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Expression of a truncated hepatitis E virus capsid protein in the protozoan organism *Leishmania tarentolae* and its application in a serological assay

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A B S T R A C T

Zoonotic infections with hepatitis E virus (HEV) genotype 3 are presumably transmitted via contaminated pig meat products, which raises the necessity for enhanced serological surveillance of pig herds. The aim of the study was to set up a novel protein expression system to overcome the well-known problems in (HEV-) protein expression using the standard *Escherichia coli* tools such as inclusion body formation and loss of protein conformation. A recombinant strain of the protozoan organism *Leishmania tarentolae* (*L. tarentolae*) was therefore established. A fragment of HEV ORF2 coding for a truncated capsid protein of a porcine HEV strain was cloned and parts of the plasmid DNA were introduced into the Leishmania genome, resulting in stably transformed cells. Via a C-terminal His-tag the recombinant HEVΔORF2 protein could be purified and concentrated directly from the medium, resulting in a total protein amount of approximately 1.4 mg/l Leishmania culture. The recombinant protein was coated on ELISA plates and was proven to be highly reactive and well-suited to be applied in a serological assay. By investigating 144 porcine sera, the in-house assay detected specific antibodies in 43.1% of the samples and demonstrated a higher sensitivity than a commercially available antibody test. Taken together, it was shown that *L. tarentolae* exhibits a remarkable alternative expression strategy for viral antigens with considerable advantages of a eukaryotic protein expression host.

1. Introduction

Hepatitis E virus (HEV), a non-enveloped RNA virus is responsible for large water-borne outbreaks of acute hepatitis in different parts of the world, mainly in developing regions where HEV genotypes 1 and 2 are predominant. In industrial countries, hepatitis E is believed to be caused mainly by a zoonotic infection with HEV genotypes 3 and 4 (Aggarwal, 2011). In the case of Germany, there is a growing body of evidence for high HEV seroprevalences in the adult population (Faber et al., 2012). In addition to acute infections, HEV genotype 3 is known to cause persistent disease in immunocompromised patients such as organ transplant recipients (Kamar et al., 2008; Legrand-Abravanel et al., 2010; Pischke et al., 2012). Certainly, wild boars as well as domestic pigs serve as reservoir hosts in Europe (Pavio et al., 2010) and several reports support the possibility of HEV transmission via pork food products (Colson et al., 2010; Wenzel et al., 2011).

The prevalence of anti-HEV antibodies in domestic pigs was investigated largely in the past few years (Baechlein et al., 2010; Breum et al., 2010; Peralta et al., 2009; Rose et al., 2011; Seminati et al., 2008; Wacheck et al., 2012). In serological assays, the viral capsid protein, which is encoded by the second open reading frame (ORF2) was most often used as antigen since it is known to harbour several neutralizing epitopes (Meng et al., 2001). To date, various expression systems for HEV antigens have been used to set up serological tests including the use of prokaryotic as well as eukaryotic organisms (Dremsek et al., 2012; Jiménez de Oya et al., 2009; Peralta et al., 2009). In addition to this, the aim was to establish an expression system which combines all the benefits of the yet recognized approaches.

The non-human pathogenic species *Leishmania tarentolae* (*L. tarentolae*) is a protozoan parasitic organism which was found to infect naturally the lizard *Tarentola annularis* (Elwasila, 1988). In 2002, Breitling et al. generated a protein expression system based on

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the stable transformation of *L. tarentolae* cells, which ensures the constant expression of foreign proteins after homologous recombination of the respective DNA with the Leishmania small subunit RNA gene. In this case, the signal peptide for secreted acid phosphatase of *L. mexicana* ensures transportation of the recombinant protein to the medium (Breitling et al., 2002). Since then, a broad range of proteins have been produced via this expression system (Basile and Peticca, 2009). Apart from the ability to form native disulfide bonds, the strong resemblance of the N-linked glycosylation pattern of *L. tarentolae* in particular to the glycosylation form of mammalian cells is of considerable interest when conformation becomes crucial for protein functionality (Phan et al., 2009; Soleimani et al., 2007). In addition, protein structure is extremely important when investigating antigen-antibody binding reactions. In particular for HEV it was shown that neutralizing sites are characterized by discontinuous epitopes (Zhou et al., 2005; Zhang et al., 2012). Therefore, in the present study, the *L. tarentolae* expression system was used to produce a novel HEV antigen and to explore if it could be advantageous to established recombinant HEV proteins.

