HUMAN ENTEROVIRUS SPECIFIC ANTIBODIES AND THEIR USES IN DIAGNOSTICS

FIELD OF THE INVENTION

The present invention relates generally to the field of immune mediated therapies for the detection and/or treatment of Enterovirus 71 (EV71) and EV71-related diseases and to methods of preparing these therapies. In particular, the immune mediated therapies may be in the form of EV71 specific monoclonal antibodies.

BACKGROUND TO THE INVENTION

Enteroviruses are a heterogeneous group of pathogens responsible for a broad spectrum of human and nonhuman diseases. Enteroviruses belong to a large genus within the family Picornaviridae; other genera within this family include rhinoviruses, hepatoviruses, cardioviruses, and aphthoviruses. The enterovirus genus encompasses polio viruses, coxsackie A viruses (CAV), coxsackie B viruses (CBV), echoviruses, and enteroviruses 68-71, as well as a number of uncharacterized enteroviruses isolated from humans and other primates. Like other picornaviruses, enteroviral virions comprise an icosahedral capsid with no envelope enclosing a core comprising infectious, single- stranded genomic sense RNA (ssRNA), about 7-8.5 kb in size. Enteroviruses are distinguished from other members of the picornaviridae by their stability in acid and their fecal-oral route of passage and transmission. Virus entry into cells is believed to involve specific cellular receptors. Virion proteins include multiple copies of four capsid proteins (VP1, VP2, VP3 and VP4). A small protein, VPg (Mr about 24 x 10^3), is linked covalently to the 5' terminus of the genomic RNA. Other proteins of the enteroviruses include the predominantly non-structural proteins, P2 and P3.

Enterovirus 71 (EV71) belongs to the human Enterovirus A species of the Enterovirus genus. EV71 is not a zoonotic agent and is the causative agent of a number of neurological diseases including aseptic meningitis, encephalitis, cranial nerve palsies, Guillan-Barre syndrome, and poliomyelitis-like syndrome.

This virus is also associated with large outbreaks of hand foot and mouth disease (HFMD) in human beings. Outbreaks of EV71 associated illness are common especially in Asian countries such as Taiwan, Malaysia, Singapore, Japan, Korea, Thailand, Vietnam, Hong Kong and even Australia.

The largest EV71 outbreaks have occurred in Bulgaria in 1975 with 44 deaths; Hungary in 1978 with 45 deaths; Malaysia in 1997 with 30 deaths; and in Taiwan in 1998, 2000 and 2001 with 78, 25 and 26 deaths respectively. The 1998 outbreak in Taiwan involved over 130,000 reported cases, including over 400 severe neurological cases and 78 deaths, of which 91% were children under five years of age. There have also been more recent outbreaks in early 2005 in Hong Kong, Vietnam and Taiwan in which a combined total of 31 people died and in Malaysia in early 2006 with 8 deaths. Considerable public concern has been raised about EV71 because of its high incidence of mortality and because its victims are commonly young children.

EV71 exhibits a wide variation in clinical presentation. Because of this wide variation in clinical presentation as well as increased public health concerns associated with EV71 and its potential for greater neurovirulence, it is important to be able to rapidly identify the specific strain of EV71 during an HFMD outbreak and to be able to rapidly discriminate between EV71 and CA16 (another leading agent responsible for large outbreaks of HFMD). Since early symptoms are similar for HFMD associated with infections of different strains of EV71 or CA16, the current etiologic diagnosis is dependent on virus isolation and serotyping. Standard serotyping involves neutralization tests with monospecific antiserum, which are quite limited in their availability. Further, antigenic typing is often hampered by non-neutralizable virus due to aggregation. Currently, there are two diagnostic tests kits on the market, one based on real time RT-PCR and the other based on anti-EV71 IgM detection in patient serum. Both these methods have great disadvantages. For example, real time PCR requires a well maintained laboratory and expensive equipment while detection of serum antibodies is only possible some weeks after infection which is a drawback as severe disease of EV71 has a rapid onset and progression.

SUMMARY OF THE INVENTION

The present invention addresses the problems above, and in particular provides at least one novel antibody that is specific to at least one EV71 and/or EV71 related disease.

According to a first aspect, the present invention provides at least one isolated monoclonal antibody or a fragment thereof that is capable of specifically binding to at least one conformational and/or linear epitope of EV71. In particular, the antibody may be selected from the group consisting of:

(a) antibody produced by hybridoma cell line CBA20110001, CBA20110003,or CBA20110002;

(b) antibody having the immunological binding characteristics of the antibody produced by hybridoma cell line CBA20110001, CBA20110003,or CBA20110002; and

(c) antibody that binds to an antigen capable of binding the antibody produced by the hybridoma cell line CBA20110001, CBA20110003,or CBA20110002.

According to another aspect, the present invention provides at least one isolated hybridoma cell line deposited with ATCC (10801 University Blvd., Manassas, VA 20110) on 27 July 2011 with accession number CBA20110001, CBA20110003,or CBA20110002.

According to other aspects, the present invention provides a method of detecting and/or quantifying the presence of EV71, a method of treating EV71 and/or at least one EV71-linked disease, the antibody or fragment thereof of the present invention for use as medicine, use of the antibody or fragment thereof of the present invention for the preparation of a medicament, kits, nucleic acids and uses thereof.

As will be apparent from the following description, preferred embodiments of the present invention allow an optimal use of the isolated antibodies to take advantage of their accuracy and specificity to at least one epitope of EV71. This and other related advantages will be apparent to skilled persons from the description below.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the results of characterization of Mab 1C6 by IFA. Cells were labeled with Mab 1C6 followed by anti-mouse FITC secondary antibody. (A) Vero cells infected with EV71-B5 (NUH0083). (B) Non-infected vero cells. (C) Insect cells expressing virus-like particles (VLPs) of EV71.

Figure 2 shows the results related to characterization of Mab 7C7. IFA (A), Western blot (B), Dot blot (C) of EV71 virus with Mab 7C7. (A) IFA of EV71 infected vero cells. Cells were labelled with Mab 7C7 followed by anti-mouse FITC secondary antibody. Top panel: Vero cells infected with EV71-B5 (NUH0083). Bottom panel: Non-infected vero cells. (B) Western blot of sucrose purified EV71-C4 strain (75-Yamagata-03). Blots were labelled with primary antibodies and HRP coupled secondary antibodies and bands were developed

by ECL. Lane 1: Polyclonal anti-EV71 (B5 strain) antibody detects all four capsid proteins. Lane 2: Monoclonal anti-VP1 antibody specifically labels the VP1 band. Lane 3: Mab 7C7 recognizes VP2 at about 26kD and its precursor VP0 at around 40kD (arrows). (C) Sensitivity of 7C7 in dot blot. EV71 B5 virus was dotted at 10-fold dilutions of TCID50 units per dot, labelled by Mab 7C7 and detected by HRP-coupled secondary antibody. H7N1 virus served as negative control. The detection limit was around 10^5 TCID50 units.

