# A West Nile Virus (WNv) Human Vaccine:

# From Research to Vaccination

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# Overview of the Project

This project will educate readers about the West Nile virus (WNv), and the vaccine research approaches currently underway. A narrative review used an instrument to synthesize WNv vaccine research studies, into an easy to understand format. Results indicated the instrument had a high magnitude of reliability ($r = 0.90$, $p<0.0001$). The size and frequency of future epidemics of the rare occurrence WNv is uncertain. Research for a WNv vaccine for humans is a responsible public health initiative. The rationale for preplanning a vaccination program is discussed as a future application of this research. The public expects preventative and therapeutic solutions to infectious diseases, and the project's conclusion indicates that for WNv this can be achieved.

## *Goal*

100% of participants who read the project "A West Nile Virus (WNv) Human Vaccine: From Research to Vaccination" will have an immediate understanding of the WN virus, the current vaccine research approaches, and the public health implications of vaccination upon completion.

## *Objectives*

- To utilize extensive peer-reviewed literature of the WN virus
- To discuss the impact of WNv, and define the "at-risk" population
- To summarize study results of WNv vaccine approaches
- To recommend preparedness planning for a vaccination program, as a future application.

# Introduction

The West Nile virus (WNv) is a threat to population health, and research for a WNv vaccine for humans is a responsible public health initiative. While the size and frequency of future epidemics of this rare occurrence infectious disease is uncertain, the virus will continue to adapt and thrive. Industrialized nations have come to expect preventative and therapeutic solutions to preventable diseases. WNv research is advancing in the scientific development and formulation of customized vaccines, specific for disease challenge situations. Planning now for a WNv vaccination program to utilize a future vaccine, would add credible preparedness information as evidence to support or refute current and future public health policy considerations.

In Ontario, the 36 Health Units are guided by the *West Nile Virus Preparedness and Prevention Plan 2006* for Ontario (Ministry of Health and Long Term Care [MOHLTC], 2006), a reference document that assists with the implementation of *Ontario Regulation 199/03 Control of West Nile Virus* (Canadian Legal Information Institute [CanLII], n.d., a.) under the *Health Protection and Promotion Act* (CanLII, n.d., b.). WNv is a rare occurrence infectious disease that since its emergence in 1999 in New York has caused ~ 20,000 human cases (800 deaths), and 25,000 equine cases (5000 cases) to 2005 (Dauphin and Zientara, 2007). The virus affects over 250 species of birds, 35 species of mammals and 2 species of reptiles over a large geographic area[1] (MOHLTC, 2006). As shown in Table 1, from 2002 - 2007 there have been 4,331 cases reported in Canada, with 552 in Ontario (Public Health Agency of Canada [PHAC],

1 In Canada WNv has been seen from Nova Scotia to Alberta. Newfoundland and Labrador continue to remain free of infection. In BC in 2007, 13 cases were reported (7 neurological, 3 non-neurological and 3 unclassified) however, all cases were likely related to travel outside the Province. See PHAC, (2007b).

2007a). There is no way to predict how serious WNv will be in any given year, as fluctuations in climate – temperature, precipitation, humidity and wind all influence the mosquitoes linked to this infectious disease.[2] Illness and death from infectious diseases are particularly tragic, because they are largely preventable. Public health activities related to WNv surveillance and control have traditionally been reactive, and at times controversial. Population-based programs must be innovative and should acknowledge the complex ecosystem based transmission cycles, if they are to be successful.

**Table 1. West Nile Virus Cases from 2002-2007.**

| Year | Neurological | | Non-neurological | | Unclassified | | Asymptomatic | | Total[2] | Total[2] | Deaths |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Canada | Ont[1] | Canada | Ont | Canada | Ont | Canada | Ont | Canada | Ont | Canada |
| 2007[5] | 106 | 1 | 974 | 9 | 955 | 0 | 26 | 3 | 2035 | 10 | [4] |
| 2006 | 38 | 17 | 112 | 24 | 1 | 1 | 3 | 1 | 151 | 42[3] | [4] |
| 2005 | 49 | 3 | 172 | 1 | 4 | 0 | 13 | 1 | 225 | 4 | 12 |
| 2004 | 13 | 10 | 12 | 3 | 0 | 0 | 1 | 1 | 25 | 13 | 2 |
| 2003 | 217 | 47 | 1248 | 42 | 16 | 0 | 14 | 0 | 1481 | 89 | 12 |
| 2002 | 259 | 243 | 18 | 18 | 137 | 133 | 0 | 0 | 414 | 394 | 20 |

1 Includes probable/confirmed cases.
2 Total doesn't include asymptomatic infection.
3 Some totals include some cases related to travel outside province or territory.
4 Information not available
5. As of September 15, 2007.

Source: From "*Human Surveillance (2002-2006)*" and "*Human Surveillance 2007*" West Nile Virus MONITOR. Public Health Agency of Canada, 2007a, b. 

2 As of September 6, 2007, 65 equine WNv cases were reported in Alberta, Saskatchewan, and Manitoba. More than 1,200 human WNv cases were reported in Canada (3 deaths in Saskatchewan and 2 in Manitoba). In the US, 906 human infections (26 fatalities) were reported in 33 States, with 56 equine cases. See BC Escapes West Nile Again (2007, October/November).

## Framework

The development and implementation of innovations is a vital aspect of public health services. This project utilized the Diffusion of Innovations Theory (see Appendix A, Figure 1) to support preparedness planning of a community-based public health prevention initiative, as a future application of the research that is currently underway to develop and market a WNv vaccine for humans.

The original diffusion research was done as early as 1903 by the French sociologist Gabriel Tarde, who plotted an S-shaped diffusion curve that reflects the S-shaped rate of adoption for new innovations (University of Twente, 2004). According to Rogers (1995b), Ryan and Gross studied the phenomenon in 1943 using agricultural innovations; however, the application of the diffusion theory in public health immunization campaigns can be found prior to 1960. The diffusion model has been used successfully for public health education and promotion programs for innovations that are preventive in nature, that have been perceived as having a lower degree of relative advantage over current ideas and practices (Rogers, 1995b). These programs, according to Rogers (1995b), often require individuals to adopt a new idea at a certain time in order to avoid the potential occurrence of some unwanted health event later, in which the future outcome cannot be predicted with certainty. For this reason, preventive health innovations generally diffuse slowly and require greater promotion to reach an acceptable level of adoption, and this may be particularly true for a novel vaccine. Canadians can be characterized as having positive opinions about vaccine effectiveness and research. However, attitudes about safety,

efficacy, and self-perceived knowledge are all associated with the willingness to get a vaccine and this would be especially likely for one currently in development (Ritvo, et. al., 2003).

