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Description

RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 61/294,048, filed January 11, 2010, and U.S. Provisional Application No. 61/316,193, March 22, 2010.

FIELD OF THE INVENTION

[0002] The invention relates to methods for analyzing samples for the presence of JC virus antibodies.

BACKGROUND

[0003] Progressive Multifocal Leukoencephalopathy (PML) is an opportunistic infection of the central nervous system (CNS) that is associated with exposure to the JC virus (JCV), a polyoma virus that is believed to be pathogenic in humans only under conditions of persistent immune suppression or immune modulation. While the presence of JCV is required for development of PML, PML risk is considered, in a not well-understood way, to be associated with the convergence of multiple viral and host-related factors that cause the virus to become pathogenic (Major, "Progressive Multifocal Leukoencephalopathy in Patients on Immunomodulatory Therapies" Annu. Rev. Med. 61:35-47 (2010) [2009 Aug. 31, Epub ahead of print]). Published studies reporting the prevalence of JCV infection in the human population are varied. This information is based on various types of studies including PCR analysis for viral DNA and detection of antibodies to JCV. Despite the prevalence of JCV in the population, infection with JCV rarely results in PML, even in individuals with documented immunosuppression.

[0004] Published reports on JCV DNA detection suggest the method to be insensitive and of limited use for assessing exposure to JCV because JCV DNA has been rarely and inconsistently detected in the plasma, serum or peripheral blood mononuclear cells of JCV-infected PML patients. Detection of anti-JCV antibodies appears to be a more sensitive marker of JCV infection; however the reported results are variable. In 1973, Padgett and Walker published a study reporting a JCV seroprevalence of 65-84% using a haemagglutination inhibition (HI) assay (Padgett and Walker, "Prevalence of antibodies in human sera agains JC virus, an isolate from a case of progressive multifocal leukoencephalopathy" J. Infect. Dis. 127:467-70, 1973). Later reports of JCV seroprevalence rates using the HI assay or ELISA have varied between 33-91%. The variable seroprevalence rates among these studies are likely due to marked differences in the size and demographics of the studies, and, perhaps most importantly, differences in assay methods.

[0005] It is therefore desirable to implement a reliable and sensitive assay for determining the presence of JCV antibodies that can be used, for example, for assessing whether an individual has been exposed to JCV.

SUMMARY OF THE INVENTION

[0006] The invention relates to the development of an analytically validated, sensitive assay for detecting the presence of JCV antibodies in a biological fluid, *e.g.*, serum or plasma. The scope of the invention is defined by the appended claims. Embodiments, disclosures, aspects, instances and descriptions no longer falling under the scope of the appended claims are considered merely as examples suitable for understanding the invention.

[0007] The present invention is an in vitro method for identifying a subject at risk of developing progressive multifocal leukoencephalopathy (PML) comprising a primary and secondary assay; wherein the primary assay comprises: (a) contacting a biological sample obtained from a subject with highly purified VP1 particles (HPVLPs), consisting predominantly of the VP1 protein of the JCV, immobilized on a solid substrate under conditions suitable for binding a JC virus (JCV) antibody in the sample to an HPVLP; (b) detecting the level of JCV antibody binding in the sample to HPVLPs; and (c) correlating the detected level with a reference level derived from a control sample or set of samples that is processed with the sample from the subject; and wherein the secondary assay comprises: (a) contacting a portion of the biological sample obtained from a subject with highly purified virus-like particles (HPVLPs), consisting predominantly of the VP1 protein of JCV, in solution under conditions suitable for binding of a JCV antibody in the sample to an HPVLP, thereby providing a pre-incubated sample; (b) contacting the pre-incubated sample with HPVLPs, consisting predominantly of the VP1 protein of the JCV, immobilized on a solid substrate under conditions suitable for binding of a JCV antibody in the sample to an HPVLP; (c) detecting the level of JCV antibody in the preincubated sample binding to the immobilized HPVLPs; and (d) comparing the detected level of JCV antibody in the pre-incubated biological sample to the level of JCV antibody detected in a biological sample obtained from the subject that was pre-incubated in a solution without HPVLPs, and was contacted with HPVLP immobilized on a solid substrate under conditions suitable for binding of a JCV antibody in the sample to an HPVLP, wherein the HPVLPs are composed of more than 5, at least 50, 150 or 360 VP1 polypeptides; wherein a decrease in the detected level of JCV antibody in the pre-incubated biological sample compared to the biological sample obtained from the subject that was pre-incubated in a solution without HPVLPs indicates that the sample is positive for JCV antibody and the subject is at an increased risk for developing PML, and a change in the detected level of JCV antibody below a specified percentage indicates that there is no JCV-specific antibody present in the sample.

[0008] In one embodiment, contacting the biological sample with HPVLPs in solution is for a period of time selected from 30 minutes, one hour, or overnight at 4°C.

[0009] In a preferred embodiment, the specified percentage is 40% and the method uses an HPVLP ELISA. Preferably, the reference level is selected to provide a false negative rate of 3% or less for detection of JCV antibodies in the sample obtained from the subject. Most preferably, the reference level is selected to provide a false negative rate of 1% or less for detection of JCV antibodies in the sample obtained from the subject.

[0010] In one embodiment, the biological sample obtained from the subject is classified as negative for JCV antibody when the level of JCV antibody in the pre-incubated biological sample binding to the immobilized HPVLPs is approximately the same as the level of JCV antibody detected in the biological sample obtained from the subject that was pre-incubated in a solution without HPVLPs.

[0011] In a preferred embodiment, the HPVLPs contain more than 1, at least 5, 10, 20, 30, 40, 50, 60, 70 or 72 VP1 pentamers. In some embodiments an HPVLP further comprises at least one of a JCV VP2, or a JCV VP3; or the VP1 in an HPVLP is a recombinant VP1; or at least one VP1 in the HPVLP is a mutant VP1.

[0012] Preferably, the biological sample is serum. In some embodiments, the biological sample is from a subject prescribed an immunomodulator or a subject considering taking an immunomodulator, wherein the immunomodulator is selected from an anti-VLA-4 therapy, and anti-CD20-therapy, an anti-CD11a therapy, or mycophenolate mofetil. The immunomodulator is preferably natalizumab.In some embodiments, the subject prescribed an immunomodulator, or considering taking an immunomodulator, has not previously been administered the immunomodulator. In other embodiments, the subject has previously received one or more doses of the immunomodulator.

[0013] In some embodiments, detection of JCV antibody binding to the HPVLPs indicates that the subject: (i) is not a candidate to receive treatment with an immunomodulator if the biological sample is positive for a JCV antibody; or (ii) is a candidate to receive treatment with an immunomodulator and enhanced monitoring for adverse symptoms upon treatment with the immunomodulator, optionally wherein the adverse symptoms indicate the development of PML.

[0014] In some embodiments, failure to detect JCV antibody binding to HPVLPs indicates that the subject is a candidate to receive treatment with an immunomodulator. The immunomodulator is preferably natalizumab.

[0015] In some embodiments, a subject having a biological sample determined not to have JCV antibodies in an initial testing is re-tested at least annually for the presence of JCV antibodies after the initial testing. In some embodiments, the subject who on a first date has been determined to have JCV antibodies is re-tested at a later date to determine if the subject does not have JCV antibodies.

[0016] In highly preferred embodiments, the subject has multiple sclerosis (MS) or Crohn's Disease (CD).

[0017] In some embodiments, the reference is selected to indicate a false negative rate not greater than 3% and minimal cross reactivity to other components of the sample such as antibodies against other polyoma viruses, *e.g.*, BK virus (BKV). The reference is derived from a control sample or set of samples that is processed with the sample from the subject. In some embodiments, the reference is selected such that the false negative rate of the assay is not greater than 1%.

[0018] In one instance , at least about 10% of the HPVLPs in a preparation of purified HPVLPs contain more than five VP1 polypeptides per HPVLP. In other instances , at least about 15%, about 20%, about 25%, about 30%, about 40%, about 50%, about 60%, about 65%, about 70%, about 80% or about 90% of the HPVLPs in a preparation of purified HPVLPs contain more than five VP1 polypeptides per HPVLP.

[0019] The HPVLP is immobilized on a solid substrate such as a microtiter plate or slide. The HPVLP consists essentially of VP1 viral protein. The HPVLP can further include other viral proteins, for example at least one of a JCV VP2 or a VP3. The viral protein(s) in the HPVLP can be recombinantly derived (*e.g.,* a MAD1 strain VP1) or can be a naturally-occurring viral protein (*e.g.*, derived from a naturally-occurring source). The method can be performed using, for example, a biological sample obtained from a subject currently being treated with an immunomodulatory drug, a subject considering initiating treatment with an immunomodulatory drug, or a subject suspected of having Progressive Multifocal Leukoencephalopathy (PML). The claimed method

[0020] is a two-step assay that includes a secondary confirmation assay process that includes contacting a portion of the biological sample from the subject with HPVLP in solution (prior to incubating the sample with the HPVLP attached to a solid substrate), thereby providing a secondary sample; contacting the secondary sample with HPVLP under the same conditions used for the primary assay; detecting the level of JCV antibody binding to HPVLP in the secondary sample; and comparing the detected level of JCV antibody in the secondary sample to the level of JCV antibody in the sample that was not preincubated with soluble HPVLP, such that a decrease in the detected level in the secondary assay sample compared to the sample that was not preincubated indicates the sample is positive for JCV antibody, and a change in the detected level below a specified percentage indicates that there is no JCV-specific antibody present in the sample.

[0021] An assay described herein can be used to assay for the presence of JCV antibodies in a subject who has never received treatment with an immunomodulator; or in a subject who has previously received an immunomodulator, but who is no longer receiving treatment with the immunomodulator; or in subject who is presently undergoing treatment with an immunomodulator.

[0022] Detection of JCV antibodies binding to the HPVLPs in an assay featured in the invention can indicate that a subject is at an increased risk for PML. Detection of JCV antibodies can also indicate that the subject is at an increased risk for adverse symptoms, such as the development of PML, upon administration of certain therapeutic agents, such as certain immunomodulators, and therefore the subject is not a candidate for treatment with these agent. For example, detection of JCV antibodies in a sample from a subject can indicate that the subject is not a candidate for treatment with an anti-VLA-4 therapeutic, such as natalizumab. Detection of JCV antibodies in a biological sample may indicate that the subject is a candidate for treatment with an immunomodulator, such as natalizumab, except that the subject will undergo enhanced monitoring during treatment than a subject who does not have detectable JVC antibodies. For example, the enhanced monitoring can include observation for adverse symptoms, such as symptoms that may indicate the development of PML.

[0023] Failure to detect JCV antibodies binding to HPVLPs in an assay featured in the invention can indicate that the subject is a candidate to receive treatment with an immunomodulator, such as natalizumab, and in one instance , the subject is further administered the immunomodulator. A subject determined not to have JCV antibodies can be re-tested at least annually (e.g., at least every 3 months, every 6 months, every 9 months, or every 12 months) to determine whether the subject has developed JCV antibodies, which may indicate that the subject has been infected with JCV. A subject who previously did not have detectable JCV antibodies in a biological sample, and who subsequently develops JCV antibodies in a biological sample, can stop receiving treatment with an immunomodulator.

