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(54) Title: CHICKEN VIRUS VACCINE AND DIAGNOSTIC

(57) Abstract: A novel astrovirus designated chicken astrovirus type 3 has been isolated and characterised. Nucleotide sequences and polypeptide sequences of this astrovirus are provided with uses of the same and the isolated astrovirus in assay kits and vaccines.

Chicken virus vaccine and diagnostic

FIELD OF THE INVENTION

The present invention relates to vaccines, assay kits and detection methods for a novel astrovirus, in particular chicken astrovirus type 3 (CAstV-3). Infections of broiler chickens with this chicken astrovirus type 3 are associated with enteritis and growth depression and possible adverse effects on chick embryo development.

BACKGROUND

Growth depression problems in young chickens, known in the poultry production industry by various terms such as “stunting”, “runting-stunting” or “uneven growth” syndrome, result in considerable economic costs for affected farms. Such growth depression has been associated with infections with a variety of viruses including rotaviruses and as yet uncharacterised, small round viruses, known as enterovirus like viruses (ELV). Since the clinical problems caused by specific viruses are ill-defined due to the lack of specific diagnostic tests, it is difficult to accurately estimate the demand for a vaccine to protect against virus-induced growth depression problems.

Virus infections of chickens can be horizontally transmitted from virus that may be contaminating the chicken house; for example, with enteric infections, faecal-oral spread is likely to be common. It is also recognised that vertical transmission of enteric infections via the embryo from virus-infected parent chickens may also occur.

Previous studies (Veterinary Laboratories Agency (VLA), Weybridge), have shown that an enterovirus like virus (ELV), designated FP3, could be detected in the meconium (gut contents) of dead-in-shell chicks, suggesting that this ELV was infecting the embryo and was vertically transmitted from infected parents [Spackman et al. 1984].

Enterovirus-like viruses include picornaviruses, astroviruses, caliciviruses and the like and small non-enveloped spherical viruses which replicate in the cytoplasm. They have an RNA genome and are stable at pH3. Several ELVs which are antigenically distinct from each other have been suggested to cause growth depression in avians.

SUMMARY OF INVENTION

Following extensive virological investigation of sick birds from UK flocks in 2004-5, the present inventors have successfully isolated an infectious agent from weak chicks. Characterisation of this agent suggests that this viral agent causes growth depression in young chickens and possible adverse effects on chick embryos.

According to a first aspect of the present invention there is provided an isolated novel astrovirus strain designated, CAstV-3. A sample of this strain has been deposited under Accession number CNCM I-3541 at CNCM, Institut Pasteur on 15 December 2005.

Astroviruses are small spherical viruses which typically have 5 or 6 pointed star morphology and a positive sense single strand RNA genome of around 7kb.

A number of astroviruses have been previously characterized in duck, turkeys and chickens, however, the astrovirus characterized by the present inventors is antigenically distinct as demonstrated by indirect immunofluorescence assays.

A first aspect of the present invention relates to an isolated astrovirus strain, wherein said strain is that deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005.

A substantial region (~3.3 kb) of the genome of CAstV-3 has been sequenced. This region comprises part of the astrovirus ORF1 b, the astrovirus RNA dependent RNA polymerase, a small intergenic sequence of 24 nucleotides, and astrovirus ORF 2, which encodes the capsid protein region, and the 3' untransiated region.

According to a second aspect, the present invention provides an isolated nucleotide sequence which has at least 85%, preferably at least 90%, preferably at least 93%, more preferably at least 95%, more preferably at least 98%, even more preferably at least 99%, and most preferably 100% sequence identity to

(a) a nucleotide sequence as set out in any one of SEQ ID NOs 1 to 4,

(b) a nucleotide sequence that hybridises under stringent conditions to a portion of SEQ ID NO 4 wherein the portion of SEQ ID NO 4 encodes an amino acid sequence of at least one antigenic site provided by the capsid protein of the strain deposited under

Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005, or

(c) a fragment of a nucleic acid comprising a nucleotide sequence SEQ ID NO 4 wherein said fragment encoding encodes an amino acid sequence of at least one antigenic site provided by the capsid protein of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005.

Suitably the immunogenic response is against at least one antigenic site provided by a capsid protein of chicken astrovirus type 3(CAstV-3).

The invention further provides a gene construct including at least one nucleotide sequence of the second aspect of the invention and a control sequence, for example a promoter.

There is further provided a vector including the isolated nucleotide sequence according to the second aspect of the invention and a promoter which is operably linked to said nucleotide sequence. Suitable vectors include viruses (eg. Vaccinia virus, adenovirus, baculovirus etc), yeast vectors, phage, chromosomes, artificial chromosomes, plasmids or cosmid DNA.

According to a third aspect of the invention there is provided a method of producing a polypeptide encoded by the nucleotide sequence of the invention, or fragment thereof, including the steps of:

(a) contacting at least one of a bacterial cell, an insect cell via a baculovirus, a yeast cell, and a plant cell with a vector as described herein, and

(b) cultivating at least one of said bacterial cell, an insect cell, a yeast cell, and a plant cell under conditions suitable for the production of polypeptide or fragment thereof.

Suitably the polypeptide is encoded by the nucleotide sequence of SEQ ID NO 4.

The invention further provides a polypeptide produced substantially from the above method. As will be understood by those of skill in the art such a polypeptide may be isolated or substantially purified from the mixture in which it is expressed.

According to a fourth aspect of the present invention there is provided a polypeptide sequence encoded by any nucleotide sequence of the second aspect of the invention.

Preferably there is provided a polypeptide sequence encoded by SEQ ID No. 4.

Suitably a polypeptide of the invention may be antigenic in that it exhibits at least one antigenic site provided by a capsid protein of CastV-3.

Antigenic polypeptides derived from CAstV-3, for example a capsid protein (SEQ ID NO 5) or fragments thereof, are within the scope of the present invention.

Suitably there is provided a polypeptide which has at least at least 85%, preferably at least 90%, preferably at least 93%, more preferably at least 95%, more preferably at least 98%, even more preferably at least 99%, and

most preferably 100% sequence identity to a polypeptide with an amino acid sequence as set forth in SEQ ID NO: 5.

Polyclonal and monoclonal antibodies which specifically bind to the polypeptide of the present invention are also within the scope of the invention as are the use of said antibodies. As will be understood by those of skill in the art such antibodies may be isolated or substantially purified from the mixture in which they are provided.

According to a fifth aspect of the invention there is provided

(i) the use of an isolated novel astrovirus strain designated, CAstV-3 of the first aspect of the invention,

(ii) the use of a nucleic acid comprising a nucleotide sequence of the second aspect of the invention, or

(iii) the use of a polypeptide of the fourth aspect of the invention in the preparation of a composition which is capable of mediating an immune response in an avian.

According to a sixth aspect of the invention there is provided a composition prepared according to the fifth aspect of the invention.

Suitably said composition includes at least part of the isolated novel astrovirus of the first aspect of the invention, at least one nucleotide sequence of the second aspect of the invention, or at least one polypeptide of the fourth aspect of the invention.

Vaccination, the induction of adaptive immunity, of broiler chickens is advantageous as vaccination can be used to control clinical disease. Chicks produced from vaccinated parents are likely to be less susceptible

to adverse clinical effects caused by the infectious agent vaccinated against during the early growing period of the broiler chicken.

Accordingly a seventh aspect of the present invention provides a vaccine for immunisation against growth depression in an avian wherein said vaccine comprises a composition according to the sixth aspect for inducing an immune response in avians.

The present invention also provides a method of vaccinating avians against CAstV-3 by providing an immunologically effective amount of said vaccine.

One or more of Isolated CAstV-3, nucleotides of the invention, polypeptides, and antibodies provided by the invention may be used for the preparation of a diagnostic assay of the invention. Such assays can be used to detect avian astrovirus in samples, for example tissues, faeces and serum from avians suspected of being infected with the virus.

According to an eighth aspect of the present invention there is provided a diagnostic assay for the detection of chicken astrovirus type 3 in samples from avians suspected of being infected with the virus comprising the steps:

(i) contacting avian physiological material with a probe wherein said probe is selected from:

(a) the nucleic acid comprising a nucleotide sequence as set out in any one of SEQ ID NOs 1 to 4,

(b) a nucleic acid comprising a nucleotide sequence that hybridises to a portion of SEQ ID NO 4 and encodes an amino acid sequence of at least one antigenic site provided by the capsid protein of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005,

(c) a nucleic acid comprising a nucleotide sequence which has at least 85%, preferably at least 90%, preferably at least 93%, more preferably at least 95%, more preferably at least 98%, even more preferably at least 99% and most preferably 100% sequence identity to a nucleotide sequence as set out in SEQ ID NO 4 wherein said nucleotide sequence encodes an amino acid sequence or a fragment thereof wherein said fragment is an amino acid sequence of at least one antigenic site provided by a capsid protein of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005,

(d) a fragment of the nucleic acid comprising a nucleotide sequence of SEQ ID NO 4 wherein said fragment encodies an amino acid sequence of at least one antigenic site provided by the capsid protein of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005CAstV,

e) the polypeptide encoded by the nucleic acid of (d), or

(f) an antibody with binding specificity to the polypeptide of (e), and

(ii) detecting a successful binding event between the probe and at least one component of the sample.

In a particular embodiment a probe may be a suitable primer set for use in RT-PCR to amplify a selected target sequence SEQ ID NO 4 of chicken astrovirus type 3.

Suitably the binding event is detected using a marker associated with the probe, for example a fluorescent marker, a radioisotope marker or the like.

In one embodiment the assay comprises the steps:

(i) contacting avian physiological material, for example blood, containing antibodies which specifically bind to at least part of chicken astrovirus type 3, with at least part of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005, such that antibody present in the sample with binding specificity to at least part of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005 binds the at least part of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005, and

(ii) detecting the presence of said antibody bound to the at least part of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005.

In a further embodiment a diagnostic assay method may comprise the steps;

i) contacting the sample of avian physiological material with an antibody with binding specificity to an amino acid sequence of at least one antigenic site provided by the capsid protein of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005,

ii) incubating the avian physiological material with said antibody such that chicken astrovirus present in the sample binds said antibody, and

iii) detecting the presence of said chicken astrovirus present in the sample.

The captured virus may be detected through the use of antibody with specificity to the virus in conjugated form as would be known to those of skill in the art.

According to a further aspect of the present invention there is provided a diagnostic kit for use in the diagnosis of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005, comprising a substrate for supporting a test sample; and a probe wherein said probe is at least one of

(a) a nucleic acid comprising the sequence as set out in any one of SEQ ID NOs 1 to 4,

(b) a nucleic acid comprising a sequence that hybridises under stringent conditions to a portion of SEQ ID NO 4, wherein the portion of SEQ ID NO 4 encodes an amino acid sequence of at least one antigenic site provided by the capsid protein of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005,

(c) a nucleic acid comprising the a nucleotide sequence which has at least 95% sequence identity to a nucleotide sequence as set out in SEQ ID NO 4 wherein said nucleotide sequence encodes an amino acid sequence or a fragment thereof wherein said fragment is an amino acid sequence of at least one antigenic site provided by a capsid protein of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005,

(d) a fragment of the nucleic acid comprising a nucleotide sequence of SEQ ID NO 4 wherein said fragment encodes an amino acid sequence of at least one antigenic site provided by the capsid protein of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005,

(e) a polypeptide encoded by nucleic acid of (d), or

(f) an antibody with binding specificity to the polypeptide of (e).

DETAILED DESCRIPTION

Virus

The invention relates to isolated chicken astrovirus type 3 and characterisation of the nucleotide sequences and polypeptide of said virus.

Pairwise amino acid identity comparison by the inventors of CAstV-3 with other avian astroviruses suggests that the closest homology it shares with known astroviruses is with CAstV-2. The pairwise amino acid identity between CAstV-3 and CAstV-2 is 84.6%.