According to Rogers, (1995a) diffusion is the process by which an innovation is communicated through certain channels over time, among the members of a social system. This type of communication is concerned with the spread of messages, which will be received with a degree of uncertainty. The elements in the diffusion of new ideas are:

- innovation
- communication channels
- time
- social system.

Rogers (1995a) describes an innovation as an idea, practice, or object that is perceived as new, where the characteristics of relative advantage,[3] compatibility,[4] complexity,[5] trialability,[6] and observability[7] determine its rate of adoption. While it can be measured in economic terms, social prestige, convenience, and satisfaction are also important factors in whether individuals perceive the innovation as advantageous. Ideas that are incompatible with the values and norms of a social system will not be adopted rapidly, as they require the prior adoption of a new value system. Complicated innovations will not be adopted rapidly, especially if they require people to develop new skills and understanding. New ideas that are simple and can be tried on a limited

3 Relative advantage of an innovation, reflects the degree to which it is perceived as better than the idea it supercedes.
4 Compatibility is the degree to which an innovation is perceived as being consistent with the existing values, past experiences, and needs of potential users.
5 Complexity refers to the degree to which an innovation is perceived, as difficult to understand and use.
6 Trialability is the degree to which an innovation may be experimented with, on a limited basis.
7 Observability is the degree to which the results of an innovation are visible.

basis will represent less uncertainty to individuals who can learn by doing, and will be adopted more rapidly. In addition, innovations with visible results are more likely to be adopted. A key concept in the diffusion process is the critical mass, which occurs at the point in which enough individuals have adopted an innovation so that further adoption becomes self-sustaining (Rogers, 1995a).

Communication is the process, by which participants create and share information with one another in order to reach a mutual understanding (Rogers, 1995a). Social in nature, the channel is the means by which messages get from one individual to another. Mass media channels are effective in creating knowledge, whereas interpersonal channels are effective in forming and changing attitudes towards a new idea by influencing the decision to adopt or reject it.

The time dimension according to Rogers (1995a) is involved in three ways. First it is the mental process through which a decision-maker passes from first knowledge of an innovation to forming an attitude toward it; to the decision to adopt or reject it; to the implementation of the new idea; and finally to confirmation of the decision to adopt the innovation. The second dimension is how early the decision-maker adopts the new idea, and is classified in five adopter categories:

- innovators
- early adopters
- early majority
- late majority
- laggards.

Innovators the first 2.5% of the individuals in a system to adopt an innovation are cosmopolites, who play an important role in the diffusion process. Early adopters, the next 13.5% of the individuals to adopt an innovation are localites, and have the greatest degree of opinion leadership in most systems. Outreach activities should be focused on these individuals, the change agents and gatekeepers, as they serve as role models and are instrumental in getting an innovation to the point of critical mass. The early majority category contains the next 34% of individuals in a system to adopt an innovation. They adopt new ideas just before the average member does but rarely hold positions of opinion leadership, nevertheless, they function as links in the diffusion process. The late majority make up 34% of individuals who do not adopt until most others have done so, therefore system norms must favour the innovation, and peer pressure is necessary for motivation. Laggards are the final 16% of individuals who have little opinion leadership, and function in near isolation to the social networks of their system. The final time dimension is the rate or relative speed in which an innovation is adopted, and is measured as the number of members of the system that adopt it in a given time period (Rogers, 1995a).

A social system is defined as a set of interrelated units that are engaged in joint problem solving, to accomplish common goals (Rogers, 1995a). Members may be individuals, groups, organizations, and/or subsystems that constitute a boundary within which an innovation diffuses. Diffusion is affected by norms or established behaviour patterns and opinion leadership, which influences the attitudes or the behaviour of others in a desired way.

Diffusion research has focused on five elements that support the goal of public health prevention research: (1) the characteristics of an innovation which may influence its adoption;

(2) the decision-making process; (3) the characteristics of individuals that make them likely to adopt an innovation; (4) the consequences for individuals and society of adopting an innovation; and (5) communication channels used in the adoption process (University of Twente, 2004). In order to generate a strong science base that supports practice and includes measures to translate science into programs and policy, community-based prevention research entails preparedness planning in communities where cultural and social factors are considered (Baker, White, and Lightveld, 2001). In their study Waterman, et al., 2007, argued that there is no single way to develop and diffuse an innovation. They stated "[a]dopters...need to be viewed as active participants in the adoption process who, given the right circumstances and reasons, might innovate (Waterman, et al., 2007, p. 377). Preplanning to explore what works in communities will fill a gap in the research base that will lead to improved public health vaccine programs and policies that rely on science to develop, test, and validate new interventions and methods (Baker et al., 2001).

The project's comprehensive literature review utilizes peer-reviewed literature to illustrate the impact of WNv, a disease that strikes urban and relatively affluent areas with strong public health infrastructures, as the rationale for the development of a human WNv vaccine. It critically examines the knowledge, trends, attitudes, and cost-benefits of vaccination in support of the research to create a vaccine that could be used in both an endemic and an impending epidemic to provide preventative immunization. For a disease in which there is no cure and clinical presentation (encephalitis) can be severe and potentially fatal, vaccination provides the ultimate measure for personal protection against the WNv (Monath, 2001). The needs assessment determined the at-risk population – the elderly or immunocompromised, as well as residents of

areas with evidence of high virus transmission – as candidates for vaccination The project's methodology utilized a narrative systematic review of heterogeneous studies to describe the range of available evidence to identify, appraise, and synthesize current WNv vaccine research Preparedness planning for a potential vaccine frames the importance of using theory to initiate ideas that increase knowledge, develop evidence-based protocols, and best practice models for universal public health application in the event of epidemic WNv disease.

