Detection of Retroviral Infections and their Application to the Detection of a Novel Retrovirus isolated in Ostriches diagnosed with Ostrich Fading Syndrome

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Introduction

Retrovirus infections are common throughout much of vertebrate phylogeny, and are responsible for diseases of varied pathology. Infections, if not completely asymptomatic, may result in symptoms ranging from slowly progressive wasting diseases often associated with immunosuppression, to acute lymphoid malignancies. Infections with retroviruses may also involve extended periods of latency, during which time no infectious virus is detectable within the usually asymptomatic animal.[1]

Virally encoded reverse transcriptase mediates the conversion of the viral RNA genome into a DNA intermediate, enabling integration into the host genome. During this period the virus remains latent as a DNA provirus in infected cells. Subsequent activation of the virus then leads to the manifestation of the disease and presence of infectious viral particles in various clinical samples.

Various retrovirus detection methods are available, most having been developed through the substantial research into HIV detection. These detection methods can be divided into four groups, depending on the use of, either **viral culture**, **viral antigen detection**, **serological detection**, or **nucleic acid detection**. The viability of each detection method is dependent on the data already elucidated from genetic and antigenic studies on the virus in question.

Each method and its data requirements will be outlined, as well as its use as it pertains to the detection of a newly isolated, and uncharacterized ostrich retrovirus.

Ostrich Fading Syndrome

Ostrich Fading Syndrome (OFS) was first recognised in an Australian outbreak in 1995, resulting in an average morbidity of 71% (range 13 to 100%), and average mortality of 29% (range 9 to 50%), on 13 Victorian farms.

In Western Australia the mortality range was 20 to 80%, affecting chicks between 3 and 16 weeks of age. Since 1995, the number of notifications has steadily declined. The syndrome is generally characterised by a progressive wasting of chicks less than 6 months of age. The duration of clinical signs and survival rates increase with the age of the affected bird. Presence

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of anorexia, non-regenerative anemia, abdominal ascites, and dispersed necrotic and inflammatory lesions are all characteristic of the syndrome. [2]

Histologically, a consistent non-suppurative lymphoplasmacytic lesion can be seen in the small intestine, accompanied with intestinal epithelial hyperplasia and effusion with an amyloid-like protein. It is postulated that the presence of this lesion may induce a mal-adsorption syndrome.[2]

Affected ostriches older than 6 months show milder symptoms (anorexia, decreased activity) but tend to recover fully after several weeks[2]. Diverse aetiological investigations so far conducted on affected ostriches resulted in isolation of several bacterial, fungal, and viral species (e.g. avirulent Newcastle disease virus), none of which, however, could be convincingly linked to the syndrome. The majority of these isolates were also not considered to be virulent primary pathogens of avian species.[2]

Due to the presence of progressive wasting, anemia, poorly responsive bone marrow, and opportunistic pathogen infections, the involvement of an immunosuppressive agent such as a retrovirus is suspected.[2]

The Retrovirus Isolate

Primary leukocyte cultures derived from OFS-affected ostriches were established at the Animal Health Laboratories, Agriculture Western Australia by Dr Trevor Ellis, and found to contain a retrovirus. Electron micrograph analysis showed budding from the cell membrane similar to that of three retrovirus families: mammalian type-C, avian type-C, and lentiviruses, classified as C-type budding.

Reverse transcriptase activity was also detected in the culture supernatant. This is not unique to retroviruses, with hepadnaviruses such as Hepatitis B virus also coding for this enzyme. Hepadnaviruses, however, differ morphologically from retroviruses, and bud via a different mechanism.

As yet, any disease correlation studies using the isolated retrovirus have been hindered by the fact that it takes approximately 2 months to ascertain if infection is present. This is with the use of viral culture, the only current method available for detection. Since the syndrome affects chicks, rapid methods capable of confirming virus presence in chicks prior to experimental inoculation are not yet available.

Presently, primary macrophage cultures, derived from the ostrich chicks are established, and examined for evidence of infection for up to 60 days. If infection results, cytopathic effect (CPE) is usually noticed between days 35 to 45 of culture, but may take up to 60 days.[2] If the cell culture is continued for 60 days without evidence of CPE, the ostrich is then considered virus free.

It is because of the length of time taken to determine whether virus is present, that a more rapid assay technique must be developed. The required time also does not allow for determination of viral titres by conventional methods such as cell culture plaque assays.

Based on the premise that the isolate is a retrovirus, various detection methods that have so far been developed for detection of other retroviruses, can be analysed for their potential to

detect its presence in ostriches.

Viral Culture

In general, incubation of infection-permissive cell cultures with either; cell-free clinical samples, leukocytes (co-cultivation), or establishment of primary leukocyte cell cultures from the animal in question, allows for the detection of infectious virus particles with a relatively high degree of sensitivity.

