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Review article

West Nile virus infection of horses

Javier CASTILLO-OLIVARES*, James WOOD

Centre for Preventive Medicine, Animal Health Trust, Newmarket, Suffolk CB8 7UU, United Kingdom

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Abstract – West Nile virus (WNV) is a flavivirus closely related to Japanese encephalitis and St. Louis encephalitis viruses that is primarily maintained in nature by transmission cycles between mosquitoes and birds. Occasionally, WNV infects and causes disease in other vertebrates, including humans and horses. West Nile virus has re-emerged as an important pathogen as several recent outbreaks of encephalomyelitis have been reported from different parts of Europe in addition to the large epidemic that has swept across North America. This review summarises the main features of WNV infection in the horse, with reference to complementary information from other species, highlighting the most recent scientific findings and identifying areas that require further research.

West Nile virus / flavivirus / horses / encephalitis

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* Corresponding author: javier.castillo-olivares@aht.org.uk

1. INTRODUCTION

West Nile virus (WNV) was first isolated in the West Nile district of Uganda in 1937 from the blood of a woman suffering from a mild febrile illness [83]. Since then, sporadic and major outbreaks, mainly in humans, but also in horses, have been reported during the 1960's in Africa, the middle East and Europe (reviewed in [67]). In the last decade, WNV has re-emerged as an important pathogen for humans and horses, as frequent outbreaks with increased proportion of neurological disease cases have been reported [23, 27, 39, 66, 98, 99]. Indeed, outbreaks in Romania and Morocco in 1996, Tunisia in 1997, Italy in 1998, Russia, United States and Israel in 1999, and France, United States and Israel in 2000 presented either an increase in the number of severe human cases, an increase in the severity of neurological disease in horses or high bird mortality [14, 32]. In some instances these three features were present, as was the case in the USA outbreaks. West Nile virus-related disease in humans and horses is still being reported from the USA and lately confirmed human cases have been reported from France [56]. It has also been introduced during 2003 in Canada [97], Mexico and the Caribbean region [14, 32]. Furthermore, recent serological investigations in birds in Britain suggest that a WN-like virus has been circulating amongst resident bird populations [21].

2. AETIOLOGY

West Nile virus is a positive sense single-stranded RNA enveloped virus of the genus *Flavivirus*, family Flaviviridae [64]. Amongst the 12 *Flavivirus* sero-groups, classified according to cross-reactivity in virus neutralisation assays, WNV belongs to the Japanese encephalitis sero-complex group together with Japanese encephalitis (JE), Murray Valley encephalitis (MVE), St Louis encephalitis (SLE), Kunjin (KUN), Usutu (USU), Koutango (KOU), Cacipacore (CPC), Alfuy (ALF) and Yaounde (YAO) viruses [38].

2.1. Virion structure

Consistent with the typical virion architecture of flaviviruses, the WNV virion comprises an icosahedral core composed of multiple copies of a highly basic capsid protein (C) of 12 kDa. This is surrounded by a host-cell envelope modified by the insertion of two virus encoded proteins, the major envelope protein E (53 kDa) and the membrane protein M (8 kDa). The latter derives from a precursor protein, prM (18–20 kDa), that is cleaved before virion release from the infected cell [18, 31].

The capsid encloses a single-stranded RNA molecule with positive polarity which lacks a polyadenylate tract at the 3'end. The genome contains a 5' and a 3' noncoding region of 96 and 631 nucleotides respectively, flanking one single open reading frame of 10 302 nucleotides encoding a polyprotein. This is processed by viral and cellular proteases into three structural proteins (C, E and M or pr-M) and 5 non-structural proteins (NS1, NS2a / NS2b, NS3, NS4a / NS4b and NS5). The ends of the genome contain tertiary structures that play important regulatory functions in replication and assembly.

The non-structural proteins of flaviviruses have virus replication and assembly functions. Thus, NS1 and NS4A participate in virus replication, NS2A is involved in assembly and virion release, NS3 and NS2B have proteolytic activities and NS5 acts as an RNA-dependent RNA polymerase and methyltransferase participating in the methylation of the 5'-cap structure [18].

2.2. The envelope protein E

Many biological properties of flaviviruses, such as tropism, cell binding, virulence, haemagglutination and antigenicity, are associated with the envelope protein E. This protein contains 12 conserved cysteine

residues involved in the formation of intramolecular disulphide bridges and forms head-to-tail rod-shaped curved homodimers which do not protrude from the surface of the virion [18]. The protein E of the flavivirus of tick-borne-encephalitis contains three antigenic domains. A central domain I (previously referred to as domain C) is formed by the 50 N-terminal amino acids folded as an eight-stranded β-barrel. This is flanked by the antigenic domain II (previously domain A), structured in two loops and containing a highly conserved sequence among all flaviviruses and possibly a fusion sequence, and a domain III (previously domain B) composed of seven antiparallel β-strands resembling the constant region of immunoglobulins and which contains important neutralising epitopes and cell binding receptors [64]. Recently, neutralising epitopes of WNV have been mapped to residues 307, 330 and 332 of protein E which correspond to domain III [8].

