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ABSTRACT

“PORCINE TORQUE TENO VIRUS VACCINES AND DIAGNOSIS”

The present invention provides four purified preparation containing a polynucleic acid molecule encoding porcine Torque teno virus (PTTV) genotypes or subtypes PTTV1a-VA, PTTV1b-VA, PTTV2b-VA, and PTTV2c-VA. The present invention also provides infectious DNA clones, biologically functional plasmid or viral vector containing the infectious nucleic acid genome molecule of the same. The present invention further provides live, attenuated, vector-expressed and purified recombinant capsid subunit or killed viral vaccines for protection against PTTV infection. The present invention additionally provides subunit vaccines comprising PTTV specific gene products, especially ORF1 capsid gene product for protection against PTTV infection. Further, the present invention provides methods for diagnosing PTTV infection via polymerase chain reaction (PCR) using specific primer for PTTV1, PTTV2, and individual PTTV1 genotypes. Finally, the present invention provides methods for diagnosing PTTV infection via immunological methods, e.g., enzyme-linked immunoabsorbent assay (ELISA) and Western blot using PTTV specific antigens for detecting serum PTTV specific antibodies.

We Claim:

1. An infectious nucleic acid molecule of porcine Torque teno virus (PTTV) comprising a nucleic acid molecule encoding an infectious PTTV which contains at least one copy of genomic sequence having at least 85% homology to a genomic sequence selected from the group consisting of PTTV1a-VA, PTTV1b-VA, PTTV2b-VA, and PTTV2c-VA.
2. The infectious nucleic acid molecule of claim 1, wherein the at least one copy of genomic sequence having at least 95% homology to the genomic sequence selected from the group consisting of PTTV1a-VA, PTTV1b-VA, PTTV2b-VA, and PTTV2c-VA.
3. The infectious nucleic acid molecule of claim 1, wherein the genomic sequence is selected from sequences set forth in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12.
4. A biologically functional plasmid or viral vector containing the infectious nucleic acid molecule according to claim 3.
5. The biologically functional plasmid or viral vector of claim 4, containing more than one copy of the infectious nucleic acid molecule.
6. A suitable host cell transfected by the vector comprising the infectious nucleic acid molecule according to claim 4.

7. An avirulent, infectious PTTV produced by cells containing the infectious nucleic acid molecule according to claim 6.
8. A vaccine comprising a nontoxic, physiologically acceptable carrier and an immunogenic amount of a member selected from the group consisting of:
- (a) a nucleic acid molecule containing at least one copy of genomic sequence having at least 80% homology to a genomic sequence selected from the group consisting of PTTV genotypes or subtypes PTTV1a-VA, PTTV1b-VA, PTTV2b-VA, and PTTV2c-VA, or its complementary strand;
 - (b) a biologically functional plasmid or viral vector containing a nucleic acid molecule containing at least one copy of genomic sequence having at least 80% homology to a genomic sequence selected from the group consisting of PTTV genotypes or subtypes PTTV1a-VA, PTTV1b-VA, PTTV2b-VA, and PTTV2c-VA, or its complementary strand;
 - (c) an avirulent, infectious nonpathogenic porcine Torque teno virus, which is derived from and contains a genomic sequence having at least 85% homology to a genomic sequence selected from the group consisting of PTTV genotypes or subtypes PTTV1a-VA, PTTV1b-VA, PTTV2b-VA, and PTTV2c-VA; and
 - (d) an immunogenic fragment of a polypeptide sequence or a complete protein translated according to a polynucleotide sequence selected from the group consisting of ORF1, ORF2, ORF1/1, and ORF2/2 of PTTV genotypes or subtypes PTTV1a-VA, PTTV1b-VA, PTTV2b-VA, and PTTV2c-VA.

9. The vaccine according to claim 8, wherein the vaccine contains live PTTV virus.
10. The vaccine according to claim 8, wherein the vaccine contains killed PTTV virus.
11. The vaccine according to claim 8, wherein the immunogenic fragment of a polypeptide sequence or a complete protein comprising purified bacterial expressed or baculovirus-expressed recombinant proteins expressed from ORF1, ORF2, ORF1/1 and ORF2/2 of PTTV genotypes or subtypes PTTV1a-VA, PTTV1b-VA, PTTV2b-VA, and PTTV2c-VA.
12. The vaccine according to claim 11, wherein the immunogenic fragment of a polypeptide sequence or a complete protein comprising purified bacterial expressed or baculovirus-expressed recombinant proteins expressed from ORF1 of PTTV genotypes or subtypes PTTV1a-VA, PTTV1b-VA, PTTV2b-VA, and PTTV2c-VA.
13. The vaccine according to claim 8, further contains an adjuvant.
14. A method of immunizing a pig against PTTV viral infection, comprising administering to a pig an immunologically effective amount of the vaccine according to claim 8.
15. The method according to claim 14, which comprising administering the vaccine parenterally, intranasally, intradermally, or transdermally to the pig.
16. The method according to claim 14, which comprising administering the vaccine

intralymphoidly or intramuscularly to the pig.

17. An isolated polynucleotide consisting of a poly nucleic acid sequence having 85% homology to a nucleotide sequence of PTTV1a-VA set forth in SEQ ID NO:9.
18. The isolated polynucleotide of claim 17, wherein the poly nucleic acid sequence having 95% homology to the nucleotide sequence of PTTV1a-VA set forth in SEQ ID NO:9.
19. The isolated polynucleotide of claim 17, wherein the poly nucleic acid sequence consisting the nucleotide sequence of PTTV1a-VA set forth in SEQ ID NO:9.
20. An isolated polynucleotide consisting of a poly nucleic acid sequence having 85% homology to a nucleotide sequence of PTTV1b-VA set forth in SEQ ID NO:10.
21. The isolated polynucleotide of claim 20, wherein the poly nucleic acid sequence having 95% homology to the nucleotide sequence of PTTV1b-VA set forth in SEQ ID NO:10.
22. The isolated polynucleotide of claim 20, wherein the poly nucleic acid sequence consisting the nucleotide sequence of PTTV1b-VA set forth in SEQ ID NO:10.
23. An isolated polynucleotide consisting of a poly nucleic acid sequence having 85% homology to a nucleotide sequence of PTTV2b-VA set forth in SEQ ID NO:11.

24. The isolated polynucleotide of claim 23, wherein the poly nucleic acid sequence having 95% homology to the nucleotide sequence of PTTV2b-VA set forth in SEQ ID NO:11.
25. The isolated polynucleotide of claim 23, wherein the poly nucleic acid sequence consisting the nucleotide sequence of PTTV2b-VA set forth in SEQ ID NO:11.
26. An isolated polynucleotide consisting of a poly nucleic acid sequence having 85% homology to a nucleotide sequence of PTTV2c-VA set forth in SEQ ID NO:12.
27. The isolated polynucleotide of claim 23, wherein the poly nucleic acid sequence having 95% homology to the nucleotide sequence of PTTV2c-VA set forth in SEQ ID NO:12.
28. The isolated polynucleotide of claim 23, wherein the poly nucleic acid sequence consisting the nucleotide sequence of PTTV2c-VA set forth in SEQ ID NO:12.
29. A vaccine comprising an immunogenic fragment of a polypeptide sequence or a complete protein translated according to a polynucleotide sequence selected from the group consisting of ORF1, ORF2, ORF1/1, and ORF2/2 of PTTV genotypes or subtypes PTTV1a-VA, PTTV1b-VA, PTTV2b-VA, and PTTV2c-VA.

30. The vaccine of claim 29, wherein the immunogenic fragment or complete protein is a capsid protein of PTTV genotypes or subtypes PTTV1a-VA, PTTV1b-VA, PTTV2b-VA, and PTTV2c-VA.
31. The vaccine of claim 29, wherein the polynucleotide sequence is selected from the group consisting of ORF1 of PTTV genotypes or subtypes PTTV1a-VA, PTTV1b-VA, PTTV2b-VA, and PTTV2c-VA.
32. The vaccine of claim 31, wherein the polynucleotide sequence is ORF1 of PTTV genotype PTTV1a-VA.
33. The vaccine of claim 31, wherein the polynucleotide sequence is ORF1 of PTTV genotype PTTV1b-VA.
34. The vaccine of claim 31, wherein the polynucleotide sequence is ORF1 of PTTV subtype PTTV2c-VA.
35. The vaccine of claim 29, wherein the polypeptide sequence is selected from the group consisting of sequence set forth in SEQ ID No:13, SEQ ID No:14, SEQ ID No:15, SEQ ID No:16, SEQ ID No:17, SEQ ID No:18, SEQ ID No:19, SEQ ID No:20, SEQ ID No:21, SEQ ID No:22, SEQ ID No:23, SEQ ID No:24, SEQ ID No:25, SEQ ID No:26, SEQ ID No:27, and SEQ ID No:28.

36. The vaccine of claim 35, wherein the polypeptide sequence is set forth in SEQ ID No:13.
37. The vaccine of claim 36, wherein the polypeptide sequence is a C-terminal region (aa 317-635) of SEQ ID No:13.
38. The vaccine of claim 35, wherein the polypeptide sequence is set forth in SEQ ID No:14.
39. The vaccine of claim 38, wherein the polypeptide sequence is a C-terminal region (aa 322-639) of SEQ ID No:14.
40. The vaccine of claim 35, wherein the polypeptide sequence is set forth in SEQ ID No:16.
41. The vaccine of claim 40, wherein the polypeptide sequence is C-terminal region (aa 310-625) of SEQ ID No:16.
42. The vaccine of claim 35, wherein the polypeptide sequence is set forth in SEQ ID No:20.
43. The vaccine according to claim 29, further contains an adjuvant.
44. A method of immunizing a pig against PTTV viral infection, comprising administering to a pig an immunologically effective amount of the vaccine according to claim 29.
45. The method according to claim 44, which comprises administering the immunogenic

fragment or complete protein to a pig.

46. The method according to claim 44, which comprises administering the vaccine parenterally, intranasally, intradermally, or transdermally to the pig.

47. The method according to claim 44, which comprises administering the vaccine intralymphoidly or intramuscularly to the pig.

48. A method for diagnosing PTTV1 infection and quantification of PTTV1 load, comprising:

extracting DNA from a sample suspected of PTTV1 infection;

performing polymerase chain reaction (PCR) using primers comprising the sequences set forth in SEQ ID NO:29 and SEQ ID NO:30; and

detecting PTTV1 specific amplification.

49. The method of claim 48, wherein the polymerase chain reaction is a SYBR green real-time PCR.

50. A method for diagnosing PTTV2 infection and quantification of PTTV2 load, comprising:

extracting DNA from a sample suspected of PTTV2 infection;

performing polymerase chain reaction (PCR) using primers comprising the sequences set forth in SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31 and SEQ ID

NO:32; and

detecting PTTV2 specific amplification.

51. The method of claim 50, wherein the polymerase chain reaction is a SYBR green real-time PCR.

52. A method for simultaneously detecting and diagnosing PTTV1 and PTTV2 infection, comprising:

extracting DNA from a sample suspected of PTTV infection;

performing polymerase chain reaction (PCR) using primers comprising the sequences set forth in SEQ ID NO:31 and SEQ ID NO:32; and

detecting PTTV1 and PTTV2 specific amplification.

53. The method of claim 52, wherein the polymerase chain reaction is a SYBR green real-time PCR.

54. A method for simultaneously detecting and diagnosing PTTV1a and PTTV1b infection, comprising:

extracting DNA from a sample suspected of PTTV1 infection;

performing a first polymerase chain reaction (PCR) using primers comprising the sequences set forth in SEQ ID NO:33 and SEQ ID NO:34;

performing a second PCR using primers comprising the sequences set forth in SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, and SEQ ID NO:38; and

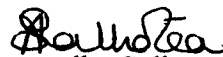
detecting PTTV1a and PTTV1b specific amplification.

55. A method for diagnosing PTTV infection, comprising
- immobilizing an immunogenic fragment or a complete protein of a polypeptide sequence translated according to a polynucleotide sequence selected from the group consisting of ORF1, ORF2, ORF1/1, and ORF2/2 of PTTV subtypes PTTV1a-VA, PTTV1b-VA, PTTV2b-VA, and PTTV2c-VA;
 - contacting a serum sample from a pig suspected of PTTV infection with the immobilized immunogenic fragment or complete protein; and
 - detecting captured antibody specific to the immunogenic fragment.
56. The method of 55, wherein the polynucleotide sequence is selected from the group consisting of ORF1 of PTTV genotypes or subtypes PTTV1a-VA, PTTV1b-VA, PTTV2b-VA, and PTTV2c-VA.
57. The vaccine of claim 56, wherein the polynucleotide sequence is ORF1 of PTTV genotype PTTV1a-VA.
58. The method of claim 56, wherein the polynucleotide sequence is ORF1 of PTTV genotype PTTV1b-VA.

59. The method of claim 56, wherein the polynucleotide sequence is ORF1 of PTTV subtype PTTV2c-VA.
60. The method of claim 55, wherein the polypeptide sequence is selected from the group consisting of sequence set forth in SEQ ID No:13, SEQ ID No:14, SEQ ID No:15, SEQ ID No:16, SEQ ID No:17, SEQ ID No:18, SEQ ID No:19, SEQ ID No:20, SEQ ID No:21, SEQ ID No:22, SEQ ID No:23, SEQ ID No:24, SEQ ID No:25, SEQ ID No:26, SEQ ID No:27, and SEQ ID No:28.
61. The method of claim 60, wherein the polypeptide sequence is set forth in SEQ ID No:13.
62. The vaccine of claim 60, wherein the polypeptide sequence is set forth in SEQ ID No:16.
63. The vaccine of claim 62, wherein the immunogenic fragment is C-terminal region (aa 317-635) of SEQ ID No:13, (aa 322-639) of SEQ ID No:14, or (aa 310-625) of SEQ ID No:16.
64. The vaccine of claim 60, wherein the polypeptide sequence is set forth in SEQ ID No:20.
65. The method of claim 55, wherein the detecting captured antibody is via Western blot.
66. The method of claim 55, wherein the detecting captured antibody is via enzyme-linked

immunosorbent assay (ELISA).

Dated this 15th day of March, 2012.



(Anuradha Salhotra)

Of LALL LAHIRI & SALHOTRA
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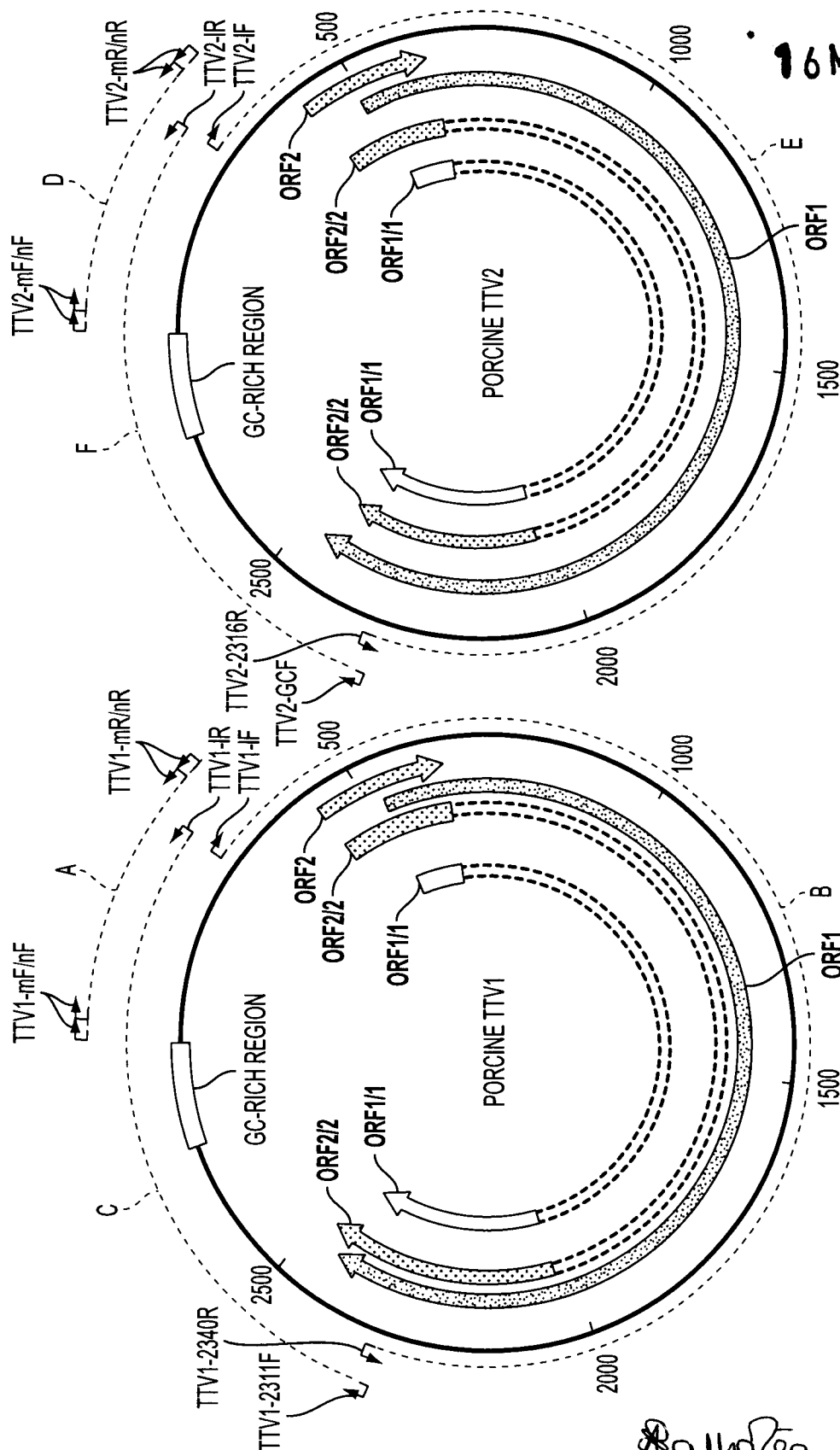


FIG. 1A

Salhotra
(Anuradha Salhotra)
of LALL LAHIRI & SALHOTRA
AGENTS FOR THE APPLICANT

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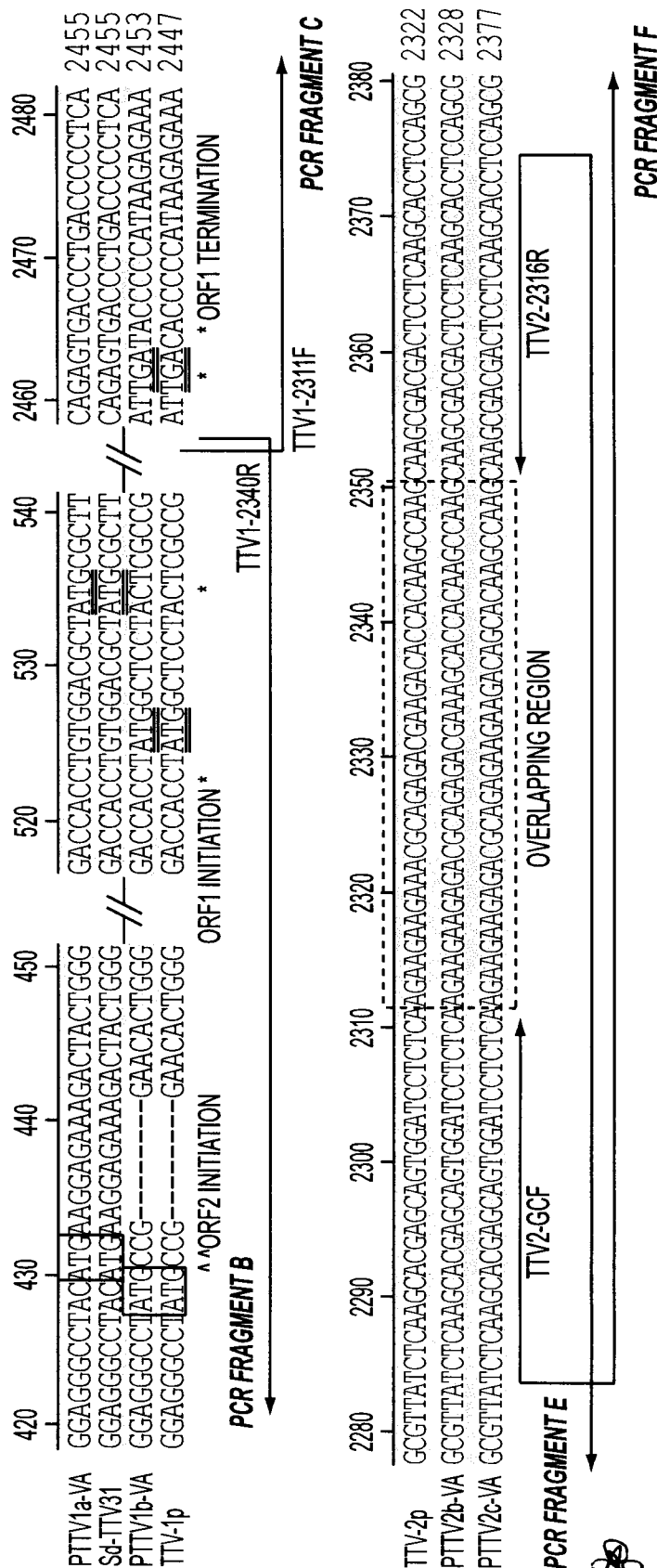


FIG. 1B

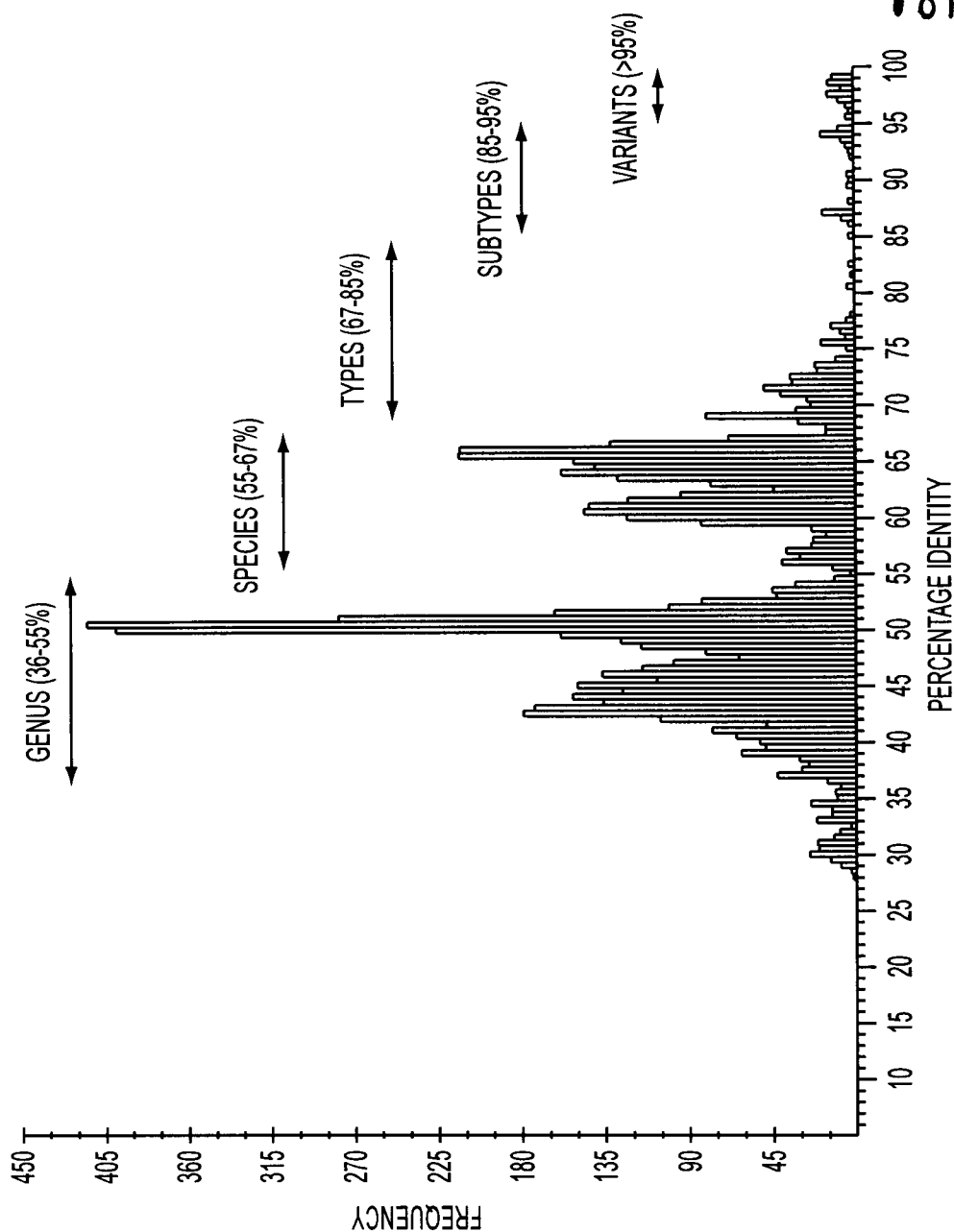
TTV- _____
 PTV- _____
 PTE- _____
 PCR- _____

(Anuradha Salhotra)
 of LALL LAHIRI & SALHOTRA
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(Anuradha Salhotra)
of LALL LAHIRI & SALHOTRA
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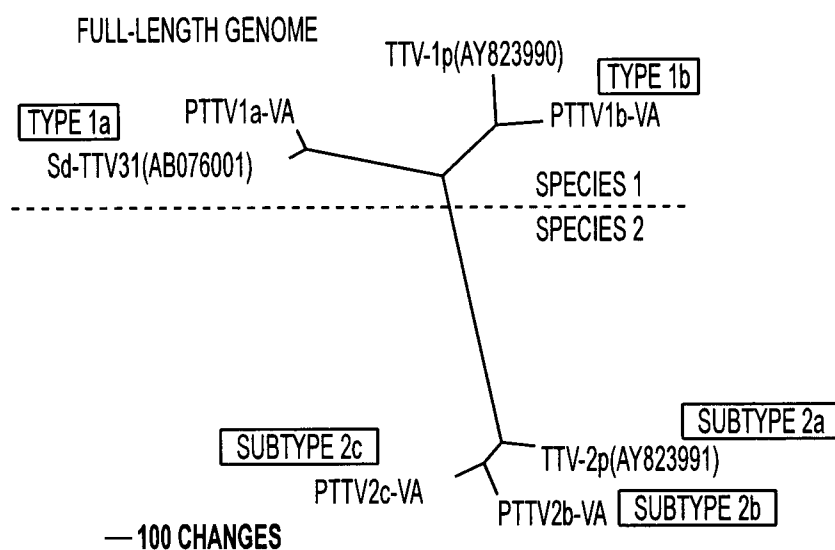


FIG. 3A

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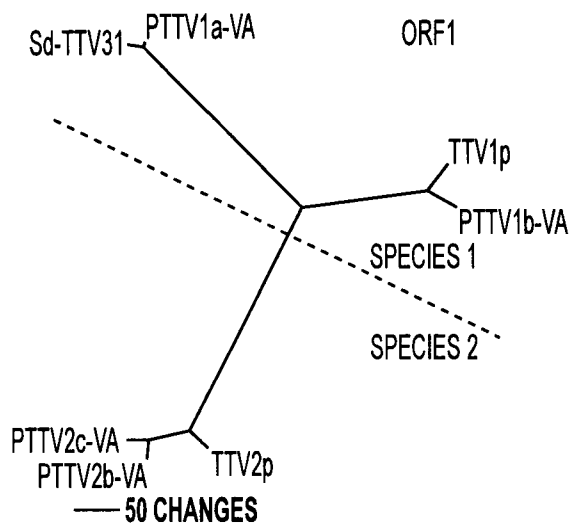


FIG. 3B

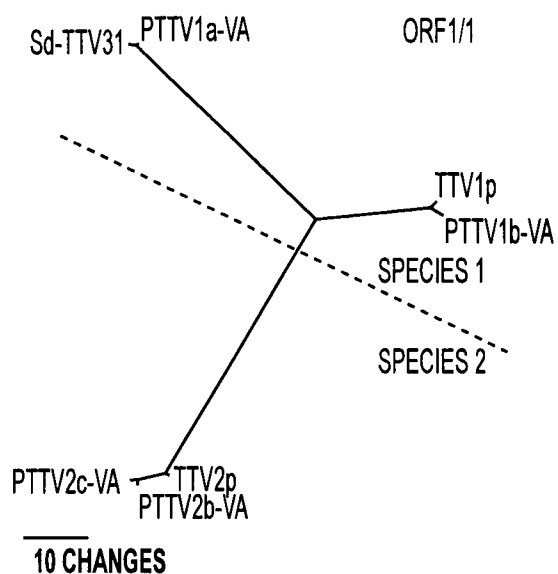


FIG. 3C

Anuradha Salhotra
(Anuradha Salhotra)
of LALL LAHIRI & SALHOTRA
AGENTS FOR THE APPLICANT

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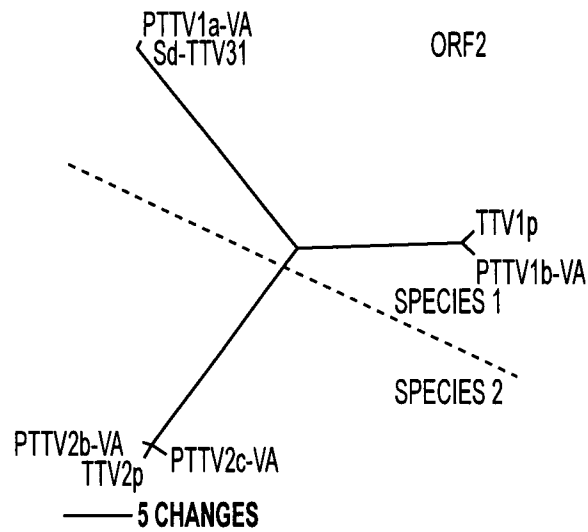


