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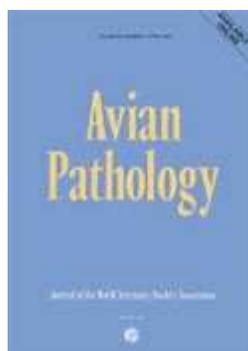
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**Detection of West Nile Virus in the tissues of SPF chickens and serological response to laboratory infection: A comparative study.**

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**L P Phipps\*, R E Gough, Vanessa Ceeraz, W J Cox and I H Brown**

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Detection of West Nile Virus in the tissues of SPF chickens and serological response to laboratory infection: A comparative study.

L P Phipps\*, R E Gough, Vanessa Ceeraz, W J Cox and I H Brown

**Abstract**

Using an isolate of West Nile virus (WNV) from lineage 1 (Goose/Israel 1998), groups of specific pathogen free (SPF) chickens were experimentally infected via the subcutaneous or intravenous routes. To evaluate the relative efficiency of detecting the virus in the infected chickens, samples from a range of tissues and organs were examined by virus isolation tests in tissue culture, including Vero, primary chicken embryo liver and fibroblast cells, and polymerase chain reaction (PCR) analyses. Additionally, in order to investigate the serological response of the chickens and produce WNV monospecific antibodies, serum samples were collected from the birds during the trial and analysed for antibodies by virus neutralisation (VN) and plaque reduction neutralisation tests (PRNT). No clinical signs or gross pathological changes were seen in any of the inoculated chickens throughout the study. The nested PCR used in the study appeared to be significantly more sensitive at detecting the presence of the virus in both the tissues and the inoculated Vero cell cultures compared to the detection of gross cytopathic changes as observed in infected Vero cell culture. No cytopathic changes were seen in the inoculated avian cell cultures. Following primary inoculation of the chickens there was a weak antibody response 15 days post inoculation, however following reinoculation with inactivated WNV and adjuvant

there was a substantial increase in the neutralising antibody titres when tested two weeks later. The results obtained suggested that the PRNT was more sensitive than the conventional VN test.

Based on detection of virus and serology there was no evidence of viral transmission to the close contact controls. It can be concluded that the PCR used in this study was more sensitive than virus isolation for the detection of WNV whilst the PRNT also appeared more sensitive than the conventional VN test.

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## Introduction

West Nile virus (WNV) is an arthropod-borne flavivirus primarily maintained in natural transmission cycles between mosquito vectors and birds. The virus may also infect humans, horses and other animals causing a range of symptoms from fever to neurological signs including severe meningoencephalomyelitis and at least 150 bird species and 30 other vertebrates have been reported to be susceptible to infection with WNV (van der Meulen, *et al.*, 2005). In mammals, a transient viraemia of low virus titre precedes clinical signs and they are generally considered “dead end” hosts (Komar, 2000; Ostlund *et al.*, 2001). Sporadic outbreaks of disease in humans and horses have been regularly reported throughout the Middle East, Asia and eastern and southern Europe (Weinberger *et al.*, 2001; Murgue *et al.*, 2001; George *et al.*, 1984 ). In 1996 an epidemic in Romania involved hundreds of human cases with 17 fatalities and recent serological surveys have detected WNV antibodies in wild birds in Poland, the Czech Republic and the United Kingdom (Buckley *et al.*, 2003; Buckley *et al.*, 2006). The disease has reached epidemic proportions throughout North America following its introduction to New York in 1999 (Nash *et al.*, 2001) and although severe disease in humans is only seen in about 1% of infections, 564 deaths were attributed to WNV infection in the USA between 1999 - 2003 (Briese & Bernard, 2005). WNV infections in wild birds are useful risk indicators for human infection therefore diagnosis and avian disease surveillance is extremely important (Steele *et al.*, 2000). A range of tests have been developed for the laboratory detection of WNV including direct methods of virus isolation, reverse transcription PCR (RT PCR) and realtime RT PCR as well as indirect serological methods such as virus neutralisation

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(VN), plaque reduction neutralisation test (PRNT), IgM capture ELISA (MAC-ELISA), IgG ELISA and immunohistochemistry. The aim of this study was to

evaluate the efficiency of detection of virus in the tissues of SPF chickens infected with a lineage 1 WNV strain (Goose Israel 1998). This was carried out using virus isolation procedures in primary avian cell cultures and a mammalian cell line and by comparing the results obtained with molecular detection methods. The serological response of the infected chickens was also investigated using VN and PRNT.