2. Materials and methods

2.1. Cloning of the HEV fragment

Total RNA was isolated from an HEV genotype 3 positive liver sample derived from a domestic pig. Complementary DNA (cDNA) was synthesized with random primers and SuperScript III Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA). Next, primers HEVΔORF2_XbaI (5′-CTGCGCCTCTAGACGCCATATCACCTGCTCCCGAT-3′) and HEVΔORF2_KpnI (5′-TCTTCAAGGGTACCAAGAGCCGAATGTGGG GCTAAAA-3′) were used to amplify a 1516 bp long fragment of the HEV ORF2. The restriction sites required for in-frame-cloning into the vector pLexsyHyg2 (Jena Bioscience, Jena, Germany) are underlined. After purification of the PCR product, a treatment with *Xba*I and *Kpn*I ensured a correct ligation with the help of the *T4* ligase (all enzymes from Thermo Fisher Scientific, Waltham, MA, USA). After ligation overnight at 15 °C the construct was introduced into *Escherichia coli* SURE Electroporation-Competent Cells (Agilent Technologies, Santa Clara, CA, USA). Ampicillin resistant clones were confirmed by sequencing to carry the plasmid with the desired fragment.

2.2. Leishmania culture and transformation

L. tarentolae strain P10 (Jena Bioscience, Jena, Germany) was cultured in brain heart infusion (BHI) medium (37 g/l) with penicillin (50 U/ml), streptomycin (50 μg/ml) and hemin (5 μg/ml) (Sigma–Aldrich, St. Louis, MO, USA) at 27 °C according to the manufacturer's instructions. To select for recombinant cells, hygromycin was added to a final concentration of 50 μg/ml (PAA Laboratories, Pasching, Austria). To maintain a culture, cells were passaged twice a week with dilutions of 1:40.

To transfer the DNA into the Leishmania cells, 20 μg of the plasmid pLexsyHyg2_HEVΔORF2 were digested with *Swa*I. 3 ml of a two-day-old culture were centrifuged at 330 × *g* for 7 min and resuspended in 1 ml of *cytomix* electroporation buffer (Van den Hoff et al., 1992). Of these, 350 μl were mixed with 50 μl digested plasmid DNA and kept on ice for 10 min. Cells were then transferred to a 4.0 mm cuvette (Bio-Rad, Hercules, CA, USA) and electroporated by two pulses (0.3 ms) at 1500 V. After 10 min incubation on ice, electroporated cells were transferred to 8 ml of BHI-medium.

2.3. Clonal selection of recombinant cells and verification of protein expression

Two days after electroporation, 2 ml of the culture were pelleted by centrifugation and resuspended in 80 μl medium. They were spread on agar plates containing BHI, antibiotics, hemin, the selection marker hygromycin, and 10% foetal bovine serum as well as 1 M HEPES. Plates were sealed and incubated upside-down at 27 °C. Approximately one week later, individual colonies were picked and transferred into 100 μl BHI medium containing hygromycin. Subsequently, proliferating cells were grown and scaled up before storage as glycerol stocks at −80 °C.