Antigenic relationships between flaviviruses have been studied using immune polyclonal antisera in virus neutralisation tests. Monoclonal antibody studies have revealed the complexity of these relationships, showing the existence of flavivirus-group, subgroup, sero-complex, type-specific and strain-specific antigenic determinants [38]. The identification and classification of WNV isolates has been made using these reagents in virus neutralisation and indirect immunofluorescence tests, but the analysis of nucleotide sequences encoding the protein E is perhaps what has revealed more clearly the relationship between various WNV isolates. Thus, it has been found that WNV viruses fall into two distinct lineages [11, 78, 80]. Lineage 1 includes viruses isolated outside and inside the African continent which have been associated with recent epidemics of increased severity in humans and horses, whereas lineage 2 comprises viruses that have only been found to circulate in enzootic cycles in birds in Africa.

Apart from protein E, flavivirus proteins prM, NS1, NS3 and NS5 have also been identified as antigens. For example, prM specific monoclonal antibodies of dengue 3 and dengue 4 virus with neutralising activity have been obtained [46], and NS3 specific monoclonal antibodies of dengue 1 virus have shown some protective effect in passive immunisation experiments in mice [86]. Likewise, the prM and NS3 proteins of WNV can be recognised by WNV-specific mouse and horse antisera [29] and the antigenic nature of WNV NS5 was demonstrated in a recent study [101] that shows that WNV human patient sera recognise this antigen consistently.

The NS1 protein is a glycosylated protein that is expressed on the surface of infected cells and can also be secreted. Polyclonal and monoclonal antibodies to the NS1 protein have identified type-, complex-, and flavivirus-specific epitopes [34], which are mainly conformation dependant but are not virus neutralising. The NS1 protein of WNV has also been demonstrated to be antigenic and has been used as a diagnostic reagent in antibody capture ELISA procedures [44].

2.3. Virus replication in the cell

West Nile virus, like many other flaviviruses, grows in a wide variety of primary cells and continuous cell lines from mammalian (Vero cells, BHK-21, RK-13, SW-13) and mosquito species (C6/36, *Aedes albopictus*, *Aedes Aegypti cells*) [64]. In vivo it can grow in a variety of cells from different tissues depending on the host species. These tissues include neurons, glial cells, and cells from spleen, liver, heart, lymph nodes and lung [24]. Virus replication takes place in the perinuclear region of the rough endoplasmic reticulum (ER) where the newly synthesised E, NS1 and prM are translocated to the ER lumen where prM and E heterodimerise [30, 31, 100]. The immature virions are transported through the secretory pathway to the cell membrane where the final cleavage of the prM protein takes

place by the action of furin. The virions are finally released by exocytosis or by budding.

Cell infection with WNV can result in cell lysis, syncytia formation or may result in virus persistence. This phenomenon has been observed in vitro in neuroblastoma cell lines infected with JE and in vivo in many flavivirus related diseases [64] including WNV infections in monkeys where virus was recovered up to five months post-infection [77], and in hamsters with viruses being isolated up to day 53 post-challenge [103].

3. ECOLOGY

The natural cycle of all members of the JE antigenic complex of flaviviruses involves birds as the main amplifying host and several species of mosquitoes as vectors. The natural cycle of WNV typically involves ornithophilic mosquitoes, particularly, but not exclusively, *Culex* species. The identity of the primary vectors and vertebrate host species is dependent on the geographic area and the levels of virus that are circulating [47]. However, there is a vast range of both avian and mosquito species that can be infected by WNV [9, 12].

During periods of adult mosquito blood feeding, WNV can be transmitted continuously between mosquito vectors and avian reservoir hosts. Infectious mosquitoes carry WNV in salivary glands and infect susceptible vertebrate hosts at feeding. Competent vertebrate hosts will sustain a viraemia, typically for up to five days and, if the virus is to be transmitted on, other insect vectors must feed on these viraemic hosts during this period to become infected. In common with many other arboviruses, a temperature dependent "extrinsic period" then ensues, during which virus must replicate and enter the salivary glands of the mosquitoes. Typically, this period is around two weeks during warm periods, but is sensitive to both temperature and humidity [9, 28]. After this period, if the cycle is to be maintained, sufficient numbers of infected mosquitoes must then feed again on susceptible hosts. The temperature dependence of both mosquito reproduction and viral replication in insect vectors results in highly seasonal variation in WNV transmission and disease outbreaks. In temperate regions such as Europe, Canada and the northern states of USA, most encephalitis cases are seen in late summer or autumn when insect numbers and temperatures are high [39].

