULTS

Expression of HEV genotype 3 ORF-2r proteins in Trichoplusia ni larvae.

Inoculation of T. ni larvae with Bac1-ORF2r resulted in expression of a 65-68 kDa protein that appeared as the major species recognized by an anti-His monoclonal antibody (Figure 1A). The same species, as well as some minor cell associated immunoreactive proteins, was observed with anti-HEV IgG positive swine (Figure 1B) and human sera (Figure 1C). These specific proteins were not present in uninfected larvae (mock) or in extracts obtained from larvae infected with a baculovirus expressing an irrelevant protein fused to an His tail (Bac-Irr-His). Likewise, Western blot staining using anti-HEV IgG negative pig or human sera did not detect any specific band of the expected mass (data not shown).

On the other hand, infection of T. ni larvae with Bac1-∆-ORF2r yield a species of 65-68 kDa, similar to that observed upon infection with the baculovirus that expresses the full-length protein, as well as some other protein species of lower molecular mass, which were also recognized by swine and human sera (Figure 1A-C).

HEV specific proteins obtained after infection with both recombinant baculoviruses, Bac1-ORF2r and Bac1-∆-ORF2r, were difficult to dissolve, but the yield of soluble protein was higher when the truncated form was expressed, as shown in figure 1.

Optimization of the production and purification of HEV genotype 3 ∆-ORF-2 protein.

To optimize protein expression, T. ni larvae were inoculated with different doses (1 to 3x10^6 pfu/larvae) of Bac1-∆-ORF2r recombinant baculovirus and processed at day 1 to 3 post inoculation. Optimal protein yields were obtained in larvae infected with a
multiplicity of infection of $1.5 \times 10^6$ pfu/larva that were harvested three days after infection (data not shown).

HEV $\Delta$-ORF-2 protein from recombinant baculovirus infected larvae extracts was partially purified in its native form as described in material and methods. As displayed in figure 2, Western-blot analyses with an anti-His monoclonal antibody, or with positive swine or human sera, show the partially purified protein plus some degraded and/or proteolysed products of lower molecular masses.

**ELISA based on the $\Delta$-ORF-2 expressed proteins**

To optimize the ELISA, and since both recombinant proteins, ORF-2 and $\Delta$-ORF-2 expressed in insect cells reacted similarly in ELISA (13), different concentrations of partially purified $\Delta$-ORF2r recombinant protein were tested with previously characterized positive and negative swine and human sera as described in material and methods. Optimal conditions of antigen concentration and serum dilution were obtained with 15 ng of antigen/well (Figure 3) and with a 1/100 serum dilution (Figure 4). The same panel of sera was tested with partially purified proteins obtained from larvae inoculated with an irrelevant recombinant baculovirus. As shown in figure 4, HEV positive sera produced $A_{495}$ values significantly higher in Ag positive than in Ag negative coated wells, while negative sera showed similar $A_{495}$ values in both cases and these values were significantly lower than that of positive serum samples.

**Criteria for determining positive reactivity in anti-HEV ELISA.**

To establish the cut-off of the assay, eight previously characterized negative swine and human sera (5, 27) with $A_{495}$ values below 0.15 (between 0.05-0.14) were pooled and used as controls. These values were similar to those obtained with purified
proteins from extracts from larvae infected with an irrelevant baculovirus. None of these sera reacted in Western blot with the partially purified Δ-ORF2r recombinant protein. Absorbance values were expressed as P/N (Absorbance value of the test sample/Absorbance value of the negative control). Then, the cut-off value was established as a P/N ≥ 2.5. Samples above the cut-off value were considered positive.

Comparison of Δ-ORF2r based ELISA with a commercial kit.

A battery of 40 human and 66 previously characterized swine sera (5, 10, 27) were tested by ELISA using the partially purified Δ-ORF2r protein as antigen and the commercial kit, although, in the case of swine sera the commercial procedure had to be modified to use a conjugated anti-swine IgG as described in material and methods and, therefore, swine results should be interpreted with caution. A comparison of the ELISA results is shown in figure 5. Concordance between both assays was 80% (kappa value=0.60) and 92.5% (kappa value=0.82) for swine and human sera, respectively (Table 1).