Two pairs of primers were used to prove the genomic integration of the expression cassette. After DNA preparation, the primers A3804 (5′-CCGATGGCTGTGTAGAAGTACTCG-3′) and F3002 (5′-CTGCAGGTTCACCTA CAGCTAC-3′) (Jena Bioscience, Jena, Germany) were applied in the first PCR reaction to verify the integration of the fragment including the marker gene into the chromosomal 18S rRNA locus (*ssu*). Thereby, A3804 binds to the hygromycin phosphotransferase gene whereas F3002 attaches to the *ssu* outside the site of recombination. To prove the specificity of the inserted DNA, a second PCR reaction was performed with the primers F3001 (5′-GATCTGGTTGATTCTGCCAGTAG-3′) binding to the Leishmania *ssu* and a particular HEV ORF2 primer HEV5826 (5′-TAGAGTTCATATCAACAGACGTGGG-3′).

To investigate the protein expression, total protein was precipitated from 9 ml of the supernatant by 1.8 ml trichloroacetic acid followed by centrifugation at 15,000 × *g* and 4 °C. The pellet was washed with 80% acetone and resuspended in 2× SDS sample buffer. After SDS gel electrophoresis and Western blotting the HEVΔORF2 protein was detected with mouse anti-His_6 (Roche Applied Science, Penzberg, Germany) or with mouse anti-HEV (United States Biological, Salem, MA, USA). Polyclonal rabbit anti-mouse immunoglobulin/HRP (Dako, Glostrup, Denmark) was applied as secondary antibody in both cases.

2.4. Expression and purification of the HEVΔORF2 protein

A frozen glycerol stock of a recombinant clone was thawed on ice and transferred into BHI medium complemented with 50 μg hygromycin/ml medium. When the culture had reached an optical density of about 1.5, cells were separated from the medium by centrifugation for 20 min at 4000 × *g*. Protein purification was carried out under native conditions using a liquid chromatography system and a column pre-packed with Ni-NTA agarose (Macherey-Nagel, Düren, Germany). Subsequently, the protein was eluted from the column with a buffer containing 50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazol and was stored at 4 °C.

2.5. ELISA development and comparison to a commercially available test

To investigate the reactivity of the novel antigen, different amounts of the ΔORF2 antigen ranging from 200 ng/well to 6.25 ng/well were tested. For this, 96-well plates (Medisorp, Thermo Fisher Scientific, Waltham, MA, USA) were coated at room temperature or 37 °C in a 0.1 M carbonate/bicarbonate buffer. Further on, seven anti-HEV-IgG-negative as well as seven positive serum samples were tested in different concentrations. The antibody status of these samples was previously determined by ELISA based on a recombinant ORF2 protein expressed via a baculovirus system. For the detection of specific serum IgG antibodies, rabbit anti-pig IgG peroxidise conjugate (Sigma–Aldrich, St. Louis, MO, USA) was applied, followed by an incubation step with the peroxidase substrate 3,3′,5,5′-tetramethylbenzidine (TMB) (Promega, Fitchburg, WI, USA).

To characterize the properties of the novel antigen, 144 porcine field sera were tested in parallel with the commercially available ELISA PrioCHECK® HEV Ab porcine (Prionics, Schlieren, Switzerland). This test was carried out according to the manufacturer's instructions.

2.6. Immunoblot analysis

To confirm the specificity of the ELISA reaction, the HEVΔORF2 protein was applied in a concentration of 500 ng per lane in SDS gel electrophoresis. After Western transfer, pig sera were applied in a dilution of 1:40. Bound serum antibodies were detected by rabbit anti-pig IgG peroxidase (1:3000) (Sigma–Aldrich, St. Louis, MO, USA).

3. Results

3.1. Cloning and confirmation of genomic integration

Application of the primer pair HEVΔORF2_XbaI and HEVΔORF2_KpnI resulted in a fragment which encodes a capsid protein encompassing a truncation of 111 aa at the N-terminus and a truncation of 53 aa at the C-terminus. After bacterial cloning, plasmid DNA preparation and transformation of the cells, the correct insertion of the HEVΔORF2 fragment into the protozoan genome could be verified via two PCR reactions. Amplification with the primers A3002 and F3804 resulted in a fragment measuring 1819 bp in length, whereas execution of the second PCR reaction using the primers F3001 and HEV5826 proved the specificity of the recombination with a 1665 bp product (data not shown). Several recombinant *L. tarentolae* clones were thereby shown to have correctly integrated the expression cassette with the HEVΔORF2 fragment into their *ssu* locus.