In relation to nucleotide sequences provided by the invention, sequence identity is determined using a suitable mathematical algorithm. Computer implementations of such mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, California); the ALIGN program (Version 2.0) and GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Version8 (available from Genetics Computer Group (GCG), 575 Science Drive, Madison, Wisconsin, USA).

Suitably alignments using these programs may be performed using the default parameters.
As used herein, "sequence identity" or "identity" in the context of two nucleotide or polypeptide sequences makes reference to a specified percentage of residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison

window, as measured by sequence comparison algorithms or by visual inspection.

Suitably, a specified comparison window is selected from a sequence encoding or representing at least 50, at least 100, at least 150, at least 200, at least 250, or most preferably all of the amino acids of a specified polypeptide being aligned.

When percentage of sequence identity is used in reference to proteins it will be understood by those of skill in the art that residue positions which are not identical often differ by conservative amino acid substitutions, i.e. wherein amino acids are substituted with amino acids which have similar chemical properties to those amino acids which are replaced. The percent sequence identity may be adjusted upwards to correct for the conservative nature of a substitution.

Hybridisation refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent conditions when that sequence is present in a complex mixture (e.g., total cellular) DNA or RNA.

Stringent hybridisation occurs when a nucleic acid binds the target nucleic acid with minimal background. Typically, to achieve stringent hybridisation, temperatures of around 1 C to about 20 C, more preferably 5 C to about 20 C below the Tm (melting temperature at which half the molecules dissociate from their partner) are used. However, it is further defined by ionic strength and pH of the solution.

An example of highly stringent wash conditions is 0.15 M NaCl at 72 C for about 15 minutes. An example of a stringent wash condition is a 0.2X SSC

wash at 65 C for 15 minutes (see, Sambrook and Russell, infra, for a description of SSC buffer). Often, a high stringency wash is preceded by a low stringency wash to remove background probe signal. An example of a medium stringency wash for a duplex of, for example, more than 100 nucleotides, is 1X SSC at 45 C for 15 minutes. An example of a low stringency wash for a duplex of for example more than 100 nucleotides, is 4-6X SSC at 40 C for 15 minutes. For short probes (for example about 10 to 50 nucleotides), stringent conditions typically involve salt concentrations of less than about 1.5 M, more preferably about 0.01 to 1.0 M, Na ion concentration (or other salts) at pH 7.0 to 8.3, and the temperature is typically at least about 30 C and at least about 60 C for long probes (for example, > 50 nucleotides).

Suitably nucleotide sequences of the present invention encode antigenic polypeptides of chicken astrovirus type 3.

Suitably the present invention provides an isolated nucleotide sequence selected from

(a) a nucleotide sequence as set out in any one of SEQ ID NOs 1 to 4,

(b) a nucleotide sequence that is capable of hybridising to any one of SEQ ID NOs 1 to 4 under stringent conditions,

(c) or a fragment of (a) or (b).

In specific embodiments nucleotide sequences of the invention consist of any one of nucleotide sequences selected from SEQ ID NO 1, 2, 3 and / or 4.

Suitably, the nucleotide sequences of the present invention may be expressed to provide the encoded polypeptides.

Preferably polypeptides of the present invention include polypeptides encoded by a nucleotide sequence selected from

(a) a nucleotide sequence as set out in any one of SEQ ID NOs 1 to 4,

(b) a nucleotide sequence that is capable of hybridising to any one of SEQ ID NOs 1 to 4 under stringent conditions,

(c) or a fragment of (a) or (b).

Polypeptide fragments of the present invention may be a variant of any one of the polypeptides encoded by any one of SEQ ID NOs: 1 to 4 which include one or more truncations, substitutions, deletions or insertions wherein said polypeptides provide an antigenic response similar to that of any one of the polypeptides encoded by SEQ ID NOs: 1 to 4. Advantageously these variations may be made to the protein to enhance the efficacy of the protein in stimulating immune response or to make it safer for use in an avian.

The length of such a fragment which provides an immune response comprises at least 6, up to 15, preferably 25, and more preferably 50 contiguous amino acids encoded by any one of SEQ ID No 1 to SEQ ID No 4. An antigenic fragment may be generated using for example C terminal deletion of any one of the polynucleotide sequences of SEQ ID No 1 to SEQ ID No 4 and said C. terminal deletion constructs may then be inserted into suitable prokaryotic or eukaryotic expression plasmid. The antigenic activity of the expression products derived from the polynucleotide fragments may then be tested by assessing reactivity with antisera from naturally and/ or experimentally infected chickens using immunoblotting methods.

Alternatively a series of overlapping synthetic peptides specified by the sequence of the CAstV-3 proteins, preferably the capsid protein could be generated. These peptides may then be reacted with antisera from naturally or experimentally infected chickens using an ELISA method to determine which peptides are antigenic. Additionally, synthetic peptides may be used to immunise mice, rabbits, chickens and the antisera produced can be assessed for reactivity with CAstV-3 using indirect immunofluorescence assays. In this way immunogenic peptides may be identified and virus-specific antisera can be elicited. These two latter approaches described are particularly advantageous for small peptides that contain linear, continuous epitopes.

Suitably the invention provides a polypeptide of amino acid sequence wherein between 1 to 5, 1to 10, 1 to 15, or 1 to 20 amino acid residues are deleted, substituted, and / or added to the amino acid sequence encoded by the nucleotide sequences of any one of SEQ ID NOs:1 to 4 and wherein said polypeptides stimulate an immune response (i.e. have antigenic activity).

In specific embodiments nucleotide sequences of the invention encode a polypeptide of the following (a) or (b):

(a) a polypeptide comprising of an amino acid sequence of a SEQ ID NO: 5

(b) a protein comprising of an amino acid sequence when one or several amino acid residues are deleted, substituted, and / or added to the amino acid sequence of SEQ ID NO:5 wherein said protein has a similar antigenic response as SEQ ID NO:5.

In particular embodiments nucleotide sequences of the invention encode a polypeptide of the following (a) or (b):

(a) a polypeptide consisting of an amino acid sequence of a SEQ ID NO: 5

(b) a protein consisting of an amino acid sequence when one or several amino acid residues are deleted, substituted, and / or added to the amino acid sequence of SEQ ID NO 5 wherein said protein has a similar antigenic response as SEQ ID NO:5.

Nucleotide sequences may be codon-optimised or otherwise modified to increase the efficiency of expression of the polypeptides.

Suitably the invention provides polypeptides consisting of an amino acid sequence SEQ ID NO 5.

Polyclonal antibody sera may be produced through the use of at least part of CAstV-3 to raise an immune response. The immunising preparation could be purified virus from cell culture, virus-specified synthetic peptides, polypeptides produced by expression vectors etc; DNA expression plasmids. After repeated challenge, portions of the blood serum can be removed and antigenically purified. The semi-purified sera may additionally be purified using chromatography, for example, a saccharide gel column and suitable buffer to separate the components of the sera according to molecular weight.

Suitably the invention provides polyclonal antibodies which have binding specificity to at least one polypeptide of the invention.

Suitably the invention provides polyclonal antibodies which have binding specificity to

(a) at least one polypeptide sequence encoded by a nucleotide sequence of any one of SEQ ID Nos 1 to 4,

(b) at least one polypeptide sequence comprising of an amino acid sequence when one or several amino acid residues are deleted, substituted, and / or added to the amino acid sequence encoded by any one of SEQ ID Nos 1 to 4 wherein said polypeptide has a similar antigenic response as a polypeptide encoded by any of nucleotide sequences SEQ ID NO: 1 to 4, or

(c) a fragment of the polypeptide of (a), (b) which has a similar antigenic response as a polypeptide encoded by any of nucleotide sequences SEQ ID NO: 1 to 4.

Suitably the invention provides polyclonal antibodies which have binding specificity to

(a) at least one polypeptide sequence encoded by a nucleotide sequence of any one of SEQ ID Nos 1 and / or 2 and / or 4,

(b) at least one polypeptide comprising of an amino acid sequence when one or several amino acid residues are deleted, substituted, and / or added to the amino acid sequence encoded by any one of SEQ ID Nos 1 and / or 2 and / or 4 wherein said polypeptide has a similar antigenic response as a polypeptide encoded by any of nucleotide sequences SEQ ID NO: 1 and / or 2 and / or 4, or

(c)a fragment of the polypeptide of (a), (b) which has a similar antigenic response as a polypeptide encoded by any of nucleotide sequences SEQ ID NO: 1, 2 or 4.

Suitably such a polyclonal antibody does not have binding specificity to polypeptides encoded by SEQ ID NO: 3

Preferably the invention provides polyclonal antibodies which have binding specificity to (a), (b), or (c) wherein

(a) is at least one polypeptide with amino acid sequence SEQ ID NO 5,

(b) is at least one polypeptide comprising of an amino acid sequence when one or several amino acid residues are deleted, substituted, and / or added to the amino acid sequence of SEQ ID NO 5 wherein said polypeptide has a similar antigenic response as SEQ ID NO 5

(c) is a fragment of the polypeptide of (a), (b) or (c) which has a similar antigenic response as SEQ ID NO 5.

Monoclonal antibodies may be produced by the hybridoma technique, for example, immunisation of a mouse may be used to generate mouse monoclonal antibodies

Suitably the invention provides a monoclonal antibody which has binding specificity to at least one polypeptide of the invention.

Suitably the invention provides a monoclonal antibody which has binding specificity to

(a) at least one polypeptide sequence encoded by a nucleotide sequence of any one of SEQ ID Nos 1 to 4,

(b) at least one polypeptide comprising of an amino acid sequence when one or several amino acid residues are deleted, substituted, and / or added to the amino acid sequence encoded by any one of SEQ ID Nos 1 to 4 wherein said protein has a similar antigenic response as a polypeptide encoded by any of nucleotide sequences SEQ ID NO: 1 to 4, or

(c) a fragment of the polypeptide of (a), (b) which has a similar antigenic response as a polypeptide encoded by any of nucleotide sequences SEQ ID NO: 1 to 4.

Suitably the invention provides a monoclonal antibody which has binding specificity to

(a) at least one polypeptide sequence encoded by a nucleotide sequence of any one of SEQ ID Nos 1 and / or 2 and / or 4,

(b) at least one polypeptide comprising of an amino acid sequence when one or several amino acid residues are deleted, substituted, and / or added to the amino acid sequence encoded by any one of SEQ ID Nos 1 and / or 2 and / or 4 wherein said polypeptide has a similar antigenic response as a polypeptide encoded by any of nucleotide sequences SEQ ID NO: 1 and / or 2 and / or 4, or

(c) a fragment of the polypeptide of (a), (b) which has a similar antigenic response as a polypeptide encoded by any of nucleotide sequences SEQ ID NO: 1, 2 or 4.

Suitably such a monoclonal antibody does not have binding specificity to polypeptides encoded by SEQ ID NO: 3

Preferably the invention provides a monoclonal antibody which has binding specificity to (a), (b), or (c) wherein

(a) is a polypeptide with amino acid sequence SEQ ID NO 5,

(b) is at least one polypeptide comprising of an amino acid sequence when one or several amino acid residues are deleted, substituted, and / or added to the amino acid sequence of SEQ ID NO 5 wherein said protein has a similar antigenic response as SEQ ID NO 5, or

(c) is a fragment of the polypeptide of (a), (b) or (c) has a similar antigenic response as SEQ ID NO 5.

Immunisation to provide monoclonal antibodies may be performed, for example as detailed below or may involve a combination of more than one of these three methods.

1) CAstV-3 may be purified from embryo-grown virus or virus present in infected avian faeces, for example chicken faeces, and used for immunisation.

2) Mice may be immunised with recombinant chicken astrovirus type 3, for example CAstV-3, protein produced by expression of chicken astrovirus type 3 polynucleotide sequence (preferably a polynucleotide sequence encoding a capsid protein) in E. coli, yeast, plant or insect cells infected with a recombinant baculovirus.

