EUROPEAN PATENT SPECIFICATION

THE POLYPEPTIDE FRAGMENTS OF HEPATITIS E VIRUS, THE VACCINE COMPOSITION COMPRISING SAID FRAGMENTS AND THE DIAGNOSTIC KITS

THE POLYPEPTIDFRAGMENTE DES HEPATITIS-E-VIRUS, DIE DIESE FRAGMENTE ENTHALTENDE IMPFSTOFFZUSAMMENSETZUNG UND DIE DIAGNOSTISCHEN KITS

FRAGMENT POLYPEPTIDIQUE DU VIRUS DE L'HEPATITE E, COMPOSITION VACCINALE CONTENANT LEDIT FRAGMENT, AGENT DIAGNOSTIC ET LEURS APPLICATIONS

Designated Contracting States:
DE FR GB IT

Priority: 30.09.2000 CN 00130634

Date of publication of application: 30.07.2003 Bulletin 2003/31

Divisional application: 10183466.3 / 2 298 793

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The present invention relates to a polypeptide consisting of the amino acid sequence as of amino acid residues 414 to 660 from SEQ ID No. 1 of hepatitis E virus ORF 2 which is in the form of N-polymeric polypeptide, wherein N is an integer from 2-180.

The present invention further relates to a vaccine composition against hepatitis E virus which comprises the above-mentioned N-polymeric polypeptide, or diagnostic kit for hepatitis E virus infection comprising the above-mentioned N-polymeric polypeptide, which includes IgG, IgM, or total antibody diagnostic kit for hepatitis E virus, and to the use of vaccine composition and diagnostic kit for prophylaxis, diagnosis and/or treatment of hepatitis E virus infection.

BACKGROUND OF THE INVENTION

Hepatitis E Virus (HEV) was firstly recognized as a pathogen to enterically transmitted non-A, non-B hepatitis in 1983 (Balayan et al., 1983. Intervirology 20:23). Hepatitis E is mainly endemic in developing countries in Asia, Africa and Middle America. In developed countries, hepatitis E cases were mostly found in immigrants or traveler from abroad. Both sporadic cases and large epidemic have been documented. During the period from 1950s to 1990s, several hepatitis E outbreaks happened sequentially due to polluted drinking water (Visvanathan, 1957, Indian J. Med. Res.(Suppl.). 45:1-30; Wong et al., 1980 Lancet., 2: 882-885; Myint et al., 1985, Am J Trop Med Hyg., 34:1183-1189; Belabbes et al., 1985 J Med Virol., 16:257-263; Hau et al., 1999, Am J Trop Med Hyg., 60:277-280). Most hepatitis E infection was self-limited and scarcely developed into chronic; but for the pregnant, the sequel was severe with a mortality rate above 17% (Tsenga et al., 1992, Clin. Infec Dis., 14:961-965; Dilawari et al., 1994, Indian J Gastroenterol., 13:44-48; Hussaini et al., 1997, J Viral Hepat., 4:51-54).

In 1991, researchers got the first complete genome sequence of HEV, a single-strand non-enveloped positive RNA virus (Tam et al., 1991, Virology 185:120-131). Sequence analysis showed the genome was 7.2kb with three open reading frames. ORF1 which located at 5' end encodes non-structural protein of the virus, ORF2 which located at 3' end encodes major structural protein of the virus. The full length amino acid sequence of the ORF2 protein (amino acids 1 to 660) is disclosed for example in US 6,054,567 as SEQ ID No: 2. In this document the sequence has been used in the development of vaccines and diagnostic methods, e.g. in immunoassays for the detection of IgG and IgM anti-HEV, for Hepatitis E virus. At ORF3 5' end, there is one bp overlapped with ORF1 3' end. At ORF3 3' end, there are 339 bp overlapped with ORF2 end. It's acknowledged that ORF3 encodes another structure protein with unknown function (Tam et al., 1991, Virology, 185:120-131; Aye et al., 1992, Nucleic Acids Res., 20:3512; Aye et al., 1993, Virus Genes., 7:95-109; Huang et al., 1992, Virology, 191:550-558; Reyes et al., 1993, Arch Virol Suppl., 7:15-25).

The detection of HEV infection mainly depended on Immunological Electron Microscope (IEM) or Immunological Fluorescence Technique for a long time, but those techniques are very complicated, expensive and hard to be fulfilled in many laboratories. After the clone and sequencing of HEV genome, more sensitive techniques like ELISA, Western Blot, PCR, etc. were developed to be used in the detection of HEV infection.

It is well recognized that the development of serum HEV antibody kits is absolutely necessary, but due to very low-concentration HEV virus secreted by the infected human or animals, thus it is impossible to use sera as the source of antigen. Till now, the efficiency of HEV cell culture is still very low, which limited the availability of enough antigens for HEV detection. Thus, the detection of HEV antibody is still depending on synthesized polypeptides or recombinant antigens. Unfortunately, many serological studies showed greatly varied consistence based on synthesized polypeptides or recombinant antigens from different HEV genome regions. For example, Goldsmith et al., (1992, Lancet, 399:328-331) used ORF2 3-2(M)antigen(a.a. 613-660, Mexico strain)to detect in-hospital hepatitis E virus infection cases. The detecting rate of IgG was 91%, and fell to 27-50% after 6-12 month. When he used 3-2(B) (The same ORF2 fragments from Burma strain) in detection, the rate was just 64% and no positive result was found after 6-12 months. On the contrary, 3-2(M) could not react with the convalescent serum in a Pakistan subject, but 3-2(B) could react with the serum of the same case 4.5 years later. For those proteins, when the antibody in some cases turned negative, in others it still remained in high titer. The results were similar when the mosaic protein with several linear epitopes expressed in E. Coli was used. Lack of good HEV antibody kits limited deep research on the dynamic of antibody during HEV infection. In general, during HEV infection, specific IgG antibody is detectable in early stage, peaks after 2-4 weeks and declines quickly. Most turned to negative after 9 months, but some patients kept positive many years later. Recently, several recombinant antigens have been expressed in both baculovirus and E. coli, which reacts strongly with sera from both acute and convalescence phase. In principle, these antigens are more suitable for sero-epidemiological survey: the titer of serum HEV IgG fell rapidly after acute stage, but it still retained at a detectable level. It's worthy of notification that the antibody is related to the protection from infection during the disease epidemic.
established HEV IgM kit is ever developed around world. With respect to the indirect methods, on the one hand, the
detect result is affected by various factors and reproduction is poor; on the other, the reliability of the result is poor for
high possibility of false positive, higher negative value, or lower sensitivity. According to recent reports, during the
detection of clinical samples, when the result for IgM is positive, IgG is generally positive too, thus its value on early
diagnosis is limited but may be helpful in elevation of the specificity of diagnosis of acute infection.

It's reported that the antibody to several synthesized peptide and some recombinant antigens will disappear
quickly in many infected subjects, so clinical diagnosis on acute hepatitis E virus infection is generally based on IgG
antibody with a higher clinical concordance, but the most important defect of this method is that it can not distinguish
past infection from recent infection, which will lead to both false diagnosis in high hepatitis E virus endemic area and
under evaluation of the prevalence of hepatitis E virus infection during epidemiological studies. Therefore, there is urge
to develop a reliable and sensitive anti-μ chain IgM diagnostic kit and IgG diagnostic kit which is characterized by
high sensitivity toward convalescence sera.

In recent years, there was some progress in developing a highly sensitive IgG diagnostic kit. Mast et al. (1998,
Hepatology, 27: 857-861) provided a comprehensive evaluation of 10 major IgG antibody EIAs around world. The con-
cordance of many kits was fairly good in detecting known positive sera, but great difference existed among different kits
in detecting American blood donors. It implied that the reliability of the results is worse in HEV prevalence studies in
non-endemic areas. Among those kits, most antigens are based on linear epitopes of HEV, but two kits used conformational epitopes as antigens. First is ORF2.1 (aa394-660), the other is baculovirus expressed VLP (aa112-607). Both antigens can detect convalescence antibody, but direct data on the comparison of the concordance between those two antigens is not available till now. It’s possible that those two antigens identified different antibodies. In addition, nearly 20% prevalence was reported in non-endemic America using VLP kit, which aroused the suspicion of its specificity. But
with the reported positive HEV infection in swine, goats, cows, chickens, rats, wild monkeys and enclosed monkeys,
this could be caused due to its virulence. (Kabrane-Lazizi et al., 1999 Am J Tropic Medicine, 61:331-335). And ORF2.1 kit can detect higher positive rate among CMV infection and autoimmunological diseases. In addition, the reported ORF2.1 polypeptide, which
is a GST-conchimeric protein or polyarginie conchimeric protein, intends to obtain false positive results in practice.

Both cell and tissue culture for HEV have ever been successful, and practical methods to get a large amount of
virus is not yet available, so it’s the only research way to switch from tradition killed or attenuated vaccine to subunit
or DNA vaccine through genetic engineering.

HEV ORF2, beginning at the base positioned at 5147, has 1980 nucleotides, which encodes a polypeptide with
660 amino acids presumed to be major structural protein and constitutes the capsid of virus. At N terminal of ORF2
protein, there is a classical signal sequence followed by a region rich in arginine, which is highly positive charged region
and believed to involve in genomic RNA encapsidation during virus assembly. During translation process, ORF2 entered
endoplasmic reticulum (ER) by a mechanism of signal peptide recognized protein (SRP), and is further glycosylated
and accumulated in ER, then probably formed the capsomer of capsid in suit. Three N-glycosylated sites, Asn-137, Asn-
310 and Asn-662, are located at ORF2. They are highly conservative among different virus strains, and Asn-310 is the
major glycosylated site. ORF2-transfected mammalian cell COS, human hepatocellular carcinoma Huh-7, HepG2 can
thereby express a 88kD glycoprotein which can be found in both cytoplasm and membrane. The mutation in those
glycosylated sites did not affect the location of PORF2 onto cell membrane. However after the signal peptide sequence
was removed therefrom, PORF2 can only be found in cytoplasma. This implied that the shift of PORF2 instead of
glycosylation is necessary to protein location onto cell membrane. Like MS protein in HBV, PORF2 is possibly secreted
to cell membrane directly through ER instead of Golgi body. On the surface of transfected cell, gpORF2 is not randomly
distributed, but concentrated in some zone, which implied an active combination process of a protein subunit and maybe
aggregate into some more ordered advance forms. The final assembly / maturation of the virus need the encapsidation
of genomic RNA, thus it must be occurred in cytoplasma outside of ER or endo-wall of cell membrane. The accumulation
of gpORF2 in membrane may imply the assembly of virus. At the same time, the location of capsid protein on membrane
also implied the possibility of secretion of matured virus out of the cell through budding. One more attention should be
drawn that, the in vitro transcript and translation of PORF2 using in-vitro translation system with translating and modifying
function can produce 88kD of gpORF2 in forms of both monomers and dimmers. It illustrated that gpORF2 was prone to
form homologous dimmer, and capsomer of capsid may be constituted by said homologous dimer of gp ORF2 (Jameel
et al., 1996. J. Virol., 70:207-216.). Through Frost Etching electron microscope, Li et al. found that recombinant HEV
VLPs which is expressed by baculovirus had an icosahedral symmetry viroms (T=1), which was made up of sixty p50
subunits with 22-23 nm in diameter. Since the inner space of this size particle can contain about lkb RNA, and HEV
genome is 7.5kb in length, it is speculated natural HEV should be a crystal lattice structure with T=3, but the topological
structure of capsomer is similar. The total number of T=3 subunit is 90 capsomers (Li et al., 1999. virology, 265:35-45.) .

According to the above, HEV is a non-enveloped virus. Virus capsid is made up of ORF2-encoded protein. The
protein embodies major immunological epitopes and some neutralizing epitopes, thus it became the most favorable
In U.S. patent 5,885,768, Reyes et al. firstly reported that 4 cynomolgus monkeys were injected i.m. with recombinant protein trpE-C2 expressed in E. Coli comprising HEV Burma strain ORF2 C terminal 2/3 (aa225–660), wherein said protein is formulated with an alum adjuvant, by administering at 0, 30 day for 50 μg /dose. Another 2 monkeys were used as controls with adjuvant only. Four weeks later, no positive result regarding raised antibody from collected bloods is found by Western Blotting. A third-time immunization on two monkeys among them by administering 80 μg unsolvable recombinant protein without adjuvant. Four weeks later, both monkeys were positive (WB). Then the six monkeys were grouped into first and second group, each included three monkeys, two of them is immunized with either three-times or two-times inoculation, and one is control. The first group was attacked with Burma HEV, and the second group Mexico HEV. The results were, (1) ALT kept normal all the time in the immunized group, it but increased 6 – 10 times higher than before immunization in control; (2) Liver biopsy sample was detected by Immunological Fluorescence method. The antigen can be found in all other monkeys except those immunized with three doses and attacked by Burma strain. (3) Virus excretion in feces can be found sequentially in all other monkeys except those immunized with three doses and attacked by Burma strain. This research sample is small, but it implied that recombinant protein from ORF2 can block the occurrence of biochemical indexes of virus hepatitis and protected completely from infection when the monkeys were attacked by wild HEV.

Tsarev et al. (1994, Proc. Nat. Acad. Sci. USA., 91:10198-10202; Tsarev et al., 1993, J. Infect. Dis., 168: 369-378; Tsarev et al., 1997, Vaccine 15:1834-1838) used baculovirus in insect (SF cell) to express HEV ORF2 and got protein particles with various size from 20nm–30nm in cell solution. The percentage of smaller particles is substantively increased during anaphase of infected cells. WB method was used to detect baculovirus expressed ORF2 with many specific different-size bands at 25kD, 29kD, 35kD, 40–45kD, 55–70kD, 72kD. Ion exchange and molecular screening method were used to purify HEV specific protein. One day after recombinant virus infected the cells, the whole ORF2 peptide of 72kD firstly appeared and then disappeared gradually. On the second day, the peptides of 63kD and 55kD appeared. On the first day, 53kD peptide appeared in cell solution with large amount and peaked on the third day. This implied the primary 72kD protein was randomly cut into HEV protein with 55kD (in cell lysis solution) and / or 53kD (cell solution). The sequencing to those two proteins showed 55kD located at ORF2 aa112–607, but 53kD located at aa112–578 and 63kD at aa112–660. The results of ELISA showed the activity of 55kD was apparently stronger than 53kD. If aa112–660 fragment was expressed in baculovirus insect, 63kD and 55kD recombinant HEV protein can also be found.

Li et al, J. Virol., 1997, 7207-7213 further describes cleavage of the aa 112–660 fragment to a polypeptide of 50 kD. The cleavage product has been observed to self-assemble into virus-like particles (VLPs) composed of 30 dimers. It was further shown that the VLPs have an antigenic similarity to an authentic Hepatitis E virus and are immunogenic in rabbits and guinea pigs.

SF9 cells were collected at day 7 from the cells had been infected. The protein was primarily purified and absorbed with alum adjuvant. Then cynomolgous monkeys were immunized i.m. with 50 μg protein per dose. Four received 1 dose, the other four two doses (0d, 28d). After the final dose, all monkeys were attacked with dose 1000–10000CID50 i.v. of the same HEV strain (SAR-55, from a Pakistan patient). Within 15 weeks, liver biopsy, sera and feces were collected every week. Before virus attack, antibody titer in one-dose monkeys were 1:100–1:10000, but in two-dose group they were all 1:10000 (coated with purified 55kD). In one-dose group, one monkey was dead due to anesthesia accident 9 weeks after virus attack (still calculated in the results). In two-dose group, two monkeys died soon after the virus attack (no calculated in the results) due to unknown reason. Six monkeys after immunization were found no ALT elevation or liver biopsy pathological change, and no viremia. Among four monkeys in one-dose group, three has virus excretion, but two monkeys in two-dose group no virus excretion was found.