## Literature Review

### *West Nile Virus*

Emerging and reemerging infectious diseases have received increased attention in recent years with the realization that "...the concept of globalization include[s] global exposure to disease agents formerly confined to small, endemic or remote areas" (Lashley, 2003, p. 258). WNv is an Old World virus that was first isolated in 1937, in the West Nile province of Uganda (Sampathkumar, 2003). Outbreaks occur infrequently in humans, however, the most notable epidemics causing encephalitis and death have been in Israel (1950's), France (1962), South Africa (1974), India (1980/1981) and Romania (1986) (Lanciotti, et al., 2002). Since its 1999 North American appearance in New York, the diversity, distribution, and effects of WNv have made efforts to understand its human-ecosystem interaction difficult. It is important to recognize that although WNv is not new, it is being found in new geographic areas and it is bringing new complexities. According to Petersen and Roehrig (2001), three epidemiologic trends for WNv have emerged: 1) increase in frequency of outbreaks in humans and horses,[8] 2) apparent

8 Romania 1996; Morocco 1996; Tunisia 1997; Italy 1998; Russia, USA and Israel 1999; Israel, France and USA 2000.

increase in severe human disease[9] and 3) high avian death rates accompanying the human outbreaks in Israel and the US. Petersen and Roehrig (2001) further suggested that recent outbreaks of WNv have been accompanied by an apparent evolution of a new virus variant.[10]

The most common route of WNv infection to humans is through a bite from an infected mosquito. After infection, the viral incubation period in humans ranges from 2 to 15 days (usually 2 - 6 days) with most infections clinically inapparent[11] (Sampathkumar, 2003). WNv illness consists of two clinical pictures, and it is classified according to laboratory criteria as either "confirmed" or "probable" (Department of Health and Human Services [CDC], 2006). Approximately 20% of infected individuals will develop "WNV Non-Neurological Syndrome"[12] (MOHLTC, 2006) and will experience only mild symptoms such as fever, headache, body aches, skin rash, and swollen lymph glands (National Institute [NIAID], 2006). However, 1 in 150 (0.7 %) of those infected will develop "WNV Neurological Syndrome" (MOHLTC, 2006). If WNv enters the brain, it causes life-threatening encephalitis, meningitis, or meningoencephalitis, the most common type of central nervous system (CNS) presentation (Sampathkumar, 2003). WNv has been associated with acute flaccid paralysis with damage localized to spinal anterior horn cells, which results in a poliomyelitis-like syndrome. Movement disorder syndromes[13] have also been reported (Sampathkumar, 2003).

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9 Confirmed human infections in recent outbreaks: Romania, 393 cases, Russia [Volgograd], 942 cases, US, 62 cases in 1999 and 21 in 2000; Israel, 2 cases in 1999 and 417 in 2000.

10 NY99.

11 Serological testing of residents in the most heavily affected area of New York City (Queens) in 1999, showed that ~ 2.6% of the population might have been positive for WNv. Estimates of WN in horses are less precise, but only 0.4 % of the horses in the most heavily affected area (Suffolk County, Long Island), exhibited clinical signs of infection in 1999. See Stringham and Watson, 2002.

12 Formerly known as West Nile Fever.

13 Tremors and myoclonus, parkinsonism, rhabdomyolysis, ataxia, cranial nerve involvement, optic neuritis and polyradiculopathy. Involvement of the CNS is associated with mortality of up to 10%. See Sampathkumar, 2003.

Rainham (2005) suggested that transmission cycles are connected to local and global climate variability, where weather pattern changes, slight global warming, or receding freezing zones favour mosquitoes and the expansion of their range. Natural disasters with heavy rain and flooding, alterations in the environment from both agricultural practices and urban purposes can alter water levels, which affect mosquito populations and disease occurrence. The interactions between vectors and hosts are also complex, since each element may adapt and/or reorganize in response to an intervention (Rainham, 2005). Kondro (2006) suggested that several variables add to this complexity by noting that WNv infects numerous different species of mosquitoes, which suggests that it has a large environmental niche in addition to a great geographic distribution. Further, he stated "[i]t also seems very hardy, in that it can tolerate continental climates that are quite north" (Kondro, 2006, p. 570). Another interesting factor suggested by Lashley (2003), is that genetic adaptive changes are important in the emergence of infectious diseases. This is further supported by Kondro (2006) who stated "...birds carrying the virus are becoming 'more immune as a population'" (p. 570). In addition, he proposed that there is a genetic transmission of the virus within mosquito larvae that acts as an over-wintering mechanism, which suggests the spread of WNv is no longer limited to annual reintroduction from migratory birds (Kondro, 2006).

Infectious mosquitoes carry virus particles in their salivary glands, and infect susceptible bird species during blood-meal feeding. The primary or "reservoir" host birds sustain an infectious viremia for 1 - 4 days after exposure, after this time they either die or develop immunity. Bridging species of mosquitoes that feed upon both birds and mammals transmit the virus to humans and vertebrates (animals with backbones), which function as "incidental" or "dead-end"

hosts (Department of Health and Human Services [CDC], n.d.). According to Brinton (2002), the minimum level required to infect a feeding mosquito is ~$10^5$ PFU/ml of serum.[14] It appears unlikely, however, that humans exhibit WNv viremia levels of sufficient magnitude to infect mosquitoes (Hayes, et al., 2005). Kondro (2006) reported that, unlike birds, only 3% of humans remain immune after WNv infection.

Mosquitoes of the genus *Culex* are the principal maintenance vectors in the Old World.[15] In North America, WNv has been found in 59 mosquito species, however <10% are considered as principal vectors (Hayes et al., 2005). The most common vectors are: in the northeast US *Cx. pipiens*, *Cx. quinquefasciatus* in the south US, and *Cx. tarsalis* and *Cx. restuans* to the west of the Mississippi River (Hayes, et al., 2005). The intensity of WNv transmission to humans is dependent on abundance and feeding patterns of infected mosquitoes, local ecology, and behaviour that influences human exposure (Hayes, et al., 2005). The incidence of WNv disease is seasonal in temperate zones, with peak activity from July through October (Hayes et al., 2005). Temperature profoundly influences mosquito-to-vertebrate transmission rates, therefore summer temperatures are likely one of the most important environmental variables modulating WNv infection (Savage, et al., 1999). According to Hayes et al. (2005), in the US the transmission season has lengthened as the virus has moved south, with the trend of onset of human illness beginning earlier.

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14 Plaque forming unit (PFU), is a measure of infectious virus particles. One plaque forming unit is equivalent to one infectious virus particle.

15 In Europe and Africa the primary vectors *are Cx. pipiens, Cx. Univittatus* and *Cx. Antennatus*, in India the *Cx. vishnui*, and in Australia the Kunjin virus is transmitted primarily by *Cx. annulirostris*. See Hayes et al., 2005.

Recent lower WNv incidence may be due to mosquito abatement programs, public education campaigns, or to the fact that physicians are more familiar with the virus and likely to diagnose and treat milder infections resulting in fewer instances of progression to neuroinvasive disease and death (Kondro, 2006). Of unknown implication are reported nonmosquitoborne WNv transmission among US farmed alligators, domestic turkeys, and domestic geese in Canada, and the close contact transmission in both birds and alligators in laboratory conditions (Hayes et al., 2005).