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## Materials and Methods

**West Nile virus:** The reference strain of WNV used in this study was Goose Israel 1998 (tissue culture passage 5), a lineage 1 strain of the virus and phylogenetically very similar to the strain of virus which appeared in North America in 1999 and recent European strains. Virus propagation was undertaken in Vero C1008 (ATCC) cells which were cultivated at 37 °C in Eagles Minimal Essential medium (EMEM) containing 10% foetal bovine serum in a 5% CO<sub>2</sub> in air atmosphere according to ATCC recommendations.

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**Experimental design:** Fourteen three-week-old SPF white leghorn chickens were randomly assigned to two groups of five and one group of four birds. The birds were housed within a single isolator at ACDP Category 3 within a high biosecure facility for the period of the study, commercial feed and fresh water were provided *ad libitum*. The groups of chickens were inoculated with WNV as follows; one group of five birds (Group 1) received 10<sup>4.0</sup> median tissue culture infectious doses (TCID<sub>50</sub>) in

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0.2ml subcutaneously (s/c) in the base of the neck. One group of five birds (Group 2) received  $10^{4.0}$  TCID<sub>50</sub> WNV in 0.2ml intravenously (i/v) in the brachial vein and four birds (Group 3) remained uninoculated yet in direct contact with the inoculated birds.

The birds were monitored daily for clinical signs during the course of the study. Prior to inoculation and at three and 15 days post-infection (pi) 1ml of blood was taken

from the brachial vein of each bird, the blood sample was allowed to clot at room temperature and serum separated for serological tests. At three days p.i. one bird from

each group was ethanized and tissue samples including brain, blood, kidney, trachea, heart, liver, spleen, caecum, bursa, skin and lung were collected aseptically from each

bird using separate sets of instruments for each individual organ for virological investigations as follows: RT-PCR examination together with virus isolation in Vero

C1008 cells and chicken embryo liver and fibroblast cells. At 21 days pi the

remaining birds in groups 1 and 2 were inoculated intra-muscularly in the thigh

muscle with 0.5ml inactivated WNV in an oil emulsion in order to prepare

hyperimmune WNV antiserum. The oil emulsion vaccine was prepared using 20%

WNV in a mineral oil adjuvant, which prior to inactivation had a titre in Vero cell

cultures of  $10^{5.8}$  median tissue culture infective doses per ml.

At two and three weeks after re-inoculation blood samples were taken from

the brachial vein of each bird and at three weeks the birds were euthanized and bled

out. Serum was removed from the blood clot, centrifuged, heat inactivated at 56 °C for

30 minutes and stored at -70°C prior to testing. The sera, together with reference

WNV positive and negative control sera (USDA, PO Box 844, Ames, Iowa 50010,

USA), were individually tested by VN test, and pools of sera with similar neutralising

titres were subsequently tested by PRNT.

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**Deleted:** Two and three weeks after re-inoculation, 1ml of blood was taken from all remaining birds for serology and at three weeks all birds were exsanguinated

**Cell cultures:** Fresh confluent monolayers of Vero C1008 cell cultures were used for attempted virus isolation as well as chicken embryonic liver (CEL) and fibroblast (CEF) cultures from SPF chicken embryos prepared and inoculated as described (Gough *et al* 1988).

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**Virus Isolation on tissues.** Twenty per cent w/v suspensions of tissue homogenate were made in antibiotic PBS pH 7.2 and allowed to stand at room temperature for one hour. Following centrifugation to clarify the suspensions, supernatants were filtered through a disposable 0.45µm filter and inoculated onto fresh confluent Vero, CEL and CEF cell monolayers in 25ml flasks. The cultures were incubated for one hour at 37°C and then overlaid with 8 – 10ml of fresh maintenance medium warmed to 37°C. Cultures were incubated in a 5% CO<sub>2</sub> in air atmosphere at 37°C, examined daily for cytopathic effects (CPE) using an inverted microscope and frozen at -70°C after 7 days. Cultures in which a CPE was observed were frozen at -70°C and subsequently tested by RT-PCR for the presence of WNV. Cultures in which no CPE was detected were frozen and thawed, and clarified by centrifugation. The supernatants were passaged once more onto similar cell cultures by the same procedure and again monitored daily for CPE for a further 7 days. All the Vero cultures in which no CPE was observed were further tested by RT-PCR for the presence of WNV.

