

Serological Immunoassay for Detection of Hepatitis E Virus on the Basis of Genotype 3 Open Reading Frame 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 suboptimal sanitary conditions. In many industrialized countries, HEV strains of genotype 3 are highly endemic in swine, and an increased number of autochthonous infections with HEV genotype 3 strains have been reported lately. Serological studies of HEV infection are often conducted with commercial assays based on peptides and recombinant proteins of HEV genotype 1 and 2 strains. For some patients with proven HEV genotype 3 infections, these assays failed to detect specific antibodies, and they are not applicable or validated for the detection of anti-HEV antibodies in swine. To elucidate the incidence of hepatitis E in regions where HEV genotype 3 infections can be expected, and to study the seroprevalence of HEV in swine, new tools with broad specificity for all genotypes of HEV are needed. We present the expression and partial characterization of recombinant HEV genotype 3 open reading frame 2 (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 relatively high yields and at low cost upon infection of *Trichoplusia ni* larvae with recombinant baculoviruses expressing recombinant HEV genotype 3 ORF-2 proteins. The enzyme-linked immunosorbent assay based on the recombinant proteins showed good specificity and sensitivity for anti-HEV genotype 3 immunoglobulin G 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 in order to develop a broadly sensitive new tool for the diagnosis of hepatitis E.

Hepatitis E virus (HEV) causes an acute liver disease in humans that is endemic 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 (gt1) strains. gt2 has caused epidemics in Mexico and some regions of Africa. In China, epidemics were caused mainly by gt1, but recently gt4 has become the dominant cause of sporadic hepatitis E. gt3 is widely distributed and is the cause of sporadic cases worldwide.

HEV is a spherical, nonenveloped virus with a diameter of around 32 to 34 nm, whose genome is a single-stranded RNA molecule of approximately 7.2 kb with positive polarity, containing three 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. HEVs infecting mammals have been classified into four different genotypes: 1, 2, 3, and 4 (8, 20, 24, 32). Characterized HEV strains from regions where only sporadic cases of hepatitis E have been reported represent a diverse cluster of sequences largely grouped into gt3.

Since in many industrialized countries an increased number of autochthonous infections with HEV gt3 strains have been reported lately (4, 6, 11, 12, 17, 23, 25, 31), HEV gt3 is now considered an emerging pathogen. gt3 strains detected in humans and swine from the same geographical areas are genetically closely related (20, 23, 30, 31). This fact, together with the descriptions of cases of acute hepatitis in people who had eaten uncooked deer meat, pork liver, or wild boar liver (19, 28, 33), and the recent detection of HEV RNA and infectious virus in commercial pig livers sold in local grocery stores in the United States, The Netherlands, and Japan (3, 9, 33), has led to the hypothesis of a zoonotic potential for HEV (20).

Since viremia is limited to the acute phase of the infection, the diagnosis of the disease is dependent mainly on serology, especially when only convalescent-phase 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 antibodies 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 genotype in swine and humans in industrialized countries (20, 24, 32). Various reports indicate that commercial assays have sometimes failed to detect specific antibodies in sera from patients with proven HEV gt3 infections (7, 10,

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34). Therefore, the number of autochthonous HEV infections in industrialized regions may have been underestimated (18). These considerations 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 method for the production of HEV gt3 ORF-2 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 the detection of HEV antibodies.

MATERIALS AND METHODS

Protein expression and analysis of recombinant ORF-2 proteins in insect larvae. The construction of recombinant baculoviruses, Bac1-ORF2r and Bac1- Δ -ORF2r, expressing the entire histidine-tagged gt3 ORF-2 product and a truncated form of the ORF product lacking the first 111 amino acids at the N terminus, respectively, and their production upon infection of Sf21 insect cells have been reported by us recently (13). To obtain both recombinant proteins on a larger scale in an easy and economical way, *Trichoplusia ni* larvae were reared under biosafety level 2 conditions as previously described (2, 22). Briefly, larvae were sedated by incubation on ice for 15 min, injected with different doses (1×10^6 to 3×10^6 PFU/larva) of recombinant baculoviruses (Bac1-ORF2r or Bac1- Δ -ORF2r) near the proleg (forward along the body cavity), kept in growth chambers at 28°C, harvested at different times postinoculation, and frozen immediately at -20°C until they were processed as described previously (22).