Figure 3 shows the results related to characterization of mAb 4B12. IFA (A) and Western blot (B) of EV71 virus with the anti-3CD monoclonal antibody 4B12. (A) IFA of infected versus uninfected Vero cells labeled with either polyclonal anti-3CD serum or monoclonal 4B12 antibody. Cells were infected with the EV71 C4 subgenotype. (B) Western blot of whole cell lysates of EV71 infected (lane 2) and un-infected RD cells (lane 3). 4B12 monoclonal antibody was able to detect a linear epitope of the 3CD proteinase at 73 kDa, as well as of the 3D RNA-dependent RNA polymerase at 52kDa (arrowheads). Both proteins could also be detected in sucrose gradient purified EV71 virus particles (lane 4).

Figure 4 is the Epitope Mapping of Mab 7C7. (A) Fragmentation of the VP2 protein into 7 continual overlapping segments. Western blotting of the fragments with anti-GST showed expression of all constructs, while blotting with 7C7 did not recognize fragments F and G, indicating that the epitope lies between amino acids 130 and 162 of VP2. (B) Fragmentation of amino acids 1-159 of VP2 into 6 continual overlapping segments. Again, blotting with anti-GST confirms protein expression, while blotting with 7C7 indicates that the epitope lies on fragment "d" between amino acids 145-159 of VP2. (C) Fragmentation of "d" into 6 continual overlapping peptides. Anti-GST blotting shows expression while the epitope of 7C7 is restricted to peptide "4" corresponding to the epitope EDSHPP. (D) Mutational analysis of 7C7 epitope. Mutated epitopes were expressed as GST fusion proteins and expression was confirmed by Western blotting with anti-GST antibody. The minimal epitope EDSHP as well as the aspartic acid to asparagine substitution (E**N**SHPP) present in CA16 virus could be recognized by our mAB 7C7. However, the threonine to serine mutation (EDTHPP) of some C4 strains and the double mutation of human echoviruses (ED**NA**PP) could not be recognized.

Figure 5 is the Epitope mapping of 4B12. (A) Fragmentation of the 3CD protein into 5 continual overlapping segments. Western blotting of the fragments with anti-GST showed expression of all constructs, while blotting with 4B12 did not recognize 3C, indicating that the epitope lies on the 3D1 segment. (B) Fragmentation of 3D1 into 4 continual overlapping

segments. Again, blotting with anti-GST confirms protein expression, while blotting with 4B12 indicates that the epitope lies on fragment 3D1.2. (C) Fragmentation of 3D1.2 into 8 continual overlapping peptides. Anti-GST blotting shows expression while the epitope of 4B12 is restricted to peptide "H".

Figure 6 is the finalizing of 4B12 Epitope. (A) The proposed 6 (EQALFS) and 8 (DFEQALFS) amino acid long epitopes of peptide "H" were expressed as GST fusion proteins. Expression was confirmed by anti-GST antibody. (B) Western blotting with 4B12 detected only the longer 8aa epitope at position 1784-1791 of the EV71 polyprotein (lane 2) but not the shorter putative 6aa epitope (lane 1). (C) Mutational analysis of the 4B12 Epitope. Two naturally occurring EV71 variations were introduced into the identified epitope. Both the D to E and the Q to H mutations were detected by the 4B12 Mab. (D) The Ev71 3D epitope was blasted against hepatitis A virus and poliovirus 1 and their corresponding epitopes were expressed as GST-fusion proteins in E. coli. Expression of the fusion proteins was confirmed by Western blotting with anti-GST antibody. (E) The hepatitis A and polio 1 epitopes were not recognized by 4B12 Mab, confirming its specificity. The previously characterized Ev71 3D epitope was loaded as positive control. GST alone was expressed as negative control.

Figure 7 shows the specificity of 7C7 and 1C6 to EV71 subgenogroups. Vero cells were infected with the EV71 wild-type strains A (BrCr), B2 (7423-MS-87), B4 (NUH-HFM41), B5 (NUH0083-SIN-08), C1 (Y90-3761), C2 (NUH0075-SIN-08), C4 (75-Yamagata-03), and C5 (3437-SIN-06). IFA was conducted with Mab 7C7 or 1C6 and FITC labeled secondary antibody. All tested strains were recognized.

Figure 8 shows the specificity of 4B12 to EV71 subgenogroups. Vero cells were infected with the EV71 wild-type strains B5 (NUH0083-SIN-08), A (BrCr), B2 (7423-MS-87), B4 (NUH), B5 (#363, Malaysia, unpublished sequence), C1 (Y90-3761), C2 (NUH0075-SIN-08), C4 (75-Yamagata-03), C5 (3437-SIN-06) and RG virus C4 (Fuyang-08). IFA was conducted with Mab 4B12 and FITC labeled secondary antibody. All tested strains were recognized by 4B12.

Figure 9 shows the specificity of 4B12 to EV71 subgenogroups. Western Blot (A) and Dot Blot (B) of sucrose purified EV71 particles from the subgenogroups A (BrCr), B2 (7423-MS-87), B5 (#363, Malaysia, unpublished sequence), B5 (NUH0083-SIN-08), C1 (Y90-3761), C2 (NUH0075-SIN-08), and C4 (75-Yamagata-03). ECL reagent was used for developing.

A concentrated preparation of influenza virus strain H7N1 served as a negative control in the dot blot.

Figure 10 shows the sensitivity of 4B12 to EV71 in dot blot assay. (A) Purified recombinant 3D protein was dotted at decreasing concentrations from 1000pg to 10pg and detected with Mab 4B12 followed by HRP-coupled secondary antibody and ECL development (ECL) or infrared-coupled secondary antibody followed by scanning in an Odyssey reader (IR). The detection limit was 10pg for both methods. (B) The IR method was used to establish the detection limit of serial dilutions of EV71 in TCID50 units. The detection limit for virus particles was 10^4 TCID50 units. Recombinant 3D protein was used as a positive control.

Figure 11 is a graph showing the sensitivity and specificity of the EB-ELISA. Performance of the EB-ELISA with sera from guinea pigs immunized with different strains of EV71, or mice with different coxsackievirus. Sera were collected days after the second immunization and normalized to a virus neutralization titer of before the EB-ELISA. Inhibition above the cut-off value of 30% blocking was considered as positive; i.e. antibodies to EV71 were present. The results were expressed as the arithmetic mean of percent blocking values (n = 4 per group, with each error bar representing the standard error of the mean). NS: Normal pre-immune serum; GP: guinea pig; MS: mouse. Dotted line: cutoff value.

Figure 12 is a graph showing the comparison of EB-ELISA to microneutralization assay. The EV71 antibody detection sensitivity and specificity of the EB-ELISA and microneutralization assays were compared using guinea pig immune sera collected 14 days after the second immunization to determine the endpoint of inhibition. The EB-ELISA titer was determined as Mab titer giving $\geqslant$30% blocking. The neutralization assays were performed using B5 NUH0083 EV71 virus. The results were expressed as the geometric mean titers (n = 4 per group, with each error bar representing the standard error of the mean).

DETAILED DESCRIPTION OF THE INVENTION

Bibliographic references mentioned in the present specification are for convenience listed in the form of a list of references and added at the end of the examples. The whole content of such bibliographic references is herein incorporated by reference.

Definitions

For convenience, certain terms employed in the specification, examples and appended claims are collected here.