Further purification was done in 55kD protein expressed in baculovirus system through ion exchange and molecular sifting methods to make its purification reachable above 99%. After absorbed with alum adjuvant, the protein with dose 50 μg, 10 μg, 2 μg, 0.4 μg, 0 μg(control)was each injected into 4 rhesus monkeys administered 0 and 28 day. Four weeks after the last dose, the monkeys were attacked with the same virus (SAR-55). Sixteen monkeys in the immunized group were all normal, and just one monkey with 2 μg dose and the other with 0.4 μg dose appeared very light pathological change. Though the immunized can prevent from hepatitis but not infection. All sixteen monkeys immunized appeared virus excretion, also viremia except one monkey with 50 μg dose and the other with 10 μg dose. And the amount of virus was limited in most monkeys, but the duration has not been shortened. Another four monkeys were immunized with 2 × 50 μg, and attacked with 100,000 MID50 other HEV 4 weeks after the final dose. The results were similar. All four monkeys did not show ALT elevation and pathological change, but only one monkey did not show virus excretion and viremia. The amount of virus decreased apparently, but the duration has not been shortened. According to the author’s opinion, the effect of complete protection on those monkeys was worse that previous time. It’s possibly attributable to the amount of virus used in attack. The amount of virus in this experiment reached 300,000, but was 1000–10000MID50 last time. One more, the titer of antibody among groups from 0.4 μg to 50 μg has showed no difference before attack.
The staffs in Genelabs company expressed ORF2 aa112-660 using the same baculovirus in insect and got a large amount solvable recombinant 62kD protein. After purification, the cytomolgous monkeys were immunized and protected from the attack of virus (Mexico strain) with dose 1000CID50 (3 monkeys immunized with 20 μg did not get disease. Virus excretion was not found in two monkeys, and the amount of virus excretion decreased in one monkey). (Zhang et al., 1997, Clin Diagn Lab Immunol.;4:423-8.)

McAttee et al. (1996, Protein Expr. Purif., 8:262-270) prepared Burma ORF2 62kD dimersexpressed in recombinant baculovirus. The dimer was dissociated into two peptides separately with 56.5kD and 58.1kD through HPLC-MS. Peptide mass fingerprint analysis showed the N terminal of those two peptides was the same aa112, and the C terminal is separately aa637 and aa6852. And 56.5kD protein was a very good immunogen.

Anderson group in Australia (Anderson et al., 1999. J. Virol. Methods., 81:131-142; Li et al., 1994, J Clin Microbio.)32:2060-2066; Li et al., 1997 J. Med. Virol., 52:289-300 Li et al., 2000, J. Med. Virol., 60;379-386 Riddel et al., J. Virol., 74, 8011-8017) used ORF2 aa394-660 (ORF2.1) expressed in E. Coli. The product is a GST-conchimeric or poly arginine protein which can form a highly conformational convalescence epitope. This epitope can detect a very high-rate convalescence sera, but it will disappear when the fragment was extended or truncated towards N terminal. The serum at 30 weeks after rats were immunized with recombinant ORF2.1 protein was used to block the serum from high-rate convalescence sera, but it will disappear when the fragment was extended or truncated towards N terminal.

The serum at 30 weeks after rats were immunized with recombinant ORF2.1 protein was used to block the serum from convalescence patients with VLP expressed in baculovirus as the coated antigen. The blocking rate will reach 81%.

Monoclonal antibody was prepared with ORF2.1 protein and two monoclonal antibody 2E2 and 4B2 which can identify ORF2.1 conformational epitopes, and five possible identifiable monoclonal antibodies were obtained. The blocking rate can reach 60% whether 2E2 or 4B2 was used to block convalescence sera with VLPs as antigen. This implied that those two monoclonal antibodies can identify the epitopes which was major components of antibody identified epitopes in convalescence sera. Different data showed that ORF2.1 had major epitope structure rather similar to VLP. The antibody to the epitopes can exist for a long time in HEV infected serum. It’s probably an important protective epitope, but animal protection experiment about ORF2.1 has not been reported till now.

WO 96/12807 discloses an antigen fragment corresponding to amino-acids 334-660 of the HEV ORF2 protein which was expressed recombinantly and used in immunoassays using various human acute phase and convalescent phase sera taking during various hepatitis E epidemics. The sera were tested by ELISA for IgG and IgM antibodies to hepatitis E virus and a 62 kD fragment of HEV ORF2 antigen was used to immunize cynos.

Finally a, WO 95/17501 discloses monoclonal antibodies for the detection of HEV ORF2 antigens and competitive immunoassays using these antibodies.

SUMMARY OF THE INVENTION

In one aspect of the present invention, it provides a polypeptide consisting of the amino acid sequence of amino acid residues 414 to 660 from the amino acid sequence of hepatitis E virus open reading frame (ORF) 2 (as set forth in SEQ ID No. 1) which is in the form of n-polymeric polypeptide, wherein n is an integer from 2-180.

In another aspect of the present invention, it further provides a polypeptide having at least 80% identity to the aforementioned polypeptides and having identical antigenicity or immunogenicity.

In another aspect of the present invention, it further provides a vaccine composition for prophylaxis and/or treatment of hepatitis E virus infection in mammals, which comprises at least one of the polypeptides of the present invention or any combination thereof, and optionally, pharmaceutically acceptable vehicles and/or adjuvant.

In another aspect of the present invention, a multimer formed by 2-180 purified monomeric polypeptides via self-aggregation, wherein each said purified monomeric polypeptide is a chimeric protein formed by linking polypeptide of the present invention and a conserved fragment of hemagglutin antigen from influenza virus.

In another aspect of the present invention, it further provides a vaccine composition for prophylaxis and/or treatment of hepatitis E virus infection in mammals, which comprises chimeric protein consisting of one of polypeptides of the present invention and a conserved fragment of hemagglutin antigen from influenza virus, and optionally, pharmaceutically acceptable vehicles and/or adjuvant.

In another aspect of the present invention, it further provides use of the above-mentioned vaccine compositions for vaccinating mammals to prevent from hepatitis E virus infection.

In another aspect of the present invention, it further provides a method for prophylaxis and/or treatment of hepatitis E virus infection in mammals, which comprises administrating to the subject with a prophylaxis and/or treatment effective amount of at least one of the above-mentioned polypeptide or chimeric protein multimers consisting of at least one of the above-mentioned polypeptide and a conserved fragment of hemagglutin antigen from influenza virus.

In another aspect of the present invention, it further provides a diagnostic kit for the diagnosis of hepatitis E virus infection in biological sample, which comprises a diagnosis effective amount of at least one of the polypeptide multimers of the present invention or any combination thereof.

In another aspect of the present invention, it further provides a diagnostic kit for the diagnosis of hepatitis E virus infection in biological sample, which comprises a diagnosis effective amount of at least one of the polypeptide
multimers of the present invention or any combination thereof, and further comprises the polypeptide containing immunogenic epitope from hepatitis E virus ORF3 or an immunogenic fragment thereof, wherein said polypeptide containing immunogenic epitope from hepatitis E virus ORF3 or an immunogenic fragment thereof is, optionally, covalently bound to said polypeptide.

[0033] The diagnostic kit can be used in a method for diagnosis hepatitis E virus infection in biological samples, comprising contacting the above-mentioned diagnostic kit with sample to be detected under the conditions suitable for the interaction of antigen and antibody.

[0034] In another aspect of the present invention, it further provides a method for detecting total antibodies against hepatitis E virus, a method for detecting antibody IgG against hepatitis E virus, and a method for detecting antibody IgM against hepatitis E virus in biological samples.

BRIEF DESCRIPTION OF THE DRAWINGS

[0035] Figure 1 presents a schematic diagram of the construction of plasmid pTO-T7-ORF2-201 for expression of the polypeptide 201.

Figure 2 shows the results of analysis by 12% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) (Coomassie brilliant blue R250 stain) regarding culture lysates of the induced E.coli transformed with expression vector pTO-T7-ORF2-201 (with steps of: centrifuging culture medium, collecting precipitated cell, then resuspending pellet with loading buffer including 0.1% SDS, further treating it in boiling water 10 min, then centrifuging under 12,000 rpm for 10min, taking supernatant for determination). Lanes 1 and 2 respectively contain two different bacteria culture lysates. Expressed products take up around 35% of total protein as analyzed by Uvi gel imaging system (UVitec, ltd., model DBT-08).

Figure 3 shows the analysis results of Coomassie blue R250-stained 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) regarding 2M and 4M Urea solution of purified polypeptide 201 inclusion body from four batches of polypeptide 201, wherein said samples are obtained from recombinant E. coli embodying expression vector pTO-T7-ORF2-201. The results show that some of the polypeptide 201 had undergone renaturation to form dimmer polypeptide with a proportion for the dimmer polypeptide varied from 10% to 60%. The proportion for renaturation is lower than that of sample renatured in 1/3 PBS (1L):Na2HPO4-12H2O, 73.344g; KH2PO4, 4g; NaCl, 163.632g; KCl, 4.024g, pH7.45). As shown in Figure 4, percent of dimmer is 99%.

Figure 4 shows the results of Western blotting analysis of polypeptide 201 with serum from HEV-infected patient. Lanes 1-3 is SDS PAGE control, wherein lane 1, protein molecular weight marker; lane 2, renatured sample of polypeptide 201 in 1 X PBS; Lane 3, renatured polypeptide 201 treated in boiling water bath for 10 min; Lane 4 and 5, the respective Western blot results corresponding to the sample of Lanes 2 and 3.

Figure 5 shows the results from hydrated dynamic semi-diameter by dynamic light scattering instrument of aforementioned polypeptide 201, wherein polypeptide 201 is in advance purified by gel filtration HPLC, and centrifuged for 10 min under 20000g, filtrated with 0.1 um filter membrane.

Figure 6 shows Western blot results of the reaction of polypeptide 208N, 209N and 225N with mouse Mab 1F6, 2C9 and 3F5. Lanes 1, 2, 3, respectively, corresponds to the renatured sample being treated in boiling water bath for 10 min, renatured sample and the precipitated renatured samples of polypeptide 208N; Lanes 4, 5, 6, respectively, corresponds to the renatured sample being treated in boiling water bath for 10 min, renatured sample and the precipitated renatured samples of polypeptide 209N; Lanes 7, 8, 9, respectively, corresponds to the renatured sample being treated in boiling water bath for 10 min, renatured sample and the precipitated renatured samples of polypeptide 225N; and Lane 10 is monomer polypeptide 201 as control.

Figure 7 illustrates the profile of HEV antibodies raised in sera from mice following immunization with vaccine of polypeptide 201 (containing Feund’s adjuvant) in various dosages. The horizontal coordinate is defined as the days after the first immunization. The vertical coordinate is defined as the OD450nm/620nm measured by ELISA.

Figure 8 illustrates the profile of HEV antibodies raised in sera from mice following immunization with vaccine of polypeptide 201 (containing no adjuvant) in various dosages. The horizontal coordinate is defined as the days after the first immunization. The vertical coordinate is defined as the OD450nm/620nm of ELISA.
DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

[0036] Unless otherwise indicated, all the terms or nomenclatures used herewith are the same as those conventionally used in the art. The conventional manufacturers in cell culturing, molecular genetics, nucleic acid chemistry, and immunological procedure are carried out as routine technique in the art. In the present invention, unless otherwise indicated, these terms used herewith have the meanings as follows:

[0037] "hepatitis E virus" or "HEV" refers to a virus, virus type or virus class, which i) causes water-borne, infectious hepatitis; ii) it distinguishes from hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), or hepatitis D virus (HDV) in terms of serological characteristics; iii) contains a genomic region that is homologous to a 1.33 kb cDNA inserted in pTZKF1(ET1.1), said plasmid is embodied in a E.coli strain deposited in American Type Culture Collection (ATCC) with accession number 67717.

The polypeptide of the present invention

[0038] In one aspect, the present invention surprisingly provides a multimer of a polypeptide fragment of HEV with satisfied antibody reactivity and/or immunogenicity, wherein said fragment consists of amino acid residues 414 to 660 from the amino acid sequence of HEV ORF2, as set forth in SEQ ID NO:1. The name of individual fragment can be found in table 2 of example 6.

[0039] In the present invention, the numbering of the amino acid residue by position in the amino acid sequence is in accordance with the numbering manner of International Union of Pure and Applied chemistry and International Union of Biochemistry, Joint commission on biochemical Nomenclature, "Nomenclature and symbolism for Amino Acids and Peptides", Pure Appl. Chem., 56, 595-624 (1984). Specifically, the coding start site Met in Seq Id No: 1 is designed as position 1, increased in the direction from 5’ to 3’.

[0040] In one aspect of the present invention, a polypeptide is provided, which consists of amino acid residues 414 to 660 of the amino acid sequence as set forth in SEQ ID No. 1 of hepatitis E virus ORF2 in the form of n-polymeric polypeptide, wherein n is an integer from 2-180. When n is 2, said polypeptide is a dimmer polypeptide; when n is 3, said polypeptide is a trimmer polypeptide; when n is 4, said polypeptide is a tetrammer polypeptide, and so on.

[0041] In the present invention, the amino terminus (5’ end) of said polypeptide fragment comprising amino acid sequence as set forth in SEQ ID NO: 1 starts at amino acid residue 414 and the carboxyl terminus (3’ end) of said polypeptide ends at amino acid residue 660. Specifically, the polypeptide of the present invention is polypeptide 247.

[0042] In another aspect of the present invention, it further relates to a multimer of polypeptide having at least 80% identity to the preceding polypeptide and having identical antigenicity or immunogenicity, i.e., the derivatives of the polypeptide of the present invention. Specifically, the polypeptide is considered as derivatives of polypeptide of present invention under the condition that the amino acid of said polypeptide comprises the amino acid sequence of aforementioned polypeptide with other amino acid than a natural sequence neighboring to the present polypeptide at N-terminus and/or C-terminus thereof, but it still remain the similar antigenicity and/or immunogenicity etc. to the present polypeptide. Consequently, DNA fragment corresponding to the same is called derive DNA of present invention. For example, for the purpose of expression and/or purification, it would be facilitate to purification by adding start amino acid (Methionine) or other leading peptide and/or signal peptide at N-terminus thereof, or by adding several Histidines at C-terminus thereof.

[0043] The term "percentage identity " is intended to denote a percentage of nucleotides or of amino acid residues which are identical between the two sequences to be compared, obtained after the best alignment, this percentage being purely statistical and the differences between the two sequences being distributed randomly and over their entire length. Sequence comparisons between two nucleotide or amino acid sequences are conventionally carried out by comparing these sequences after having aligned them optimally, said comparison being carried out be segment or by "window of comparison" in order to identify and compare local regions of sequence similarity. The optimal alignment of the sequences for comparison may be produced, besides manually, by means of the local homology algorithm of Smith and Waterman.

[0044] The percentage identify between two nucleic acid or amino acid sequences is determined by comparing these two sequences aligned in an optimal manner in which the nucleic acid or amino acid sequence to be compared may comprise additions or deletions compared to the reference sequence for optimal alignment between these two sequences. The percentage identify is calculated by determining the number of identical positions for which the nucleotide or the amino acid residue is identical between the two sequences, dividing this number of positions compared and multiplying the result obtained by 100 so as to obtain the percentage identity between these two sequences.