3) Mice may be immunised with a DNA expression plasmid capable of expressing avian astrovirus type 3 polynucleotide sequence, for example a polynucleotide sequence of CAstV-3 including at least one of SEQ ID NO 1 to 4.

As indicated above, these preparations can also be used to produce polyclonal antibodies.

The presence of antibodies to avian astrovirus type 3, for example CAstV-3 in the immunised mice can be detected using an indirect immunofluoresence (IIF) test.

Hybridoma cells can be prepared from the spleens removed from the immunised mice and cloned cell cultures can be screened for their abilities to secrete virus-specific antibodies using an IIF test.

Antibodies produced in an animal treated with a protein of the present invention can be isolated and used as an assay and / or for assay purposes.

In particular the invention provides the use of an antibody which has binding specificity to polypeptides encoded by any one of SEQ ID No 1 to 4 in a diagnostic assay.

As will be appreciated by those in the art an "antibody" should be construed as covering any binding member or substance having a binding domain with the required specificity. An antibody may be natural or partly or wholly synthetically produced. The term also covers any polypeptide, protein or peptide having a binding domain that is, or is homologous to, an antibody binding domain. These can be derived from natural sources, or they may be partly or wholly synthetically produced. Examples of antibodies are the immunoglobulin isotypes and their isotypic subclasses and fragments which comprise an antigen binding domain such as Fab, scFv, Fv, dAb, Fd, and diabodies.

An avian may be a chicken, turkey, duck, quail, goose, ostrich, pheasant, peafowl, guinea fowl, pigeon, swan, bantam and / or penguin.

Treatment

A virus, nucleic acid sequence, protein or antibody of the invention may be used to modify the immune system of an avian. Such modulation may be used to treat an avian.

Treatment includes any regimen that can benefit an avian. The treatment may be in respect of an existing condition or may be prophylactic

(preventative treatment). Treatment may include curative, alleviation or prophylactic effects.

Treatment may be provided via any suitable route. The precise dose will depend upon a number of factors, for example the precise nature of the antigen of the vaccine or the use of particular adjuvants.

Vaccine or Composition to Mediate an Immune Response

A vaccine or composition to mediate an immune response of the present invention can comprise live virus, live attenuated virus or inactivated CAstV-3. Suitably, a vaccine of the present invention may comprise immunogenic derivatives and / or at least part of CAstV-3, including, for example, antigenic subunits, vectors able to express nucleotide sequences of the invention, including CAstV-3 nucleotide sequences, SEQ ID NO 1 to 4, for example a DNA vaccine encoding a polypeptide of the invention, for example a capsid protein of CAstV-3 (SEQ ID NO 5), recombinant chicken astrovirus type 3, synthetic peptide vaccines, or the like.

Suitably to inactivate the virus a standard chemical inactivating agent, such as aldehyde reagent including formalin, acetaldehyde and the like may be used. Alternatively, irradiation (for example, ultraviolet or Gamma irradiation) of the virus may be used or the virus may be repeatedly grown in cell culture from non-avian origin such that its ability to virulently reproduce is lost.

A vaccine of the present invention may be used in avians for immunisation against growth depression.

Derivatives of polypeptides of the present invention may be used to mediate immune response. Polypeptides of the invention may be suitably linked to a coupling partner, e.g. an effecter molecule, a label, a drug, a toxin and/or a carrier or transport molecule. Techniques for coupling the polypeptides of the invention to both peptidyl and non-peptidyl coupling partners are well known in the art.

Suitably the vaccine may comprise a pharmaceutical carrier or diluent, for example physiological saline, propylene glycol and the like.

Suitably the vaccine may comprise an adjuvant, for example, Freund's incomplete adjuvant.

Method of vaccination

The vaccine may be delivered orally, parenterally, intranasally or intravenously. The dosage of the vaccine provided will typically take into account the age and / or weight and / or physical condition of the avian.

Suitably the vaccine may be provided in drinking water, spray or as an aerosol, for mass vaccination of poultry such as, but not limited to, chickens and turkeys.

In one embodiment a vaccine may be prepared as a live vaccine, which will be administered via drinking water or by a spray. Such a vaccine may be non-attenuated, since it is likely that infections of the growing breeder birds (say 10 weeks and older) will not result in pathogenic effects. (Infections of very young chicks (e.g. 0-3 weeks) are likely to be pathogenic.)

In a second embodiment a vaccine may be prepared as an attenuated virus which may be produced by growing the virus in embryos or cell culture. Such an attenuated vaccine could also be given by drinking water or spray. It is also possible that an attenuated or non-attenuated CAstV-3 virus could be given by inoculation (e.g. subcutaneous route).

In a third embodiment a vaccine may be prepared as a dead or inactivated virus. Inactivated (dead) virus may be administered by inoculation. The adjuvant used with inactivated virus will likely be important in order to maximise the immune response elicited.

In a fourth embodiment a vaccine may be prepared as a recombinant subunit vaccine. This approach may be adopted, for example, if live vaccines are not efficacious and if inactivated vaccines are too expensive to produce. A recombinant subunit vaccine may be based on expression of capsid protein in E. coli, yeast, plant or insect cells infected by a recombinant baculovirus. In such an embodiment at least part of CAstV-3 may be used to prepare the vaccine, for example a protein (preferably a structural protein) of CAstV-3 may be used. Such a protein may be produced by recombinant DNA expression methodologies or by culturing the virus.

Suitably the vaccine to avian astrovirus type 3, for example CAstV-3 may further comprise antigens of other agents, for example other avian, more specifically other chicken viruses, as part of a combination vaccine.

Assay method

In particular embodiments, an assay of the invention comprises the steps:

- providing a sample from an avian,

- bringing into contact with the sample an antibody capable of binding specifically to at least part of chicken astrovirus type 3 (CAstV-3),
- detecting binding of the antibody to CAstV-3 in the sample wherein the binding of the antibody to chicken astrovirus type 3 in the sample is indicative of the presence of CAstV-3, in the avian.

Suitably the antibody capable of binding specifically to chicken astrovirus type 3 is capable of binding a polypeptide encoded by SEQ ID NOs 1 to 4, more preferably a polypeptide encoded by SEQ ID NOs 1,2 or 4, more preferably a polypeptide with an amino acid sequence SEQ ID NO 5 or at least part of the polypeptide of SEQ ID NO 5 which is antigenic.

In alternative embodiments, an assay of the invention comprises the steps:

- bringing into contact with the sample at least part of chicken astrovirus type 3,
- detecting the presence or absence of binding of at least part of chicken astrovirus type 3 to an antibody in the sample wherein the binding of the at least part of the isolated chicken astrovirus type 3 to an antibody is indicative of the presence of antibodies to chicken astrovirus type 3 in the avian.

In further alternative embodiments, an assay of the invention comprises the steps:

(i) providing genetic material, and

(ii) testing the genetic material to detect any genetic material from chicken astrovirus type 3 .

Suitably the genetic material is from an avian and may be RNA from an avian, for example a chicken.

Suitably the genetic material may be taken from feathers, eggs, blood, faeces, intestines, and intestinal contents, tissue or the like from an avian.

A number of commercial kits are available to extract RNA from tissue samples. These are well known to those skilled in the art.

The skilled person will be aware of a range of detection method for detecting genetic material in samples.

Suitably the method involves the use of a reverse transcription PCR (RT-PCR) reaction. In a particularly preferred embodiment the detection method comprises the steps

(i) providing forward and reverse primers for a nucleic acid polymerase, which primers are capable of binding specifically to a chicken astrovirus type 3 polynucleotide sequence;

(ii) amplifying polynucleotide sequence between the primers;

(iii) detecting amplified polynucleotide sequence;

wherein detection of multiple copies of amplified polynucleotide sequence in the sample is indicative of the presence of chicken astrovirus type 3 in the sample.

The basis of this test is that a positive cDNA product will be produced if the two small primer sequences find exact or very close sequence matches in the RNA extracted from the test samples.

Forward and reverse primers for a nucleic acid polymerase suitable for use in the invention may be selected from any suitable sequences from within the CAstV-3 genome.The skilled person would appreciate the factors required to be taken into account when designing suitable primers for example, the use of sequence comparisons to determine conserved regions of sequence to which forward and reverse primers may be designed.

Suitably the primers may have the sequence
Forward: 5`- AGC CTC AAA GTA TAA GAC GCA G-3` SEQ ID No 6.
Reverse: 5`- CCA TGC TAT TTC AAA GGT GGT T-3` SEQ ID NO 7.

Advantageously using such primers a test between 10, preferably 20, more preferably 30 and most preferably 100-fold more sensitive than a method using detection by indirect immunofluorescence of virus antigen produced by inoculating primary chick embryo liver cells with virus-containing samples is provided.

Preferably, the assay method provides quantitative information on the amount of virus present in the sample.

Advantageously the primers may be labelled fluorescently and the assay comprises the further step of determining if the primers have bound to the polynucleotide sequence by determining the fluorescent emissions of the primer.

Suitably fluorescent labels are within the common general knowledge of skilled persons.

Preferably the assay method of the invention is specific for chicken astrovirus type 3 as deposited under Accession number CNCM I-3541 at CNCM, Institut Pasteur on 15 December 2005.

Any suitable sample may be used in an assay of the present invention, for example, a sample from an avian, for example a chicken may be used and a tissue where the virus replicates, for example, gut may be used. Alternatively, the sample may be blood or faeces.

Preferably a sample for diagnosis may be selected from faeces, gut contents or faecal swab.

Suitably when the sample is faeces and / or gut contents, crude virus suspensions are prepared as 10% homogenates in phosphate buffered saline (PBS). These may be clarified using 3000g for 30 minutes and an aliquot (eg 200 microlitre) of clarified extract is extracted. With swabs, suspensions in 1-2ml PBS may be made and clarified as above prior to extraction.

Diagnostic kit

In particular examples of diagnostic assay kits provided by the invention for the detection of chicken astrovirus type 3 in samples from avians suspected of being infected with the virus, the assay kit comprises at least part of chicken astrovirus type 3, a nucleotide sequence of the invention, a polypeptide of the invention, a polyclonal antibody of the invention or a monoclonal antibody of the invention.

The at least part of the chicken astrovirus type 3, nucleotide sequence of the invention, polypeptide of the invention, polyclonal antibody of the invention or monoclonal antibody of the invention may be bound to a substrate such that a test sample may be placed on or washed over the at least part of the chicken astrovirus type 3, nucleotide sequence of the invention, polypeptide of the invention, polyclonal antibody of the invention or monoclonal antibody of the invention.

In specific examples, a diagnostic assay kit of the invention for the detection of chicken astrovirus type 3 in samples from avians suspected of being infected with the virus comprises antibodies with binding specificity to at least part of chicken astrovirus type 3. Suitably, the antibodies may be bound to a substrate such that a test sample may be placed on or washed over the bound antibodies.

Suitably the antibodies have binding specificity to polypeptides encoded by any one of SEQ ID NOs: 1 to 4. In a particular embodiment the antibodies have a binding specificity to a polypeptide with an amino acid sequence SEQ ID NO: 5.

In a further example, a diagnostic assay kit of the invention for the detection of chicken astrovirus type 3 in samples from avians suspected of being infected with the virus comprises a nucleic acid probe capable of hybridising to any one of SEQ ID NOs 1 to 4.

In particular examples a nucleic acid probe capable of hybridising to any one of SEQ ID NOs 1, 2 or 4 is provided.

In one embodiment, a probe may be a nucleic acid sequence and the hybridisation of said probe to a nucleotide sequence in the sample may be detected by dot blot hybridisation.

Suitably the probe may be a primer set.

When a primer set is used, said primer set may be used amplify a selected sequence via RT- PCR if a particular nucleotide sequence is present in a sample.