As used herein, the term "antibody" refers to any immunoglobulin or intact molecule as well as to fragments thereof that bind to a specific epitope. Such antibodies include, but are not limited to polyclonal, monoclonal, chimeric, humanised, single chain, Fab, Fab', F(ab)' fragments and/or F(v) portions of the whole antibody. The term "monoclonal antibody" may be referred to as "mAb". For example, the antibody "1C6 mAb" used interchangeably with "1C6", is capable of specifically binding to EV71 including but not limited to a conformational epitope comprising at least one capsid protein of EV71 and includes monoclonal antibodies, polyclonal antibodies, single-chain antibodies, and fragments thereof which retain the antigen binding function of the parent antibody. Similarly, the antibody "7C7 mAb" used interchangeably with "7C7", is capable of specifically binding to EV71, including but not limited to VP2 of EV71 and includes monoclonal antibodies, polyclonal antibodies, single-chain antibodies, and fragments thereof which retain the antigen binding function of the parent antibody. Also, the antibody "4B12 mAb" used interchangeably with "4B12", is capable of specifically binding to EV71 including but not limited to at least one epitope comprising a non-structural protein of EV71, for example 3CD, and/or 3D and includes monoclonal antibodies, polyclonal antibodies, single-chain antibodies, and fragments thereof which retain the antigen binding function of the parent antibody.

The term "antibody fragment" as used herein refers to an incomplete or isolated portion of the full sequence of the antibody which retains the antigen binding function of the parent antibody. Examples of antibody fragments include Fab, Fab', F(ab')2, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments. Fragments of the 1C6, 7C7 and 4B12 are encompassed by the invention so long as they retain the desired affinity of the full-length antibody. In particular, it may be shorter by at least one amino acid. For example, the fragment of a 1C6 antibody comprises the antigen binding function that enables it to bind to a conformational epitope of a capsid protein of EV71, the fragment of a 7C7 antibody comprises the antigen binding function that enables it to bind to capsid protein VP2 of EV71, or fragment thereof and the fragment of 4B12 antibody comprises the antigen binding function that enables it to bind to a non-structural protein, for example 3CD and/or 3D of EV71, or fragment thereof.

The term "antigen" as used herein, refers to a substance that prompts the generation of antibodies and can cause an immune response. It may be used interchangeably in the present invention with the term "immunogen". In the strict sense, immunogens are those substances that elicit a response from the immune system, whereas antigens are defined as substances that bind to specific antibodies. An antigen or fragment thereof may be a molecule (i.e. an epitope) that makes contact with a particular antibody. When a protein or a fragment of a protein is used to immunize a host animal, numerous regions of the protein may induce the production of antibodies (i.e. elicit the immune response), which bind specifically to the antigen (given regions or three-dimensional structures on the protein). The antigen may include but is not limited to a capsid protein and/or non-structural proteins of EV71. In particular, the term "epitope" refers to a consecutive sequence of from about 6 to about 13 amino acids which form an antibody binding site. The epitope in the form that binds to the mAb or binding protein may be in a denatured protein that is substantially devoid of tertiary structure. The epitope may be a conformational epitope. A "conformational epitope" is herein defined as a sequence of subunits (usually, amino acids) composing an antigen that comes in direct contact with a receptor of the immune system. Whenever a receptor interacts with an undigested antigen, the surface amino acids that come in contact may not be continuous with each other if the protein is unwound. Such discontinuous amino acids that come together in three dimensional conformation and interact with the receptor's paratope are called conformational epitopes. In contrast, if the antigen is digested, small segments called peptides are formed, which bind with major histocompatibility complex molecules, and then later with T cell receptors through amino acids that are continuous in a line. These are known as linear epitopes.

The term "comprising" is herein defined to be that where the various components, ingredients, or steps, can be conjointly employed in practicing the present invention. Accordingly, the term "comprising" encompasses the more restrictive terms "consisting essentially of" and "consisting of."

The term "derivative," as used herein, refers to the chemical modification of at least one EV71 epitope. Chemical modifications of a polynucleotide sequence can include, for example, replacement of hydrogen by an alkyl, acyl, or amino group. A derivative polynucleotide encodes a polypeptide which retains at least one biological or immunological function of the natural molecule. A derivative polypeptide is one modified by glycosylation, pegylation, or any similar process that retains at least one biological or immunological function of the polypeptide from which it was derived.

The term "humanized antibody," as used herein, refers to at least one antibody molecule in which the amino acid sequence in the non-antigen binding regions has been altered so that the antibody more closely resembles a human antibody, and still retains its original binding ability.

As used herein, the term "hybridoma" refers to cells that have been engineered to produce a desired antibody in large amounts. For example, to produce at least one hybridoma, B cells are removed from the spleen of an animal that has been challenged with the relevant antigen and fused with at least one immortalized cell. This fusion is performed by making the cell membranes more permeable. The fused hybrid cells (called hybridomas), will multiply rapidly and indefinitely and will produce at least one antibody.

"Immortalised cells" as used herein are also known as transformed cells - i.e. cells whose growth properties have been altered. This does not necessarily mean that these are "cancer" or "tumour" cells, i.e. able to form a tumour if introduced into an experimental animal, although in some cases they may do. Immortalised cell lines include but are not limited to NS1, Jurkat, HeLa, HepG2, SP2/0, Hep-3b and the like.

The term "immunological binding characteristics" of a mAb or related binding protein, in all of its grammatical forms, refers to the specificity, affinity and cross-reactivity of the mAb or binding protein for its antigen.

The term "isolated" is herein defined as a biological component (such as a nucleic acid, peptide or protein) that has been substantially separated, produced apart from, or purified away from other biological components in the cell of the organism in which the component naturally occurs, i.e., other chromosomal and extrachromosomal DNA and RNA, and proteins. Nucleic acids, peptides and proteins which have been isolated thus include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids, peptides and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acids.

The term "sample," as used herein, is used in its broadest sense. A biological sample suspected of containing nucleic acids encoding at least one EV71 derived peptide, or fragments thereof, or EV71 itself may comprise a bodily fluid, an extract from a cell,

chromosome, organelle, or membrane isolated from a cell, a cell; genomic DNA, RNA, or cDNA (in solution or bound to a solid support), a tissue, a tissue print and the like.

As used herein, the terms "specific binding" or "specifically binding" refer to the interaction between a protein or peptide and an agonist, an antibody, or an antagonist. In particular, the binding is between an antigen and an antibody. The interaction is dependent upon the presence of a particular structure of the protein recognized by the binding molecule (i.e., the antigen or epitope). For example, if an antibody is specific for epitope "A", the presence of a polypeptide containing the epitope A, or the presence of free unlabeled A, in a reaction containing free labeled A and the antibody will reduce the amount of labeled A that binds to the antibody. For example, 1C6 may specifically bind a conformational epitope on the capsid 7C7 may specifically bind the linear epitope VP2 and 4B12 may specifically bind the linear epitope 3CD and/or 3D.

The term "subject" is herein defined as vertebrate, particularly mammal, more particularly human. For purposes of research, the subject may particularly be at least one animal model, e.g., a mouse, rat and the like.

A person skilled in the art will appreciate that the present invention may be practised without undue experimentation according to the method given herein. The methods, techniques and chemicals are as described in the references given or from protocols in standard biotechnology and molecular biology text books.