Protein analyses were carried out by Western blotting. Briefly, processed protein extracts from larvae were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Richmond, CA), which were then blocked for 1 h with phosphate-buffered saline-0.05% Tween 20 (PBST) containing 5% skim milk. The membranes were subsequently incubated at 4°C overnight with a commercial mouse monoclonal antibody against histidine (Clontech Laboratories, Inc., Mountain View, CA) and then with a horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (Sigma-Aldrich, St. Louis, MO), or they were incubated with either positive and negative human sera or positive and negative swine sera before the addition of HRP-conjugated goat anti-human or goat anti-pig immunoglobulin G (IgG)-specific immunoglobulin (MorphoSys AG, Martinsried-Plangg, Germany) as a secondary antibody. 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. Larval extracts obtained upon inoculation with an irrelevant baculovirus, and similarly processed, were used as controls.

Partial purification of recombinant Δ -ORF2r protein. Recombinant HEV Δ -ORF2r protein from larval extracts was partially purified in its native form with imidazole (Merck, Mollet del Vallés, Spain) by means of the histidine tails using His-GraviTrap columns (GE Healthcare, Alcobendas, Spain) according to the manufacturer's instructions. Imidazole concentrations of 10, 20, and 500 mM were used in the binding, washing, and elution buffers. Protein concentrations were determined by gel densitometry using known amounts of purified bovine serum albumin as a control. The integrity of partially purified proteins was analyzed by Western blotting as described above.

Δ -ORF-2-based ELISA. Partially purified Δ -ORF2r protein was tested as an enzyme-linked immunosorbent assay (ELISA) antigen. Microplates (Polysorp; Nunc, Roskilde, Denmark) were coated with 50 μ l/well of serial dilutions of purified protein (2.5 to 80 ng/well) in 50 mM carbonate/bicarbonate buffer (pH 9.6) and were incubated overnight at 4°C. All subsequent incubations were carried out for 1 h at 37°C under constant shaking. After three washes with PBST, plates were incubated with 100 μ l/well of blocking solution (PBST-3% skim milk); then serum diluted in blocking buffer was added to duplicate wells. Plates were washed again with PBST before the addition of 50 μ l/well of an HRP-conjugated goat anti-swine or anti-human IgG secondary antibody diluted 1:30,000 (0.03 μ g/ml) or 1:10,000 (0.06 μ g/ml), respectively (MorphoSys, Martinsried-Plangg, Germany). After incubation with the secondary antibody, plate wells were washed three times before 50 μ l/well of the substrate solution (*o*-phenylenediamine-0.056% H₂O₂) was added. Plates were incubated (in the dark at room temperature for 10 min); then the reaction was stopped by the addition of 50 μ l/well of 3 N H₂SO₄, and plates were read at 495 nm in an ELISA microplate reader (Tecan Genios Ag, Vienna, VA). Previously characterized negative and positive swine and human (5, 27) sera were included in the assay as controls.

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

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

RESULTS

Expression of HEV gt3 ORF-2r proteins in *Trichoplusia ni* larvae. Inoculation of *T. ni* larvae with Bac1-ORF2r resulted in the expression of a 65- to 68-kDa protein that appeared as the major species recognized by an anti-His monoclonal antibody (Fig. 1A). The same species, as well as some minor cell-associated immunoreactive proteins, was observed with anti-HEV IgG-positive swine (Fig. 1B) and human (Fig. 1C) sera. 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 a His tail (Bac-Irr-His). Likewise, Western blotting with anti-HEV IgG-negative pig or human sera failed to detect any specific band of the expected mass (data not shown).

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

HEV-specific proteins obtained after infection with either recombinant baculovirus, Bac1-ORF2r or Bac1- Δ -ORF2r, were difficult to dissolve, but the yield of soluble protein was higher when the truncated form was expressed, as shown in Fig. 1.