While experimental studies suggest that, as for many other arboviruses, transmission from infected mosquito to susceptible hosts is very efficient if feeding occurs [22, 69], transmission from vertebrate host to mosquito is very dependent on the level of viraemia in the vertebrate. Duration and titre of viraemia are typically both much greater in birds than in mammals, but both vary hugely between avian species. Mammalian species, including both horse and man, are thought rarely to develop titres sufficient to infect mosquito species and so are unlikely to be able to sustain infectivity cycles. Although they are typically referred to as "dead-end" hosts [47], occasional individuals, given sufficient numbers, may in fact be able to infect mosquitoes. Modes of transmission other than via insect vectors may occur and direct bird to bird spread may be possible under some circumstances [9], although again it is perhaps unlikely that such a mechanism is important in the ecology of the disease.

Identifying the avian hosts principally responsible for amplifying WNV during periods of both epidemic and endemic viral activity is not straightforward. Serological studies in themselves only serve to identify which species are becoming infected and cannot be used to determine major amplifying hosts. Not only must these hosts be capable of sustaining high-level viraemia for sufficient periods of time, they must also be fed on by sufficient numbers of competent insect vectors during this period. The relative importance of different mosquito species in the transmission cycle, whilst affected by the competence of each species

to become infected with and transmit virus, is also determined by host feeding preferences, longevity and contact rates between vector and competent host [9]. It is likely that of the (at least) 16 species of mosquito reported to be virologically competent vectors of WNV [47], far fewer are likely to be important.

Detailed studies of WNV have now suggested that transmission cycles of WNV in both Europe and North America are typically maintained in passerine birds, particularly the house sparrow (*Passer domesticus*) [47, 79], which is the only New World avian host in which a prolonged (5-6 day) and high titre viraemia has been reported [48, 79]. However, care needs to be taken when interpreting and extrapolating these results, as viraemia may be shorter and of a lower titre when other strains of WNV are considered [59]. *Cx. pipiens*, thought to be the major insect vector of WNV in both North America and Europe [9, 47], feed almost exclusively on passerine and columbiform birds [9]. Other *Culex* species, including *Cx. nigripalpus* and *Cx. tarsalis* in North America feed predominantly on birds in the early part of the transmission season, then increasingly switch to mammalian hosts during the summer months; other *Culex* species feed indiscriminately on both avian and mammalian hosts, some preferring multiple hosts [9]. These less discriminant species may function as important bridge vectors that spread infection to horses and man from birds. Understanding of host feeding preferences of species involved in transmission of WNV is critical to the understanding of WNV ecology and has been little researched, particularly in Europe; knowledge of preferences in species involved in transmission amongst birds as well as to man and horse is vital to inform control strategies.

While WNV transmission may be maintained by continuous circulation between avian and mosquito species in tropical or sub-tropical areas, different mechanisms may be important in more temperate regions between periods of continuous transmission. Presence of virus in hibernating [102] or overwintering mosquitoes [68], or continuous, but low level, transmission in vertebrate hosts have been proposed as possible means of persistence, but the mechanism(s) currently remain unknown [47]. Many authors have speculated on the role of migratory birds in repeated re-introduction of WNV to temperate areas where transmission occurs sporadically, such as the Camargue in southern France, where irregular epidemics in horses have been reported and where there are large populations of migratory birds from areas of endemic activity in Africa, [66, 67]. Migratory storks and other species may also be important in introducing the infection to the Middle East [57] and seropositive birds have also recently been reported in the United Kingdom [21]. The speed and pattern of spread across North America makes the role of migratory birds less likely than that from dispersal movements from non-migratory birds such as the house sparrow [79].

Transovarial transmission of WNV has been identified in Kenya in one species of mosquito [62] and has been demonstrated experimentally in a range of *Culex* and *Aedes* species mosquitoes [7], as well as in north American *Cx. pipiens* [94]. It has been suggested that this mode of transmission of WNV is probably unimportant as it is usually inefficient in flaviviruses [9], but it does provide a source of WNV persistence. The development of quantitative, biologically parameterised mathematical models describing transmission of WNV would hugely inform evaluation of the significance of such mechanisms and rates of transmission.

Following the introduction of WNV to North America in 1999, avian mortality has been extensive [10] and crow deaths in the USA have been one of the most sensitive sentinel systems for appearance and spread of West Nile virus, as well as for both equine and human cases of disease [33, 49]. Other than outbreaks in Israel in 1998 and

1999 involving mortality in geese [71], reports of extensive avian mortality are more or less unique to the Western hemisphere; the similarity of the viruses involved in the North American and Israeli outbreaks [51] suggests strongly that this is likely to be a feature of the virus strain involved. Current evidence suggests that monitoring avian mortality in areas where other strains of virus are involved may not detect virus transmission.