### *Vaccination trends and principles*

Research and development of new vaccines are costly in terms of time and money, and historically it has not been a profitable business. According to Carey and Capell (2002), progressive US policy moves, advances in science, and a big change in the economics of the business has brought about resurgence. The ability of vaccine makers to command premium prices has prompted renewal in research and development spending that has resulted in growth for the industry (Carey and Capell, 2002). Carey and Capell (2002) reported that interest in vaccines as a business as opposed to [just] a public health intervention, has led to profits for the industry leaders. Vaccines are becoming more versatile, and able to tackle diseases beyond the infection itself (Carey and Capell, 2002). This has created a potential for basic vaccines that can be customized for a range of diseases, or when novel diseases suddenly emerge (Carey and Capell, 2002).

Infectious diseases are caused by organisms that are able to exploit the body, so that they may grow and reproduce (see Appendix B, Figure 2). According to the NIP *Reference Guide on*

*Vaccines and Vaccine Safety*, humans are protected against infectious disease by various physical and biochemical factors, such as the skin and its acidic secretions, tears and mucous membranes. If an infectious agent gets past the first line of defense, a second tier of defense is provided by natural or innate immune mechanisms. Our own cells and the chemicals they produce seek out, identify, and eliminate the pathogen. When stronger protection is needed, an acquired immune reaction (antibody and cell-mediated responses) specific to the pathogen are mounted (see Appendix B, Figure 3). This response can result in either short or long-term protection against a specific pathogen, and often to some of its close relatives. Re-exposure to the same pathogen reactivates the response mechanisms laid down during the original exposure, which leads to the rapid effective elimination of the agent often without symptoms or signs of infection. Specific immunity that results in protection from exposure to agents in the environment is referred to as being passively acquired,[16] whereas intentional exposure to the agent or through vaccination results in actively acquired immunity (Department of Health [CDC], 2007).

The immune system is a complex network of molecules, cells, and tissues that respond when a pathogen or vaccine enters the body. The cells in the surrounding tissues release chemokines[17] and cytokines[18] that attract white blood cells (WBC's, e.g., neutrophils, monocytes, lymphocytes) to the area that leads to the destruction of the pathogen. WBC's release other chemicals that constitute the natural immune response. This innate immunity is not specific or long lasting, and

16 Passive immunity results when antibodies are produced by one individual, and then acquired by another. Naturally acquired passive immunity occurs with the acquisition of antibodies in colostrum and breast milk by an infant, or by maternal antibodies crossing the placenta. Artificially acquired passive immunity occurs when antiserum or antibodies produced by one individual, are transfused into a second individual.

17 Chemokines are a family of structurally related glycoproteins with potent leukocyte activation and/or chemotactic activity. They are 70 to 90 amino acids in length and approximately 8 to 10 kDa in molecular weight.

18 Cytokines are small secreted proteins, which mediate and regulate immunity, inflammation, and hematopoiesis.

occurs each time when there is a threat of infection from any pathogen. Acquired immunity enhances this natural response by directing interactions among macrophages that engulf an infectious organism or vaccine, which is broken down chemically into constituent proteins and other biochemical components (see Appendix B, Figure 4). Further degraded the protein fragments (antigens) associate with major histocompatibility complex (MHC)[19] Class I[20] or Class II[21] molecules. The antigen and the MHC molecules are arrayed on the surface of macrophages and other antigen-presenting cells where the antigen can be "presented" to T lymphocytes (T cells), that circulate in the blood and reside in the spleen and lymph nodes (see Appendix B, Figure 5). B cells have receptors on their surface membranes that recognize and adhere to proteins that make up the pathogen or vaccine. This activates the B cell causing it to divide rapidly, differentiate into antibody-producing cells, and the latter release large amounts of antibody molecules that can specifically attack the pathogen. Helper T cells recognize and adhere to the antigen and MHC complex presented by the antigen-presenting cells, which activates the lymphocyte causing it to release cytokines. The cytokines stimulate cells (particularly antigen-stimulated B cells) to divide, and become mature (see Appendix B, Figure 6). This results in the production and release of antibodies that recognize the distinct antigenic components of the pathogen. Antibody molecules that encounter the pathogen adhere to it enabling macrophages to attach to the pathogen, resulting in its destruction (CDC, 2007).

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19 The major histocompatibility complex (MHC) is a large genomic region or gene family found in most vertebrates, and plays an important role in the immune system, autoimmunity, and reproductive success. The proteins encoded by the MHC are expressed on the surface of cells and display both self antigens (peptide fragments from the cell itself) and nonself antigens (e.g. fragments of invading microorganisms) to T cells.

20 Class I molecules consist of two polypeptide chains. Humans synthesize three different types designated HLA-A, HLA-B, and HLA-C (HLA = human leukocyte antigen).

21 Class II molecules designated HLA-D, consist of two transmembrane polypeptides and are only found on B-cells, macrophages and other "antigen-presenting cells" (APCs). They present "exogenous" antigen to helper T-cells (TH-cells). Exogenous antigens might be fragments of bacterial cells or viruses that are engulfed and processed by e.g. a macrophage, and then presented to helper T-cells. The TH-cells in turn, could activate B-cells to produce antibody that would lead to the destruction of the pathogen.

In the case of viruses, they take over the machinery of the cell using it to produce more copies of themselves (see Appendix B, Figure 7). This will cause fragments of virus protein to become attached to the cell's own MHC Class I molecules.[22] This complex attaches to the surface of the cell, where the antigen is presented to cytotoxic T cells bearing receptors for the antigen and the Class I molecules. The interaction between the antigen-presenting cell and the cytotoxic T cell causes the cell to divide and destroy the virus-infected cells. This process of interactions of the T cells responsible for specific cell-mediated immunity with the pathogen is referred to as the cell-mediated response. As the amount of antibodies and cytotoxic T cells increase, the infection or the response to the immunization diminishes; some of the B cells become memory B cells[23] with receptors specific for the antigen that originally stimulated the parent cell. If the individual is subsequently re-exposed to the agent, the B memory cell will respond by dividing and releasing antibodies. Vaccination establishes a pool of memory cells that can produce pathogen-specific responses, to largely prevent development of the disease (CDC, 2007).