FIG. 3D

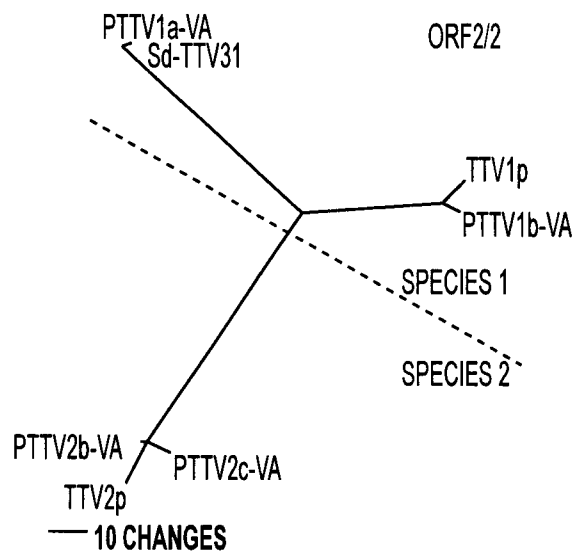


FIG. 3E

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FIG. 4A

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of LALL LAHIRI & SALHOTRA
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	210	220	230	240	250	260	270	280	290	300
PTTV1a-VA	VMLQAKNSRLIVCKQKMP	IRRRVK--SIFIPPPAQLTTQWK	FQQLCQFPLEFWACICIDMDTPFDYNG--AWRNAWLMRRLQNG-----NMEYIERW	286						
Sd-TTV31	VMLQAKNSRLIVCKQKMP	IRRRVK--SIFIPPPAQLTTQWK	FQQLCQFPLEFWACICIDMDTPFDYNG--AWRNAWLMRRLQNG-----NMEYIERW	286						
PTTV1b-VA	VMMWAKRTRLVIARDRAPHRRVR--KIFIPPPSRD	TTQWQFQTD	FCRPLFTWAAGLIDMQKPF	DANG--AFRNAWLETRNDQ-----EMKYIELW	286					
TTV-1p	VMMWAKRTRIVIAERAPHRKVR--KIFIPPPSRD	TTQWQFQTD	FCRKLFTWAAGLIDMQKPF	DANG--AFRNAWLETRNDQ-----EMKYIELW	284					
TTV-2p	IMLLKKQHKIVLSQNCNPNRKQK	PVTLKFKPPK	LSQWRLSRELA	KMPLIRLGVSFIDLT	EPWVEGNAFYSVLGYEAVKDQ	CHWSNWTQIKYFWIY	263			
PTV-2b-VA	IMLLKKQHKIVLSQKDCNPNRKQK	PVTLKFRPPK	LSQWRLSRELSKIPI	RLGLISLIDLSEPWLEGNFYSVLGYEASKHSGRWSNWTQIKYFWIY	263					
PTV-2c-VA	IMLLKKQHKIVLSQKDCNPNRRQK	PVTLKIRPPK	LSQWRLSRELA	KMPLVRLGVSLIDLSEPWLEGNFYSVLGYEASKHSGRWSNWTQIKYFWIY	263					
	310	320	330	340	PTTV1-HVR2	360	370	380	390	400
PTTV1a-VA	GRIPMTGDTLPPADDFKAGGVNKNFKPTG--IQRIYPIVAVCLVEGNKRVV	KWATVHNGPI	DRWRKKQTGTILKLSNLRG-----LVLRVCSSEITYK	378						
Sd-TTV31	GRIPMTGDTLPPADDFKAGGVNKNFKPTG--IQRIYPIVAVCLVEGNKRVV	KWATVHNGPI	DRWRKKQTGTILKLSALRR-----LVLRVCSSEITYK	378						
PTTV1b-VA	GRVPPQGDTELPKQSEF	FKGDNPNVNT	TEGHEKNIYP	ELIYVDQDKQTRKKYCV	CYNKTLNRWRKAQASTLAIGDLQ-----LVLRLQIMNQEMTYI	380				
TTV-1p	GRVPPQGDSELPKKEF	STGTDNPNVNQDNEEKNIYP	ELIYVDQDKQPRKKYCV	CYNKTLNRWRKAQASTLAIGDLQ-----LVLRLQIMNQEMTYI	378					
TTV-2p	DTGVGNNAVYVILLKKDVT	DPGNMATTFKAS--GGQHPDAIDHIELINQ	GWPLYFYKSEQDIKEAHS	AEISREYTRDPKSKKLKIGIVGWASSNYT	361					
PTTV2b-VA	DTGVGNNAVYVILLKKDVS	NDPGMATQFVTG--SGQHPDAIDHIELINQ	GWPLYFYKSEQDIKEAHS	AEISREYTRDPKSKKLKIGIVGWASSNYT	361					
PTTV2c-VA	DTGVGNNAVYVILLKQEV	DDNPGMATQFVTG--PGQHPDAIDRIEON	EGWPWLFYFGQSEODIKLAHDO	EIAREYANNPKSKKLKIGIVGWASSNYT	361					

FIG. 4B

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FIG. 4C

PTTV1a-VA	WIGSEFTGAFOQDWWPVG--TEYPLCTIKMPEYENPVEVWSWKANIPITSG-TLKDYFGLST--GQWMDTDFARLQIPRSSHNVDFGHKARFGPFCVKK	475
Sd-ITV31	WIPASEFTGAFOQDWWPVG--TEYPLCTIKMEPEFENPVEVWSWKATIPITAG-TLKDYFGLSS--GQWMDTDFGRQLPSSHNVDFGHKARFGPFCVKK	475
PTTV1b-VA	WIGSEFSSPFLQWKK---G-TRLITIDAR-KADTENPKYSSWEGQNNWNTSGTIVLOEVENISLNTQIRDDFAKLTLPKSPHDIDFGHHSRFGPFCVKN	475
TTV-1p	WKEGEYSAPFVORMK---G-SREFAVIDAR-KADOENPKVSTWPIEGTWNTOITVLKDVGINLQNOQFRAADFGKLTLPKSPHDIDFGHHSRFGPFCVKN	473
TTV-2p	TTIGSDQNSGGST--SAIQGGYVAYAGSGVIGAGSIGNLQOQWPSNQWPNIN-RDKTNFDWGTGRLCILR---DNMHLGSQELDDDECTMLTLFGPFVEKA	456
PTTV2b-VA	TAGSNQNSVLQTPFAIQGGYVAYAGSRIPGAGSITNLQMGWPGDQNWPPIN-QDQTNFNWGLRGLCVLR---DNMKLGAQELDDDECTMLSLFGPFVEKA	457
PTTV2c-VA	TAGSSQNTPTPTFAIQGGYVAYAGSKIQAGAITNLITDAMPGDQNWPPIN-REQTNFNWGLRGLCIMR---DNMKLGAQELDDDECTMLTLFGPFVEKA	457
PTTV2-RVR1	PTTV2-HVR2	
PTTV1a-VA	PPVEFRDTA-----PNPLNIWVKYTFYFQFG--GMVQPPGTGIQDPCISNPTYPV--MVGAVTHPKYAGCGGITTQIGDQGITAAASIRAI SAAPPTY	564
Sd-ITV31	PPVEFRDTA-----PNPLNIWVKYTFYFQFG--GMVQPPGTGIQDPCISNPTYPV--MVGAVTHPKYAGCGGITTQIGDQGITAAASIRAI SAAPPTY	564
PTTV1b-VA	EPLFQQLP-----PTPTNLWFQYKFLFQFG--GEYQPPGTGIRDPICIDTPAYPVP---QSGSVTHPKFAGKGGMLTETDRWGITAAASRTLSADTPTTEA	564
TTV-1p	EPLFQQLP-----PEPTNLWFQYKFFQFG--GEYQPPGTGIRDPICIDTPAYPVP---QSGSVTHPKFAGKGGMLTETDRWGITAAASRTLSADTPTTEA	562
TTV-2p	NPIFATTDPKFFKPELKDYNIIMKVAEKFQWGGHGTFRFKNIGDPSTIPCPFEPGDRFHSIGQDPKSVQNTVLNPDWDYDCDGIVRKDTILKRLLELPTET	556
PTTV2b-VA	NTAFATNDPKFFKPELKDYNVVMKVAEKFQWGGHGTFRFKNIGDPSTIPCPFEPGERVHHGVQDPKAVQNTVLNPDWDYDCDGIVRTDILKRLLELPTET	556
PTTV2c-VA	NTAFATNDPKYFRPELKDYNIIMKVAEKFQWGGHGTFRFKNIGDPSTIPCPFEPGERVHHGVQDPKAVQNTVLNPDWDYDCDGIVRTDILKRLLELPTET	557
PTTV1a-VA	TQSAFLKAP---ETEKEEERESETSFSAESSSEGGSSDDQAERRAARK--RVIKLLKRLADRPVDNKRREFSE	635
Sd-ITV31	TQSAFLKAP---ETEKEEERESETSFSAESSSEGGSSDDQAERRAARK--RVIKLLKRLADRPVDNKRREFSE	635
PTTV1b-VA	AQSALLRGDAEKKEETEETASSSITSAESSTEGDSDDDEETIRRRRTWKRLRMVRRQQLDRMDHKRQLRH	639
TTV-1p	AQSALLRGDSEAKGEETEETASSSITSAESSTEGDSDDDEETIRRRRTWKRLRMVRRQQLDRMDHKRQLRH	637
TTV-2p	EETEKAFLPL---LGQKTEKEPLSDSDEESVISSTSSGSSQEEETQRRHH--KPSKRRLIKHLQRVVWRMKTIL	634
PTTV2b-VA	EETEKAFLPL---LGQKTEKEPLSDSDEESVISSTSSGSSQEEETQRRKH--KPSKRRLIKHLQRVVWRMKTIL	635
PTTV2c-VA	EETEKAFLPL---LGQKTEKEPLSDSDEESVISSTSSGSSQEEETQRRQH--KPSKRRLIKHLQRVVWRMKTIL	625

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FIG. 5


(Anuradha Salhotra)
of LALL LAHIRI & SALHOTRA
AGENTS FOR THE APPLICANT

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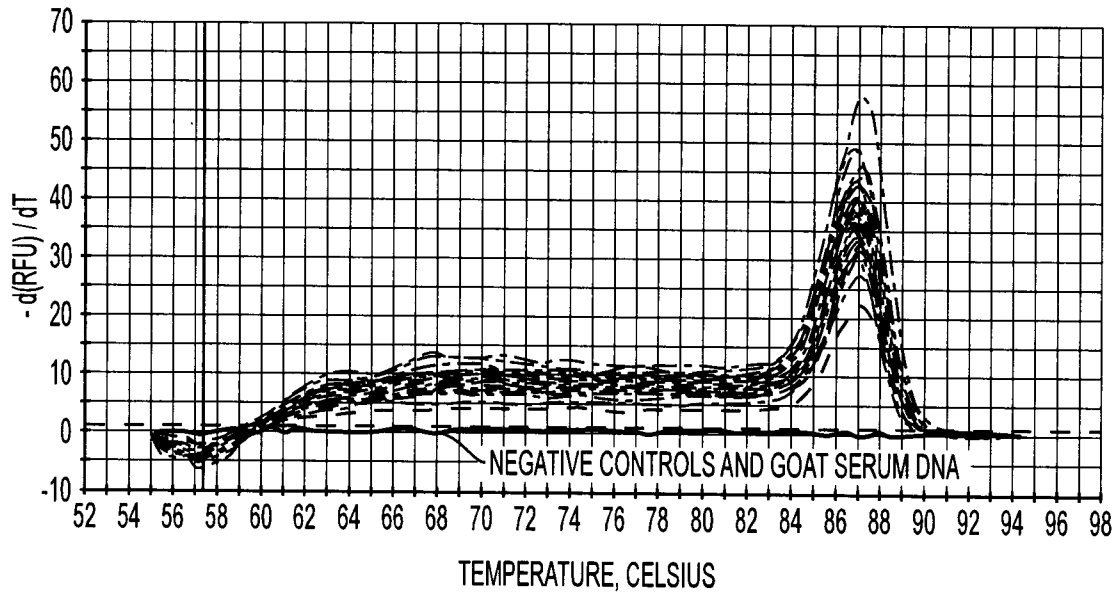


FIG. 6A

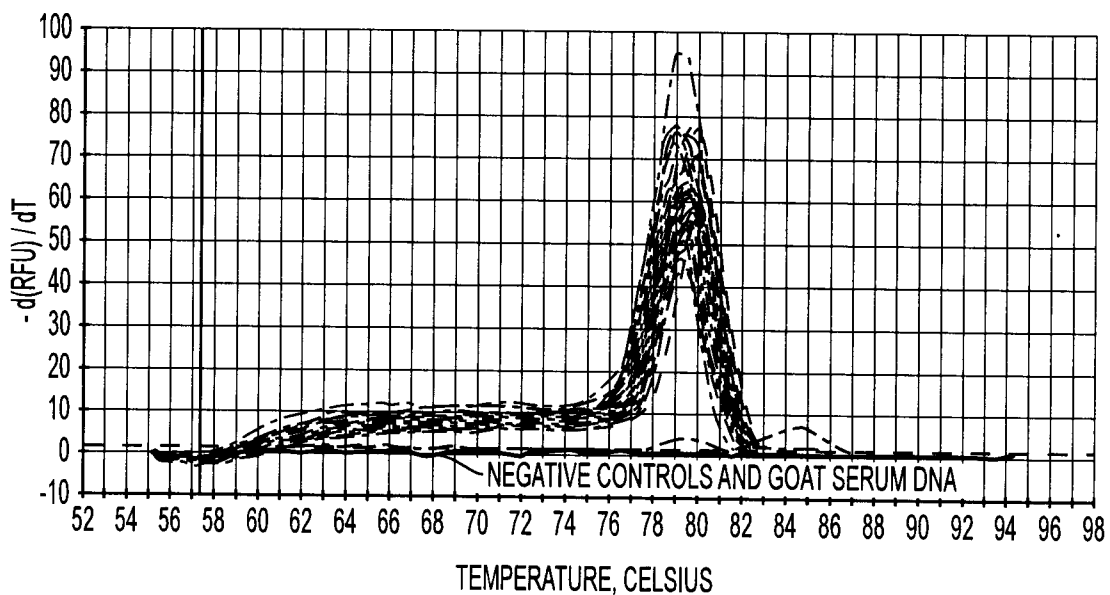


FIG. 6B

Anuradha Salhotra
(Anuradha Salhotra)
of LALL LAHIRI & SALHOTRA
AGENTS FOR THE APPLICANT

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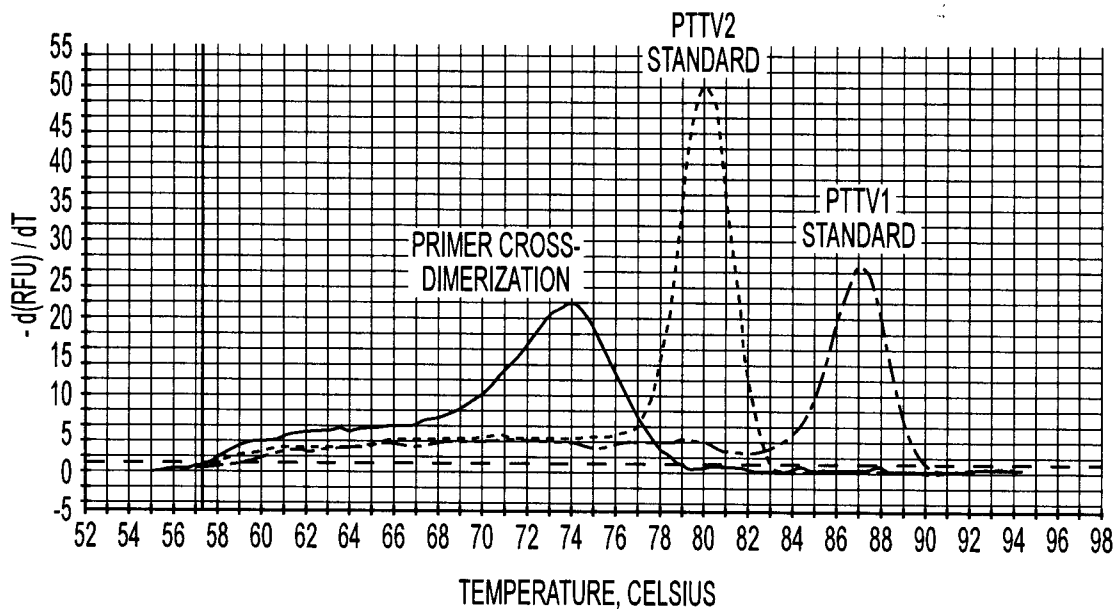


FIG. 7A

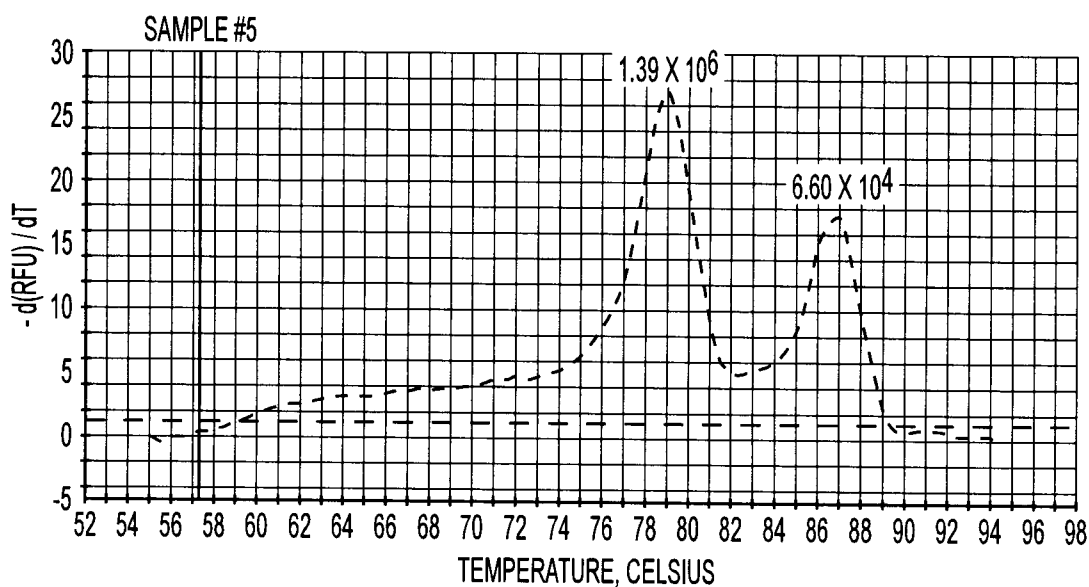


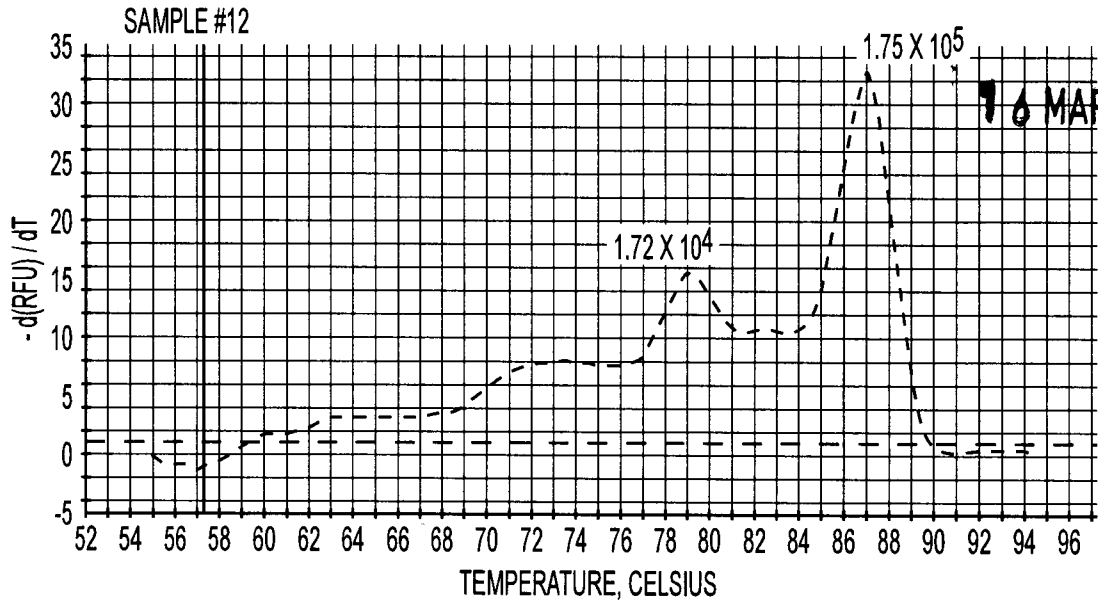
FIG. 7B

Anuradha Salhotra
(Anuradha Salhotra)
of LALL LAHIRI & SALHOTRA
AGENTS FOR THE APPLICANT

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FIG. 7C

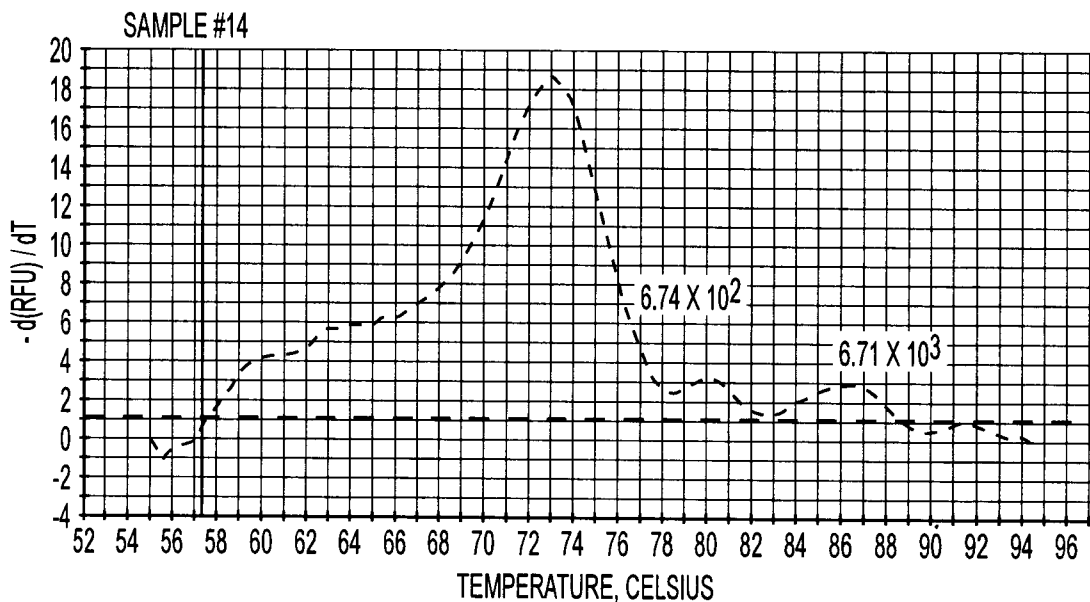


FIG. 7D

Anuradha Salhotra
(Anuradha Salhotra)
of LALL LAHIRI & SALHOTRA
AGENTS FOR THE APPLICANT

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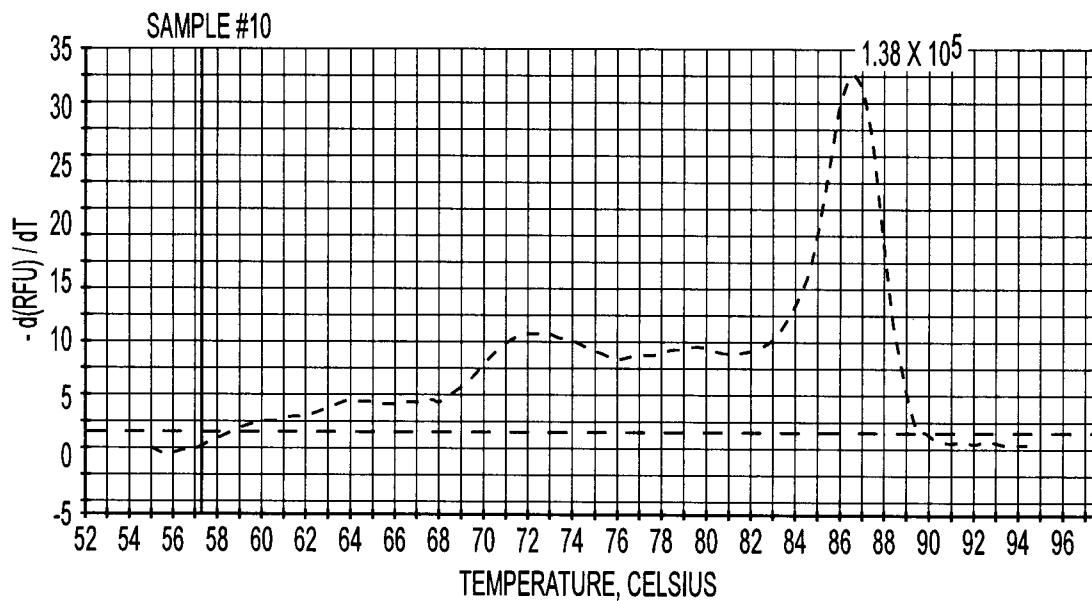


FIG. 7E

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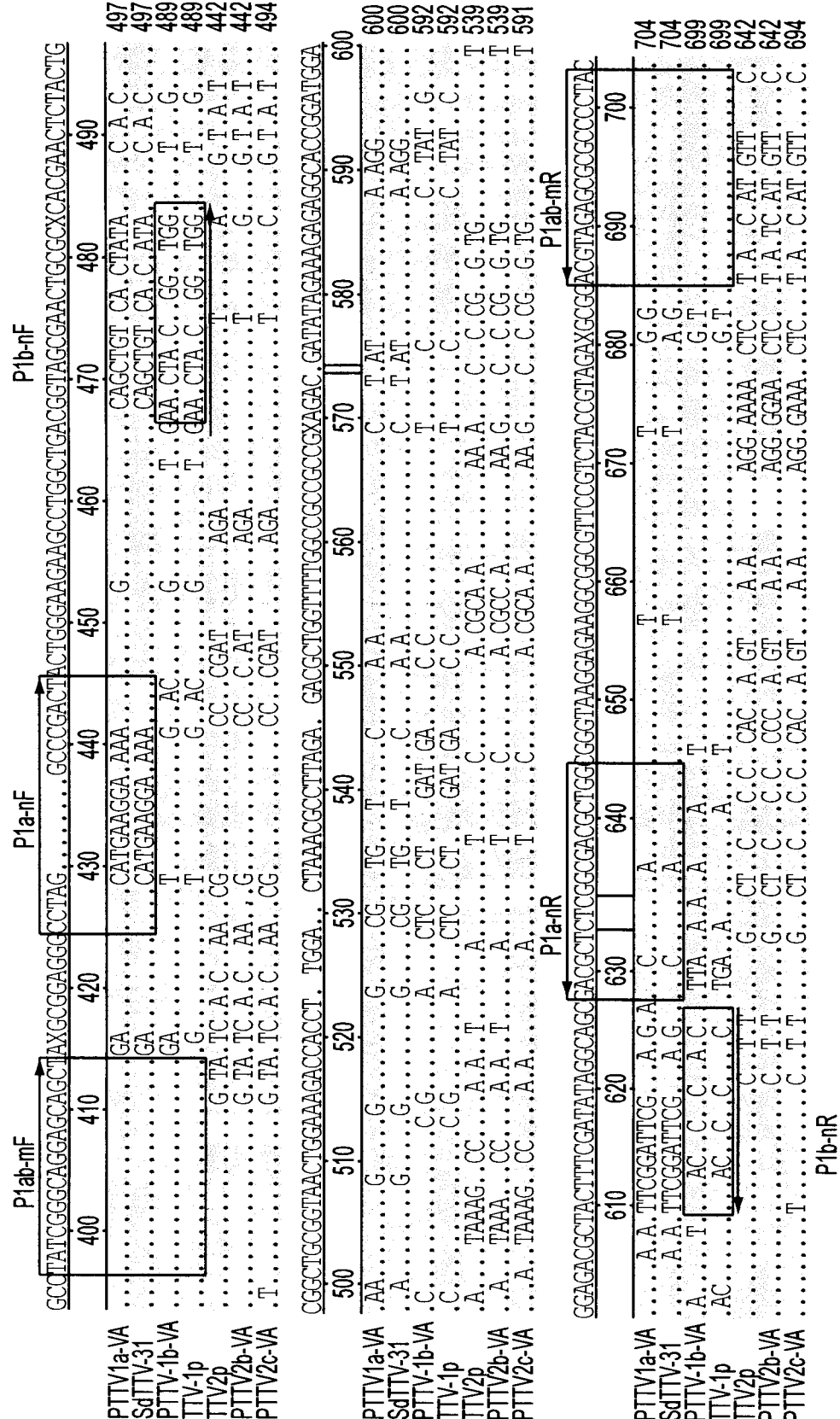


FIG. 8

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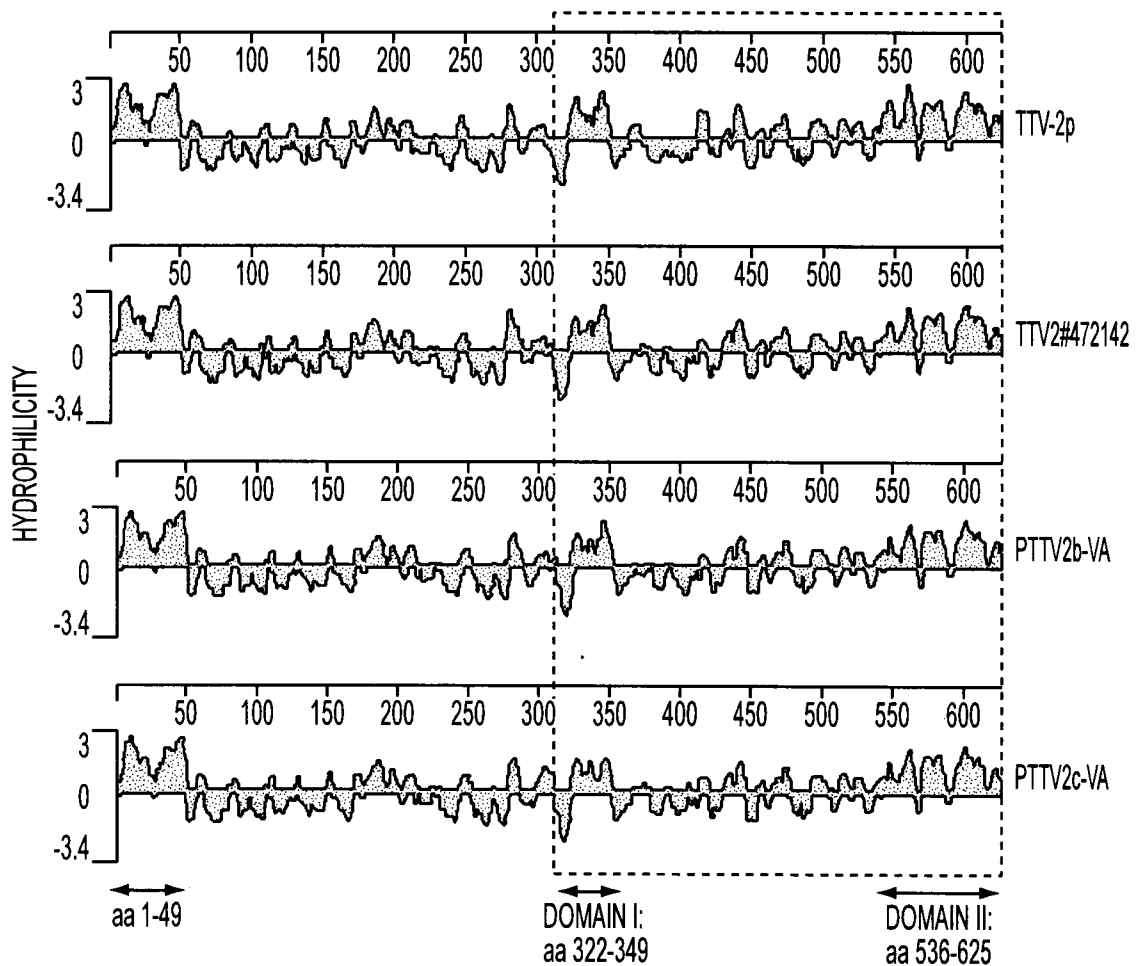


FIG. 9A

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DOMAIN I 322 349
↓
CONSENSUS SEQDIKKLAHQXIAREVARDPKSKKLK
PTTV2c-VAE.....NN.....
PTTV2b-VAD.V.....
TTV2#472142D.....
TTV-2pE.SAE.S...T.....