4. CLINICAL SIGNS IN HORSES

WNV infection in horses is usually not accompanied by presentation of clinical illness. However, the latest outbreaks of WNV saw an increased proportion of neurological disease in both humans and horses [75]. Approximately 10% of horses and around 1% of humans infected with WNV presented neurological disorders. These epidemiological observations have been corroborated by experimental infections of equines [22] where only one animal out of 12 displayed clear neurological symptoms. Except for fever, clinical signs of WNV in horses are almost exclusively of neurological nature and reflect the pathology in the central nervous system (CNS). These occur predominantly in the spinal cord, rhombencephalon and mesencephalon being the cerebral cortex less often affected [24]. A transitory febrile period might occur after infection although this is not always observed in some epidemics, e.g. outbreak in Italy 1998 [23]. The most common symptoms are associated with spinal cord injury like ataxia, paresis or paralysis of the limbs, which can affect one, two (usually the hindlimbs) or all four limbs, the latter usually progress to recumbency. Often these signs are accompanied by skin fasciculations, muscle tremors and muscle rigidity. In addition to the above, the USA outbreaks saw a proportion of horses displaying symptoms derived from damage of the medulla oblongata, pons, thalamus, the reticular formation, cerebellum and brain cortex [73, 74]. Thus, horses affected during these outbreaks presented with ataxia, dysmetria, abnormal mentation ranging from somnolence to hyperexcitability or even aggression, and hyperaesthesia. Some animals presented with facial nerve paralysis, paresis of the tongue and dysphagia resulting from deficits in cranial nerves VII, XII and IX.

A proportion of horses suffering from WNV infection do not recover and die spontaneously or, more often, are euthanased on humane grounds. Mortality rates among clinically affected horses have been estimated around 38%, 57.1% and 42% during outbreaks in the USA in 2000, France in 2000 and Italy in 1998 respectively [24, 66, 74]. In contrast to the human disease, severe neurological disease in horses does not appear to occur preferentially in old individuals.

Treatment guidelines for horses with WNV encephalomyelitis can be found in recent publications [54, 78]. Treatment is aimed at reducing CNS inflammation, preventing self-inflicted injuries and providing fluid and nutritional care.

5. EPIDEMIOLOGY

Before 1994, WNV was not perceived as a serious public health problem. Rather, it was regarded as a mosquito-borne infection of birds, which occasionally infected humans and equines causing illness on rare occasions. Indeed, the first epidemiological studies, performed in the Nile delta in Egypt [87], demonstrated viral activity in birds without any epidemics in horses. A study in the same area [81] recorded an equine seropositivity rate of 54% with only one fatal case. Between 1950 and 1994, WNV was isolated in different parts of Europe (Spain, Portugal, Russia, the former Czechoslovakia, Romania, France), Africa (South Africa, Senegal, Kenya) and Asia (Israel, Iran, India) but was rarely associated with clinical disease in either horse or man. The principle exception to this was the epidemic in the

Camargue in the south east of France in 1962 and 1963 [43]. During this epidemic, at least 80 horses were affected with ataxia and weakness and 25–30% died. Other wild Camargue horses were apparently also affected but the number affected was never reported. There was another important outbreak in South Africa in 1974, in which 18 000 people were affected, although no deaths were recorded; no information about equine disease was reported [45, 59].

Since the mid 1990's, the number of outbreaks and their severity has increased. Human cases with deaths were reported from Algeria in 1994 [52], Romania in 1996 [93], Morocco in 1996 [88], Tunisia in 1997 [91], the Democratic Republic of Congo in 1998 [70], Russia in 1999 [76] and in Israel from 1998 to 2000 [57]. In the 1996 Morocco outbreak, WNV fever was also described in 94 horses of which 42 died. In the Romanian and Russian epidemics a relatively high number of people were affected clinically but there is little or no published information about horse disease. In the outbreaks in Israel, WNV infections were reported in over 400 people, 325 of whom were hospitalised and 33 died. At least 75 horses were affected with encephalitis and 15 died, but, very unusually, there was also extensive mortality in birds, particularly geese [6, 85].

In addition to these outbreaks, there were limited outbreaks restricted to horses in northern Italy in 1998 [23] and in France in 2000 [66]. The activity in Italy during 1998 resulted in 14 horses displaying neurological signs. Follow up serological studies [5] revealed a 58% equine seroprevalence in the area of the outbreak, a far higher frequency than that for those with clinical signs. The clinical disease in horses described during the French outbreak in 2000 was in the Hérault and Gard department, close to where the outbreak in 1962–1963 occurred. Approximately 131 equines developed neurological disease, in 76 of which WNV infection was confirmed. WNV human cases were not reported from the WNV outbreaks of Italy and France, despite the equine cases.