In the case of WNv disease, the immune response to WNv is essentially mediated by neutralizing antibodies,[24] (N Abs) limiting viral dissemination (Dauphin and Zientara, 2007). According to Sampathkumar (2003), CNS infection is presumed to occur when the virus crosses the blood-brain barrier,[25] with infection confirmed by detecting virus or antibodies against the

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22 Class I molecules are found on virtually every cell in the human body, and present "endogenous" antigen to cytotoxic T-cells (CTLs). An endogenous antigen might be fragments of viral or tumor proteins, and presentation of such antigens would indicate internal cellular alterations that if not contained could spread throughout the body. Hence, destruction of these cells by CTLs is advantageous to the body as a whole.

23 Certain pathogen-specific cytotoxic T cells also persist as memory T cells, that are available to respond quickly upon re-exposure.

24 A form of antibody that reacts with an infectious agent (usually a virus), and destroys or inhibits its infectivity and virulence.

25 By endothelial replication or axonal transport through olfactory neurons.

virus in serum or cerebral spinal fluid (CSF). Immunoglobulin M (IgM)[26] associated antibodies are detected 2 - 8 days after the onset of clinical signs, peak at 2 weeks post infection, and then decrease over several weeks or months (Dauphin and Zientara, 2007). According to Sampathkumar (2003), IgM antibody does not usually cross the blood-brain barrier,[27] therefore "(t)he detection of IgM antibodies [to WNv] in CSF[28] is...direct evidence of CNS infection" (Dauphin and Zientara, 2007, p. 5568). Immunoglobulin G (IgG)[29] antibodies appear after IgM Abs ~ 12 days after onset of symptoms. According to Samuel and Diamond (2006), IgG can protect against flavivirus (WNv) infection,[30] however the function of IgG during primary infection is not clear and requires more study. According to Dauphin and Zientara (2007), T-cell response mediated mostly by $CD8^+$ lymphocytes play a crucial role in WNv recovery, particularly in viral clearance. According to Kimball (2007), most of the T cells of the body belong to one of two distinct subsets: $CD4^+$ or $CD8^+$, which are surface glycoproteins and have an antigen receptor (TCR) that "sees" an epitope. CD8 molecules on $CD8^+$ T cells bind to a site found only on class I histocompatibility molecules,[31] therefore, $CD8^+$ T cells are only able to respond to antigens presented by class I molecules. Most $CD8^+$ T cells are cytotoxic T lymphocytes (CTLs), and contain the machinery for destroying cells whose class I epitope they recognize. Upon recognition of a WNv-infected cell that expresses class I MHC molecules,

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26 IgM binds complement, and is the largest antibody. They are found in blood and lymph fluid, and are the first type of antibody made in response to an infection. IgM antibodies are about 5% to 10% of all the antibodies in the body.

27 Antibodies are too large to cross the blood-brain barrier, therefore infections of the brain which do occur, are often very serious and difficult to treat.

28 MAC-ELISA (IgM antibody capture enzyme-linked immunosorbent assay) with WNv, gives a rapid and reasonably accurate determination of the identity of the infecting virus. See Martin et al., 2002.

29 IgG antibodies are found in all body fluids. They are the smallest but most common antibody (75% to 80%) of all the antibodies in the body. They facilitate the phagocytic destruction of foreign microorganisms, that bind to and activate complement.

30 Passive transfer of polyclonal or monoclonal immunoglobulin G (IgG) prior to infection, protects mice against lethal flavivirus challenge. See Samuel and Diamond, 2006.

31 CD4 molecules on $CD4^+$ T cells, bind to a site found only on class II histocompatibility molecules.

antigen-restricted CTLs proliferate and release special molecules that lyse cells[32] (Samuel and Diamond, 2006).

As explained by Kimball (2007), the CD4 molecules expressed on the surface of $CD4^+$ T cells enable them to bind to cells presenting antigen fragments in class II molecules but not in class I. Certain types of cells such as macrophages, B lymphocytes (B cells) and dendritic cells specialized for taking up antigen from extracellular fluids, express class II molecules. Therefore, $CD4^+$ T cells see antigen derived from extracellular fluids, processed by specialized antigen-presenting cells. A $CD4^+$ T cell must also have a TCR able to recognize an epitope comprising an antigenic fragment displayed by a class II molecule, and bind a site on the class II molecule with its CD4. If these conditions are met the T cell becomes activated, and enters the cell cycle leading to the growth of a clone of identical T cells that begin to secrete cytokines. Cytokines activate other cells in their microenvironment, particularly B cells enabling them to develop into a clone of antibody-secreting cells. The $CD4^+$ T cells that activate B cells are called helper T cells, T lymphocytes that belong to the CD4+ subset. Th1 participate in cell-mediated immunity. They are essential for controlling intracellular pathogens, viruses and certain bacteria. Th2 provide help for B cells and, in so doing, are essential for antibody-mediated immunity. Antibodies are needed to control extracellular pathogens, which unlike intracellular pathogens, are exposed to antibodies in blood and other body fluids. De Groot et al. (2001) suggested that cell-mediated immunity to the WNv may prove to be an important barrier to infection in the CNS, and vaccines that promote the development of T-effector cells: T helper cells ($CD4^+$), and cytotoxic T cells ($CD8^+$) may provide protection for WNv encephalitis.

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32 Through the delivery of perforin and granzymes A and B, or via Fas-Fas ligand interactions. See Samuel and Diamond, 2006.

WNv infection can be suspected in a person based on clinical symptoms and patient history; however, laboratory testing is required for a confirmed diagnosis. Serological testing remains the primary method of diagnosis, using a West Nile Virus Antibody Panel, ELISA for serum or CSF samples. ELISA an enzyme immunoassay (EIA) requires the separation of reagents to measure macromolecules such as antigens and antibodies, to diagnose infectious disease. WNv IgM is usually detectable by the time disease symptoms appear, but IgG may not be detectable until day 4 or 5 of illness. In acute WNv infection, specific antibodies are sometimes detectable in CSF before they are detectable in serum (Focus Diagnostics, 2007). In preparation for future outbreaks, commercial assays using the ELISA format for the detection of IgG and IgM antibodies against WNv have been developed.[33] According to Focus Diagnostics (2007), the IgG and IgM capture assay tests[34] detect IgG and IgM antibodies respectively, to aid in the presumptive laboratory diagnosis of WNv. IgG antibody is present in serum by 3 weeks post infection, and often earlier. Using the West Nile Virus IgG DxSelect™ test, an index value of $\geq$ 1.50 indicates IgG antibodies to WNv, presumptive evidence that the patient was or is currently infected with (or exposed to) WNv or another flavivirus. An index value of $\geq$ 1.30 and < 1.50 is considered an equivocal result. These samples must be tested using a different method, or re-drawn two or more weeks later and re-tested with this assay. An index value of <1.30 indicates antibodies to WNv (or another flavivirus) were not detected. The sample may have been drawn