[0024] In some embodiments, a subject who was previously identified as having JCV antibodies, can be subsequently tested at a later date and determined not to have JCV antibodies. These subjects can be determined to be candidates to receive treatment with an immunomodulator, such as natalizumab. In one instance , a subject who previously tested positive for the presence of JCV antibodies and who subsequently tested negative for JCV antibodies can be administered the immunomodulator, and undergo enhanced monitoring as compared to a subject who never tested positive for JCV antibodies, such as to monitor for symptoms that may indicate the development of PML.

[0025] An assay featured in the invention is useful to treat a subject having an immunological disease or disorder, such as multiple sclerosis (MS) or Crohn's Disease The claimed assay has been validated for use in MS and CD patients, and is effective to detect JCV antibodies in MS and CD patients in a controlled test environment, such as in a clinical trial.

[0026] In one aspect, the disclosure features a method of identifying a subject at risk of developing PML, such as by obtaining a biological sample from the subject; contacting the biological sample with HPVLPs under conditions suitable for binding of a JC Virus (JCV) antibody in the sample to an HPVLP; detecting the level of JCV antibody binding in the sample to HPVLPs; and correlating the detected level with a reference set, wherein the subject is at increased risk of PML if JCV antibody binding is detected. The reference set is selected to indicate a false negative rate of about 5%, about 3%, about 1% or less.

[0027] In another aspect, the disclosure features a method of identifying PML risk in a subject by determining the level of anti-JCV antibodies in a sample from the subject, such as from a plasma, blood or serum sample; and assigning a risk level to the subject according to the level of anti-JCV antibodies in the sample. The subject may be receiving an immunomodulatory therapy, such as an anti-VLA4 treatment, *e.g.*, natalizumab, or may be a candidate for receiving an immunomodulatory thereapy. In some instances , the subject has been diagnosed with an immunological disease or disorder, such as multiple sclerosis or Crohn's disease. In the invention , the level of anti-JCV antibodies is determined using a two-step assay. The two-step assay may include an ELISA assay.

[0028] The claimed method includes determining the level of anti-JCV antibodies in the subject in a sample from a date subsequent to the initial sample; comparing the level of anti-JCV antibodies in the sample from the subsequent date to the level in the sample from the initial sample; and determining whether the subject is at increased risk of PML at the subsequent date compared to the time of the initial sample.

[0029] The claimed method may be used for monitoring PML risk in a subject, the method comprising determining the level of anti-JCV antibodies in a subject using a sample from a first date; assigning a risk of PML (*e.g.*, high, or moderate or low risk) based on the level of anti-JCV antibodies in the subject on the first date; determining the level of anti-JCV antibodies in the subject using a sample from a second date; and assigning a risk of PML (*e.g.,* high, or moderate or low risk) based on the level of anti-JCV antibodies in the subject on the second date.

[0030] As used herein, an "HPVLP" is a highly purified VLP ("virus-like particle") consisting predominantly of the VP1 protein. An "HPVLP" featured in the invention is composed mainly of the major capsid protein "VP1," which can be a naturally-occurring VP1 or a recombinant VP1, from the polyomavirus, JC Virus (JCV). An HPVLP can be composed of, e.g., more than one pentameric subunit, at least 10 pentameric subunits, at least 20 pentameric subunits, at least 30 pentameric subunits, at least 50 pentameric subunits, at least seventy-two pentameric subunits or more of VP1. An

HPVLP may contain VP1 polypeptides in an undetermined configuration *(e.g.,* the polypeptides may or may not be organized in pentamers), in which case an HPVLP can be composed of more than 5 VP1 polypeptides, at least 50 VP1 polypeptides, at least 150 VP1 polypeptides, at least 360 VP1 polypeptides or more. HPVLPs include capsomeres, which contain about 10 to 24 pentamers. An HPVLP featured in the invention can bind antibodies against naturally-occurring, intact JC virus. In some embodiments, an HPVLP includes a second, and optionally a third, type of polypeptide that is a minor capsid protein of JC virus, *e.g.*, at least one VP2 or VP3 polypeptide. The VP2 or VP3 can be recombinant or naturally-occurring or naturally-derived polypeptides.

[0031] Such "highly purified" particles contain more than one VP1 pentamer, *e.g.*, at least 5, 10, 20, 30, 40, 50, 60, 70, 72 VP1 pentamers, or less than 100 VP1 pentamers. Such highly purified particles can be obtained, for example, by a method that involves double filtration. For example, a highly purified preparation of VLPs is obtained by purifying the particles at least twice by centrifugation, *e.g.*, through a sucrose cushion. In general, an HPVLP preparation can be identified by its activity in an ELISA assay using defined control samples. In some cases, such control samples are negative controls and/or control samples containing low levels of JCV antibodies.

[0032] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The materials, methods, and examples are illustrative only and not intended to be limiting.

[0033] The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. The present invention and embodiments thereof are set out in the appended claims.

DESCRIPTION OF THE DRAWINGS

[0034]

FIG. 1 is a graph depicting the results of an HPVLP ELISA on samples from subjects positive for JCV DNA in their urine (Uropositive) and negative for JCV DNA in their urine (Uronegative). The box represents the interquartile (IQR) range with the median line in the center; brackets represent observations within 1.5 times the IQR. "+" signs represent observations beyond 1.5 times the IQR (outliers). *Mann-Whitney U test.

FIG. 2 is a graph depicting anti-JCV antibody levels as measured by ELISA against urinary JCV DNA level as measured by qPCR (n=204). Open circles represent urine and serum samples collected at matched STRATA time points. Closed circles represent samples collected at different time points. For 17 samples with DNA test results below the level of quantitation (<500 copies/mL) the level was set to the detection limit.

FIG. 3 is a graph depicting BKV-JCV cross-reactivity data from one rabbit immunized with BKV. Antisera from the BKV-immunized rabbit bound BKV VLPs with high affinity (EC50 = 1:100,000) and cross-reacted with JCV VLPs (EC50 = 1:5,000).

FIGs. 4A and 4B depict the anti-JCV assay reactivity of serum samples from uronegative (n=311) (FIG. 4A) and uropositive (n=204) (FIG. 4B) patients in the screening and confirmation ELISAs. Distribution of serological reactivity of the samples in the screening ELISA are shown, with lower (nOD_{450} = 0.10) and upper (nOD_{450} = 0.25) cut points highlighted (left panels). In the supplemental confirmation ELISA (right panels), a 40% inhibition cut point is highlighted (vertical line) with shaded regions denoting samples that did not confirm to have anti-JCV specific antibodies (nOD_{450} ≤ 0.25 and percent inhibition ≤ 40%).

FIGs. 5A and 5B are histograms depicting the frequency of observations within each 10% inhibition range for all patients (n=515) (FIG. 5A) and uropositive patients (n=204) (FIG. 5B). The distribution consisted of two clearly defined peaks, most optimally separated at 40% inhibition. A 40% inhibition level corresponded to approximately the lower fifth percentile of the response distribution of uropositive samples.

FIGs. 6A and 6B are plots of nOD_{450} values from the screening ELISA (FIG. 6A) versus percent inhibition values from the confirmation ELISA (FIG. 5B) for the 11 pre-PML samples. Horizontal lines represent nOD_{450} values of 0.10 and 0.25, the vertical line represents percent inhibition of 40%.

DETAILED DESCRIPTION OF THE INVENTION

[0035] A sensitive assay for JCV antibodies that minimizes false negatives and minimizes detection of cross-reacting antibodies is useful for identification of individuals that have been exposed to JCV. Deployment of such a test may be useful in the identification of individuals who have a current JCV infection or have had sufficient past exposure to JCV to develop antibodies against the virus. Such an assay may also provide a tool to assist clinicians with PML clinical vigilance and risk stratification. For example, such a test may be useful for practitioners and patients as part of an evaluation of a patient's risk of developing PML by accurately assessing whether a subject has been exposed to JCV. In some cases, the analysis may include determining JCV antibody levels in a biological sample from the patient.

[0036] Certain difficulties lie in development of a useful assay for JCV antibodies, for example, the establishment of validated cut points. Applicants have solved this problem using data derived from assays of urine and plasma samples from patients that are uropositive or uronegative for JCV DNA. Another problem is developing an assay with specificity and reproducibility. Applicants have solved this problem by using a highly purified viral protein-containing particle in an antibody assay. In addition, applicants have discovered that the use of a secondary assay to resolve samples with ambiguous results in the primary assay improves the utility of the assay for providing a useful result for such samples.

[0037] Accordingly, an analytically validated assay that uses a highly purified VP1-containing virus-like particle (VLP) has been developed to detect the presence of JCV antibody in a body fluid, such as serum, plasma, urine, CSF, or other body fluid that contains antibodies. In experiments to validate the new assay, an approximately 54% prevalence of JCV antibodies in a population of MS patients enrolled in a clinical study was identified. A key feature of the assay described herein is the use of a highly purified viral-like particle (HPVLP).

[0038] One advantage of the assay described herein is that it has a relatively low false negative rate, *e.g.*, a false negative rate of about 10%, about 8%, about 6%, about 4%, about 3%, about 1% or less for the detection of antibodies to JCV. In general, the assay has a false negative rate of only about 3% or less for the detection of antibodies to JCV. As described herein the new assay can be used to monitor the serconversion rate for JCV. For example, the assay has been used to discover an annual seroconversion rate of no more than about 2% in a tested cohort of subjects who were initially negative for JCV antibody. This demonstrates that the assay can be useful for monitoring the JCV exposure status of an individual over time.

[0039] The assay can be used for the detection of JCV antibodies in any human subject, including a subject considering treatment with an immunomodulator, for example an anti-VLA-4 therapy (*e.g.,* natalizumab), an anti-CD20 therapy (*e.g.,* rituximab), an anti-CD11a therapy (*e.g.*, efalizumab), or mycophenolate mofetil; in a subject currently being treated with an immunomodulator; or a subject that has ceased treatment with an immunomodulator. The assay may be useful to others who may be susceptible to PML, such as individuals having lymphoproliferative disorders, such as multiple myeloma or a lymphoma; individuals infected with human immunodeficiency virus (HIV), or having acquired immune deficiency syndrome (AIDS), hematologic malignancies, or an autoimmune disease such as systemic lupus erythematosus (SLE), an inflammatory bowel disease, such as Crohn's Disease (CD) or ulcerative colitis, multiple sclerosis (MS) or arthritis, *e.g.*, rheumatoid arthritis (RA). The assay may also be useful to subjects receiving immunosuppressive or immunomodulatory therapies, such as transplant patients. Exemplary immunosuppressive or immunomodulatory therapies include natalizumab, rituximab, efalizumab, and mycophenolate mofetil. The assay can be useful for detection of JCV antibodies in a subject having a disorder, or being treated with a drug, disclosed in Piccinni et al. "Stronger association of drug-induced progressive multifocal leukoencephalopathy (PML) with biological immunomodulating agents" Eur. J. Clin. Pharmacol. 66:199-206, 2010.