In the late summer of 1999, WNV infections were recorded for the first time in the western hemisphere when cases in people and horses occurred in New York. Over a period of eight weeks 59 people had to be hospitalised with severe neurological illness, including seven deaths, in the New York City metropolitan area [2, 4]. Simultaneously, approximately 20 horses from Long Island were confirmed as WNV encephalitis cases [92]. Later investigations indicated that in contact animals were also seropositive to WNV (approximately 20%). As in Israel, there was substantial avian mortality, in particular in crows, which in this case preceded the human and horse disease reports.

Despite the cold New York winter, the infection did not disappear and virus activity was first detected in 2000 when WNV was isolated from a Red-tailed hawk in New York in February 2000, as well as from adult *Culex* mosquitoes overwintering in protected areas in January and February 2000 [1]. By the end of the year, the Centers for Disease Control and Prevention had received reports of 21 human cases, 63 equine cases, 4 304 infected dead birds and 6 infected other mammals [75] in a total of 7 other northeastern states.

WNV spread further to all the USA, Mexico, Canada and the Caribbean during the next three years. Indeed, in 2001 WNV was reported from 20 states. During this time, 738 horses were confirmed with WNV by the laboratory, most of which (550) came from the state of Florida.

The outbreak exploded in 2002 across the USA and into Canada and by the end of the year, WNV had been identified in 44 states, being associated with over 4 000 confirmed encephalitis cases in people, including 284 deaths, nearly 15 000 laboratory confirmed equine encephalitis cases and 16 500 dead birds [3]. Many equine cases were also diagnosed in five Canadian provinces [97].

In 2003, WNV encephalitis was diagnosed by USDA in more than 4 000 horses in 41 states, with a preponderance of cases in Colorado, Montana, New Mexico and Wyoming. In contrast to the equine situation, there were more human cases in 2003, with nearly 9 000 being diagnosed. One reason for the relative reduction in equine cases compared to human cases could have been that widespread vaccination of horses was practiced. In addition to the continued Western hemisphere outbreak in 2003, further cases were diagnosed in France (Var department), where confirmed cases in three horses and six people and probable cases in one horse and one person were reported [56] (S. Zientara personal communication), although detailed investigations do not currently appear available.

6. PATHOLOGY IN HORSES

The pathology of WNV in horses has been studied in natural cases during the epidemics of Italy in 1998 and the northeastern states of the USA in 1999 [23, 24]. As briefly indicated earlier in the text, lesions of WNV infection in the horse are predominantly, if not exclusively, limited to the CNS, and lesions affecting extraneural tissues are rarely described, which contrasts with widespread lesions observed in many internal organs of WNV infected birds [84]. WNV causes polioencephalomyelitis in horses, particularly evident in the lower brain stem and ventral horns of the spinal cord. The lesions are characterised by inflammatory changes accompanied by scant viral antigen staining. Lesions include perivascular cuffs of lymphocytes and macrophages with frequent haemorrhages, but generally in the absence of viral infection of vessel walls; scattered foci of microgliosis; and, in the most severe cases, neuronal degeneration with cytoplasmic swelling and chromatolysis. These lesions are less commonly observed in the cortex of cerebellum and cerebrum. These observations contrast with the findings observed in hamsters following experimental intraperitoneal infection [103]. These animals often showed, in addition to the lesions of the lower brain stem and spinal cord, inflammatory changes as well as antigen staining in regions of the cerebral cortex and cerebellum. However, the extent of the inflammatory changes was not prominent despite the frequent antigen staining of neurons. Abnormalities in extraneural tissues were not significant.

7. PATHOGENESIS

Flavivirus infections are initiated after virus inoculation into the skin by an infected arthropod (mosquito or tick). The virus then replicates in local tissues and in regional lymph nodes and is transported via lymphatic vessels to the blood stream. Langerhans cells of the skin have been associated with this virus transport to the lymph nodes in WNV infections in mice [42]. This virus replication and viraemia may seed infection in extraneural tissues, increasing the virus titres in blood and perhaps preceding the invasion of the CNS. Neuroinvasion pathways for flaviviruses are not well defined, but may involve passive diffusion across the capillary endothelium, virus replication in endothelial cells and budding of virus into the CNS parenchyma, or retroaxonal virus transport of infected neurons of the olfactory epithelium. The low titre and short duration of viraemia in horses [22], as opposed to birds, and failure to detect WNV antigen in vascular endothelial cells [24] make the first two neuroinvasion pathways unlikely. However, in the hamster model of WNV infection [103], West Nile antigen was not detected in the olfactory bulb of any of the infected hamsters.