When establishing primary cell cultures, or co-cultivating leukocytes, it should be realized that these also have the potential to detect latent virus. The use of virus culture for the detection of retroviral infection is also limited because of the need for infection-permissive cell lines, which are not available for all retroviruses.

Bioassays

The use of genetically engineered infection-permissive cell lines for retrovirus detection largely overcomes the problem of time taken for infection to be visualized. Rather than depending on the production of CPE to indicate infection, reporter genes have been integrated into the cell line genome. These reporter genes are attached to a promoter which activates the reporter gene only in the presence of viral proteins such as tat (transactivator) in HIV. Activation of the reporter gene will therefore only occur in infected cells.[9]

Reporter genes so far utilized include the Chloramphenicol Acetyl Transferase (CAT) gene, the Acetyl Transferase gene, and the β-galactosidase gene. The promoter used is the Long Terminal Repeat (LTR) region found in all retroviruses which, as a promoter, responds specifically to the presence of a virally encoded protein expressed only in infected cells.[9]

Testing of clinical samples for infectious retrovirus can then be completed in less time than a classical viral culture, and to the same or even greater sensitivity. This technique obviously requires a great deal of molecular data on the virus in question, and therefore cannot be used for detection of newly isolated, un-characterised retroviruses. This technique is also relatively expensive, and hence more suited to research purposes.

Serological detection

Several solid-phase immunoassays have been developed for the detection of circulating anti-viral antibodies.

ELISA

The Enzyme Linked Immunosorbant Assay (ELISA) is commonly used for detection of past and present retrovirus infections due to its speed, ease, and relatively low cost. This assay relies on the highly specific antigen/antibody interaction between a viral antigen, and its specific antibody, for the detection of circulating anti-retroviral antibodies in clinical samples..

Serum from the infected animal is incubated with prepared (recombinant or native) viral antigen bound to a hydrophilic surface (plastic or nitrocellulose membrane). Any anti-viral antibodies present in the sera bind to the immobilized viral antigen, and marker enzyme-conjugated antibodies specific for the already bound antibodies, then bind to the antigen-bound

antibodies. Binding of the antibody-conjugated enzyme results in an enzymatic colour reaction confirming presence of antiviral antibodies.

As viral infections often take months to elicit an immunological response, and sometimes never do, as is the case in many immunocompromised hosts (anti-HIV antibody detection in progressed AIDS is however successful). In these cases viral culture, nucleic acid detection, or antigen capture must be utilized.

Problems in detecting antibodies in mammal infants arise when transplacental maternal IgG transfer is considered. The presence of these (up to 18 months in circulation in humans) will obscure the presence of infant-produced antibodies.[3]

The above problem, in the case of HIV infection, has been overcome with the use of *in vitro* anti-HIV production (IVAP). This process involves the culture and washing of infant-derived peripheral blood mononuclear cells (PBMC's) and detection of anti-HIV antibody production from the infant's plasma cells once in *in vitro* culture.[3]

The same problem exists for avian species, due to the presence of maternally derived antibodies in the yolk sac and circulation of the chick for several weeks.

Western Blot (Immunoblot)

The Western Blot gives more information regarding antibody specificity to each particular protein making up the retrovirus than does ELISA. Whole virus proteins are fractionated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to their sizes, and are blotted directly onto a nitrocellulose membrane. Incubation of these strips (available commercially for HIV and HTLV detection) with clinical serum allows for any anti-viral protein antibody to bind to each protein. Binding of antibody is then visualized in a similar manner to ELISA.[8]

Immunofluorescence and Immunohistochemistry

Antibody-marker conjugates specific for viral proteins can also be utilised to detect infection in histological sections and cell culture. Markers may include fluorescent labels, or colour producing enzymes. Histological examination with either marker system enables localisation of the infection to certain cell types.

Virus Neutralizing Antibodies

With the availability of infectious, titratable virus, and a virus propagation system, virus-neutralizing antibody titres can be determined from clinical sera. Briefly, the incubation of known and constant titres of virus, with increasing dilutions of clinical serum leads to the neutralization of virus up until the point at which the antibody dilution is too great to neutralize.

Neutralization is determined by inoculation of the virus propagation system (infection permissive cell culture, or healthy animal). In the case of cell culture, infection can be visualised by detecting cells expressing viral protein. For this, any in-situ method utilising ant-viral protein can be used, namely the indirect immune fluorescent assay.