[0045] For example, program BLAST "BLAST 2 sequences" can be used which is obtained from website http://www.ncbi.nlm.nih.gov/gorf/b12.html, wherein parameter is default (particularly, open gap penalty is 5, extension gap penalty is 2; matrix is provided by such as program "blosum 62"), the percentage of identity between two sequences to be aligned is directly calculated by the program.

The preparation of polypeptide precursor of the multimers of the present invention


[0047] In one embodiment of the present invention, E.coli is used to individually express the polypeptide 247.

[0048] The ratio between monomer and dimer of said polypeptide, the formation of polymeric peptide and individual radium thereof are determined (see, Example 6 for detail). As the result shows that the expressed product is prone to refold. The capability for self-forming stable polymers in solution after refolding makes the polypeptide particularly suitable to be used as vaccine, for the prophylaxis and/or treatment of HEV. In one of the embodiment in the present invention, the polymer including dimmer, trimmer, and tetramer has been detected in one test. Limited by the methodology up to date, there is possibility that the deduced trimmer is in fact composed of the mixture of dimmer and tetramer in a suitable ratio. It is also contemplated that the polypeptide is able to form even larger polypeptide due to the improvement on methodology. Deducing from published reference (Jameel, et al., 1996, J. Virology, 70:207-216; Li, et al., 1999, Virology, 265:35-45), there is high possibility that natural HEV is composed of 90 sub-particles, wherein each sub-particles is dimmer of ORF2 polypeptide. Therefore, it is reasonably contemplated that the polypeptide is able to form a polymer of up to 180-meric polypeptide, or even larger polymers, with the development on the understanding of virus structure.

[0049] Polypeptide 201 is expressed by E.coli in form of inclusion body with high yield, and the present inventor surprisingly finds that said inclusion body is able to spontaneously self-renatured in the PBS buffer of pH7.45, which avoids the time-costing and tedious conventional denature/renature process that substantively reduce recovering rate, including the steps of adding guanidine hydrochloride and then undergoing multi-steps of dialysis. In addition, since other non-desired protein inclusion body simultaneously expressed by E.coli is unable to spontaneously self-renature, the protein of interest in the present invention can be substantially purified simply by centrifugation and recovering supernatant.

[0050] The chimeric protein consisting of polypeptide and conserved fragment of hemagglutinin antigen from influenza virus

[0051] In another aspect of the present invention, chimeric protein consisting of the above-mentioned polypeptides and conserved fragment of hemagglutinin antigen from influenza virus is also provided. Hemagglutinin antigen (hereinafter designed as HA) is one the two surface antigens of influenza virus, and is the most imported antigen used in specific detection for antibody against influenza virus in the serum of subject. It is known that antibody raised by vaccinating animal with HA can effectively prevent the receipt from re-infection of influenza virus. Therefore, it is reasonable to believe that antibody against HA is presented in most of population. According to previous report (Mcewen J. et al., Vaccine, 1992;10 (6):405-11), epitope 91-108 aa is the conserved amino sequence in HA gene among all the H3 strains of influenza virus type A. In one preferred embodiment of the present invention, firstly, chimeric expression in prokaryotic expression system, especially in E.coli is established by flexibly linking HA gene (91-108aa) to the polypeptide fragment of HEV ORF2 gene of the present invention that is highly immunogenic, such as Gly-Gly-Ser by genetic engineering. Then boost with HA antibody raised by previously infection of influenza virus, so as to generate high-titer protective anti-
HEV antibody. In this way, a HEV vaccine is obtained that is super to the vaccine containing ORF2 fragment of polypeptide of the present invention alone.

[0052] In light of the teachings in the present invention, epitopes useful to the vaccine composition of the present invention can be selected from other conserved fragment in HA gene by skilled person in the art. As to the specific flexible linker linking specific epitope of HA and polypeptide, it can be consisted of suitable peptide fragment or analog thereof, provided that it facilitate linkage between polypeptide and selected fragment of HA and that does not substantively affect the use of polypeptide multimers of the present invention for prophylaxis/treatment HEV infection in mammals. It should be understood, selection of linker for linking polypeptide and HA mainly depends on the specific property of the selected polypeptide. For example, different linkers might be selected according to selected polypeptide to be linked with HA. Preferably, the conserved fragment of HA used in the present invention is a fragment from amino acid residue 91-108.

[0053] Linker for the linkage of polypeptide of the present invention as immunogen and selected fragment of HA can be synthesized preferably by conventional synthesis technique, such as chemical synthesis technique. In addition, any peptide can be synthesized by skilled person in the art in accordance with standard chemical method, such as t-BOC method by automatic peptide synthesisor (see, e.g., L.A. Carpino, J. Am. Chem. Soc., 79:4427, 1957). However, peptide can also be produced by chemically hydrolysis of protein or other known methods.

[0054] Alternatively, the chimeric protein of polypeptide with HA can be produced by host cell transformed with nucleic acid sequence of a DNA molecule, wherein said DNA molecule comprise a sequence encoding fragment of HA and polypeptide which is obtained by cloning in host microorganism or cell through conventional genetic engineering method, such as recombinant DNA technique. When it is generated by recombinant technique in the transformed cell, the resulted chimeric protein can be purified and recovered by routine method from culture medium, host cell or from the both. Said chimeric protein produced by recombination method is isolated so that the resulted peptide can be substantively separated from cell substance or culture medium during the recombinant production by recombinant DNA techniques. In addition, coding sequence for the resulted peptide could also be prepared by synthesis, or by using virus RNA in accordance known method or the available plasmid containing cDNA thereof.

[0055] For use in the present method, the above-mentioned chimeric protein can be designed to generally known construct or other construct in order to increase the production thereof or facilitate purification of the same. The suitable system and vectors is known and public available, or commercial available for cloning and expression chimeric peptide in various microorganisms and cells, including such as E.coli, Bacillus, Streptomyces, Saccharomyces, mammals, yeast, insect cell and plant cell.

[0056] The chimeric protein produced by either recombination or synthesis can be purified by routine purification method. The skilled person in the art can easily determine a desired purity for the polypeptide according to the use of interest.

Vaccine composition

[0057] In another aspect of the present invention, it further provides a vaccine composition for prophylaxis and/or treatment of hepatitis E virus infection in mammals, which comprises at least one multimer of polypeptides of the present invention or any combination thereof, and optionally, pharmaceutically acceptable vehicles and/or adjuvant.

[0058] In still another aspect of the present invention, it further provides a vaccine composition for prophylaxis and/or treatment of hepatitis E virus infection in mammals, which comprises multimers of chimeric protein containing a polypeptide as described above and a conserved fragment from hemagglutinin antigen of influenza virus, and optionally, pharmaceutically acceptable vehicles and/or adjuvant.

[0059] In still another aspect of the present invention, use of above vaccine compositions for vaccinating mammals to prevent from hepatitis E virus infection is provided.

[0060] In present invention, mammals to be inoculated or treated includes but not limits to human being and other primates, such as baboon, ape, monkey, etc.; economic animals, such as, bovine, caprine, swine, rabbit, murine, as well as pets, such as feline, canine, etc. Said vaccine composition contains treatment and/or prophylaxis effective amount of at least one of polypeptide multimer of the present invention, wherein said effective amount is the amount that sufficient for effectively treat subject infected by HEV or prevent subject from HEV infection after administrating for a certain time.

[0061] The vaccine composition of present invention could be used either alone or as part of the formulation for medicament or prophylaxis, which optionally contains pharmaceutically acceptable vehicles, including release-controlling agent. Said vehicles might further include pharmaceutically acceptable vectors or diluents suitable for the administration for treatment and/or prophylaxis of HEV infection. Suitable pharmaceutically acceptable vectors refer to those biologically inert and/or non-toxic. Various vectors known in the art can be selected according to desired use. Typically, said vector can be selected from but not limit to the group consisting of: sterile saline, lactose, sucrose, calcium orthophosphate, gelatin, dextrin, agar, alum, aluminum oxide, aluminum hydroxide, peanut oil, olive oil, sesame oil, and water. Additionally, vector or diluents can further include controlled released substance, such as glyceryl monostearate/glyceryl distearate,
The polypeptide multimer of the present invention can be used for detection of presence of antibody IgG, IgM or total antibody against HEV in biological sample. The polypeptide multimer of the present invention is amino acid fragment from 91-108 amino acid residues. The inventive polypeptide multimers can be used in a method for prophylaxis and/or treatment of hepatitis E virus infection in mammals, which comprises administrating to the subject with a prophylaxis and/or treatment effective amount of polypeptide multimers of the present invention or chimeric protein multimers consisting of at least one of polypeptides as described above and conserved fragment of hemagglutinin of influenza virus. In particularly, said method comprises the step of administrating to subject with the vaccine composition of the present invention. Preferably, the conserved fragment selected by the present invention is amino acid fragment from 91-108 amino acid residues.

Suitable amounts of these compositions may be determined based on the level of response desired. In general, compositions comprising the polypeptide multimer of present invention may contain between about 5 ug and about 200 ug of the particles. Such compositions may be administered as one or a series of inoculations, for example, three inoculations at intervals of two to six months. Suitable dosage may also be determined by judgment of the treating physician, taking into account factors such as the patient's health status, weight or age, as well as the conventional dosage of a component immunogen, when administered as a monotherapy. Upon improvement of a patient's condition or likelihood of increase exposure to a given pathogen, a maintenance dose of a composition comprising polypeptide of present invention may be administered, if necessary. Subsequently, the dosage or frequency of administration, or both, may be reduced to a level at which the desired effect is retained. At that point, treatment should cease. Individuals may, however, require intermittent treatment on a long-term basis upon recurrence of a given unwanted condition.

Compositions comprising polypeptide multimers of present invention may be administered by any suitable route, such as, for example, parenteral administration, particularly intramuscular or subcutaneous, as well as oral administration. Other routes, may also be used, such as pulmonary, nasal, aural, anal, ocular, intravenous, intrarterial, intraperitoneal, mucosal, sublingual, subcutaneous and intracranial.

Preparation of vaccine composition of present invention may be carried out to formulate injectable compositions or vaccine, either as liquid solutions or suspensions. Solid forms suitable for solution or suspension in liquid prior to injection may also be prepared. Preparations also may, in certain embodiments, be emulsified or encapsulated in liposomes, or in soluble glasses, for controlled released and/or prolonged delivery. Alternatively, preparations may be in aerosol or spray form. They may also be included in trans-dermal patches. The active ingredient may be mixed with any number of excipients which are pharmaceutically acceptable and compatible with the active ingredient or ingredients. The excipients include, for example, Freund's incomplete adjuvant, bacterial lipopolysaccharides, ion exchanger, alumin, aluminum stearate, muramyl dipeptide, lecithin, buffer substance, cellulose-base substances and polyethylene glycol.

Diagnostic kit and method for detecting antibody IgG, IgM or total antibody against HEV in biological sample

Still further, when desired, the vaccine composition of the present invention comprising at least one polypeptide multimer of present invention or any combination thereof can further comprise other treatment / prophylaxis agent. For example, said composition could comprise a "cocktail mixture" of various agent that is useful in the treatment or prophylaxis for HEV infection. Such cocktail mixture could further include other agents, such as interferon, nucleotide analogs and/or N-acetyl-cysteine.

Optionally, the vaccine composition of the present invention comprising at least one polypeptide multimer of present invention might further comprise immune system modifiers, such as, adjuvants or cytokines useful for further induction of antibody and T cell response in subject. Said modifier includes conventionally alum-based adjuvants, muramyl dipeptides, preservatives, chemical stabilizers or other antigenic protein. Generally, stabilizers, adjuvants or preservatives and the like are optimized of dertrim the best formulation for efficacy in the desired application. Suitable preservatives may include chlorobutynol, potassium sorbate, sorbaic acid, sulphur dioxide, propyl galade, parabens, glycerine, and phenol.

Method for prophylaxis and/or treatment of HEV infection in mammals using vaccine composition of the present invention

Diagnostic kit and method for determining the presence of antibody IgG against hepatitis virus.
E virus in the biological sample is provided, which comprises at least one of the polypeptide multimers of the present invention, if desired, said polypeptide multimer is pre-coated on the surface of a suitable support; and further comprises commercial available or routinely generated, detectable labeled antibody anti-IgG that is directed against IgG from biological sample to be detected, and detection agent corresponding to said detectable label; and if desired, further comprises a suitable buffer system.

[0072] In one embodiment of present invention, said biological sample to be detected is derived from human being, wherein antibody is anti-human IgG antibody. More specifically, the diagnostic kit for IgG antibody of the present invention further includes a polypeptide having immunogenic epitope in HEV ORF3 or an immunogenic fragment thereof, wherein said immnogenic epitope in HEV ORF3 or an immunogenic fragment thereof is optionally covalently bound to the polypeptide of present invention.

[0073] For the situation that said immnogenic epitope in HEV ORF3 or an immunogenic fragment thereof is optionally covalently bound to the polypeptide as described above, the chimeric polypeptide is preferably produced by genetic recombination method. Chemical method can also by used to covalently bind said immnogenic epitope in HEV ORF3 or an immunogenic fragment thereof to the aforementioned polypeptide.

[0074] In another aspect of the present invention, a diagnostic kit for determination of antibody IgM against hepatitis E virus in the biological sample is provided, which comprises commercially available or routinely generated, detectable labeled antibody anti-IgM as capture antibody that is directed against IgM from biological sample to be detected, if desired, said capture antibody is pre-coated on the surface of a suitable support; and further comprises detectable labeled at least one of the polypeptide multimers of the present invention, and detection agent corresponding to said detectable label; if desired, further comprises a suitable buffer system.

[0075] In one embodiment of present invention, said biological sample to be detected is derived from human being, wherein antibody is anti-human IgM antibody. More specifically, the diagnostic kit for IgM antibody of the present invention further includes a polypeptide having immunogenic epitope in HEV ORF3 or an immunogenic fragment thereof, wherein said immnogenic epitope in HEV ORF3 or an immunogenic fragment thereof is optionally covalently bound to the polypeptide as described above.

[0076] For the situation that said immnogenic epitope in HEV ORF3 or an immunogenic fragment thereof is optionally covalently bound to the polypeptide as described above, the chimeric polypeptide is preferably produced by genetic recombination method. Chemical method can also by used to covalently bind said immnogenic epitope in HEV ORF3 or an immunogenic fragment thereof to the aforementioned polypeptide.

[0077] In another aspect of the present invention, a diagnostic kit for determination of antibody IgM against hepatitis E virus in the biological sample is provided, which comprises commercially available or routinely generated, detectable labeled antibody anti-IgM as capture antibody that is directed against IgM from biological sample to be detected, if desired, said capture antibody is pre-coated on the surface of a suitable support; and further comprises detectable labeled at least one of the polypeptide multimers of the present invention, and detection agent corresponding to said detectable label; if desired, further comprises a suitable buffer system.

[0078] In one embodiment of present invention, said biological sample to be detected is derived from human being, wherein antibody is anti-human IgM antibody. More specifically, the diagnostic kit for IgM antibody of the present invention further includes a polypeptide having immunogenic epitope in HEV ORF3 or an immunogenic fragment thereof, wherein said immnogenic epitope in HEV ORF3 or an immunogenic fragment thereof is optionally covalently bound to the polypeptide as described above.

[0079] For the situation that said immnogenic epitope in HEV ORF3 or an immunogenic fragment thereof is optionally covalently bound to the polypeptide as described above, the chimeric polypeptide is preferably produced by genetic recombination method. Chemical method can also by used to covalently bind said immnogenic epitope in HEV ORF3 or an immunogenic fragment thereof to the aforementioned polypeptide.