DOMAIN II 536 625
↓
CONSENSUS YDCDGIVRTDTLKRLLLELPTETETEKAYPLLGGQKTEKEPLSDSDEESVISSSSGSSQEEETQRRRXHKPSKRRLLKHLQRVVKRMKTL
PTTV2c-VAQ.....
PTTV2b-VAKH.....
TTV2#472142Q.....
TTV-2pK.....E.....H.....

FIG. 9B


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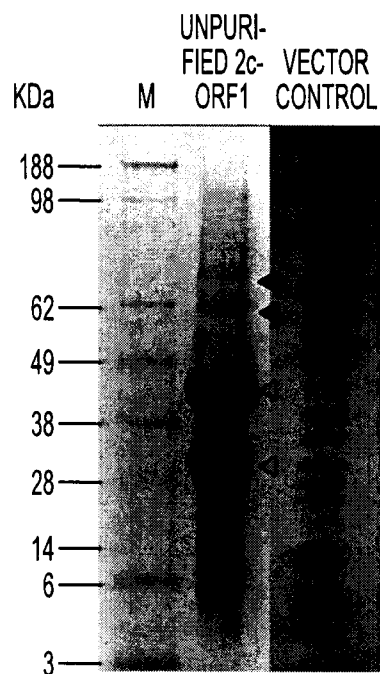


FIG. 10A

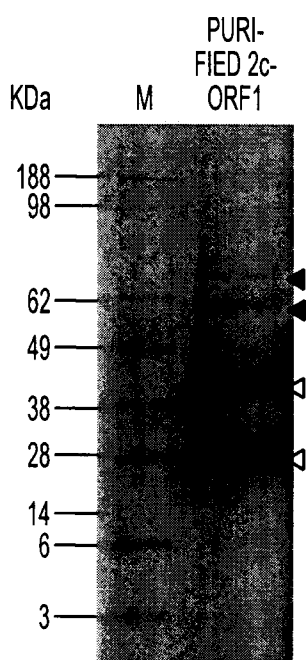


FIG. 10B

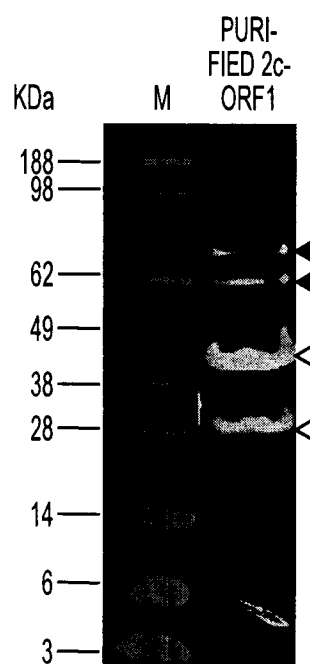


FIG. 10C

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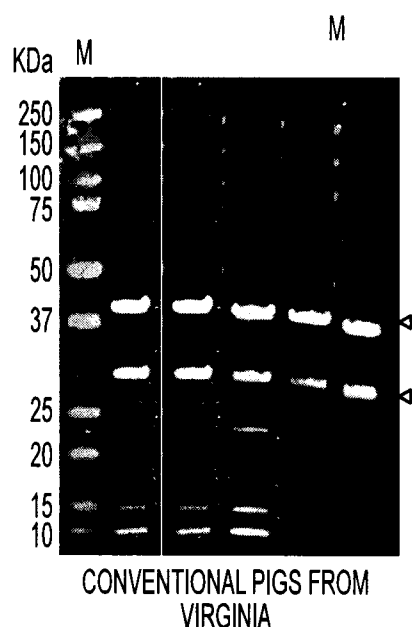
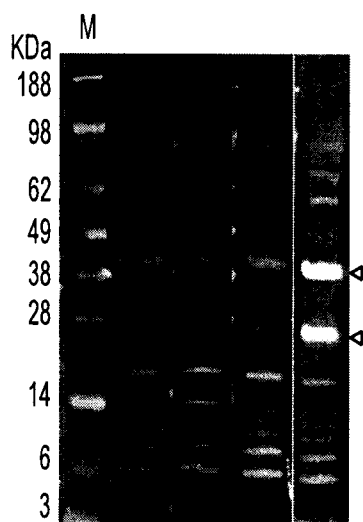
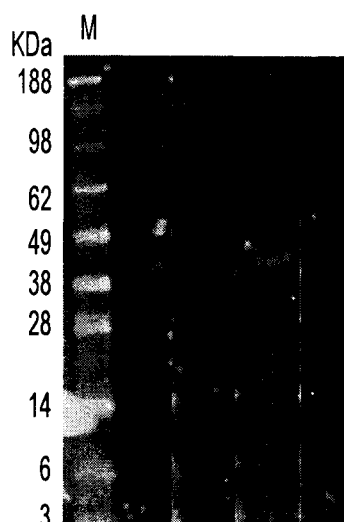


FIG. 11A



CD/CD PIGS

FIG. 11B



GNOTOBIOTIC PIGS

FIG. 11C

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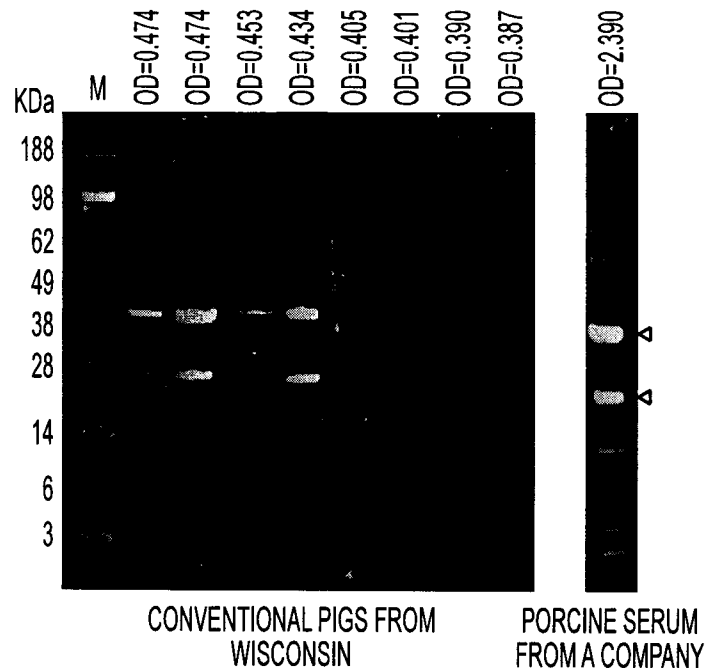


FIG. 12

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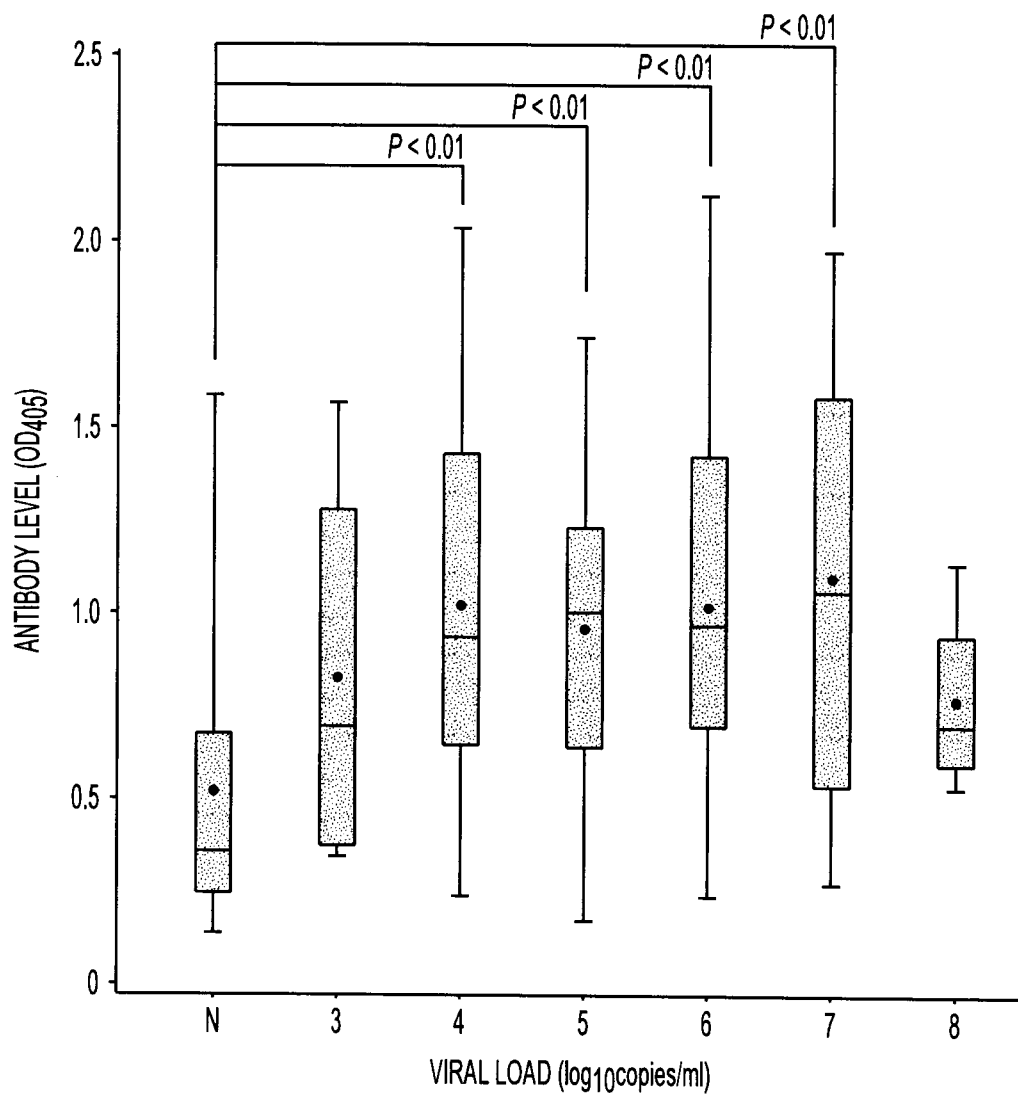


FIG. 13

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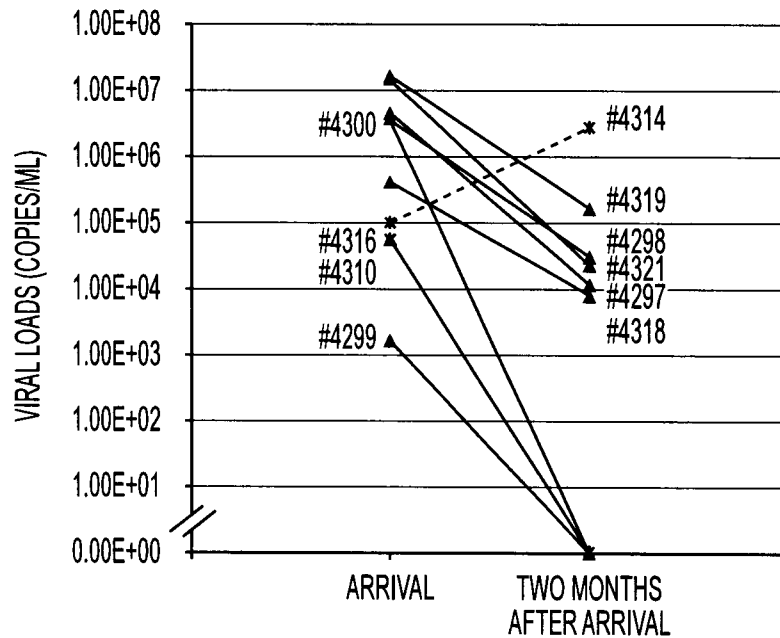


FIG. 14A

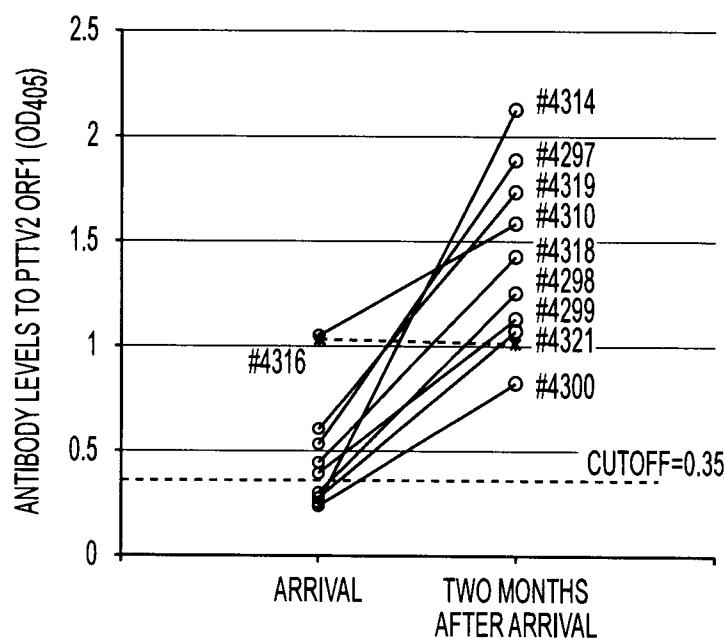


FIG. 14B

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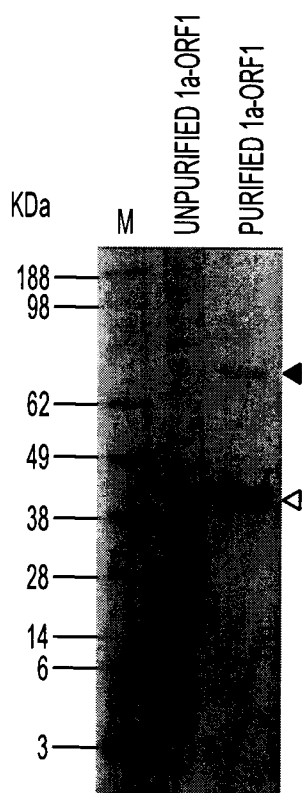


FIG. 15A

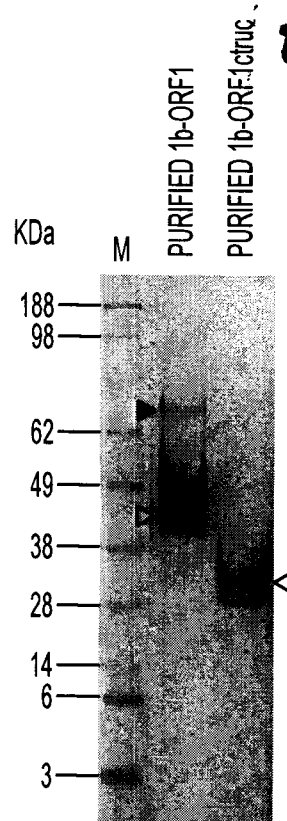


FIG. 15B

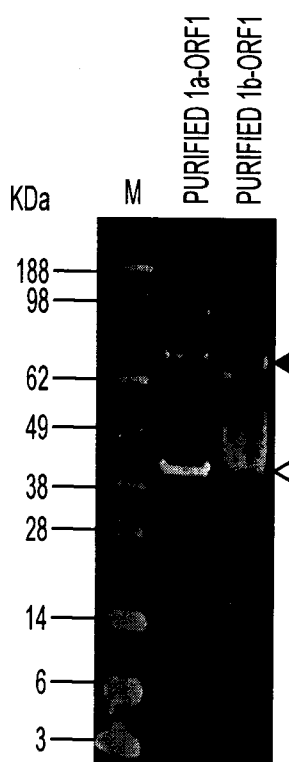


FIG. 15C

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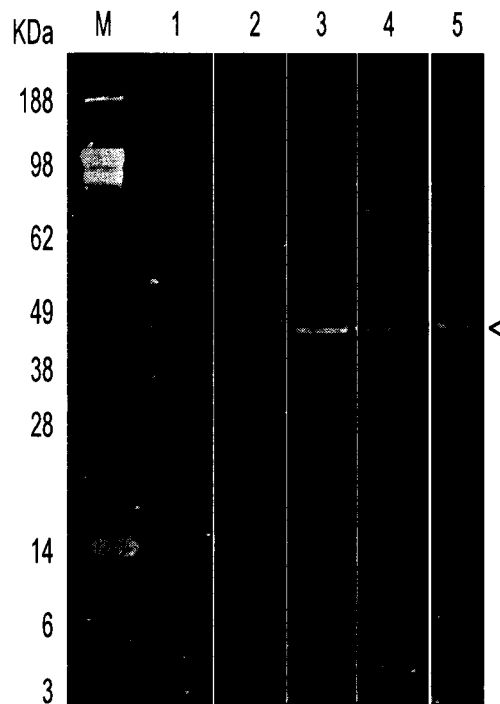
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WISCONSIN

FIG. 16

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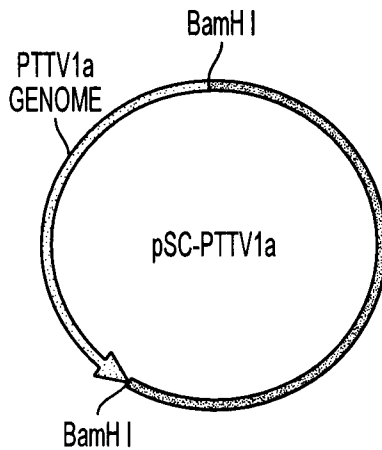


FIG. 17A

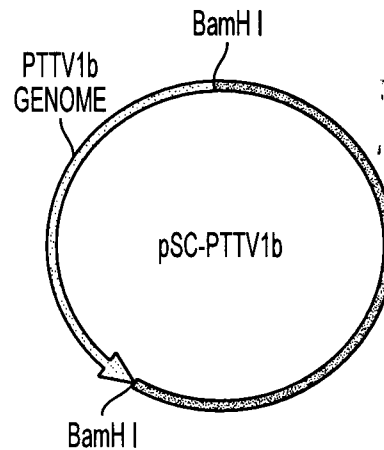


FIG. 17B

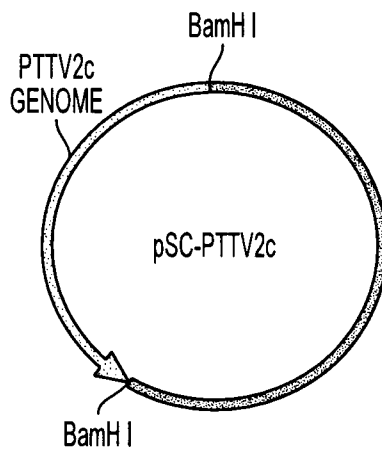


FIG. 17C

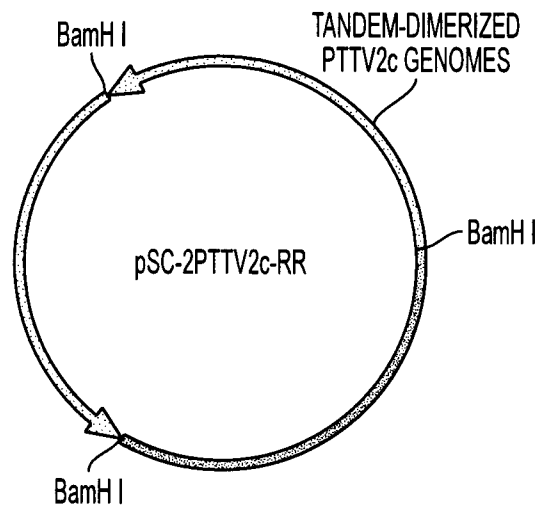


FIG. 17D

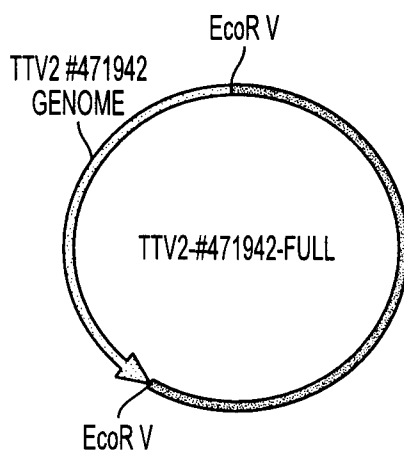


FIG. 17E

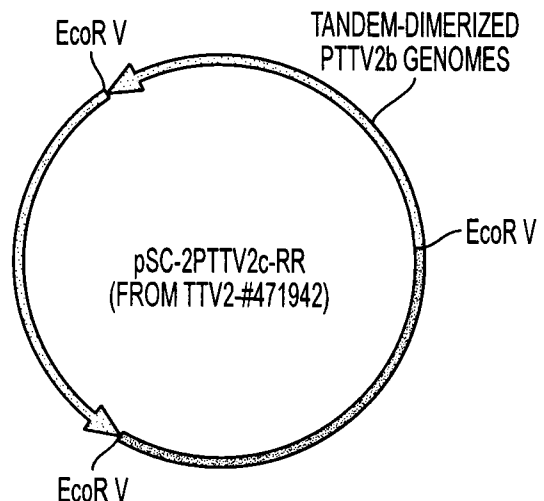


FIG. 17F

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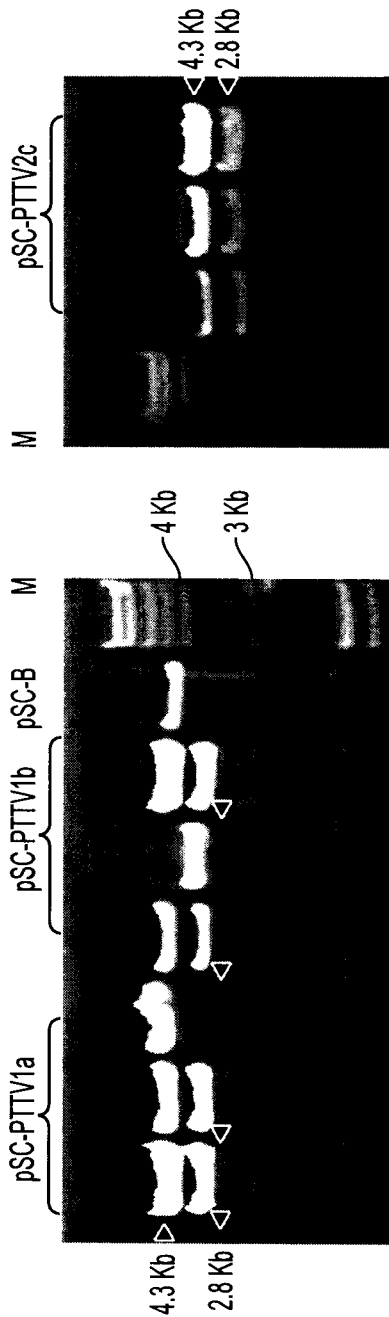


FIG. 18A



FIG. 18B

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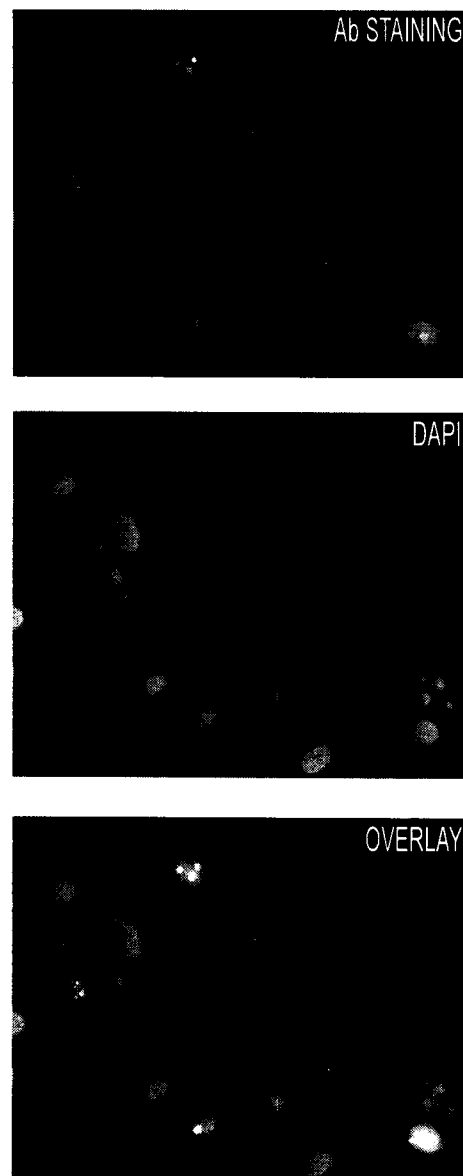
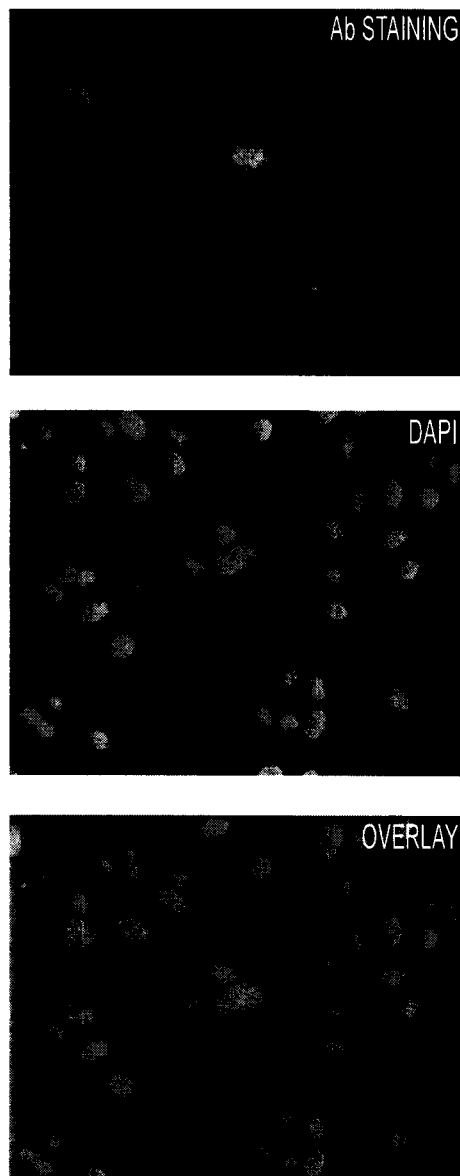
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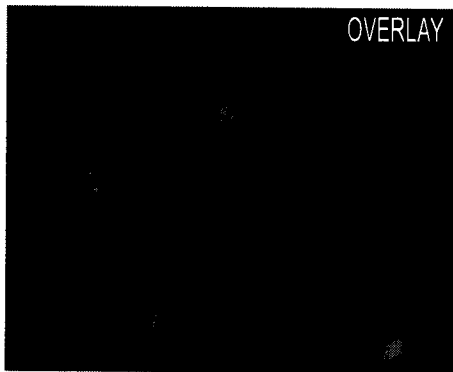
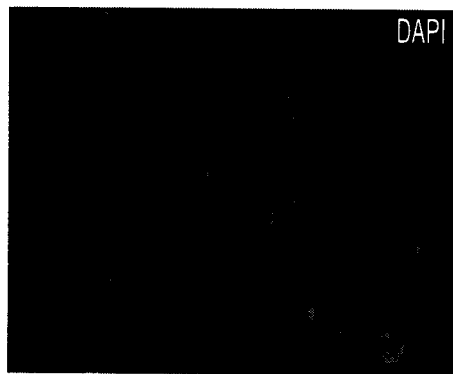
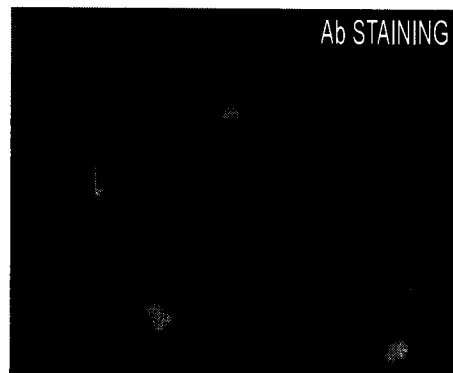


FIG. 20A

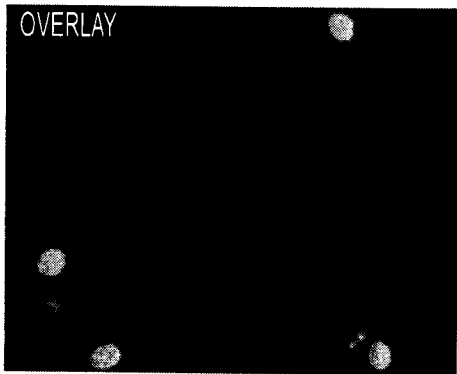
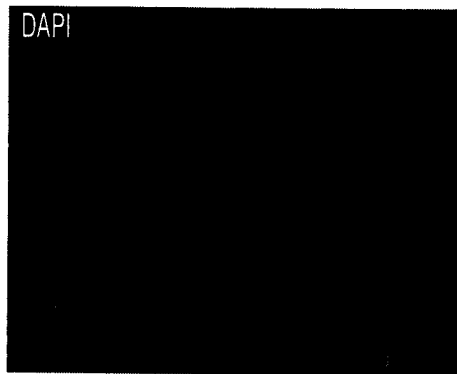


FIG. 20B


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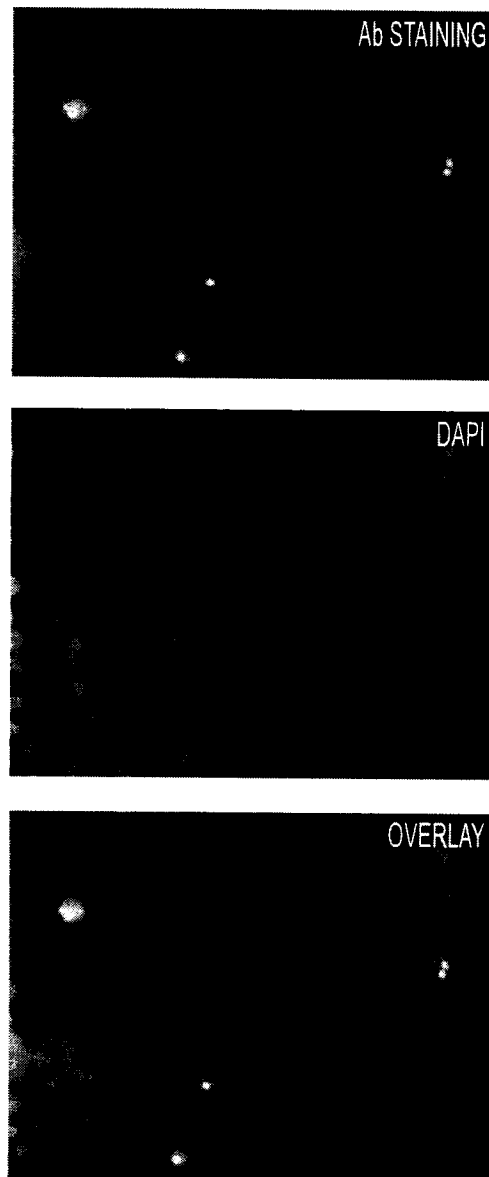