This method quantifies anti-viral antibodies and can, therefore, be used to determine whether a rising or waning antibody response is present. It has been successfully used in detection and quantification of human antibodies to HIV-1. [10]

Viral Antigen Detection

Antigen Capture

The principle is similar to the serological ELISA, however in this case, the presence of viral antigen in clinical samples is tested for instead. Antibodies from clinical samples, as used in serological detection, are this time replaced by commercially available, monoclonal anti-viral antibodies. (e.g. anti-p24 in HIV detection). These antibodies are immobilized on a surface, and incubated with the test serum. Any viral antigen bound to these antibodies are picked up by enzyme-conjugated antibody to the antigen and visualised by the colour reaction of the enzyme.[8]

Serum, plasma, CSF, and cell culture supernatant have all been used as suitable HIV antigen sources for ELISA. Even though there is a certain amount of viral antigen not associated with infectious particles, antigen levels are an adequate approximation of viral load and disease progression in HIV[5]. Also, this method, as with all antigen detection methods, benefits from its ability to detect virus in hosts not eliciting an immune response, but cannot be used for situations in which virus load is low.

Nucleic Acid Detection

Retroviral nucleic acid exists essentially as two entities; first as a linear single stranded RNA molecule within the infectious virion and infected cell cytoplasm, and secondly as a double stranded DNA provirus integrated into the cell genome, and to small extent free in the cytoplasm prior to genomic integration.[1]

PCR

The Polymerase Chain Reaction (PCR) is an enormously sensitive method of detecting and amplifying known sequences of DNA. The principle rests on the ability of short strands of synthesised DNA (primers) to hybridize to complementary sequences on target DNA (e.g. proviral DNA) and initiate DNA polymerization. When two such primers are designed to hybridize short distances apart (40 to 5,000bp) from each other, on opposite strands of the template DNA, their direction of polymerization is essentially towards each other, and replication of the region in between occurs.

Once this process is repeated several times (20-40 cycles), exponential amplification of the fragment in between the primers occurs due to the ability of the newly synthesised fragment to also act as a template, essentially doubling the amount of template each time. [11]

With the exponential increase in the amount of amplified fragment, the presence of original template DNA becomes negligible. In the case of retroviral DNA, primers can be designed based on viral DNA sequence data already elucidated from previous studies[11]. Any amplification of expected size fragments that occurs in clinical samples using these primers can usually be assumed to denote the presence of viral DNA. The presence of amplified fragments can be shown with DNA electrophoresis. Confirmation of the viral origin of the

amplified fragment can be made by DNA sequencing or commercial viral DNA probe hybridization.

RT-PCR

Reverse Transcriptase Polymerase Chain Reaction is a useful tool in the detection of viral RNA. It is essentially PCR, with its initial step utilizing the reverse transcriptase enzyme to convert RNA to cDNA. A PCR is then used to detect the cDNA. Where DNA detection is mainly an indicator of integrated virus, RNA detection is an indicator of active viral replication.[8,11] RT-PCR has become increasingly important in the detection of infectious HIV-1 in humans, and has also in the last few years, enabled quantification of HIV-1 viral load[12]. In the detection of HIV-1 in semen samples, RT-PCR was shown to be 5-125 times more sensitive than viral culture[6].

In Situ Hybridization

Hybridization of histological sections with labeled, characterised viral DNA fragments is a method generally confined to research, as it is time consuming, and technically difficult. Its ability to detect latently infected cells, and also localize infection to specific cell types, however, makes it a very useful research tool.[8]

The Un-characterised Isolate

The main aim of our current research is to design a rapid detection method, capable of detecting the virus in eggs or chicks. Essentially all that is known about the virus isolate (presumed retroviral), is that it expresses the reverse transcriptase enzyme, it has a diameter of 100nm (consistent with it being a retrovirus), shows C-type budding, and it replicates (be it tediously slow) in ostrich macrophages.[2]

Since no genetic or antigenic data has so far been derived, and the production of antigen (from viral culture) is minimal and time consuming, the initial detection method used must orientated towards detection of viral nucleic acid, and be based upon the assumption that the isolate is a retrovirus. The presence of maternally derived anti-viral antibodies in the eggs and chicks also eliminates the possibility of serological detection.

It is known that all retrovirus studied to date have conserved regions in the gene coding for reverse transcriptase. PCR Primers have previously been designed by Donehower, L.A. et al. 1990, to amplify a 135bp fragment between two of these regions. A major problem exists with the use of these primers on genomic DNA because of the presence of hundreds of endogenous retroviral sequences within the genome of most vertebrates, and some invertebrates, during evolution. These interfere with the use of any primers designed to amplify conserved retrovirus regions.[7]

It is hoped that we can then use these primers to amplify fragments, firstly from reverse transcribed viral RNA extracted from cell culture supernatant . If unsuccessful, cytoplasmic RNA or DNA will then be used as an amplification template. If that again does not lead to fragment amplification, genomic DNA will be used as the PCR template. Specificity of any genomic DNA amplified for the ostrich virus will then be tested for (as it is possibly amplified endogenous sequence), by *in situ* hybridization of infected and uninfected cells. Successful design of a rapid detection method will allow for the commencement of a disease association

study of the virus.

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