The mechanism of neurological damage is uncertain. In the WNV infection model in hamsters it was observed that many degenerating neurons underwent apoptosis but this was not associated with inflammatory cells and the authors suggested cellular damage was caused by the WNV infection. In contrast, Cantile et al. [24] suggested that

neurological damage in natural WNV infection of horses has an immunopathological component since inflammatory changes were present in the absence of abundant detectable WNV antigen.

The outcome of WNV infection probably depends on host factors and virus strain. It has been demonstrated in experimental mouse models of Murray Valley encephalitis (MVE) that pathogenicity is dependent on innate immune responses, especially type I interferon responses (IFN-α/β) [53]. A deficit in these responses during the acute phase of the infection results in increased virus replication in extraneural tissues with the subsequent rapid entry of virus into the brain. Further work would be required to elucidate whether a deficient innate immune response may result in the development of equine neurological disease in WNV infection.

Neurovirulence and neuroinvasiveness of flaviviruses are typically associated with sequence variation of the protein E although other viral proteins such as NS1 and NS2b might also play a role. McMinn [60] reviewed comprehensively the molecular basis of flaviviruses pathogenicity. Accordingly, it has been shown that recent WNV outbreaks of increased virulence are caused by strains of lineage 1 rather than lineage 2, this classification being based on nucleotide sequences of the genome region encoding the E protein [51].

Persistent infections of flaviviruses, including WNV, have been documented in vitro and in vivo [26, 50, 77, 95, 103]. Persistent WNV infections have been associated with the generation of defective interfering particles, temperature sensitive mutants and non-plaquing mutants [15–17, 19]. Whether persistent infections occur in vivo during WNV infection of horses and these genetic variations represent immune evasion strategies employed by the virus is not known. Recurrent neurological disease in children previously exposed to JE and chronic progressive encephalitis after tick-borne encephalitis in humans may be due to persistent infections of the CNS. It is not known whether this occurs in the horse.

8. DIAGNOSIS

Laboratory procedures are necessary to confirm the presence of WNV due to the subclinical nature of many WNV infections and the similar symptomatology of WNV encephalomyelitis to other equine neurological syndromes. These include JE, alphavirus encephalitides (VEE, WEE, EEE), protozoal meningoencephalitis, EHV-1 myelitis, rabies and Borna Disease. Other less likely causes of disease that may be considered in some cases include botulism, hypocalcaemia, leukoencephalomalacia and hepatoencephalopathy.

The confirmation of WNV infection can be made directly by identification of the virus or indirectly by testing for antibodies in clinical specimens which include, post-mortem tissues, cerebrospinal fluid, whole blood or serum.

8.1. WNV detection

Detection of WNV in field cases in horses is hampered by the typically short duration and low level of the viraemia in horses. Negative virus detection test results should thus never be regarded as evidence of absence of WNV.

Virus isolation can be attempted from cerebrospinal fluid (CSF), blood or tissues in Vero, RK-13 cells [74] or mosquito cell lines. However, cytopathic effect is not always evident, especially in mosquito cells, and indirect immunofluorescence using a monoclonal antibody (MAb) of well defined specificity is necessary to confirm the presence of and/or to identify virus isolates.

Alternatively, the presence of the virus can be confirmed by nucleic acid detection. A sensitive and WNV-specific reverse transcription and nested polymerase chain reaction method has been used successfully as a diagnostic tool in clinical samples from

suspected cases of WNV encephalitis [41, 74]. This method detected WNV nucleic acid in all brain tissues of serologically confirmed cases of WNV encephalitis. Results from plasma samples from some confirmed WNV cases were negative in contrast to those collected from experimentally infected horses during the first six days post-inoculation. It is important to note that samples from natural clinical cases were collected after the onset of neurological signs, when virus is unlikely to be present in blood. The presentation of clinical signs in infected hamsters also coincides with the end of the viraemic phase [103]. Non-nested PCR techniques, such as those in routine use for diagnosis of infection in man and birds, are unsuitable for use in horses due to their relative insensitivity at detecting low titres of virus.

Immunocytochemistry on CNS tissues (including cortex, cerebellum, brain stem and spinal cord) using WN-specific MAb, can also be used [24].

Antigen capture ELISA tests have been used to confirm the presence of WNV in avian tissues and mosquito pools [40] but are unsuitable for use in horses due to the low level of viraemia.