FIG. 21

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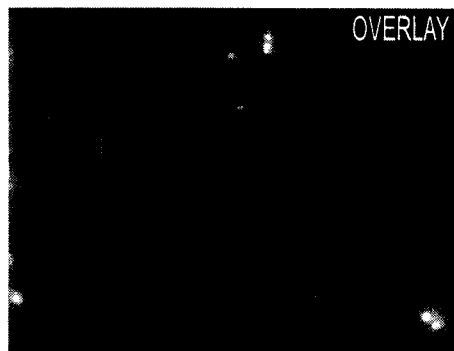
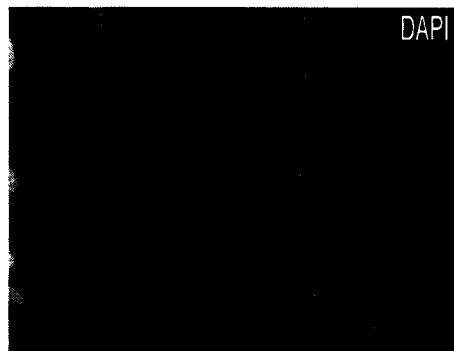
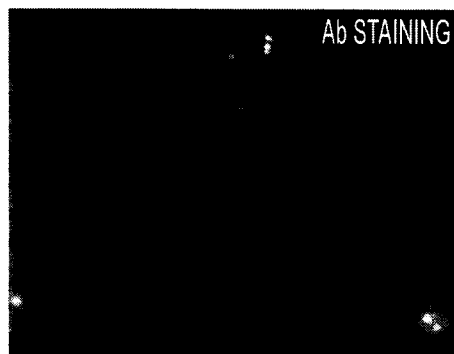


FIG. 22A

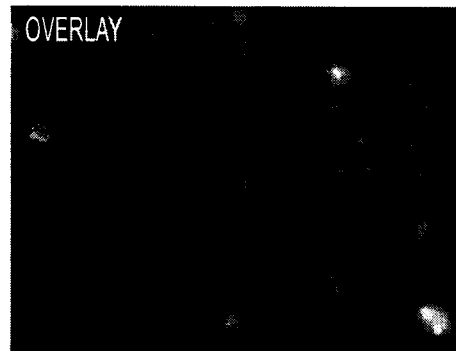
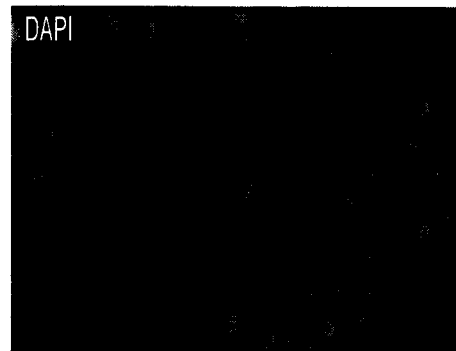


FIG. 22B

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of LALL LAHIRI & SALHOTRA
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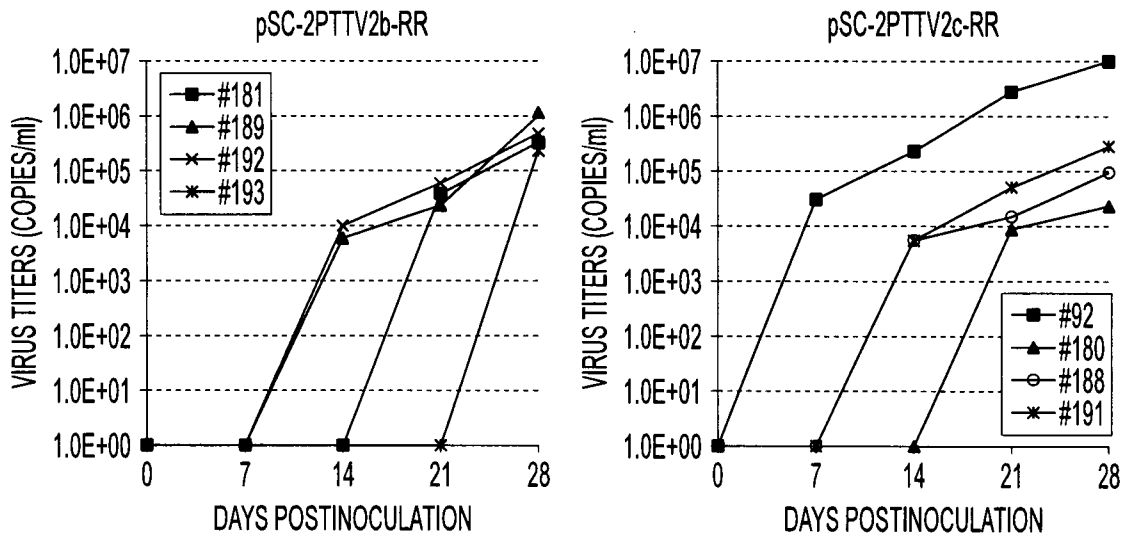


FIG. 23A

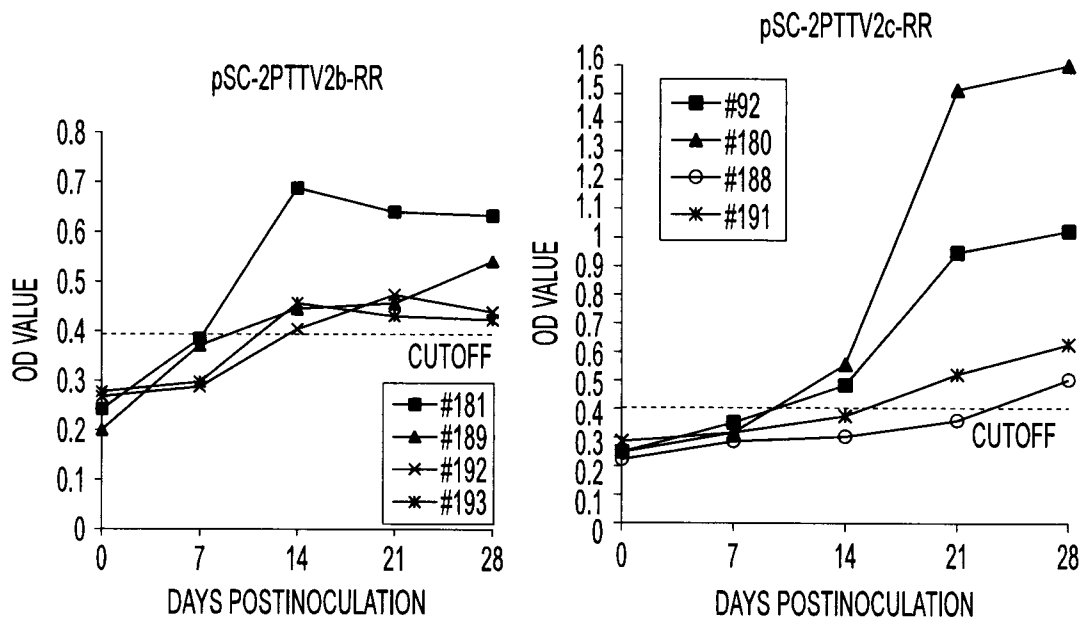


FIG. 23B

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(Anuradha Salhotra)
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REFERENCE TO RELATED APPLICATION

[0001] This patent application claims the benefit of U.S. Provisional Patent Application No. 61/235,833, filed on August 21, 2009, and U.S. Provisional Patent Application 61/316,519, filed on March 23, 2010, whose disclosures are hereby incorporated by reference in their its entirety into the present disclosure.

FIELD OF INVENTION

[0002] The present invention relates to vaccines for protecting against porcine Torque teno virus (TTV) infection, and infectious DNA clones of porcine TTV (PTTV) and their uses thereof. The present invention also relates to diagnosis of porcine Torque teno virus (PTTV) infection, particularly diagnosis of species- or type-specific PTTV infection, and simultaneous infection of multiple strains from different genotypes.

BACKGROUND OF THE INVENTION

[0003] Torque teno virus (TTV) was first discovered in a Japanese patient with post-transfusion non-A-E hepatitis in 1997 (Nishizawa, T., Okamoto, H., Konishi, K., Yoshizawa, H., Miyakawa, Y., and Mayumi, M. (1997). A novel DNA virus (TTV) associated with elevated transaminase levels in posttransfusion hepatitis of unknown etiology. *Biochem Biophys Res Commun* **241**(1), 92-7.). Since then, a large number of human TTV strains and two groups of TTV-related viruses, designated subsequently as Torque teno mini virus (TTMV) and Torque teno midi virus (TTMDV), have been identified with high prevalence in serum and other tissues from healthy humans (Hino, S., and Miyata, H. (2007). Torque teno virus (TTV): current status. *Rev Med Virol* **17**(1), 45-57; Okamoto, H. (2009a). History of discoveries and pathogenicity of TT viruses. *Curr Top Microbiol Immunol* **331**, 1-20). Human TTV, TTMV and TTMDV are

non-enveloped spherical viruses with circular single-stranded DNA (ssDNA) genomes of 3.6-3.9, 2.8-2.9 and 3.2 kb in length, respectively, and they are currently classified into a newly-established family *Anelloviridae* by the International Committee on Taxonomy of Viruses (ICTV; <http://www.ictvonline.org/virusTaxonomy.asp?bhcp=1>) (Biagini, P. (2009). Classification of TTV and related viruses (anelloviruses). *Curr Top Microbiol Immunol* **331**, 21-33). These three groups of TTV-related viruses exhibit a high degree of genetic heterogeneity, each consisting of many genogroups and genotypes (Biagini, P., Gallian, P., Cantaloube, J. F., Attoui, H., de Micco, P., and de Lamballerie, X. (2006). Distribution and genetic analysis of TTV and TTMV major phylogenetic groups in French blood donors. *J Med Virol* **78**(2), 298-304; Jelcic, I., Hotz-Wagenblatt, A., Hunziker, A., Zur Hausen, H., and de Villiers, E. M. (2004). Isolation of multiple TT virus genotypes from spleen biopsy tissue from a Hodgkin's disease patient: genome reorganization and diversity in the hypervariable region. *J Virol* **78**(14), 7498-507). The prevalence of multiple infections of TTV with different genotypes as well as dual or triple infections of TTV, TTMV and TTMDV have been documented in humans, and are considered to be a common event in healthy human adults (Niel, C., Saback, F. L., and Lampe, E. (2000). Coinfection with multiple TT virus strains belonging to different genotypes is a common event in healthy Brazilian adults. *J Clin Microbiol* **38**(5), 1926-30; Ninomiya, M., Takahashi, M., Hoshino, Y., Ichiyama, K., Simmonds, P., and Okamoto, H. (2009). Analysis of the entire genomes of torque teno midi virus variants in chimpanzees: infrequent cross-species infection between humans and chimpanzees. *J Gen Virol* **90**(Pt 2), 347-58; Okamoto, H. (2009a). History of discoveries and pathogenicity of TT viruses. *Curr Top Microbiol Immunol* **331**, 1-20; Takayama, S., Miura, T., Matsuo, S., Taki, M., and Sugii, S. (1999). Prevalence and persistence

of a novel DNA TT virus (TTV) infection in Japanese haemophiliacs. *Br J Haematol* **104**(3), 626-9).

[0004] TTV infects not only humans but also various other animal species as well including non-human primates, tupaia, pigs, cattle, cats, dogs and sea lions (Biagini, P., Uch, R., Belhouchet, M., Attoui, H., Cantaloube, J. F., Brisbarre, N., and de Micco, P. (2007). Circular genomes related to anelloviruses identified in human and animal samples by using a combined rolling-circle amplification/sequence-independent single primer amplification approach. *J Gen Virol* **88**(Pt 10), 2696-701; Inami, T., Obara, T., Moriyama, M., Arakawa, Y., and Abe, K. (2000). Full-length nucleotide sequence of a simian TT virus isolate obtained from a chimpanzee: evidence for a new TT virus-like species. *Virology* **277**(2), 330-5; Ng, T. F., Suedmeyer, W. K., Wheeler, E., Gulland, F., and Breitbart, M. (2009). Novel anellovirus discovered from a mortality event of captive California sea lions. *J Gen Virol* **90**(Pt 5), 1256-61; Okamoto, H. (2009b). TT viruses in animals. *Curr Top Microbiol Immunol* **331**, 35-52; Okamoto, H., Nishizawa, T., Takahashi, M., Tawara, A., Peng, Y., Kishimoto, J., and Wang, Y. (2001). Genomic and evolutionary characterization of TT virus (TTV) in tupaia and comparison with species-specific TTVs in humans and non-human primates. *J Gen Virol* **82**(Pt 9), 2041-50; Okamoto, H., Nishizawa, T., Tawara, A., Peng, Y., Takahashi, M., Kishimoto, J., Tanaka, T., Miyakawa, Y., and Mayumi, M. (2000a). Species-specific TT viruses in humans and nonhuman primates and their phylogenetic relatedness. *Virology* **277**(2), 368-78; Okamoto, H., Takahashi, M., Nishizawa, T., Tawara, A., Fukai, K., Muramatsu, U., Naito, Y., and Yoshikawa, A. (2002). Genomic characterization of TT viruses (TTVs) in pigs, cats and dogs and their relatedness with species-specific TTVs in primates and tupaia. *J Gen Virol* **83**(Pt 6), 1291-7). In addition, chimpanzees are also infected with TTMV and TTMDV (Ninomiya, M., Takahashi, M.,

Hoshino, Y., Ichiyama, K., Simmonds, P., and Okamoto, H. (2009). Analysis of the entire genomes of torque teno midi virus variants in chimpanzees: infrequent cross-species infection between humans and chimpanzees. *J Gen Virol* **90**(Pt 2), 347-58; Okamoto et al., 2000a, *supra*). Although the genomic sizes of the identified animal TTV strains, especially non-primate animal TTV, are relatively smaller than that of human TTV, they share the same genomic structure with a minimum of two partially overlapping open reading frames (ORF1 and ORF2) translated from the negative ssDNA as well as a short stretch of untranslated region (UTR) with high GC content (~90%) (Okamoto, 2009b, *supra*). The arrangement of TTV ORFs is quite similar to that of chicken anemia virus (CAV) belonging to the genus *Gyrovirus* in the family *Circoviridae* but is different from porcine circovirus (PCV) types 1 (PCV1) and 2 (PCV2), which are also classified into the same family (Davidson, I., and Shulman, L. M. (2008). Unraveling the puzzle of human anellovirus infections by comparison with avian infections with the chicken anemia virus. *Virus Res* **137**(1), 1-15; Hino, S., and Prasetyo, A. A. (2009). Relationship of Torque teno virus to chicken anemia virus. *Curr Top Microbiol Immunol* **331**, 117-30). The genomes of PCV1 and PCV2 are ambisense, in which the ORF1 is coded for by the genomic strand and the ORF2 is coded for by the antigenomic strand (Hino and Miyata, 2007, *supra*). Recently, the transcription pattern and translated products of both human TTV genotypes 1 and 6 have been identified by transfection of the respective TTV infectious DNA clones into cultured cells (Mueller, B., Maerz, A., Doberstein, K., Finsterbusch, T., and Mankertz, A. (2008). Gene expression of the human Torque Teno Virus isolate P/1C1. *Virology* **381**(1), 36-45; Qiu, J., Kakkola, L., Cheng, F., Ye, C., Soderlund-Venermo, M., Hedman, K., and Pintel, D. J. (2005). Human circovirus TT virus genotype 6 expresses six proteins following transfection of a full-length clone. *J Virol* **79**(10), 6505-10). Expression of at least six proteins, designated ORF1, ORF2, ORF1/1, ORF2/2,

ORF1/2 and ORF2/3, from three or more spliced mRNAs, have been reported (Kakkola, L., Hedman, K., Qiu, J., Pintel, D., and Soderlund-Venermo, M. (2009). Replication of and protein synthesis by TT viruses. *Curr Top Microbiol Immunol* **331**, 53-64; Mueller et al., 2008, *supra*; Qiu et al., 2005, *supra*). Accordingly, it is likely that, when more data regarding the animal TTV become available, the presumed genome structure of animal TTV will need to be modified.

[0005] Although TTV was first identified in a cryptogenic hepatitis patient, subsequent studies were not able to produce evidence of a significant role of TTV in the pathogenesis of hepatitis or other diseases (Hino and Miyata, 2007, *supra*; Maggi, F., and Bendinelli, M. (2009). Immunobiology of the Torque teno viruses and other anelloviruses. *Curr Top Microbiol Immunol* **331**, 65-90; Okamoto, 2009a, *supra*). While human TTV is not considered to be directly associated with a disease, porcine TTV (PTTV) was recently shown to partially contribute to the experimental induction of porcine dermatitis and nephropathy syndrome (PDNS) combined with porcine reproductive and respiratory syndrome virus (PRRSV) infection (Krakowka, S., Hartunian, C., Hamberg, A., Shoup, D., Rings, M., Zhang, Y., Allan, G., and Ellis, J. A. (2008). Evaluation of induction of porcine dermatitis and nephropathy syndrome in gnotobiotic pigs with negative results for porcine circovirus type 2. *Am J Vet Res* **69**(12), 1615-22), and also to the experimental induction of postweaning multisystemic wasting syndrome (PMWS) combined with PCV2 infection in a gnotobiotic pig model (Ellis, J. A., Allan, G., and Krakowka, S. (2008). Effect of coinfection with genogroup 1 porcine torque teno virus on porcine circovirus type 2-associated postweaning multisystemic wasting syndrome in gnotobiotic pigs. *Am J Vet Res* **69**(12), 1608-14). The data suggested that porcine TTV is pathogenic in pigs. However, further in-depth studies with a biologically pure form of PTTV virus to definitively characterize the diseases and lesions associated with PTTV infection are needed.

[0006] Compared to human TTV, the genomic information of PTTV is very limited. Currently, only one full-length and two near full-length genomic sequences of PTTV are reported from pigs in Japan and Brazil, respectively (Niel, C., Diniz-Mendes, L., and Devalle, S. (2005). Rolling-circle amplification of Torque teno virus (TTV) complete genomes from human and swine sera and identification of a novel swine TTV genogroup. *J Gen Virol* **86**(Pt 5), 1343-7; Okamoto et al., 2002, *supra*). Among the three known PTTV strains, the Sd-TTV31 and TTV-1p strains were clustered together into the genogroup 1 (PTTV1), whereas TTV-2p was the sole strain classified into the genogroup 2 (PTTV2) (Niel et al., 2005, *supra*). However, genogroup classification is a vague concept in the taxonomy of virology, and further and more accurate classification of PTTV is needed but can only be performed when more full-length genomic sequences of new PTTV strains representing multiple genotypes become available.

[0007] It was previously showed that PTTV infections were widespread in pigs from six different countries including the United States, Canada, Spain, China, Korea and Thailand (McKeown, N. E., Fenaux, M., Halbur, P. G., and Meng, X. J. (2004). Molecular characterization of porcine TT virus, an orphan virus, in pigs from six different countries. *Vet Microbiol* **104**(1-2), 113-7).

[0008] Whether porcine TTVs play a significant role in pathogenesis of specific swine diseases is still debatable. In a gnotobiotic pig model, it was shown that PTTV1 infection alone did not develop any clinical diseases but induced mild histological lesions (Krakowka, S. and Ellis, J.A., 2008. Evaluation of the effects of porcine genogroup 1 torque teno virus in gnotobiotic swine. *Am J Vet Res* **69**, 1623-9). Gnotobiotic pigs that were experimentally inoculated with both PTTV1 and porcine reproductive and respiratory syndrome virus (PRRSV) developed clinical porcine dermatitis and nephropathy syndrome (PDNS) (Krakowka, S.,

Hartunian, C., Hamberg, A., Shoup, D., Rings, M., Zhang, Y., Allan, G. and Ellis, J.A., 2008. Evaluation of induction of porcine dermatitis and nephropathy syndrome in gnotobiotic pigs with negative results for porcine circovirus type 2. *Am J Vet Res* **69**, 1615-22), whereas pigs inoculated with both PTTV1 and porcine circovirus type 2 (PCV2) developed acute postweaning multisystemic wasting syndrome (PMWS) (Ellis et al., 2008, *supra*). Although PCV2 is considered as the primary causative agent for clinical PMWS or PCV-associated diseases (PCVAD), a higher prevalence of PTTV2 infection in PMWS-affected pigs with low or no PCV2 than that in non-PMWS-affected pigs was observed in Spain (Kekarainen et al., 2006, *supra*). The data collectively suggest that porcine TTVs may serve as co-factors involved in triggering or exacerbating diseases in pigs.

[0009] Porcine TTV has been detected in porcine serum, fecal, saliva, semen and tissue samples of infected pigs, indicating its diverse transmission routes including both horizontal and vertical transmissions (Kekarainen et al., 2007, *supra*; Pozzuto, T., Mueller, B., Meehan, B., Ringler, S.S., McIntosh, K.A., Ellis, J.A., Mankertz, A. and Krakowka, S., 2009. In utero transmission of porcine torque teno viruses. *Vet Microbiol* **137**, 375-9; Sibila, M., Martinez-Guino, L., Huerta, E., Llorens, A., Mora, M., Grau-Roma, L., Kekarainen, T. and Segales, J., 2009. Swine torque teno virus (TTV) infection and excretion dynamics in conventional pig farms. *Vet Microbiol* **139**, 213-8). However, current detection of porcine TTV infection was mainly based upon conventional PCR assays. Thus far, neither serological assay nor viral culture system has been established. In particular, nested PCR amplifications of the conserved regions in the UTR of PTTV1 and PTTV2, respectively, developed by a Spanish group, have become widely used (Kekarainen et al., 2006, *supra*). Since the amount of virus is likely associated with the severity of clinical diseases, as demonstrated for PCV2-induced PCVAD (Opriessnig, T.,

Meng, X.J. and Halbur, P.G., 2007. Porcine circovirus type 2 associated disease: update on current terminology, clinical manifestations, pathogenesis, diagnosis, and intervention strategies. *J Vet Diagn Invest* **19**, 591-615), it will be important to determine the viral load of porcine TTV by quantitative real-time PCR than the presence of TTV DNA by conventional PCR. In addition, real-time PCR is more reliable, rapid and less expensive than conventional PCR. Recently, two TaqMan probe-based real-time PCR assays were described for detection and quantification of two porcine TTV species (Brassard, J., Gagne, M.J., Houde, A., Poitras, E. and Ward, P., 2009. Development of a real-time TaqMan PCR assay for the detection of porcine and bovine Torque teno virus. *J Appl Microbiol*. Nov 14, 2009, Epub ahead of print; Gallei, A., Pesch, S., Eising, W.S., Keller, C. and Ohlinger, V.F., 2009. Porcine Torque teno virus: Determination of viral genomic loads by genogroup-specific multiplex rt-PCR, detection of frequent multiple infections with genogroups 1 or 2, and establishment of viral full-length sequences. *Vet Microbiol*. Dec 21, 2009, Epub ahead of print). A main drawback of probe-based assays is that the false-negative results may be obtained if the probe-binding sequences contain mutations (Anderson, T.P., Werno, A.M., Beynon, K.A. and Murdoch, D.R., 2003. Failure to genotype herpes simplex virus by real-time PCR assay and melting curve analysis due to sequence variation within probe binding sites. *J Clin Microbiol* **41**, 2135-7). Considering the high degree of heterogeneity among the sequences of known porcine TTV strains, variations in the probe-binding sequences are expected for field strains of PTTVs. The SYBR green-based real-time PCR is an alternative method avoiding this potential problem, in spite of its relatively lower specificity, which provides a universal way to detect and quantify the potential porcine TTV variants. Moreover, melting curve analysis (MCA) following SYBR green real-time PCR ensures reaction specificity and also allows multiplex detection of distinct types of virus (Ririe, K.M., Rasmussen, R.P. and

Wittwer, C.T., 1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal Biochem* **245**, 154-60). MCA-based SYBR green real-time PCR methods have been successfully applied to various human and veterinary viruses (Gibellini, D., Gardini, F., Vitone, F., Schiavone, P., Furlini, G. and Re, M.C., 2006. Simultaneous detection of HCV and HIV-1 by SYBR Green real time multiplex RT-PCR technique in plasma samples. *Mol Cell Probes* **20**, 223-9; Martinez, E., Riera, P., Sitja, M., Fang, Y., Oliveira, S. and Maldonado, J., 2008. Simultaneous detection and genotyping of porcine reproductive and respiratory syndrome virus (PRRSV) by real-time RT-PCR and amplicon melting curve analysis using SYBR Green. *Res Vet Sci* **85**, 184-93; Mouillesseaux, K.P., Klimpel, K.R. and Dhar, A.K., 2003. Improvement in the specificity and sensitivity of detection for the Taura syndrome virus and yellow head virus of penaeid shrimp by increasing the amplicon size in SYBR Green real-time RT-PCR. *J Virol Methods* **111**, 121-7; Wilhelm, S., Zimmermann, P., Selbitz, H.J. and Truyen, U., 2006. Real-time PCR protocol for the detection of porcine parvovirus in field samples. *J Virol Methods* **134**, 257-60).

[0010] Currently, little is known about PTTV-specific humoral response. Since PCR-based assays do not reflect the course of PTTV infection in pigs, an efficient enzyme-linked immunosorbent assay (ELISA) for detection of PTTV serum antibody is necessary to evaluate seroprevalence of PTTV and help characterize the role of PTTV in porcine diseases.

[0011] Thus far, no subunit, killed and live vaccines for porcine TTVs are available. It will be desirable and advantageous to express recombinant PTTV capsid proteins from different genotypes for development of PTTV subunit vaccines, and to construct infectious PTTV molecular DNA clones from different genotypes for propagating biological pure form of PTTVs in cell culture system that are used for killed and live vaccines development.

SUMMARY OF THE INVENTION

[0012] The present invention provides an infectious nucleic acid molecule (“infectious DNA clone”) of porcine Torque teno virus (PTTV) comprising a nucleic acid molecule encoding an infectious PTTV which contains at least one copy of genomic sequence having at least 80% homology to a genomic sequence selected from the group consisting of genotypes of PTTV1a-VA, PTTV1b-VA, PTTV2b-VA, and PTTV2c-VA.

[0013] According to one aspect of the present invention, the infectious DNA clones of PTTV of set forth in claim 1, wherein the genomic sequence is selected from sequences set forth in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12.

[0014] The present invention provides a biologically functional plasmid or viral vector containing the infectious PTTV genomes.

[0015] The present invention provides a suitable host cell transfected with the infectious clone DNA plasmid or viral vector.

[0016] The present invention provides an infectious PTTV produced by cells transfected with the PTTV infectious DNA clones.

[0017] The present invention also provides a viral vaccine comprising a nontoxic, physiologically acceptable carrier and an immunogenic amount of a member selected from the group consisting of (a) a nucleic acid molecule containing at least one copy of genomic sequence having at least 80% homology to a genomic sequence selected from the group consisting of PTTV genotypes or subtypes PTTV1a-VA, PTTV1b-VA, PTTV2b-VA, and PTTV2c-VA, or its complementary strand, (b) a biologically functional plasmid or viral vector containing a nucleic acid molecule containing at least one copy of genomic sequence having at least 80% homology

to a genomic sequence selected from the group consisting of PTTV genotypes or subtypes PTTV1a-VA, PTTV1b-VA, PTTV2b-VA, and PTTV2c-VA, or its complementary strand, and (c) an avirulent, infectious nonpathogenic PTTV which contains at least one copy of genomic sequence having at least 80% homology to a genomic sequence selected from the group consisting of PTTV genotypes or subtypes PTTV1a-VA, PTTV1b-VA, PTTV2b-VA, and PTTV2c-VA.

[0018] According to one aspect of the present invention, the vaccine contains live PTTV virus derived from the PTTV infectious clones. According to another aspect of the present invention, the vaccine contains killed PTTV virus derived from the PTTV infectious clones.

[0019] The present invention provides purified recombinant proteins expressed from the ORF1 capsid genes of PTTV genotypes or subtypes PTTV1a-VA, PTTV1b-VA, and PTTV2c-VA in bacterial expression system, and the use of these recombinant capsid proteins as subunit vaccines against PTTV infections. In one embodiment of the present invention, the recombinant capsid proteins for the use as subunit vaccines are expressed in baculovirus expression system and other expression vector systems.

[0020] According to a further aspect of the present invention, further contains an adjuvant.

[0021] The present invention further provides a method of immunizing a pig against PTTV viral infection, comprising administering to a pig an immunologically effective amount of the viral vaccine.

[0022] According to one aspect of the present invention, the method comprising administering the recombinant subunit capsid protein, the infectious nucleic acid molecule or live PTTV virus to the pig.

[0023] According to another aspect of the present invention, the method comprising administering the vaccine parenterally, intranasally, intradermally, or transdermally to the pig. According a further aspect of the present invention, the method comprising administering the vaccine intralymphoidly or intramuscularly to the pig.

[0024] The present invention also provides an isolated polynucleotide consisting of the sequence of the nucleotide sequence of PTTV1a-VA set forth in SEQ ID NO:9.

[0025] The present invention also provides an isolated polynucleotide consisting of the sequence of the nucleotide sequence of PTTV1b-VA set forth in SEQ ID No:10.

[0026] The present invention also provides an isolated polynucleotide consisting of the sequence of the nucleotide sequence of PTTV2b-VA set forth in SEQ ID No:11.

[0027] The present invention also provides an isolated polynucleotide consisting of the sequence of the nucleotide sequence of PTTV2c-VA set forth in SEQ ID No:12.

[0028] The present invention further provides a subunit vaccine comprising an immunogenic fragment of a polypeptide sequence or a complete protein translated according to a polynucleotide sequence selected from the group consisting of ORF1, ORF2, ORF1/1, and ORF2/2 of PTTV genotypes or subtypes PTTV1a-VA, PTTV1b-VA, PTTV2b-VA, and PTTV2c-VA, particularly the ORF1 encoding the capsid protein.

[0029] According to one aspect of the present invention, the polynucleotide sequence is selected from the group consisting of ORF1 of PTTV genotypes or subtypes PTTV1a-VA, PTTV1b-VA, PTTV2b-VA, and PTTV2c-VA.