**RT-PCR.** The RT-PCR used in this study employed segment specific primers directed at the C-terminal of the C gene and the N-terminal of the prM gene (nt 233-640). Oligonucleotides included forward primer 5`- TTGTGTTGGCTCTCTTGGCGTTCTT-3` and reverse primer 5`- CAGCCGACAGCACTGGACATTCATA-3`. This first round one tube reaction was

followed by a second (nested) PCR using forward primer 5`- CAGTGCTGGATCGATGGAGAGG-3` and reverse primer 5`- CCGCCGATTGATAGCACTGGT-3` (nt 287-390) to produce a WNV specific 104 base pair (bp) product (Shi *et al.*, 2001).

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First round RT-PCR: The master mix was prepared on ice using the QIAGEN OneStep RT-PCR kit and 5µl of sample RNA was added to 20µl of master mix in 200µl reaction tubes. The following thermal cycling conditions were used: 50 °C for 30 min, 95 °C for 15 min., 35 cycles of: 94 °C for 45 secs., 56 °C for 45 secs., 72 °C for 1 min. followed by a single step of 72 °C for 10 min. Positive control reactions using RNA derived from Goose Israel 1998 WNV together with negative controls using Rnase-free water instead of RNA template were included in each run.

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Second round nested PCR: The master mix was prepared on ice using the QIAGEN Taq PCR Core kit and 0.2µl of first round product was added to 24.8µl of master mix in 200µl reaction tubes. The following thermal cycling conditions were used: 95 °C for 3mins, 25 cycles of: 94 °C for 45 secs., 58 °C for 45 secs., 72 °C for 1 min., followed by a single step of 72 °C for 10 mins. The PCR product was visualised over UV light following electrophoresis in 2% agarose gel incorporating ethidium bromide.

## Serology

**Virus neutralisation:** Sera were tested by the “diluted sera/constant virus” method in 96 well micro-neutralisation plates using two-fold dilutions of sera in MEM from 1/2 to 1/256 and an equal volume of MEM containing 100 median tissue culture infective doses of WNV. Following a virus-serum reaction time of one hour at 37 °C in a 5%

CO<sub>2</sub> in air atmosphere, 25µl volumes of the virus-serum mixtures were inoculated into wells containing fresh confluent monolayers of Vero C1008 cells. Following gentle rocking of the plates to spread the inoculum over the monolayer, the cultures were incubated at 37 °C for one hour in a 5% CO<sub>2</sub> in air atmosphere to allow adsorption to the monolayer of any virus that had not been neutralised by the WNV antibodies. After adsorption, the cultures were overlaid with 200µl of Vero maintenance medium, sealed and incubated at 37 °C in a 5% CO<sub>2</sub> in air atmosphere. The Vero cell monolayers were examined microscopically after 4 to 5 days for CPE and the virus neutralising titre of the serum calculated as the reciprocal of the highest serum dilution that completely neutralised the virus.

**Plaque reduction neutralisation test (PRNT):** Test serum was serially diluted in MEM using doubling dilutions from 1/10 – 1/2560 to produce 125µl volumes in sterile 96well microplates. WNV diluted in MEM to contain 60 -70 plaque forming units (pfu) / 125µl was added to the serial dilutions of test serum, the plates were sealed and incubated for 1hour at 37 °C in a 5% CO<sub>2</sub> in air atmosphere to allow for virus neutralisation. Fresh confluent Vero cell cultures in 12 well microdishes were washed once with MEM, the serum /virus mixture was then added to each of 10 wells of the plate and incubated for 1 hour at 37 °C in a 5% CO<sub>2</sub> in air atmosphere. A virus only control well containing 60/70 pfu of virus in 250µl of MEM and a negative control well containing 250µl of MEM only were also included on each plate. Following incubation, 1ml of 50% carboxymethyl cellulose/ 50% double strength MEM overlay was added to each well and the sealed plates incubated at 37 °C in a 5% CO<sub>2</sub> in air atmosphere. After 4 days incubation, 1ml of 10% neutral buffered formalin was added to each well and the plates fixed for at least 3 hours. Each well of the