Optimization of the production and purification of HEV gt3 Δ -ORF-2 protein. To optimize protein expression, *T. ni* larvae were inoculated with different doses (1×10^6 to 3×10^6 PFU/larva) of the Bac1- Δ -ORF2r recombinant baculovirus and were processed at day 1 to day 3 postinoculation. Optimal protein yields were obtained from larvae infected at a multiplicity of infection of 1.5×10^6 PFU/larva and harvested 3 days after infection (data not shown).

HEV Δ -ORF-2 protein from extracts of recombinant baculovirus-infected larvae was partially purified in its native form as described in Materials and Methods. As displayed in Fig. 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 proteolyzed products with lower molecular masses.

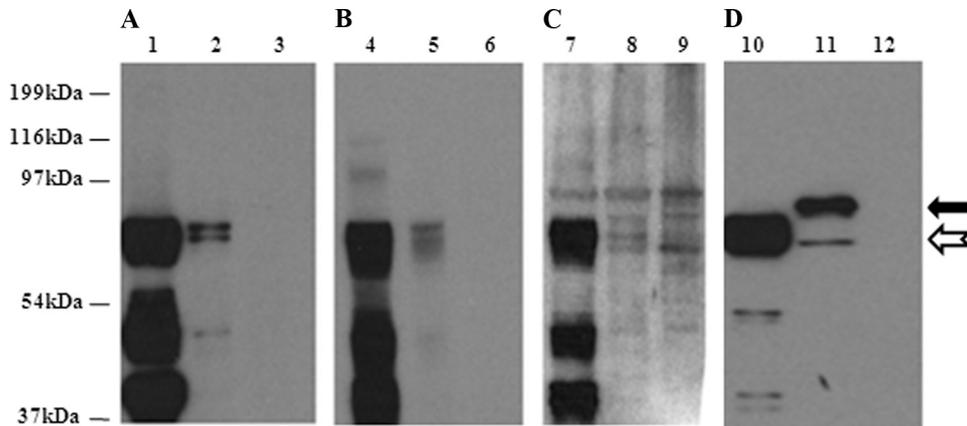


FIG. 1. Western blot analysis of HEV ORF-2r and Δ -ORF-2r proteins. Western blots were stained with an anti-His monoclonal antibody (A and D), swine serum (B), or human serum (C). Lanes 1, 4, and 7, Bac1- Δ -ORF-2-infected larval extracts; lanes 2, 5, and 8, Bac1-ORF2r-infected larval extracts; lanes 3, 6, and 9, mock-infected larval extracts; lanes 10, 11, and 12, Bac1- Δ -ORF-2-, Bac1-ORF2r-, and mock-infected Sf21 cell extracts, respectively. Molecular size markers are shown on the left. Filled and open arrows mark the positions of the HEV ORF-2r and Δ -ORF-2r proteins, respectively.

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

Criteria for determining positive reactivity by an anti-HEV ELISA. To establish the cutoff of the assay, eight previously characterized negative swine and human sera (5, 27) with A_{495}

values below 0.15 (between 0.05 and 0.14) were pooled and used as controls. These values were similar to those obtained with purified proteins from extracts of larvae infected with an irrelevant baculovirus. None of these sera reacted in Western blotting with the partially purified Δ -ORF2r recombinant protein. Absorbance values were expressed as P/N and calculated as (absorbance value of the test sample)/(absorbance value of the negative control). Then the cutoff value was established as a P/N of ≥ 2.5 . Samples with absorbance values above the cutoff were considered positive.

Comparison of a Δ -ORF2r-based ELISA with a commercial kit. A battery of previously characterized sera consisting of 40 human and 66 swine sera (5, 10, 27) was tested by an ELISA using the partially purified Δ -ORF2r protein as an antigen and the commercial kit, although for the swine sera the commercial procedure had to be modified to use a conjugated anti-swine IgG as described in Materials and Methods; therefore, the results for swine sera should be interpreted with caution. A comparison of the ELISA results is shown in Fig. 5. The con-