[0080] In still another aspect of present invention, a diagnostic kit for determination of total antibodies against hepatitis E virus in the biological sample is also provided, which comprises at least one of the polypeptides multimers of present invention, if desired, said polypeptide is pre-coated on the surface of a suitable support; and further comprises detectable labeled at least one of polypeptides according to claim 1, and detection agent corresponding to said detectable label; wherein said polypeptide multimer selected from polypeptide multimers according to claim 1 for pre-coating the surface of a support and the detectable labeled polypeptide multimer selected from polypeptide multimers according to claim 1 could be the same polypeptide, or different one.

[0081] More specifically, the diagnostic kit for total antibodies of the present invention further includes a polypeptide having immunogenic epitope in HEV ORF3 or an immunogenic fragment thereof, wherein said immnogenic epitope in HEV ORF3 or an immunogenic fragment thereof is optionally covalently bound to the polypeptide as described above, the chimeric polypeptide is preferably produced by genetic recombination method. Chemical method can also by used to covalently bind said immnogenic epitope in HEV ORF3 or an immunogenic fragment thereof to the aforementioned polypeptide.

[0082] For the situation that said immnogenic epitope in HEV ORF3 or an immunogenic fragment thereof is optionally covalently bound to the polypeptide as described above, the chimeric polypeptide is preferably produced by genetic recombination method. Chemical method can also by used to covalently bind said immnogenic epitope in HEV ORF3 or an immunogenic fragment thereof to the aforementioned polypeptide.
It is well known in the art that in the aforementioned diagnostic kit, anti-human IgG or anti-human-IgM which can be generated by various commercial available or routinely generated method taking advantage of various animals are used. Alternatively, anti-IgG or anti-IgM against the specific animals from which biological sample to be detected derived are used which can be generated by taking advantage of relating animals. For the purpose of preparing antibody, selected animal includes but not limit to goat, sheep, rat, mouse, rabbit, guinea pig, swine, etc. Said detectable label for labeling can be used alone or in combination with other composition or compound for providing detectable signal to visualize the presence of substance of interest in sample. Said detectable label can be those known and easily available materials in the art of detection field, including but not limited to enzyme marker, fluorescent marker, radioactive marker, etc. Thus, the present invention is not limited to specific selection of detection label, and contemplates that it includes all those detection method known in the art. For the purpose of convenience, said detection agent can be provided in the form of kit.

Optionally, said kit further includes micro-titer plate pre-coated with polypeptide multimer of present invention, various suitably formulated diluent and/or buffer, labeled substance or other signal-generating agent for detection of specifically bound antigen/antibody complex, such as, enzymetic substrate, co-factor and chromophore. Other components therein could be easily selected by skilled person in the art.

Additionally, a method for detecting antibody IgG against hepatitis E virus in biological samples is provided, which comprises the step of: immobilizing at least one of the polypeptide multimers of present invention on the surface of a support; then washing with a suitable buffer; contacting it with sample to be detected under the conditions suitable for the interaction of antigen and antibody; washing again with a suitable buffer; and then incubating with commercial available or routinely generated, detectable labeled anti-IgG antibody a certain time sufficient for antigen/antibody interaction, wherein said anti-IgG is against the animal from which the biological sample to be detected derived; after that, detecting the antigen/antibody complex on the surface of a support by using detect agent corresponding to said detectable label, calculating the amount of antibody IgG in the sample.

In one embodiment of present invention, said biological sample to be detected is derived from human being, said antibody as used is anti-human IgG.

In a non-inventive embodiment of present invention, polypeptide NE2I as antigen is pre-coated to the surface of a predetermined support. In an embodiment of present invention, polypeptide 247 coupled with epitope in HEV ORF3 is used as antigen to be pre-coated with specific surface of support.

In another aspect of present invention, a method for detecting antibody IgM against hepatitis E virus in biological samples is provided, which comprises the step of: immobilizing commercial available or routinely generated antibody anti-IgM on the surface of a support, wherein said anti-IgM is against the animal from which biological sample to be detected derived; washing suitably; contacting it with sample to be detected, preferably, under the conditions suitable for the interaction of antigen and antibody; washing again with a suitable buffer; and then incubating with detectable labeled at least one of polypeptide multimers of present invention for a time sufficient for the interaction between antigen and antibody, after that, detecting the antigen/antibody complex on the surface of a support by using detect agent corresponding to said detectable label, calculating the amount of antibody IgM in the sample.

In one embodiment of present invention, said biological sample to be detected is derived from human being, said antibody as used is anti-human IgM.

In a non-inventive embodiment, said IgM antibody in sample is detected by polypeptide 225N coupled with horseradish peroxidase. In an embodiment of present invention, polypeptide 247 coupled with epitope of HEV IRF3 is further coupled with horseradish peroxidase to detect IgM contained in sample.

Additionally, a method for detecting total antibodies against hepatitis E virus in biological samples is also provided, which comprises the step of: immobilizing at least one of the polypeptide multimers of present invention on the surface of a support; washing with a suitable buffer; contacting it with biological sample to be detected under the conditions suitable for the interaction of antigen and antibody; optionally, washing again with a suitable buffer; incubating with detectable labeled one of present polypeptide for a time sufficient for the interaction between antigen and antibody; and detecting antigen/antibody complex on the surface of a support by using antigen of hepatitis E virus with a detectable label and corresponding detect agent, calculating the amount of total antibodies in the sample.

In a non-inventive embodiment, NE2I is pre-coated on the surface of support, and total antibodies in the sample is detected by polypeptide 225 previously coupled with horseradish peroxidase. In another non-inventive embodiment, NE2I coupled with epitope from HEV ORF3 is pre-coated on the surface of support, and total antibodies in the sample is detected by horseradish peroxidase-bound polypeptide 225 which is previously coupled with epitope from HEV ORF3.

The present invention is further illustrated in details with reference to the following description of drawings and examples, which should not in any way be interpreted as the limitation to the protection scope of the present invention.

Unless specifically indicated, experiment methods of molecular biology and immunoassays of the present
Example 1 Preparation of the genes encoding the polypeptides of the present invention and construction of expression vector containing the same

Preparation of the fraction of HEV ORF2 as template.

To prepare gene of interest, polymerase chain reaction (PCR) is used with a full-length HEV gene cloned from HEV-infected patient in Xin Jiang provine, China as template (Aye, T.T., Uchida etc., Nucleic Acids Research, 20(13), 3512(1992); GeneBank accession number D11092), together with two primers, ORF2 FP:5'-atgccctctggccca-3' as upper primer and ORF2 RP: 5'-aaataaactataactccgga-3' as lower primer. PCR reaction is carried out in PCR thermal cycler (BIOMETRA I3) under following condition: 94°C 5 min; then 25 circles of: 94°C 50 sec, 57°C 50 sec and 72°C 2.5 min; ended by 72°C 10 min. A DNA fragment about 2 kb is obtained, which is from HEV ORF 2 as the template for preparation of polypeptide multimers of the present invention. The above-mentioned PCR product is further linked into commercial available vector pMD18-T (TAKARA CO.) and then digested with BamHI/Hind III, so as to identify the positive clone inserted with ORF2 gene. Using M13 (+/-) as primer, the resultant is sequenced and thereby identify the two DNA fragment of HEV ORF 2 which is used as the template for preparing polypeptide of present invention, one of them is a conservative sequence (Template 1, SEQ ID NO; 5), the other is a mutant sequence (Template 2, SEQ ID NO: 6).

By sequence alignment and analysis of ORF, it is found that the mutant sequence of HEV ORF2 (SEQ ID NO: 6) as template for preparing the polypeptide of present invention has a base A deleted, compared to conservative sequence (SEQ ID NO:5), which resulted in shift mutation that amino acid residues 604-605 in ORF2 are mutated from His-Ser-Val to Pro-Pro-Arg, and the translation for said polypeptide is thereby stopped by stop code tag formed by such mutation.

By way of example, the polypeptide 201 is used hereafter to illustrate the preparation of nucleotide encoding polypeptide 201 and the expression vector comprising the same.


The preparation of nucleotide encoding polypeptide 201 of present invention and the expression vector containing the same.

The gene is synthesized using polymerase chain reaction (PCR), wherein the above-obtained sequence SEQ ID NO:5 is used as template, together with forward primers, 201FP: 5'-ggatcccatatggttattcaggattatgac-3' (see, Table 1), in which BamHI sites, Ndel sites (CAT ATG), and ATG as translation start codon in E.coli system are introduced; and 201RP: 5'-ctcgagaaataaactataactccgga-3' (see, Table 1) as reversed primer, in which stop codon and EcoR I site are introduced. The PCR is carried out in thermo cycler as follows: heat-denatured for 5 min at 94°C, then amplified for 30 circles: 50 sec at 94°C, 40 sec at 57 °C and 40 sec at 72°C, finally 10 min at 72 °C. The resulted ~ 600 bp PCR product is identified as the nucleotide sequence encoding polypeptide 201 of present invention.

The construction of expression vector pTO-T7 for expressing the polypeptide of present invention is following the method in reference document of LUO Wen-Xin, et al., Chinese Journal of Biotechnology, 2000,16:53-57. Said method includes steps of: cloning aforementioned PCR product into commercial available pMD18-T vector (TAKARA company), and digesting with BamHI/Hind III to identify and obtain the positive subclone inserted with nucleotide encoded polypeptide 201; further digesting said positive subclone with Ndel and EcoR I site are introduced. The nucleotide encoding polypeptide 201 of present invention is illustrated in Figure 1.

Similarly, other polypeptides of present invention whose carboxyl terminus is other than Pro-Pro-Arg can be acquired according to the above method by using sequence of SEQ ID NO:6 as template, and using primers listed in Table 1, which is specifically designed against individual polypeptide of interest. The nucleotide encoding polypeptides of present invention whose carboxyl terminus is Pro-Pro-Arg and expression vectors containing the same.

The reference polypeptides whose carboxyl terminus is Pro-Pro-Arg is expressed by transforming E.coli ERR 2566 with expression vector obtained according to the above-mentioned method for expression vector of ORF2-201. Specifically, taken above-mentioned HEV ORF2 mutant sequence SEQ ID NO:6 as template, using individual forward/reverse primer specifically designed against individual polypeptide (see, Table 1), corresponding expression vector is obtained by PCR under similar the condition for generating expression vector of reference polypeptide 201. In this way, a series of polypeptide of present invention with good immnogenicity and immunoreactivity is obtained, wherein the
resulted polypeptide has Met added at its N-terminus, and has amino acid sequence -Pro-Pro-Arg added in direction from 5'-3' at 3' end of amino acid 603, Pro, at its carboxyl terminus.

<table>
<thead>
<tr>
<th>polypeptide</th>
<th>position HEV ORF2 in template</th>
<th>forward primer (FP) and reversed primer (RP)</th>
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<tbody>
<tr>
<td>NE2</td>
<td>394-630pp*</td>
<td>HEFP:5'-ggatccatagctgactactctgct-3'</td>
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<td></td>
<td></td>
<td>HERP:5'-ctcgagaatataactataactccga-3'</td>
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<tr>
<td>217C</td>
<td>390-603ppr</td>
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</table>
Example 2: Expression of reference polypeptide 201

[0102] 1uL plasmid pTO-T7-ORF2-201 (0.15mg/mL) was added into 40uL competent cells of E. coli ERR2566 (generated under calcium chloride method) for transformation. Then the mixture was scrawled onto a kanamycin-LB plate, and the plate was incubated at 37°C for 10–12 hours till the present of individual clones. The individual clones were picked and further inoculated in 4mL LB culture medium in tubes, 220rpm shaking at 37 °C for 10 hours, until the OD550nm value of the culture is about 1.5. Then 1mL culture medium was stored at 4°C for later use, and 2uL 0.5M IPTG was added into the rest 3 ml of culture medium (final content is 0.3 mM). The culture medium contain IPTG was keep on incubating at 37°C for 4 hours with 220rpm shaking for inducing the expression of polypeptide of interest. 1.5mL induced culture medium was centrifuged at 12000g for 30 seconds. The precipitated cells were re-suspended in 100uL protein loading buffer (50mM Tris Cl pH6.8, 100mM DTT, 2% SDS, 0.1% Bromophenol Blue, 10%Glycerol), and further boiled for 10 minutes, then centrifuged at 12000g for 10 minutes. 10ul supernatant was load onto 12% SDS-PAGE for analysis of the expression of polypeptide 201. The clone which express in highest yield was used for future fermentation.

[0103] 200uL seek culture medium was added into 500mL LB culture medium containing in 1L Erlenmeyer flask. After incubating at 37°C with 190rpm shaking for about 11 hours till the OD550nm value of the culture reached 2.0, 300uL 0.5M IPTG was added to the final content is 0.3 mM. And the mixture was further incubated for 4 hours under aforementioned condition. 1.5mL induced culture was centrifuged at 12000g for 30 seconds. The cells were resuspended in 100uL protein loading buffer, and boiled for 10 minutes, then centrifuged at 12000g for 10 minutes. 10ul supernatant was load onto 12% SDS-PAGE for analysis of expression of polypeptide 201. The result of the analysis of the SDS-PAGE (staining with Coomassie Brilliant blue R250) showed the expression of polypeptide 201 is about 35% of total expressed cell proteins as UVI gel imaging instrument shown (UVItech, model DBT-08).
Example 3: Purification of reference polypeptide 201 inclusion body expressed in *E. coli*

[0104] The culture medium of *E. coli* containing recombinant polypeptide 201 obtained in example 2 was centrifuged at 4000 rpm for 15 minutes, and each 500mL culture's precipitate was re-suspended in 15mL lysis buffer (50mM Tris Cl, 10mM EDTA and 300mM NaCl in dH2O, pH7.2). The cells were sonicated in ultrasonic instrument (Ulbra-Cell VCX500, SONICS&MATERIALS company, power 70%; 40 seconds on; 60 seconds off; sonicated for 20 minutes totally.). The sonicated mixture was centrifuged 12000rpm for 10 minutes at 4°C, and the pellet was resuspended in buffer I solution (200mM Tris Cl, pH8.5; 5mM EDTA; 100mM NaCl) containing 2% Triton-X100, and the final volume is the same as the original lysis. The mixture was shaking at 200rpm for 30 minutes at 37°C, then centrifuged at 10000rpm for 10 minutes at 4°C. The pellet was resuspended in equal volume of buffer I, and the mixture was sonicated (40 seconds on; 60 seconds off; power 70%; sonicated for 3 minutes totally). After that the mixture was centrifuged (10000rpm) for 10 minutes at 4°C. The pellet was resuspended in buffer I containing 2% Triton-X100 to the final volume is the same as before. After the mixture was shake (200rpm) for 30 minutes at 37°C, it was centrifuged (10000rpm) for 10 minutes at 4°C. The pellet was resuspended in equal volume of buffer I, shaking (200rpm) for 30 minutes at 37°C. Then the mixture was centrifuged 10000rpm for 10 minutes at 4°C. The pellet was resuspended in buffer I which containing 2M Urea to the final volume is the same as original mixture. After shaking 200rpm for 30 minutes at 37°C, the mixture was centrifuged 10000rpm for 10minutes at 4°C. This supernatant is marked with 201-2M. The purity of all the above samples is analyzed by 12% SDS-PAGE. The results are shown in Fig. 3.