According to a first aspect, the present invention provides isolated monoclonal antibodies and related binding proteins that bind specifically to EV71. Monoclonal antibodies also known as “mAbs” may be a substantially homogeneous population of antibodies derivable from a single antibody-producing cell. Thus, all antibodies in the population may be identical and may have the same specificity for a given epitope. The specificity of the mAb responses provides a basis for an effective diagnostic reagent. Monoclonal antibodies and binding proteins derived therefrom also have utility as therapeutic agents.

In particular, the isolated monoclonal antibody or a fragment thereof of the present invention may be capable of specifically binding to at least one conformational epitope of Enterovirus 71 (EV71). This is advantageous as epitopes usually exist in nature in a three dimensional conformation and the antibodies may thus be more efficient and effective in detecting the presence of EV71. These antibodies may thus be able to bind and to

recognize viral antigens without prior treatments of a tissue section. The conformational epitope may comprise at least one capsid protein and/or at least one non-structural protein. The capsid protein may be an intact virus capsid protein. The capsid protein may comprise one or more proteins selected from the group consisting of VP1, VP2 VP3, VP4 and VP0 precursor.

In particular, the antibody according to any aspect of the present invention may comprise the immunological binding characteristics of monoclonal antibody 1C6. These immunological binding characteristics of mAb 1C6 are produced by hybridoma 1C6, deposited with the CellBank Australia, 214 Hawkesbury Road, Westmead NSW 2145, Australia on 27 July 2011, in accordance with the provisions of the Budapest Treaty, and assigned Accession Number CBA20110001. The hybridoma provides a continuous source of the mAbs and binding proteins of the invention.

The antibody according to one aspect of the present invention may be selected from the group consisting of:

(a) antibody produced by hybridoma cell line CBA20110001;

(b) antibody having the binding characteristics of the antibody produced by hybridoma cell line CBA20110001; and

(c) antibody that binds to an antigen capable of binding the antibody produced by the hybridoma cell line CBA20110001.

According to another aspect, the present invention provides an isolated monoclonal antibody or a fragment thereof that may be capable of specifically binding to at least one linear epitope. In particular, the epitope may be a capsid protein and/or a non-structural protein derived from EV71. Even more in particular, the capsid protein may be VP2 and/or the non-structural protein may be 3CD and/or 3D.

In particular, the antibody according to an aspect of the present invention may comprise the immunological binding characteristics of monoclonal antibody 7C7, as produced by hybridoma 7C7, deposited with the CellBank Australia, 214 Hawkesbury Road, Westmead NSW 2145, Australia on 27 July 2011, in accordance with the provisions of the Budapest Treaty, and assigned Accession Number CBA20110003. The hybridoma provides a continuous source of 7C7 mAbs and binding proteins of the invention.

The antibody according to one aspect of the present invention may be selected from the group consisting of:

(a) antibody produced by hybridoma cell line CBA20110003;

(b) antibody having the binding characteristics of the antibody produced by hybridoma cell line CBA20110003; and

(c) antibody that binds to an antigen capable of binding the antibody produced by the hybridoma cell line CBA20110003.

In particular, the antibody according to an aspect of the present invention may have comprise the immunological binding characteristics of monoclonal antibody 4B12, as produced by hybridoma 4B12, deposited with the CellBank Australia, 214 Hawkesbury Road, Westmead NSW 2145, Australia on 27 July 2011, in accordance with the provisions of the Budapest Treaty, and assigned Accession Number CBA20110002. The hybridoma provides a continuous source of 4B12 mAbs and binding proteins of the invention.

The antibody according to one aspect of the present invention may be selected from the group consisting of:

(a) antibody produced by hybridoma cell line CBA20110002;

(b) antibody having the binding characteristics of the antibody produced by hybridoma cell line CBA20110002; and

(c) antibody that binds to an antigen capable of binding the antibody produced by the hybridoma cell line CBA20110002.

The antibody according to any aspect of the present invention may be capable of recognizing EV71 of any genotype. In particular, the antibody may be capable of recognizing EV71 of a genotype selected from the group consisting of A, B2, B4, B5, C1, C2, C4 and C5.

According to another aspect, the present invention provides methods for preparing monoclonal antibodies having binding characteristics of mAb 1C6 or mAb 7C7 by immunizing an animal with an EV71-B5 strain or preparing monoclonal antibodies having the binding characteristics of mAb 4B12 by immunizing an animal with a purified and isolated antigen, 3CD and/or 3D of EV71. Any such antigen can be used as an immunogen to generate antibodies with the desired immunological binding characteristics. Such antibodies include, but are not limited to, monoclonal antibodies, human antibodies,

humanized antibodies, chimeric antibodies, single chain antibodies, Fab fragments and proteins comprising the antigen binding sequence of mAb 1C6, 7C7 or 4B12.

The mAbs of the present invention can also be produced by any technique that provides for the production of antibody molecules by continuous cell lines in culture. Such methods include, but are not limited to, the hybridoma technique originally developed in 1975 by Kohler and Milstein (Nature 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., in Monoclonal Antibodies and Cancer Therapy Alan R. Liss, Inc., pp 77-96 (1985)). Human antibodies can be used and can be obtained by using human hybridomas (Cote et al., 1983, *Proc.* Nat. Acad. Sci. U.S.A., 80:2026-2030).

Techniques developed for the production of "chimeric antibodies" (Morrison, et al., 1984 incorporated herein by reference in their entirety) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. For example, the genes from a mouse antibody molecule such as mAbs 1C6, 7C7 or 4B12 can be spliced together with genes from a human antibody molecule of appropriate biological activity. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region. Chimeric antibodies are also those that contain a human Fc portion and a murine (or other non-human) Fv portion.

Techniques have been developed for the production of humanized antibodies (e.g., US 5,585,089 and/or US 5,225,539, which are incorporated herein by reference in their entirety). An immunoglobulin light or heavy chain variable region consists of a "framework" region interrupted by three hypervariable regions, referred to as complementarity determining regions (CDRs). Briefly, humanized antibodies are antibody molecules from non-human species having one or more CDRs from the non-human species and a framework region from a human immunoglobulin molecule. Both chimeric and humanized antibodies may be monoclonal. Such human or humanized chimeric antibodies may be preferred for use in *in vivo* diagnosis or therapy of human diseases or disorders.

Antibody fragments that contain the idiotype of the antibody molecule can be generated by known techniques. For example, such can be produced by pepsin digestion of the antibody molecule; the Fab fragments can be generated by reducing the disulfide bridges of the

$F(ab)_2$ fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent. Such antibody fragments can be generated from any of the polyclonal or monoclonal antibodies of the invention.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art. For example, these techniques may include but are not limited to radioimmunoassay, enzyme-linked immunosorbent assay (ELISA), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, in situ immunoassays (using colloidal gold, enzyme, radioisotope labels or the like), western blots, precipitation reactions, agglutination assays (gel agglutination assays, hemagglutination assays or the like), immunofluorescence assays, immunoelectrophoresis assays and the like. For example, the antibody binding may be detected by detecting a label on the primary antibody. In another example, the primary antibody may be detected by detecting binding of a secondary antibody or other reagent to the primary antibody. The secondary antibody may be labeled.