3.2. Expression of the HEVΔORF2 protein by L. tarentolae

To confirm the expression and secretion of the requested protein by the recombinant Leishmania clones, the protein was precipitated from the supernatant and examined by immunoblot. Thereby it could be shown that clones which had undergone genomic integration of the desired fragment also constantly expressed the truncated ORF2 protein. The molecular weight of the HEVΔORF2 protein including the N-terminal signal peptide and the C-terminal His-tag was calculated to be 58 kDa.

In the following experiments, the clone *L. tarentolae*/pLexsyhyg2/HEVΔORF2/H3 was chosen for high yield protein production. Passaging the static culture twice with a dilution of 1:10 followed by an incubation step at 27 °C and 200 rpm for 40 h were found to be the best conditions for expression of the recombinant protein. Under these conditions, approximately 700 μg of purified protein could be recovered from a 500 ml Leishmania culture. Thus, the protein concentration of the different elution fractions varied between 50 and 225 μg/ml. The high purity of the ΔORF2 protein was proven by Coomassie blue staining (Fig. 1).

3.3. ELISA development and comparison to a commercially available test

Titration of the antigen from 200 ng/well to 6.25 ng/well showed a decrease in optical density (OD) values proportionally to the decrease of the antigen concentration (Fig. 2). By comparing the mean negative and positive OD values, a concentration of 100–50 ng antigen/well was found to be most favourable. The final protocol comprised coating the antigen for 1 h at 37 °C, followed by an overnight blocking step with 2% BSA in PBS-Tween (0.02%)

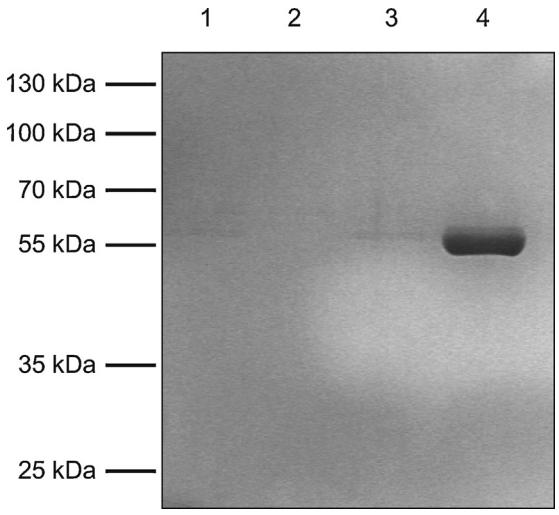

Fig. 1. Purification of the recombinant HEVΔORF2 protein via the C-terminal His-tag. (1) *L. tarentolae* supernatant prior to FPLC purification; (2) flow-through; (3) washing buffer; (4) pooled elution fractions.

at 4 °C. The next two steps were carried out at 37 °C for 1 h with a three-fold washing procedure between each incubation period. Sera were diluted 1:400, whereas bound serum antibodies were detected with rabbit anti-pig IgG at a dilution of 1:35,000 before TMB was added. After 10 min of incubation in the dark, the enzymatic reaction was stopped with 1 M hydrochloric acid and the OD was read at 450 nm.