Example 4: Renaturation of recombinant polypeptide 201

[0105] 100ml of sample 201-4M prepared according to Example 3 was loaded into 2 dialysis bags (36 DM, retentate MW: 8000–10000, United Carbon Compound, U.S.A.) and dialyzed under stirring in a 1L beaker, with 900ml of 1/3 PBS (20xPBS (1L) containing 73.344g of Na2HPO4.12H2O, 4g of KH2PO4, 163,632g of NaCl, 4.024g of KCl and pH 7.45) at 25°C over night (10 hours), and then white precipitates were observed in the dialysis bags. Refreshing the dialysate and going on the dialysis, and then the dialysate was refreshed every 3 hours for 4 times. In principle, the content of urea in the sample would be 4×10^{-6}M when the dialysis is over. The dialysate sample was centrifuged at 25°C, 12000 rpm for 10 minutes; the supernatant was filtrated with 0.22μm filter membrane for further purification; the pellet resuspended in 4M urea/buffer I can be used in a new dialysis during which precipitates would also appear, but the concentration of the obtained protein sample would be lower than that of the first obtained sample.

Example 5: Purification of recombinant reference polypeptide 201 with Gel filtration HPLC

[0106] The renatured 201 sample prepared according to the methods of Example 4 was further purified by HPLC as below:

Instrument: Beckman System Gold Nouveau 125NMP/166NMP HPLC,
Column: TSK GEL SW3000 21.5mm×60cm,
Elution: 1×PBS pH 7.45,
Flow Rate: 4ml/min,
Detection: UV at 280nm,
Sample: 2ml of 4M NE2 (8mg/ml),
Collection: automatic apex collection of window mode,
Collection time: 1 tube/20 seconds,
Collection delay: 6 seconds.

[0107] The result shows that the molecule filtering is very effective in the chromatogram but that the apex component contains monomers and dimers as well as proteins distributed equably between them. After treated in boiling water for 10 minutes, the sample protein was analyzed by SDS-PAGE with 12% acrylamide for the monomer purity of the object protein peak, which is up to more than 95%. This demonstrates that in addition to self-aggregated, ORF2-201 monomer also aggregates with other small proteins and interactions occur also among the multimers which were eluted together during chromatogram.

Example 6 Characterization of the recombinant polypeptide products of the invention

[0108] The recombinant polypeptide of the invention was constructed and expressed according to the methods of
Examples 1-5. Furthermore, each recombinant peptide was washed and dialyzed according to the methods of Examples 3-4. In Table 2 the corresponding amino acid position of each recombinant peptide in hepatitis E virus, the renaturation property of the expressed recombinant products and the proportions of the monomers and dimers in their SDS-PAGE as well as the formation of the multimers are provided.

Table 2. Corresponding amino acid position of each recombinant peptide in hepatitis E virus, the renaturation property of the expressed recombinant products and the proportions of the monomers and dimers in their SDS-PAGE as well as the formation of the multimers.

<table>
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<tr>
<th>name of polypeptide</th>
<th>sequence no.</th>
<th>percent of monomer</th>
<th>percent of dimer</th>
<th>Multimerization</th>
<th>renaturable by dialysis</th>
</tr>
</thead>
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<td>Yes</td>
</tr>
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<td>95%</td>
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<td>Yes</td>
</tr>
<tr>
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</tr>
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</tr>
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<td>0%</td>
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<td>247 2</td>
<td>aa414-aa660</td>
<td>10%</td>
<td>90%</td>
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<td>Yes</td>
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As shown in Table 2, the polypeptides which is included in the amino acid sequence of SEQ ID NO: 1 of HEV ORF2 has the ability to be well-renatured, which is intend to exhibits a dimensional structures close to natural HEV protein, when their carboxyl terminals locate between aa 601 (Leu) and aa 660 of SEQ ID NO: 1. Specifically, the peptides 247, 232, 222, 201, 235N, 225N, 209N, NE2I, 217D, 205, 189, 188, NE2 (SEQ ID NO:2) and 193C (SEQ ID NO: 3) was found to have expression bonds in the position corresponding to monomer molecule weight and 2 folds of monomer molecule weight while the amounts of dimmers are apparently over those of monomers. NE2 and 193C was found to have obvious bonds in positions of bigger molecule weights, which suggests that said peptides tend to multimerize spontaneously.

After renaturable recombinant peptides in Table 2, 193C, 201, 208N, 209N, NE2, 222, 225N, 232 and 247, were further purified by Gel filtration HPLC and centrifuged each for 10min at 20000g and filtered with 0.1 μm Al2O3 filter membrane, the dynamic radiuses of these peptides was measured by dynamic light scattering instrument (DYNAPRO99-D-50 dynamic light scattering instrument, produced by PROTEIN SOLUTIONS) and their assemble status were speculated in Table 3. The obtained molecule radius of each recombinant peptide is apparently bigger than the predicted radius of the monomer. According to the putative molecule weights, it can be concluded that those peptides form at least dimers in the solution, and most of them form higher order multimers, which are in conformity with their behavior in SDS-PAGE. In fact, the polypeptides prepared with the above methods can form multimers of up to 180 or more monomers. It was further demonstrated that the polypeptides have unexpected property, i.e. above said recombinant peptides expressed by E. coli system tends multimerize spontaneously in PBS solution free of denaturing reagents and this is advantageous for increasing its immunogenicity as vaccine.

Table 3 Detection of the aggregate status of renaturable recombinant peptides of the invention by dynamic light scattering instrument.

<table>
<thead>
<tr>
<th>polypeptide</th>
<th>theoretical molecule weight of monomer (KD)</th>
<th>measured radius (nm)</th>
<th>putative molecule weight (KD)</th>
<th>Putative aggregate states</th>
</tr>
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<tbody>
<tr>
<td>193C 1</td>
<td>21.2</td>
<td>3.44</td>
<td>47</td>
<td>dimer (21.2×2 = 42.4)</td>
</tr>
<tr>
<td>201 1</td>
<td>22.1</td>
<td>3.08</td>
<td>62.7</td>
<td>trimer (22.1×3 = 66.3)</td>
</tr>
<tr>
<td>208N 1</td>
<td>22.9</td>
<td>3.57</td>
<td>66.1</td>
<td>trimer (22.9×3 = 68.7)</td>
</tr>
<tr>
<td>209N 1</td>
<td>23</td>
<td>4.10</td>
<td>91</td>
<td>tetramer (23×4=92)</td>
</tr>
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</table>
Example 7: The physicochemical properties of polypeptide

Renaturation from the inclusion body

[0111] The inclusion body of the recombinant polypeptide prepared as described in examples 1-3 was denatured by 4M urea, then dialyzed with over 100 volumes of PBS, as described in example 4. The dialysate was centrifuged at 12,000rpm for 10 min. The supernatant contains some or all of the recombinant polypeptides, thereby demonstrating that said recombinant polypeptides are capable of renaturation.

The polymerization of the recombinant peptides

[0112] In the analysis of the supernatant using conventional SDS-PAGE, the bands corresponding respectively to the monomer, dimer and polymer were identified. The specificity of said bands were further confirmed by conventional Western blotting, thereby demonstrating that recombinant polypeptide 201 forms a polymer after renaturation (see Figure 4).

The determination of the molecular size of polypeptide 201 by light-scanning technique

[0113] According to Example 6, polypeptide 201 was centrifuged at 20,000rpm for 10 min after purification with HPLC, then filtered with 0.1um Millipore membrane of aluminia. The filtrate was measured by dynamic light-scanning instrument (DYNAPOR99-D-50, PROTEIN SOLUTION Com. Ltd. U.S.A) at 824.0nm. The Regulation algorithm was used for calculation, and its applicability was confirmed by many standard samples. The radius of the molecule is calculated from the dynamic radius corresponding to the % Intensity peak. The solvent was set as sample buffer PBS. The measured results shown in Figure 5 indicated that the mean radius of polypeptide 201 in denaturant-free solution was 3.08nm, with the calculated MW 62.7KD(corresponding to the trimer). It is known to those skilled in the art that said polypeptide of the invention could actually form polymers of 180 monomers or more.

Example 8: Preparation of mouse anti-NE2 monoclonal antibodies

Establishment of the hybridoma cell line

[0114] For the primary immunization, each Balb/C female mouse (6-8 weeks old) was inoculated with 5ug recombinant antigen NE2 emulsified Freund’s incomplete adjuvant (the total volume is 50uL). Fifteen days later, the mouse was intramuscularly immunized for the second time with the same mount of NE2 emulsified in incomplete Freud’s adjuvant. 30 days later, the mouse was then boosted intravenously (via the tail vein) with 5ug antigens without the adjuvant. The mice were sacrificed 72-96 hours after booster immunization. The blood was then collected and the spleen was resected to prepare the suspension of the splenocyte (suspending in RPMI 1640 medium). The splenocytes were counted with a cell counter. Then the splenocytes were mixed in a 6:1 ratio with the SP2/0 mouse myeloma cells and centrifuged.
The cells were fused with PEG (PEG 1500), then mixed with the equal volume of feeder cells, and transferred to 96-well plate (200uL/well). At the atmosphere of 5% CO₂, the 96-well plate was incubated in a incubator (ESPEC BNA-31) at 37°C. 3 days later, half of the culture medium was replaced with fresh HT medium (1.361mg hypoxanthin and 0.388mg thymidine, with the addition of RPMI 1640 medium (GIBCO Int.) to 100mL, dissolved at about 45-50 °C and filtered for sterilization.). 7 days later, the 96-well plate was coated with NE2, and ELISA assay was performed on the hybridoma cell culture as described below. The cells positive to ELISA assay were cloned by limiting dilution means.

**ELISA assay**

[0115] 100uL NE2 were purified by HPLC described in Example 5 at 37°C, and then dissolved in 0.05mol/L CB (20.02g Na₂CO₃ and 2.52g NaHCO₃, with the addition of ddH₂O to 1L, pH9.5) to a final concentration of 0.3ug/mL. The 96-well polyvinyl microtiter plate was treated with the resulting solution for 2 hours at 37°C and then overnight at 4°C. The microtiter plate was washed with PBST (8.0g NaCl, 0.2g KH₂PO₄, 2.9g Na₂HPO₄•12H₂O, 0.2g KCl and 0.5mL Tween-20, with the addition of ddH₂O to 1L, pH7.4) to remove the unabsorbed antigens. Then 200uL blocking solution (2% glutin, 0.2% casein and 2% sucrose in 1XPBS) were added per well and incubated for 2 hours. Then pour off the solution, dry the well and store in vacuum at 4°C.

[0116] To assay, 100uL cell culture were added to each well, and set one positive control (add 100uL 1:1 diluted polyclonal anti-NE2 serum) and one negative control (add 100uL HT medium) for each plate. After incubating at 37°C for 30 min, the plate was washed with PBST for 5 times and then dried. HRP-GAM Ig (DAKO company) was added and incubated for another 30 min at 37°C. The plate was washed with PBS- Tween-20 again for 5 times and dried. 50uL substrate solution A (13.42g Na₂HPO₄•12H₂O, 4.2g citricacid•H₂O and 0.3g H₂O₂, with the addition of ddH₂O to 700mL) and 50uL substrate solution B (0.2g TMD and 20mL dimethylformamide, with the addition of ddH₂O to 700mL) were added to the plate and incubated for 10 min at 37°C. 50uL stop solution was used to terminate the reaction. The OD₄₅₀ value of each well was read by an ELISA reader. In general, the OD450 value at least twice higher than that of the negative control can be considered as positive.

The preparation of the ascites and the purification of the monoclonal antibodies

[0117] Each 10-week-old Balb/C mouse was inoculated intraperitoneally with 0.5mL incomplete Freud’s adjuvant. 2-7 days later, the hybridoma cells were collected and centrifuged. Then discard the supernatant and add serum-free medium (1.361mg hypoxanthin and 0.388mg thymidine, with the addition of normal saline to 100mL). (NH₄)₂SO₄ was then added dropwise with gently stirring until 50% saturation, and kept at 4°C overnight. The solution was centrifuged (12,000rpm) at 4°C for 15 min, and the supernatant was discarded. The pellet was dissolved in PBS (2 volumes of the ascites used), (NH₄)₂SO₄ was added dropwise again to the resulting solution with stirring until 33% saturation, and kept overnight at 4°C. The solution was centrifuged (12,000rpm) at 4°C for 15 min, and the supernatant was discarded. The pellet was dissolved in PBS (2 volumes of the ascites used), (NH₄)₂SO₄ was added dropwise with gently stirring until 50% saturation, and kept at 4°C overnight. The solution was centrifuged (12,000rpm) at 4°C for 15 min, and the supernatant was discarded. The pellet was then dissolved in proper amounts of PBS in a dialysis bag and dialyzed in 50-100 volumes of 120 mmol/L Tris-HCl buffer (containing 20mmol/L NaCl, pH7.8) for about 12 hours at 4°C with stirring. Replace the buffer for more than three times. The dialysate was stored at -20°C.

[0118] According to the method described above, monoclonal antibodies were prepared by immunizing Balb/C mice with polypeptide NE2 of the invention, and 8 anti-NE2 monoclonal antibodies were identified (1F6, 2C9, 3F5, 8C11, 8H3, 13D8, 15B2 and 16D7). Coat the eppendorf tubes with said 8 antibodies respectively, and test the capability of said antibodies binding native HEV by capture RT-PCR (see Example 9). As a result, 8C11, 8H3 and 13D8 shown significant activity of binding HEV, which indicated that their recognition sites were the native epitopes on the surface of the viral coating. Said three antibodies were used in Example 10.

Example 9: Testing the capability of mAb binding HEV by antibody-capturing RT-PCR

[0120] The 1.5mL eppendorfs were irradiated by ultraviolet for 30 min, and then added 500uL mAb 1:1000 diluted in CB (20.02g Na₂CO₃ and 2.52g NaHCO₃, with the addition of ddH₂O to 1L, pH9.5). After incubating overnight at 37°C, pour off the buffer, and add 1.5mL blocking buffer (1X PBS with 2% albumin, pH7.4) to block 2 hours at 37°C. Then pour off the blocking buffer and add 500uL 10% dejecta in sterilized normal saline which is positive for HEV. After reaction
at 37°C for 2 hours, wash the eppendorf with PBST for 6 times and then add 250uL ddH2O to each eppendorf. The RT-PCR assay was then performed according to Example 14. As a result, the monoclonal antibodies of 8C11, 8H3 and 13D8 were capable of binding HEV, while 1F6, 2C9, 3F5, 15B2 and 16D7 were not capable of binding HEV.

Example 10: ELISA of the polypeptides of the present invention with the serum from positive rhesus monkey, serum from human and murine derived monoclonal antibodies and the Dot blotting of the polypeptides of the present invention with murine derived monoclonal antibodies

ELISA of the recombinant polypeptide with serum from positive rhesus monkey, serum from human and murine derived monoclonal antibodies

[0121] The polypeptides of the present invention shown in Table 2 are produced and purified according to the methods mentioned in examples 1-6. The resulted purified recombinant protein samples with concentration of 1mg/ml are diluted 1:500 with PBS buffer (20Mm, pH7.4), and coated 100µl/well on the 96-well microtitre plate under the following condition: incubation at 37°C for 2 hours and then incubation overnight for about 12 hours at 4°C. After washing once with PBS-Tween20 washing solution (8.0g NaCl, 0.2g KH2PO4, 2.9g Na2HPO4,12H2O, 0.2gKCl and 0.5ml Tween20, adding non-ionic H2O to final volume 1L, pH7.4) on the automatic washer (TECAN, M12/4R Columbus plus), and drying, blocking solution (2% glutin, 0.2% casein and 2% sucrose in PBS) was added, 200µl/well, incubation at 37°C for 30mins. Then the properly diluted anti-serum or monoclonal antibody was added, at 37°C for 30mins. After washing 5 times with PBS-Tween20 washing solution on the automatic washer at 20 seconds interval and drying, the properly diluted HRP-labelled second antibody (goat anti-human, mouse IgG antibody) was added, at 37°C for 30mins. After washing 5 times with PBS-Tween20 washing solution on the automatic washer at 20 seconds interval and drying, a drop of the stop solution (2M H2SO4) was added. The OD450mn was measured on a microplate reader (TECAN, Sunrise Remote/ Touch Screen) (with reference wavelength of 620nm). 3 times of the mean value of the negative control was set as the positive threshold value, and the result is positive when the OD value thereof is higher than the threshold value.