The antibody may be labelled with at least one radionuclide and/or fluorochrome in order to improve targeting of EV71 *in vivo* in at least a diagnostic and/or therapeutic capacity. For example, detecting EV71 by Positron Emission Tomography (PET) may be used. The antibody labeled with the radionuclide may enable better targeting of EV71 for detection and/or treatment. The antibody may be further labelled with at least one drug, anti-viral drug and/or toxin for treatment of EV71. In particular, the antibodies may be used to deliver drugs to the infected cells with a high degree of specificity to suppress viral infections. The drugs may include but are not limited to ribavirin and or other potent inhibitors of EV71 infection such as bovine or human lactoferrins and the like. The antibody of the present invention linked with at least one anti-viral drug, may increase the drug availability for EV71 infected cells which may enhance the efficiency of the drug and also reduce side effects usually caused by the anti-viral drugs. This may lead to a new treatment for EV71 possibly reducing other complications associated with EV71 infection.

The mAbs 1C6, 7C7 and/or 4B12 may be linked with at least one traceable agent and may be potentially used to quantify EV71 infected cells *in vitro* or *in vivo*. The traceable agent may be any biological or chemical component which is traceable. The traceable agent may include, but is not limited to environmental agents, blood markers, antigens, pesticides, drugs, chemicals, toxins, PCBS, PBBS, lead, neurotoxins, blood electrolytes, metabolites, analytes, NA+, K+, CA+, urea nitrogen, creatinine, biochemical blood markers and

components, ChE, AChE, BuChe, tumour markers, PSA, PAP, CA 125, CEA, AFP, HCG, CA 19-9, CA 15-3, CA 27-29, NSE, hydroxybutyrate, acetoacetate, anti-malarial drugs such as amodiaquine, artemether, artemisinin, artesunate, atovaquone, cinchonine, cinchonidine, chloroquine, doxycycline, halofanthne, mefloquine, primaquine, pyrimethamine, quinine, quinidine, and sulfadoxine; anti-biotic drugs such as ampicillin, azithromycin, doxycycline, erythromycin, penicillin, and tetracycline; anti-retroviral drugs such as abacavir, adefovir, didanosine, entecavir, indinavir, lamivudine, nevirapine, remofovir, ritonavir, saquinavir mesylate, telbivudine, tenofovir, zalcitabine, and zidovudine.

The antibodies according to any aspect of the present invention may be used in methods known in the art relating to the detection or localization of EV71. For example, these methods may include but are not limited to Western blotting, ELISA, radioimmunoassay, immunofluorescence assay, immunohistochemical assay, and the like. The method of detecting and/or quantifying according to any aspect of the present invention may be a non-invasive method and may also be useful for studying several immunological aspects of EV71.

In particular, the present invention provides a method of detecting and/or quantifying the presence and distribution of at least one EV71 infected cell in a subject, the method comprising:

a. contacting at least one antibody or fragment thereof according to any aspect of the present invention with at least one sample obtained from at least one subject; and
b. detecting and quantifying binding of the antibody to the EV71 infected cell.

The step of detecting may comprise contacting the sample with a binding protein that contains or is conjugated to a detectable element. In particular, the antibody according to any aspect of the present invention may be immobilized onto a solid surface. More in particular, the binding protein may contain a radioactive atom, may be conjugated to a fluorescent molecule, or may be conjugated to an enzyme.

The present invention also includes assay and test kits for the qualitative and/or quantitative determination of EV71. Such kits may contain at least the mAb or related binding protein according to any aspect of the present invention, means for detecting immunospecific binding of the mAb or related binding protein to EV71 in a biological sample, and instructions for use, depending upon the method selected, e.g., "competitive," "sandwich," "DASP" and the like. The kits may also contain positive and negative controls.

They may be configured to be used with automated analyzers or automated immunohistochemical slide staining instruments.

In particular, an assay kit of the invention may further comprise a second antibody or binding protein that may be labelled or may be provided for attachment to a solid support (or attached to a solid support). Such an antibody or binding protein may be, for example, one that binds to EV71. Such second antibodies or binding proteins may be polyclonal or monoclonal antibodies.

MAbs 1C6, 7C7 and 4B12 are highly efficacious in detecting EV71. They may be used separately and/or together for efficient and effective early detection of EV71. The antibodies according to any aspect of the present invention provide convenient, highly specific and sensitive means for detecting EV71. One such means is the ELISA format. Each of MAb 1C6, 7C7 and 4B12 can be used as a capture antibody, either alone or in combination. If used alone, the selected antibody can be used as the capture antibody and that same antibody conjugated with horseradish peroxidase (HRP) can be used as detecting antibody.

Other immunological methods to detect EV71 viruses include, for example, dot-blot and *in situ* hybridization.

According to another aspect, the present invention provides at least one method of treatment and/or protection of EV71 and/or at least one EV71-linked disease, the method comprising administering to a subject in need thereof at least one antibody or a fragment thereof according to any aspect of the present invention. The antibody of the present invention may be administered in combination with other similar antibodies targeting different EV71 epitopes. The viruses may thus be given no opportunity to adapt to this form of therapy.

According to another aspect, the present invention provides at least one antibody or a fragment thereof according to any aspect of the present invention for use in medicine.

According to yet another aspect, the present invention provides at least one use of the antibody or a fragment thereof according to any aspect of the present invention for the preparation of a medicament for treatment of EV71 and/or at least one EV71-linked disease. EV71-linked diseases include but are not limited to aseptic meningitis, encephalitis, cranial

nerve palsies, Guillan-Barre syndrome, poliomyelitis-like syndrome, Hand, Foot and Mouth disease and the like.

According to yet another aspect, the present invention provides at least one pharmaceutical composition comprising an antibody according to any aspect of the present invention and a pharmaceutically acceptable carrier.

The EV71 mAbs according to any aspect of the present invention have advantages over other current methodologies as diagnostic tools. For example, the mAbs are highly specific for EV71 which is still not well understood in the field of virology. Such highly specific mAbs represent a breakthrough in the field of EV71 diagnosis. The mAbs according to any aspect of the present invention may recognize all, or essentially all, of the EV71 genotypes. These mAbs also provide a safe and convenient diagnostic approach for the detection of EV71. The antibodies are useful for diagnosis and for the preparation of recombinant antibodies for treatment, and as such will be very useful tools in restraining a potential EV71 outbreak.

Also, despite the existence of typing methods, there remains a need for reagents which can be used in a diagnostic assay to rapidly and accurately distinguish EV71 from CA16, and which can be used to identify the strain of EV71 specifically. The mAbs according to any aspect of the present invention may be used to rapidly and accurately identify the strain of EV71 and/or differentiate between EV71 and CA16. Such reagents and methods will allow the clinician to improve the speed and accuracy of processing large numbers of clinical samples. Such reagents and methods will also aid the clinician in patient management, eliminate unnecessary tests, improve the speed and accuracy of diagnosis and prognosis, help control EV71 infection, and reduce the use of unnecessary antibiotics. The mAbs of according to any aspect of the present invention may be used for direct detection of EV71 viral particles from blister or oral swabs.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention.