Compared to the bacterially expressed antigens HEV ORF2 and ORF3 used in the commercially available ELISA PrioCHECK® HEV Ab porcine, the novel in-house HEV ELISA performed very well. To gain a first impression of the reactivity of the HEVΔORF2 protein with field samples, 144 serum samples from slaughter pigs from Germany were tested in parallel with the commercial test as well as the novel in-house HEV described here. While the HEVΔORF2 in-house ELISA detected antibodies specific to HEV in 62 cases (43.1%), the commercial test did so for 59 sera (41%). Thereby, four samples tested positive with the PrioCHECK® assay, but tested negative in the in-house test. On the contrary, the positive reaction of seven sera with the novel antigen could not be confirmed by the commercial assay. In total, the tests revealed a result concordance of 92.4% (Table 1).

3.4. Immunoblot analysis

In addition, immunoblot analysis proved the specificity of the antigen-antibody reaction seen by the in-house ELISA. Initially, it could be shown, that three HEV antibody positive sera used for

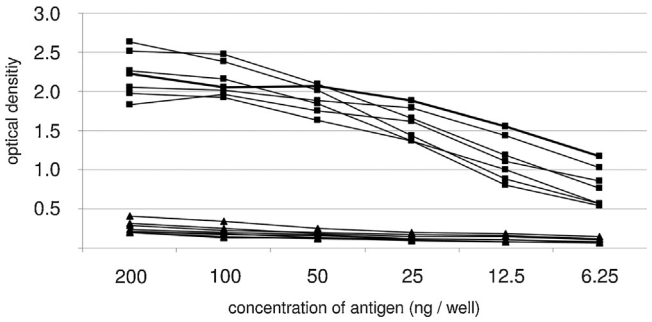

Fig. 2. Titration of the HEVΔORF2 antigen on ELISA plates. Seven anti-HEV antibody negative and positive pig sera were applied. (■) Anti-HEV antibody positive sera; (▲) anti-HEV antibody negative sera.

Table 1
Comparison of the ELISA results obtained by the commercial antibody test and the in-house assay.

	In-house assay: HEVΔORF2		Total
	Positive	Negative	
PrioCHECK® HEV Ab porcine			
Positive	55	4	59
Negative	7	78	85
Total	62	82	144

setting up the ELISA bound to the HEVΔORF2 antigen, while one negative reference serum and a serum sample from an antibody-deprived piglet did not react (Fig. 3a). Further on, the serum samples reacting differently in the two ELISA tests were subsequently investigated by immunoblot for their specific reaction to the HEVΔORF2 antigen. Two of the four serum samples which tested negative in the in-house ELISA test were clearly non-reactive to the antigen, whereas two exhibited a very weak reaction. In contrast, the reactivity to the HEVΔORF2 antigen of four of the seven serum samples which tested positive in the in-house-ELISA but negative in the PrioCHECK® assay could be confirmed by immunoblot (Fig. 3b).

4. Discussion

It has been well-documented for several years now that hepatitis E is not longer a disease occurring exclusively in developing regions. In fact, in Germany as well as in other European countries, HEV genotype 3 is believed to be transmitted zoonotically, possibly through the consumption of contaminated or even infected meat products (Colson et al., 2010; Feagins et al., 2007; Wenzel et al., 2011). Due to the potential for severe infections with chronic manifestations, it is of greatest importance to gain more knowledge on the behaviour of HEV in its natural host and the distribution in pig herds in Germany. Direct proof of infection via PCR methods is a reliable and well established tool to detect HEV RNA in faecal or liver samples of animal origin. However, monitoring of pig herds is much more suitable through serological assays. Great