VP1

[0040] It was discovered that the use of HPVLPs in an assay for JCV antibodies can improve the accuracy of the assay and is useful in an assay suitable for analytic and diagnostic purposes. VP1 for use in producing HPVLPs can be generated using methods known in the art and can be either naturally-occurring VP1 or recombinantly produced VP1, *e.g.,* a VP1 from a JCV virus. In general, the VP1 used is VP1 from MAD1 strain of JCV. The VP1 used in the assay may comprise VP1 from more than one JCV strain, for example, from one or more of strains 1A, 1B, 2A, 2B, 3, 4, and 7. After preparation of VP1, *e.g.*, recombinantly synthesized VP1, the VP1 for use in the assays described herein is then further purified through standard biochemical methods including density-gradient/ultracentrifugation methods, or a series of chemical precipitation steps, concentration/diafiltration and ion-exchange chromatography. The purification methods typically include a step to remove smaller proteins including monomer VP1 polypeptides, or pentamer VP1. The removal of these smaller particles can be done in, for example, in one step or in two steps (e.g., a first filtration step to remove VP1 monomers, and then a second filtration step to remove pentamer VP1 particles). Such biochemical purification methods are known to those in the art. Examples 1 and 7 provide two different methods of JCV VP1-VLP purification. The HPVLP can be prepared from recombinant VP1 or naturally-occurring VP1 (*e.g.*, isolated from virus or virus capsid). In some embodiments, additional JCV components, such as one or both of the minor coat proteins from JC virus, *e.g.*, VP2 or VP3, are included in the HPVLP particle or are associated with the substrate.

[0041] In some cases, recombinantly expressed VP1 may not assemble into pentamers or HPVLPs that resemble naturally-occurring viral capsids, for example, recombinantly expressed VP1 may assemble into tubes or other non-spherical geometries.

Methods of making HPVLP

[0042] HPVLPs can be made, for example, by transforming a baculovirus with a vector expressing a VP1 gene, such as a VP1 gene from a JC virus. The baculovirus is used to infect a cell culture, such as an insect cell culture (*e.g.*, SF9 cells) or a mammalian cell culture, and the cells express the VP1 protein. HPVLPs are isolated by lysing the cells, and purifying the particles through a series of centrifugation and ultrafiltration steps. In general, the purification is performed using methods such as sucrose cushion sedimentation, isopycnic ultracentrifugation and extensive ultrafiltration or other methods known to those in the art. The purification may include twice centrifuging the particles through a sucrose cushion. In an alternative purification method, cells are lysed, and particles are isolated by a series of precipitation and concentration/diafiltration steps with a final ion-exchange step.

[0043] Purity can be assessed using any suitable techniques known in the art, for example, analytical ultracentrifugation, electron microscopy, PAGE analysis, mass spectrometry, protein concentration, or activity in an ELISA with control sera. Insufficiently purified VLPs result in a high background yielding falsely high JCV antibody levels or calculated exposure rates.

[0044] In some embodiments, the HPVLPs contain VP1 as the sole JC virus protein.

[0045] In some embodiments, the HPVLPs are heterogeneous particles, and therefore include VP1 protein, and at least one of the minor coat proteins of JC virus, *e.g.*, VP2 or VP3. In another embodiment, the HPVLP includes VP1, VP2 and VP3 proteins. An HPVLP that includes VP1 and VP2 can be produced using methods known in the art, for example, by transforming a baculovirus with a nucleic acid including a VP1 and a VP2 gene, such as under the control of the same or different promoters. A cell culture is infected with the baculovirus, and the cells express VP1 and VP2, and HPVLPs form which include both types of proteins. The VP1 and VP2 genes are-on different DNA molecules, the DNA molecules are transformed into different baculoviruses and the baculoviruses are used to transfect cells in the same culture. The cells express the VP1 and VP2 proteins, and HPVLPs form which include both types of protein. In some cases, a heterogeneous HPVLP will include, e.g, one or two VP2 polypeptides for every five VP1 polypeptides. In general, an HPVLP will contain more VP1 polypeptides than VP2 polypeptides, as is the case in naturally-occurring JC virus.

[0046] An HPVLP that includes both VP1 and VP3 or both VP1 and VP2 molecules can be produced, for example, by transforming a baculovirus with a nucleic acid including a VP1 and a VP3 gene or a VP1 and VP2 gene, respectively, under the control of the same or different promoters. A cell culture is infected with the baculovirus, and the cells express VP1 and VP3 or VP1 and VP2, and HPVLPs form which include both types of proteins. The VP1 and VP3 or VP1 and VP2 genes may be on different DNA molecules, the DNA molecules are transformed into different baculoviruses, and the baculoviruses are used to transfect cells in the same culture. The cells express the VP1 and VP3 proteins or VP1 and VP2 genes, respectively, and HPVLPs form which include both types of protein. HPVLP particles can be isolated from such preparations using methods known in the art such as those used to isolate JCV capsids.

[0047] Typically, a VP1 pentamer that is in a heterogeneous HPVLP will include, *e.g,* five VP1 polypeptides and one VP3 polypeptide and/or one VP2 polypeptide, depending on whether a VP3 gene or VP2 gene was used to make the constructs. There will typically be more VP1 polypeptides than VP3 or VP2 polypeptides in an HPVLP. In some instances , the VP2 or VP3 is from a polyoma virus that is not a JC virus, *e.g.*, a BK virus polypeptide.

[0048] An HPVLP that includes all three of VP1 and VP2 and VP3 molecules can be produced by transforming a baculovirus with a nucleic acid (*e.g.,* a circular DNA, *e.g.,* < 5.5 kb) including a VP1, VP2 and VP3 gene, such as under the control of the same or different promoters. A cell culture, such as a mammalian cell culture, is infected with the baculovirus, and the cells express VP1, VP2 and VP3 proteins. HPVLPs consequently form which include all three types of proteins. The VP1, and either or both of the VP2 and VP3 genes may be on different DNA molecules, the DNA molecules are transformed into the same or different baculovirus, and the baculovirus are used to infect cells in the same or separate cultures. The cells express the VP1, VP2 and VP3 proteins, and HPVLPs form which include both types of protein. A heterogeneous HPVLP can include, *e.g*, five VP1 polypeptides and one each of VP2 and VP3 polypeptides, although the ratios may vary within a preparation. There will typically be more VP1 polypeptides than VP2 and VP3 polypeptides in an HPVLP.

[0049] The HPVLP may be greater in size than a VP1 pentamer. By greater in size, it is meant that the mass of protein contained in an HPVLP particle is greater than a pentamer containing solely VP1.

[0050] In general, an HPVLP preparation suitable for use in an assay will contain at least 20% HPVLPs, at least 25% HPVLPs, at least 40% HPVLPs, at least 60% HPVLPs, at least 65% HPVLPs, at least 70% HPVLPs, at least 80% HPVLPs, at least 85% HPVLPs, at least 90% HPVLPs, at least 95% HPVLPs, or at least 99% HPVPLs compared to non-HLVLP particles (*e.g.*, by percent of pentamers compared to VP1 monomers and aggregates containing fewer than five VP1 molecules).

Cut point

[0051] The invention provides methods of analysis that employ "cut points" to reduce false negative and false positive rates. The cut points are established based on data from the HPVLP assays (*e.g.*, to detect JCV antibodies in a biological sample), averaged, for example, between duplicate test samples and multiple replicates (for example, at least two, at least four, or at least eight replicates of control samples).

[0052] In one version of an assay according to the present disclosure, results from initial HPVLP screening assays, *e.g.*, ELISA assays, will cause a test sample to be classified as having or not having JCV-specific antibodies, or, if the sample does not fall under one of these two classifications, then the sample will be subjected to a supplemental confirmation assay. For example, samples that produce a result in an HPVLP ELISA assay featured in the invention less than an established level (*e.g.*, an $nOD_{450} < 0.1$) will be classified as lacking JCV-specific antibodies, and samples that provide a result in the ELISA greater than an established level (*e.g.*, an $nOD_{450} > 0.25$) will be classified as positive for JCV-specific antibodies. Samples that do not clearly fall into one of these classifications (*e.g.*, 0.1 < OD450 < 0.25) can be tested in a confirmatory assay. In the claimed method, In the claimed method, the confirmatory assay requires a pre-incubation step, where the test sample is pre-incubated with buffer (or other suitable solution) control or with HPVLPs (in buffer or other suitable solution) to pre-adsorb JCV-specific antibodies prior to analysis in an HPVLP ELISA, as described in further detail below. In one embodiment, if after pre-incubation with HPVLP if the reaction in the primary assay decreases by less than 40% compared to buffer control, then the sample is interpreted to be negative for the presence of JCV-specific antibodies. If the results show a ≥40% reduction in reaction compared to buffer control in the primary assay after pre-incubation with HPVLP then the sample is interpreted to contain JCV specific antibodies.

[0053] An example of a method for selecting and verifying suitable cut points is provided in Example 4.

Substrate

[0054] Any suitable solid substrate can be used for the HPVLP assay format. The substrate may be a microtiter plate (*e.g.,* a 96-well plate) a slide, a bead, or a column. The substrate can be suitable for chromogenic or chemiluminescent detection methods.

Assay

[0055] Assays are conducted by adding a biological sample to a substrate that has been coated with an HPVLP and detected using methods known in the art. In general, a solid base platform is used such as a microtiter plate (for example, a 96 well plate); although other formats known in the art can be used. The biological sample may be diluted prior to use in an assay.

[0056] The assay format may be an enzyme-linked immunoassay (ELISA). Broadly, the method typically includes coating the substrate with capture antigen such as HPVLP, incubating sample containing binding antibodies directed to capture reagent, washing to remove non-specifically bound species, and detecting the bound immune complexes, *e.g.*, by a chromogenic or chemiluminescent assay. Chromogenic substrates produce a colored end product, which can be detected and measured visually or with the use of a spectrophotometer. Chemiluminescent substrates produce light, which can be measured using a luminometer.

[0057] Coating a plate with HPVLP generally includes incubating the solid substrate (such as wells of a microtiter plate) with a solution of HPVLP at a suitable concentration (*e.g.,* 1 μg/ml), either overnight or for a specified number of hours. The HPVLP can include VP1 as the only JCV viral component, or the HPVLP can be a heterologous particle, that contains at least one of VP2 or VP3 per particle or at least one each of VP2 and VP3 per particle. After coating with the HPVLP, the wells of the plate are washed. The substrate is then "coated" with a nonspecific protein that is antigenically neutral with regard to the samples to be tested. Suitable coating materials are known in the art and include bovine serum albumin (BSA), casein or solutions of milk powder.

[0058] The sample or reference is incubated on the prepared substrate under conditions effective to permit complex formation (HPVLP/JCV antibody), thus forming a bound complex. Detection of the bound complex is performed using a labeled antibody that can bind to human antibody. In general, the labeled antibody can detect human IgG or human IgG and IgM. In some cases, the assay can be performed using secondary or tertiary detection methods.

[0059] A reference sample can be of the same biological material (*e.g.*, plasma, serum, urine, or CSF) isolated from an individual known to be infected with JC virus based on the presence of JCV DNA in urine of the individual (uropositive). A reference sample is used to establish the assay cut point such that the false negative rate of the assay is not greater than 1%-3%.