A person skilled in the art will appreciate that the present invention may be practised without undue experimentation according to the method given herein. The methods, techniques and chemicals are as described in the references given or from protocols in standard biotechnology and molecular biology text books.

EXAMPLES

Standard molecular biology techniques known in the art and not specifically described were generally followed as described in Sambrook and Russel, Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (2001).

EXAMPLE 1

Preparation and purification of Mabs 1C6 and 7C7 from whole EV71 virus.

Hybridomas secreting specific Mabs were derived from BALB/c mice which had been immunized twice intramuscularly with purified EV71-B5 strain in 0.1 ml of PBS, emulsified with an equal volume of adjuvant (SEPPIC, France). An intraperitoneal booster of the same dose of virus was given three days before splenocytes were fused to the SP/2.0 myloma cells which were purchased from ATCC. Hybridomas identified to produce specific antibody, were cloned by limiting dilution and expanded in $75cm^2$ flasks. One week later, the hybridoma suspension was harvested and cell debris pelleted by centrifugation at 400 g for 10 min, followed by collection of the supernatant and storage at -20℃. Mab concentrations were determined spectrophotometrically (Nanodrop, DE, USA).

Antigen capture ELISA.

96-well, round-bottom microtiter plates (Nunc, Roskilde, Demark) were coated with $500ng^{-1}$ ug/well of capture antibody in 100µl of carbonate buffer (73 mM sodium bicarbonate and 30 mM sodium carbonate, pH 9.7) overnight at 4℃ or 37℃ for 2 h. The plates were washed twice with PBST, followed by two washes with PBS after each incubation with antibody or antigen. The antibody-coated plates were blocked with 100µl of blocking buffer (PBS containing 5% milk) for 1 h at room temperature and then incubated at 37℃ for 1 h with 100µl of virus-containing samples diluted in PBST. Virus binding was detected by incubation with 100µl of horseradish peroxidase-conjugated detection MAb (in-house labeling; Pierce) for 1 h at 37℃. Chromogen development was mediated by the addition of 100µl of freshly prepared substrate solution (o-phenylenediamine-dihydrochloride; Sigma). The reaction was stopped by adding 0.1 N sulfuric acid, and the optical density at 490 nm was recorded. The detection limit was determined as the optical density value that gave a signal-to-noise ratio of 3.

Characterization of Mab 1C6.

Mab 1C6 belongs to Isotype IgG2a, as identified with an isotyping Kit (GE). As shown in Figure 1A, 1C6 could detect EV71 viral protein expression in infected vero cells as shown

by immuno-fluoresence assay (IFA). AS shown in Figure 1B, no signal was detected in non-infected vero cells, confirming that it is an EV71-specific mAb. mAb 1C6 was tested to be negative in Western blot against EV71 whole virus lysate, indicating that it recognized a conformational epitope instead of a linear epitope. Attempts to assign the epitope to a specific EV71 capsid protein using recombinant baculovirus that express VP0, VP1, VP2, VP3, or VP4 in insect cells failed. However, 1C6 could detect virus-like particles of EV71 generated in insect cells co-infected with VP0, VP1, and VP3 proving that 1C6 is specific for the EV71 capsid as shown in Figure 1C. No virus neutralizing activity was observed with 1C6 in EV71 virus neutralization test.

Characterization of Mab 7C7.

The mAb 7C7 has been isotyped as IgG1 with kappa light chain by Isostrip (Santa Cruz). The antibody was able to detect EV71 in infected vero cells as seen by IFA. No labeling was observed in uninfected cells as shown in Figure 2A. Western blotting of sucrose-gradient purified EV71 virus particles from the C4 strain revealed that the Mab 7C7 is specific for VP2 and also recognizes the precursor protein VP0 as shown in Figure 2B. To test the sensitivity of Mab 7C7, a dot blot assay was performed. EV71 B5 virus was dotted at dilutions of $10^{6.6}$ to $10^{3.6}$ TCID50 units per dot and labeled with 7C7. The results of the dotblot are shown in Figure 2C. The detection limit was around 10^5 TCID50 units. No neutralization activity was observed in a virus neutralization assay.

EXAMPLE 2

Expression and purification of recombinant 3CD from EV71 subtype C4.

The non-structural protein 3CD from the EV71 C4 subgenogroup was expressed to use as an antigen to raise monoclonal antibodies. The 3CD DNA was amplified from a synthetic gene designed according to the published sequence of the EV71 C4 subgenogroup (Fuyang-08). The sequence is denoted with NCBI accession number EU703813.1 and was, cloned into pGex-4T-1 vector and transformed to *E. coli*. Total lysates of induced und un-induced cells were analyzed by SDS-PAGE where the expressed 3CD-GST fusion protein could be detected at 98.5 kDa. The fusion protein could be enriched by extraction with 8M urea and was shown to be GST positive by Western Blot. The enriched protein was then purified by gel cutting and the concentration of the recombinant proteins was determined by BCA assay.

Mab production against EV71-3CD

Protein injected	No. of mouse	1st injection	1st booster	2nd booster	3rd booster
EV71: 3CD	3	28/09/09	12/10/09	26/10/09	09/11/09

Polyclonal and monoclonal antibodies against purified EV71 3CD protein were developed. Mice were immunized with the purified antigen according to standard protocols. Briefly, 100µg of gel eluted 3CD-GST fusion protein was injected subcutaneously in a 1:1 emulsion with Seppic adjuvans. Three boosters were administered in 14 day intervals and blood was collected to monitor polyclonal antibody production as shown in Figure 3A. Spleen cells were then harvested and fused with hybridoma cells. Monoclonal antibody screening and subsequent subcloning resulted in the isolation of the 3CD specific monoclonal antibody 4B12.

Characterization of Mab 4B12

The Mab 4B12 was of the IgG1 isotype as determined by Isostrip (Santa Cruz). The specificity of the 4B12 mAb was assayed by Western blotting and IFA. 4B12 mAb specifically labeled EV71 infected Vero cells in the IFA as shown in Figure 3A. Furthermore, 4B12 recognized a linear epitope on 3CD as demonstrated by Western blotting in Figure 3B. In total RD cell lysates from EV71 infected cells the antibody detected 3CD, the precursor of 3C proteinase and 3D polymerase, at 73 kDa as well as the RNA-dependent RNA polymerase 3D at 52 kDa as shown in Figure 3C. No unspecific bands were observed in total cell lysates from uninfected RD cells. Both 3CD and 3D proteins could also be detected in preparations of sucrose gradient purified EV71 virus particles as confirmed in lane 3 of Figure 1B.

EXAMPLE 3

Epitope mapping 7C7

In order to characterize the 7C7 epitope, the VP2 protein was fragmented into GST-tagged continual overlapping peptides and cloned into pGex-4T-1 vector and expressed in *E. coli* BL21 cells and tested in Western blots using Mab 7C7. Gradual reduction of the fragment length led to the identification of a linear epitope of 7C7 mapped to amino acid positions 142-147 of the VP2 protein or amino acid positions 211-216 of the Ev71 open reading frame. The minimal epitope consists of the six amino acids EDSHPP (B5) (SEQ ID NO:1) or ENSHPP (A, CA16) (SEQ ID NO:2) as shown in Figure 4.