To further confirm the accuracy of the assays, all samples providing discordant results, and randomly selected samples providing concordant results, were further analyzed by Western blot. None of the 12 swine and 6 human sera tested, that were negative with both assays, reacted in Western blot, while all swine (n=17) and human (n=3) sera tested, which were positive in both ELISAs, were also positive by Western blot. Among those samples that were positive in the in house test and negative in the commercial assay, all 8 swine sera reacted positively in Western blot, as well as did 2 of the 3 human sera. On the other hand, 4/5 swine sera that reacted positively by the commercial kit and negatively by the in house assay were also negative by Western blot.
Since Western blot is more sensitive than ELISA (Herremans et al., 2007), we used it as gold standard. Thus, based on this data, and comparing to the Western blot results, the sensitivity for detecting anti-HEV gt3 antibodies of the in house assay was 100% and 96.9%, for human and pig sera, respectively, while that of the commercial kit was 83.3% and 75%, respectively. Likewise, the specificity of the in house test was 96.4% and 100% for human and pig sera, respectively, while that of the commercial kit were 96.4% and 88.2%. So that, when human sera were tested, one false positive (2.5%) and none false negative sera were observed with the in house test, while the commercial kit resulted in detection of one false positive (2.5%) and 2 (4.5%) false negative samples. Similarly, when swine sera were assayed, none false positive and one (1.5%) false negative results were recorded with the ELISA developed here, while the commercial kit produced 4 (6%) false positive and 8 (12%) false negative results.

DISCUSSION

We have characterized ORF-2 recombinant proteins from HEV gt3, produced at a relative low cost and quite large scale, upon infection of Trichoplusia ni larvae with recombinant baculoviruses. Partially purified ∆-ORF2r protein has been tested for its usefulness as diagnostic antigen.

We have previously shown (13) that infection of Sf21 cells with Bac1-ORF2r recombinant baculovirus produced a major protein of around 78 kDa (figure 1D). Here we show that when the same recombinant baculovirus was used to infect T. ni larvae the major species detected was of 65-68 kDa, together with some cell associated immunoreactive proteins. These species were similar to that observed when the ∆-ORF-2 protein was expressed upon inoculation of Bac1-∆-ORF2r recombinant baculovirus.
These lower proteins were most probably due to the proteolytic processing of the complete ORF-2 that occurs by truncation of the N-terminus of the protein (15, 26, 29). Although the reason for this different expression pattern of full-length ORF-2 in Sf21 cells and T. ni larvae is unclear, a different processing of HEV ORF-2 protein expressed either in suspension or monolayers of insect cells have been described (34), as well as a different processing of ORF-2 in Sf-9 and in Tn5 cells (14, 15). In addition, previous studies have also reported the production of multiple immunoreactive proteins with a wide size range in insect cells infected with recombinant ORF-2 baculoviruses (1, 15, 26, 29). In any case, the specificity of the expressed proteins reported here was confirmed by Western blot analysis using well characterized human and pig sera.

Partially purified Δ-ORF-2 protein was used to set up an ELISA test. The conditions applied to the production of HEV gt3 antigens in T. ni larvae allows yields between 0.2-0.3 mg of Δ-ORF2r protein per larvae to be obtained in 72 h. The amount of partially purified protein used in the ELISA assay was ≈15 ng/well, which indicates a yield of an estimated 20,000 individual ELISA determinations from a single larva.

The accuracy of the recombinant Δ-ORF-2 antigen as diagnostic tool was further confirmed by comparison of the in house ELISA with a broadly used commercial kit. Our results showed a concordance between both assays of 92.5% when human sera were tested. Likewise, a concordance of 80% was observed when swine sera were analyzed, although in this case data should be taken with caution since the kit had to be slightly modified to test these sera. A similar concordance (85%) between three different commercial kits tested with human samples obtained during an outbreak has been reported (21). Likewise, ELISA concordance for human samples between the commercial kit tested here and either human HEV gt1 or swine HEV gt4 ORF-2 recombinant proteins has been shown to be 90.3% and 92%, respectively (1).
Taking the Western blot as a gold standard, our results showed a fairly good specificity of the in house and commercial assays tested here (96.4%) for human sera and a higher one of the in house test for pig sera (100% vs 88.2%, respectively). Specificities for IgG detection in human sera (92%-93%), similar to that found here and lower than that provided by the manufacturers of the commercial kit have been previously reported (10, 16). Likewise, the sensitivity observed for pigs (96.9%) and human (100%) samples was also fairly good with the in house assay and higher than that of the commercial kit (75% and 83% for human and pig sera, respectively). Figures found here for human sera with the commercial kit are also in agreement with the previously described sensitivity (86%-88%) reported for gt3 infected patients from non endemic areas (10, 16). However, we cannot discard that some of the swine sera that resulted positive with the commercial test, but did not recognize HEV ORF-2 gt3 recombinant proteins in ELISA or Western blot, could be due to the presence of antibodies against for example ORF-3. In any case, and although further analysis of patients infected with different genotypes will be needed to fully confirm it, our results indicate that the developed ELISA could be useful to the overall detection of HEV infection.