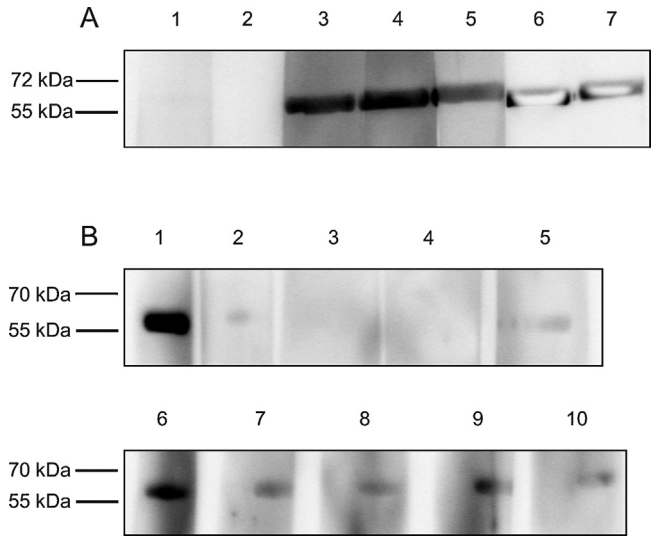

Fig. 3. (A) Characterization of the HEVΔORF2 antigen via immunoblot. (1) Anti-HEV IgG negative reference pig serum; (2) serum of colostrum-deprived piglet; (3–5) anti-HEV IgG positive reference pig sera; (6) monoclonal anti-His antibody; (7) monoclonal anti-HEV antibody. (B) Analysis of the serum reactivity to the HEVΔORF2 antigen. (1 and 6) Monoclonal anti-His antibody; (2–5) serum samples reacting negative in the HEVΔORF2 in-house ELISA but positive in the commercial test; (7–10) four of the seven serum samples reacting positive in the HEVΔORF2 in-house ELISA but negative in the commercial test.

variations concerning sensitivity and specificity of different assays have been observed not only in veterinary but also in human diagnostics (Baechlein et al., 2010; Wenzel et al., 2013). The aim of this study was therefore the expression of a highly reactive antigen with conserved antigenic structures and its application in a serological test.

The performance of serological assays relies mostly on the characteristics and properties of the applied antigen. In addition to aspects of quality, the ability to produce considerable amounts of antigen affects the decision for an expression system for recombinant proteins. In terms of the latter aspect, expression of antigens in *E. coli* would be the best choice. Nevertheless, the isolation and enrichment of recombinant proteins can be challenging. Protein expression in *E. coli* is frequently hampered by the accumulation in inclusion bodies within bacterial cells (Hartley and Kane, 1988; Petro and Raben, 2013). Non-soluble proteins can often be recovered only by washing steps with highly concentrated denaturants (Kumar et al., 2012). Taking advantage of mild solubilization of inclusion bodies enhances the recovery of bioactive recombinant proteins (Singh and Panda, 2005), although protein refolding remains cumbersome. Nonetheless, protein conformation can be crucial for antigen-antibody binding reactions in serological tests including assays which investigate serological responses to HEV, as neutralizing epitopes of the capsid protein are reported to be conformation-dependent (Zhou et al., 2004).

To overcome this issue, the production of foreign proteins in the parasitic organism *L. tarentolae* is an ideal alternative, since it combines several advantages. On the one hand, cloning the requested DNA fragment in frame with the Leishmania signal peptide ensures protein secretion directly into the culture medium. The disruption of cells with subsequent steps of washing and centrifugation steps is not required. On the other hand, the culturing of cells is very easy: it allows the growing of the promastigote phenotype in a complex medium as static or agitated culture without supplementation of CO_2. Furthermore, a Leishmania culture can be straightforwardly scaled up to reach several milligrams of protein, which makes the organism attractive for industrial-scale protein production. However, culturing of Leishmania cells is as much as five times more expensive than a culture of *E. coli*. Higher costs can mostly be attributed to the selection marker hygromycin. Apart from this, it has to be stressed that, according to the German Central Committee on Biological Safety (ZKBS), the organism can be handled permanently under S1 biosafety conditions. Whole-genome sequencing indicates a reduced ability to proliferate in mammalian macrophages. Furthermore, several genes determining high virulence are underrepresented in *L. tarentolae* compared to human pathogenic Leishmania species (Raymond et al., 2012).