[0060] "Under conditions effective to permit complex formation" generally means conditions in which the reagents have been diluted to reduce background and provide readouts of results that lie within a specified range. Diluents can include, in non-limiting examples, solutions that include BSA, phosphate buffered saline (PBS), or PBS containing Tween.

[0061] "Suitable" conditions also include conditions that are at a temperature and/or for a period of time sufficient to allow effective binding. Incubations are typically from about one to two hours or one to four hours, at temperatures of approximately 25°C to 27°C, or may be overnight at about 4°C. However, those in the art will understand that other conditions may be suitable.

[0062] In general, one or more washes are conducted between the incubations of the assay. Appropriate wash solutions include diluent buffer (*e.g.*, PBS or PBS/Tween) or borate buffer.

[0063] In general, the detection of antibody bound to HPVLP is performed using methods well known in the art. In general, such methods are based on the detection of a label or marker, such as a radioactive, fluorescent, biological or enzymatic tag. U.S. patents concerning the use of such labels include, for example, U.S. Pat. Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149 and 4,366,241. In general, the detection of JCV antibody binding is detected using a secondary antibody that is labeled. In general, the secondary antibody is specific for detecting human IgG. Quantification is achieved by measuring the degree of color generated, *e.g.*, using a visible spectra spectrophotometer.

[0064] Example 2 illustrates a method of performing the assay and those in the art will understand that suitable modifications can be made.

[0065] The assay may be performed in a medical office, such as by a healthcare provider, *e.g.*, a doctor, a nurse or a technician, working in a facility where the biological sample is obtained from a patient. The biological sample obtained from a patient may be transported to another facility, *e.g.*, to a third party facility, where the assay is performed. In this latter case, the results of the assay can be reported back to the healthcare provider, such as through a form, which can be submitted by mail or electronically (*e.g.*, through facsimile or e-mail) or through an on-line database. The results of the assay (including the screening assay and, optionally, a confirmatory assay) may be stored in a database and can be accessed by a healthcare provider, such as through the worldwide web.

Secondary Test

[0066] In the present invention , a secondary test (also referred to herein as a "confirmatory assay") of the sample is employed. For the secondary test, two aliquots of a biological sample are used. The first is prepared prior to use in the assay by preincubating the sample in the presence of assay buffer in solution for a period of time *(e.g.,* for 30 minutes, one hour, or longer such as overnight at 4°C). The second aliquot is prepared prior to use in the assay by preincubating the sample in the presence of HPVLP in solution for a period of time *(e.g.,* for 30 minutes, or one hour or longer). The two aliquots are then used in the HPVLP assay as described herein, and the assignment of the sample to JCV antibody positive or antibody negative is made. If the assay results for the aliquot incubated with HPVLP in solution is the same as for the first aliquot incubated with buffer in the primary assay (*i.e.*, approximately the same OD), then the sample is interpreted to be negative for the presence of JCV-specific antibodies. If the assay results are lower after pre-incubation (*i.e.*, in the secondary assay), then the sample is interpreted to contain JCV specific antibodies.

[0067] The assay of the invention is also referred to herein as a "two-step test" or a "two-step assay."

Reporting of Assay Results

[0068] The assay includes a read out that can be a level (*e.g.*, OD) relative to a reference or a read out that is an evaluation of whether the sample is positive, negative, or indeterminate for the presence of JCV antibodies.

[0069] The HPVLP can be provided in any form, *e.g.*, liquid, dried, semi-dried, or lyophilized form, or in a form for storage in a frozen condition. Prepared HPVLPs may be pelleted and stored in a semi-solid form.

[0070] Typically, HPVLPs are provided in a form that is sterile. When HPVLP is provided in a liquid solution, the liquid solution generally is an aqueous solution, *e.g.*, a sterile aqueous solution. When the HPVLP is provided as a dried form, reconstitution generally is accomplished by the addition of a suitable solvent. The solvent, *e.g.*, sterile buffer, can optionally be provided in the kit.

[0071] In some instances , a biological sample is provided to an assay provider, *e.g.*, a service provider (such as a third party facility) or a healthcare provider, who evaluates the sample in an assay and provides a read out. For example, in one instance , an assay provider receives a biological sample from a subject, such as a plasma, blood or serum sample, and evaluates the sample using an assay described herein, and determines that the sample contains JCV antibodies. The assay provider, *e.g.*, a service provider or healthcare provider, can then conclude that the subject is at increased risk for PML. The assay provider can further determine that the subject is not a candidate to receive treatment with an immunomodulator, such as an anti-VLA therapy, such as natalizumab, or that the subject is a candidate to receive treatment with an immunomodulator, but the candidate will have enhanced monitoring as compared to a subject who is determined not to have JCV antibodies. For example, the candidate will be examined more frequently for the development of adverse symptoms, such as symptoms that may indicate the development of PML.

[0072] The assay provider may perform an assay described herein and determines that a subject does not have detectable JCV antibodies. The assay provider further determines that the subject is a candidate to receive treatment with an immunomodulator, such as natalizumab. The assay provider may inform a healthcare provider that the subject is a candidate for treatment with the immunomodulator, and the candidate is administered the immunomodulator.

[0073] The assay provider can provide the results of the evaluation, and optionally, conclusions regarding one or more of diagnosis, prognosis, or appropriate therapy options to, for example, a healthcare provider, or patient, or an insurance company, in any suitable format, such as by mail or electronically, or through an online database. The information collected and provided by the assay provider can be stored in a database.

[0074] The invention is further illustrated by the following examples, which should not be construed as further limiting.

EXAMPLES

Example 1: Synthesis and Purification of Highly Purified VP1 Particles

[0075] HPVLPs consisting of JCV or BKV capsid protein VP1 were produced in SF9 insect cells transfected with a recombinant baculovirus. In the case of JCV VP1 containing particles, recombinant baculovirus was transformed with a nucleic acid expressing VP1 from the Mad-1 strain of JCV. The recombinant VLP was harvested prior to cell lysis and was purified by differential ultracentrifugation, detergent washing and ultrafiltration.

[0076] Briefly, baculovirus infected cells were harvested about three days post infection by centrifugation at 3000 × G and stored frozen until purification of HPVLPs. Purification was performed using about 100 grams of frozen cell pellets. Thawed cells were lysed in 500 ml of PBS supplemented with 0.1 mM $CaCl_2$ (PBS-C). The cells were disrupted by passing the cell suspension twice through a Microfluidics Microfluidizer®. Cell debris was removed by pelleting at 8000 × G for 15 minutes. The supernatant volume was adjusted to 720 ml with PBS-C and loaded onto 5 ml 40% sucrose cushions. HPVLPs were twice pelleted through the sucrose cushions in a SW28 rotor at 100,000×G for 5 hours. The HPVLP pellets were resuspended in PBS-$CaCl_2$ and then treated with 0.25% deoxycholate for 1 hour at 37°C followed by the addition of 4 M NaCl supplemented with 0.1 mM $CaCl_2$ for 1 hour at 4°C. Precipitated material was removed by centrifugation at 8000 × G for 15 minutes. The resulting supernatant was concentrated and buffer exchanged by ultra-filtration through a Pelicon-2 500,000 MWCO membrane (Millipore). The concentrated VLPs were applied to the center of a 25-40% step gradient of Optiprep™ (Sigma, St. Louis, MO) and banded at 190,000 g for 17 hours in a Type 50.2 rotor. VLP bands were collected and then concentrated and buffer exchanged in an Amicon stirred cell (Millipore) with a 300,000 MWCO (molecular weight cut-off) membrane. The concentrated material was filtered through a 0.22 μ PES (polyethersulfone) filter and stored at 4°C. VLPs prepared in this way are termed HPVLPs herein. VLP quality is generally determined by gel electrophoresis and electron microscopy.

[0077] To denature the VLPs for protein determination, EDTA, DTT and SDS were added to final concentrations of 2mM, 2mM and 2% respectively. The concentration of the fully denatured protein was determined by using the Pierce BCA (bicinchoninic acid) assay.

[0078] For analysis by gel electrophoresis, a sufficient volume to give 2 μg to 5 μg of total protein was loaded on precast 4% to 20% polyacrylamide gels (NOVEX, San Diego, CA) by using a NuPAGE® morpholineethanesulfonic acid-SDS buffer system (Invitrogen, Carlsbad, CA). The gels were electrophoresed at a constant current of 70 mA/gel to 80 mA/gel for 30 minutes. Protein bands were fixed with 50% methanol and 10% acetic acid in distilled water and visualized with a commercial colloidal Coomassie blue reagent (Invitrogen) according to the recommendations of the manufacturer.

[0079] VLPs were evaluated using electron microscopy. VLP samples were placed on carbon grids, briefly washed in water and negatively stained with uranyl acetate and allowed to dry. The grids were viewed and imaged on a Tecnai™ G2 Spirit BioTWIN TEM.

[0080] An alternative JCV VP1-VLP purification method is presented below, at Example 7.

Example 2: Example of an HPVLP Antibody assay not claimed as the invention

[0081] A sensitive assay for anti-JCV antibodies was developed using the HPVLPs and can be used in connection with the confirmatory assay that is the claimed method. It is described herein and is referred to herein as an HPVLP assay. In an example of the assay, 96 well microtiter plates were prepared by adding a solution containing HPVLP at a concentration of 1 μg/ml and incubating the plate overnight at 4°C. The wells were rinsed with diluent buffer and then blocked for one hour at room temperature with Casein Blocking Buffer and rinsed with diluent buffer. The assay controls and serum or plasma samples were diluted 1:200 in assay diluent. The diluted samples and controls were added to wells and incubated for one hour at room temperature and washed with diluent buffer. Detection was performed using donkey anti-human-HRP antibody (IgG), which was added to the wells and incubated at room temperature for one hour. Plates were then washed and TMB (3,3',5,5'-tetramethylbenzidine) buffer (Chromagen, Inc., San Diego, CA) was added. After a development for a time suitable to permit color to develop (about 20 minutes), the reaction was stopped with 1 N H_2SO_4, and the absorbance at 450 nm was read. Levels of anti-JCV antibody in the samples were expressed as OD units.

[0082] The assay was interpreted as described below using the OD units to determine levels.

[0083] If unknown samples produced greater than 40% competitive inhibition of binding with HPVLP in solution, the sample was considered JCV+ (JCV positive), with <40% inhibition being scored as JCV- (JCV negative).

[0084] Initially, samples with OD values greater than the cut point OD (mean Negative Control OD × 1.23) were defined as positive for the presence of JCV antibodies, whereas samples with OD values equal to or less than the cut point OD were defined as negative.

[0085] Controls used in the assay were selected based on target OD and specificity (as determined in the secondary confirmation assay for specificity (described *infra*) and included Positive Control 1, which was pooled donor sera with high reactivity in the assay defined as having target OD value of about 1.0 and for specificity, competed with JCV >80%; Positive Control 2, which contained pooled donor sera with lower reactivity in assay defined as having a target OD value of about 0.25 in the assay; and for specificity competed with JCV >80%; and Negative Control, which was pooled donor sera with reactivity similar to buffer control in assay having a target OD value of approximately 0.07 (note that the assay buffer has an O.D. value of approximately 0.045).