In conclusion, we have successfully expressed and partially characterized ORF-2 HEV gt3 proteins upon inoculation of recombinant baculoviruses in T. ni larvae. This system allows for a relatively large scale production of the proteins at low cost. We show that the recombinant HEV gt3 capsid protein can be used as antigen in an ELISA and that it can be used to detect anti-HEV gt3 IgG in humans and swine.
ACKNOWLEDGMENTS.

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REFERENCES


food-borne, as suggested by the presence of hepatitis E virus in pig liver as food.


FIGURES TO THE LEGENDS

Figure 1. Western blot analysis of HEV ORF-2r and Δ-ORF-2r proteins. Western blots were stained with: A and D) an anti-His Mab; B) Swine serum; and C) Human serum. Lanes 1, 4 and 7 Bac1-Δ-ORF-2 infected larvae extracts. Lanes 2, 5 and 8 Bac1-ORF2r infected larvae extracts. Lanes 3, 6 and 9 mock infected larvae extracts. Lanes 10, 11 and 12 Bac1-Δ-ORF-2, Bac1-ORF2r and mock infected Sf21 cells extracts. Molecular weight markers are shown. Black and white arrows mark the position of the HEV ORF-2r and -Δ-ORF-2r proteins, respectively.

Figure 2. Analysis of partially purified Δ-ORF2 HEV recombinant protein obtained from infected T. ni larvae. Lines 1 and 2; Coomassie blue stained SDS-PAGE of Bac1-Δ-ORF2r and mock infected T. ni larvae partially purified extracts, respectively. Lines
3-4, 5-6, and 7-8 Western blot analysis of the same partially purified extracts revealed with an anti-His monoclonal antibody, a swine serum and a human serum, respectively.

**Figure 3.** Optimization of antigen concentration. Previously characterized positive (solid circles) and negative (open squares) swine (left panel) and human (right panel) sera were tested by ELISA against two-fold serial dilutions (2.5 to 80 ng/well) of partially purified ∆-ORF2r recombinant protein (A) or an irrelevant protein (B) obtained from extracts of infected larvae, as described in material and methods. Data in each panel are shown as the average A₄₉₅ of 4 negative and 4 positive sera. Standard deviations for each point are shown.

Figure 4. Optimization of sera dilution. Different two-fold serial dilutions of sera (1/25 to 1/800) were tested by ELISA against 15 ng of partially purified ∆-ORF2r recombinant protein (A), or irrelevant proteins (B), obtained from extracts of infected larvae, as described in material and methods, using previously tested positive (solid lines) and negative (dotted lines) swine (left panel) and human (right panel) sera. Data in each panel are shown as the average A₄₉₅ of 4 negative and 4 positive control sera. Standard deviations for each point are shown.

**Figure 5.** Comparison of ELISA immunoreactivity of ∆-ORF2r recombinant protein based ELISA and a commercial kit with swine (A) and human (B) sera. ELISAs were performed as described in material and methods. Data are expressed as P/N ratio, which were calculated as described in material and methods. Vertical and horizontal dotted lines represent the cut off value of the assays (P/N ≥ 1 for the in house ELISA and P/N ≥ 2.5, for the commercial kit). Open and solid squares represent sera that reacted...
positively and negatively, respectively, with both assays. Open circles and triangles represent sera that were positive only with the in house assay or with the commercial kit, respectively.
Table 1. Concordance between the *in house* and a commercial kit for the detection of IgG antibody to Hepatitis E virus.

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* Concordance 80% (kappa=0.60). ** Concordance 92.5% (kappa = 0.82)
Figure 1.
Figure 2.
Figure 3.
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Figure 5

A

\[
\Delta \text{ORF-2 (P/N)}
\]

Commercial kit (P/N)

1 2 3 4 5

B

\[
\Delta \text{ORF-2 (P/N)}
\]

Commercial kit (P/N)

1 2 3 4 5 6 7