[0030] According to another aspect of the present invention, the polynucleotide sequence is ORF1 of PTTV genotype PTTV1a-VA. According to a further aspect of the present invention,

the polynucleotide sequence is ORF1 of PTTV genotype PTTV1b-VA. According to yet another aspect of the present invention, the polynucleotide sequence is ORF1 of PTTV subtype PTTV2c-VA.

[0031] According to one aspect of the present invention, the polypeptide sequence is selected from the group consisting of sequence set forth in SEQ ID No:13, SEQ ID No:14, SEQ ID No:15, SEQ ID No:16, SEQ ID No:17, SEQ ID No:18, SEQ ID No:19, SEQ ID No:20, SEQ ID No:21, SEQ ID No:22, SEQ ID No:23, SEQ ID No:24, SEQ ID No:25, SEQ ID No:26, SEQ ID No:27, and SEQ ID No:28.

[0032] According to another aspect of the present invention, the polypeptide sequence is set forth in SEQ ID No:13. According to another aspect of the present invention, the polypeptide sequence is set forth in SEQ ID No:14. According to a further aspect of the present invention, the polypeptide sequence is set forth in SEQ ID No:16. In one specific embodiment of the present invention, the polypeptide sequence is C-terminal region (aa 310-625) of SEQ ID No:16. According to yet another aspect of the present invention, the polypeptide sequence is set forth in SEQ ID No:20.

[0033] According to an additional aspect of the present invention, the vaccine further contains an adjuvant.

[0034] The present invention further provides method of immunizing a pig against PTTV viral infection, comprising administering to a pig an immunologically effective amount of the vaccine comprising an immunogenic fragment of a polypeptide sequence or a complete protein translated according to a polynucleotide sequence selected from the group consisting of ORF1, ORF2, ORF1/1, and ORF2/2 of PTTV genotypes or subtypes PTTV1a-VA, PTTV1b-VA, PTTV2b-VA, and PTTV2c-VA.

[0035] According to one aspect of the present invention, the method comprises administering the immunogenic fragment or recombinant capsid protein to the pig.

[0036] According to another aspect of the present invention, the method comprises administering the vaccine parenterally, intranasally, intradermally, or transdermally to the pig. According to a further aspect of the present invention, the method comprises administering the vaccine intralymphoidly or intramuscularly to the pig.

[0037] The present invention additionally provides a method for diagnosing PTTV1 infection and quantification of PTTV1 load, comprising extracting DNA from a sample suspected of PTTV1 infection, performing polymerase chain reaction (PCR) using primers comprising the sequences set forth in SEQ ID NO:29 and SEQ ID NO:30, and detecting PTTV1 specific amplification. According to one aspect of the present invention, the polymerase chain reaction is a SYBR green real-time PCR.

[0038] The present invention further provides a method for diagnosing PTTV2 infection and quantification of PTTV2 load, comprising extracting DNA from a sample suspected of PTTV2 infection, performing polymerase chain reaction (PCR) using primers comprising the sequences set forth in SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31 and SEQ ID NO:32, and detecting PTTV2 specific amplification. According to one aspect of the present invention, the polymerase chain reaction is a SYBR green real-time PCR.

[0039] The present invention also provides a method for simultaneously detecting and diagnosing PTTV1 and PTTV2 infection, comprising extracting DNA from a sample suspected of PTTV infection, performing polymerase chain reaction (PCR) using primers comprising the sequences set forth in SEQ ID NO:31 and SEQ ID NO:32, and detecting PTTV1 and PTTV2

specific amplification. According to one aspect of the present invention, the polymerase chain reaction is a SYBR green real-time PCR.

[0040] The present invention, in addition, provides a method for simultaneously detecting and diagnosing PTTV1a and PTTV1b infection, comprising extracting DNA from a sample suspected of PTTV1 infection, performing a first polymerase chain reaction (PCR) using primers comprising the sequences set forth in SEQ ID NO:33 and SEQ ID NO:34, performing a second PCR using primers comprising the sequences set forth in SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, and SEQ ID NO:38, and detecting PTTV1a and PTTV1b specific amplification.

[0041] The present invention provides a method for diagnosing PTTV infection, comprising immobilizing an immunogenic fragment of a polypeptide sequence translated according to a polynucleotide sequence selected from the group consisting of ORF1, ORF2, ORF1/1, and ORF2/2 of PTTV genotypes or subtypes PTTV1a-VA, PTTV1b-VA, PTTV2b-VA, and PTTV2c-VA; contacting a serum sample from a pig suspected of PTTV infection with the immobilized immunogenic fragment, and detecting captured antibody specific to the immunogenic fragment.

[0042] According to one aspect of the present invention, the polynucleotide sequence is selected from the group consisting of ORF1 of PTTV genotypes or subtypes PTTV1a-VA, PTTV1b-VA, PTTV2b-VA, and PTTV2c-VA.

[0043] According to one embodiment of the present invention, the polynucleotide sequence is ORF1 of PTTV genotype PTTV1a-VA. According to another embodiment of the present invention, the polynucleotide sequence is ORF1 of PTTV genotype PTTV1b-VA. According to a further embodiment of the present invention, the polynucleotide sequence is ORF1 of PTTV subtype PTTV2c-VA.

[0044] According to another aspect of the present invention, the polypeptide sequence is selected from the group consisting of sequence set forth in SEQ ID No:13, SEQ ID No:14, SEQ ID No:15, SEQ ID No:16, SEQ ID No:17, SEQ ID No:18, SEQ ID No:19, SEQ ID No:20, SEQ ID No:21, SEQ ID No:22, SEQ ID No:23, SEQ ID No:24, SEQ ID No:25, SEQ ID No:26, SEQ ID No:27, and SEQ ID No:28.

[0045] According to one embodiment of the present invention, the polypeptide sequence is set forth in SEQ ID No:13. According to another aspect of the present invention, the polypeptide sequence is set forth in SEQ ID No:14. According to another embodiment of the present invention, the polypeptide sequence is set forth in SEQ ID No:16. According to a further embodiment of the present invention, the immunogenic fragment is C-terminal region (aa 310-625) of SEQ ID No:16. According to yet another embodiment of the present invention, the polypeptide sequence is set forth in SEQ ID No:20.

[0046] The present invention provides three standardized enzyme-linked immunosorbent assays (ELISA) to diagnose PTTV infections and detect antibodies in serum of pigs infected by PTTV genotypes PTTV1a-VA, PTTV1b-VA, and all known subtypes in PTTV species 2.

[0047] The ELISA diagnostic tests are based on the bacterial-expressed or baculovirus-expressed recombinant ORF1 capsid protein of PTTV genotypes PTTV1a-VA, PTTV1b-VA, and PTTV2c-VA.

[0048] According to another aspect of the present invention, the detecting captured antibody is via Western blot. According to yet another aspect of the present invention, the detecting captured antibody is via enzyme-linked immunosorbent assay (ELISA).

BRIEF DESCRIPTION OF THE DRAWINGS

[0049] The above-mentioned features of the invention will become more clearly understood from the following detailed description of the invention read together with the drawings in which:

[0050] Figure 1A and 1B represent the schematic diagram of genomic structures, strategies for genomic cloning and assemblies of four prototype U.S. strains of porcine TTV virus group 1 (species 1) and group 2 (species 2) strains;

[0051] Figure 2 represents PASC (pairwise sequences comparisons) distribution of nucleotide sequence comparisons of 121 TTV strains available in GenBank database. The genus, species, types, subtypes and variants and their corresponding percentage of nucleotide sequence identities are displayed;

[0052] Figure 3A illustrates a phylogenetic tree constructed by the neighbor-joining method based upon the full-length genomic nucleotide sequences;

[0053] Figure and 3B illustrates a phylogenetic trees constructed based upon deduced amino acid sequences of ORF1 among seven porcine TTV strains;

[0054] Figure and 3C illustrates a phylogenetic trees constructed based upon deduced amino acid sequences of ORF1/1 among seven porcine TTV strains;

[0055] Figure 3D illustrates a phylogenetic trees constructed based upon deduced amino acid sequences of ORF2 among seven porcine TTV strains;

[0056] Figure and 3E illustrates a phylogenetic trees constructed based upon deduced amino acid sequences of ORF2/2 among seven porcine TTV strains;

- [0057] Figure 4 represents an alignment of the full-length amino acid sequences of ORF1 among seven PTTV strains;
- [0058] Figure 5 represents an alignment of the full-length amino acid sequences of ORF2 among seven PTTV strains;
- [0059] Figure 6A illustrates melting curves of PTTV1 real-time PCR products after 40 cycles of amplifications of respective standard template (indicated in blue) and 20 porcine serum samples;
- [0060] Figure 6B illustrates melting curves of PTTV2 real-time PCR products after 40 cycles of amplifications of respective standard template and 20 porcine serum samples;
- [0061] Figures 7A-7E illustrate melting curve analysis (MCA) of PTTV1/PTTV2 SYBR green-based duplex real-time PCR;
- [0062] Figure 8 represents an alignment of nucleotide sequences located at the N-terminal part of the putative ORF1 among seven PTTV strains;
- [0063] Figures 9A and 9B represent hydrophilicity profiles and conserved regions of the four known porcine TTV2;
- [0064] Figures 10A-10C illustrate the expression and purification of recombinant PTTV2c ORF1 capsid protein;
- [0065] Figures 11A-11C show representative results of Western blot analyses of selected porcine serum samples;
- [0066] Figure 12 illustrates the consistency of PTTV2c-ORF1-based Western blot and ELISA;

- [0067] Figure 13 shows Box-and-Whisker-plots of PTTV2 serum antibody level by viral load in 138 pigs from different sources;
- [0068] Figure 14A illustrates a retrospective evaluation of the viral load of PTTV2;
- [0069] Figure 14B illustrates antibody level to PTTV2 ORF1 capsid protein in 10 pigs growing from arrival to two months after arrival;
- [0070] Figures 15A-15C illustrate the expression and purification of PTTV1a and PTTV1b recombinant ORF1 capsid protein; and
- [0071] Figure 16 shows examples of PTTV1a-ORF1-based Western blot analyses of selected porcine serum samples from a farm of Wisconsin.

DETAILED DESCRIPTION OF THE INVENTION

[0072] In accordance with the present invention, in one specific example, the aforementioned four novel porcine TTV subtypes are isolated from a single boar in Virginia.

[0073] In Fig. 1A, both the PTTV1 and PTTV2 genomes are shown in bold and the sizes and directions of the four putative ORFs (ORF1, ORF2, ORF1/1 and ORF2/2) are indicated by arrows. The GC-rich regions are also shown. Dashed-line arcs A and D represent the regions used for detection of PTTV1 and PTTV2 from serum and semen samples by nested PCR, respectively. Dashed-line arcs B and C represent the two overlapping PCR fragments for genomic cloning of PTTV1 whereas dashed-line arcs E and F represent the two overlapping PCR fragments for genomic cloning of PTTV2. The locations of the primers used in the study (see Table 1) are also shown in the corresponding positions.

[0074] One boar serum sample (SR#5) that was shown to be positive for both PTTV1 and PTTV2 in the first-round PCR, thus indicative of higher virus load, was used for subsequent full-length genomic cloning of PTTV. Surprisingly, initial attempts to utilize two primer sets (NG372/NG373 and NG384/NG385) of an inverse PCR (Okamoto et al., 2002, *supra*) designed for cloning of the first PTTV strain Sd-TTV31 to amplify the virus genomic DNA were not successful. No PCR product was obtained after several trials. Based upon the initial sequence of the region A of PTTV1 and the region D of PTTV2, two new pairs of primers (TTV1-If(SEQ ID NO:1)/TTV1-2340R(SEQ ID NO:2) and TTV1-2311F(SEQ ID NO:3)/TTV1-IR(SEQ ID NO:4)) were subsequently designed to amplify regions B and C spanning the assumed PTTV1 genome, and two additional pairs of primers (TTV2-IF(SEQ ID NO:5)/TTV2-2316R(SEQ ID NO:6) and TTV2-GCF(SEQ ID NO:7)/TTV2-IR(SEQ ID NO:8)) to amplify regions E and F spanning the

assumed PTTV2 genome, respectively (Fig. 1A and Table 1). Primers TTV1-2340R(SEQ ID NO:2) and TTV1-2311F(SEQ ID NO:3) were deduced from a common sequence in PTTV1 stains Sd-TTV31 (Okamoto et al., 2002, *supra*) and TTV-1p (Niel et al., 2005) that is absent in PTTV2 strain TTV-2p (Niel et al., 2005, *supra*), whereas primers TTV2-2316R(SEQ ID NO:6) and TTV2-GCF(SEQ ID NO:7) were deduced from a sequence of strain TTV-2p that is absent in the two PTTV1 strains. The resulting four different PCR products with expected sizes were each inserted into a blunt-end cloning vector, and the resulting recombinant plasmids were transformed into *Escherichia coli*. Eight to fifteen positive (with white color) bacterial clones for each construct representing fragments B, C, E and F were identified and subsequently sequenced.

Table 1. Oligonucleotide primers used for nested PCR and genomic PCR amplifications of porcine TT viruses

Primer ID	Sequence (5' to 3')	Used for:
TTV1-mF (SEQ ID NO:45)	TACACTTCCGGGTTCAAGGAGGCT	Detection of porcine TTV1
TTV1-mR (SEQ ID NO:46)	ACTCAGCCATTCGGAACCTCAC	Detection of porcine TTV1
TTV1-nF (SEQ ID NO:47)	CAATTTGGCTCGCTTCGCTCGC	Detection of porcine TTV1
TTV1-nR (SEQ ID NO:48)	TACTTATATTCGCTTTCGTGGGAAC	Detection of porcine TTV1
TTV2-mF (SEQ ID NO:49)	AGTTACACATAACCACCAAACC	Detection of porcine TTV2
TTV2-mR (SEQ ID NO:50)	ATTACCGCCTGCCCCGATAGGC	Detection of porcine TTV2
TTV2-nF (SEQ ID NO:51)	CCAAACCACAGGAACTGTGC	Detection of porcine TTV2
TTV2-nR (SEQ ID NO:52)	CTTGACTCCGCTCTCAGGAG	Detection of porcine TTV2
TTV1-IF (SEQ ID NO:1)	CATAGGGTGTAACCAATCAGATTTAAGGCGTT	Genomic cloning (fragment B)
TTV1-2340R (SEQ ID NO:2)	GGTCATCAGACGATCCATCTCCCTCAG	Genomic cloning (fragment B)
TTV1-2311F (SEQ ID NO:3)	CTTCTGAGGGAGATGGATCGTCTGATGA	Genomic cloning (fragment C)
TTV1-IR (SEQ ID NO:4)	TTGAGCTCCCGACCAATCAGAATTGACT	Genomic cloning (fragment C)
TTV2-IF (SEQ ID NO:5)	TTGTGCCGGAGCTCCTGAGAGC	Genomic cloning (fragment E)
TTV2-2316R (SEQ ID NO:6)	AGGTGCTTGAGGAGTCGTCGCTTG	Genomic cloning (fragment E)
TTV2-GCF (SEQ ID NO:7)	CTCAAGCACGAGCAGTGGATCCTCTCA	Genomic cloning (fragment F)
TTV2-IR (SEQ ID NO:8)	TACCCAGGCGGTTAGACACTCAGCTCT	Genomic cloning (fragment F)

[0075] Unexpectedly, two groups of sequence data from each construct were identified, indicating that there exist two types of PTTVs in genogroup 1 and genogroup 2 from the same pig. In order to differentiate and assemble the four PTTV strains, sequence comparisons were performed together with the three known PTTV strains, Sd-TTV31, TTV-1p and TTV-2p (Fig. 1B and 1C).

[0076] Fig. 1B illustrates differentiation and assembly of full-length genomic sequences of PTTV1 strains PTTV1a-VA and PTTV1b-VA with PCR fragments B and C that were subsequently cloned. The initiation codons of ORF1 and ORF2 in the fragment B as well as the termination codons of ORF1 in the fragment C are marked by “^” or “*”. The corresponding sequences of two known PTTV1 strains, Sd-TTV31 and TTV-1p, are also shown. Conserved sequences are shaded, and dashes indicate nucleotide deletions.

[0077] For PTTV1, the initiation codon ATG and the termination codon TGA of the putative ORF1 were located in fragments B and C, respectively (Fig. 1B). The positions of the codons were differed in two PTTV1 groups, the first one identical to Sd-TTV31 and the second one identical to TTV-1p (Fig. 1B). In addition, the ORF2 initiation codons in the two groups were also located at different positions consistent with that of ORF1. Moreover, phylogenetic analyses using four different sequences of the region B (two from the sequencing data and two from strains Sd-TTV31 and TTV-1p) and four different sequences of the region C supported that the first sequence was clustered with Sd-TTV31 and the second was clustered with TTV-1p (data not shown). Therefore, we were able to differentiate and assemble two groups of sequence data from both fragments B and C into two full-length PTTV1 genomes that were designated as strains PTTV1a-VA (SEQ ID NO:9) and PTTV1b-VA (SEQ ID NO:10), respectively (Fig. 1B).

[0078] Fig. 1C illustrates differentiation and assembly of full-length genomic sequences of PTTV2 strains PTTV2b-VA and PTTV2c-VA with PCR fragments E and F that were subsequently cloned. The corresponding sequence of TTV-2p strain is included and the conserved sequences are shaded. Dashes indicate nucleotide deletions. The unique nucleotides within the overlapping region (boxed with dashed-line) for each strain (a continuous “AG”

nucleotides for PTTV2b-VA (SEQ ID NO:11) and two single “A” and “G” nucleotides for PTTV2c-VA (SEQ ID NO:12)) are shown, respectively.

[0079] Differentiation of the two PTTV2 strains was easier. A unique continuous “AG” nucleotides located in the overlapping region of two PCR fragments was shared by two groups of sequence data from fragments E and F, respectively (Fig. 1C). The assembled full-length genomic sequence represented a PTTV2 strain and was designated as PTTV2b-VA (SEQ ID NO:11). Similarly, the complete genomic sequence of a second strain designated as PTTV2c-VA (SEQ ID NO:12) was assembled based upon two unique single “A” and “G” nucleotides shared in the overlapping region by another set of sequence data from fragments E and F, respectively (Fig. 1C). Phylogenetic analyses using four sequences from fragments E and F together with the two corresponding sequences from TTV-2p also supported this assignment (data not shown).

[0080] The present invention provides four isolated porcine TTV virus genotypes or subtypes that are associated with viral infections in pigs. This invention includes, but is not limited to, porcine TTV virus genotypes or subtypes PTTV1a-VA, PTTV1b-VA, PTTV2b-VA, and PTTV2c-VA, the virus genotypes or subtypes which have nucleotide sequences set forth in SEQ ID NO:9 (PTTV1a-VA), SEQ ID NO:10 (PTTV1b-VA), SEQ ID NO:11 (PTTV2b-VA), and SEQ ID NO:12 (PTTV2c-VA), their functional equivalent or complementary strand. It will be understood that the specific nucleotide sequence derived from any porcine TTV will have slight variations that exist naturally between individual viruses. These variations in sequences may be seen in deletions, substitutions, insertions and the like.

[0081] The proposed genomic structure for each of the four PTTV strains was analyzed in detail and summarized in Table 2, together with the three known PTTV strains, Sd-TTV31, TTV-1p and TTV-2p. All the four U.S. strains of PTTV have a similar genomic size of 2,878 bp

(PTTV1a-VA SEQ ID NO:9), 2,875 bp (PTTV1b-VA SEQ ID NO:10), 2,750 bp (PTTV2b-VA SEQ ID NO:11), and 2,803 bp (PTTV2c-VA SEQ ID NO:12), respectively. Both PTTV1a-VA (SEQ ID NO:9) and Sd-TTV31 have the same genomic length. The published sequences of the strains TTV-1p and TTV-2p all have many undetermined nucleotides in the GC-rich region of the UTR. After artificial filling of these nucleotides with the consensus sequences corresponding to PTTV1 and PTTV2, it was shown that the TTV-1p is more closely-related to PTTV1b-VA (SEQ ID NO:10), and that TTV-2p is more closely-related to PTTV2b-VA (SEQ ID NO:11) in genomic length, respectively (data not shown).

[0082] The assembled genomic sequences of porcine TTV virus genotypes or subtypes PTTV1a-VA (SEQ ID NO:9), PTTV1b-VA (SEQ ID NO:10), PTTV2b-VA (SEQ ID NO:11), and PTTV2c-VA (SEQ ID NO:12) are submitted to Genbank® (*Nucleic Acids Research*, 2008 Jan; 36(Database issue):D25-30) with accession numbers GU456383, GU456384, GU456385, and GU456386, respectively.

Table 2. Comparison of the genomic organization and ORFs of the seven porcine TTV

strains

Virus	Porcine TTV species 1				Porcine TTV species 2		
	Type 1a		Type 1b		Subtype 2a	Subtype 2b	Subtype 2c
Strain	PTTV1a-VA	Sd-TTV31	PTTV1b-VA	TTV-1p	TTV-2p	PTTV2b-VA	PTTV2c-VA
Country	USA	Japan	USA	Brazil	Brazil	USA	USA
Full-length (nt)	2878	2878	2875	Uncompleted	Uncompleted	2750	2803
GenBank accession #	GU456383	AB076001	GU456384	AY823990	AY823991	GU456385	GU456386
TATA box	288-291	288-291	288-291	288-291	233-236	233-236	285-288
Putative mRNA 5'-end	316	316	316	316	261	261	313
ORF1							
Size (aa)	635	635	639	637	624	625	625
Exon #	1	1	1	1	1	1	1
Initiation	534	534	517	517	476	476	528
Termination	2441	2441	2436	2430	2350	2353	2405
ORF2							
Size (aa)	73	73	72	72	68	68	68
Exon #	1	1	1	1	1	1	1
Initiation	430	430	428	428	393	393	445
Termination	651	651	646	646	599	599	651
ORF1/1							
Size (aa)	174	174	182	182	178	178	178
Exon #	2	2	2	2	2	2	2
Initiation	534	534	517	517	476	476	528
Splicing	647/648 2030/2031	647/648 2030/2031	642/643 2013/2014	642/643 2007/2008	595/596 1933/1934	595/596 1936/1937	647/648 1988/1989
Termination	2441	2441	2436	2430	2350	2353	2405
ORF2/2 (ORF3)							
Size (aa)	224	224	228	228	199	199	199
Exon #	2	2	2	2	2	2	2
Initiation	430	430	428	428	393	395	445
Splicing	647/648 2030/2031	647/648 2030/2031	642/643 2013/2014	642/643 2007/2008	595/596 1933/1934	595/596 1936/1937	647/648 1988/1989
Termination	2487	2487	2485	2479	2330	2333	2385
Polyadenylation signal (AATAAA)	2458-2463	2458-2463	2462-2467	2456-2461	2473-2478	2476-2481	2528-2533

The numbers (except sizes of the full-length genome, ORFs and the exon numbers) indicate the nucleotide (nt) positions on the genome of respective PTTV strains.

[0083] Two recent studies have identified the transcribed viral mRNAs and the expression of at least six viral proteins during human TTV replication (Mueller et al., 2008, *supra*; Qiu et al.,

2005, *supra*), which is more than the predicted number of ORFs encoded by human TTV (Okamoto, H., Nishizawa, T., Tawara, A., Takahashi, M., Kishimoto, J., Sai, T., and Sugai, Y. (2000b). TT virus mRNAs detected in the bone marrow cells from an infected individual. *Biochem Biophys Res Commun* **279**(2), 700-7), therefore we included the new human TTV genomic information for comparison with the PTTV sequences. The 5'-ends of the mRNA transcripts of human TTV strain P/1C1 were mapped to an "A" that is 25 nt downstream of the TATA-box (Mueller et al., 2008, *supra*). This starting point, its adjacent sequence (CGAATGGCTGAGTTTATGCCGC (SEQ ID NO:39); the starting point was underlined) and the distance to the upstream TATA-box (24 nt; Table 2) are very conserved in all seven PTTV strains, suggesting that PTTV and human TTV may utilize a common 5'-end of mRNA for translation.

[0084] Five additional completely-conserved regions were identified in the vicinity of the TATA-box among all seven PTTV strains. Two regions of 11 nt each (AGTCCTCATTT (SEQ ID NO:40) and AACCAATCAGA (SEQ ID NO:41)) are located in the upstream of the TATA-box, whereas the remaining three regions (CTGGGCGGGTGCCGGAG of 17 nt (SEQ ID NO:42); CGGAGTCAAGGGGC of 14 nt (SEQ ID NO:43); TATCGGGCAGG of 11 nt (SEQ ID NO:44)) are located between the proposed 5'-end of mRNA and the initiation codon of ORF2. These conserved PTTV-specific sequences may contain the common elements regulating the viral gene expression.

[0085] Previously, three ORFs (ORFs 1-3) were proposed in the genome of the three known PTTV strains, respectively (Niel et al., 2005, *supra*; Okamoto et al., 2002, *supra*). The four prototype U.S. strains of PTTV identified in this study possess this structure. The corresponding ORF3 in human TTV has been renamed as ORF2/2 since it initiates at the same ATG in ORF2

and remains in the same ORF (extending ORF2) after the splicing (Fig. 1A) (Mueller et al., 2008, *supra*; Qiu et al., 2005, *supra*). We follow the nomenclature of human TTV for revising PTTV classification in this study. Human TTV ORF1/1 is a newly identified viral protein that is encoded by two exons in ORF1 (Qiu et al., 2005, *supra*). ORF1/1 share the identical N- and C-terminal part with ORF1. The PTTV ORF1/1 counterpart was readily identified in all seven PTTV strains (Fig. 1A and Table 2).

[0086] The ORF1 and ORF2 are encoded by a ~2.8 kb viral mRNA whereas the ORF1/1 and ORF2/2 are encoded by a spliced viral mRNA with ~1.2 kb in human TTV (Mueller et al., 2008, *supra*; Qiu et al., 2005, *supra*). Since these four ORFs were also deduced in PTTV genomes, and since the sequences and positions of the putative splice donor and acceptor sites in the seven PTTV strains are very conserved (Table 2), it is speculated that porcine TTV probably also encodes the two corresponding mRNAs.

[0087] Most of the human TTV strains share a genetic similarity with the CAV, encoding a TTV apoptosis-inducing protein (TAIP) in which its CAV counterpart was named apoptin (de Smit, M. H., and Noteborn, M. H. (2009). Apoptosis-inducing proteins in chicken anemia virus and TT virus. *Curr Top Microbiol Immunol* **331**, 131-49). The ORF of TAIP is embedded within the ORF2. However, the corresponding TAIP does not exist in porcine TTV. A recent study showed that the expression of apoptin or TAIP was required for CAV replication in cultured cells (Prasetyo, A. A., Kamahora, T., Kuroishi, A., Murakami, K., and Hino, S. (2009). Replication of chicken anemia virus (CAV) requires apoptin and is complemented by VP3 of human torque teno virus (TTV). *Virology* **385**(1), 85-92).

[0088] Pairwise sequence comparisons (PASC) is a useful method that plots the frequency distribution of pairwise nucleotide sequence identity percentages from all available genomic

sequence of viruses in the same family (Bao, Y., Kapustin, Y., and Tatusova, T. (2008). Virus Classification by Pairwise Sequence Comparison (PASC). In "Encyclopedia of Virology, 5 vols." (B. W. J. Mahy, and M. H. V. Van Regenmortel, Eds.), Vol. 5, pp. 342-8. Elsevier, Oxford). The different peaks generated by the PASC program usually represent groups of virus genera, species, types, subtypes and strains (Fig. 2). In this study, we performed PASC analyses of TTV using 121 full-length genomic sequences of human and animal TTV-related strains available in GenBank database (Fig. 2). Assuming that TTV members are classified into a separate family, *Anelloviridae*, the two major peaks, at 36-55% and 55-67% nucleotide sequence identities, represent groups of genera and species, respectively (Fig. 2). Accordingly, a TTV type is defined as a group of TTV having 67-85% nucleotide sequence identity whereas a TTV subtype may be defined as a group of TTV sequences sharing 85-95% nucleotide sequence identity. TTV strains sharing more than 95% nucleotide sequence identity may be further classified into variants (Fig. 2). A similar classification has been proposed using sequences of 103 TTV isolates by Jelcic *et al* (Jelcic, I., Hotz-Wagenblatt, A., Hunziker, A., Zur Hausen, H., and de Villiers, E. M. (2004). Isolation of multiple TT virus genotypes from spleen biopsy tissue from a Hodgkin's disease patient: genome reorganization and diversity in the hypervariable region. *J Virol* **78**(14), 7498-507).

[0089] This proposed criteria of TTV classification were applied to phylogenetic analyses of the genomic sequences of the 4 prototype U.S. strains of PTTV and the 3 other known PTTV strains. Pairwise comparison of full-length nucleotide sequences among these strains showed that the four PTTV1 strains have 54.0-56.4% nucleotide sequence identity compared to the three PTTV2 strains (Table 3). Therefore, the previously designated “genogroup” of PTTV in the literature will probably be more appropriate to designate as “species”, and PTTV1 and PTTV2

probably should represent porcine TTV species 1 and species 2, respectively. PTTV species 1 consists of two types of viruses designated as type 1a (including Sd-TTV31 and PTTV1a-VA (SEQ ID NO:9)) and type 1b (including TTV-1p and PTTV1b-VA (SEQ ID NO:10)), respectively, since the nucleotide sequence identity between these two types of viruses is between 69.8-70.7% (Table 3). Sd-TTV31 and TTV1a-VA (SEQ ID NO:9) are recognized as variant strains of the same species due to their higher sequence identity (95.1%). However, the two type 1b strains, TTV-1p and PTTV1b-VA (SEQ ID NO:10), may belong to two different subtypes (nucleotide sequence identity=86.4%). For PTTV species 2, three strains are likely to be classified into separate subtypes (TTV-2p for subtype 2a, PTTV2b-VA (SEQ ID NO:11) for subtype 2b, and PTTV2c-VA (SEQ ID NO:12) for subtype 2c, respectively) based upon their 86.5-90.9% nucleotide sequence identity. This proposed new classification system for PTTV was clearly evident in the phylogenetic tree (Fig. 3A). Phylogenetic trees constructed based upon the deduced amino acid sequences of ORF1, ORF1/1, ORF2 and ORF2/2 of PTTV were also consistent with this proposed classification (Figs. 3B to 3E).

Table 3. Pairwise sequence comparison of the full-length genomic sequence of the seven porcine TTV strains

	Porcine TTV species 1				Porcine TTV species 2		
	Type 1a		Type 1b		Subtype 2a	Subtype 2b	Subtype 2c
	PTTV1a-VA	Sd-TTV31	PTTV1b-VA	TTV-1p	TTV-2p	PTTV2b-VA	PTTV2c-VA
Type 1a							
PTTV1a-VA	—	95.1	70.5	69.8	55.7	55.1	56.2
Sd-TTV31		—	70.7	70.1	55.9	56.0	56.4
Type 1b							
PTTV1b-VA			—	86.4	54.0	54.7	55.2
TTV-1p				—	55.2	54.7	55.4
Subtype 2a							
TTV-2p					—	86.5	86.8
Subtype 2b							
PTTV2b-VA						—	90.9
Subtype 2c							
PTTV2c-VA							—

The data were generated by using the PASC program, and the values indicate % nucleotide sequence identities.