[0086] In some cases, a titration assay was conducted in which positive samples were tested at multiple dilutions, and the highest dilution giving an OD value greater than the cut point OD was defined as the JCV IgG titer.

[0087] The assays have been validated from the perspective of specificity, precision, matrix interference, robustness, and reagent stability.

Example 3: Secondary Confirmation Assay

[0088] In the method according to the invention a secondary confirmation assay (secondary assay) was carried out in addition to the test described *supra.* In the confirmation assay, samples (plasma or serum) were incubated with HPVLP (final VLP concentration = 1 μg/mL; final sample dilution = 1:200) for one hour at room temperature prior to use in the assay. Control samples were incubated in assay buffer, and not in the presence of HPVLP. The assay was then conducted as described above. A percent nOD_{450} inhibition was calculated as: % inhibition = 100 × [1- (average nOD_{450}) (JCV MAD-1 VLP pre-incubated samples) ÷ (average nOD_{450}) (buffer incubated samples)].

[0089] If the assay results were the same after pre-incubation with buffer as in the primary assay *(i.e.,* approximately the same O.D.), then the sample was interpreted to be negative for the presence of JCV-specific antibodies. If the assay results were lower after pre-incubation with HPVLPs *(i.e.,* in the secondary assay), then the sample was interpreted to contain JCV-specific antibodies.

Example 4: Screening/Confirmation Assay Cut Point Algorithm

[0090] The serological test (JCV antibody test) was configured as a two-step assay: a screening ELISA and a supplemental confirmation ELISA (secondary assay).

[0091] For comparison of results between assay plates, assay runs, and analysts, sample results were normalized to the optical density (OD_{450}) value of the positive control on the plate and reported as normalized OD_{450} as described below.

[0092] To implement the utility of the HPVP assay, cut points were derived using a Weibull three component mixture-distribution model. In these determinations, the following definitions were used:

$$\text{Screening assay normalized OD (nOD)}=\frac{avg(sample\_OD\_duplicates)}{avg(PC1\_OD\_replicates)};$$

[0093] For example:

Average (sample_OD_duplicates) = 0.60
Average (Positive Control 1 OD_replicates) = 1.20
Normalized OD=0.60/1.20=0.50.

For the Confirmation Assay

[0094]

$$\text{Confirmation assay \% inhibition}= 100\% \times (1-\frac{competition\_sample\_OD}{noncompetition\_sample\_OD}$$

[0095] In the supplemental confirmation ELISA, soluble HPVLP was used to pre-adsorb high affinity antibodies against JCV in samples prior to evaluation of the samples in the screening ELISA. Results were calculated as percent inhibition to determine decreases in reactivity in the screening ELISA after the samples were pre-adsorbed with HPVLP [% inhibition = 100 × [1- (average nOD_{450} HPVLP pre-incubated samples) ÷ (average nOD_{450} buffer incubated samples)].

[0096] False positive and false negative rates were defined as follows. The false negative rate is the proportion of true JC virus positive samples that are determined to be antibody negative by the assay. The sero-positive rate is the proportion of samples determined to be sero-positive (*i.e.*, have JCV antibodies as determined using the anti-JCV screening/confirmation cut point algorithm).

[0097] Data were analyzed using SAS v9. Data not demonstrating a normal distribution were analyzed by the Mann-Whitney U test. Categorical data were analyzed using Pearson's $\chi 2$ test or Fisher's exact test depending on the sample size. Pearson's correlation coefficient was used to asses the relationship between nOD_{450} and urinary JCV DNA levels. All tests were two-sided at an alpha level of 0.05. Confidence limits for the seroprevalence and false-negative rates were obtained by the bootstrap percentile method (6) using 10,000 bootstraps.

Example 4(a): Serological Reactivity to JCV

[0098] A study was conducted to establish an assay to detect anti-JCV antibodies in MS patients and to conduct a preliminary evaluation of the potential clinical utility of the assay for PML risk stratification. To characterize antibody responses against infectious agents in humans, it was critical to have reference sera from both infected and noninfected individuals. While the asymptomatic nature of JCV infection makes it impossible to identify "true" negative individuals, Applicants were able to identify a population of "true" positive individuals by measuring JCV DNA in the urine of "uropositive" individuals.

[0099] Urinary JCV DNA levels (collected in the STRATA (natalizumab reinitiation of dosing) clinical trial protocol) were determined by a quantitative real-time polymerase chain reaction (q-PCR) assay (ViraCor Laboratories, Lee's Summit, MO) with a limit of quantitation of 500 copies/mL and a limit of detection of 50 copies/mL.

[0100] The anti-JCV antibody status of 831 MS patient serum samples, which included samples from 204 JCV uropositive patients, was initially evaluated for anti-JCV antibodies in a screening ELISA to determine the distribution of serological responses. The assay results by urinary DNA status showed the presence of two overlapping yet distinct populations of JCV IgG reactivity (FIG. 1). The median level of reactivity for JCV DNA uropositive MS patients (nOD_{450} = 0.895) was significantly higher than for JCV DNA uronegative MS patients (nOD_{450} = 0.131; p<0.001), and no uropositive patient showed assay reactivity below a nOD_{450} of 0.10. Therefore, a lower assay cut point was established at nOD_{450} 0.10, wherein the empirical false-negative rate in the negative zone was 0%.

[0101] Many patients with no detectable JCV DNA in the urine (uronegatives) had serological reactivity similar to that of uropositive patients. These results are consistent with the assumption that a urine JCV DNA test is likely to fail to detect all JCV infected individuals.

Example 4(b): Urinary JCV DNA Load and Serological Activity

[0102] To address the potential concern that JCV infected patients with low levels of viral replication may have low serum antibody levels that are not detected in the serological assay (potential false negatives) the correlation between viral levels and antibody reactivity were examined. FIG. 2 shows data from the 204 JCV DNA uropositive STRATA patients, and illustrates that there is no detectable relationship between urinary JCV DNA levels and anti-JCV antibody levels in samples with nOD_{450} below 0.60 (Pearson's correlation coefficient = 0.048, p=0.751). This result holds true even if the urine and serum were collected at the same STRATA study time point (Pearson's correlation coefficient = 0.002,p=0.993). At nOD_{450}> 0.60, a stronger correlation was observed with a higher proportion of serum samples from individuals with high JCV DNA copies/mL exhibiting higher nOD_{450} values, consistent with literature reports (*e.g.*, Egli et al., J. Infect. Dis. 199:837-846, 2009). These data suggest that seronegative results are likely due to an absence of JCV infection, rather than to very low viral levels.

Example 4(c): Assessment of BKV-JCV Cross Reactivity

[0103] Assignment of a single conservative cut-point that controls the false-negative rate at 0% is unlikely to exclude detection of antibodies that cross-react to other common polyoma viruses (false positives), such as anti-BKV antibodies, which share high identity to JCV in the VP1 capsid protein. Additionally, such antibody cross-reactivity may occur through exposure of conserved viral epitopes when the HPVLP is directly coated onto the ELISA plate. Because dual infections with BKV and JCV may occur in humans and it is not possible to reliably identify patients who have been infected with BKV and not JCV, the issue of cross-reactivity was examined in rabbits, a species in which natural infection with either BKV or JCV cannot occur.

[0104] Rabbits were immunized with BKV by subcutaneous injection of proteins in phosphate-buffered saline without adjuvant, followed by three booster injections over a three month period. Serum samples were assayed for direct binding to JCV or BKV by ELISA. Antisera from BKV-immunized rabbits bound BKV VLPs with high affinity (EC50 = 1:100,000) and cross-reacted with HPVLPs with lower affinity (EC50 = 1:5,000). Pre-immune sera showed no reactivity. Representative data from one rabbit are shown in FIG. 3.

[0105] Because BKV antibodies cross-reacted with JCV, thus producing a false positive signal in the anti-JCV assay (FIG. 3), low level reactivity against JCV in humans could represent low affinity anti-BKV antibodies that cross-react with JCV to produce falsepositive signals.

Example 4(d): Measuring JCV-Specific Antibody Response (Supplemental Confirmation ELISA)

[0106] To distinguish patients with JCV-specific antibodies from those with potentially low affinity, cross reactive antibodies, a competition ELISA was developed using soluble HPVLP (secondary assay). JCV-specific higher affinity antibodies were expected to be more effectively competed by the soluble antigen, whereas lower affinity antibodies may detach from the complexes formed with the JCV antigen in solution and bind to the JCV VLP coated on the ELISA plate. A subset of 515 serum samples from uropositive (n=204) and uronegative (n=311) patients was systematically and non-proportionally sampled for evaluation in the ELISA after pre-adsorption with either soluble JCV VLP or assay buffer. In FIGs. 4A and 4B, the reactivity of serum samples from uronegative or uropositive patients in the screening and confirmation assays are shown side by side. Samples with strong JCV reactivity were highly inhibited by pre-adsorption of antibodies with soluble JCV, while samples with low levels of JCV antibodies showed differential competition. The antibody responses in most uropositive patients were strongly competed (FIG. 4B). These results support the idea that a significant proportion of the low serum reactivity to JCV may be due to cross-reactivity of antibodies not specific to JCV.

[0107] The distribution of the serum responses in the confirmation ELISA consisted of two defined peaks, most optimally separated at 40% inhibition (FIG. 5A) corresponding approximately to the lower 5th percentile of the response distribution of uropositive samples (FIG. 5B). Therefore, the 40% inhibition level was selected as the cut point for the confirmation ELISA.

Example 4(e): Finalized Two-Step Anti-JCV Serological Assay

[0108] By combining the screening and confirmation assays, the chance of detecting samples with "true" JCV-specific antibodies is greatly enhanced. In the final analysis, samples with nOD_{450} values <0.10 in the screening ELISA are considered negative for JCV antibodies, and those with nOD_{450} values >0.25 in the screening ELISA are considered positive for JCV antibodies. Samples with reactivity between nOD values 0.10 to 0.25 were further tested in the confirmation ELISA. In the confirmation ELISA, all samples exhibiting > 40% inhibition are classified as positive (FIG. 4). At nOD_{450} values >0.25 the probability of observing >40% inhibition was approximately 95%.

Example 4(f): JCV Seropositivity in the STRATA Cohort and False-Negative Rate

[0109] Based on the above algorithm, the seroprevalence rate in STRATA population was estimated as 53.6% with bootstrap determined 95% confidence limits ranging from 49.9% to 57.3% [0.536 = 0.451 (probability of the screening ELISA nOD_{450} >0.25) + 0.085 (probability of screening ELISA nOD_{450} falling between 0.10 and 0.25, and the supplemental confirmation ELISA %-inhibition >40%)]. This seroprevalence calculation assumed confirmation of anti-JCV antibodies in equal proportions of samples from uropositive and uronegative subjects in the nOD region between 0.10 and 0.25. (percent inhibition >40%); this assumption was supported by a 2-sided Fisher's exact test with a p-value of 0.702.

[0110] Of the 204 uropositive patients, five had nOD_{450} between 0.10 and 0.25 and did not confirm as having anti-JCV specific antibodies (percent inhibition ≤40%; FIG. 4B).