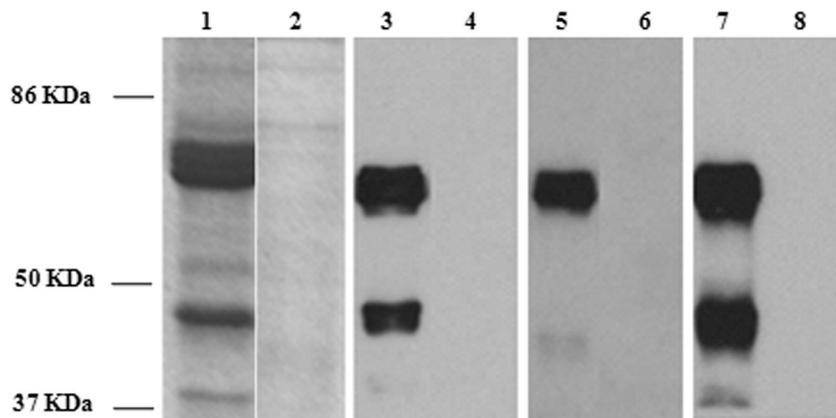


FIG. 2. Analysis of partially purified HEV Δ -ORF2 recombinant protein obtained from infected *T. ni* larvae. Lanes 1 and 2, Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gels of partially purified extracts from Bac1- Δ -ORF2r-infected and mock-infected *T. ni* larvae, respectively. Lanes 3 to 8, Western blot analyses of the same partially purified extracts revealed with an anti-His monoclonal antibody (lanes 3 and 4), swine serum (lanes 5 and 6), and human serum (lanes 7 and 8).

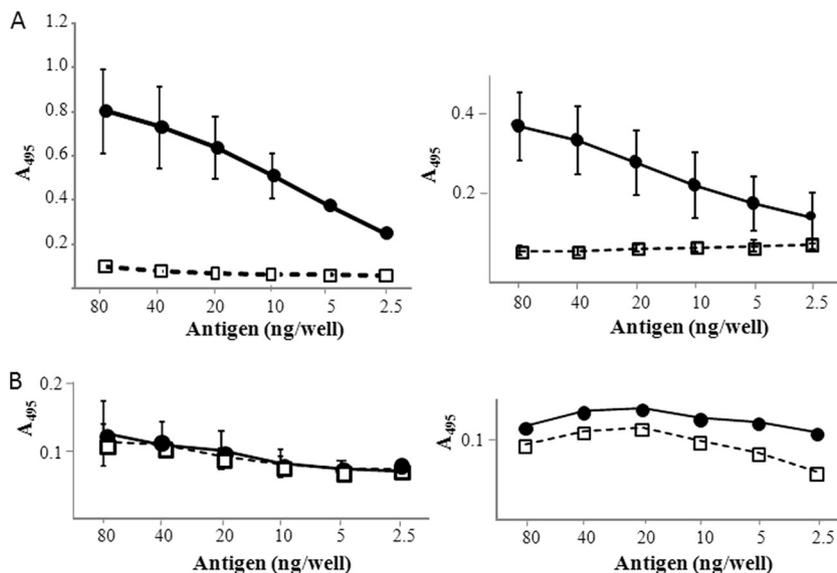


FIG. 3. Optimization of antigen concentration. Previously characterized positive (filled circles) and negative (open squares) swine (left) and human (right) sera were tested by an ELISA against twofold serial dilutions (2.5 to 80 ng/well) of partially purified Δ -ORF2r recombinant protein (A) or an irrelevant protein (B) obtained from extracts of infected larvae, as described in Materials and Methods. Data in each panel are shown as the average A_{495} of four negative or four positive sera. Standard deviations for each point are shown.

cordances between the two assays were 80% ($\kappa = 0.60$) and 92.5% ($\kappa = 0.82$) 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 blotting. None of the 12 swine or 6 human sera tested that were negative by both assays reacted in Western blot analyses,

while all swine ($n = 17$) and human ($n = 3$) sera tested that were positive in both ELISAs were also positive by Western blotting. Among those samples that were positive by the in-house test and negative by the commercial assay, all eight swine sera reacted positively in Western blotting, as did two of the three human sera. On the other hand, four of the five swine sera that reacted positively with the commercial kit and negatively with the in-house assay were also negative by Western blotting.