Dot blotting of the polypeptides of the present invention with various murine derived monoclonal antibodies

[0122] 10µl (mg/ml) of each of the polypeptides listed in Table 2 which were produced according to the methods of examples 1-5 and purified by HPLC gel filtration was dotted respectively and slowly on the nitrocellulose membrane and air-dried. After blocking with 5% skim milk for 1.5 hours at room temperature, various murine derived monoclonal antibodies produced as mentioned in example 8 (the cell supernatant secreted by monoclonal B lymphocytes at 1:100 dilution with 5% skim milk) were added, reacting at room temperature for 1 hour. Then the membrane was washed 3 times using TNT (10mM Tris.Cl, pH8.0, 150Mm NaCl, 0.05% Tween20) at 5mins interval. The HRP-labelled Goat anti-mouse IgG (produced by JINGMEI Biological Company, diluted in 1:1000 with 5% skim milk) was added, and reacted at room temperature for 1 hour. After washing 3 times with TNT at 5mins interval, NBT/BCIP (C40H30N10O6Cl2/C8H6BrClNO4P.C7H9N) was added to develop color. The dots were scanned with gel imagining system and diverted into the values of grey degree and divided into five positive grades as ++++, ++, +, + and negative grade as -. Compared with classic Western blotting, this method can reflect more really the immunoreactivity in the absence of denaturing agent due to not subject to be denatured with SDS.

Table 4 The reactivity of polypeptide of present invention against murine derived monoclonal antibody, serum from HEV patient in recovery phase, and serum from HEV-infected rhesus monkey in acute phase

<table>
<thead>
<tr>
<th>polypeptide</th>
<th>ELISA</th>
<th>dot blotting</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>monkey</td>
<td>human</td>
</tr>
<tr>
<td>NE2¹</td>
<td>++</td>
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</tr>
<tr>
<td>193C¹</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>178C¹</td>
<td>+</td>
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<td>148C¹</td>
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<tr>
<td>138C¹</td>
<td>-</td>
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</tr>
</tbody>
</table>
Respectively, the purified recombinant polypeptides listed in Table 4 were coated on microtitre plate, and the reactivities thereof to three murine derived monoclonal antibodies 8C11, 8H3 and 13D8 mentioned in example 8, serum of HEV patient in recovery phase and serum from HEV-infected rhesus monkey in acute phase were examined by ELISA, and the reactivities thereof to the used three monoclonal antibodies were examined by Dot blotting assay. The results showed that the polypeptides NE2, 193C, 178C, NE2I, 235N, 225N, 209N, 247, 232, 222 and 201 had better reactivities to each of the serum/monoclonal antibody. This suggested that the polypeptides had better native HEV epitope and can be used to diagnostic kit and/or vaccine for HEV.

The reference polypeptides 138C, C160, 150, 142 and 134 had poor reactivities to various antibodies. It showed that the formation of the major native epitope ORF2 involved at least the fragment of aa469 to aa600.

The sequences of the polypeptides 170, C160, N160, 144, 142 and 134 were those that aa459-aa628, aa469-aa628, aa459-aa618, aa469-aa618, aa459-aa602, aa459-aa600 of the ORF2 were linked respectively to the initial amino acid (Met).
The immunity assay of the reference polypeptide 201 without adjuvant

Preparation of the vaccine containing the reference polypeptide 201 with aluminum adjuvant

Example 11: The Western blotting of the polypeptides of the present invention with murine derived monoclonal antibodies

Preparation of the vaccine containing reference polypeptide 201 and the assay of the immunizing mouse with it

Preparation of the vaccine containing reference polypeptide 201 with Freund’s adjuvant

Example 12: preparation of the vaccine containing reference polypeptide 201 and the assay of the immunizing mouse with it

A desired amount of the original aluminum adjuvant (A13+ 13.68%, Na+3.36%, pH5.55), which was from the Lanzhou Biological Product Institute of China, was adjusted with IN NaOH till to produce precipitate. After mixing completely, 1×PBS was added to reach the double volume. Then centrifuge was performed at 10,000 for 1min, and the supernatant was discarded. The precipitate was resuspended with 1×PBS to the double volume again, and centrifuged at 10,000 for 1 min and the supernatant was discarded. Such process was repeated several times until the pH reached 7-7.4. Finally, the precipitate was resuspended with equal volume of 1×PBS, and the solution was sterilized and stored at 4°C, and used as 9×store solution.

Similarly, the polypeptide201 produced as above-mentioned and purified by HPLC (purity > 95% and the concentration of the protein 1.02mg/ml) was diluted with PBS, and the equivalent volume of complete Freund’s adjuvant (containing BCG) was added to reach a desired final concentration of the polypeptide201 (for example, if each mouse was desired to be immunized 100μl, 5μg, the concentration of the polypeptide 201 would be prepared into 0.05mg/ml). The solution was mixed and emulsified for 30mins until no separating liquid phase appeared after keeping still for 30mins.

Using Freund’s adjuvant as vaccine adjuvant, four groups mice (each group of 3 Kunming White mice), each was injected intramuscularly with 0.5, 1, 2, 5μg in 100μl/mouse according to the immunizing schedule of 0, 7, 28 days. The results were shown in Fig.7. The results indicated that ORF2-201 vaccine prepared with Freund’s adjuvant with does above 2μg had very strong immunogenicity, and the antibody was started to produce at the second week after immunization and to reach the highest titre at the forth week. It is thought that the antibody with higher titre can be produced only in the mice immunized with protein antigen at the does of 30-70μg/mouse according to the common books and literatures on immunology. Therefore, the results show that the vaccine, the polypeptide 201 of the present invention combined with Freund’s adjuvant, has remarkably high immunogenic effect compared with the available vaccine.

The immunity assay of the reference polypeptide 201 without adjuvant

The polypeptide 201 obtained as above-mentioned was solved in 4M urea, and the supernatant was dialyzed with PBS (pH7.45) to renature, with the purity of about 95%. Using Freund’s adjuvant as vaccine adjuvant, each group...
of 3 Kunming White mice, each was injected intramuscularly with 5, 25, 50μg /mouse (the control group with 5μg /mouse) according to the immunizing schedule of 0, 7, 28 days. The results were shown in Fig.8. The results indicated that the remarkable antibodies were produced in the mice immunized with non-adjuvant ORF2-201 vaccine, and the used dosage thereof was comparable to that conventional antigen with Freund’s adjuvant. It further shows that the polymer polypeptide of the present invention has higher immunogenicity compared with the conventional antigen.

**Example 13:** Immunization of rhesus monkeys with the vaccine comprising a recombinant polypeptide.

- **[0132]** Six rhesus monkeys with normal ALT and negative HEV were selected and divided into two groups, one group designated HF1, HF2 and HF3 and the other group designated HF4, HF5 and HF6. The two groups of the subject rhesus monkeys were vaccinated by intradeltoidal injection with the aluminum adjuvant containing polypeptide NE2I vaccine and polypeptide 201 vaccine prepared as described in the examples 1-5 and 12 at the dosages of 20 μg, respectively. Such vaccinations were carried out on days 0, 10, and 30 respectively. Three weeks after the last vaccination, the titers of the anti-NE2I IgG antibody from the animals were tested by indirect ELISA with the results as follows: HF1(1:16000), HF2(1:4000), HF3(1:8000), HF4(1:2000), HF5(1:3000) and HF6(1:5000).

- **[0134]** The above results illustrated by polypeptides NE2I and 201 indicated that the recombinant polypeptides of the invention have better immunogenicity compared with HEV ORF2 polypeptide trpE-C2 (amino acids 225-660 of SEQ ID NO: 1) used as immunogen in US Patent No. 5885768. In U.S. Patent No.5885768, Reyes, et al. vaccinated 4 cynomolgus monkeys by intravenous injection with 50μg of HEV ORF2 polypeptide trpE-C2 expressed from E. coli in combination with an improved aluminum adjuvant on days 0 and 30 respectively. Two cynomolgus monkeys injected with adjuvant alone were used as control. No antibody to HEV was detected in the group of the vaccinated monkeys 4 weeks after the second vaccination. Two of these vaccinated monkeys were selected to receive a third vaccination with 80μg of insoluble trpE-C2 polypeptide without adjuvant on day 58, and the anti-HEV antibody was detected only 4 weeks later.

**Example 14:** Challenging with HEV of the rhesus monkeys immunized with vaccine comprising the recombinant polypeptide of the present invention

**Preparation and quantification of hepatitis E virus (HEV)**

- **[0135]** A fecal from a HEV patient from Xinjiang, China, was formulated to 10% suspension in sterile physiological saline solution. The suspension was centrifuged at 12000g for 20 minutes at 4°C, and the supernatant was filter-sterilized with 0.2 μm sterile filter (NALGENE® Cat.No.190-2520). HEV in a PCR-detectable amount was used as an infection dosage.

- **[0136]** Extraction, reverse transcription and PCR of RNA of HEV from fecal: HEV RNAs were extracted from the 10% fecal suspension using Trizol reagent (GIBCOL) according to its manipulation instructions, and were subjected to reverse transcription in a 20 μl reaction volume at 42 °C for 40 minutes with the specific primer A3 (4566-4586, 5’-gtgttcttcgagtgttcttc-3’) as RT primer using AMV reverse transcriptase. Then, the first round of RT-PCR was carried out in a final volume of 20ul using 2 ul RT product as template and using A3 primer and A5 primer (4341-4362, 5’-ctgtggtgcagccttcgcttg-3’) under the following reaction conditions: pre-denaturing at 94°C for 5 min; 35 cycles of denaturing at 94°C for 40s and extending at 68°C for 40s; extending at 75°C for 5min. The second round of PCR was carried out in a final volume of 20ul using 2 ul of the first round reaction product as template and using primers B5 (4372-7392, 5’-ggtggtcagcagggtcagcagtgg-3’) and B3 (4554-4575, 5’-tgtggtggttcttctggctgccg-3’) under the following reaction conditions: pre-denaturing at 94°C for 5 min; 35 cycles of denaturing at 94°C for 40s, annealing at 56°C for 40s and extending at 72°C for 1min 20sec; extending at 75°C for 5min.

- **[0137]** Grouping of rhesus monkeys used in this experiment: immunized group 1 including rhesus monkeys V10, V11 and V12 corresponding to animals HF1, HF2 and HF3 in Example 2, respectively; immunized group 2 including rhesus monkeys V13, V14 and V15 corresponding to animals HF4, HF5 and HF6 in Example 2, respectively; a control group including three non-immunized rhesus monkeys designated V16, V17 and V18.

**Challenging with HEV**

- **[0138]** HEV in a PCR-detectable amount was used as one infection dosage. Three weeks after the last vaccination of the rhesus monkey HF1-6 with vaccine comprising the recombinant polypeptides of the present invention as described
in Example 13, the above monkeys were challenged with 1,000 infection dosages of HEV. After challenge ALT of every monkey did not increase. Anti-NE2I-IgG in animals V16 and V17 was detected at week 4, and Anti-NE2-IgG was detected in animal V18 at week 5. From days 1 to 37, no HEV excretion was detected in animals V10-15; HEV excretion in animal V16 started on day 5 and ended on day 30; and HEV excretion in animals V17 and V18 began on day 5 too, but didn’t stop on day 37. These results indicated that the polypeptide of the present invention as vaccine possessed better immunogenicity and produced better protection compared with the polypeptide trpE-C2 of HEV ORF2 in U.S. Patent No. 5885768.

[0139] From these results, it could be seen that when low dosage of vaccine of this invention was used to vaccinate monkeys, the vaccinated monkeys could produce excellent antibody response to HEV and abnormal ALT and virus excretion in fecal were not observed after challenge with HEV. Thus better immunoprotection was produced compared with the reported vaccination results of the vaccines prepared using polypeptide trpE-C2 from HEV ORF2 (U.S. Patent No. 5885768) and of the vaccines comprising the polypeptides prepared by Tsarev et al. In addition, the baculovirus expression system applied by Tsarev and Genelabs co. has potential harm to the human body, so there was no report hitherto that any recombinant protein expressed by this system was approved as a commercialized drug or a commercialized vaccine used in human. On the contrary, several of the recombinant proteins expressed by E.coli expression system according to the present invention were approved as commercialized in vivo drugs used in human, and have more reliable safety.

Example 15: Preparation of a chimeric polypeptide comprising polypeptide 247 of the invention linked with the epitope in HEV ORF3

[0140] PCR reaction was performed with the full length genome of hepatitis E virus (HEV) isolated from a HEV patient of XinJiang, China. (Aye, T.T., Uchida et al. Nucleic Acids Research, 20(13), 3512 (1992); GenBank accession number: GI221701) as template using the forward primer, 372FP (5’-ggatccatatagaacatgtcttttgct-3’), introducing restricted endonuclease sites BamHI and NdeI at its 5’-terminal, and the reverse primer, 372BRP (5’-ggatcctcggcgcggcc-3’), introducing a restricted endonuclease site BamHI in its 5’-terminal, under the following reaction condition: 94°C 5min, 30 cycles of 94°C 50sec, 56°C 50sec and 72°C 30sec, and 70°C 10min. A specific DNA fragment with the size of about 370bp encoding the epitope in HEV ORF3 was obtained. The PCR product obtained above was ligated into commercial pMD 18-T plasmid (TAKARA co.). A positive sub-clone in which the epitope gene in HEV ORF3 was inserted was identified by digestion with BamHI. DNA sequencing indicated no mutation in the clone, and thereby the amino acid sequence of the epitope gene in HEV ORF3 was obtained, as set forth in SEQ ID NO: 11.

[0141] The HEV-ORF3 gene fragment was obtained by digestion with BamHI, and ligated into pTO-T7-ORF2-247 expression plasmid vector (prepared according to the method described in Example 1) which had been digested with BamHI. A positive expression clone, pTO-T7-ORF3-247, in which the HEV ORF3 gene fragment was inserted, was identified by digestion with BamHI. The clone was transformed into E.coli ERR2566 strain, which was used to express the ORF3-247 chimeric polypeptide.

Example 16: Preparation of a chimeric reference polypeptide of NE2D linked with hemagglutin antigen from influenza virus

[0142] The nucleotide sequence of the chimeric peptide was obtained by PCR amplification with a HEV-ORF2 mutant sequence (SEQ ID NO:6) prepared in example 1 as template by using primer pairs HAFP/E2RD. The PCR reaction is carried out as follows: pre-heating at 94°C for 5 minutes; 30 cycles of denaturation at 94°C for 50 seconds, annealing at 56°C for 50 seconds, extension at 72°C for 70 seconds; and extension at 72°C for 10 minutes at last. The resulted PCR product is an about 800 bp DNA fragment, encoding chimeric polypeptide comprising HA from influenza virus and polypeptide NE2D of present invention. The forward primer HAFP contains BamHI and NdeI restriction sites. The reverse primer E2RD contained EcoRI restriction site and a stop translation codon TAA. The sequence for NdeI site is CAT ATG, wherein ATG is an initial translation codon. Moreover, in order to maintain the exact conformations of the two peptides HA and NE2D, respectively, a flexible linker Gly-Gly-Ser coded by CAG CTG TTC was introduced between HA and NE2D peptides. Therefore, the chimeric polypeptide HA-NE2D could be suitably employed in HEV vaccine. The sequences of primer pairs are as follows:
Preparation of expression vector comprising a nucleic acid construction encoding resulted chimeric polypeptide

[0143] The aforementioned PCR product was cloned into commercial available plasmid pMD 18-T (TAKARA com. Ltd.). The interest sequence then was acquired by BamHI/EcoRI digestion from pMD 18-T-HA-ORF2-NE2D plasmid, and integrated into expression vector with BamHI/EcoRI digestion and ligation. An HA-ORF2-NE2D chimeric peptide was isolated from E.coli ERR2566 host cells transformed with the pTO-T7-HA-ORF2-NE2D expression plasmid. The amino acid sequence was designated as SEQ ID NO: 12.