33 Focus Technologies (Cypress, California), using flavivirus and WNV recombinant protein technology licensed from the CDC, and PANBIO, Inc., using inactivated purified native WNV antigen, have formulated IgM-capture immunoassays for the detection of WNV-specific IgM. IgG enzyme immunoassays are also available from both companies. The Food and Drug Administration (FDA) has granted clearance for the PANBIO WNV IgM assay, and the Focus Technologies WNV IgM and IgG assays. These assays appeared to offer highly sensitive and specific testing platforms with decreased turnaround time, while providing a method to effectively test high numbers of samples. These assays are comparable to immunofluorescence assay (IFA), but require much less time and labour. See Malan et. al., 2003.

34 Focus Diagnostics West Nile Virus IgG DxSelect™ and West Nile Virus IgM Capture DxSelect™ for *in vitro* diagnostic use are available as FDA-cleared kits for laboratories that choose to run the test in-house, as well as referral tests from Focus Diagnostics' reference laboratory. See Focus Diagnostics, 2007.

before antibodies were detectable,[35] therefore another sample should be drawn 7 to 14 days later and re-tested if infection is suspected. IgM antibody is present in serum by the 8th day of infection, and will be detectable for at least 1 to 2 months after illness onset, and up to 500 days or longer. Using the West Nile Virus IgM Capture DxSelect™ test, an index value of > 1.10 indicates IgM antibodies to WNv were detected, presumptive evidence that the patient was recently or is currently infected with WNv or another flavivirus.[36] An index value of ≥ 0.90 but ≤1.10 is considered an equivocal result. These samples should be tested using a different method, or re-drawn two or more weeks later, and re-tested with this assay. An index value of < 0.90 indicates IgM antibodies to WNv were not detected, presumptive evidence that the patient was not recently infected with WNv or another flavivirus. The sample may have been drawn before antibodies were detectable[37] therefore, another sample should be drawn 7 to 14 days later and tested if infection is suspected (Focus Diagnostics, 2007).

According to Focus Diagnostics (2007), closely related arboviruses exhibit serologic cross-reactivity, which can result in positive results occurring in persons vaccinated for flaviviruses (e.g. yellow fever, Japanese encephalitis, dengue), with persons infected with other flaviviruses, or previously infected with WNv. Cross-reactivity has been observed in some specimens containing antibody to cytomegalovirus (CMV) and bunyaviruses (e.g. La Crosse virus). In addition to using the current CDC guidelines for diagnosing WN encephalitis, positive results must be confirmed by Plaque Reduction Neutralization Test (PRNT). The PRNT is used to

35 Or the patient may be immunosuppressed. See Focus Diagnostics, 2007.
36 IgM antibodies from previous infections may be present for over 500 days. IgM positive results reported for children must contain a caution statement regarding possible cross-reactivity with enteroviruses. See Focus Diagnostics, 2007.
37 Or the patient may be immunosuppressed. See Focus Diagnostics, 2007.

confirm the presence in a serum specimen of neutralizing antibodies specific for WNv, versus St. Louis Encephalitis virus (SLE). The test is considered positive for WNv at a titer 1:5 or greater, with a 4-fold higher titer than SLE[38] (Focus Diagnostics, 2007). The WNv IgG Avidity ELISA test uses serum or plasma, to distinguish current season from prior season WNv infection in patients with complicated medical histories and/or presentations. Avidity, a measure of the strength with which IgG antibodies attach to antigen, matures with the length of time since infection. IgG produced early in an infection exhibits low avidity, whereas IgG produced a few months later exhibits high avidity. A low IgG avidity value is an extremely accurate indicator of WNv infection, within the previous 3 - 4 months[39] (Focus Diagnostics, 2007). WNv RNA Real-Time Reverse Transcription, Polymerase Chain Reaction (RT-PCR)[40] testing on CSF, plasma, or serum samples is performed as an adjunct to serologic testing, and is considered the most sensitive laboratory tool to diagnose WNv. The detection of WNv RNA is based upon reverse transcription of specific WNv genomic RNA sequences, followed by PCR amplification (Focus Diagnostics, 2007). Due to the sensitivity of PCR tests, they are the test of choice when little of the target organism is expected in tissue, and maximum sensitivity is required (Indiana Animal Disease Diagnostic Laboratory [ADDL], 2002). If WNv nucleic acid is not detected by RT-

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38 The PRNT test satisfies the CDC requirement for performing a WNV-specific serology test. Positive results will be reported to the Health Department. See Focus Diagnostics, 2007..

39 The interpretation must be made with a clear appreciation of the assay's limitations. An AI <0.50 indicates with 98% accuracy that WNv infection occurred within the last 3 or 4 months; however, an AI >0.50 is not useful for estimating the time since WNV infection. See Focus Diagnostics, 2007.

40 PCR is done with DNA not RNA, which is the genetic material of the virus. Therefore, the first step is to turn the viral RNA sequence into a DNA sequence, which is done with the enzyme Reverse Transcriptase. The RT-PCR test to detect RNA is composed of: extraction of the DNA from the sample, addition of sample DNA (one set of DNA nucleotide primers and other reagents to a PCR-cycler machine for amplification of target DNA), detection of target DNA by gel-electrophoresis and using reverse-transcriptase enzyme, to synthesize complementary DNA from the target RNA. This complementary DNA is then run in the PCR test. See ADDL, 2002.

PCR, the test result is negative.[41] If WNv nucleic acid is detected, the test is positive[42] (ARUP Laboratories, 2007).