[0090] Unique mutations and deletions and/or insertions are scattered throughout the genomes between PTTV species, types and subtypes. For example, the location of ORF1 initiation and termination codons and the ORF2 initiation codons between PTTV type 1a and 1b, which was shown in Fig. 1B as mentioned above, are different. The two PTTV1b strains also have a 2-codon deletion after the ORF2 initiation compared to PTTV1a (Fig. 1B).

[0091] Remarkably, both TTV-2p and PTTV2b-VA have a large 52-nt deletion, which is 39 nt upstream of the first 11-nt conserved sequence (AGTCCTCATTT (SEQ ID NO:40)) in the UTR, compared to PTTV2c-VA. Due to this deletion, the genomic size of PTTV2b-VA (probably TTV-2p as well) was significantly smaller than that of PTTV2c-VA (Table 2). A number of “subviral” human TTV clones have been isolated from serum samples that are considered as full-length TTV genomes since the ORFs in a majority of these subviral molecules

usually remain intact (de Villiers et al., 2009; Leppik et al., 2007). They have variable lengths in the UTR that are completely or partially deleted. The situation of TTV-2p and PTTV2b-VA appears to resemble that of the human TTV subviral molecules, implying that subtypes PTTV2a and PTTV2b might be the subviral molecules derived from subtype PTTV2c. Of note, the 3'-terminal sequence of a nested-PCR primer TTV2-nF (Table 1) that is commonly used for detection of the PTTV2 from field samples by other groups (Ellis et al., 2008, *supra*; Kekarainen et al., 2007, *supra*; Kekarainen et al., 2006, *supra*; Krakowka et al., 2008, *supra*) is located at both sides of the deletion. Therefore, the current nested-PCR assay for PTTV2 detection is likely not sufficient to identify the genetically diverse strains of PTTV2c subtype.

[0092] The source of the isolated virus strain is serum, fecal, saliva, semen and tissue samples of pigs having the porcine TTV viral infection. However, it is contemplated that recombinant DNA technology can be used to duplicate and chemically synthesize the nucleotide sequence. Therefore, the scope of the present invention encompasses the isolated polynucleotide which comprises, but is not limited to, a nucleotide sequence set forth in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12, or its complementary strand; a polynucleotide which hybridizes to and which is at least 67% complementary to the nucleotide sequence set forth in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12, preferably 85% complementary, or more preferably 95% complementary; or an immunogenic fragment selected from the group consisting of an amino acid sequence of ORF1 protein set forth in SEQ ID NO:13 (PTTV1a-VA), SEQ ID NO:14 (PTTV1b-VA), SEQ ID NO:15 (PTTV2b-VA), SEQ ID NO:16 (PTTV2c-VA), an amino acid sequence of ORF2 protein set forth in SEQ ID NO:17 (PTTV1a-VA), SEQ ID NO:18 (PTTV1b-VA), SEQ ID NO:19 (PTTV2b-VA), SEQ ID NO:20 (PTTV2c-VA), an amino acid sequence of ORF1/1 protein set forth in SEQ ID NO:21 (PTTV1a-

VA), SEQ ID NO:22 (PTTV1b-VA), SEQ ID NO:23 (PTTV2b-VA), SEQ ID NO:24 (PTTV2c-VA), an amino acid sequence of ORF2/2 protein set forth in SEQ ID NO:25 (PTTV1a-VA), SEQ ID NO:26 (PTTV1b-VA), SEQ ID NO:27 (PTTV2b-VA), SEQ ID NO:28 (PTTV2c-VA). The immunogenic or antigenic coding regions or fragments can be determined by techniques known in the art and then used to make monoclonal or polyclonal antibodies for immunoreactivity screening or other diagnostic purposes. The invention further encompasses the purified, immunogenic protein encoded by the isolated polynucleotides. Desirably, the protein may be an isolated or recombinant ORF1 protein or an ORF2 protein of at least one of the above isolated porcine TTV subtypes, more desirably ORF1 protein.

[0093] The ORF1 of porcine TTV is believed to encode a structural and replication-associated protein (Maggi, F., and Bendinelli, M. (2009). Immunobiology of the Torque teno viruses and other anelloviruses. *Curr Top Microbiol Immunol* **331**, 65-90). The ORF1-encoding products of seven PTTV strains have 624-635 aa in length and possess a high number of arginine residues at the N-terminus that are thought to have the DNA-binding activity (Fig. 4). In Fig. 4, conserved sequences are shaded. Dashes indicate amino acid deletions. The RCR motifs are boxed with solid lines. Three HVRs (PTTV1-HVRs 1, 2 and 3) of PTTV1 strains and two HVRs (PTTV2-HVRs 1 and 2) of PTTV2 strains are boxed with dashed lines. The connection boundaries of ORF1/1 are indicated by arrows. The predicted rolling-circle replication (RCR) motifs (Ilyina, T. V., and Koonin, E. V. (1992). Conserved sequence motifs in the initiator proteins for rolling circle DNA replication encoded by diverse replicons from eubacteria, eucaryotes and archaebacteria. *Nucleic Acids Res* **20**(13), 3279-85) are presented at different positions in different PTTV types and subtypes that may be type- or subtype-specific. RCR motif-III (YxxK) is conserved in the PTTV type 1a (aa position 14-17 of PTTV1a-VA SEQ ID

NO:13) and type 1b strains (aa position 379-382 of PTTV1b-VA SEQ ID NO:14), respectively, whereas the same conserved motif identified in all three PTTV2 strains is located at aa position 482-485 of PTTV2b-VA SEQ ID NO:15 (Fig. 4). Both PTTV2b-VA SEQ ID NO:15 and PTTV2c-VA SEQ ID NO:16 also have a conserved RCR motif-II (HxQ) at aa position 331-333 of PTTV2b-VA that is absent in TTV-2p (Fig. 4).

[0094] The ORF1 proteins of PTTV strains between species 1 and species 2 share very low aa sequence identity with only 22.4 to 25.8%, which makes it difficult to identify significantly conserved aa sequences between the two species (Fig. 4). In PTTV species 1, the aa identity of ORF1 between type 1a and 1b strains are 50.3-52.7%. Three major hypervariable regions (HVR), PTTV1-HVRs 1 to 3, with a relatively high number of aa substitutions, were identified among the four PTTV1 strains, whereas two HVRs (PTTV2-HVRs 1 and 2) were observed among the three PTTV2 strains (Fig. 4). The three PTTV2 strains have an approximately 20-aa deletion in the corresponding PTTV1-HVR1 region. Moreover, the two HVRs of PTTV2 are within the corresponding PTTV1-HVR3 region (Fig. 4). These HVRs are located only in the ORF1 but not in the truncated ORF1/1. They likely play a role in evading the host immune surveillance and helping PTTV to establish a persistent infection, as suggested by studies of human TTV.

[0095] The aa sequences of ORF2 differed considerably between the four PTTV1 (PTTV1a-VA SEQ ID NO:17; PTTV1b-VA SEQ ID NO:18) and three PTTV2 (PTTV2b-VA SEQ ID NO:19; PTTV2c-VA SEQ ID NO:20) strains (Fig. 5). However, they share a conserved protein-tyrosine phosphatase (PTPase)-like motif (W_x₇H_x₃C_xC_x₅H) at the N-terminus (Fig. 4). This motif is also conserved among all human TTV, TTMV and TTMDV strains as well as CAV. The TTMV or CAV ORF2 protein also exhibited a serine/threonine phosphatase (S/T PPase) activity (Peters, M. A., Jackson, D. C., Crabb, B. S., and Browning, G. F. (2002). Chicken anemia virus

VP2 is a novel dual specificity protein phosphatase. *J Biol Chem* **277**(42), 39566-73). The dual specificity of the ORF2 protein is thought to regulate host gene transcription, signal transduction and cytokine responses during viral replication. Recently, mutagenesis analyses of two conserved basic aa residues before the last histidine residue of the motif in CAV revealed that the two residues affect virus replication, cytopathology *in vitro* and attenuation *in vivo* (Peters, M. A., Crabb, B. S., Washington, E. A., and Browning, G. F. (2006). Site-directed mutagenesis of the VP2 gene of Chicken anemia virus affects virus replication, cytopathology and host-cell MHC class I expression. *J Gen Virol* **87**(Pt 4), 823-31; Peters, M. A., Crabb, B. S., Tivendale, K. A., and Browning, G. F. (2007). Attenuation of chicken anemia virus by site-directed mutagenesis of VP2. *J Gen Virol* **88**(Pt 8), 2168-75). The two basic aa residues (“KK”) are conserved in the three PTTV2 strains. However, only the first basic residue (“R”) is retained in the two PTTV1a strains whereas both basic residues are substituted in the PTTV1b strains (Fig. 5). In Fig. 5, dashes indicate amino acid deletions. The five conserved amino acids within the common motif W_{x7}Hx₃CxCx₅H (underlined) identified in TTV, TTMV and CAV are shaded. The positions of the two basic aa residues before the last histidine of the motif, which have been shown to affect virus replication, cytopathology or *in vivo* attenuation in CAV, are indicated by “^”.

[0096] In summary, the present invention has determined the full-length genomic sequences of four porcine TTV strains representing different genotypes or subtypes in a serum sample of a single boar in Virginia. The finding from this study clearly indicates that, similar to human TTV, multiple PTTV infections with distinct genotypes or subtypes exist and probably are common in pigs. We have also provided new information regarding the genomic organization, the degree of variability and the characteristics of conserved nucleotide and amino acid motifs of PTTV, which

will improve the current PCR detection assay, aid in developing reagents for serological diagnostics and help initiate the structural and functional study of PTTV. A new classification of PTTV is also proposed in this study based upon the phylogenetic and genetic analyses of the genomic sequences of seven known PTTV strains.

[0097] The present invention also provides methods for diagnostics of porcine TTV infection by detecting viral DNA in samples of porcine TTV infected pigs or other mammals. One preferred embodiment of the present invention involves methods for detecting porcine TTV nucleic acid sequences in a porcine or other mammalian species using oligonucleotide primers for polymerase chain reaction (PCR) to further aid in the diagnosis of viral infection or disease. The diagnostic tests, which are useful in detecting the presence or absence of the porcine TTV viral nucleic acid sequence in the porcine or other mammalian species, comprise isolating viral DNA from samples of porcine TTV infected pigs or pigs suspected of infection of TTV, and performing SYBR green real-time quantitative PCR using PTTV1-specific (SEQ ID NO:29/ SEQ ID NO:30) or PTTV2-specific (SEQ ID NO:31/ SEQ ID NO:32) primers.

[0098] In another embodiment of the present invention, the diagnostic method may be adapted to simultaneously detect PTTV1 and PTTV2 by using PTTV1/PTTV2 duplex real-time PCR. More specifically, the method comprises isolating viral DNA from samples of porcine TTV infected pigs or pigs suspected of infection of TTV, performing real-time PCR using both PTTV1-specific (SEQ ID NO:29/ SEQ ID NO:30) or PTTV2-specific (SEQ ID NO:31/ SEQ ID NO:32) primers in the same real-time PCR reaction. Since the T_m value between PTTV1 and PTTV2 can be distinguished by MCA, the presence of PTTV1 and PTTV2 DNA can be simultaneously detected.

[0099] In a further embodiment of the present invention, the diagnostic method may employ duplex nested PCR. The method comprises isolating viral DNA from samples of porcine TTV infected pigs or pigs suspected of infection of TTV, performing a first round of PCR using one pair of primers P1ab-mF (SEQ ID NO:33)/P1ab-mR (SEQ ID NO:34), and performing a second round of PCR using a mixture of two pairs of primers, P1a-nF (SEQ ID NO:35)/P1a-nR (SEQ ID NO:36) for detection of PTTV1a, and P1b-nF (SEQ ID NO:37)/P1b-nR (SEQ ID NO:38) for detection of PTTV1b, and visualizing the PCR products.

[00100] The above diagnostics methods maybe optimized by one skilled in the art according to well known methods in the art.

[00101] Accordingly, an embodiment of the present invention develops two novel singleplex SYBR green real-time PCR assays to quantify the viral loads of two porcine TTV species, respectively. PTTV1- and PTTV2-specific primers were designed to target the extremely conserved regions across six PTTV1 and four PTTV2 full-length genomes available to date, respectively. Another embodiment of the present invention combines the two singleplex assays into a duplex real-time PCR assay followed by MCA of the viral amplicons that can be identified by their distinct melting temperatures for simultaneous detection of the two porcine TTV species, PTTV1a and PTTV1b. In a third embodiment, a duplex nested PCR assay for simultaneous amplification of the viral DNAs from two types of PTTV1 in the first round PCR and differential detection of types 1a and 1b in the second round PCR was developed for the identification of two types of porcine TTV species, PTTV1a and PTTV1b, in a single sample. These assays represent simple and practical tools for diagnoses of species- or type-specific porcine TTVs.

[00102] Potential primers sequences were identified by multiple sequence alignments of 10 available porcine TTV full-length genomes. PTTV1-specific primers TTV1F (SEQ ID NO:29)

and TTV1R (SEQ ID NO:30) were designed based upon two conserved genomic regions immediately before the putative ORF2 across six PTTV1 genomes, whereas PTTV2-specific primers TTV2F4 (SEQ ID NO:31) and TTV2R4 (SEQ ID NO:32) were designed based upon two conserved genomic regions immediately after the putative ORF2/2 across four PTTV2 genomes (Table 4). Primers showed no potentials for self- and cross-dimerization. The expected amplicon sizes were a 118-bp fragment from the PTTV1 primers corresponding to the PTTV1b-VA genome and a 200-bp fragment from the PTTV2 primers corresponding to the PTTV2c-VA genome, respectively.

Table 4. Oligonucleotide primers used for real-time PCR and duplex nested PCR detections of porcine TTVs.

Primer ID	Sequence (5' to 3')	Purpose
TTV1F SEQ ID NO:29	TCCGAATGGCTGAGTTTATGC	PTTV1-specific real-time PCR
TTV1R SEQ ID NO:30	TCCGCTCAGCTGCTCCT	PTTV1-specific real-time PCR
TTV2F4 SEQ ID NO:31	GGTGGTAAAGAGGATGAA	PTTV2-specific real-time PCR
TTV2R4 SEQ ID NO:32	AATAGATTGGACACAGGAG	PTTV2-specific real-time PCR
P1ab-mF SEQ ID NO:33	TATCGGGCAGGAGCAGCT	Duplex nested PCR
P1ab-mR SEQ ID NO:34	TAGGGGCGCGCTCTACGT	Duplex nested PCR
P1a-nF SEQ ID NO:35	CCTACATGAAGGAGAAAGACT	Duplex nested PCR
P1a-nR SEQ ID NO:36	CCAGCGTCTCCAGGGTC	Duplex nested PCR
P1b-nF SEQ ID NO:37	AAGCTACCAAGGGCTGG	Duplex nested PCR
P1b-nR SEQ ID NO:38	GCGGTC(T/G)GTAGCGGTAGT	Duplex nested PCR

[00103] According to one specific embodiment of the present invention, SYBR green simplex real-time PCR using PTTV1- and PTTV2-specific primers can be used specifically to detect

porcine TTV1 and TTV2 DNA, respectively. For PTTV1, a standard curve was established over a range of target DNA concentrations per 25 μ l. The linear range was shown to span 4.4×10^1 to 4.4×10^8 copies. The minimum detection limit (44 copies) corresponded to a threshold cycle (C_t) of 37.57. For PTTV2, standard curve was also generated and used to detect DNA concentration ranging from 8.6×10^0 to 8.6×10^8 copies per 25 μ l reaction. The corresponding C_t of minimum detection limit (8.6 copies) was 36.53.

[00104] According to another specific embodiment of the present invention, SYBR green duplex real-time PCR is utilized for the simultaneous detection of porcine TTV1 and TTV2 DNA. The 7-degree difference of T_m value between PTTV1 (87.0°C) and PTTV2 (80.0°C) made it feasible to distinguish them from one another by the MCA. Therefore, two singleplex assays can be coupled into a duplex real-time PCR assay for the simultaneous detection of PTTV1 and PTTV2. A positive sample was one that had a symmetrical melt peak within the known T_m for that product. This new assay was first validated by using a 10-fold dilution of PTTV1 and PTTV2 standards mixture. The non-template negative control using sterile water as the template showed a non-specific amplification caused by cross-dimerization between the PTTV1 and PTTV2 primers not seen in the singleplex assays (Fig. 7a). This produced a distinct melt peak between 72.0°C and 76.0°C. Figure 7A shows melt peaks of PTTV1 standard (red; T_m =87.0°C), PTTV2 standard (green; T_m =80.0°C) and non-template negative control (caused by primer cross-dimerization; black). Figures 7B-7E show melt peaks of representative serum samples with distinct viral loads of PTTV1 and PTTV2. Figure 7B shows boar serum sample no. 5: relatively high viral loads of both PTTV1 and PTTV2, but PTTV2>PTTV1; Figure 7C shows boar serum sample no. 12: relatively high viral loads of both PTTV1 and PTTV2, but PTTV1>PTTV2; Figure 7D shows boar serum sample no. 14: low viral loads of both PTTV1 and PTTV2; Figure

7E shows boar serum sample no. 10: PTTV1 positive, but PTTV2 negative. The viral loads (unit: genomic copies/ml) of PTTV1 and PTTV2 in each sample that were determined by singleplex real-time PCR were indicated at the top of the corresponding melt peak.

[00105] In one example, when the duplex real-time assay was applied to the 20 serum samples of adult boars, samples with relatively high viral loads of both PTTV1 and PTTV2 displayed two distinct melt curves corresponding to PTTV1 and PTTV2 without a non-specific melt peak (Fig. 7B & 7C), whereas samples with low viral load of either PTTV1 or PTTV2 showed virus-specific as well as non-specific melt curves (Fig. 7D & 7E). Although the two melt peaks in sample #14 were very small, they were considered positive since they displayed a visually distinct and symmetrical rise and fall at the appropriate T_m of PTTV1 and PTTV2 (Fig. 7D). In contrast, sample #10 was considered only PTTV1 positive because a symmetrical PTTV2 melt peak was not evidently present (Fig. 7E). These results were consistent with that of the two singleplex assays (Table 5). Moreover, the size and shape of melt peaks qualitatively reflected the corresponding viral load in the detected sample.

[00106] According to another aspect of the present invention, duplex nested PCR is used for differential detection of two porcine TTV types, PTTV1a and PTTV1b.

[00107] The inventor of the present invention demonstrated the existence of two distinct genotypes, tentatively named PTTV1a and PTTV1b, in porcine TTV species 1. To further determine whether the co-infection of PTTV1a and PTTV1b is common in pigs, a novel duplex nested PCR assay to quickly distinguish between the two was developed. Alignment of porcine TTV genomic DNA sequences identified a conserved genomic region located at the N-terminal part of the putative ORF1 encoding the viral capsid protein (Fig. 8). This region also contains the entire ORF2 and the partial UTR in the upstream. Primers P1ab-mF (SEQ ID NO:33)/P1ab-mR

(SEQ ID NO:34) were designed to simultaneously amplify both PTTV1a and PTTV1b DNAs in the first-round PCR. A mixture of PTTV1a-specific primers P1a-nF (SEQ ID NO:35)/P1a-nR (SEQ ID NO:36) and PTTV1b-specific primers P1b-nF (SEQ ID NO:37)/P1b-nR (SEQ ID NO:38) was used to differentially amplify each genotype in the second-round PCR. The final PCR products of PTTV1a and PTTV1b were 162 bp and 96 bp in sizes, respectively, which could be easily distinguished by gel electrophoresis on a 1% agarose gel stained with ethidium bromide. This assay was not expected to detect PTTV2 DNA due to the specificity of primers (Fig. 8). In Fig. 8, conserved sequences were indicated by dots and shaded. Dashes indicated nucleotide deletions. The locations and directions of three pairs of primers used for duplex nested PCR were marked by arrows.

[00108] In one example, the 20 serum samples from adult boars that were subjected to the duplex nested PCR assay were all found to be positive for both PTTV1a and PTTV1b, as determined by visualizing two bands of the expected sizes and subsequent sequencing confirmation of PCR products (data not shown). No PCR products were amplified in the 19 semen samples, which was consistent with the results of PTTV1 conventional nested PCR and real-time PCR assays described above.

[00109] Infection of pigs with the two species of porcine TTV has been found back to 1985 in Spanish pig farms according to a retrospective investigation (Segales et al., 2009, *supra*). However, whether porcine TTVs are associated with any particular pig diseases remains elusive. Since both of porcine TTV species have a high prevalence in domestic pigs, determination of TTV viral loads is presumably more important than assessing the presence of TTV DNA. The level of viral loads in serum and semen samples has been indicated as an important marker for PCVAD in PCV2 infection (Opriessnig et al., 2007, *supra*). Therefore, establishment of

quantitative PTTV-specific real-time PCR assays would help identify potential disease conditions associated with porcine TTVs.

[00110] Two TaqMan probe-based real-time PCR assays have recently been described. The singleplex assay developed by a Canadian group was not species-specific and was only designed to quantify the total viral loads of two PTTV species (Brassard et al., 2009, *supra*). The duplex assay established by a Germany group allowed the specific and simultaneous detection of both species (Gallei et al., 2009, *supra*). The target sequences of primers used in those two assays were determined by alignment of the three porcine TTV genomic sequences (Sd-TTV31, TTV-1p and TTV-2p) and were located in the UTR. In the present study, with 7 additional complete PTTV genomic sequences available (4 PTTV1 and 3 PTTV2 sequences), we analyzed and re-determined the conserved regions across the 10 full-length PTTV genomes. Based upon the updated alignment result from this study, two species-specific singleplex SYBR green-based real-time PCR assays were developed to quantify the viral loads of PTTV1 and PTTV2, respectively. The primers used in our assays were designed to bind to conserved genomic regions distinct from the previous studies, which may increase the accuracy of quantification. Our assays showed a considerable species-specificity and sensitivity of detection with 44 genomic copies for PTTV1 and 8.8 genomic copies for PTTV2 per 25- μ l reaction, whereas the detection limit of 10 genomic copies per reaction was reported in the TaqMan probe-based duplex real-time PCR (Gallei et al., 2009, *supra*). In addition, the SYBR green-based real-time PCR assay is a flexible and inexpensive approach that can be directly carried out without the need to use fluorescently labeled probes. Finally, considering porcine TTVs exhibit a high degree of genetic diversity, the results from SYBR green-based assays are unlikely affected by the different genetic background

of porcine TTV variants that likely contain mutations in the probe-binding sequences in the TaqMan probe-based assays.

[00111] In spite of the presence of TTV DNA, all serum samples from healthy pigs tested in this study had low amounts of PTTV1 and PTTV2 that were less than 2×10^6 copies/ml. Moreover, only an extremely low titer of PTTV2 DNA was detected in three semen samples. Most of the tested serum samples were also positive for PCV2 DNA as determined by conventional nested PCR (data not shown). Many PCV2-positive pigs with low viral load do not develop clinical PCVAD. A proposed threshold for developing PCVAD is 10^7 or greater PCV2 genomic copies/ml of serum (Opriessnig et al., 2007, *supra*). In addition, semen PCV2 DNA-positivity is also a notable marker of diseased status (Opriessnig et al., 2007, *supra*; Pal, N., Huang, Y.W., Madson, D.M., Kuster, C., Meng, X.J., Halbur, P.G. and Opriessnig, T., 2008. Development and validation of a duplex real-time PCR assay for the simultaneous detection and quantification of porcine circovirus type 2 and an internal control on porcine semen samples. *J Virol Methods* 149, 217-25). The situation of species-specific PTTV may be analogous to that of PCV2 and a high PTTV titer greater than 10^7 copies/ml may be required for the induction of porcine diseases. The species-specific real-time PCR assays developed in this study will offer simple and practical tools for future investigations of PTTV association with diseases using a large number of clinical samples from various disease conditions.

[00112] Furthermore, by coupling the two species-specific singleplex assays, we developed and validated a quick, inexpensive and reliable screening for the simultaneous detection and differentiation of the two porcine TTV species, PTTV1 and PTTV2, in a MCA-based duplex real-time PCR assay. Although this assay is not intended for accurate quantification of both PTTV species, it is a more convenient approach that could replace the conventional nested PCR

for detection purpose. In comparison with real-time PCR, the conventional nested PCR assay for porcine TTVs detection is time-consuming (requiring total 4 rounds of PCR), laborious and prone to sample contamination occurring during multiple rounds of PCR processing. Due to the difference of T_m value between PTTV1 and PTTV2 species, an MCA following duplex PCR amplification is able to ensure distinct reaction specificity. Another advantage of this duplex real-time assay is that inclusion of PTTV1 and PTTV2 standards is dispensable when performing the described protocol, which makes it easier for much wider use in any diagnostic labs equipped with an automated real-time PCR instrument.

[00113] Multiple infection of porcine TTVs with distinct genotypes or subtypes of the same species has been demonstrated (Gallei et al., 2009, *supra*). In particular, our previous study showed that porcine TTV species 1 consists of two distinct types, PTTV1a (including strains Sd-TTV31 and PTTV1a-VA) and PTTV1b (including strains TTV-1p and PTTV1b-VA). The two newly published PTTV1 isolates with full-length genomes, swSTHY-TT27 (GQ120664) from Canada and TTV1 #471819 (GU188045) from Germany, were both classified into type 1b based upon the phylogenetic analysis (data not shown). The duplex nested PCR described in this study confirmed that dual infection of two PTTV1 genotypes frequently occurred in pigs. This novel assay is the first diagnostic PCR approach developed to distinguish between PTTV1a and 1b so far. Since it is currently not known whether one or both of PTTV1a and PTTV1b infection represents a relevant factor associated with diseases, our differential PCR assay should be of great value for future potential disease associations of these two PTTV types.

[00114] According to another aspect of the invention, porcine TTV ORF proteins were expressed and used in immunodetection assays to detect the presence of porcine TTV specific antibodies. In one embodiment of the present invention, three truncated and Histidine-tagged

ORF1 proteins of PTTV1a, PTTV1b and PTTV2, were expressed and purified in *Escherichia coli* (*E. coli*), respectively. Furthermore, both serum Western blot and ELISA assays based on these recombinant antigens were developed and validated using porcine serum samples from different sources. In particular, serological testing using the PTTV1a-, PTTV1b- and PTTV2-specific ELISA provides an accurate and simple tool for revealing the association of porcine TTV infection with diseases.

[00115] According to a further aspect of the invention, porcine TTV ORF proteins were expressed and purified as recombinant ORF1 capsid protein in *E.coli* expression system (Fig. 10, Fig. 15). Three truncated and His-tagged ORF1 capsid proteins of PTTV1a, PTTV1b and PTTV2, were expressed and purified in *Escherichia coli* (*E. coli*), respectively, and served as recombinant capsid subunit vaccines against PTTV infection.

[00116] Four porcine TTV2 strains, TTV-2p, TTV2#472142, PTTV2b-VA and PTTV2c-VA, had available complete genomic sequences to date. Although they are phylogenetically classified into three putative subtypes, a comparative analysis of hydrophilicity profiles of the ORF1 encoding amino acids from four PTTV2 showed that they shared three hydrophilic regions, an arginine-rich region from aa 1-49 at the N-terminal and two particular domains (I and II) located at the middle and C-terminal part, respectively (Fig. 9A). The C-terminal region used for truncated PTTV2c-VA ORF1 expression and the corresponding regions shared in other three PTTV2 strains were indicated by a dashed box. Alignments of amino acid sequences demonstrated high levels of sequence conservation of domains I (aa 322-349) and II (aa 536-625) across the four PTTV2 strains (Fig. 9B).

[00117] Since hydrophilic domains are believed to be important for the antigenicity of many proteins, the C-terminal region (aa 310-625) of the PTTV2c-VA ORF1 SEQ ID NO:16

containing the two domains was chosen for protein expression, which would be used as antigen for PTTV2-specific antibody detection in porcine serum. According to one aspect of the invention, expression of the truncated PTTV2c ORF1 was sufficient for detection of all PTTV2 subtypes (2a, 2b and 2c; also see Fig. 3A).

[00118] According to one embodiment of the present invention, the C-terminal part of the PTTV2c ORF1 gene fused with 8 × His-tags was constructed and expressed in *E.coli*. The recombinant protein was insoluble and expressed within the bacterial inclusion bodies. Figure 10A shows SDS-PAGE of unpurified 2c-ORF1 products. Figure 10B shows SDS-PAGE of purified 2c-ORF1 products. Figure 10C shows Western blot analysis of purified 2c-ORF1 products using an anti-His-tagged mAb. White arrowheads indicated the ORF1 protein with the expected size and its truncated product whereas black arrowheads show the putative dimers of the expected and truncated proteins. M: protein markers. In Fig. 10A, two significant polypeptides (white arrowheads) were produced in the 2c-ORF1 unpurified sample in comparison with the control sample. The band of ~40 KDa was consistent with the expected size of 2c-ORF1 whereas the ~30 KDa polypeptide was probably an N-terminally truncated product from the former. After purification with a nickel-affinity column, four polypeptides including the two described significant bands were showed in SDS-PAGE (Fig. 10B). They were also detected by western blot using an anti-His-tagged mAb (Fig. 10C). Two high-molecular-mass bands (black arrowheads) were the homodimers formed by the two polypeptides of ~40 KDa and ~30 KDa, respectively, based on the predicted sizes (~80 KDa and ~60 KDa). The results demonstrated that the purified C-terminal PTTV2c-ORF1 was successfully produced and could be used for porcine TTV2 antibody detection in porcine sera.

[00119] According to another aspect of the present invention, porcine TTV2 antibodies in various porcine serum samples can be detected by Western blot using purified C-terminal PTTV2c-ORF1. White arrowheads indicated the ORF1 protein with the expected size and its truncated product. It should be noted that only the bands in green color were recognized as positive. A total of more than 200 serum samples of conventional pigs (healthy or diseased), CD/CD pigs and gnotobiotic pigs from different sources were collected. Samples were randomly selected for detection of anti-PTTV2c-ORF1 IgG antibodies using the purified C-terminal PTTV2c-ORF1 as antigen. Figure 11A shows results of Western blot analyses of selected porcine serum samples of conventional pigs, Figure 11B shows CD/CD pigs, and Figure 11C shows gnotobiotic pigs. Purified PTTV2c-ORF1 products were used as the antigens. The two marked ~40 KDa and ~30 KDa bands were detected in most samples of the conventional pigs (Fig. 11A) and CD/CD pigs (Fig. 11B), indicating widely PTTV2 infection in these pigs. However, all the gnotobiotic pigs from two different sources (Blacksburg, VA and Ames, IA) had no detectable PTTV2 antibody (Fig. 11C). Additional low-molecular-mass bands were also observed (Fig. 11A and 11B). They were likely from non-specific reactivity in the Western blot.