Example: 17 Detecting IgG for HEV in biological samples with the indirect Elisa kit based on reference polypeptide NE2I.

[0144] The kit detecting IgG for HEV with polypeptide NE2I of this invention comprises: microtiter plate coated with recombination polypeptide NE2I and blocked with blocking solution (20mM pH7.2 PB, 0.5% Casein, 2% Gelatin); sample diluent (20mM pH7.2 PB, 1% Casein); working conjugate (goat anti-human IgG (DAKO) labeled with HRP diluted with enzyme diluent (20mM pH7.2 PB, 0.5% Casein, 10% NBS)); nonbioactivity material such as 20x PBST, chromatogen A, chromatogen B and stop solution (Beijing wantai).

[0145] Series of monkeys' sera were detected with anti-HEV IgG kit based on NE2I compared with two commercial anti-HEV IgG kits from Beijing wantai and Singapore Genelabs. The monkeys' sera were the sera of monkeys Nol, No2, No3 and No13 mocking natural infected monkey by HEV from 0 to 18 weeks after intravenous challenge.

[0146] The manipulating procedure is as follows: add 100ul of sample solvent to each microtiter; add 10ul of specimen to the microtiter; mix thoroughly by tapping gently on all sides of microplate; Incubate for 30 minutes at 37°C; wash the microplate with 1.times. PBST five times; blot dry by inverting the microplate and tapping firmly onto absorbent paper; add 100ul of working conjugate to all wells and incubating the microplate for 30 minutes at 37°C; wash the microplate five times again and blotting dry; add 50ul of chromatogen A and 50ul of chromatogen B and mixing thoroughly by tapping gently; incubate the microplate for 10 minutes at 37°C in dark; add 50ul of stop solution to each well and mixing gently by tapping the plate; determine the absorbance for each well at 450nm/620nm. The two commercial anti-HEV IgG kits was assayed accurately according to each assay procedure.

[0147] The result is as follows: the kit based on NE2I detected seroconversion earlier than the two commercial kits from Beijing wantai and Singapore Genelabs by 7-14 days; the duration of anti-NE2I-IgG was longer than Genelabs anti-HEV-IgG and Wantai anti-HEV-IgG; the detectable rate of anti-NE2I-IgG was higher than the two commercial anti-HEV-IgG kits (see figure 10, raw data shown in table 5). When 34 random sera of normal people were detected, the positive rates of anti-NE2I-IgG and of Genelabs anti-HEV-IgG were 35% and 11% respectively, and the later was absolutely included in the former. When 263 clinical sera of hepatitis patients, the positive rates of anti-NE2I-IgG and of Wantai anti-HEV-IgG were 27.2% and 10.6% respectively. When 91 sera of non-A, non-B, non-C hepatitis patients, the positive rates of anti-NE2I-IgG and of Genelabs anti-HEV-IgG were 69.2% and 24.2% respectively. In a word, the anti-HEV-IgG based on NE2I of this invention is better sensitive than the commercial anti-HEV-IgG kits.
Table 5 Comparison of sensitivity for detection HEV infected monkey serum by anti-HEV IgG antibody detection kit of present invention (NE2I-IgG) and by two commercial available detection kit (Genelabs; WANTAI)

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Example 18: Method for labeling the recombinant proteins of present invention with HRP

[0148] Dissolving 1 mg of HRP (Biozyme R/Z>3) and NaIO4, respectively, in ultra-pure water (UPW); adding drop-wise NaIO4 solution with agitation; the mixture solution is stand for 30 minutes in dark at room temperature; step-wise add 100μl of 1% ethylene glycol solution, mixing; allow it to stand for 30 minutes in dark at 4°C. Dialye recombinant protein against carbonate buffer (10mM pH9.6) 3 hours; add appropriate diazyed recombinant protein to the oxygenized HRP, dialyze for 6 hours at room temperature (or 4°C) in carbonate buffer (10mM pH9.5) with gently stirring; add 20ul of freshly prepared 0.1M NaBH4 solution to the above-mentioned blending; allow it to stand for 2 hours at 4°C in darkness, gently vortex once each 30 minutes; dialyze it in PBS (10mM pH7.2) overnight at 4°C.

Example 19: Diagnostic kit for detecting antibody IgM against HEV in biological samples and capture assay for detecting antibody IgM against HEV in biological samples

[0149] Reference Polypeptide 225N was labeled with HRP according to the method described in example 18.

[0150] The diagnostic kit for detecting antibody IgM against HEV containing HRP-labeled polypeptide NE2I of present invention comprises: microtiter strip that is pre-coated with mouse anti-human IgM μ chain polyclonal antibody (Dako company, Denmark) and blocked with blocking solution; sample diluent (20mM pH7.2 PB, 1% Casein); working conjugate (HRP-labeled polypeptide 225N that is suitably diluted with enzyme diluent (20mM pH7.2 PB, 0.5% Casein, 10% NBS)); non-bioactivity material, such as 20x PBST, chromatogen agent A, chromatogen agent B and stop solution (WANTAI company, Beijing).

[0151] The capture assay using present diagnostic kit is carried out as follows: adding 100ul of diluent buffer to each well which is pre-coated with mouse anti-human IgM μ chain polyclonal antibody; adding lul of sample to be detected into the diluent buffer; mixing thoroughly by tapping gently; incubating for 30 minutes at 37°C; washing with PBST for five times; blot dry by inverting the microplate up-side-down and tapping firmly onto tissue; adding 100ul of working conjugate (HRP-labeled polypeptide 225N that is suitably diluted) to each well and incubating the microplate for 30 minutes at 37°C; washing five times again and blotting dry; then adding 50ul of chromatogen A and 50ul of chromatogen B and mixing thoroughly by tapping gently; incubating for 10 minutes at 37°C in dark; add 50ul of stop solution to each well and mixing gently by tapping the plate; determine the absorbance for each well at OD450nm/620nm.

[0152] Using the diagnostic anti-HEV-IgM kit as prepared in accordance with present invention, 263 clinical sera of hepatitis patients were detected by aforementioned capture assay with the positive rate of 11%; and 91 sera of non-A, non-B, non-C hepatitis patients were also detected by said capture assay with the positive rate of 48.4%. Meanwhile, the positive rate for these 91 sera sample detected by diagnostic kit of anti-HEV-IgG from Genelabs was merely 24.2%. As indicated by the above results, the positive sample detected by diagnostic anti-HEV-IgM kit of present invention using capture assay is in good accordance with the clinical diagnosis. Moreover, most of positive samples detected by Genelabs anti-HEV-IgG kit are also positive in the present capture assay. That is to say, the anti-HEV-IgM kit of present invention as well as said capture assay possess higher sensitivity and specificity in clinical HEV diagnosis than that of existing commercial available kits.
the positive rate detected by Genelabs anti-HEV-IgG was merely 24.2%. As indicated by the above data, detection with the present diagnostic kit is superior to that of existing commercial available kits in clinical diagnosis.

SEQUENCE LISTING

[0157]

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<140> PCT/CN01/01469

<141> 2001-09-30

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Claims

1. A multimer of purified monomeric polypeptides, wherein said multimer is formed by 2-180 purified monomeric polypeptides via self aggregation, and each said purified monomeric polypeptide is selected from the group consisting of
   1) A polypeptide consisting of the amino acid sequence of amino acid residues 414 to 660 from SEQ ID NO: 1, of 247 amino acids; and
   2) A polypeptide having at least 80% identity to the above 1) and having identical antigenicity or immunogenicity.

2. A vaccine composition for prophylaxis and/or treatment of hepatitis E virus infection in mammals, which comprises the multimer according to claim 1, and optionally, pharmaceutically acceptable vehicles and/or adjuvant.

3. A multimer of purified monomeric polypeptides, wherein said multimer is formed by 2-180 purified monomeric polypeptides via self aggregation, and each said purified monomeric polypeptide is a chimeric protein formed by linking a polypeptide as defined in claim 1 and a conserved fragment of hemagglutin antigen from influenza virus, optionally via a flexible linker.

4. A vaccine composition for prophylaxis and/or treatment of hepatitis E virus infection in mammals, which comprises the multimer according to claim 3, and optionally, pharmaceutically acceptable vehicles and/or adjuvant.

5. A multimer according to claim 1 or a multimer according to claim 3 for vaccinating mammals to prevent from hepatitis E virus infection.

6. Use of vaccine composition according to claim 2 or 4 in the manufacture of a medicament for vaccinating mammals to prevent from hepatitis E virus infection.

7. Use of a prophylaxis and/or treatment effective amount of a multimer as claimed in claim 1 or 3 for the manufacture of a medicament for prophylaxis and/or treatment of hepatitis E virus infection in mammals.

8. A diagnostic kit for the diagnosis of hepatitis E virus infection in biological sample, which comprises a diagnosis effective amount of multimer according to claim 1 or 3.

9. The diagnostic kit according to claim 8, which further comprises the polypeptide containing immunogenic epitope from hepatitis E virus ORF3 or an immunogenic fragment thereof, wherein said polypeptide containing immunogenic epitope from hepatitis E virus ORF3 or an immunogenic fragment thereof is, optionally, covalently bound to the selected polypeptide defined in claim 1.

10. The diagnostic kit according to claim 8 or 9 for determination of antibody IgG against hepatitis E virus in the biological sample, which comprises the multimer according to claim 1 or 3, if desired, said multimer is pre-coated on the surface of a suitable support; and further comprises detectable labeled antibody anti-IgG that is directed against IgG from biological sample to be detected, and detection agent corresponding to said detectable label; and if desired, further comprises a suitable buffer system.

11. The diagnostic kit according to claim 8 or 9 for determination of antibody IgM against hepatitis E virus in the biological sample, which comprises detectable labeled antibody anti-IgM as capture antibody that is directed against IgM from biological sample to be detected, if desired, said capture antibody is pre-coated on the surface of a suitable support; and further comprises the detectable labeled multimer according to claim 1 or 3, and detection agent corresponding...
to said detectable label; if desired, further comprises a suitable buffer system.

12. The diagnostic kit according to claim 8 or 9 for determination of total antibodies against hepatitis E virus in the biological sample, which comprises at least one of the multimer according to claim 1 or 3, if desired, said multimer is pre-coated on the surface of a suitable support; and further comprises a detectable labeled multimer, and detection agent corresponding to said detectable label; whereas said multimer selected from multimer according to claim 1 or 3 for pre-coating the surface of a support and the detectable labeled multimer selected from multimer according to claim 1 or 3 could be the same polypeptide, or different one.

13. A method for diagnosis hepatitis E virus infection in mammals, comprising contacting the diagnostic kit according to claim 8 or 9 with sample of mammal to be detected under the conditions suitable for the interaction of antigen and antibody.

14. A method for detecting total antibodies against hepatitis E virus in biological samples, comprising the step of: immobilizing at least one of the multimer according to claim 1 or 3 on the surface of a support; contacting it with sample to be detected under the conditions suitable for the interaction of antigen and antibody; washing with a suitable buffer; and detecting antigen/antibody complex on the surface of a support by using antigen of hepatitis E virus with a detectable label and corresponding detect agent.

15. A method for detecting antibody IgG against hepatitis E virus in biological samples, comprising the step of: immobilizing at least one of the multimer according to claim 1 or 3 on the surface of a support; contacting it with sample to be detected under the conditions suitable for the interaction of antigen and antibody; washing with a suitable buffer; and detecting the antigen/antibody complex on the surface of a support by using detectable labeled antibody anti-IgG against hepatitis E virus and corresponding detect agent.

16. A method for detecting antibody IgM against hepatitis E virus in biological samples, comprising the step of: immobilizing antibody anti-IgM on the surface of a support; contacting it with sample to be detected under the conditions suitable for the interaction of antigen and antibody; washing with a suitable buffer; and detecting the anti-IgM/IgM complex on the surface of a support by using detectable labeled at least one of the multimer according to claim 1 or 3 and corresponding detect agent.

17. A method for detecting antibody IgM against hepatitis E virus in biological samples, comprising the step of: immobilizing antibody anti-IgM on the surface of a support; contacting it with sample to be detected under conditions suitable for the interaction of antigen and antibody; washing with a suitable buffer; and then contacting with at least one of multimer according to claim 1 or 3 under conditions suitable for the interaction of antigen and antibody; washing with a suitable buffer; and then detecting antigen/antibody complex on the surface of a support by using detectable labeled anti-HEV polyclonal or monoclonal antibody and corresponding detect agent.

18. A multimer of purified monomeric polypeptides, wherein said multimer is formed by 2-180 purified monomeric polypeptides via self aggregation, and each said purified monomeric polypeptide is a chimeric protein comprising a polypeptide as defined in claim 1 and immunogenic epitope from hepatitis E virus ORF3 or an immunogenic fragment thereof.

19. A multimer of claim 18 for use in prophylaxis and/or treatment of hepatitis E virus infection in mammals or for use in the diagnosis of hepatitis E virus infection.

Patentansprüche

1. Multimer von gereinigten monomeren Polypeptiden, wobei das Multimer durch Selbstaggregation von 2 bis 180 gereinigten monomeren Polypeptiden gebildet wird und jedes dieser gereinigten monomeren Polypeptide aus der Gruppe ausgewählt ist, bestehend aus:

   1) einem Polypeptid, das aus der Aminosäuresequenz der Aminosäurereste 414 bis 660 der Sequenzidentifikation 1, aus 247 Aminosäuren, besteht; und

   2) einem Polypeptid, das dem vorstehenden 1) zu mindestens 80 % identisch ist und eine identische Antigenität oder Immunogenität aufweist.
2. Impfstoffzusammensetzung für die Prophylaxe und/oder Behandlung einer Infektion mit dem Hepatitis E-Virus bei Säugern, die das Multimer gemäß Anspruch 1 und gegebenenfalls pharmazeutisch akzeptable Träger und/oder Adjuvantien aufweist.


4. Impfstoffzusammensetzung für die Prophylaxe und/oder Behandlung einer Infektion mit dem Hepatitis E-Virus bei Säugern, die das Multimer gemäß Anspruch 3 und gegebenenfalls pharmazeutisch akzeptable Träger und/oder Adjuvantien aufweist.

5. Multimer nach Anspruch 1 oder Multimer nach Anspruch 3, zum Impfen von Säugern, um eine Infektion mit dem Hepatitis E-Virus zu verhindern.


7. Verwendung einer für die Prophylaxe und/oder Behandlung wirksamen Menge eines Multimers nach Anspruch 1 oder 3 für die Herstellung eines Medikamentes für die Prophylaxe und/oder Behandlung einer Infektion mit dem Hepatitis E-Virus bei Säugern.

8. Diagnose-Kit für die Diagnose eine Infektion mit dem Hepatitis E-Virus bei einer biologischen Probe, das eine für die Diagnose wirksame Menge eines Multimers nach Anspruch 1 oder 3 aufweist.