plates was then washed in cold running tap water to remove the overlay and fixative, the plates were blotted dry and 250µl of 0.1% crystal violet in PBS pH 7.2 added to each well to stain the Vero monolayers and visualise the plaques formed within. The plates were then air dried and the plaques counted in each well. The PRNT titre was calculated as the reciprocal of the dilution of test serum that produced a 90% reduction in the number of plaques formed in the virus control well (PRNT<sup>90</sup>). If plaques were seen in the negative control well, the test was deemed invalid.

## Results

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**Clinical and Postmortem findings:** All the birds remained clinically normal throughout the period of the study and no gross pathological lesions were seen in the chickens from which samples were taken.

**Virus Isolation:** Four days after inoculation a widespread CPE was seen in the Vero cultures inoculated with samples of kidney and spleen from the s/c inoculated chicken and from the kidney of the i/v inoculated chicken. The CPE was characterised by the appearance of rounded, refractile cells which progressed to complete destruction of the cell monolayer. On passage no additional cultures produced a CPE following inoculation with the other tissues examined. The cultures inoculated with samples from the uninoculated contact bird remained normal through two passages. No CPE was detected in any of the CEL or CEF cultures following two passages.

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**RT-PCR on tissues and cell cultures:** PCR amplicons of the predicted size (104 bp) were detected in RT-PCR undertaken on RNA extracted from kidney, spleen, caecum,

heart, trachea and lung but not from brain, liver, skin, bursa and blood of the i/v infected chicken (table 1). Amplicons were also detected in RT-PCR undertaken on RNA extracted from skin, spleen, kidney, lung and heart from the s/c infected chicken but not from caecum, trachea, brain, liver, bursa and blood. Amplicons of strong intensity were detected in RNA samples derived from heart muscle of both infected birds. No product was amplified from RNA extracted from the tissues of the uninfected in-contact birds. PCR amplicons of the predicted size were also detected in RNA extracts from the infected Vero cultures in which a CPE was seen, (table1). The CEL and CEF cultures were not available for examination by PCR.

### **Serological results.**

**Virus neutralisation (VN) tests:** The results are presented in table 2. No neutralising antibody was detected in any of the serum samples three days after inoculation. By 15 days three of the four s/c inoculated birds had titres of 2 and one of <2, in the i/v inoculated group three birds had titres of 2 and one of 8. Two weeks after re-inoculation with inactivated virus (35 days) the birds showed an enhanced antibody response with antibody titres ranging from 16 to 64 in the s/c group and 8 to 128 in the i/v group. When sampled and tested a week later (42 days) the antibody titres in the birds showed a slight increase. No WNV antibody was detected in any of the serum samples from the in-contact control birds.

**Plaque reduction neutralisation (PRNT):** The results are presented in table 3. As can be seen no PRNT antibodies were detected in pool11 sera, which was from the



suggest that WNV was concentrated in spleen and kidney tissue in infected birds and are comparable with the findings of Steele *et al.* (2000) where kidney, spleen and heart samples from both North American and exotic birds, infected during the 1999 outbreak in New York, were consistently found to be positive by RT-PCR, virus isolation and immunohistochemistry respectively. Virus was neither isolated in cell culture nor detected by RT-PCR in any tissues of the uninfected control birds (Table 1). In the study by Senne *et al.* (2000), the isolation of WNV in Vero 76 cell cultures from the plasma of all infected birds was reported from 3 to 5 days pi. and from spleen, kidney and lung up to 10 days pi but not from brain or liver. In the study reported here no virus was detected by virus isolation or PCR in whole blood samples taken from the s/c and i/v birds 3 days after infection. This may have been due to the fact that only one bird from each group was sampled at this time or that the isolate of WNV used in this study was less invasive than the virus used by Senne *et al.* (2000). It is probable that the latter explanation is the more likely as the dose and s/c route of inoculation used in this study were similar to those reported by Senne *et al.* (2000). It is interesting to note that in the present study WNV specific RNA was detected both directly from skin tissue taken from the s/c infected bird and from Vero cell cultures in which skin tissue homogenate from the same bird had been passaged but not from skin of the i/v infected bird. The detection of WNV in skin tissue is perhaps not surprising and concentration of virus in peripheral tissue during the relatively short period which virus is present in birds may aid transmission by arthropod vectors. There was a good correlation between the PCR positive results obtained from the infected Vero cell cultures and the post-mortem tissues although CPE was only detected in the cultures inoculated with samples of kidney and spleen. This may have been due to the presence of higher concentrations of virus in these tissues as reported