[00120] According to yet another aspect of the present invention, PTTV2-specific ELISA can be used as a porcine TTV2 serological test. Seronegative results were also shown in a few samples from conventional pigs of a Wisconsin farm (Fig. 12). These negative samples were pooled and used as a negative reference in development of a PTTV2-specific ELISA. The remaining samples from this source were positive (Fig. 12, the four lanes in the left). In addition, porcine sera from a commercial company used in cell culture (supposed to be OIE diseases-free) also displayed strong anti-PTTV2-ORF2 positivity (Fig. 12), which was used as a positive control for ELISA. The concentrations of purified 2c-ORF1 antigen, porcine sera and IgG

conjugate were determined by checkboard titration to present low background signal and give the highest difference of OD₄₀₅ value between the positive and negative controls. The optimal antigen amount was 69 ng per well, and the optimal ELISA results were obtained by use of a 1:100 dilution of serum samples and a 1:4000 dilution of IgG conjugates. The ELISA cutoff values ranged from 0.25 to 0.5 in each trial. Figure 4 shows a representative result reflecting the consistency of serum western blot and the developed ELISA.

[00121] 138 conventional pig sera samples from 3 herds were chosen to analyze the correlation between PTTV2 viral load by real-time PCR and anti-PTTV2 IgG antibody level by ELISA. The results showed that pigs with undetectable or higher PTTV2 viral load (10^8 copies/ml) were more likely to have a lower serum PTTV2 antibody titer than pigs with middle values of PTTV2 viral load (Fig. 13).

[00122] In particular, sera from 10 pigs in the same herd were also analyzed by comparing the PTTV2 viral loads and anti-PTTV2 antibody levels of their sera from their arrival in the new facility to two months after arrival. Nine of the 10 pigs had decreased viral loads (three had no detectable virus) after 2 months whilst the anti-PTTV2 antibody titers increased in nine of 10 pigs (Fig. 14A and 14B). The results suggested that the 10 pigs acquired PTTV2 infection at early stage, which induced humoral response and produced anti-ORF1 capsid IgG antibody progressively. The PTTV2-ORF1 IgG antibody was able to neutralize or even clear the virus, indicating the ORF1 indeed encode a viral capsid protein and may contain neutralizing epitopes against PTTV2.

[00123] According to one embodiment of the present invention, the C-terminal PTTV1a- and PTTV1b-ORF1 proteins were expressed and purified in *E.coli* system, respectively. SDS-PAGE and western blot analysis using an anti His-tagged mAb showed that both 1a- and 1b-ORF

products had two polypeptides, one with expected size (~40 KDa) and another as the putative homodimer (~80 KDa) (Fig. 15A-C). Figure 15A shows SDS-PAGE of unpurified and purified 1a-ORF1 products. Figure 15B shows SDS-PAGE of purified 1b-ORF1 and 1b-ORF1ctruc products. Figure 15C shows Western blot analysis of purified 1a- and 1b-ORF1 products using an anti-His-tagged mAb. White arrowheads indicate the ORF1 protein with the expected size whereas black arrowheads show the putative dimer of the ORF1 proteins. Compared to 2c-ORF1 expression, no truncated polypeptide was observed. As a comparative control, expression of a C-terminal-truncated 1b-ORF1 region (1b-ORF1ctruc) resulted in a lower-molecular-mass polypeptide compared to its C-terminal-non-truncated counterpart 1b-ORF1 (Fig. 15B).

[00124] According one embodiment of the present invention, the purified C-terminal PTTV1a- and PTTV1b-ORF1 proteins were used to develop genotype-specific serum Western blots and ELISA as described for PTTV2 above. Figure 16 shows negative (lanes 1-2) and positive (lanes 3-5) examples of serum Western blot using 1a-ORF1 as antigen. The same antigen amount (69 ng), dilution of sera (1:100) and dilution of IgG conjugate (1:4000) as PTTV2-ORF1 were used in PTTV1a- and PTTV1b-specific ELISA (data not shown).

[00125] Additionally, the present invention provides a useful diagnostic reagent for detecting the porcine TTV infection which comprise a monoclonal or polyclonal antibody purified from a natural host such as, for example, by inoculating a pig with the porcine TTV or the immunogenic composition of the invention in an effective immunogenic quantity to produce a viral infection and recovering the antibody from the serum of the infected pig. Alternatively, the antibodies can be raised in experimental animals against the natural or synthetic polypeptides derived or expressed from the amino acid sequences or immunogenic fragments encoded by the nucleotide sequence of the isolated porcine TTV. For example, monoclonal antibodies can be produced

from hybridoma cells which are obtained from mice such as, for example, Balb/c, immunized with a polypeptide antigen derived from the nucleotide sequence of the isolated porcine TTV. Selection of the hybridoma cells is made by growth in hypoxanthine, thymidine and aminopterin in a standard cell culture medium like Dulbecco's modified Eagle's medium (DMEM) or minimal essential medium. The hybridoma cells which produce antibodies can be cloned according to procedures known in the art. Then, the discrete colonies which are formed can be transferred into separate wells of culture plates for cultivation in a suitable culture medium. Identification of antibody secreting cells is done by conventional screening methods with the appropriate antigen or immunogen. Cultivating the hybridoma cells in vitro or in vivo by obtaining ascites fluid in mice after injecting the hybridoma produces the desired monoclonal antibody via well-known techniques.

[00126] For another alternative method, porcine TTV capsid protein can be expressed in a baculovirus expression system or *E. coli* expression system according to procedures known in the art. The expressed recombinant porcine TTV capsid protein can be used as the antigen for diagnosis in an enzyme-linked immunoabsorbent Assay (ELISA). The ELISA assay based on the porcine recombinant capsid antigen, for example, can be used to detect antibodies to porcine TTV in porcine and mammalian species. Although the ELISA assay is preferred, other known diagnostic tests can be employed such as immunofluorescence assay (IFA), immunoperoxidase assay (IPA), etc.

[00127] Desirably, a commercial ELISA diagnostic assay in accordance with the present invention can be used to diagnose porcine TTV infection in pigs. The examples illustrate using purified ORF1 and ORF2 proteins of porcine TTV to develop an ELISA assay to detect anti-TTV antibodies in pigs. Sera collected from pigs infected with porcine TTV, and negative sera

from control pigs are used to validate the assay. PTTV2 specific, PTTV1a specific, and PTTV1b specific antibodies were demonstrated to specifically recognize PTTV ORF proteins. Further standardization of the test by techniques known to those skilled in the art may optimize the commercialization of a diagnostic assay for porcine TTV.

[00128] Another aspect of the present invention is the unique immunogenic composition comprising the isolated porcine TTV or an antigenic protein encoded by an isolated polynucleotide described hereinabove and its use for raising or producing antibodies. The composition contains a nontoxic, physiologically acceptable carrier and, optionally, one or more adjuvants. Suitable carriers, such as, for example, water, saline, ethanol, ethylene glycol, glycerol, etc., are easily selected from conventional excipients and co-formulants may be added. Routine tests can be performed to ensure physical compatibility and stability of the final composition.

[00129] In accordance with the present invention, there are further provided infectious molecular and nucleic acid molecules of porcine Torque teno (TTV), live viruses produced from the nucleic acid molecule and veterinary vaccines to protect pigs from porcine TTV viral infection or disease caused by porcine TTV co-infection with other viruses. The invention further provides immunogenic polypeptide expression products that may be used as vaccines.

[00130] The novel infectious DNA molecule of porcine TTV comprises a nucleic acid molecule encoding at least a portion of an infectious PTTV1a-VA (SEQ ID NO:9), PTTV1b-VA (SEQ ID NO:10), PTTV2c-VA (SEQ ID NO:11), or PTTV2c-VA (SEQ ID NO:12) genome. The infectious PTTV DNA clone preferably contains at least one of ORF1, ORF2, ORF1/1, and ORF2/2 gene of the PTTV1 or PTTV2. Multiple copies of the PTTV1a-VA (SEQ ID NO:9), PTTV1b-VA (SEQ ID NO:10), PTTV2c-VA (SEQ ID NO:11), or PTTV2c-VA (SEQ ID

NO:12) genome may be inserted into a single DNA molecule to construct tandem infectious PTTV clones.

[00131] The cloned genomic DNA of PTTV, particularly PTTV1a-VA, PTTV1b-VA, PTTV2c-VA, and tandem PTTV2b-RR, PTTV2c-RR, described herein is shown to be *in vitro* or *in vivo* infectious when transfected into PK-15 cells and given to pigs. This new, readily reproducible pathogenic agent lends itself to the development of a suitable vaccination program to prevent PTTV infection in pigs.

[00132] According to a further embodiment of the present invention, three one-genome-copy PTTV DNA clones were derived from the prototype US isolates PTTV1a-VA, PTTV1b-VA and PTTV2c-VA by fusion PCR, respectively. Each of the full-length genomic DNA was inserted into a cloning vector pSC-B-amp/kan by blunt-end ligation. The restriction site BamH I is the unique site on the three PTTV genomes, which was engineered at both ends of the three genomes to facilitate the generation of concatemers and thus mimic the TTV circular genome. BamH I single digestions of the selected plasmid DNA of each clone clearly resulted in two different fragments of 4.3-Kb and 2.8-Kb in size (Fig. 18A). The 4.3-Kb fragments represented the backbone vector whereas the 2.8-Kb fragments represented the inserted PTTV genomic DNA. The empty vector pSC-B-amp/kan digested with the same enzyme only showed a 4.3-Kb band (Fig. 18A). The resulting PTTV clones were designated pSC-PTTV1a, pSC-PTTV1b and pSC-PTTV2c, respectively (Fig. 17A-C).

[00133] Furthermore, two copies of the full-length PTTV2c-VA genome derived from the clone pSC-PTTV2c were ligated in tandem into the pSC-B-amp/kan vector to generate the clone pSC-2PTTV2c-RR (Fig. 17D). Comparison of the Afl II single digestion patterns between pSC-PTTV2c and pSC-2PTTV2c-RR showed that the latter plasmid had an additional 2.8-Kb

fragment representing the second copy of PTTV2c genome (Fig. 18B, right panel). Subsequently, we utilized the same cloning strategy to produce a tandem-dimerized PTTV2b DNA clone derived from the Germany TTV clone TTV2-#471942-full. An additional 2.8-Kb fragment representing the second copy of PTTV2b genome was presented in this construct, designated pSC-2PTTV2b-RR (Fig. 17F), which was digested with the Hind III alone when compared to its one-genome-copy counterpart (Fig. 18B, left panel), confirming the successful construction.

[00134] The replication competencies of the constructed PTTV infectious clones were tested by *in vitro* transfection of PK-15 cells. IFA using the commercially generated rabbit polyclonal antibodies against PTTV2c ORF1 confirmed that both the concatemers of clones TTV2-#471942-full and pSC-PTTV2c were replication competent, respectively (Fig. 19A and Fig. 20A). Passaging of the transfected cells did not eliminate or reduce the fluorescent signals (Fig. 19B and Fig. 20B), suggesting that the expression of ORF1 proteins was resulted from the PTTV2 concatemers that mimicked the natural PTTV2b or PTTV2c circular molecules. No fluorescent signals was observed in mock-transfected cells or DNA-transfected cells using pre-immune rabbit serum as the antibody for IFA detection (data not shown). The concatemers of the clone pSC-PTTV1a also showed to be replication-competent using an anti-PTTV1a ORF1 antibody (Fig. 21). The positive fluorescent signals were located in the nucleus of transfected or passaged cells, indicating that porcine TTVs likely replicate in the cell nucleus. It is not unexpected because porcine circovirus (PCV) has a similar expression pattern *in vitro*.

[00135] Direct transfection of the tandem-dimerized clone pSC-2PTTV2b-RR or pSC-2PTTV2c-RR in PK-15 cells results in viral replication and produces the ORF1 capsid antigen.

IFA using antibodies against PTTV2 ORF1 confirmed that both clones were also replication-competent and the positive ORF1 antigens were localized in the nuclei (Fig. 22A and B).

[00136] According to one embodiment of the present invention, infectious clones of porcine TTV can be used to inoculate pigs, which will then elicit an immune response of the host animal and stimulate production of neutralizing antibodies. In one particular embodiment of the present invention, the two tandem-dimerized PTTV2 clones were infectious when injected into the lymph nodes and muscles of conventional pigs.

[00137] To test the *in vivo* infectivity of PTTV2 molecular clones, conventional pigs were inoculated with the clone pSC-2TTV2b-RR or pSC-2TTV2c-RR. Serum samples were collected from animals at 0, 7, 14, 21 and 28 days post-inoculation (DPI). PTTV2 DNA was detected in pSC-2TTV2c-RR-inoculated pigs beginning at 7 DPI (#92), 14 DPI (#188 and #191) and 21 DPI (#180), respectively (Fig. 23A, right panel). PTTV viremia appeared late for pigs inoculated with the clone pSC-2TTV2b-RR: two began at 14 DPI (#189 and #192), one at 21 DPI (#181) and one at 28 DPI (#193) (Fig. 23A, left panel). The viral loads increased during the course in all inoculated pigs that had the highest viral loads at 28 DPI before necropsy, as determined by PTTV2-specific real-time PCR (Fig. 23A). The real-time PCR products amplified from selected pigs were sequenced and found to have identical sequences to the corresponding regions of pSC-2TTV2b-RR or pSC-2TTV2c-RR (data not shown).

[00138] All inoculated pigs were negative for PTTV2 ORF1 antibodies at 0 and 7 DPI. At 14 DPI, all the four pSC-2TTV2b-RR-inoculated pigs seroconverted to anti-PTTV2 ORF1 IgG, whereas pigs in pSC-2TTV2c-RR-inoculated group seroconverted at 14 (#92 and #180), 21 (#191) and 28 (#188) DPI, respectively (Fig. 23B). The results indicated that active porcine TTV2b or TTV2c infection had occurred.

[00139] Vaccines of the infectious viral and infectious molecular DNA clones, and methods of using them, are also included within the scope of the present invention. Inoculated pigs are protected from viral infection and associated diseases caused by TTV2 infection or co-infection. The novel method protects pigs in need of protection against viral infection by administering to the pig an immunologically effective amount of a vaccine according to the invention, such as, for example, a vaccine comprising an immunogenic amount of the infectious PTTV DNA, a plasmid or viral vector containing the infectious DNA clone of PTTV, the recombinant PTTV DNA, the polypeptide expression products, the bacteria-expressed or baculovirus-expressed purified recombinant ORF1 capsid protein, etc. Other antigens such as PRRSV, PPV, other infectious swine agents and immune stimulants may be given concurrently to the pig to provide a broad spectrum of protection against viral infections.

[00140] The vaccines comprise, for example, the infectious viral and molecular DNA clones, the cloned PTTV infectious DNA genome in suitable plasmids or vectors such as, for example, the pSC-B vector, an avirulent, live virus, an inactivated virus, expressed recombinant capsid subunit vaccine, etc. in combination with a nontoxic, physiologically acceptable carrier and, optionally, one or more adjuvants. The vaccine may also comprise the infectious TTV2 molecular DNA clone described herein. The infectious PTTV DNA, the plasmid DNA containing the infectious viral genome and the live virus are preferred with the live virus being most preferred. The avirulent, live viral vaccine of the present invention provides an advantage over traditional viral vaccines that use either attenuated, live viruses which run the risk of reverting back to the virulent state or killed cell culture propagated whole virus which may not induce sufficient antibody immune response for protection against the viral disease.

[00141] Vaccines and methods of using them are also included within the scope of the present invention. Inoculated mammalian species are protected from serious viral infection, may also provide protection for disease related to co-infection of PTTV, such as porcine dermatitis and nephropathy syndrome (PDNS), postweaning multisystemic wasting syndrome (PMWS), and other related illness. The vaccines comprise, for example, an inactivated or attenuated porcine TTV virus, a nontoxic, physiologically acceptable carrier and, optionally, one or more adjuvants.

[00142] The adjuvant, which may be administered in conjunction with the vaccine of the present invention, is a substance that increases the immunological response of the pig to the vaccine. The adjuvant may be administered at the same time and at the same site as the vaccine, or at a different time, for example, as a booster. Adjuvants also may advantageously be administered to the pig in a manner or at a site different from the manner or site in which the vaccine is administered. Suitable adjuvants include, but are not limited to, aluminum hydroxide (alum), immunostimulating complexes (ISCOMS), non-ionic block polymers or copolymers, cytokines (like IL-1, IL-2, IL-7, IFN- α , IFN- β , IFN- γ , etc.), saponins, monophosphoryl lipid A (MLA), muramyl dipeptides (MDP) and the like. Other suitable adjuvants include, for example, aluminum potassium sulfate, heat-labile or heat-stable enterotoxin isolated from *Escherichia coli*, cholera toxin or the B subunit thereof, diphtheria toxin, tetanus toxin, pertussis toxin, Freund's incomplete or complete adjuvant, etc. Toxin-based adjuvants, such as diphtheria toxin, tetanus toxin and pertussis toxin may be inactivated prior to use, for example, by treatment with formaldehyde.

[00143] The vaccines may further contain additional antigens to promote the immunological activity of the infectious PTTV DNA clones such as, for example, porcine reproductive and

respiratory syndrome virus (PRRSV), porcine parvovirus (PPV), other infectious swine agents and immune stimulants.

[00144] The new vaccines of this invention are not restricted to any particular type or method of preparation. The cloned viral vaccines include, but are not limited to, infectious DNA vaccines (i.e., using plasmids, vectors or other conventional carriers to directly inject DNA into pigs), live vaccines, modified live vaccines, inactivated vaccines, subunit vaccines, attenuated vaccines, genetically engineered vaccines, etc. These vaccines are prepared by standard methods known in the art.

[00145] As a further benefit, the preferred live virus of the present invention provides a genetically stable vaccine that is easier to make, store and deliver than other types of attenuated vaccines.

[00146] Another preferred vaccine of the present invention utilizes suitable plasmids for delivering the nonpathogenic DNA clone to pigs. In contrast to the traditional vaccine that uses live or killed cell culture propagated whole virus, this invention provides for the direct inoculation of pigs with the plasmid DNA containing the infectious viral genome.

[00147] Additional genetically engineered vaccines, which are desirable in the present invention, are produced by techniques known in the art. Such techniques involve, but are not limited to, further manipulation of recombinant DNA, modification of or substitutions to the amino acid sequences of the recombinant proteins and the like.

[00148] Genetically engineered vaccines based on recombinant DNA technology are made, for instance, by identifying alternative portions of the viral gene encoding proteins responsible for inducing a stronger immune or protective response in pigs (e.g., proteins derived from ORF1,

ORF1/1, ORF2, ORF2/2, etc.). Such identified genes or immuno-dominant fragments can be cloned into standard protein expression vectors, such as the baculovirus vector, and used to infect appropriate host cells (see, for example, O'Reilly et al., "Baculovirus Expression Vectors: A Lab Manual," Freeman & Co., 1992). The host cells are cultured, thus expressing the desired vaccine proteins, which can be purified to the desired extent and formulated into a suitable vaccine product. The recombinant subunit vaccines are based on bacteria-expressed (Fig. 10, Fig. 15) or baculovirus-expressed ORF1 capsid proteins of PTTV1a, PTTV1b and PTTV2.

[00149] If the clones retain any undesirable natural abilities of causing disease, it is also possible to pinpoint the nucleotide sequences in the viral genome responsible for any residual virulence, and genetically engineer the virus avirulent through, for example, site-directed mutagenesis. Site-directed mutagenesis is able to add, delete or change one or more nucleotides (see, for instance, Zoller et al., DNA 3:479-488, 1984). An oligonucleotide is synthesized containing the desired mutation and annealed to a portion of single stranded viral DNA. The hybrid molecule, which results from that procedure, is employed to transform bacteria. Then double-stranded DNA, which is isolated containing the appropriate mutation, is used to produce full-length DNA by ligation to a restriction fragment of the latter that is subsequently transfected into a suitable cell culture. Ligation of the genome into the suitable vector for transfer may be accomplished through any standard technique known to those of ordinary skill in the art. Transfection of the vector into host cells for the production of viral progeny may be done using any of the conventional methods such as calcium-phosphate or DEAE-dextran mediated transfection, electroporation, protoplast fusion and other well-known techniques (e.g., Sambrook et al., "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Laboratory Press, 1989). The cloned virus then exhibits the desired mutation. Alternatively, two oligonucleotides can be

synthesized which contain the appropriate mutation. These may be annealed to form double-stranded DNA that can be inserted in the viral DNA to produce full-length DNA.

[00150] An immunologically effective amount of the vaccines of the present invention is administered to a pig in need of protection against viral infection. The immunologically effective amount or the immunogenic amount that inoculates the pig can be easily determined or readily titrated by routine testing. An effective amount is one in which a sufficient immunological response to the vaccine is attained to protect the pig exposed to the PTTV virus. Preferably, the pig is protected to an extent in which one to all of the adverse physiological symptoms or effects of the viral disease are significantly reduced, ameliorated or totally prevented.

[00151] The vaccine can be administered in a single dose or in repeated doses. Dosages may range, for example, from about 1 microgram to about 1,000 micrograms of the plasmid DNA containing the infectious chimeric DNA genome (dependent upon the concentration of the immuno-active component of the vaccine), preferably 100 to 200 micrograms of the porcine TTV DNA clone, but should not contain an amount of virus-based antigen sufficient to result in an adverse reaction or physiological symptoms of viral infection. Methods are known in the art for determining or titrating suitable dosages of active antigenic agent to find minimal effective dosages based on the weight of the pig, concentration of the antigen and other typical factors. Preferably, the infectious viral DNA clone is used as a vaccine, or a live infectious virus can be generated in vitro and then the live virus is used as a vaccine. In that case, from about 50 to about 10,000 of the 50% tissue culture infective dose (TCID₅₀) of live virus, for example, can be given to a pig.

[00152] The new vaccines of this invention are not restricted to any particular type or method of preparation. The vaccines include, but are not limited to, modified live vaccines, inactivated vaccines, subunit vaccines, attenuated vaccines, genetically engineered vaccines, etc.

[00153] The advantages of live vaccines are that all possible immune responses are activated in the recipient of the vaccine, including systemic, local, humoral and cell-mediated immune responses. The disadvantages of live virus vaccines, which may outweigh the advantages, lie in the potential for contamination with live adventitious viral agents or the risk that the virus may revert to virulence in the field.

[00154] To prepare inactivated virus vaccines, for instance, the virus propagation and virus production can occur in cultured porcine cell lines such as, without limitation PK-15 cells. Serial virus inactivation is then optimized by protocols generally known to those of ordinary skill in the art or, preferably, by the methods described herein.

[00155] Inactivated virus vaccines may be prepared by treating the porcine TTV with inactivating agents such as formalin or hydrophobic solvents, acids, etc., by irradiation with ultraviolet light or X-rays, by heating, etc. Inactivation is conducted in a manner understood in the art. For example, in chemical inactivation, a suitable virus sample or serum sample containing the virus is treated for a sufficient length of time with a sufficient amount or concentration of inactivating agent at a sufficiently high (or low, depending on the inactivating agent) temperature or pH to inactivate the virus. Inactivation by heating is conducted at a temperature and for a length of time sufficient to inactivate the virus. Inactivation by irradiation is conducted using a wavelength of light or other energy source for a length of time sufficient to inactivate the virus. The virus is considered inactivated if it is unable to infect a cell susceptible to infection.

[00156] The preparation of subunit vaccines typically differs from the preparation of a modified live vaccine or an inactivated vaccine. Prior to preparation of a subunit vaccine, the protective or antigenic components of the vaccine must be identified. In the present invention, antigenic components of PTTV were identified as the ORF1 capsid proteins of PTTV1a, PTTV1b and PTTV2, which were expressed and purified in *Escherichia coli* (*E. coli*) in this invention, and other expression system, such as baculovirus expression system, for use as subunit recombinant capsid vaccines. Such protective or antigenic components include certain amino acid segments or fragments of the viral capsid proteins which raise a particularly strong protective or immunological response in pigs; single or multiple viral capsid proteins themselves, oligomers thereof, and higher-order associations of the viral capsid proteins which form virus substructures or identifiable parts or units of such substructures; oligoglycosides, glycolipids or glycoproteins present on or near the surface of the virus or in viral substructures such as the lipoproteins or lipid groups associated with the virus, etc. Preferably, the ORF1 protein is employed as the antigenic component of the subunit vaccine. Other proteins may also be used such as those encoded by the nucleotide sequence in the ORF2, ORF1/1, and ORF2/2 gene. These immunogenic components are readily identified by methods known in the art. Once identified, the protective or antigenic portions of the virus (i.e., the “subunit”) are subsequently purified and/or cloned by procedures known in the art. The subunit vaccine provides an advantage over other vaccines based on the live virus since the subunit, such as highly purified subunits of the virus, is less toxic than the whole virus.

[00157] If the subunit vaccine is produced through recombinant genetic techniques, expression of the cloned subunit such as the ORF1, ORF2, ORF1/1, and ORF2/2 genes, for example, may be expressed by the method provided above, and may also be optimized by

methods known to those in the art (see, for example, Maniatis et al., "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Laboratory, Cold Spring Harbor, Mass. (1989)). On the other hand, if the subunit being employed represents an intact structural feature of the virus, such as an entire capsid protein, the procedure for its isolation from the virus must then be optimized. In either case, after optimization of the inactivation protocol, the subunit purification protocol may be optimized prior to manufacture.

[00158] To prepare attenuated vaccines, the live, pathogenic virus is first attenuated (rendered nonpathogenic or harmless) by methods known in the art or, preferably, as described herein. For instance, attenuated viruses may be prepared by the technique of the present invention which involves the novel serial passage through embryonated pig eggs. Attenuated viruses can be found in nature and may have naturally-occurring gene deletions or, alternatively, the pathogenic viruses can be attenuated by making gene deletions or producing gene mutations. The attenuated and inactivated virus vaccines comprise the preferred vaccines of the present invention.

[00159] Genetically engineered vaccines, which are also desirable in the present invention, are produced by techniques known in the art. Such techniques involve, but are not limited to, the use of RNA, recombinant DNA, recombinant proteins, live viruses and the like.

[00160] For instance, after purification, the wild-type virus may be isolated from suitable clinical, biological samples such as serum, fecal, saliva, semen and tissue samples by methods known in the art, preferably by the method taught herein using infected pigs or infected suitable cell lines. The DNA is extracted from the biologically pure virus or infectious agent by methods known in the art, and purified by methods known in the art, preferably by ultracentrifugation in a CsCl gradient. The cDNA of viral genome is cloned into a suitable host by methods known in the art (see Maniatis et al., id.), and the virus genome is then analyzed to determine essential regions

of the genome for producing antigenic portions of the virus. Thereafter, the procedure is generally the same as that for the modified live vaccine, an inactivated vaccine or a subunit vaccine.

[00161] Genetically engineered vaccines based on recombinant DNA technology are made, for instance, by identifying the portion of the viral gene which encodes for proteins responsible for inducing a stronger immune or protective response in pigs (e.g., proteins derived from ORF1, ORF2, ORF1/1, and ORF2/2, etc.). Such identified genes or immuno-dominant fragments can be cloned into standard protein expression vectors, such as the baculovirus vector, and used to infect appropriate host cells (see, for example, O'Reilly et al., "Baculovirus Expression Vectors: A Lab Manual," Freeman & Co. (1992)). The host cells are cultured, thus expressing the desired vaccine proteins, which can be purified to the desired extent and formulated into a suitable vaccine product.

[00162] Genetically engineered proteins, useful in vaccines, for instance, may be expressed in insect cells, yeast cells or mammalian cells. The genetically engineered proteins, which may be purified or isolated by conventional methods, can be directly inoculated into a porcine or mammalian species to confer protection against porcine TTV.

[00163] An insect cell line (like sf9, sf21, or HIGH-FIVE) can be transformed with a transfer vector containing polynucleic acids obtained from the virus or copied from the viral genome which encodes one or more of the immuno-dominant proteins of the virus. The transfer vector includes, for example, linearized baculovirus DNA and a plasmid containing the desired polynucleotides. The host cell line may be co-transfected with the linearized baculovirus DNA and a plasmid in order to make a recombinant baculovirus.

[00164] Alternatively, DNA from the isolated porcine TTV which encode one or more capsid proteins can be inserted into live vectors, such as a poxvirus or an adenovirus and used as a vaccine.

[00165] An immunologically effective amount of the vaccine of the present invention is administered to an porcine or mammalian species in need of protection against said infection or syndrome. The “immunologically effective amount” can be easily determined or readily titrated by routine testing. An effective amount is one in which a sufficient immunological response to the vaccine is attained to protect the pig or other mammal exposed to the porcine TTV virus, or porcine TTV co-infection, which may cause porcine dermatitis and nephropathy syndrome (PDNS), postweaning multisystemic wasting syndrome (PMWS) or related illness. Preferably, the pig or other mammalian species is protected to an extent in which one to all of the adverse physiological symptoms or effects of the viral disease are found to be significantly reduced, ameliorated or totally prevented.

[00166] The vaccine can be administered in a single dose or in repeated doses. Dosages may contain, for example, from 1 to 1,000 micrograms of virus-based antigen (dependent upon the concentration of the immuno-active component of the vaccine), but should not contain an amount of virus-based antigen sufficient to result in an adverse reaction or physiological symptoms of viral infection. Methods are known in the art for determining or titrating suitable dosages of active antigenic agent based on the weight of the bird or mammal, concentration of the antigen and other typical factors.

[00167] The vaccine can be administered to pigs. Also, the vaccine can be given to humans such as pig farmers who are at high risk of being infected by the viral agent. It is contemplated that a vaccine based on the porcine TTV can be designed to provide broad protection against

both porcine and human TTV. In other words, the vaccine based on the porcine TTV can be preferentially designed to protect against human TTV infection through the so-called “Jennerian approach” (i.e., cowpox virus vaccine can be used against human smallpox by Edward Jenner). Desirably, the vaccine is administered directly to a porcine or other mammalian species not yet exposed to the TTV virus. The vaccine can conveniently be administered orally, intrabuccally, intranasally, transdermally, parenterally, etc. The parenteral route of administration includes, but is not limited to, intramuscular, intravenous, intraperitoneal and subcutaneous routes.

[00168] When administered as a liquid, the present vaccine may be prepared in the form of an aqueous solution, a syrup, an elixir, a tincture and the like. Such formulations are known in the art and are typically prepared by dissolution of the antigen and other typical additives in the appropriate carrier or solvent systems. Suitable carriers or solvents include, but are not limited to, water, saline, ethanol, ethylene glycol, glycerol, etc. Typical additives are, for example, certified dyes, flavors, sweeteners and antimicrobial preservatives such as thimerosal (sodium ethylmercurithiosalicylate). Such solutions may be stabilized, for example, by addition of partially hydrolyzed gelatin, sorbitol or cell culture medium, and may be buffered by conventional methods using reagents known in the art, such as sodium hydrogen phosphate, sodium dihydrogen phosphate, potassium hydrogen phosphate, potassium dihydrogen phosphate, a mixture thereof, and the like.