Epitope mapping of Mab 4B12

In order to characterize the 4B12 epitope, the 3CD protein was fragmented into GST-tagged continual overlapping peptides and cloned into pGex-4T-1 vector and expressed in *E. coli* BL21 cells. Gradual reduction of the fragment length led to the identification of an 8 amino acid long epitope on peptide "H" (DFEQALFS) (SEQ ID NO:3) which corresponds to amino acid positions 1784-1791 of the EV71 polyprotein as shown in Figure 5C. It was further confirmed that the 4B12 epitope indeed comprised these 8 amino acids and that the antibody does not recognize the shorter peptide EQALFS (SEQ ID NO:4) as shown in Figure 6B. The epitope lies at the N-terminal region of the 3D polymerase, close to the polymerase active site, and the 3C proteinase is not recognized by 4B12 Mab. The epitope DFEQALFS was blasted against EV71 sequences and two single amino acid variations were identified: Aspartic acid to Glutamic acid at position 53 of 3CD and Glutamine to Histidine at position 56. These mutations were introduced into our identified 4B12 epitope by site-directed mutagenesis and assessed whether 4B12 would still recognize the epitope. Western blotting of the mutated epitope with 4B12 showed in Figure 6C, that both common amino acid substitutions could be recognized, opening up the way to a universal EV71 detection kit.

To further confirm the specificity of the 4B12 Mab the Ev71 3D epitope was blasted against hepatitis A virus and poliovirus 1 of the enterovirus family and their corresponding epitopes were expressed as GST-fusion proteins in E. coli. Expression of the fusion proteins was confirmed by Western blotting with anti-GST antibody. The hepatitis A and polio 1 epitopes were not recognized by 4B12 mAb as shown in Figure 6.

EXAMPLE 4

Specificity of 7C7 and 1C6 to EV71 subgenogroups by IFA

To corroborate the findings of the mutational analysis of the epitope, we infected African green monkey kidney cells (vero) were infected with wild-type EV71 subgenogroups A (BrCr), B2 (7423-MS-87), B4 (NUH-HFM41), B5 (#363, Malaysia, unpublished sequence), B5 (NUH0083-SIN-08), C1 (Y90-3761), C2 (NUH0075-SIN-08), C4 (75-Yamagata-03), and C5 (3437-SIN-06). Both Mabs 7C7 and 1C6 reacted to all the tested EV71 subgenogroups in IFA as shown in Figure 7.

Specificity of 4B12 to EV71 subgenogroups by IFA

To corroborate the findings of the mutational analysis of the epitope, African green monkey kidney cells (vero) were infected with wild-type EV71 from different subgenogroups and an IFA conducted. The selected strains were A (BrCr), B2 (7423-MS-87), B4 (NUH-HFM41),

B5 (#363, Malaysia, unpublished sequence), B5 (NUH0083-SIN-08), C1 (Y90-3761), C2 (NUH0075-SIN-08), C4 (75-Yamagata-03), and C5 (3437-SIN-06) previously grown in rhabdomysarcoma (RD) cells. All tested strains were positively identified by Mab 4B12 as shown in Figure 8. 4B12 can thus be used as a universal detection antibody for EV71.

Specificity of 4B12 to EV71 subgenogroups by Western blotting

To confirm the presence of 3D protein in the virus particles, virus particles from the different EV71 subgenogroups were purified. RD cells were infected with virus and the supernatant was collected after 48 h when more than 90% of the cells showed cytopathic effect. Cell debris were removed by a clarification spin and microfiltration through a 0.2µm cut-off filter, followed by concentration by ultraspin at 100'000g for 3 h. Virus particles were purified by centrifugation in a 20% to 60% sucrose gradient for 3h at 27'000g. Several bands were detected and the virus band at the 40 to 60% sucrose meniscus was collected. Western blotting using 4B12 as primary antibody was performed and both 3CD as well as 3D protein bands were detected in the viral fractions as shown in Figure 9A.

Specificity of 4B12 to EV71 subgenogroups by dot blotting

The next step was to develop a dot blot assay for the detection of EV71. The purified virus strains were diluted in a 2% SDS containing buffer and dotted on a nitrocellulose membrane. The blot was then incubated with 4B12 as primary antibody and HRP-coupled secondary antibody. The signal was developed using conventional ECL chemo-luminescence reagent (Amersham). Again, all tested strains were positively identified by our Mab, making dot blots a viable strategy to detect EV71 antigens as shown in Figure 9B.

EXAMPLE 5

Sensitivity of 4B12 against recombinant 3D protein.

The sensitivity of the dot blot system was assessed using both ECL and infrared (IR) detection methods which have been shown to detect such low concentrations of antigens at ≥20pg and ≥0.6pg protein respectively (Qdot, Nature Methods; IR detection, LI-COR). Recombinant 3D protein was expressed in E. coli BL21 cells and purified by His-beads and protein concentration was measured by Bradford assay. 3D protein was diluted from 1ng to 10pg, dotted and labeled by 4B12. Using either HRP-coupled secondary antibody and ECL detection or infrared (IR) labeled secondary antibody followed by scanning on a LI-COR Odyssey reader as little as 10pg of protein could be detected as shown in Figure 10A.

Sensitivity of 4B12 against whole EV71 virus.

The EV71 wild-type strains A, B2, B5#557, C1, C2, C5, and B5#363 were serially diluted from 10^7 to 10^1 TCID50 per dot labeled with Mab 4B12 followed by IR-coupled secondary antibody and detected in the Odyssey reader. The detection limit for all tested strains was 10^4 TCID50 units of EV71 virus as shown in Figure 10B.

EXAMPLE 6

Development of an antigen-capture ELISA to detect EV71 with specific Mabs.

Two polyclonal anti-sera were screened as capture antibodies for EV71 AC-ELISA. Both guinea pig and rabbit anti-EV71 serum showed high readings, but the background of the guinea pig serum was higher. Therefore, rabbit anti-EV71 polyclonal serum was used as the capture antibody to evaluate the Mabs as detection antibodies. 4B12, 7C7, and 1C6 were screened as detection antibodies for EV71 AC-ELISA. Mab 4B12, targeting the 3D polymerase, had a low activity. However, the Mabs 7C7 and 1C6, targeting viral capsid proteins, were able to detect captured EV71 virus. Based on this result, the Mabs 7C7 and 1C6 were further tested as capture antibodies with HRP-labeled Mab 1C6 as the detection antibody. Both Mabs tested were able to capture EV71 virus. To test the specificity of the AC-ELISA Mab 1C6 was used both as the capture and the detection antibody. Different EV71 strains were added to the test. 1C6 based AC-ELISA could successfully detect all virus strains without any cross-reaction with the culture medium or other non-related viruses, such as the porcine virus PCV.

EXAMPLE 7

Development of epitope-blocking ELISA for universal detection of serum antibodies to human EV71.