In particularly preferred examples a primer set may be selected from:

Forward: 5`- AGC CTC AAA GTA TAA GAC GCA G-3` SEQ ID No 6.
Reverse: 5`- CCA TGC TAT TTC AAA GGT GGT T-3` SEQ ID NO 7.

Preferably the assay kit is specific for chicken astrovirus type 3 as deposited under Accession number CNCM I-3541 at CNCM, Institut Pasteur on 15 December 2005.

Suitably the virus, nucleotide sequences, polypeptide sequences, modulators of the immune system, vaccines and kits of the present invention may be used in relation to avians, more preferably any birds which are produced commercially, more preferably poultry such as chickens, turkeys, ducks, geese, pheasants, pigeons, guinea fowl, yet more preferably chickens.

Definitions

As used herein, the term “isolated” refers to an in vitro preparation, isolation and / or purification of a peptide, polypeptide, protein, antibody,

virus or nucleic acid molecule of the invention, such that it is not associated with in vivo substances or is substantially purified from in vivo substances.

As used herein the term "aerosol" includes finely divided solid or liquid particles that may be created using a pressurized system such as a nebuliser.

As used herein the terms "nucleic acid" or "nucleotide sequence" includes genomic DNA, cDNA or RNA.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention described herein will now be exemplified with reference to the following non-limiting examples and figures. Other embodiments of this invention will be apparent to those of ordinary skill in the art in view of this description.

Figure 1 shows pairwise amino acid identity comparison of ELVs and avian astroviruses;

Figure 2 shows phylogenetic analysis of Human, chicken and turkey astrovirus sequences; and

Figure 3 shows agarose gel electrophoresis of RT-PCR results obtained with gut samples from day-old chicks, wherein the RT-PCR product is 187bp.

Infection of young chickens with CastV-3

Experimental and preliminary filed investigations suggest that infections with the CastV-3 are associate with growth retardation or growth depression in young chickens.

Growth depression is a characteristic of disease states including stunting in which young birds fail to grow at the expected rate. This can be transient lasting days after which the affected birds will grow normally and "catch up" with their non-affected hatchmates. Alternatively, the birds may remain relatively small ("runting") and lag behind their non-affected hatchmates. Thus, the flock as a whole might be described as displaying "uneven growth".

Experimentally, it has been shown that oral inoculation of 1-day-old specific pathogen free (SPF) chickens with chicken astrovirus 3 resulted in a 17% depression in growth over a 14-day period. Histological changes were observed in the intestine, kidney and pancreas indicating that this virus replicated in the intestine but additionally had the ability to spread beyond the intestinal tract. Virus antigen was detected in a wide range of tissues. It is suggested that the observed growth depression may have been due to the combined effects on both the intestine and the other organs e.g. pancreas.

Evidence from the field involved investigation of clinical samples from flocks exhibiting "uneven growth", which showed that the gut contents from some clinically affected chickens contain large numbers of enterovirus-like viruses (ELV), including CAstV-3, together with rotaviruses and reoviruses. It is recognised that chicken astrovirus type 3 may be contributing to the clinical problem. In addition, oral infection of 1-day-old SPF chickens with an inoculum prepared from the gut contents from a sick chicken from a flock exhibiting uneven growth resulted in approximately 30% growth depression at 5 weeks post infection. Serological testing showed that the inoculated chickens had seroconverted to CAstV-3 and another immunologically distinct ELV identified previously. This finding indicated

that the inoculum contained CAstV-3 and suggested that this virus may have been contributing to the growth retardation.

It is probable that the pathogenic effects caused by infections with enteric viruses such as CAstV-3 will be more severe in younger chickens and in chickens without maternal antibodies to the infecting viruses. It is recognised that the presence of maternal antibody may not prevent infections of the chicken intestinal tract due to the absence of antibody at this site, but may prevent or reduce the spread of the infection beyond the intestine.

Infection of Embryo with CAstV-3

Evidence for embryo transmission of CAstV-3 was observed in recent work by the inventors, which has demonstrated the presence of virus in gut tissues prepared from 1-day-old chicks obtained from several breeder flocks. Serological testing showed that the majority of breeder flocks displayed partial seroconversion to chicken astrovirus type 3 when tested at 22 weeks (just before they come into lay). Taken together these results support the view that many breeder birds become infected during lay and, that, as a consequence, are capable of transmitting CAstV-3 to their embryos and progeny chicks.

Inoculation of 6 or 7-day-old embryos with isolates of CAstV-3 including FP3, caused embryo death, dwarfing and liver and skin necrosis in embryos and reduced hatchability. Therefore, in the field infections with CAstV-3 have the potential to damage the developing embryo and to cause reduced hatchability.

Irrespective of whether CAstV-3 causes disease problems for the embryo, it is likely that vertically-transmitted virus will pose a threat to the newly-hatched chick, particularly since these chicks will not have maternally-derived antibody to the virus. Virus replication may cause pathogenic effects to these directly infected chicks. Virus excreted by these birds, often in the first few days, will in turn horizontally infect hatchmates that will either have maternal antibody (if the egg was produced by a previously-infected, antibody-positive breeder), or not have maternal antibody (if the egg was produced from an uninfected, antibody-negative breeder). The protective effect of maternal antibody is likely to lead to variation in the pathogenic effects observed and uneven growth within the broiler flock may be observed.

Isolation and preliminary characterisation of the infectious agent

Using the chick embryo inoculation method, a number of successful attempts were made to isolate infectious agents from weak 1-day-old chickens from flocks displaying reduced hatchability and higher proportions of weak chicks. The following is one such example:

Weak chicks from sample submission "11672" were homogenised as whole chicks and inoculated into 7-day-old embryos via the yolk sac. No embryo deaths were recorded after 10 days of incubation, but the embryos were smaller than the controls. Samples of allantoic fluid from the eggs were processed and examined by EM, but no virus particles were seen. The livers from these embryos were pooled, homogenised and re-inoculated by the yolk sac route (1st pass) into 7-day-old eggs. With these samples, embryo deaths were recorded between 9 and 12 days after inoculation. The embryos were dwarfed, compared to the controls, and white lesions (areas of necrosis) were seen around the margins of the liver

lobes. In these eggs, the allantoic fluid had a distinct green colour, and this was collected and stored (-70°C). EM examination failed to reveal any evidence of virus particles.

Livers were removed from the dead embryos, pooled, homogenised and examined by electron microscopy but no virus particles were seen. The pooled liver was re-inoculated into 7-day-old eggs (2nd pass), and all embryos died with deaths recorded from day 4 post inoculation. The embryos were dwarfed and as before the allantoic fluid was a distinct green colour. Livers and kidneys were removed from the dead embryos and cryostat sections cut. The liver sections were stained by immunofluorescence for infectious bronchitis virus, but no positive fluorescence was observed. Liver and kidney cryostat sections were retained for staining with additional antisera at a later date. White necrotic lesions were seen on the livers from one set of samples, and some of this material was processed for thin section EM, but no evidence of virus replication was seen. Livers with necrotic lesions were homogenised and given 2 x 7 day passes in primary chick embryo liver cells. No viral CPE was observed, but the coverslips were fixed and stored at –20°C to await fluorescent staining when a conjugate becomes available.

The 2nd pass material was subjected to additional passaging in chick embryos as outlined below. The pooled liver homogenate was again inoculated by yolk sac, this time into 7- day-old SPF embryos (3rd pass). Embryo deaths were recorded from day 7 to 11 post inoculation, and embryos were stunted with gross necrotic lesions on the liver and skin. Samples of affected liver were fixed and stored for histopathological examination and the remaining livers from the dead embryos were pooled together and homogenised. Allantoic fluids were collected separately and also pooled. The allantoic fluid was inoculated (4th pass) by yolk sac

inoculation into 7-day-old embryos, resulting in embryo deaths between 7 and 10 days post inoculation. Allantoic fluids and livers were pooled and used to infect 7-day-old SPF embryos (5th pass). The embryos were collected, homogenised and stored at –70°C. This homogenate is referred to herein as the 11672 virus 1st pass Embryo Homogenate (1p EH).

Concentration and purification studies were undertaken with the homogenates derived from passaging the 11672 samples in embryos.

The homogenate of whole embryos was prepared after inoculating embryos with 11672 (1p EH), in an attempt to identify the agent responsible for the embryo mortality. Twelve 7-day-old VALO SPF eggs were inoculated and dwarfed embryos and green AF harvested after 7 days incubation. Different starting materials were used for purification. These were allantoic fluid, yolk stalks, green livers and whole embryos. A similar purification was used in each case. This involved 3 steps: (1) Clarification at 3000 rpm for 20 min, (2) Low-speed ultracentrifugation at 10000 rpm for 30 min, (3) High-speed ultracentrifugation at 30000 rpm for 3-4 hr. Centrifugations at 10,000 rpm and 30,000 rpm were performed in a Beckman ultracentrifuge (Type 35 fixed angle rotor).

With the liver and whole embryo preparations, an additional step was used. This involved resuspending the high-speed pellet, treatment or non-treatment of the suspension with the detergent sodium dodecyl sulphate (SDS) and sedimentation through 25% sucrose cushions using a 6 x 14 ml swing-out Beckman rotor centrifuged at 32000 rpm for 3-4 hr. This step was included because the high-speed pellets were thick and gelatinous and likely to contain high levels of embryo-derived contamination that would make the visualisation of virus particles very difficult. All samples of

material obtained from the above preparations were negative by negative contrast EM and by immune EM using convalescent antiserum.

To better define if the infectivity was concentrated at one or more stages of the purification process, an homogenate of stunted whole embryos, from the 1p EH passage material was treated using steps 1,2 and 3 above, with material being collected after each centrifugation step for inoculation into 7-day-old SPF embryos. All preparations produced dwarfing or death during 7 days incubation, indicating that the infectivity was present at all stages of the purification process, including the high-speed pellet. This result indicated that some infectious virus remained in association with cellular material in that infectivity was pelleted by the 10000 rpm centrifugation step (Table 1). Some infectious virus remained in the soluble 10000 rpm supernate fraction and was pelleted at 30000 rpm centrifugation. The detection of some infectivity in the 30000 rpm supernate suggested that 11672 virus may be small. However, using embryo inoculation to show virus infectivity in this way is a relatively crude method. Without titrating virus infectivity it is difficult to assess what proportions of infectivity were present in each fraction.

Table 1: Pattern of embryo deaths following inoculation of fractions from differential centrifugation.

Treatment	Days post inoculation							Findings with embryos alive at day 12
	3	4	5	6	7	10	11	
10000 rpm supernates		1	1	1	2	5		0
10000 rpm pellet (1:60)			2	1	2	3		2 very small (1 with liver necrosis)
30000 rpm supernate					1	2	1	3 small embryos 2 liver necrosis 2 liver enlarged
30000 rpm pellet (1:60)	1		1	1		4	1	2 normal

10 eggs were inoculated with each fraction.
Pellet fractions were diluted 1:60 to account for concentration effect
Embryos remaining alive at day 12 p.i. were killed and examined.

To determine if the infectious agent was chloroform sensitive i.e. if the viral agent was enveloped (sensitive to chloroform treatment) or non-enveloped (resistant to chloroform), the 11672 virus embryo homogenate (2p EH) was treated with chloroform, then inoculated into 7-day-old embryos. Embryo death and stunting was recorded in both the treated and untreated preparations, indicating that the infectious agent was not enveloped.

The 11672 virus embryo homogenate (2p EH) was diluted from 10^{-1} to 10^{-5} and each dilution was inoculated into 7-day-old embryos. Stunting and embryo deaths were recorded up to 10^{-4} dilution (Table 2). As expected, the most severe effects on the embryos, in terms of number of early deaths and dwarfing, were seen with the highest concentration of virus (i.e. at neat and 10^{-1} dilutions). There were few deaths at 10^{-2} and 10^{-3} dilutions, and none at 10^{-4} dilution. Changes such as dwarfing and liver necrosis in embryos still alive at day 11 post inoculation indicated that infectious virus was still present at these dilutions. An estimated infectivity titre of 10^4 embryo infectious doses provides an explanation for the failure so far, to see virus particles by EM, since a virus particle titre of $>10^5$ or even $>10^6$ is often stated as being required in order to see particles in EM.