The organism provides posttranslational modifications such as phosphorylation and fully complex N-linked glycosylation which strongly resembles the glycosylation pattern of the mammalian type (Breitling et al., 2002; Parodi, 1993). In terms of HEV, a glycosylation of the HEV capsid protein was postulated, as mutated glycosylation sites abolished the formation of infectious particles (Graff et al., 2008). However, in a recent study, the glycosylation of an N-terminal 111 aa truncated ORF2 mutant could not be proven after expression via a vaccinia virus system (Jiménez de Oya et al., 2012). Although there remain open questions concerning a glycosylated form of the structural protein, other benefits associated with antigen expression in *L. tarenotalae* cannot be neglected.

To explore these opportunities, a fragment of HEV coding for a truncated capsid protein was cloned into the respective vector carrying the expression cassette, followed by subsequent transformation of the *L. tarentolae* strain P10. The selection of recombinant clones, up-scaling and protein purification turned out to be uncomplicated. In the recovery of recombinant protein, the system yielded about 1.4 mg/l of culture, which corresponds well to the amount

of previously produced proteins in this expression host (Basile and Peticca, 2009). To test whether the HEVΔORF2 protein was suitable to detect porcine serum antibodies, the antigen was characterized via ELISA and Western blot. It was thus shown that anti-HEV porcine serum antibodies exhibited a specific reaction to the highly reactive recombinant protein. In an initial screening of 144 pig serum samples, we found that 43.1% contained specific anti-HEV IgG antibodies. This result resembles previous serological surveys confirming high HEV seroprevalence values in domestic pigs of all age groups (Pavio et al., 2010).

In comparison to a commercial ELISA which uses ORF2 as well as ORF3 antigen of HEV genotypes 1 and 3, the novel HEVΔORF2 antigen reacted differently to some extent. Both tests rely on the principle of indirect detection of serum antibodies, which enables a direct comparison of their performances. Although the commercial ELISA uses ORF2 and ORF3 antigen of HEV genotype 1 and genotype 3 (Wacheck et al., 2012), this test did not exhibit a higher sensitivity than the in-house ELISA based on the genotype 3 ORF2 antigen alone. Similar results were observed in a recent study using the PrioCHECK-assay as a reference test as well (Dremsek et al., 2013). Although the ORF3 protein was presumed to be associated with the virus surface (Takahashi et al., 2008), one might speculate on the relevance of the ORF3 protein for the investigation of HEV-specific serum antibodies, at least when applying an ORF2 antigen with preserved protein structure. Apart from that, the sole usage of homologous antigen appears to be appropriate, since domestic pigs in Europe are most often infected with HEV genotype 3. In fact, the sensitivity of the novel assay was higher although HEV genotype 1 derived antigen was not included. Follow-up analyses mainly confirmed the outcome of the HEVΔORF2 in-house test, thus substantiating the improved antigenic properties of the protein. In addition, a capsid protein harbouring the truncations mentioned above has been described as assembling into virus-like particles, maintaining the antigenic properties of the wild-type virus (Li et al., 2005; Xing et al., 2011). Zhou et al. (2005) also reported a capsid protein composed of aa 112–607 to be the most immunodominant region, since hepatitis E patients exhibited highest antibody titres against the truncated capsid version and against a peptide modelling the minimum neutralization epitope (aa 458–607). These observations support our hypothesis that a truncated ORF2 protein harbouring the neutralization sites that have been recorded so far is sufficient to detect a serological response to HEV.

To summarize, a recombinant *L. tarentolae* strain expressing a truncated HEV capsid protein was generated. The specificity and antigenicity of this protein were proven by ELISA and immunoblot and finally it was applied in a serological assay. To our knowledge, the study described above represents the first report on applying a recombinant Leishmania expressed protein in a diagnostic assay. Future studies will aim at the identification and monitoring of HEV positive pig herds to minimize the risk of zoonotic infections. Furthermore, investigating the HEV antibody status in the human population depicts an interesting case of application. The novel protein with its advanced antigenic characteristics might therefore help to surmount difficulties concerning strong alterations of the sensitivity of diagnostic assays.

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