Example 5: Assay Validation

[0111] Assay validation was performed by Focus Diagnostics, Inc. (Cypress, CA), where performance parameters including inter- and intra-assay precision, specificity, sensitivity and stability of assay reagents and controls were demonstrated. Assay performance parameters including inter- and intra-assay precision, specificity, sensitivity and stability of assay reagents and controls was demonstrated. Precision parameters were evaluated by three independent analysts in both plasma and serum on four different days using independent preparations of assay controls. For demonstration of assay specificity, ten individual serum and plasma samples from healthy volunteers or MS patients (TYSABRI® (natalizumab) naive) were pre-incubated with either assay buffer or a defined concentration of HPVLP or BKV VLP in solution. Robustness was evaluated by varying the upper and lower limits of incubation times for sample, conjugate, and substrate addition steps and different lots of HPVLP coating reagent were evaluated to demonstrate consistent assay control performance. Matrix interference was evaluated by determining percent recovery in samples spiked with pre-defined concentrations of anti-JCV antibodies and by spiking samples containing JCV-specific antibodies with varying concentrations of irrelevant human monoclonal antibodies.

Example 6: Determination of JCV Antibody Status in PML Patients

[0112] Plasma and serum samples (single time-points randomly selected from serial collections) were obtained from a total of 831 patients from the Safety of TYSABRI Re-dosing And Treatment (STRATA) study. STRATA is an open-label, single-arm, multinational study (North America, Europe, Australia, and New Zealand) in which all patients receive natalizumab 300 mg by intravenous infusion every 4 weeks for 48 weeks. Urine samples collected according to the STRATA protocol were analyzed for the presence of JCV DNA.

[0113] From the marketing approval of TYSABRI® in June 2006 to February 9, 2010, there were 35 reported cases of PML on natalizumab treatment. In addition, there were three PML cases in the pre-approval clinical trials of natalizumab (10, 13, 25). Stored samples were obtained from as many PML cases as possible from time points prior to PML diagnosis (pre-PML). Plasma or serum samples were only available from 11 natalizumab-treated PML patients (10 MS patients and 1 Crohn's patient: Table 1). Serum samples were tested that were obtained one to three years prior to PML diagnosis. Nearly all of these samples had been collected from patients participating in registries or clinical studies and were stored at -70C° until analysis. Notably, anti-JCV antibodies were detected in all 11 patients (100%) via the combination of the serological status screening ELISA and the supplemental confirmation ELISA (FIGs. 6A and 6B) described above. Using a one-sample Fisher's exact test, this result was significantly different from the expected proportion (53.6%) with a p-value of 0.002.

[0114] These data indicate that the assay of the present invention can be used to determine the presence or absence of JCV antibody in subjects as part of an overall evaluation of risk for contracting PML.

Table 1. Samples from 11 natalizumab-treated PML patients who had available blood samples prior to diagnosis.

Subject	Source	Geography	PML Diagnosis (date)	Natalizumab Exposure: No. of doses or months	Natalizumab Exposure: Final dose	Immunosuppressant Use: Type	Immunosuppressant Use: Duration
1	Clinical Study*	Belgium	Mar 2005	5 doses	Jun 2003	Infliximab Azathioprine	32 months 73 months
2	Clinical Study (SENTINEL)	United States	Feb 2005	28 doses	Dec 2004	None	
3	Clinical Study (SENTINEL)	United States	Feb 2005	37 doses	Jan 2005	None	
4	Post-Marketing	Sweden	Jul 2008	17 months	Jun 2008	None	
5	Clinical Study (STRATA)	Germany	Jun 2009	34 doses	Apr 2009	Mitoxantrone	11 months
6	Clinical Study (STRATA)	France	Jun 2009	35 doses	May 2009	Mitoxantrone	10 months
7	Post-Marketing	Sweden	Jun 2009	29 months	Jun 2009	None	
8	Post-Marketing	Switzerland	Aug 2009	28 doses/25 months	Jun 2009	Mitoxantrone Azathioprine	18 months 21 months
9	Post-Marketing	Switzerland	Oct 2009	36 months	Sep 2009	Mitoxantrone	4 years
10	Clinical Study (STRATA)	Czech Republic	Oct 2009	44 doses	Sep 2009	Azathioprine	3 months

(continued)

Subject	Source	Geography	PML Diagnosis (date)	Natalizumab Exposure No. of doses or months	Final dose	Immunosuppressant Use Type	Duration
11	Post-Marketing	United States	Oct 2009	33 doses	Sep 2009	Methotrexate	Unknown

*Crohn's Disease; SENTINEL = Safety and Efficacy of Natalizumab in Combination with Interferon Beta-1a in Patients with Relapsing Remitting Multiple Sclerosis; STRATA = Safety of TYSABRI Re-dosing and Treatment; qd=4 × day; qwk = 1 × week

SENTINEL = Safety and Efficacy of Natalizumab in Combination with Interferon Beta-ia in Patients with Relapsing Remitting Multiple Sclerosis; STRATA = Safety of TYSABRI® Re-dosing and Treatment; ROW = Rest of World; qd=4 × day; qwk = 1 × week; *Both prior and concurrent treatment with natalizumab

[0115] Longitudinal data from other subjects taking an immunomodulator were also evaluated (*i.e.*, multiple samples collected at different times from a single individual). The longitudinal data indicated that, unlike testing intermittent urinary DNA shedding, the HPVLP assay can reliably be used to evaluate anti-JCV antibody status, and that JCV antibody status remains relatively stable (in the absence of *de novo* infection).

Example 7: Alternate JCV VP1-VLP Purification Method

[0116] This method is an example of an alternative to the density-gradient/ultracentrifugation method described above for the purification of JCV VP1-VLP's from insect cells. The general steps in the protocol are lysis, benzonase treatment, deoxycholate precipitation, ammonium sulfate precipitation and concentration/diafiltration, with a final ion-exchange step using TMAE fractogel.

[0117] Sf9 cells infected with JCV-VP1 baculovirus were lysed in PBS, 0.1 mM $CaCl_2$ by passing twice through a microfluidizer cell disrupter at 5,000 psi. Cell debris was removed by low speed centrifugation and the supernatant treated with 40 units/ml Benzonase (EMD Biosciences 71206-3) for 1 hour at room temperature. For the deoxycholate precipitation step, one tenth volume 2.5 % deoxycholate was added to the lysate (0.25% final deoxycholate), and the lysate was incubated at 37° C for 1 hour with gentle stirring. An equal volume of 4 M NaCl, 0.1 mM NaCl was added to the lysate and the lysate was incubated on ice for 1 hour. Precipitate was removed by low speed centrifugation. The supernatant was then precipitated with 40% ammonium sulfate to remove contaminating proteins. The final 40% was achieved by using 232 g solid ammonium sulfate per liter of solution. While mixing the solution gently at 4°C, ammonium sulfate was added one fifth at a time, allowing each addition to dissolve for 10 to 15 minutes before adding the next fraction. The solution was stirred gently overnight at 4°C. The ammonium sulfate precipitate was removed by low speed centrifugation and the VP1-containing supernatant was filtered using a 0.45 μm filter and carried on to the next step. The solution was concentrated 5 to 10 fold using a 100 kDa NMWL TFF membrane (Pellicon 2 Mini UF Mod Biomax-100 C 0.1m^2, P2B100C01) and exchanged into assembly buffer (25 mM tris, 150 mM NaCl, 1 mM $CaCl_2$, pH 7.5) by diluting 5 fold and concentrating back to the starting volume twice. The solution was stored at 4°C for >/= 36 hours. The solution was then diafiltered using a 500 kDa NMWL TFF membrane (Pellicon 2 Mini UF Mod Biomax-500 V, Millipore part # P2B500V01) using 40 volumes TMA chromatography buffer (25 mM tris, 150 mM NaCl, 0.1 mM $CaCl_2$, pH 8.0). For the chromatography, approximately 1 ml resin is required per 2 g starting cell mass. The protein was loaded onto the appropriately sized TMAE column (Fractogel® EMD TMAE HiCap (M) - EMD Biosciences cat. 1.10316) and washed with 3 column volumes chromatography buffer. The VLPs were eluted with 25 mM tris, 600 mM NaCl, 0.1 mM $CaCl_2$, pH 8.0. VP1 purity was assessed by SDS-PAGE and mass spectrometry, presence of VLPs was confirmed by electron microscopy, and the percentage of total protein in the form of VLPs was determined by sedimentation velocity analytical ultracentrifugation. This method resulted in HPVLP preparations of about 80% HPVLPs.

REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

- US 61294048 **[0001]**
- US 61316193 **[0001]**
- US 3817837 A **[0063]**
- US 3850752 A **[0063]**
- US 3939350 A **[0063]**
- US 3996345 A **[0063]**
- US 4277437 A **[0063]**
- US 4275149 A **[0063]**
- US 4366241 A **[0063]**

Non-patent literature cited in the description

- **MAJOR.** Progressive Multifocal Leukoencephalopathy in Patients on Immunomodulatory Therapies. *Annu. Rev. Med.,* 31 August 2009, vol. 61, 35-47 **[0003]**
- **PADGETT ; WALKER.** Prevalence of antibodies in human sera agains JC virus, an isolate from a case of progressive multifocal leukoencephalopathy. *J. Infect. Dis,* 1973, vol. 127, 467-70 **[0004]**
- **PICCINNI et al.** Stronger association of drug-induced progressive multifocal leukoencephalopathy (PML) with biological immunomodulating agents. *Eur. J. Clin. Pharmacol,* 2010, vol. 66, 199-206 **[0039]**
- **EGLI et al.** *J. Infect. Dis.,* 2009, vol. 199, 837-846 **[0102]**

Patentkrav

1. In vitro-fremgangsmåde til identifikation af en forsøgsperson, der er i risiko for at udvikle progressiv multifokal leukoencefalopati (PML), omfattende et primært og sekundært assay;

hvor det primære assay omfatter:

(a) at bringe en biologisk prøve fra en forsøgsperson i kontakt med højtoprensede VP1-partikler (HPVLP'er), der overvejende består af VP1-proteinet fra JCV'et, immobiliseret på et fast substrat under betingelser, der er egnede til at binde et JC-virus (JCV)-antistof i prøven til en HPVLP;

b) påvisning af niveauet af JCV-antistofbinding i prøven til HPVLP'er og

(c) at korrelere det detekterede niveau med et referenceniveau, der er afledt af en kontrolprøve eller et sæt prøver, som behandles med prøven fra forsøgspersonen;

og hvor det sekundære assay omfatter:

(a) at bringe en del af den biologiske prøve fra en forsøgsperson i kontakt med højtrensede viruslignende partikler (HPVLP'er), der overvejende består af VP1-proteinet fra JCV, i opløsning under betingelser, der er egnede til binding af et JCV-antistof i prøven til en HPVLP, hvorved der tilvejebringes en præinkuberet prøve;

(b) at bringe den præinkuberede prøve i kontakt med HPVLP'er, der overvejende består af VP1-proteinet i JCV'et, immobiliseret på et fast substrat under betingelser, der er egnede til binding af et JCV-antistof i prøven til en HPVLP;

c) påvisning af niveauet af JCV-antistof i den præinkuberede prøve, der binder til de immobiliserede HPVLP'er, og

(d) sammenligning af det påviste niveau af JCV-antistof i den præinkuberede biologiske prøve med niveauet af JCV-antistof, der er påvist i en biologisk prøve fra forsøgspersonen, som blev præinkuberet i en opløsning uden HPVLP og blev bragt i kontakt med HPVLP immobiliseret på et fast substrat under betingelser, der er egnede til binding af et JCV-antistof i prøven til en HPVLP,

hvor HPVLP'erne består af mere end 5, mindst 50, 150 eller 360 VP1-polypeptider; hvor et fald i det påviste niveau af JCV-antistof i den præinkuberede biologiske prøve sammenlignet med den biologiske prøve fra forsøgspersonen, der blev præinkuberet i en opløsning uden HPVLP'er, indikerer, at prøven er positiv for JCV-antistof, og forsøgspersonen har en øget risiko for at udvikle PML, og en ændring i det påviste niveau af JCV-antistof under en bestemt procentdel indikerer, at der ikke er noget JCV-specifikt antistof til stede i prøven.