Table 2: Pattern of embryo deaths following inoculation of different infectious doses of 11672 virus.

Inoculum dilution	Days post inoculation							Findings with embryos alive at day 11
	3	4	5	6	7	10	11	
Undiluted			1	2	1	3		1 small
-1	1		1		1	5		
-2					1	1		All 6 very small 4 liver necrosis
-3					1	1		All 6 small 2 liver necrosis
-4								4 normal 4 small, liver necrosis
-5								All normal

8 eggs were inoculated for each dilution.
All dead eggs at day 7 were stunted, haemorrhagic and with liver necrosis
Remaining embryos at day 11 were killed and examined

The effect of embryo infection with 11672 virus on hatchability was investigated in a further experiment. The 11672 virus embryo homogenate (2p EH) was inoculated into 7-day-old embryos, at 3 dilutions (10^{-3}, 10^{-4}, 10^{-5}), with 30 embryos for each dilution. 24 embryos were left uninoculated as controls. All embryos which died from day 8 post inoculation were checked. All were very small compared to controls, with evidence of haemorrhaging and necrosis of the liver. In addition 8 eggs from each dilution were opened at day 12 post inoculation and the embryos examined, with results as follows:
10^{-3} dilution: 4/8 embryos were small compared to controls, 3/8 were very stunted, with obvious necrosis of the liver, 1/8 was normal.
10^{-4} dilution: 3/8 very stunted, 2 of these with liver necrosis; 5/8 normal.
10^{-5} dilution: 8/8 normal
The eggs surviving on day 13 post inoculation were transferred to an incubator and allowed to hatch.

Table 3: Effect of 11672 virus titre on hatchability

Inoculum dilution	Number of eggs incubated at 13 days post inoculation	Deaths before day 13	Number hatched	Dead in shell	% Hatched
Control	15	1	11	4	73%

(uninoculated)					
10^{-3}	11	11	3	8	27%
10^{-4}	15	7	5	10	33%
10^{-5}	17	5	5	12	29%

All chicks, which hatched, were very weak, and took a long time to get out of the egg.

All embryo inoculations so far had used the yolk sac route of inoculation, and embryo deaths and stunting had been a consistent finding. The chorioallantoic membrane (CAM) route of inoculation has been successful in some viral studies, since, if the agent grows in the CAM or can be adapted to grow in the CAM, a higher concentration of virus may result, making identification easier. Also, in the past cryostat sections from infected CAM, have been used for serology studies using indirect immunofluorescence. In addition CAMs from infected eggs are normally harvested at 4 days post inoculation, which shortens the isolation procedure (up to 12 days following yolk sac inoculation). The 11672 virus (1p EH) was therefore inoculated onto the CAMs of 9-day-old embryos, and harvested 4 days later. White foci and some thickening of the CAM was observed, indicating virus growth. Thickened areas of CAM were frozen and cryostat sections cut for immunofluorescent staining. The affected areas were excised, homogenised and re-inoculated onto the CAMs of 9-day-old embryos. Again, thickening of the inoculated CAMs was evident. The material has now been passaged 4 times by CAM inoculation. No virus was observed when CAM preparations were examined by EM, suggesting that virus yields were low.

Antigenic characterisation of infectious agent

0.1 ml of the 11672 virus whole embryo homogenate was inoculated orally and intramuscularly into 12 x 1-day-old SPF chicks in an isolator. The birds were boosted with a second oral inoculation at 6 weeks of age, then bled and sacrificed 2 weeks later. No obvious clinical signs were seen in the inoculated birds throughout this period. The serum from the birds was collected, pooled and stored at –20 °C (Antiserum 1). A second group of 1-day-birds was inoculated intramuscularly and bled out after 5 weeks. The serum was collected, pooled and stored at –20 °C, as before (Antiserum 2).

The 11672 virus embryo homogenate (2p EH) was inoculated onto SPF chick embryo liver cell cultures in 25cm^2 plastic flasks. No virus like cytopathic effect (CPE) was observed after 7 days incubation at which time the cells were scraped from the flask, resuspended and re-inoculated onto fresh cultures (second pass). No CPE was observed after the second pass, or after a third pass in CEL cultures as above.

The 11672 virus embryo homogenate (2p EH) was inoculated undiluted and at 1/10 dilution, onto SPF chick embryo liver (CEL) cell cultures growing on 13mm circular coverslips, and incubated at 30°C for 48 hours. The coverslips were then fixed in acetone for 10 minutes, air dried and stained with Antiserum 1 (above) for 1 hour at 37°C. After washing in several changes of PBS, the coverslips were stained again with an FITC anti-chicken conjugate for 1 hour, washed, mounted in buffered glycerol and examined under U.V. illumination. At both dilutions of the inoculum, positive immunofluorescence was observed in single cells. The immunofluorescence was cytoplasmic, and often granular in nature. This

result showed that embryo-passaged virus was capable of undergoing partial replication in CEL cells, but that replication did not result in CPE.

Indirect immunofluorescence (IIF), performed with Antiserum 1, was also successful in detecting virus antigen in cryostat sections of embryonic kidneys and CAM from 11672 virus-infected eggs. This confirmed that the thickening/ pocks detected in CAMs were associated with the virus and also identified a possible site (i.e. kidney) of replication of the virus in the experimentally-inoculated chick embryos.

Exploratory testing by IIF showed that Antiserum 1 stained CEL coverslip cultures that were infected with FP3 virus, an enterovirus-like virus (ELV) that was isolated from dead-in-shell chicks in the 1980s. In addition antiserum raised to FP3 in previous research (McNulty et al., 1990) was shown to react by IIF with CEL coverslip cultures infected with 11672 virus. Cross-neutralisation tests have not yet been performed.

On the basis of the IIF results, it was concluded that 11672 virus is an enterovirus-like virus (ELV), that is antigenically related to FP3 virus. Although the IIF test will detect viruses that share a common group antigen, it cannot distinguish viruses from different serotypes.

Nucleotide sequence characterisation of 11672 virus as novel chicken astrovirus

Nucleotide sequence studies of isolate 11672

The following primer sequences were obtained to allow the amplification of Astrovirus RNA polymerase gene sequence by RT-PCR

AstPol-1F
5'-GAYTGGACNMGNTAYGAYGGNACNATNCC – SEQ ID NO 8

AstPol-1R
5'-YTTNACCCACATNCCRAA – SEQ ID NO 9

Wherein Y = C or T; M = A or C; R = A or G and N = deoxyinosine (I)

One-step RT-PCR was carried out using Ready-To-GO RT-PCR beads from Amersham Pharmacia.

Temp	Time	Cycles
42 ℃	30 min	1
94 ℃	5 min	1
94 ℃	60 sec	45
45 ℃	60 sec	
72 ℃	90 sec	
72 ℃	5 min	1

F-primer	R-prime	Size
AstPol-1F	AstPol-1R	~411 bp

Seq ID No. 1 and Seq ID No. 2 are the nucleotide (nt) sequences that are specific to the chicken astrovirus type 3 (CAstV-3) (isolate 11672). This is the 391 nt that are flanked by the degenerate primers designed to amplify astrovirus sequences in the RNA polymerase gene region. The sequences of 2 clones are shown. These differ at 4 nt positions (99.0% nt identity)

The nt sequence of the corresponding region of 1 clone of isolate FP3 is also shown as Seq ID No. 3. This differs from clone 1 of 11672 virus at 20 nt positions (94.9% nt identity).

Blast searches indicate that the 391 nt region shares 65% nt identity with Turkey Astrovirus type 1 (GenBank Accession number: Y15936), 61% nt identity with avian nephritis virus (GenBank Accession number: AB033998) and 60% nt identity with human astrovirus type 1(GenBank Accession number: Z25771).

11672 and FP3 sequences ID Nos 1, 2 and 3 referred to above.

Seq ID No. 1
11672 Clone1
AAAGCCCTTGTTTTGGCGCATTAGGCAGATTCGGTTTTTTTTTTTAGC
CTCAAAGTATAAGACGCAGGAAAACAAGGAGCTGTTTGATTGGTACA
CCAAAAACCTTTTGGAGAAGGTGATATTGTTACCTACTGGGGAAGTGT
GCCAAATAAAGCGAGGAAATCCTTCAGGGCAATTTTCTACCACCGTG
GATAACAACATGTGCAACGTATGGTTAACCACCTTTGAAATAGCATGG
CTCCACCGCAAACAACGGGGCAGACTACCAACCCCAGCTGAATTGC
GTGAAAACGTTTGTTATATTTGCTACGGTGATGACAGGCTCTTATCAG
TTTCGAGAGACTTTGTCATTTATGAGCCTGAAACTGTGGTAGCAATGT
ACGCAGATGTA

Seq ID No. 2
11672 Clone 2
AAAACCCTTGTTTTGGCGCATTAGGCAGATTCGGTTTTTCTTTTTAGC
CTCAAAGTATAAGACGCAGGAAAACAAGGAGCTGTTTGATTGGTACA
CCAAAAACCTTTTGGAGAAGGTGATATTGTTACCTACTGGGGAAGTGT

GCCAAATAAAGCGAGGAAATCCTTCAGGGCAACTTTCTACCACCGTG
GATAACAACATGTGCAACGTATGGTTAACCACCTTTGAAATAGCATGG
CTCCACCGCAAACAACGGGGCAGACTACCAACCCCAGCTGAATTGC
GTGAAAACGTTTGTTATATTTGCTACGGTGATGACAGGCCCTTATCAG
TTTCGAGAGACTTTGTCATTTATGAGCCTGAAACTGTGGTAGCAATGT
ACGCAGATGTA

Seq ID No. 3
FP3 Clone 1
AAAGCCCTTGTTTTGGCGTATTAGGCAGATTCGGTTTTTCTTCTTAGC
CTCAAAGTATAAGACGCAGGAAAACAAGGACCTCTTTGATTGGTACA
CCAAAAACCTCTTGGAGAAGGTGATATTGTTACCTACTGGAGAAGTGT
GCCAAATAAAGCGAGGGAATCCTTCAGGGCAATTTTCTACTACCGTG
GATAACAACATGTGCAATGTATGGCTAACCACCTTTGAAATAGCATGG
CTTCACCGCAAACAACGGGGTAGATTACCAACCCCAGCTGAATTGCG
TGAAAATGTTTGTTATATTTGCTACGGTGATGATAGGCTCTTATCAGTT
TCAAGAGACTTTGTCATTTATGAGCCTGACACTGTGGTAGCGATGTAC
GCTGATGTA

This sequence data shows that 11672 virus and FP3 virus are very closely related at the nucleotide sequence level and that both are likely to be isolates of the same virus species. This sequence data is consistent with the finding that 11672 virus and FP3 are antigenically similar, as demonstrated by indirect immunofluorescence. On the basis of the levels of nucleotide identity shared with other astrovirus species, the virus species, of which 11672 and FP3 are isolates, is considered to be a novel chicken astrovirus, which the inventors have named chicken astrovirus type 3 (CAstV-3)

Nucleotide sequence corresponding to the 3.2kb fragment at the 3` end of the virus genome was produced by RT-PCR using an oligo dT based forward primer that binds to the Poly A tract at the 3` end of the astrovirus genome and a reverse primer selected from within the sequence of 391 nucleotide fragment amplified by the degenerate primer strategy (SEQ ID No 1-3). The 3.2kb fragment was cloned using the pTOPO vector and sequenced using a primer-walking strategy beginning with the forward and reverse M13 primers that are specific to the plasmid vector.