Vaccination is intended to elicit a specific immune response that will protect the immunized person from the pathogen should the individual be exposed to that agent. According to the NPI *Reference Guide On Vaccines And Vaccine Safety*, effective vaccines stimulate the production of antibodies that destroy the pathogen prior to its entry into cells, or elicit cytotoxic T cells that can destroy cells in which the pathogens reside (CDC, 2007). The development of a vaccine starts with pre-clinical laboratory testing to ensure that the vaccine produces the immune response needed to prevent disease, and does not have toxicities that would prevent its use. According to the *Canadian Immunization Guide* (2006), it takes many years to gather the scientific data on immunogenicity, safety, and efficacy of the vaccine needed to obtain authorization for marketing (Public Health Agency of Canada [PHAC], 2006) (see Appendix C, Table 2). Pre-marketing vaccine studies do not have sufficient numbers of subjects to detect rare adverse events, or their frequency (see Appendix C, Table 3). All potential target populations have not been fully studied prior to marketing approval, therefore post-marketing studies of vaccine safety and effectiveness are essential to gather data and monitor vaccine performance. In Canada, the Biologics and Genetic Therapies Directorate (BGTD) of Health Canada is the regulatory authority responsible for establishing the safety, efficacy, and quality of all biologics including

41 A negative result does not rule out the presence of PCR inhibitors in the patient specimen of WNv nucleic acid in concentrations below the level of detection of the assay. See ARUP Laboratories, 2007.

42 PCR tests do not differentiate vaccine from field strains of organisms. The high specificity of PCR tests means that false-positive tests due to improper test function is unlikely. False-positive PCR tests are most commonly a result of contamination of the sample with spurious target nucleic acid during sample collection, during the performance of the test or due to the nature of the sample. See ADDL, 2002.

vaccines (PHAC, 2006). The Canadian Adverse Event Following Immunization Surveillance System (CAEFISS) describes the nature of an event to facilitate monitoring[43] and follow-up at the local/provincial level, as well as causality assessment[44] and signal detection[45] at the national level (PHAC, 2006).

### *Vaccination attitude*

There is little doubt that immunization against infectious disease has saved lives, and vaccination programs are effective public health initiatives. Effectiveness of any new vaccine program will depend on the acceptance of the public, which is often challenged by concerns about safety. People vary in how they will respond to a vaccine and while most vaccinated individuals will quickly develop an effective, protective immunity to the disease agent without complications, a very small number will not, and yet others, will develop a severe adverse reaction (CDC, 2007). According to Ritvo et al. (2003), Canadians can be characterized as having positive opinions about vaccine effectiveness and research; however, insufficient knowledge and uncertainty can create fear. This can alter a person's ability to make a rational decision, by weighing both the benefits and the risks involved. Their study found 61.7% of subjects were reluctant to dismiss anti-vaccination positions, which reflects the potential for persuasion by pro-and anti-vaccine literature and argument. In addition, vaccination was seen as an act of social trust and perceived dangerousness, therefore doubts about efficacy, an unwillingness to accept vaccine-related mortality, beliefs that physicians overestimate disease risk, and perceived disease susceptibility are all factors in predicting vaccination compliance.

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43 Involves passive and active surveillance, and as necessary, focused ad-hoc studies.

44 Conducted by the Advisory Committee on Causality Assessment (ACCA), to evaluate the degree to which such events are linked to the implicated vaccine.

45 Adverse event reports are forwarded to the WHO Uppsala Monitoring Centre for entry into a global pharmacovigilance database, to identify any safety signals of potential concern.

Although Ritvo et al. (2003) concluded that Canadians indicated a willingness to take hypothetical vaccines currently in development and most are prepared to accept new vaccinations, future planning efforts by public health should be directed to improve receptivity about the necessity and safety of vaccines.

***Cost-benefit of WNv vaccination***

The emergence or resurgence of infectious disease in new geographical areas creates economic disruption, by challenging the established public health budgets. Infectious diseases are the 3rd leading cause of death in the US, the 2nd leading cause of death worldwide, and account for almost 30% of all disability-adjusted life years (Fauci, et al. 2005). Disease prevention results in substantial cost savings, and an effective vaccination program contributes to community health and quality of life (CDC, 2007). The process of vaccine licensure in the US is lengthy, taking around 5 - 10 years with costs between $300-500 million (CDC, 2007). To determine whether a vaccine is needed, studies on the prevalence of the disease, its burden on both the general population and particular risk groups, available treatments, and the costs associated with treating the disease are undertaken. This information helps advisory committees decide whether developing a vaccine would be useful, desirable, and necessary for the public, and if the vaccine would be profitable for a sponsor to produce (CDC, 2007).

Episodic infectious disease outbreaks have substantial short-term costs. In addition to its variability in transmission, WNv can cause illness that ranges from a simple fever to encephalitis. In their study Zohrabian, et al. (2004), estimated the economic impact of the June –

November 2002 WNv epidemic in Louisiana at $20.1 million.[46] Adults constituted 94% of cases, in which 204 had neuroinvasive disease with 24 deaths (median age was 78 years) that accounted for an extensive range of acute care and rehabilitative costs. Zohrabian et al. (2004) further studied the magnitude of the 2002 WNv epidemic nationwide in which of the 4,156 US cases (2,942 CNS cases, 284 deaths) the short-term cost was estimated at $140 million. In another study Zohrabian, et al. (2006) estimated the overall cost of WNv illness per person,[47] with asymptomatic illness having no cost, an uncomplicated case of febrile illness estimated at $1,000.00 USD, and a case of neuroinvasive illness with full recovery estimated at a cost of $27,500.00 USD. In that study, they found that the median age of WNv fatal cases since 1999 was 77 years, and the cost due to death estimated at $200,000.00 USD. While it is difficult to predict the future incidence of WNv in any given year and in any affected geographical area, the short-term cost attributable to an epidemic will be substantial.

The public health utility of a vaccine largely depends on incidence, geographic distribution, and the severity of the WNv disease. Zohrabian et al. (2006) estimated vaccine cost based on marketed vaccines at a baseline of $100.00 USD each, and the average cost per case of WNv illness prevented at $34,200.00 USD. The study found that the risk for WNv infection, probability of symptomatic illness after infection, and cost of the vaccine have the greatest influence on the cost-effectiveness outcome. While both the US and Canada have invested heavily in research on emerging and reemerging infectious diseases, unnecessary delays in

46 Included inpatient and outpatient costs for 329 reported cases; non-medical costs such as productivity losses caused by the illness, premature death, transportation to visit health care providers and child care expenses; the cost incurred by public health and other government agencies for epidemic control; but did not include intangible costs such as pain and suffering. The total cost of illness was $10.9 million USD or 54% of the total cost of the epidemic, with the remaining $9.2 million USD (46%) attributed to public agency cost. See Zohrabian et al, 2004.