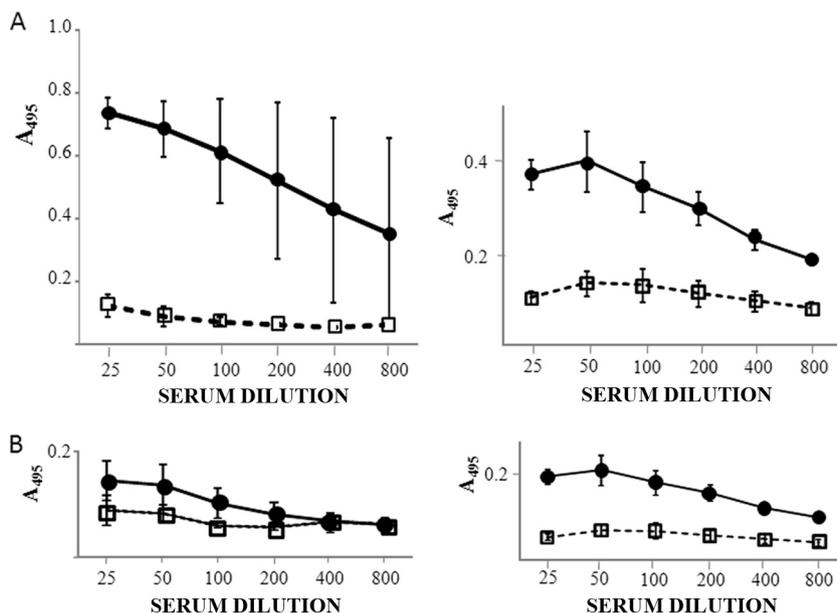


FIG. 4. Optimization of serum dilution. Different twofold serial dilutions of sera (1/25 to 1/800) were tested by an ELISA against 15 ng of partially purified Δ -ORF2r recombinant protein (A) or an irrelevant protein (B) obtained from extracts of infected larvae, as described in Materials and Methods, using previously tested positive (solid lines) and negative (dashed lines) swine (left) and human (right) sera. Data in each panel are shown as the average A_{495} of four negative or four positive control sera. Standard deviations for each point are shown.

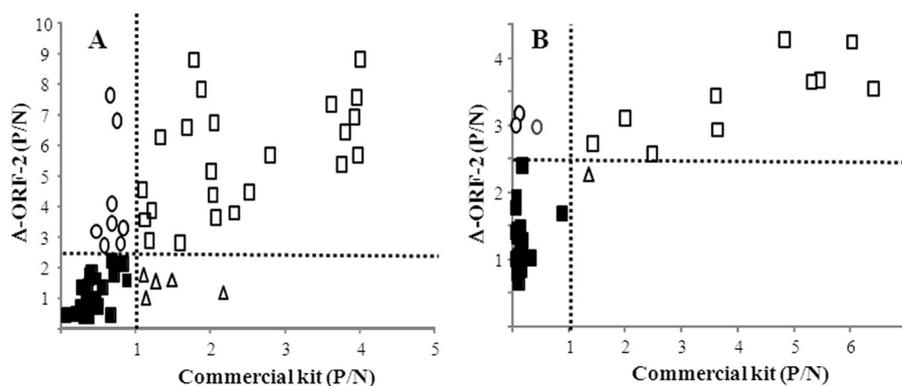


FIG. 5. Comparison of the immunoreactivities of the Δ -ORF2r recombinant-protein-based ELISA and a commercial kit with swine (A) and human (B) sera. ELISAs were performed as described in Materials and Methods. Data are expressed as P/N ratios, calculated as described in Materials and Methods. Vertical and horizontal dotted lines represent the cutoff values of the assays (P/N ratios of ≥ 1 for the in-house ELISA and ≥ 2.5 for the commercial kit). Open and filled 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 only with the commercial kit, respectively.