Combining the nucleotide sequence specific to the 391 nucleotide fragment of 11672 virus with that determined for the 3.2kb fragment gives a total sequence of 3265 nucleotides. This sequence encompasses 730 nucleotides at the 5` terminus of the astrovirus ORF 1b, which encodes the astrovirus RNA dependent RNA polymerase, a small intergenic sequence of 24 nucleotides, the complete astrovirus ORF 2 (2217 nucleotides), which encodes the capsid protein region, and the 3` untranslated region (UTR) of 276 nucleotides (excluding the Poly A tail).

The nucleotide sequence of the 3265 nucleotide fragment is as follows:-

SEQ ID No 4

AAAGCCCTTGTTTTGGCGCATTAGGCAGATTCGGTTTTTTTTTTTAGC
CTCAAAGTATAAGACGCAGGAAAACAAGGAGCTGTTTGATTGGTACA
CCAAAAACCTTTTGGAGAAGGTGATATTGTTACCTACTGGGGAAGTGT
GCCAAATAAAGCGAGGAAATCCTTCAGGGCAATTTTCTACCACCGTG
GATAACAACATGTGCAACGTATGGTTAACCACCTTTGAAATAGCATGG
CTCCACCGCAAACAACGGGGCAGACTACCAACCCCAGCTGAATTGC
GTGAAAACGTTTGTTATATTTGCTACGGTGATGACAGGCTCTTATCAG
TTTCGAGAGACTTTGTCTTTTATGAGCCTGAAACTGTGGTAGCAATGT
ACGCAGATGTATTCGGCATGTGGGTGAAGCCAGAGAATGTGAAGGTA
AGAAATACACTTAGTGGTCTCTCTTTCTGTGGTATGACAATTACAAAA
AATCAGCATGGCCGTTATGTTGGTATTCCTAATGTCAATAAAATACTG

TCCACTCTACGGTCTCCTACAAAGCGCCTTCCAAATGTAGAAGCACTA
TGGGGTAAGTTGATATCATTAAGAATTCTGTGTGAGAATGCAGATCCC
GACGTAAAGGATTACTTAGATAGGCAGATCAATTGCGTCGAGGAGTA
TGCCGCTGCTGAAGAAATACAGTTACCAGAAGTCGGGCCCGACTTCT
TTCAGAAAATCTGGTAGAGGGATGGACCGAAATATAGCAGCATGGCC
GATAAGGCTAGCGCGCAGAAGGAGAAAACAACAAGGCGCGGACGTG
GCCGTTCTCGATCTAGGTCACGTTCTCGTTCCCGTTCTAGGAATCGT
GTCAAGAAAACTGTCACGATAGTTGAATCTAAGAAAACCCCATCTAGA
TCTATATTAAGAAAAGAACTTGAAAATCATGAGAGAAGGGATAGGAGG
CGTTTTAGGAAGATTGAAAAAAAATTAAATGGCCCTAAAATACATGAT
CGCATGGCAGTCACAACTACACTTGGAGTCCTCACTGGAAATTCTGA
CAATAATTTGGAAAGGAAAATGAGAGCTCTTCTTAACCCATTGCTTTT
GAAATCTCAAAACACTGGGGCCTCAGCATCCCCACTTTCCCTTAGGG
CATCTCAGTATTCAATGTGGAAGATACAGAAATGTGTTGTAAAATTTG
TTCCCCTAGTTGGGGCTGCTAATGTGGCAGGTAGTGTATCCTTTGTG
TCTCTGGATCAGGATGCAACCTCCTCCCAGCCTGAATCACCTGATAC
GATAAAGGCAAAGGTGCATGCAGAAGTTGCAATTGGACAAAGATTTA
ATTGGAACGTTCAATCTAGATACCTGGTCGGACCCCGTTCTGGTTGG
TGGGGCATGGATACTGGTGAGTCACCAACTGATACAGTTGGACCAGC
CCTTGACTTTTGGAATCTTTATAGAACAGTGAACACACTTCAAACTGG
CTCAACATCGCAGGCATACACTGCACCATTGTTTTCTATTGAAGTGTA
TACGGTGTATGTTTTTTCAGGTTACGAACCAAAGCCTGCCCTGGCGA
CCATGACAAATTCAACTTTTGAGAGTCAGCAGGGGGTGACCATAACA
AATGGTGCTAATGGTGAACTTCTGCTCAATGTTCCACGGCGATCGAG
TCTTGCCGAAGGGCTGCGTGAAAAGGAAGTATTATACCGCGGCCAAA
ACCAAACGGGTGGTGTCGGTGAGGTACTGTGGGCGGTGGCATCAGG
AGCTGTTGAAGGAGCTGCAGAAGCGTTGGGCCCATGGGGATGGTTA
CTGAGAGGTGGCTGGTGGGTGATAAAGAAGTTGTTTGGACGGAGCG
CTGAAAATGAAAGTGACGATTATGTGATGTACTCGTCTATTGAGGATG
CCAACAAAGATAGTAGGATCTATCAAACGGTATCCAGTGCGGTCCCT

GTTCAACAAGGTCCTCTGGTTCTCACCCAAATCTCATCCCCAAATGTT
AATCAAGCTGGGGGGTGTTGTGCAGGTAGGTACAACAATTGCCACTGA
TTACTTGCCACTATCTCAGGCCCAGGTTCCGCTTTTAGAGAACATTCT
TTACTCCAGCACTGGGCAGCCTGTGACATCAACTAAGAGCCATACTA
TGAGGATCACTGGGTTTCCAGCCTCGAAATTGGTAACATCAACGTCG
TCGCAATGGTTGGGCACTACTGATACGAGTGTCCAAGCAACAAAGTG
GCTAATGTCGGATTATACAGACACTGGAGTGATATTTGGCTTTCCATA
CTCTGATGATTCCCCCGGGGGAAACTTTTGGTAACATTGGAGTAATACA
CACAGCCAAGTCGCTCATAAAAACGGTCACATCAAGGCGACAACGCG
GGCTACGCATGTCTCCACTTGTTTCGACATTGTTACCATCAACTTCTA
AGGGACCAACCCAGATGCTTAGTTGCTTTGACACGCCTTACTATTGG
ATTAGGGTTTGTGACAATACCTGCTCAAACAAACCCACAAATGGCGC
CGTGACACAGCGCTGCAATGCTTGGGGCGTTATGGTGGTGAGCTTA
GCACACAATAAAGTCTACATCTTGGCTGGTTATCCCGATTCTCAAACT
AGGGTACCACAACAACAACTAGTCTGGGACACTTTTGACTGGGATGC
TACATTTTCTACTGGCAGGATTTATAATACAACATGGCCAGGACTTTA
TGAAGAAAGTGATGATGAAACAGATGCCGAATCTGACATCTCCAGTC
TTTTTGACCCCGTTAATGAGGTTGAGCAGGACTTTCACTTCAAATGTA
GTCTGAAGACATCTGACTACTTGAAGGAGGAGGCTGATTATTGGAAA
GCAAAAGCACAACAATTGCTTATGGAGAAAGCAATGGGAAAAAATAAT
GACTCTCCTCCACTTGTCCGCTTTGAGAAGGGCGGACCTGAGCAGCA
AAAACAGCCTGCTAGCAGCCGCGGCCACGCCGAGTAGGATCGAGGG
TACAGCTGCACCTTCTTCATGGAGTTTTTATGCCATAATCAGGCTTTT
CTCCATGTAAATCAAGGCACCGGGGCCACGCCGAGCAGGAACGAGG
GTACAGTGCCGGGTTGACCCCACCTGAAAGGGGCGTCCGCCGGTGT
GATAATCACCACACCGGGGCCTGGTTTAAATCACAGATAATCACTCT
GTGTGTCAATCAGGTCTTTCGGGCGGTTTTGGAAACACTAGTTTTTAA
AACCAATTTGATTTTGAATTAGATTAATTGGCAAAAAAAAAAAAAAAA
A.

The capsid protein gene is 2217 nucleotides in length and encodes a protein region of 738 amino acids.
The amino acid sequence of the 738 amino acid capsid protein region is as follows:

SEQ ID No 5

madkasaqkekttrrgrgrsrsrsrsrsrnrvkktvtiveskktpsrsilrkelenherrdrrrfrkiekkln
gpkihdrmavtttlgvltgnsdnnlerkmrallnplllksqntgasasplslrasqysmwkiqkcvvkfvpl
vgaanvagsvsfvsldqdatssqpespdtikakvhaevaigqrfnwnvqsrylvgprsgwwgmdtg
esptdtvgpaldfwnlyrtvntlqtgstsqaytaplfsievytvyvfsgyepkpalatmtnstfesqqgvtit
ngangelllnvprrsslaeglrekevlyrgqnqtggvgevlwavasgavegaaealgpwgwllrggw
wvikklfgrsaenesddyvmyssiedankdsriyqtvssavpvqqgplvltqisspnvnqaggvvqv
gttiatdylplsqaqvpllenilysstgqpvtstkshtmritgfpasklvtstssqwlgttdtsvqatkwlmsd
ytdtgvifgfpysddspgetfgnigvihtaksliktvtsrrqrglrmsplvstllpstskgptqmlscfdtpyy
wirvcdntcsnkptngavtqrcnawgvmvvslahnkvyilagypdsqtrvpqqqlvwdtfdwdatfst
griynttwpglyeesddetdaesdisslfdpvneveqdfhfkcslktsdylkeeadywkakaqqllme
kamgknndspplvrfekggpeqqkqpassrghae

Knowledge of the nucleotide and predicted amino acid sequence of the capsid protein region can be exploited for a number of purposes including:

1) Use as a DNA vaccine, if the gene is cloned into a suitable expression vector. Such expression vectors may be also used to prime/ immunise mice for the generation of antibody reagents.
2) Use to produce recombinant protein products by prokaryotic (eg E. coli) and eukaryotic (eg recombinant baculovirus infected insect cells, yeast) expression systems. Such proteins may be of use as subunit vaccines or to immunise mice for the generation of antibody reagents.

3) Use to generate capsid protein-specific peptides for immunisation or vaccine purposes.
4) Use of specific nucleotide sequences for the development of astrovirus 3 –specific RT-PCR tests.

Serological diagnosis of chicken astrovirus type 3 in breeder flocks

Serum samples from breeder flocks aged approximately 22 weeks (just before lay) were tested for the presence of antibody to 11672 virus, an isolate of CAstV-3, by an indirect immunofluorescence assay using CEL coverslip cultures that had been infected with 11672 virus. Sera were screened at 1:500 dilution in PBS and chicken antibodies were detected using an FITC- anti chicken Ig conjugate. Antibody was detected in 62/ 211 (29.4%) birds and in samples from 13/ 17 (76.5%) flocks. The relatively low-levels of seroconversion in some flocks suggested that some flocks were experiencing on-going infections at the point of lay (22 weeks) and that further infections of breeder birds were likely to occur when the breeders were in lay.

Table 4: Serology results for breeder flocks tested at 22 week-old. CEL coverslips infected with 11672 virus were used in an indirect immunofluorescence test to detect virus-specific antibody in chicken sera.