9. Diagnose-Kit nach Anspruch 8, das ferner das Polypeptid aufweist, das ein immunogenes Epitop vom Hepatitis E-Virus ORF3 oder ein immunogenes Fragment davon enthält, wobei das Polypeptid, das ein immunogenes Epitop vom Hepatitis E-Virus ORF3 oder ein immunogenes Fragment davon enthält, gegebenenfalls kovalent an das ausgewählte Polypeptid nach Anspruch 1 gebunden ist.

10. Diagnose-Kit nach Anspruch 8 oder 9 zum Bestimmen des Antikörpers IgG für das Hepatitis E-Virus bei einer biologischen Probe, das das Multimer nach Anspruch 1 oder 3 aufweist, wobei das Multimer falls erforderlich vorher auf der Oberfläche eines geeigneten Trägers ausgestrichen worden ist; und ferner einen nachweisbaren markierten Antikörper anti-IgG, der gegen IgG aus der nachzuweisenden biologischen Probe gerichtet ist, und ein Nachweismittel aufweist, das dieser nachweisbaren Markierung entspricht; und falls erforderlich ferner ein geeignetes Puffersystem aufweist.

11. Diagnose-Kit nach Anspruch 8 oder 9 zum Bestimmen des Antikörpers IgM für den Hepatitis E-Virus in der biologischen Probe, das den nachweisbaren markierten Antikörper anti-IgM als immobilisierten Antikörper aufweist, der gegen IgM aus der nachzuweisenden biologischen Probe gerichtet ist, wobei die immobilisierte Antikörper falls erforderlich vorher auf der Oberfläche eines geeigneten Trägers ausgestrichen worden ist; und ferner das nachweisbare markierte Multimer nach Anspruch 1 oder 3 und ein Nachweismittel aufweist, das dieser nachweisbaren Markierung entspricht; und falls erforderlich ferner ein geeignetes Puffersystem aufweist.

12. Diagnose-Kit nach Anspruch 8 oder 9 zum Bestimmen aller Antikörper für das Hepatitis E-Virus in einer biologischen Probe, das zumindest eines der Multimere nach Anspruch 1 oder 3 aufweist, wobei das Multimer falls erforderlich vorher auf der Oberfläche eines geeigneten Trägers ausgestrichen worden ist; und ferner ein nachweisbares markiertes Multimer und ein Nachweismittel aufweist, das dieser nachweisbaren Markierung entspricht, wobei das Multimer, das aus dem Multimer gemäß Anspruch 1 oder 3 ausgewählt ist, zum vorherigen Ausstreichen auf der Oberfläche eines Trägers und das nachweisbare markierte Multimer, das aus dem Multimer nach Anspruch 1 oder 5 ausgewählt ist, das gleiche Polypeptid oder verschiedene Polypeptide sein können.
13. Verfahren zum Diagnostizieren einer Infektion mit dem Hepatitis E-Virus bei Säugern, das das Inkontaktbringen des Diagnose-Kits nach Anspruch 8 oder 9 mit einer nachzuweisenden Probe eines Säugers unter Bedingungen aufweist, die für die Wechselwirkung zwischen Antigen und Antikörper geeignet sind.

14. Verfahren zum Nachweisen aller Antikörper für das Hepatitis E-Virus bei biologischen Proben, das die folgenden Schritte aufweist:

- Immobilisieren von zumindest einem der Multimere nach Anspruch 1 oder 3 auf der Oberfläche eines Trägers; dessen Inkontaktbringen mit der nachzuweisenden Probe unter Bedingungen, die für die Wechselwirkung von Antigen und Antikörper geeignet sind; Waschen mit einem geeigneten Puffer; und Nachweisen des Antigen/Antikörper-Komplexes auf der Oberfläche des Trägers unter Verwendung eines Antigens des Hepatitis E-Virus mit einer nachweisbaren Markierung und eines entsprechenden Nachweismittels.

15. Verfahren zum Nachweisen des Antikörpers IgG für das Hepatitis E-Virus in biologischen Proben, das die folgenden Schritte aufweist:

- Immobilisieren von zumindest einem der Multimere nach Anspruch 1 oder 3 auf der Oberfläche eines Trägers; dessen Inkontaktbringen mit der nachzuweisenden Probe unter Bedingungen, die für die Wechselwirkung von Antigen und Antikörper geeignet sind; Waschen mit einem geeigneten Puffer; und Nachweisen des Antigen/Antikörper-Komplexes auf der Oberfläche des Trägers unter Verwendung des nachweisbaren markierten Antikörpers anti-IgG für das Hepatitis E-Virus und eines entsprechenden Nachweismittels.

16. Verfahren zum Nachweisen des Antikörpers IgM für das Hepatitis E-Virus in biologischen Proben, das die folgenden Schritte aufweist:

- Immobilisieren des Antikörpers anti-IgM auf der Oberfläche eines Trägers; dessen Inkontaktbringen mit der nachzuweisenden Probe unter Bedingungen, die für die Wechselwirkung von Antigen und Antikörper geeignet sind; Waschen mit einem geeigneten Puffer; und Nachweisen des anti-IgM/IgM-Komplexes auf der Oberfläche des Trägers unter Verwendung von zumindest einem der nachweisbaren markierten Multimere nach Anspruch 1 oder 3 und eines entsprechenden Nachweismittels.

17. Verfahren zum Nachweisen des Antikörpers IgM für das Hepatitis E-Virus in biologischen Proben, das die folgenden Schritte aufweist:

- Immobilisieren des Antikörpers anti-IgM auf der Oberfläche eines Trägers; dessen Inkontaktbringen mit der nachzuweisenden Probe unter Bedingungen, die für die Wechselwirkung von Antigen und Antikörper geeignet sind; Waschen mit einem geeigneten Puffer; und anschließendes Inkontaktbringen mit zumindest einem der Multimere nach Anspruch 1 oder 3 unter Bedingungen, die für die Wechselwirkung zwischen Antigen und Antikörper geeignet sind; Waschen mit einem geeigneten Puffer; und anschließendes Nachweisen des Antigen/Antikörper-Komplexes auf der Oberfläche des Trägers unter Verwendung eines nachweisbaren markierten polyklonalen oder monoklonalen Antikörpers anti-HEV und eines entsprechenden Nachweismittels.

18. Multimer von gereinigten monomeren Polypeptiden, wobei das Multimer durch Selbstaggregation von 2 bis 180 gereinigten monomeren Polypeptiden gebildet wird und jedes der gereinigten monomeren Polypeptide ein chimäres Protein ist, das ein Polypeptide nach Anspruch 1 und ein immunogenes Epitop vom Hepatitis E-Virus ORF3 oder ein immunogenes Fragment davon aufweist.


Revendications

1. Multimère de polypeptides monomères purifiés, ledit multimère étant formé par 2 à 180 polypeptides monomères purifiés par auto-agrégation, et chacun desdits polypeptides monomères purifiés étant choisi dans le groupe consistant en :

   1) un polypeptide consistant en la séquence d’acides aminés des résidus d’acides aminés 414 à 660 de la SEQ
2. Composition de vaccin pour la prophylaxie et/ou le traitement de l'infection par le virus de l’hépatite E chez des mammifères, qui comprend le multimère suivant la revendication 1 et, facultativement, des véhicules et/ou un adjuvant pharmaceutiquement acceptables.

3. Multimère de polypeptides monomères purifiés, ledit multimère étant formé par 2 à 180 polypeptides monomères purifiés par auto-agrégation, et chacun desdits polypeptides monomères purifiés étant une protéine chimère formée par liaison d’un polypeptide tel que défini par la revendication 1, et d’un fragment conservé d’antigène hémagglutinine du virus grippal, éventuellement par l’intermédiaire d’un groupe de liaison flexible.

4. Composition de vaccin pour la prophylaxie et/ou le traitement de l’infection par le virus de l’hépatite E chez des mammifères, qui comprend le multimère suivant la revendication 3, et, facultativement, des véhicules et/ou un adjuvant pharmaceutiquement acceptables.

5. Multimère suivant la revendication 1 ou multimère suivant la revendication 3, pour la vaccination de mammifères à des fins de prévention contre l’infection par le virus de l’hépatite E.

6. Utilisation de la composition de vaccin suivant la revendication 2 ou la revendication 4, dans la production d’un médicament pour la vaccination de mammifères à des fins de prévention contre l’infection par le virus de l’hépatite E.

7. Utilisation d’une quantité, efficace à des fins de prophylaxie et/ou de traitement, d’un multimère tel que revendiqué dans la revendication 1 ou 3, pour la production d’un médicament pour la prophylaxie et/ou le traitement de l’infection par le virus de l’hépatite E chez des mammifères.

8. Kit de diagnostic pour le diagnostic de l’infection par le virus de l’hépatite E dans un échantillon biologique, qui comprend une quantité, efficace à des fins de diagnostic, du multimère suivant la revendication 1 ou 3.

9. Kit de diagnostic suivant la revendication 8, qui comprend en outre le polypeptide contenant un épitope immunogène du virus de l’hépatite E ORF3 ou un de ses fragments immunogènes, dans lequel ledit polypeptide contenant l’épitope immunogène du virus de l’hépatite E ORF3 ou un de ses fragments immunogènes est, éventuellement, lié par covalence au polypeptide choisi défini dans la revendication 1.

10. Kit de diagnostic suivant la revendication 8 ou 9, pour la détermination de l’anticorps IgG contre le virus de l’hépatite E dans l’échantillon biologique, qui comprend le multimère suivant la revendication 1 ou 3, ledit multimère étant si cela est désiré sous forme d’un pré-revêtement sur la surface d’un support convenable ; et qui comprend en outre un anticorps anti-IgG marqué de manière détectable qui est dirigé contre la IgG de l’échantillon biologique à détecter, et un agent de détection correspondant audit marqueur détectable ; et, si cela est désiré, qui comprend en outre un système tampon convenable.

11. Kit de diagnostic suivant la revendication 8 ou 9, pour la détermination de l’anticorps IgM contre le virus de l’hépatite E dans l’échantillon biologique, qui comprend un anticorps anti-IgM marqué de manière détectable comme anticorps de capture qui est dirigé contre la IgM provenant de l’échantillon biologique à détecter, ledit anticorps de capture étant si cela est désiré sous forme d’un pré-revêtement sur la surface d’un support convenable ; et qui comprend en outre le multimère marqué de manière détectable suivant la revendication 1 ou 3, et un agent de détection correspondant audit marqueur détectable ; et qui comprend en outre, si cela est désiré, un système tampon convenable.

12. Kit de diagnostic suivant la revendication 8 ou 9, pour la détermination des anticorps totaux contre le virus de l’hépatite E dans l’échantillon biologique, qui comprend au moins un des multimères suivant la revendication 1 ou 3, ledit multimère étant si cela est désiré sous forme d’un pré-revêtement sur la surface d’un support convenable ; et qui comprend en outre un multimère marqué de manière détectable, et un agent de détection correspondant audit marqueur détectable ; dans lequel ledit multimère choisi parmi les multimères suivant la revendication 1 ou 3 pour le pré-revêtement de la surface d’un support et le multimère marqué de manière détectable choisi parmi les multimères suivant la revendication 1 ou 5 peuvent être le même polypeptide ou des polypeptides différents.
13. Procédé pour le diagnostic de l'infection par le virus de l'hépatite E chez des mammifères, comprenant la mise en contact du kit de diagnostic suivant la revendication 8 ou 9 avec un échantillon provenant d'un mammifère destiné à la détection dans les conditions convenables de l'interaction d'antigène et d'anticorps.

14. Procédé pour la détection des anticorps totaux contre le virus de l'hépatite E dans des échantillons biologiques, comprenant l'étape : d'immobilisation d'au moins un des multimères suivant la revendication 1 ou 3 sur la surface d'un support ; de mise en contact de celui-ci avec un échantillon destiné à la détection dans les conditions convenables de l'interaction d'antigène et d'anticorps ; de lavage avec un tampon convenable ; et de détection du complexe antigène/anticorps sur la surface d'un support en utilisant l'antigène du virus de l'hépatite E avec un marqueur détectable et l'agent de détection correspondant.

15. Procédé pour la détection d'un anticorps IgG contre le virus de l'hépatite E dans des échantillons biologiques, comprenant l'étape : d'immobilisation d'au moins un des multimères suivant la revendication 1 ou 3 sur la surface d'un support ; de mise en contact de celui-ci avec un échantillon destiné à la détection dans des conditions convenables de l'interaction d'antigène et d'anticorps ; de lavage avec un tampon convenable ; et de détection du complexe antigène/anticorps sur la surface d'un support en utilisant un anticorps anti-IgG marqué de manière détectable contre le virus de l'hépatite E et l'agent de détection correspondant.

16. Procédé pour détecter un anticorps IgM contre le virus de l'hépatite E dans des échantillons biologiques, comprenant l'étape : d'immobilisation d'un anticorps anti-IgM sur la surface d'un support ; de mise en contact de celui-ci avec un échantillon destiné à la détection dans les conditions convenables de l'interaction d'antigène et d'anticorps ; de lavage avec un tampon convenable ; et de détection du complexe anti-IgM/IgM sur la surface d'un support en utilisant au moins un des multimères suivant la revendication 1 ou 3, marqué de manière détectable, et l'agent de détection correspondant.

17. Procédé pour la détection d'un anticorps anti-IgM contre le virus de l'hépatite E dans des échantillons biologiques, comprenant l'étape : d'immobilisation d'un anticorps anti-IgM sur la surface d'un support ; de mise en contact de celui-ci avec un échantillon destiné à la détection dans des conditions convenables de l'interaction d'antigène et d'anticorps ; de lavage avec un tampon convenable ; et ensuite de mise en contact avec au moins un des multimères suivant la revendication 1 ou 3 dans des conditions convenables pour l'interaction d'antigène et d'anticorps ; de lavage avec un tampon convenable ; et ensuite de détection du complexe antigène/anticorps sur la surface d'un support en utilisant un anticorps polyclonal ou monoclonal anti-HEV marqué de manière détectable et l'agent de détection correspondant.

18. Multimère de polypeptides monomères purifiés, ledit multimère étant formé par 2 à 180 polypeptides monomères purifiés par auto-agrégation, et chacun desdits polypeptides monomères purifiés étant une protéine chimère comprenant un polypeptide tel que défini dans la revendication 1 et un épitope immunogène du virus de l'hépatite E ORF3 ou un de ses fragments immunogènes.

19. Multimère suivant la revendication 18 pour une utilisation dans la prophylaxie et/ou le traitement de l'infection par le virus de l'hépatite E chez des mammifères ou pour une utilisation dans le diagnostic de l'infection par le virus de l'hépatite E.
Fig. 1

Template → PCR amplification → BamHI, EcoRI digestion → ligation → selecting clone → NdeI/EcoRI digestion ↓ NdeI/EcoRI ligation → selecting clone → NdeI/EcoRI → pTO-T7-ORF2-201 → 201 recombinant protein
Fig. 5
Fig. 6

lane 1 2 3 4 5 6 7 8 9 10

SDS-PAGE

Mab 1F6

Mab 2C9

Mab 3F5
Fig. 7

- Immunized mouse 1
- Immunized mouse 2
- Immunized mouse 3

201 Freund's adjuvant immunized (0.5 μg/)

201 Freund's adjuvant immunized (1.0 μg/)

201 Freund's adjuvant immunized (2.0 μg/)

201 Freund's adjuvant immunized (5.0 μg/)

Fig. 9
REFERENCES CITED IN THE DESCRIPTION

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