2. Fremgangsmåde ifølge krav 1, hvor den biologiske prøve bringes i kontakt med HPVLP'er i opløsning i et tidsrum valgt fra 30 minutter, en time eller natten over ved 4 °C.

3. Fremgangsmåde ifølge krav 1, hvor den angivne procentdel er 40 %, og fremgangsmåden anvender en HPVLP-ELISA.

4. Fremgangsmåde ifølge krav 1, hvor referenceniveauet er valgt til at give en falsk negativ rate på 3 % eller mindre til påvisning af JCV-antistoffer i prøven fra forsøgspersonen.

5. Fremgangsmåde ifølge krav 1, hvor referenceniveauet er valgt til at give en falsk negativ rate på 1 % eller mindre til påvisning af JCV-antistoffer i prøven fra forsøgspersonen.

6. Fremgangsmåde ifølge krav 1, hvor den biologiske prøve, der er opnået fra forsøgspersonen, klassificeres som negativ for JCV-antistof, når niveauet af JCV-antistof i den præinkuberede biologiske prøve, der binder til de immobiliserede HPVLP'er, er omtrent det samme som niveauet af JCV-antistof, der er påvist i den biologiske prøve, der er opnået fra forsøgspersonen, og som blev præinkuberet i en opløsning uden HPVLP'er.

7. Fremgangsmåde ifølge krav 1, hvor HPVLP'erne indeholder mere end 1, mindst 5, 10, 20, 30, 40, 50, 60, 70 eller 72 VP1-pentamerer.

8. Fremgangsmåde ifølge krav 1, hvor
i) en HPVLP endvidere omfatter mindst én af en JCV VP2 eller en JCV VP3, eller
ii) VP1 i en HPVLP er en rekombinant VP1 eller
iii) mindst én VP1 i HPVLP er en mutant VP1.

9. Fremgangsmåde ifølge krav 1, hvor den biologiske prøve er serum.

10. Fremgangsmåde ifølge krav 1, hvor den biologiske prøve er fra en forsøgsperson, der er ordineret en immunmodulator eller en forsøgsperson, der overvejer at tage en immunmodulator, hvor immunmodulatoren er valgt fra en anti-VLA-4-terapi og anti-CD20-terapi, en anti-CD11a-terapi eller mycophenolatmofetil.

11. Fremgangsmåde ifølge krav 10, hvor forsøgspersonen, der er blevet ordineret en immunmodulator elle rder overvejer at tage en immunmodulator, ikke tidligere har fået immunmodulatoren.

12. Fremgangsmåde ifølge krav 10, hvor forsøgspersonen tidligere har modtaget en eller flere doser af immunmodulatoren.

13. Fremgangsmåde ifølge krav 1, hvor påvisning af JCV-antistofbinding til HPVLP'erne indikerer, at forsøgspersonen:
i) ikke er kandidat til behandling med en immunmodulator, hvis den biologiske prøve er positiv for et JCV-antistof, eller
(ii) er kandidat til at modtage behandling med en immunmodulator og øget monitorering for uønskede symptomer ved behandling med immunmodulatoren, eventuelt hvor de uønskede symptomer indikerer udvikling af PML.

14. Fremgangsmåde ifølge krav 1, hvor manglende påvisning af JCV-antistofbinding til HPVLP'er indikerer, at forsøgspersonen er kandidat til at modtage behandling med en immunmodulator.

15. Fremgangsmåde ifølge krav 1, hvor en forsøgsperson, hvis biologiske prøve er bestemt til ikke at have JCV-antistoffer i en indledende test, testes igen mindst en gang om året for forekomsten af JCV-antistoffer efter den indledende test.

16. Fremgangsmåde ifølge krav 1, hvor den forsøgsperson, som på en første dato er blevet bestemt ved fremgangsmåden ifølge krav 1 til at have JCV-antistoffer, testes igen på et senere tidspunkt for at bestemme, om forsøgspersonen ikke har JCV-antistoffer.

17. Fremgangsmåde ifølge et hvilket som helst af kravene 10-14, hvor immunmodulatoren er natalizumab.

18. Fremgangsmåde ifølge krav 1, hvor forsøgspersonen har multipel sklerose (MS) eller Crohns sygdom (CD).

DRAWINGS

Drawing

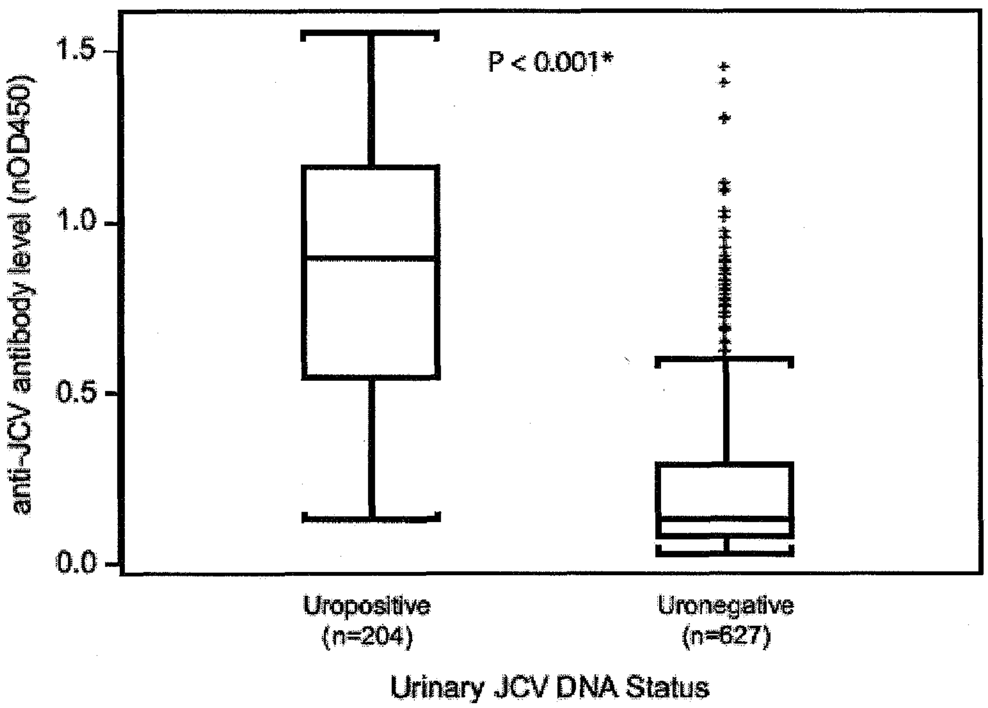

FIG. 1

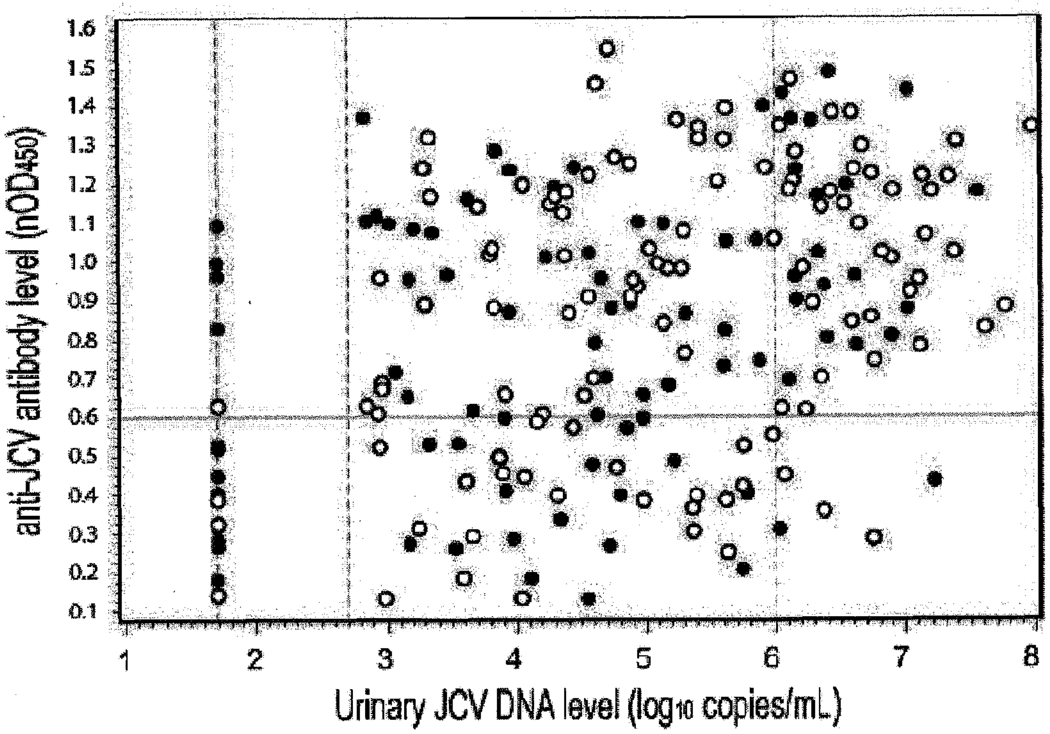
anti-JCV antibody level (nOD450)
Urinary JCV DNA level (log10 copies/mL)
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
1
2
3
4
5
6
7
8