No	**Flock**	House	Age	Res	No.	Flock	House	Age	Res
1	A	1	22	11/20	10	I	1	22	2/10
2	A	2	22	6/10	11	J	1	22	4/9
3	B	1	22	7/30	12	K	1	22	0/22
4	C		22	3/10	13	L	1	22	4/10
5	D	1	22	9/10	14	M	1	22	3/10
6	E	1	22	0/10	15	N	1	22	2/10

7	F	1	22	0/10	16	O	1	22	0/10
8	G	1	22	1/10	17	O	2	22	6/10
9	H	1	22	4/10					

Detection of chicken astrovirus type 3 in flocks exhibiting uneven growth

Gut contents from sick chickens from flocks showing "uneven growth" were investigated for the presence of virus. This involved preparing 5-10% homogenates in MEM cell culture medium, clarification at 3000 rpm for 30 min, centrifugation of the clarified extract at 10,000 rpm for 30 min, and ultracentrifugation of the supernate through a cushion of 30% (w/w) sucrose in PBS. Samples of the 10,000 rpm supernate and resuspended sucrose pellet were inoculated into CEL cells grown on glass coverslips. After washing, the coverslip cultures were incubated for 48 hr, and then fixed by treatment with acetone. Fixed coverslips were incubated with antisera against 3 immunologically different ELVs including CAstV-3. The presence of infectious CAstV-3 was detected in samples from 1 broiler flock exhibiting uneven growth at days 5, 7 and 9 post hatching. The presence of 2 other immunologically different ELVs, namely avian nephritis virus (CAstV-1) and an ELV, examplified by isolate "612", was also demonstrated. Negative contrast electron microscopy performed with crude and partially purified preparations of gut contents showed that such samples contained high levels of ELVs and rotaviruses.

An inoculum prepared from the gut contents of a sick chicken sourced from a flock exhibiting uneven growth was used to orally infect 1-day-old SPF chickens. These birds displayed feathering abnormalities at about 2-3 weeks and a 30% weight depression at 5 weeks of age. Electron

microscopic examination of gut content samples obtained from birds killed at day 4, 6, 8, 10 and 15 revealed the presence of ELVs at all timepoints. Antiserum recovered from birds at 5 week-old were shown by indirect IF to contain antibodies to CAstV-3 and to a second different ELV. This experiment showed that the gut contents of the sick chicken used for inoculation contained CAstV-3 and suggested that this ELV may have been contributing to the growth depression.

Vaccination

A vaccine to CAstV-3 is likely to protect against sickness and growth depression in young chickens, and also to protect against vertical transmission from infected breeders and possible ill-effects on the developing embryo and hatched chick that vertically transmitted virus may cause.

Vaccine for administration to breeder birds, would have health benefits for the developing embryo and young growing chick. Vaccine may also be administrated to the growing broiler chicks.

Detection of virus

The presence of virus in samples may be detected by the growth of infectious virus in embryos or cell culture. Alternatively virus (or more correctly virus antigen) can be detected inside tissue samples from infected chickens using immunohistochemistry. This involves collecting fresh tissue, fixation of tissue in formalin, paraffin embedding, the cutting of very thin tissue sections and the use of virus-specific antibodies (eg monoclonal or highly specific polyclonal antiserum) to stain virus antigen in the tissue section. The bound antibodies would be detected by a secondary enzyme-antibody conjugate.

Virus antigen can also be detected in frozen tissue sections using antibodies in a similar way.

In the case of astrovirus, which infects the intestinal tract, virus is excreted sometimes in large amounts in faeces.

The presence of virus antigen may be detected in faeces samples or swabs using an antigen-detecting ELISA. Typically, this involves the use of a virus specific antibody (immobilised onto a plastic surface) to bind to and "capture" virus particles present in diluted faeces samples. Captured virus particles are then reacted with another virus-specific antiserum that has been conjugated to an enzyme, such as horse radish peroxidase. The presence of bound conjugate is demonstrated by adding the enzyme substrate and its conversion to coloured product.

Chickens will respond to infection by producing antibodies to the CAstV-3. Antibodies in the serum can be detected using a variety of tests such as indirect immunofluorescence assay, virus neutralisation or ELISA, which is particularly useful for large sample throughput. Typically an indirect ELISA would involve immobilising virus or virus antigen to the plastic microtitre plate, reaction with serum dilutions to allow antibodies to bind, reaction with enzyme conjugated secondary anti-chicken Ig antibody, followed by reaction with enzyme substrate. Alternatively a blocking ELISA can be used. This would involve demonstrating the presence of virus-specific chicken antibodies by their ability to "block" the reaction between immobilised virus antigen and a virus-specific antibody (usually a monoclonal antibody)

Monoclonal antibody production and characterisation may be carried out and monoclonal antibodies may be used in the development of diagnostic tests such as tests used to detect serum antibody to the infectious agent.

Alternatively a PCR test may be used. Details of such a test are provided below

A prototype RT-PCR test has been developed in which Forward (SEQ ID NO 6) and Reverse primers (SEQ ID NO 7) were based on conserved sequences found within the 391nucleotide fragments specified by the 11672 and FP3 isolates (SEQ ID NO1-3) and amplified by the degenerate primer RT-PCR approach. The prototype test amplified a product of 187 base pairs (Figure 3) and was found to be 10 to 100 fold more sensitive than the method involving inoculation of cell culture followed by indirect immunofluorescence. The prototype test successfully detected CAstV-3 in gut content samples obtained from 1-day-old chicks hatched from from "sentinel" breeder chickens.

In this experiment, approximately 50 young females were sourced from a high health status flock. These were placed in pens within a diseased breeder flock, which was producing weak progeny chicks. Males from the diseased breeder flock were penned with the females. When the females began laying, eggs from the females were separately incubated, hatched and progeny chicks examined for the presence of CAstV-3. Twelve 1-day-old progeny chicks were investigated at weekly intervals. The chicks were killed and samples of intestine, liver, kidney and heart were collected from individual birds.

With each of the 12-chick submissions, the intestines from 6 chicks were pooled and processed by the ultracentrifugation method used to detect the

11672 ELV (above) in gut contents from uneven growth chicks. These preparations were inoculated onto CEL cells grown on coverslips, and, after 48 hr incubation, the inoculated cultures were stained for the presence of 11672 virus antigen using the previously described indirect IF test. No 11672 virus was detected in any of the preparations produced from the progeny submissions. (Table 5)

RNA was extracted from aliquots of the pooled intestinal samples (produced by ultracentrifugation) and tested by the prototype RT-PCR test. Of the 13 samples tested, 8 were positive for 11672 virus RNA. Positive samples were obtained intermittently from early submissions (7/3/05) to some of the late submissions (31/5/05). These results indicated that the sentinel adults had become infected with 11672 virus present in the house and that vertical transmission was likely to have occured. The detection profile, in which positive samples were detected over a 12-week period, suggested that spread of the 11672 virus was slow or that virus was transmission from the infected parent birds continued for long periods. Work is ongoing to determine whether the detection of CAstV-3 is associated with weakness or growth retardation problems in the progeny chicks.

Table 5: Detection of CAstV-3 in progeny chicks obtained from Sentinel breeder chickens

Submission/ Date	IIF with CEL cells	RT-PCR
1: 21/02/05	-	Nt
2: 28/02/05	-	Nt
3: 7/03/05	-	+
4: 14/03/05	-	+
5: 21/03/05	-	-
6 : 30/03/05	-	+
7: 4/04/05	-	-
8: 11/04/05	-	-
9: 18/04/05	-	+
10 25/04/05	-	-
11: 3/05/05	-	-
12: 9/05/05	-	+
13: 16/05/05	-	+
14: 23/05/05	-	+
15: 31/05/05	-	+
16: 6/06/05	-	Nt

Nt: not tested

The invention now being fully described, it will be apparent to one of ordinary skill in the art that changes and modifications may be made thereto without departing from the scope of the claims.

CLAIMS

1. An isolated astrovirus strain wherein said strain is that deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005.

2. An isolated nucleic acid comprising a nucleotide sequence which has at least 85% sequence identity to

(a) a nucleotide sequence as set out in any one of SEQ ID NOs 1 to 4 and encodes an antigenic polypeptide capable of eliciting an immune response against CAstV-3, or

(b) a fragment of a nucleotide sequence set forth in SEQ ID NO 4 wherein said fragment encodes an amino acid sequence of at least one antigenic site provided by a capsid protein of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005.

3. The isolated nucleic acid as claimed in claim 2 comprising the nucleotide sequence as set out in SEQ ID NO 4.

4. A gene construct comprising the isolated nucleic acid as claimed in claim 2 wherein the nucleic acid comprises a nucleotide sequence which has at least 85% sequence identity to

- the nucleotide sequence as set out in any one of SEQ ID NOs 1 to 4 and encodes an antigenic polypeptide capable of eliciting an immune response against CAstV-3, or

- a fragment of a nucleotide sequence set forth in SEQ ID NO 4 wherein said fragment encodes an amino acid sequence of at least one antigenic site provided by a capsid protein of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005, and a control sequence.

5. A vector comprising the isolated nucleic acid as claimed in claim 2

wherein the nucleic acid comprises a nucleotide sequence which has at least 85% sequence identity to

- the nucleotide sequence as set out in any one of SEQ ID NOs 1 to 4 and encodes an antigenic polypeptide capable of eliciting an immune response against CAstV-3, or

- a fragment of a nucleotide sequence set forth in SEQ ID NO 4 wherein said fragment encodes an amino acid sequence of at least one antigenic site provided by a capsid protein of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005, and a promoter which is operably linked to said nucleotide sequence.

6. A method of producing a polypeptide encoded by the nucleic acid of claim 2 wherein the nucleic acid comprises a nucleotide sequence which has at least 85% sequence identity to

- the nucleotide sequence as set out in any one of SEQ ID NOs 1 to 4 and encodes an antigenic polypeptide capable of eliciting an immune response against CAstV-3, or

- a fragment of a nucleotide sequence set forth in SEQ ID NO 4 wherein said fragment encodes an amino acid sequence of at least one antigenic site provided by a capsid protein of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005, comprising the steps of:

contacting at least one of a bacterial cell, an insect cell via a baculovirus, a yeast cell, and a plant cell with the vector as claimed in claim 5; and

cultivating at least one of said bacterial cell, said insect cell, said yeast cell, and said plant cell under conditions suitable for the production of the polypeptide.

7. A polypeptide comprising an amino acid sequence which has at least 85% sequence identity to a polypeptide with an amino acid sequence as set forth in SEQ ID NO 5 wherein the amino acid sequence provides at least one antigenic site provided by a capsid protein of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005 or a fragment thereof wherein said fragment is an amino acid sequence of at least one antigenic site provided by a capsid protein of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005.

8. The polypeptide as claimed in claim 7, comprising the amino acid sequence set forth in SEQ ID NO 5.

9. A polyclonal antibody wherein said polyclonal antibody has binding specificity to the polypeptide of claim 7 or 8.

10. A monoclonal antibody wherein said monoclonal antibody has binding specificity to the polypeptide of claim 7 or 8.

11. The use in the preparation of a composition to mediate an immune response in an avian against the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005 of:

(i) at least part of an isolated astrovirus strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005 as defined in claim 1,

(ii) the nucleic acid comprising a nucleotide sequence as claimed in claim 2 or 3, or

(iii) the polypeptide as claimed in any one of claims 7 or 8.

12. A composition comprising:

(i) an isolated astrovirus strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005 as defined in claim 1,

(ii) at least one nucleic acid comprising a nucleotide sequence as claimed in claim 2 or 3, or

(iii) at least one polypeptide as claimed in any one of claims 7 or 8; and

a pharmaceutical carrier or diluent.

13. A vaccine comprising the composition as claimed in claim 12 and an adjuvant for use in immunisation against growth depression in an avian caused by the astrovirus strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005.

14. A diagnostic assay method for the detection of chicken astrovirus in a sample from avians comprising the steps:

(i) contacting avian physiological material of the sample with a probe wherein said probe is selected from:

(a) a nucleic acid comprising a nucleotide sequence as set out in any one of SEQ ID NOs 1 to 4,

(b) a nucleic acid comprising a nucleotide sequence that hybridises under stringent conditions of a 0.2xSSC wash at 65 degrees Celsius for 15 minutes to a portion of the nucleotide sequence set forth in SEQ ID NO 4 wherein the portion of SEQ ID NO 4 encodes an amino acid sequence of at least one antigenic site provided by a capsid protein of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005,

(c) a nucleic acid comprising a nucleotide sequence which has at least 85% sequence identity to a nucleotide sequence as set out in SEQ ID NO 4 and encodes an antigenic polypeptide capable of eliciting an immune response against CAstV-3 wherein said nucleotide sequence encodes an amino acid sequence of at least one antigenic site provided by a capsid protein of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005,

(d) a fragment of a nucleotide sequence set forth in SEQ ID NO 4 wherein said fragment encodes an amino acid sequence of at least one antigenic site provided by a capsid protein of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005,

(e) a polypeptide encoded by the fragment of (d), or

(f) an antibody with binding specificity to the polypeptide of (e), and

(ii) detecting a successful binding event between the probe and at least one component of the sample.