[00169] Liquid formulations also may include suspensions and emulsions which contain suspending or emulsifying agents in combination with other standard co-formulants. These types of liquid formulations may be prepared by conventional methods. Suspensions, for example, may be prepared using a colloid mill. Emulsions, for example, may be prepared using a homogenizer.

[00170] Parenteral formulations, designed for injection into body fluid systems, require proper isotonicity and pH buffering to the corresponding levels of mammalian body fluids. Isotonicity can be appropriately adjusted with sodium chloride and other salts as needed. Suitable solvents, such as ethanol or propylene glycol, can be used to increase the solubility of the ingredients in the formulation and the stability of the liquid preparation. Further additives which can be employed in the present vaccine include, but are not limited to, dextrose, conventional antioxidants and conventional chelating agents such as ethylenediamine tetraacetic acid (EDTA). Parenteral dosage forms must also be sterilized prior to use.

[00171] The following examples demonstrate certain aspects of the present invention. However, it is to be understood that these examples are for illustration only and do not purport to be wholly definitive as to conditions and scope of this invention. It should be appreciated that when typical reaction conditions (e.g., temperature, reaction times, etc.) have been given, the conditions both above and below the specified ranges can also be used, though generally less conveniently. The examples are conducted at room temperature (about 23° C. to about 28° C.) and at atmospheric pressure. All parts and percents referred to herein are on a weight basis and all temperatures are expressed in degrees centigrade unless otherwise specified.

Example 1.

Viral DNA extraction, nested PCR and genomic PCR:

[00172] Convenient serum and semen samples from 20 conventional adult boars from a Virginia pig farm were used in the study. Total DNA was isolated from 20 serum and 19 semen samples using QIAamp DNA mini kit (Qiagen). To screen for the positive PTTV-containing samples, nested PCR amplifications of the conserved regions in the UTR of PTTV1 and PTTV2

were initially performed by using AmpliTag Gold polymerase (Applied Biosystems). The two primer pairs used to amplify the fragment A of PTTV1 were TTV1-mF (SEQ ID NO:45)/TTV1-mR (SEQ ID NO:46)(for the first-round PCR) and TTV1-nF (SEQ ID NO:47)/TTV1-nR (SEQ ID NO:48) (for the second-round PCR), whereas the two primer pairs used to amplify the fragment D of PTTV2 were TTV2-mF (SEQ ID NO:49)/TTV2-mR (SEQ ID NO:50) (for the first-round PCR) and TTV2-nF (SEQ ID NO:51)/TTV2-nR (SEQ ID NO:52) (for the second-round PCR; Fig. 1A and Table 1).

[00173] In order to amplify the full-length genomic sequences of both PTTV1 and PTTV2, we first performed an inverse genomic PCR using a pair of conserved gene-specific primers TTV1-IF (SEQ ID NO:1)/TTV1-IR (SEQ ID NO:4) located in region A for PTTV1 and another pair of gene-specific primers TTV2-IF (SEQ ID NO:5)/TTV2-IR (SEQ ID NO:8) located in region D for PTTV2, respectively, with Herculase II Fusion DNA Polymerase (Stratagene) according to the manufacturer's instructions. No PCR products with expected sizes were detected. Subsequently we designed new sets of primers to amplify two regions covering the complete PTTV1 and PTTV2 genomes in the second-round PCR, respectively (Fig. 1A). The primer pairs used to amplify fragments B and C of PTTV1 were TTV1-IF (SEQ ID NO:1)/TTV1-2340R (SEQ ID NO:2) and TTV1-2311F (SEQ ID NO:3)/TTV1-IR (SEQ ID NO:4), respectively, whereas the primer pairs used to amplify fragments E and F of PTTV2 were TTV2-IF (SEQ ID NO:5)/TTV2-2316R (SEQ ID NO:6) and TTV2-GCF (SEQ ID NO:7)/TTV2-IR (SEQ ID NO:8), respectively (Fig. 1A and Table 1). Fragments C and F contain the GC-rich regions of PTTV1 and PTTV2, respectively. The amplified PCR products were individually excised, purified, and subsequently cloned into a pSC-B-amp/kan vector (Stratagene) by StrataClone Blunt PCR

cloning strategy according to the manufacturer's instructions (Stratagene) followed by DNA sequencing.

Example 2.

Screening for porcine TTV positive samples collected from boars in a farm from Virginia:

[00174] Porcine TTV DNA was previously detected from pigs in different geographic regions by nested-PCR based on the UTR sequence of a Japanese PTTV1 strain Sd-TTV31 (McKeown et al., 2004, *supra*). With the recent identification of PTTV2, two different sets of nested-PCR primers have been used to amplify region A of PTTV1 and region D of PTTV2, respectively (Fig. 1A) (Ellis et al., 2008, *supra*; Kekarainen, T., Sibila, M., and Segales, J. (2006). Prevalence of swine Torque teno virus in post-weaning multisystemic wasting syndrome (PMWS)-affected and non-PMWS-affected pigs in Spain. *J Gen Virol* **87**(Pt 4), 833-7; Krakowka et al., 2008, *supra*). A similar detection approach was also utilized in the present study to identify PTTV strains from pigs in the United States. In order to screen for indigenous PTTV1- or PTTV2-positive samples for subsequent use to determine the full-length genomic sequences, 20 sera (SR#1-20) and 19 semen samples (SM#1-18, and SM#20) collected from 20 boars in a farm of Virginia were subjected to nested-PCR analyses. Surprisingly, all the 20 serum samples were positive for PTTV1 and 19 were also positive for PTTV2 (except for SR#18). In contrast, only 1 semen sample (SM#6) was PTTV1-positive and 3 semen samples (SM#8, 9 and 20) were PTTV2-positive. The result was consistent with a recent study in that boar semen samples were shown to be positive for PTTV DNA in Spain (Kekarainen, T., Lopez-Soria, S., and Segales, J. (2007). Detection of swine Torque teno virus genogroups 1 and 2 in boar sera and semen. *Theriogenology* **68**(7), 966-71), and thus suggesting a potential vertical transmission of PTTV.

However, the prevalence rates of both PTTV1 and PTTV2 in semen were much lower than that in sera, suggesting that there is no direct association for the presence of PTTV DNAs in sera and semen of the same pig.

Example 3.

Sequence and phylogenetic analyses:

[00175] Generic analyses and alignment of DNA and amino acid sequences were performed using Lasergene package (DNASTAR Inc., Madison, WI). The genomic sequences of three known PTTV strains and their corresponding GenBank accession numbers used for the alignment and comparison are Sd-TTV31 (AB076001), TTV-1p (AY823990) and TTV-2p (AY823991). Pairwise sequence comparisons (PASC) were performed using 121 full-length genomic sequences of human and animal TTV-related strains available in GenBank with an online program PASC (<http://www.ncbi.nlm.nih.gov/sutils/pasc/viridty.cgi?textpage=overview>) (Bao et al., 2008).

[00176] Phylogenetic trees were constructed by the neighbor-joining method in the PAUP 4.0 program (David Swofford, Smithsonian Institute, Washington, DC, distributed by Sinauer Associate Inc.) based upon the full-length genomic sequences and the deduced amino acid sequences of 4 ORFs of seven PTTV strains. The data were obtained from 1000 re-sampling.

Example 4.

Design of PCR primers for diagnosing porcine PTTV infection

[00177] Analyses and alignment of DNA sequences were performed using Lasergene package (DNASTAR Inc., Madison, WI). Full-length genomic sequences of ten porcine TTV strains and their corresponding GenBank accession numbers used for the alignment were as follows. Species PTTV1: Sd-TTV31 (AB076001), PTTV1a-VA (GU456383), TTV-1p (AY823990), PTTV1b-VA (GU456384), swSTHY-TT27 (GQ120664) and TTV1 #471819 (GU188045). Species PTTV2: PTTV2b-VA (GU456385), PTTV2c-VA (GU456386), TTV-2p (AY823991) and TTV2 #472142 (GU188046). The conserved sequences among the 6 PTTV1 and 4 PTTV2 genomes were identified, respectively, and subsequently used to guide real-time PCR primer selections using the Beacon Designer program (PREMIER Biosoft International, Palo Alto, CA). Primers used for the duplex nested PCR of PTTV1 were designed by the Lasergene package.

Example 5.

Standard curves of PTTV1 and PTTV2 real-time PCR

[00178] A region of 2091 bp corresponding to the PCR fragment B of PTTV1b-VA genome was re-amplified from the same PCR fragment using primers TTV1-IF (5'-CATAGGGTGTAACCAATCAGATTTAAGGCGTT-3') and TTV1-2340R (5'-GGTCATCAGACGATCCATCTCCCTCAG-3') as described previously (Huang et al., 2010). The resulting amplicon was gel-purified by QIAquick Gel Extraction Kit (Qiagen) and quantified by a NanoDrop spectrophotometer that was used for the real-time PCR standard template of porcine TTV species 1. A full-length DNA clone of PTTV2c-VA strain, pSC-PTTV2c, was constructed by assembling PCR fragments E and F from PTTV2c-VA in the vector pSC-B-

amp/kan (Huang et al., unpublished data). Plasmid pSC-PTTV2c (7082 bp) was used for the real-time PCR standard template of porcine TTV species 2 and the plasmid DNA concentration was measured by a NanoDrop spectrophotometer. A 10-fold dilution series of the two templates was used to generate the real-time PCR standard curves, respectively.

Example 6.

Extraction of viral DNA for PCR assays

[00179] Total DNA was isolated from 20 serum and 19 semen samples collected from 20 conventional adult boars (with no clinical syndromes) from a Virginia pig farm using QIAamp DNA mini kit (Qiagen) as described previously (Huang et al., 2010). A sample volume of 400 µl for sera and semen was used to extract DNA with a final eluate of 50 µl sterile water. All extracted DNA samples were stored at -20°C until real-time PCR testing. Detection of porcine TTVs in these samples by conventional nested PCR had been described previously (Huang et al., 2010). Total DNA extracted from a goat serum sample with the same procedure was used as the negative control.

Example 7.

SYBR green real-time quantitative PCR assays

[00180] PTTV1- and PTTV2-specific real-time PCR were performed, respectively, using SensiMix SYBR & Fluorescein kit (Quantace Ltd) and the MyiQ iCYCLER Real Time PCR instrument (BIO-RAD Laboratories). Each 25-µl reaction contained 12.5 µl of SYBR green Master Mix, 4 µl of extracted DNA, 0.5 µl of each primer (10 nM) and 7.5 µl of sterile water. The PCR condition for PTTV1 was 10 min at 95°C followed by 40 cycles of amplification (15

sec at 95°C, 30 sec at 59.4°C, 10 sec at 72°C). This was immediately followed by a melting point analysis obtained by gradually increasing the temperature from 55°C to 95°C with the fluorescence signal being measured every 0.5°C. The PCR condition for PTTV2 was the same as PTTV1 except that the annealing temperature was 56°C. PTTV1 and PTTV2 standard templates were included as positive controls in every run. Amplification and data analysis were carried out using MyiQ System software (BIO-RAD Laboratories). All samples were run in duplicate on the same plate.

Example 8.

Specificity and sensitivity of two singleplex assays

[00181] The optimal annealing temperatures for amplification of PTTV1- and PTTV2-specific assays were 59.4°C and 56°C, respectively, as determined by a 10-fold dilution of amplifications using a gradient of annealing temperatures. Amplification of the 118-bp product using primers TTV1F/TTV1R was obtained only with PTTV1 template whereas amplification of the 200-bp product with PTTV2 template was only observed when primers TTVF4/TTVR4 were used. Neither assay yielded any cross-amplification from the other, confirming the specificity of the primers and targets (data not shown).

[00182] A PTTV1 standard curve was established over a range of target DNA concentrations per 25 µl. The linear range was shown to span 4.4×10^1 to 4.4×10^8 copies. The minimum detection limit (44 copies) corresponded to a threshold cycle (C_t) of 37.57. Tested samples with $C_t > 37.57$ were considered as below the detection limit and were not quantifiable. Similarly, a PTTV2 standard curve was generated and used to detect DNA concentration ranging from 8.6×10^0 to 8.6×10^8 copies per 25 µl reaction. The corresponding C_t of minimum detection limit

(8.6 copies) was 36.53. All samples that were considered as PTTV1- or PTTV2-positive had copy numbers lower than the respective maximum detection limit. Melting curves using a 10-fold dilution of PTTV1 or PTTV2 standard template (Fig. 6a & 6b; blue curves), as well as 20 boar serum samples, displayed melting temperatures (T_m) of 87.0°C for PTTV1 and 80.0°C for PTTV2, respectively (Fig. 6a & 6b; red curves). No peaks were observed for the negative controls using sterile water or goat serum DNA as templates (Fig. 6a & 7b; black lines).

Example 9.

Quantification of porcine TTV1 and TTV2 in boar serum and semen samples

[00183] Viral load was expressed as copy numbers of PTTV1 or PTTV2 genomes per ml of original boar serum samples. PTTV1 DNA were detected in all 20 serum samples ranging from 1.91×10^3 to 3.25×10^5 copies/ml whereas PTTV2 DNA were detected in 19 serum samples (except #10) ranging from 3.59×10^2 to 1.39×10^6 copies/ml. The result was consistent to our previous study by using conventional nested PCR (Table 5). None of the semen samples were PTTV1-positive whereas three semen samples were PTTV2-positive with very low viral loads (230, 244 and 357 copies/ml, respectively).

Table 5. Comparison of porcine TTVs detection by different assays in 20 serum and 19 semen samples from adult boars in a Virginia Farm.

Samples	No. of positive / total no. tested by different assay				
	PTTV1 real-time PCR	PTTV1 nested PCR	PTTV2 real-time PCR	PTTV2 nested PCR	PTTV1/PTTV2 duplex real-time PCR
Serum PTTV1	20/20	20/20	-	-	20/20
Serum PTTV2	-	-	19/20	19/20	19/20
Semen PTTV1	0/19	1/19	-	-	-
Semen PTTV2	-	-	3/19	3/19	-

Example 10.

PTTV1/PTTV2 duplex real-time PCR assay

[00184] PTTV1/PTTV2 duplex real-time PCR assay was performed in a 25- μ l PCR system containing 12.5 μ l of SYBR green Master Mix, 0.5 μ l of each PTTV1 primers, 0.5 μ l of each PTTV2 primers, 4 μ l of DNA and 6.5 μ l of sterile water. The duplex PCR condition and melting point analysis were the same as PTTV1 except that the annealing temperature was 58°C. The melting peaks were analyzed to distinguish the PTTV1- and PTTV2-specific amplicons.

Example 11.

Duplex nested PCR

[00185] The first-round PCR was performed with a Platinum PCR HiFi Supermix (Invitrogen) using 4 μ l of extracted DNA in a total volume of 50 μ l. The PCR condition was 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec with an initial denaturation of the template DNA at 94°C for 2 min. A 4- μ l aliquot of the first-round PCR product was used for the second-round PCR with the same PCR reagents and condition. One pair of primers P1ab-mF/P1ab-mR was used in the first-round PCR whereas a mixture of two pairs of primers, P1a-nF/P1a-nR for

detection of PTTV1a, and P1b-nF/P1b-nR for detection of PTTV1b, were used in the second-round PCR (Table 1). The amplification products were visualized by gel electrophoresis on a 1% agarose gel stained with ethidium bromide and two bands specific for each type were differentiated by UV light.

Example 12.

Construction of PTTV1 and PTTV2 ORF expression plasmids

[00186] The C-terminal parts of ORF1 of PTTV1a, PTTV1b and PTTV2c were amplified from the respective full-length DNA clones (pSC-PTTV1a, pSC-PTTV1b and pSC-PTTV2c; described elsewhere). The amplified fragments were expected to encode protein products with 319 aa for PTTV1a (ORF1 aa positions 317-635 (SEQ ID NO:13); GenBank accession no. GU456383), 318 aa for PTTV1b (ORF1 aa positions 322-639 (SEQ ID NO:14); GenBank accession no. GU456384), and 316 aa for PTTV2c (ORF1 aa positions 310-625 (SEQ ID NO:16); GenBank accession no. GU456386), respectively. A C-terminal truncated fragment of PTTV1b encoding 248 aa (ORF1 aa positions 322-569 (SEQ ID NO:14)) was also amplified and used as a comparison control for SDS-PAGE analysis. All the plasmids were constructed by cloning of the PCR products into an *E.coli*/baculovirus/mammalian cells triple expression vector pTriEx1.1-Neo (Novagen) between the NcoI and XhoI restriction sites to generate C-terminally 8 × His-tagged fusion proteins. The four recombinant plasmids were designated pTri-PTTV1a-ORF1, pTri-PTTV1b-ORF1, pTri-PTTV1b-ORF1truc and pTri-PTTV2c-ORF1. All cloned sequences were confirmed by DNA sequencing.

Example 13.

Expression of recombinant PTTV1 and PTTV2 proteins

[00187] The four expression plasmids were transformed into Rosetta 2 (DE3) pLacI competent cells (Novagen), respectively, and the bacteria were plated on LB agar plates containing 100 µg/ml ampicillin overnight at 37°C. A single transformation colony for each construct was used to inoculate 3 ml of LB medium containing 100 µg/ml of ampicillin (LB/amp), and grown 6-8 hours at 37°C. The turbid 3 ml culture for each construct was then used to make bacterial stocks by adding 25% filter sterilized glycerol, and freezing the culture down at -80°C. Prior to purification, 10 µl of the frozen bacterial stock for each construct was used to inoculate a 3 ml starter culture of LB/amp, and grown for 6-8 hours at 37°C. A 100-ml of Overnight Express TB Media (Novagen) was inoculated with the starter culture to induce protein expression, and was grown 16-18 hours at 37°C. After incubation, the autoinduction culture underwent centrifugation at 3400 rpm for 15 minutes at 4°C. The resulting supernatant for each construct was discarded, and each of the bacterial pellets was reserved at -20°C until use.

Example 14.

Purification and dialysis of recombinant proteins

[00188] The recombinant proteins were insoluble and expressed within the bacterial inclusion bodies. Each of the bacterial pellets was treated with BugBuster and rLysozyme according to the manufacture's protocol (Novagen), and Benzonase Nuclease (Novagen) was added for degradation of DNA and RNA. Each of the inclusion body pellets was subsequently resuspended

with 840 µl of lysis buffer (6M Guanidine Hydrochloride, 0.1M sodium phosphate, 0.01M Tris-Chloride, 0.01M imidazole, pH 8.0), and frozen at -80°C for at least 30 minutes. It was then thawed, diluted with an additional 2.5 ml of lysis buffer and gently rotated for 30 minutes at room temperature. The lysate supernatants were collected by centrifugation at 15,000 × g for 30 minutes at room temperature. A 50%-Ni-NTA His-bind slurry (Novagen) was added to each of the decanted supernatants, and the mixtures were shaken for 60 minutes at room temperature to promote his-tag binding. The lysate/resin mixtures were loaded into an empty chromatography column. After the initial flow-through, a 7-ml of lysis buffer was added to the column and allowed to flow through. Each column was then washed 2 times with 7 mL of wash buffer (8M Urea, 0.1M Sodium Phosphate, 0.15M Sodium Chloride, 0.02M imidazole, pH 8.0). Elution of the target protein was achieved by adding 4 separate 1 ml aliquots of elution buffer (8M Urea, 0.05M Sodium Phosphate, 1M Sodium Chloride, 0.5M Imidazole, pH 8.0) to the column. The four elution fractions were analyzed by SDS Page and Coomassie Blue Staining.

[00189] The elutions containing significant concentrations of the target protein were injected into a 0.5ml – 3ml dialysis cassette with a 20,000 molecular weight cut-off (Pierce). A series of 4 dialysis buffers were used for dialysis; dialysis buffer 1 (6M Urea, 0.05M Sodium Phosphate, 0.8M Sodium Chloride, 0.3M Imidazole, pH 8.0), dialysis buffer 2 (4M Urea, 0.033M Sodium Phosphate, 0.533M Sodium Chloride, 0.2M Imidazole, pH 8.0), dialysis buffer 3 (2.67M Urea, 0.022M Sodium Phosphate, 0.356M Sodium Chloride, 0.133M Imidazole, pH 8.0) and dialysis buffer 4 (1.5M Urea, 0.0148 Sodium Phosphate, 0.237M Sodium Chloride, 0.089M Imidazole, pH 8.0). The dialysis cassette was sequentially submerged and rotated in each dialysis buffer for over 6 hours at 4°C. When dialysis was complete, the recombinant His-tagged fusion proteins were each removed from the cassettes, quantified using a NanoDrop and frozen at -80°C.

Example 15.

SDS-PAGE and anti-His-tagged Western blot

[00190] A western blot was developed to detect purified recombinant proteins by using an anti-6 × His-tagged monoclonal antibody (Rockland). Equal volumes of each of the purified truncated ORF1 proteins and LDS / 10% β-ME were mixed, and boiled at 95°C for 10 minutes. A 10-μl of the boiled sample was added to each appropriate well of a 4-12% Bis-Tris Polyacrylamide Gel (Invitrogen), and was run at 200 volts for 43 minutes in 1 × MES running buffer (Invitrogen). The proteins were transferred to a PVDF membrane (Bio-Rad) using a Trans blot semi dry transfer apparatus and 1 × transfer buffer (Invitrogen). Once transfer was complete, the PVDF membrane was incubated in Odyssey blocking buffer (Li-Cor) at room temperature for 1 hour. The anti-6 × His-tagged MAb was diluted at 1:1000 in Odyssey blocking buffer / 0.2% tween 20, and transferred to the membrane after the previous Odyssey blocking buffer was removed. The MAb was left on a rocker to incubate with the membrane for either 2 hours at room temperature or 4°C overnight, and then the membrane was washed 3 times with tris buffered saline / 0.05% tween 20 (TBS-T, Sigma). A Goat anti-rabbit IgG IRDye 800 (Li-Cor) antibody was diluted at 1:5000 in Odyssey blocking buffer / 0.2% tween 20 / 0.1% SDS. It was transferred to the freshly washed PVDF membrane, and allowed to incubate for 1 hour at room temperature while gently rocking. The membrane was washed 3 times with TBS-T, 1 time with TBS and imaged with the Li-Cor Odyssey.

Example 16.

Serum Western blot

[00191] A serum western blot was developed, and used to identify positive and negative serum controls for ELISA development. After SDS-PAGE as described above, the proteins were transferred to a PVDF membrane that was subsequently incubated in Odyssey blocking buffer (Li-Cor) at room temperature for 1 hour. A selected serum sample was diluted at 1:100 in Odyssey blocking buffer / 0.2% tween 20, and transferred to the membrane after the previous Odyssey blocking buffer was removed. The serum sample was left on a rocker to incubate with the membrane for 2 hours at room temperature, and then the membrane was washed 3 times with tris buffered saline / 0.05% tween 20 (TBS-T, Sigma). A goat anti-swine IgG IRDye 800 antibody (Rockland) was diluted at 1:2500 in Odyssey blocking buffer / 0.2% tween 20 / 0.1% SDS. It was transferred to the freshly washed PVDF membrane, and allowed to incubate for 1 hour at room temperature while gently rocking. The membrane was washed 3 times with TBS-T, 1 time with TBS and imaged with the Li-Cor Odyssey.

Example 17.

Indirect PTTV1a-, PTTV1b- and PTTV2- specific ELISA

[00192] The optimal concentrations of the antigens used to coat the plates and dilutions of antisera and conjugates were determined by checkboard titration. The ELISA was initiated by diluting each of the purified recombinant His-tagged fusion proteins (PTTV1a, PTTV1b and PTTV2c, respectively) to 680 ng/ml in 1 × Carbonate Coating Buffer (CCB) at a pH of 9.6, and coating medium binding ELISA plates (Greiner) with 100 µl/well. The plates were covered, and allowed to incubate at 37°C for 2 hours. After coating, the diluted proteins were removed, and

each well was washed 3 times with 300 μ l of 1 $\times$ TBS-T. Protein Free Blocking Buffer (Pierce) was then added at a volume of 300 μ l/well, and the plates were allowed to incubate at 37°C for 1 hour. Meanwhile, in a 96-well dilution block, the serum samples were diluted at 1:100 in 150 μ l of protein free blocking buffer. The block was then removed, and 100 μ l of each diluted serum sample was transferred to each corresponding well on the ELISA plates. The plates were allowed to incubate at 37°C for 2 hours, after which each well was washed 3 times with 300 μ l of TBS-T. Next, the HRP-conjugated anti-swine IgG antibody (Rockland) was diluted at 1:4000 in 12 ml of protein free block, and 100 μ l was added to each well of the plates. This was incubated at 37°C for 1 hour, and then each well was washed 3 times with 300 μ l of TBS-T. In order to develop the ELISA, 100 μ l of Sure Blue Reserve 1-Component (KPL) was added to each well of the plates. After 20 minutes, 100 μ l of 1N HCL was added to each well to stop development. The plates were then read at 450 nm.

Example 18.

Data analyses

[00193] Porcine sera used in cell culture research from a commercial company (manufactured in New Zealand and considered free from all OIE diseases) were used as a positive control for the three ELISA protocols because the sera were all PTTV1a-, PTTV1b- and PTTV2-positive as detected by serum western blot and displayed high OD values (> 2.0). We initially used pooled gnotobiotic pig sera as a negative control as they were negative in western blot detection. Subsequently, in comparison of the negative gnotobiotic pig sera, we screened some porcine sera collected from a conventional pig farm in Wisconsin. They were also negative in western blot detection and their OD values corresponded to that of negative gnotobiotic pig sera. These

conventional porcine sera were pooled and used as a negative control. The cutoff value for each ELISA was calculated as the mean OD value of the negative control group (n=4) plus 3 times of the standard deviation.

Example 19.

Construction of full-length genomic DNA clones of porcine TTV1a, 1b and 2c

[00194] PCR fragments B and C from the US isolate PTTV1a-VA (GenBank accession no. GU456383) were re-amplified from the constructs described previously, and were subsequently assembled into a full-length genomic DNA with a BamH I site at the both ends of the genome by overlapping PCR using the Herculanase II Fusion DNA Polymerase (Stratagene) on the vector pSC-B-amp/kan (Stratagene). The resulting construct was designated pSC-PTTV1a (Fig. 17A). Using the same strategy, the clone pSC-PTTV1b (Fig. 17B) originated from the US isolate PTTV1b-VA (GenBank accession no. GU456384) and the clone pSC-PTTV2c (Fig. 17C) originated from the US isolate PTTV2c-VA (GenBank accession no. GU456386) were constructed with the same restriction sites (BamH I) on the same backbone vector. Plasmid TTV2-#471942-full (Fig. 17E) containing a full-length genomic DNA originated from a Germany pathogenic porcine TTV2 isolate. TTV2-#471942 was a gift from Dr. Andreas Gallei (BIVI, Germany). TTV2-#471942 was classified into the porcine TTV subtype 2b together with the US isolate PTTV1b-VA based upon the phylogenetic analysis (data not shown).

Example 20.

Construction of tandem-dimerized DNA clones of porcine TTV2b and 2c

[00195] The full-length PTTV2c genome was excised from the clone pSC-PTTV2c by BamH I digestion, purified and ligated to form concatemers. Ligated concatemers were cloned into the BamH I-pre-digested pSC-B-amp/kan vector to produce a tandem-dimerized DNA clone, pSC-2PTTV2c-RR (Fig. 1D). Similarly, a tandem-dimerized DNA clone, pSC-2PTTV2b-RR, was generated from the clone TTV2-#471942-full using EcoR V restriction sites (Fig. 1F).

Example 21.

Generation of PTTV1a-, PTTV1b- and PTTV2-specific anti-ORF1 polyclonal antibodies

[00196] The ORF1-encoding product is the putative capsid protein of TTV. To generate PTTV1a-, PTTV1b- and PTTV2-specific anti-ORF1 polyclonal antibodies to detect the expression of PTTV ORF1 proteins and to determine the infectivity of PTTV DNA clones, the three ORF1 proteins from PTTV1a, PTTV1b and PTTV2c were expressed in *E.coli*, purified and were subsequently used to immunize New Zealand white rabbits, respectively, as a custom antibody production service at Rockland Immunochemicals (Gilbertsville, PA). Each anti-ORF1 polyclonal antibody was produced from serum of immunized rabbits.

Example 22.

In vitro transfection of PTTV infectious clones

[00197] PK-15 cells were seeded at 2×10^5 cells per well onto a 6-well plate and grown until 60%-70% confluency before transfection. The DNA clones pSC-2PTTV2b-RR and pSC-

2PTTV2c-RR were directly transfected into PK-15 cells, respectively, using Lipofectamine LTX (Invitrogen) according to the manufacturer's protocol. For clones pSC-PTTV1a, pSC-PTTV2c and TTV2-#471942-full, their ligated concatemers, produced as described above, were used for transfection, respectively. Cells were cultured for 3 to 5 days, and were then applied to an immunofluorescence assay (IFA) to detect the expression of ORF1 of porcine TTVs. Alternatively, transfected cells were passaged into new 6-well plates and continued to culture for 3 days before the IFA detection.

Example 23.

Immunofluorescence assay (IFA)

[00198] Transfected or passaged cells were washed 2 times with PBS and fixed with acetone. Five hundred microliters of the antibodies, specific to PTTV1a or PTTV2 at 1:500 dilution in PBS, was added over the cells and incubated for 1 hour at room temperature. Cells were washed 3 times with PBS and 500 µl Texas red- or Alexa Fluor 488-labeled goat anti-rabbit IgG (Invitrogen) at 1:200 dilution was then added. After 1-hour incubation at room temperature and washed with PBS, the cells were stained with 500 µl DAPI (KPL, Inc.) at 1:1000 dilution and visualized under a fluorescence microscope.

Example 24.

In vivo inoculation of conventional pigs with the tandem-dimerized porcine TTV2 clones.

[00199] A pig inoculation study was performed to determine the infectivities of the two tandem-dimerized porcine TTV2 clones: pSC-2TTV2b-RR and pSC-2TTV2c-RR. Briefly, eight

4-week-old conventional pigs that were seronegative and viral DNA negative for porcine TTV2 were randomly assigned into two groups of four each. Each group of pigs was housed separately and maintained under conditions that met all requirements of the Institutional Committee on Animal Care and Use.

[00200] All pigs in each group were injected by a combination of both the intra-lymph node route and intramuscular route. The four pigs (nos. 181, 189, 192 and 193) were each injected with 200 µg of the pSC-2TTV2b-RR plasmid DNA whereas another four pigs (nos. 92, 180, 188 and 191) were each inoculated with 200 µg of the pSC-2TTV2c-RR clone. Pigs were monitored daily for clinical signs of disease for a total of 28 days. All pigs were necropsied at 28 days postinoculation.

[00201] While the present invention has been illustrated by description of several embodiments and while the illustrative embodiments have been described in detail, it is not the intention of the applicants to restrict or in any way limit the scope of the appended claims to such detail. Additional modifications will readily appear to those skilled in the art. The invention in its broader aspects is therefore not limited to the specific details, representative apparatus and methods, and illustrative examples shown and described. Accordingly, departures may be made from such details without departing from the spirit or scope of applicants' general inventive concept.