8.2. Detection of antibody

Although haemagglutination inhibition and complement fixation tests have been widely used in the past for the diagnosis of flavivirus diseases [64], including Japanese encephalitis in horses (Manual of Standards for Diagnostic Tests and Vaccines of the Office International des Épizooties, 2000), and these tests are available for WNV [5, 72, 73], they are not widely used for laboratory diagnosis of WNV infection in the horse as they are laborious, time-consuming, slow and cross-reactive with other flaviviruses. However, they can be valuable in areas free of circulating WNV-related flaviviruses and also have the advantage of being species-independent. Instead, WNV-specific IgM and IgG capture ELISA tests and the plaque reduction neutralisation test (PRNT) have been preferred for serological diagnosis and surveillance during recent outbreaks of WNV fever.

The IgM antibody capture ELISA (MAC-ELISA) test is based on the capture of IgM in horse serum or CSF by an anti-IgM antibody bound to a microtitre plate, followed by demonstration of specific anti-WNV antibodies. A positive result by the MAC-ELISA test in a single sample has relevant diagnostic value since WNV-specific IgM appear in the circulation by day 6–7 post-infection [22] and are believed to last less than three months [73]. However, these antibodies have been detected for longer periods in human WNV infection and more studies are necessary to understand better the kinetics of IgM responses in the horse. Likewise, the presence in CSF of IgM is highly indicative of a recent WNV infection of the CNS since IgM cannot cross the haematoencephalic barrier.

Detection of IgG by ELISA has been performed in serosurveillance studies in Italy [5] and France [66]. ELISA plates are coated with viral antigen and the presence of serum antibodies are revealed by a peroxidase labelled anti-horse IgG. This ELISA test is particularly valuable for serosurveillance as it is very sensitive and WNV IgG lasts for at least 15 months after infection [74]. However, a positive result on a single sample has limited clinical diagnostic value and it is necessary to demonstrate a recent infection by a four-fold increase in antibody titre on paired serum samples collected 14 days apart.

Both the IgM and IgG ELISA tests appear sensitive but they cross react with antibodies against other flaviviruses. The more specific PRNT has been used successfully as a diagnostic tool for WNV infection in horses [22, 74].

More recently, an epitope blocking ELISA test has been developed which can be used to detect WNV specific antibodies in serum samples from birds and various mammalian species, including horses [13, 14].

A correct diagnosis depends, as always, on careful interpretation of combined history, clinical and laboratory data.

9. IMMUNE RESPONSES AND VACCINES

As mentioned earlier, prM, E, NS1, NS3 and NS5 are important antigens of flaviviruses, including WNV. Both humoral and cellular immune responses have been associated with protective immunity against various flavivirus infections. Effector mechanisms of immunity against flaviviruses comprise cytotoxic T lymphocytes, virus neutralising antibody and possibly non-neutralising antibody responses and antibody-dependent cell mediated cytotoxicity (ADCC) (anti-NS1 antibodies have protective efficacy) [64].

Various vaccination strategies have been devised to protect horses against WNV infection [63]. These include inactivated vaccines, DNA vaccines, live attenuated vaccines and genetically modified live vaccines.

Traditionally, inactivated vaccines against flaviviruses have been prepared in mouse brain or tissue culture followed by inactivation with formalin or betapropiolactone. A number of these vaccines have been used against other flavivirus diseases, including louping ill, JE, MV fever and tick-borne encephalitis. An inactivated vaccine against WNV in horses is available in North America and its experimental efficacy has been reported [69]. In this study, the vaccine stimulated a virus neutralising antibody response that persisted for 12 months after administration of a primary 2 doses course. Protective efficacy of the vaccine was assessed by comparing the viraemia frequencies in vaccinated and control seronegative horses after a challenge at 12 months post-vaccination. Nine of 11 control animals developed viraemia, whereas this was only detected in 1 of the 19 vaccinates.

It is not known whether previous exposure to other flaviviruses and hence heterotypic antibodies would interfere with the development of immunity. It is important to study protection against different WNV strains or even against other flaviviruses. There are reports describing cross-protective efficacy of JE vaccine against WNV infection in macaques [37] and that immunisation of mice with SL or JE viruses provided protection against WNV encephalitis [90]. However, antibody-enhancement of disease is a phenomenon observed in some flavivirus infection models. Thus, vaccination against JE did not protect against but enhanced MV encephalitis [20]. This phenomenon is due to the presence in serum of sub-neutralising levels of heterologous antibodies and has been described in vitro [36] and recently in vivo [96]. Elucidating whether this might occur in the context of WNV infections in the horse is of importance for the safety of vaccination. In particular, it would be important to know the levels of protection that inactivated vaccines prepared with North American strains provide against challenge with European viruses.