15. A diagnostic assay method to detect the presence of CastV-3 in a sample comprising the steps:

- contacting avian physiological material of the sample with the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005,

- incubating the avian physiological material and at least part of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005 such that

antibody present in the sample with binding specificity to at least part of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005 binds the at least part of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005, and

- detecting the presence of said antibody bound to the at least part of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005,

wherein the detection of the presence of said antibody bound to the at least part of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005 indicates the presence of CastV-3 in the sample.

16. The diagnostic assay method as claimed in claim 14 wherein the probe is an antibody with binding specificity to an amino acid sequence of at least one antigenic site provided by the capsid protein of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005:

- wherein step (i) comprises contacting the sample of avian physiological material with an antibody with binding specificity to an amino acid sequence of at least one antigenic site provided by the capsid protein of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005,

- wherein step (ii) comprises:

detecting a successful binding event between the probe and at least one component of the sample, which comprises incubating the avian physiological material with said antibody such that chicken astrovirus present in the sample binds said antibody, and

detecting the presence of said chicken astrovirus present in the sample.

17. A diagnostic kit for use in the detection of the presence of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005, comprising a substrate for supporting a test sample; and a probe wherein said probe is at least one of

(a) a nucleic acid comprising the sequence as set out in any one of SEQ ID NOs 1 to 4 and encoding an antigenic polypeptide capable of eliciting an immune response against CAstV-3,

(b) a nucleic acid comprising a sequence that hybridises under stringent conditions of a 0.2xSSC wash at 65 degrees Celsius for 15 minutes to a portion of SEQ ID NO 4 wherein the portion of the nucleotide sequence set forth in SEQ ID NO 4 encodes an amino acid sequence of at least one antigenic site provided by the capsid protein of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005,

(c) a nucleic acid comprising a nucleotide sequence which has at least 85% sequence identity to a nucleotide sequence as set out in SEQ ID NO 4 wherein said nucleotide sequence encodes an amino acid sequence or a fragment thereof wherein said fragment is an amino acid sequence of at least one antigenic site provided by a capsid protein of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005,

(d) a fragment of a nucleotide sequence set forth in SEQ ID NO 4 wherein said fragment encodes an amino acid sequence of at least one antigenic site provided by a capsid protein of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005,

(e) a polypeptide encoded by the fragment of (d), or

(f) an antibody with binding specificity to the polypeptide of (e).

18. A probe comprising a nucleic acid comprising a nucleotide sequence that hybridises under stringent conditions of a 0.2xSSC wash at 65 degrees Celsius for 15 minutes to a portion of the nucleotide sequence set forth in SEQ ID NO 4 wherein the portion of the nucleotide sequence set forth in SEQ ID NO 4 encodes an amino acid sequence of at least one antigenic site provided by a capsid protein of the strain deposited under Accession number CNCM 1-3541 at CNCM, Institute Pasteur on 15 December 2005.

	ELV3(a)	ELV3(b)	CAstV-1 ANV	ELV1(a)	CASTV-2	ELV4	TAstV-1	TAstV-2
ELV3(a)	-	98.5	47.7	47.7	84.6	83.8	58.8	70.8
ELV3(b)		-	48.5	48.5	84.6	85.4	58.0	70.0
CAstV-1 ANV			-	92.2	49.2	49.2	45.8	51.5
ELV1(a)				-	50.8	50.0	46.6	52.2
CAstV-2					-	94.6	57.3	70.0
ELV4						-	56.5	70.0
TAstV-1							-	57.3
TAstV-2								-

Figure 1

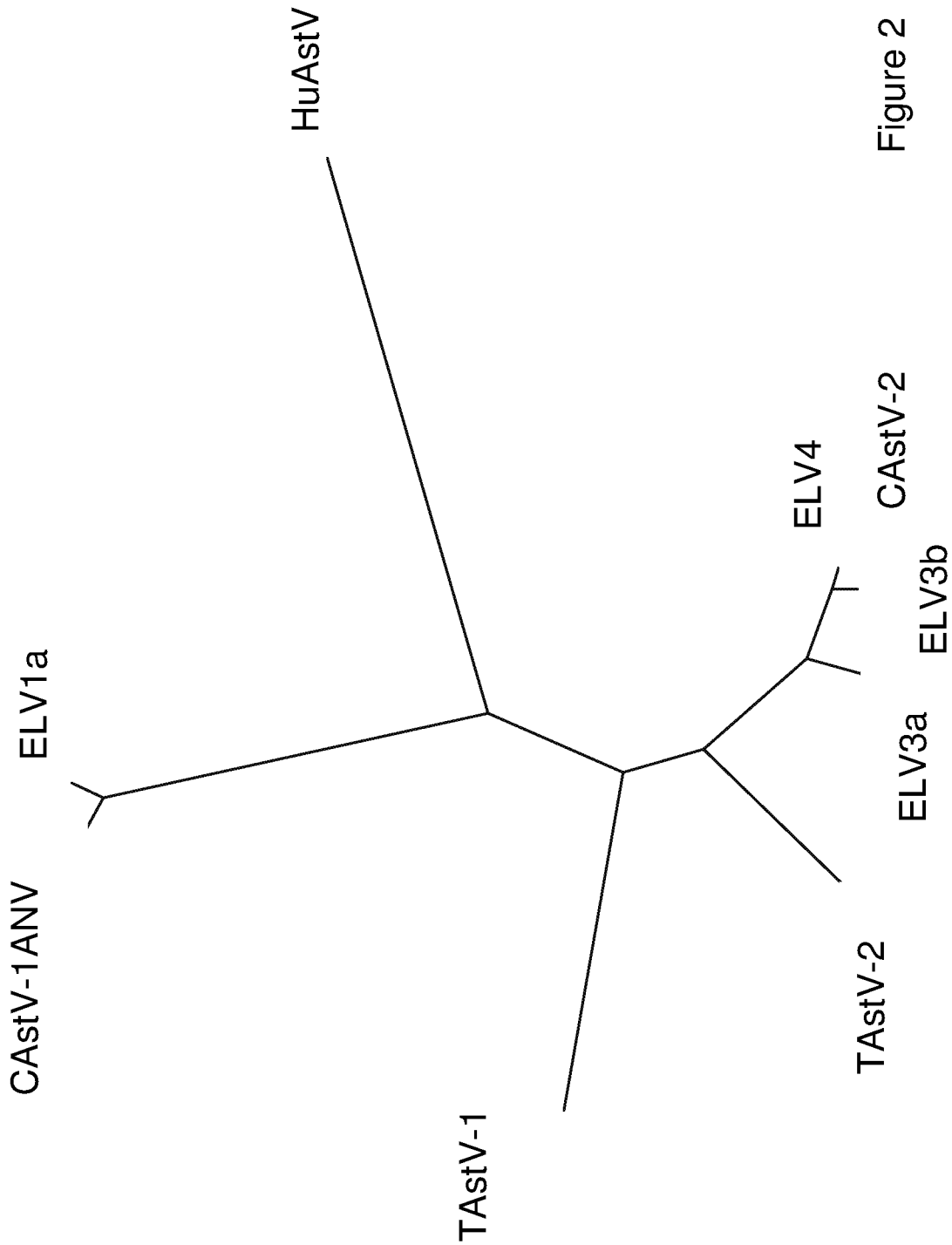
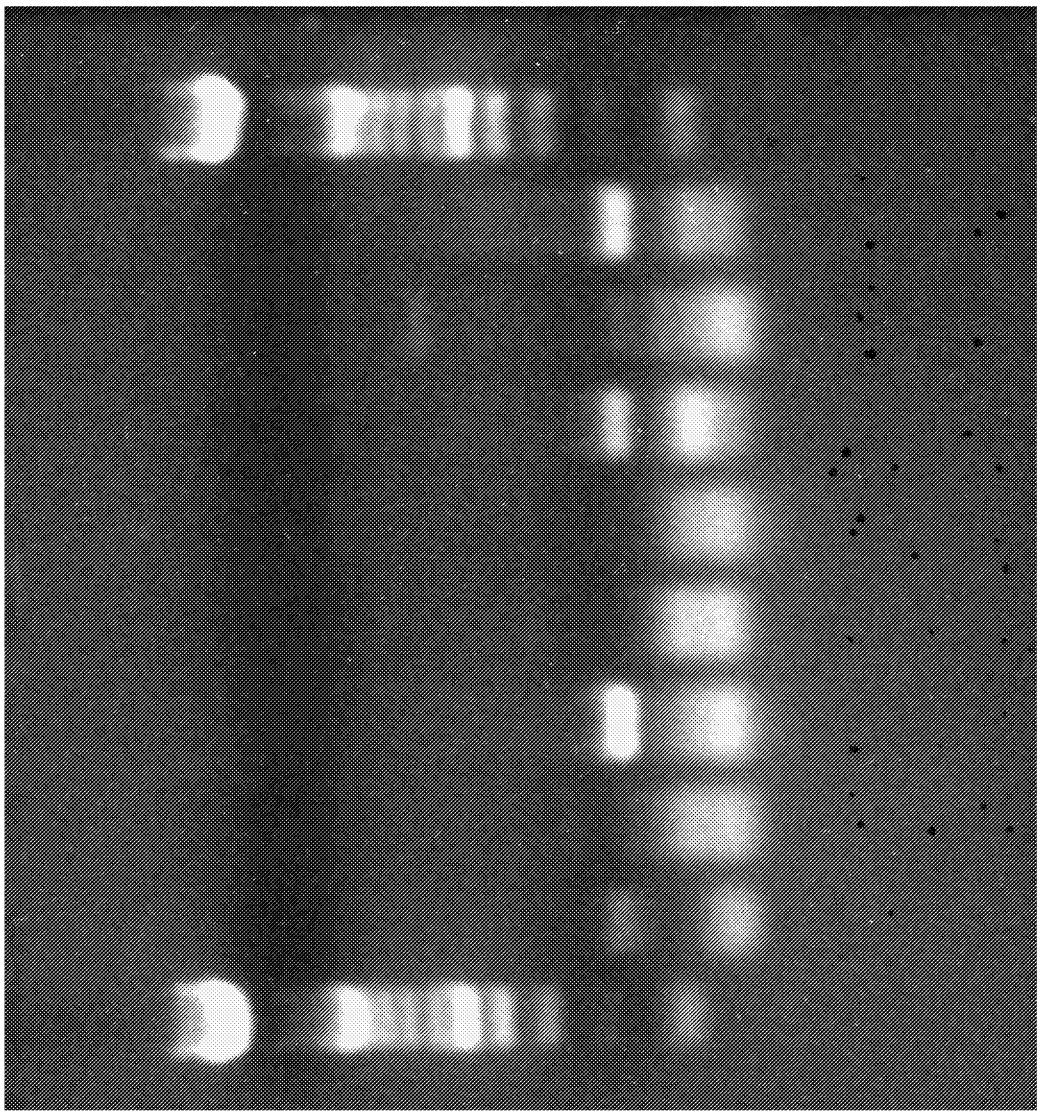

HuAstV
ELV1a
CAstV-1ANV
TAstV-1
ELV4
CAstV-2
ELV3b
ELV3a
TAstV-2

Figure 2



187bp RT-PCR product

Figure 3