in previous studies. The results of this study suggest that CEL and CEF cultures are not suitable for the detection of WNV as no CPE was detected following 2 blind passages. It is unfortunate that the inoculated cultures were not available for PCR analysis as replication of the virus may have occurred without detectable CPE, as occurred in the VERO cultures. However, in terms of comparing the sensitivity of the different assay systems there would be no practical advantage in routinely using primary avian cells for the detection of WNV if the inoculated cultures required further PCR analysis to confirm the presence of the virus.

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Virus neutralising antibodies were first detected in infected birds only at 15 days pi and following intra-muscular inoculation of inactivated virus in oil emulsion at 21 days pi, antibody titres were variable between individual birds. No virus neutralising antibody was detected in uninfected, in-contact control birds throughout the study (Table 2).

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Although the panel of positive and negative serum pools was small in number, comparison of virus neutralisation and PRNT<sup>90</sup> titres demonstrated that PRNT<sup>90</sup> was ten times more sensitive than the standard neutralisation test (Table 3). The evidence from this study suggests that the nested PCR is more sensitive than virus isolation in Vero cultures for the detection of WNV, although virus isolation may still be useful for the detection of other flaviviruses. Indeed, at the time of writing, the RT PCR described in this study has been used to test more than 2000 samples of kidney and brain tissue collected from dead wild birds in UK and to date no evidence of WNV has been recorded. The results also confirm that the strategic use of sentinel chickens would be a useful tool in monitoring the presence of WNV in this country.

## Acknowledgements

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**Table 1.** *Results of virus isolation in Vero cells, RT-PCR on RNA extracted from chicken tissues collected three days post inoculation with West Nile virus and from VI cell culture fluids.*

Tissue	Control birds			s/c infected birds			i/v infected birds		
	VI	RT-CR on VI	RT-PCR on tissue	VI	RT-PCR on VI	RT-PCR on tissue	VI	RT-PCR on VI	RT-PCR on tissue
<u>Skin</u>	=	=	=	=	±	±	=	=	=
<u>Bursa</u>	=	=	=	=	=	=	=	=	=
<u>Blood</u>	=	=	=	=	=	=	=	=	=
<u>Caecum</u>	=	=	=	=	=	=	=	±	±
<u>Trachea</u>	=	=	=	=	=	=	=	±	±
<u>Spleen</u>	=	=	=	=	+	+	CPE	+	+
<u>Kidney</u>	=	=	=	CPE	+	+	CPE	+	+
<u>Liver</u>	=	=	=	=	=	=	=	=	=
<u>Lung</u>	=	=	=	=	+	+	=	+	+
<u>Heart</u>	=	=	=	=	+	+	=	+	+
<u>Brain</u>	=	=	=	=	=	=	=	=	=

= = Negative

+ = Positive

CPE = Cytopathic effects seen in Vero C1008 cell monolayers

**Table 2. Virus neutralisation titres of WNV infected chickens and in contact controls**

*Titres expressed as the reciprocal of the highest serum dilution that completely neutralised the virus. A titre of <2 was considered negative.*

Group and ID No	3 days pi	15 days pi	35 days pi *	42 days pi
Control 434	<2	<2	<2	<2
435	<2	<2	<2	NT
436	<2**	=	=	=
437	<2	<2	<2	<2
S/C 1	<2	<2	32**	NT
2	<2	2	16	32
3	<2	2	16	32
4	<2	2	64	128
5	<2**	=	=	=
I/V 486	<2	8	128	128
487	<2	2	8	16
488	<2	2	8**	=
489	<2	2	16	16
490	<2**	=	=	=