Since Western blotting is more sensitive than ELISA (10), we used it as the gold standard. Thus, based on the data reported above, we compared the ELISA results to the Western blotting results. The sensitivities of the in-house assay for detecting anti-HEV gt3 antibodies were 100% and 96.9% for human and pig sera, respectively, while those of the commercial kit were 83.3% and 75%, respectively. Likewise, the specificities of the in-house test were 96.4% and 100% for human and pig sera, respectively, while those of the commercial kit were 96.4% and 88.2%, respectively. Thus, when human sera were tested, one false-positive serum (2.5%) and no false-negative sera were observed with the in-house test, while the commercial kit gave one false-positive (2.5%) and two (4.5%) false-negative samples. Similarly, when swine sera were assayed, no false-positive results and one (1.5%) false-negative result were recorded with the ELISA developed here, while the commercial kit produced four (6%) false-positive and eight (12%) false-negative results.

DISCUSSION

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

We have previously shown that infection of Sf21 cells with the Bac1-ORF2r recombinant baculovirus produced a major

protein of around 78 kDa (13) (Fig. 1D). Here we show that when the same recombinant baculovirus was used to infect *T. ni* larvae, the major species detected was 65 to 68 kDa, (some cell-associated immunoreactive proteins were also detected). These species were similar to those observed when the Δ -ORF-2 protein was expressed upon inoculation of the Bac1- Δ -ORF2r recombinant baculovirus. These lower-molecular-mass proteins were most probably due to proteolytic processing of the complete ORF-2, which occurs by truncation of the N terminus of the protein (15, 26, 29). Although the reason for these different expression patterns of full-length ORF-2 in Sf21 cells and *T. ni* larvae is unclear, different processing of HEV ORF-2 protein expressed in suspension versus monolayers of insect cells has been described (35), as has different processing of ORF-2 in Sf-9 versus 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. The conditions used for the production of HEV gt3 antigens in *T. ni* larvae yield 0.2 to 0.3 mg of Δ -ORF2r protein per larva in 72 h. The amount of partially purified protein used in the ELISA was ≈ 15 ng/well, which indicates an estimated yield of 20,000 individual ELISA determinations from a single larva.

The accuracy of the recombinant Δ -ORF-2 antigen as a diagnostic tool was further confirmed by comparison of the in-house ELISA with a widely used commercial kit. Our results showed a concordance of 92.5% between the two assays when human sera were tested. Likewise, a concordance of 80% was observed when swine sera were analyzed, although in this case the 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, the ELISA concordance for human samples between the commercial kit tested here and either

TABLE 1. Concordance between the in-house kit and a commercial kit for the detection of IgG antibody to HEV

Result by the in-house assay	No. of sera with the indicated result by the commercial kit					
	Swine ^a			Human ^b		
	Positive	Negative	Total	Positive	Negative	Total
Positive	23	8	31	10	3	13
Negative	5	30	35	1	26	27
Total	28	38	66	11	29	40

^a Concordance, 80% ($\kappa = 0.60$).

^b Concordance, 92.5% ($\kappa = 0.82$).

human HEV gt1 or swine HEV gt4 ORF-2 recombinant proteins has been shown to be 90.3% or 92%, respectively (1).

Taking Western blotting as a gold standard, our results showed fairly good specificity of the in-house and commercial assays tested here with human sera (96.4%) and higher specificity of the in-house test for pig sera (100% versus 88.2%, respectively). Specificities for IgG detection in human sera (92% to 93%) similar to that found here and lower than that provided by the manufacturers of the commercial kit have been reported previously (10, 16). Likewise, the sensitivities observed for pig (96.9%) and human (100%) samples were also fairly good with the in-house assay and higher than those with the commercial kit (75% and 83% for human and pig sera, respectively). The values obtained here for human sera with the commercial kit are also in agreement with the sensitivity (86% to 88%) reported previously for HEV gt3-infected patients from areas where this genotype is not endemic (10, 16). However, we cannot discard the possibility that the positive results by the commercial test for some of the swine sera that failed to recognize HEV ORF-2 gt3 recombinant proteins by ELISA or Western blotting could be due to the presence of antibodies against, for example, ORF-3. In any case, our results indicate that the ELISA developed could be useful for the overall detection of HEV infection, although further analysis of patients infected with different genotypes will be needed to fully confirm its usefulness.

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

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