FIG. 2

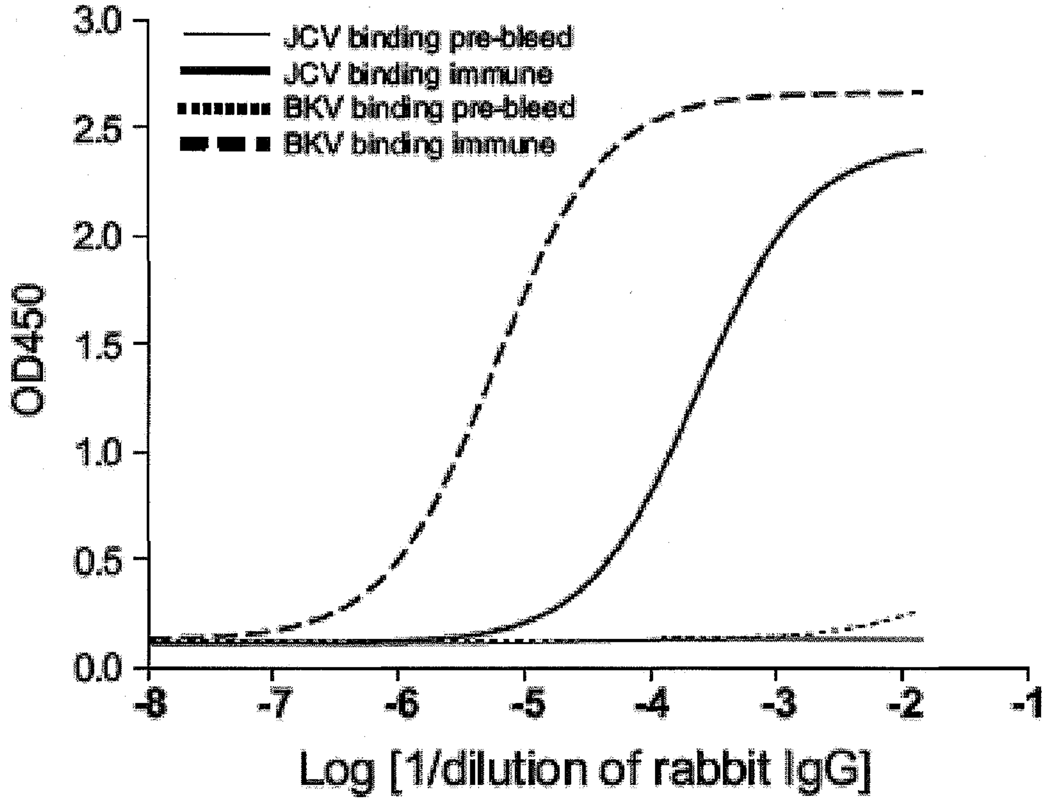
3.0
2.5
2.0
1.5
1.0
0.5
0.0
OD450
JCV binding pre-bleed
JCV binding immune
BKV binding pre-bleed
BKV binding immune
-8
-7
-6
-5
-4
-3
-2
-1
Log [1/dilution of rabbit IgG]

FIG. 3

FIG. 4A

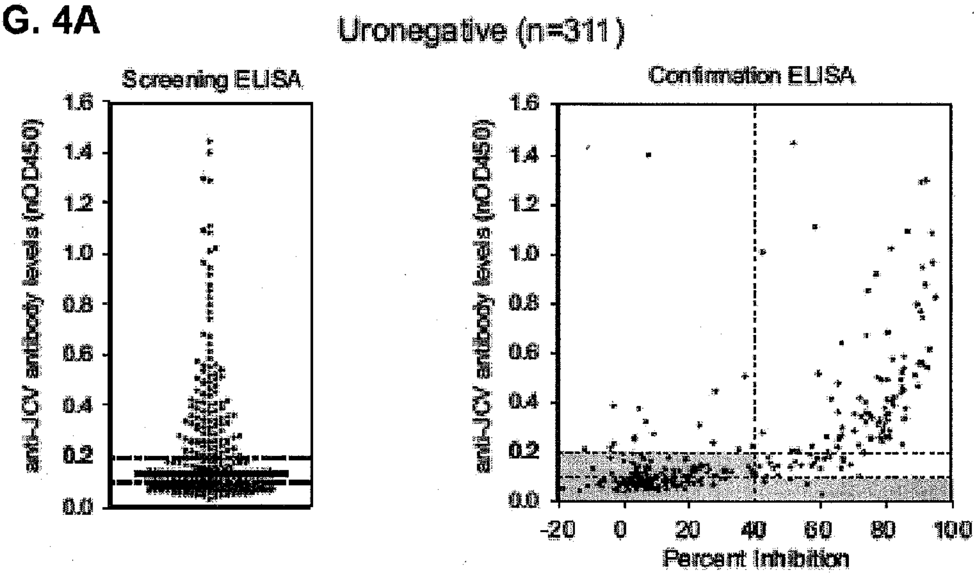
Uronegative (n=311)
Screening ELISA
anti-JCV antibody levels (nOD450)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Confirmation ELISA
-20
0
20
40
60
80
100
Percent Inhibition

FIG. 4B

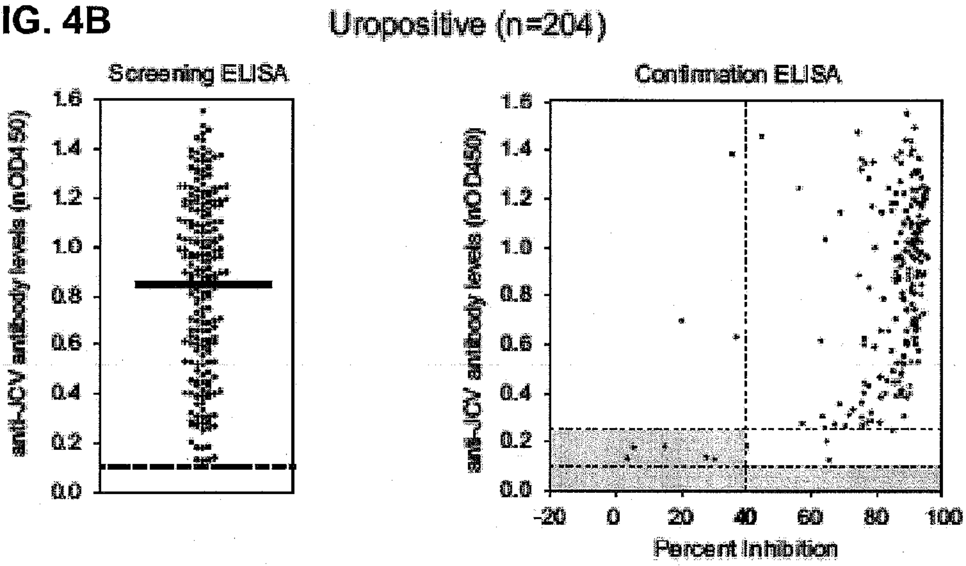
Uropositive (n=204)
Screening ELISA
anti-JCV antibody levels (nOD450)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Confirmation ELISA
-20
0
20
40
60
80
100
Percent Inhibition

FIG. 5A.

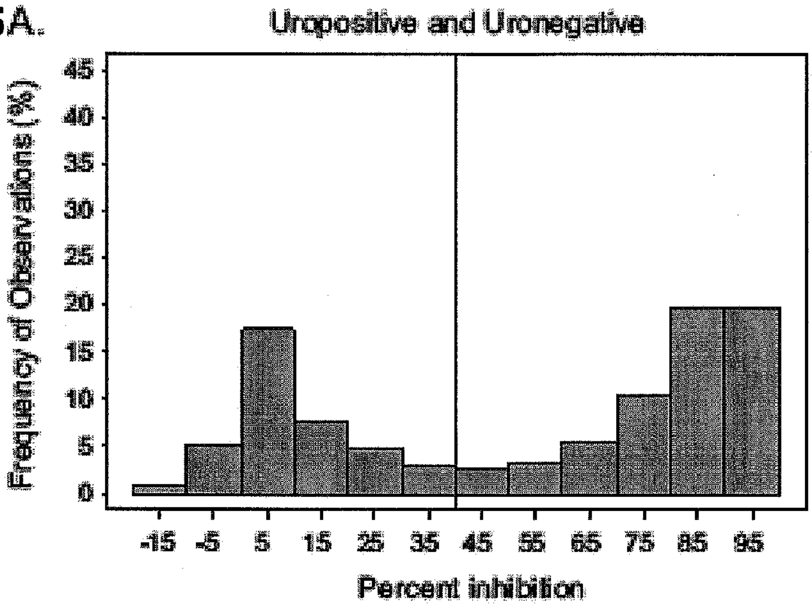
Uropositive and Uronegative
Frequency of Observations (%)
45
40
35
30
25
20
15
10
5
0
-15
-5
5
15
25
35
45
55
65
75
85
95
Percent inhibition

FIG. 5B.

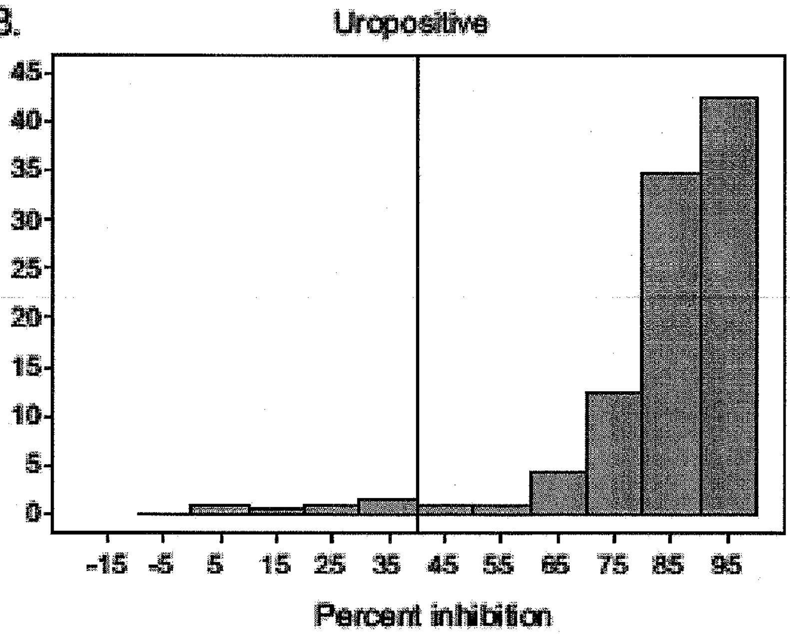
Uropositive
45
40
35
30
25
20
15
10
5
0
-15
-5
5
15
25
35
45
55
65
75
85
95
Percent inhibition

FIG. 6A.

Screening ELISA

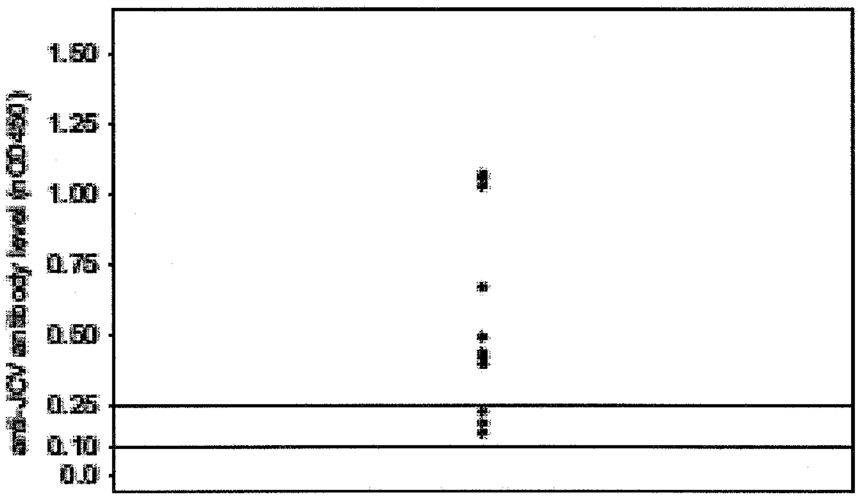

FIG. 6B.

Confirmation ELISA