\* Two weeks post revaccination

\*\* Sacrificed

NT = not tested

**Table 3. Virus neutralisation and PRNT<sup>90</sup> titres of serum pools 1, 2 and 3 together with USDA PRNT reference positive and negative sera**

*Virus neutralisation titres expressed as the reciprocal of the highest serum dilution that completely neutralised the virus. PRNT<sup>90</sup> calculated as the reciprocal of the dilution of test serum that produced a 90% reduction in the number of plaques formed in the virus control well. For both values, a titre of <2 was considered negative.*

<u>Serum</u>	<u>Virus Neutralisation Titre</u>	<u>PRNT<sup>90</sup> Titre</u>
<u>POOL 1</u>	<u>&lt;2 (Negative)</u>	<u>&lt;10 (Negative)</u>
<u>POOL 2</u>	<u>64</u>	<u>640</u>
<u>POOL 3</u>	<u>128</u>	<u>1280</u>
<u>USDA Positive</u>	<u>64</u>	<u>640</u>
<u>USDA Negative</u>	<u>&lt;2</u>	<u>&lt;10</u>

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Table 1. Results of virus isolation in Vero cells, RT-PCR on RNA extracted from chicken tissues collected three days post inoculation with West Nile virus and from VI cell culture fluids.

Tissue	Control birds			s/c infected birds			i/v infected birds		
	VI	RT-CR on VI	RT-PCR on tissue	VI	RT-PCR on VI	RT-PCR on tissue	VI	RT-PCR on VI	RT-PCR on tissue
Skin	-	-	-	-	+	+	-	-	-
Bursa	-	-	-	-	-	-	-	-	-
Blood	-	-	-	-	-	-	-	-	-
Caecum	-	-	-	-	-	-	-	+	+
Trachea	-	-	-	-	-	-	-	+	+
Spleen	-	-	-	-	+	+	CPE	+	+
Kidney	-	-	-	CPE	+	+	CPE	+	+
Liver	-	-	-	-	-	-	-	-	-
Lung	-	-	-	-	+	+	-	+	+
Heart	-	-	-	-	+	+	-	+	+
Brain	-	-	-	-	-	-	-	-	-

- = Negative

+ = Positive

CPE = Cytopathic effects seen in Vero C1008 cell monolayers

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**Table 2. Virus neutralisation titres of WNV infected chickens and in contact controls**

*Titres expressed as the reciprocal of the highest serum dilution that completely*

Group and ID	3 days pi	15 days pi	35 days pi *	42 days pi
<b>No</b>				
<b>Control</b>				
434	<2	<2	<2	<2
435	<2	<2	<2	NT
436	<2**	–	–	–
437	<2	<2	<2	<2
<b>S/C</b>				
1	<2	<2	32**	NT
2	<2	2	16	32
3	<2	2	16	32
4	<2	2	64	128
5	<2**	–	–	–
<b>I/V</b>				
486	<2	8	128	128
487	<2	2	8	16
488	<2	2	8**	–
489	<2	2	16	16
490	<2**	–	–	–

*neutralised the virus. A titre of <2 was considered negative.*

\* **Two weeks post revaccination**

\*\* Sacrificed

NT = not tested

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Table 3. Virus neutralisation and PRNT<sup>90</sup> titres of serum pools 1, 2 and 3 together with USDA PRNT reference positive and negative sera

*Virus neutralisation titres expressed as the reciprocal of the highest serum dilution that completely neutralised the virus. PRNT<sup>90</sup> calculated as the reciprocal of the dilution of test serum that produced a 90% reduction in the number of plaques formed in the virus control well. For both values, a titre of <2 was considered negative.*

Serum	Virus Neutralisation Titre	PRNT <sup>90</sup> Titre
POOL 1	<2 (Negative)	<10 (Negative)
POOL 2	64	640
POOL 3	128	1280
USDA Positive	64	640
USDA Negative	<2	<10

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