Most inactivated vaccines have the disadvantage of not eliciting cellular immunity and rely on the stimulation of strong antibody responses for protection. However, CTL responses have been detected against other flaviviruses and it would be important to establish their relationship with virus clearance and/or immunopathology in the WNV infection of the horse. DNA and live vaccines have the capacity to stimulate adaptive cellular immunity. The potential of DNA vaccination for WNV has been demonstrated in mouse and horse infection models [25, 29]. In this strategy, a c-DNA copy of the prM/E coding region of WNV is inserted into the mammalian expression plasmid vector pCBJESS, a derivative of pCBamp modified to contain the JE virus signal sequence. Single immunisation of mice resulted in the induction of neutralising antibodies (titres of 1:320–1:640 on week 9 post-immunisation) and 100% survival rate after mosquito or intraperitoneal challenge on week 9. Similarly,

horses developed neutralising antibodies (1:5–1:40 titres on day 37 post-immunisation) and were protected against viraemia when challenged by infected mosquitoes on day 39 post-immunisation.

Prototype live attenuated WNV vaccines have been developed by repetitive passage of virus in mosquito cells in the presence of a neutralising monoclonal antibody [55]. This vaccine showed capacity to protect against WNV infection in geese. The authors are not aware of extension of these studies to the horse.

One possible disadvantage of developing a live vaccine by repetitive virus passage in tissue culture is that the position of the attenuating mutations cannot be controlled and these mutations may revert to the virulent variant once the virus infects the natural host. The development of cDNA infectious clones of WNV [82] allows the attenuation of the virus by direct mutagenesis introducing the mutations in regions of the genome that are known for their genetic stability, like the 3' noncoding region. This approach has been used for Dengue and tick-borne encephalitis viruses [58, 61] by introduction of modifications in the 3' noncoding region.

Another vaccination approach for WNV is the use of chimeric yellow fever (YF) viruses expressing WNV antigens [63, 65]. This strategy has been used for JE and Dengue and the development of these vaccines are in advanced clinical trials. The recombinant virus is constructed using the cDNA backbone of the YF-17D vaccine strain which is modified by replacing the YF prM/E coding region by that of the flavivirus for which the vaccine is developed. This immunisation offers the advantages of live vaccines: stimulation of humoral as well as cellular immunity (M and E are targeted by neutralising antibodies and by CTLs) with a single dose, and long-lasting immunological memory, with the added advantage of increased safety. Thus, intracerebral inoculation of both adult and sucking mice and intracerebral and subcutaneous inoculations in rhesus macaques of chimeric YF/JE, YF/Dengue and YF-17D showed that the recombinant viruses have lower neurovirulence than the YF-17D vaccine strain in both species and that they induced self-limited viraemia in monkeys identical to those induced by the YF-17D virus. A prototype YF/WNV vaccine has been constructed and shown to be protective against WNV challenge of hamsters [89]. This vaccination approach is currently being explored in horses and humans.

10. PREVENTION AND CONTROL OF WEST NILE ENCEPHALITIS

Strategies for prevention of West Nile encephalitis in endemic areas include vaccination, reduction of virus circulation through measures influencing mosquito populations and reduction of contact with infected mosquito vectors through altered behaviour or management.

Thus far, the most widely used measure has been vaccination of equidae in north America using the licensed whole virus killed vaccine [69]. While cases of disease have been reported in horses that have received the product, most had not completed the initial course of two doses [35]. Use of this and other products, such as the recently licenced recombinant Canarypox WNV vaccine, will remain an important means of preventing the disease in horses.

There is a long history of environmental insecticide use in the management of arbovirus outbreaks, particularly in North America, and undoubtedly their use is beneficial during periods of intense challenge. However, it is unlikely that their use would be sanctioned merely to reduce equine disease outbreaks (they are principally a public health measure) and their short and long term environmental impact is uncertain. Other effective methods of reducing the local populations of some mosquito species include control of standing water and removal of larval breeding sites, but again, there is no information available on their specific efficacy against WNV transmission.

An important area in West Nile prevention is management of horses to prevent exposure to infected mosquitoes. Stabling, particularly at night in insect proof stabling may form an inexpensive, safe and effective means of preventing infection. The effectiveness of stabling in non-insect proof stabling is less clear, but probably depends on specific local environmental factors such as the local species of mosquito, the availability of other hosts for mosquitoes to feed on and the mosquito and horse population densities. Normal stabling could be enhanced by the use of insecticides within stables and topical insect repellents but the efficacy of such measures has not been fully evaluated.

11. CONCLUSION

West Nile infection has re-emerged as an important zoonosis that has demonstrated a capacity to spread over an entire continent in a few years, causing illness and deaths in humans and horses. Its future course is unpredictable but it is expected that the latest improvements in laboratory diagnosis will reveal important epidemiological features of WNV. Other recent scientific advances have also contributed to a better understanding of WNV and the disease but many aspects about immunity, pathogenesis and molecular basis of virulence, especially in the horse, are still unknown.

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