An Mab 1C6 based epitope-blocking ELISA was developed to detect specific antibodies to human EV71 viruses in human or animal sera. The sensitivity and specificity of the epitope-blocking (EB-) ELISA for EV71 was evaluated and compared to microneutralization. Further, serum samples from human individuals who were potentially infected with EV71 viruses were tested in both the blocking ELISA and microneutralization. The cut-off value for the EB-ELISA was determined using normal mouse and guinea pig sera lacking antibodies to EV71. Specific blocking activities with a 95% confidence interval were achieved by $\geqslant$30% blocking. The specificity of the EB-ELISA was tested on sera from guinea pigs immunized with different EV71 and coxsackie virus strains. Results indicated that antibodies to EV71

were readily detected in EV71 immunized animals (≥80% blocking) whereas specimens with antibodies to other enteroviruses yielded negative results (≤12% blocking). The sensitivity of the EB-ELISA was significantly higher in comparison to microneutralization ($p<0.005$). Finally, human serum samples that were virus-neutralization positive (neutralization titer >8) or negative (<8) were tested in the EB-ELISA. Human cord blood served as negative controls. The 100 samples with positive neutralization titer presented significant blocking percentages (>70%, 20 times dilution) in the 1C6 EB-ELISA. Additionally, with the cut-off value of 30% blocking, 249 out of 300 VN-negative samples were found to be positive by the 1C6 blocking ELISA, while all cord blood samples were negative (9-18% blocking). These findings further confirm that the blocking ELISA with 1C6 has better sensitivity than virus neutralization. The epitope-blocking ELISA based on a unique Mab 1C6 provided highly sensitive and 100% specific detection of antibodies to human EV71 viruses in human sera (Figures 11 and 12).

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CLAIMS

1. An isolated monoclonal antibody or a fragment thereof that is capable of specifically binding to at least one conformational epitope of Enterovirus 71 (EV71).

2. The antibody according to claim 1, wherein the conformational epitope comprises at least one capsid protein.

3. The antibody according to either claim 1 or 2, wherein the conformational epitope is an intact virus capsid.

4. The antibody according to either claim 2 or 3, wherein the capsid protein is VP1, VP2 VP3, VP4 and/or VP0 precursor.

5. The antibody according to any one of the preceding claims has substantially the immunological binding characteristics of monoclonal antibody 1C6.

6. The antibody according to any one of the preceding claims, wherein the antibody is selected from the group consisting of:
   (a) antibody produced by hybridoma cell line CBA20110001;
   (b) antibody having the binding characteristics of the antibody produced by hybridoma cell line CBA20110001; and
   (c) antibody that binds to an antigen capable of binding the antibody produced by the hybridoma cell line CBA20110001.

7. An isolated monoclonal antibody or a fragment thereof, wherein the antibody is capable of specifically binding to at least one epitope capsid protein VP2 of Enterovirus 71 (EV71).

8. The antibody according to claim 7, wherein the antibody has substantially the immunological binding characteristics of monoclonal antibody 7C7.

9. The antibody according to either claim 7 or 8, wherein the antibody is selected from the group consisting of:
   (a) antibody produced by hybridoma cell line CBA20110003;

(b) antibody having the binding characteristics of the antibody produced by hybridoma cell line CBA20110003; and

(c) antibody that binds to an antigen capable of binding the antibody produced by the hybridoma cell line CBA20110003.

10. An isolated monoclonal antibody or a fragment thereof that is capable of specifically binding to at least one epitope non-structural protein of Enterovirus 71 (EV71).

11. The antibody according to claim 10, wherein the non-structural protein is 3CD.

12. The antibody according to either claim 10 or 11, wherein the antibody has substantially the immunological binding characteristics of monoclonal antibody 4B12.

13. The antibody according to any one of claims 10 to 12, wherein the antibody is selected from the group consisting of:

(a) antibody produced by hybridoma cell line CBA20110002;

(b) antibody having the binding characteristics of the antibody produced by hybridoma cell line CBA20110002; and

(c) antibody that binds to an antigen capable of binding the antibody produced by the hybridoma cell line CBA20110002.

14. The antibody according to any one of the preceding claims, wherein the antibody is at least one human, humanized or chimeric antibody.

15. The antibody according to any one of the preceding claims, wherein EV71 is of genotype selected from the group consisting of A, B2, B4, B5, C1, C2, C4 and C5.

16. The antibody according to any one of the preceding claims, wherein the antibody is labelled with at least one radionuclide and/or fluorochrome.

17. The antibody according to any one of the preceding claims, wherein the antibody is linked with at least one drug, anti-viral drug, and/or toxin.

18. An isolated hybridoma cell line deposited with American Type Culture Collection (ATCC) with accession number CBA20110001, CBA20110003 and/or CBA20110002.

19. A method of detecting and/or quantifying the presence and distribution of at least one EV71 infected cell in a subject, the method comprising:
    a. contacting at least one antibody or fragment thereof according to any one of claims 1 to 17 with at least one sample obtained from at least one subject; and
    b. detecting and quantifying binding of the antibody to the EV71 infected cell.

20. The method according to claim 19, wherein the step of detecting comprises contacting the sample with a binding protein that contains or is conjugated to a detectable element.

21. The method according to either claim 19 or 20 wherein the antibody is immobilized onto a solid surface.

22. The method according to either claim 20 or 21, wherein the binding protein comprises a radioactive atom, is conjugated to a fluorescent molecule, or is conjugated to an enzyme.

23. A kit for diagnosing EV71 and/or at least one EV71-linked disease, the kit comprising at least one antibody or a fragment thereof according to any one of claims 1 to 17.

24. The kit according to claim 23, wherein the EV71-linked disease is at least one disease selected from the group consisting of aseptic meningitis, encephalitis, cranial nerve palsies, Guillan-Barre syndrome, poliomyelitis-like syndrome, and Hand, Foot and Mouth disease.

25. A method of treatment of EV71 and/or at least one EV71-linked disease, the method comprising administering to a subject in need thereof at least one antibody or a fragment thereof according to any one of claims 1 to 17.

26. The method according to claim 25, wherein the EV71-linked disease is at least one disease selected from the group consisting of aseptic meningitis, encephalitis, cranial nerve palsies, Guillan-Barre syndrome, poliomyelitis-like syndrome, and Hand, Foot and Mouth disease.

27. An isolated antibody according to any one of claims 1-17 for use in medicine.

28. An isolated antibody according to any one of claims 1-17 for use in treating EV71 and/or at least one EV-71 linked disease.

29. The antibody according to claim 28, wherein the EV71-linked disease is at least one disease selected from the group consisting of aseptic meningitis, encephalitis, cranial nerve palsies, Guillan-Barre syndrome, poliomyelitis-like syndrome, and Hand, Foot and Mouth disease.

30. Use of an isolated antibody according to any one of claims 1-17 for the preparation of a medicament for treating EV71 and/or at least one EV-71 linked disease.

31. The use according to claim 30, wherein the EV71-linked disease is at least one disease selected from the group consisting of aseptic meningitis, encephalitis, cranial nerve palsies, Guillan-Barre syndrome, poliomyelitis-like syndrome, and Hand, Foot and Mouth disease.

32. A method of protecting a subject from EV71 infection comprising administering to the subject at least one antibody or fragment thereof according to any one of claims 1-17 in an amount effective to protect the subject from EV71 infection.

33. A pharmaceutical composition comprising the antibody of any one of the preceding claims 1-17 and a pharmaceutically acceptable carrier.