47 Included medical treatment costs, and productivity losses due to death.

clinical research and the lack of preparedness planning can deprive the public of appropriate programs and increase costs. As these infections are often time-sensitive, the need for novel approaches that prevent delays and unnecessary costs are needed (Jester, et al., 2006). According to Nosek (2004) when research has to be accomplished while a disease is spreading, an epidemic may become a pandemic and pandemonium may ensue. Preparedness planning in a non-emergent state is an excellent method of establishing the research-based strategies that are required, for the successful implementation of a potential vaccine program. Further, planning of proposed interventions for health outcomes and cost benefits may provide evidence to support or refute current policy considerations, or add cumulative research to strengthen future health policy directions.

## Needs Assessment

According to Monath (2001) the question is often asked whether the impact of WNv disease is sufficient, to warrant the development of a vaccine for humans. The issues, trends, and challenges facing the commercialization of a human WNv vaccine include:

- low disease incidence
- uncertainties about the future medical impact
- high cost-to-benefit ratio for immunization and
- the high cost of completing vaccine development.

A more relevant question perhaps, is to consider the effect of WNv on an aging population, and the impact on government health policies and programs in the future. Seniors represent the fastest growing population group in Canada, with Health Canada (2002) estimating that by 2026

1 in 5 Canadians will have reached the age of 65 years. While all ages are equally susceptible to WNv infection, advanced age is the most important risk factor for neurologic disease with those greater than 50 years having a 10-fold higher risk of developing symptoms (Sampathkumar, 2003). The fastest growth in the seniors population is occurring among the oldest, where those aged 85 years or older are expected to grow to 1.6 million or 4% of the overall population by 2041 (Health Canada, 2002). It is important to take steps to ensure that the right health programs, services, and supports are in place considering that the risk of developing WNv neurological disease is 43 times higher in patients >80 years (Sampathkumar, 2003).

Severe disease occurs primarily in adults, but neuroinvasive disease in children has been reported. According to Hayes et al. (2005), from 2002 to 2004, 1,051 WNv disease cases among children <19 years of age occurred in the US, in which 317 (30%) had neuroinvasive disease and 106 (34%) were <10 years old. In addition, Cohen et al. have reported in a large-scale study of Israeli soldiers published in 1999,[48] that seroprevalence[49] for WNv increased with age: from 7% in the 18 - 20 year age group to 10.5% in the 21 - 30 year age group, to as high as 41.9% for people aged 40 to 55 years (Weinberger et al., 2001).

Although WNv is a rare occurrence infectious disease, Hayes et al. (2005) reported that up to 55% of affected populations have become infected during epidemics in Africa. Recent outbreaks, however have yielded much lower attack rates, such as one in New York in 1999 with ~2.6% of residents infected and one in the Middle East in 1995 with <5% affected (Hayes et al., 2005). As WNv is one of the most widespread flaviviruses worldwide, from Europe to the

48 Cohen et al. tested 1,060 stored sera samples of soldiers 18 to 55 years of age, which were collected between 1982 and 1989.

49 The rate at which a given population tests positive by ELISA for particular antibodies.

Middle East, Africa, India, parts of Asia, Australia,[50] North America, parts of Central America and the Caribbean, morbidity and mortality can be considerable. According to Lieberman et al. (2007), the proportion of reported neuroinvasive cases is now much higher, about 40% in the past few years with 5 - 14% of these cases fatal, but up to 29% fatality among patients aged 70 years or more. Lieberman et al. (2007), reported that in a high percentage of non-fatal cases, permanent neurological disabilities are common, and are significantly worse in elderly patients.

It is also important to consider immunosuppressive drugs that make hosts more susceptible, and extend the lives of those already compromised with immunodeficiency disease. Since 2002, five new modes of transmission have been reported, with confirmed cases of WNv transmission associated with blood product transfusion,[51] organ transplantation,[52] breast-feeding,[53] transplacental transmission,[54] and laboratory acquisition[55] (Sampathkumar, 2003). In addition, during WNv epidemics, as many as 1 in 150 blood donations can have detectable levels of viremia (Wolf, et al., 2006). During the 2002 - 2003 WNv epidemics in the US, Wolf et al.

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50 In the form of Kunjin virus, a subtype of WNv.

51 Between August 2002 - January 2003 there were 20 confirmed cases associated with blood product transfusion, linked to 14 infectious donor units. See Sampathkumar, 2003.

52 In August 2002, 4 transplant recipients who had received organs from a common donor developed WNv infection. The donor had received blood product transfusion from a blood donor, who had viremia at the time of donation. See Sampathkumar, 2003.

53 In September 2002, the patient developed WNv meningoencephalitis after receiving a post-partum blood transfusion from a WNv infected donor. Breast milk was positive for WNv and for WNv-specific IgG and IgM antibodies. A serum sample from the newborn showed WNV-specific IgM antibodies. See Sampathkumar, 2003.

54 An adverse pregnancy outcome was reported in August 2002 at 27 weeks gestation; serum and CSF samples were positive for WNv-specific IgM. The infant born at 32 weeks had serologic evidence of WNv infection, bilateral chorioretinitis, severe bilateral white matter loss in the temporal and occipital lobes. See Sampathkumar, 2003.

55 In 2002 there were 2 reported cases of occupationally acquired infection among laboratory workers associated with percutaneous injury, while performing autopsies on animals for WNv surveillance. See Sampathkumar, 2003.

(2006) reported that all cases of transfusion-transmission resulted from WNv IgM- negative[56] donations. These donations according to the study by Busch et al. (2005) were negative on mini-pool testing,[57] but were identified as having a low level of viremia on retrospective nucleic acid amplification testing of individual donations. Acute-phase infection is thought to be characterized by a brief period of very-low-level viremia, as reflected by the finding of IgM-negative specimens. This period is followed by a longer interval (approximately seven days) with an increasing and then decreasing viral load, which makes the viremia detectable by mini-pool testing. As IgM and IgG seroconversion evolves, the viral load decreases to a level detectable only by testing of individual donations.[58]

In 2003 the National Institutes of Health (NIH) and the Bill and Melinda Gates Foundation, considered their two highest priorities pertained to creating and improving vaccines against deadly and epidemic infectious diseases (Thomas Hegyvary, 2007). Successful translation of science into public health practice is a process that depends on needs, partnerships, and the applicability of research. Improved health programs and policies rely on relevant research to develop, test, and validate new interventions and methods of combating infectious disease (Baker, et al., 2001).

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56 IgM is the class of antibodies found in circulating body fluids. IgM antibodies are the first antibodies to appear in response to an initial exposure to an antigen.

57 The standard method of WNv testing across North America. Samples from individual units of blood are "pooled" together before testing, to enable blood operators to quickly and efficiently screen high volumes. If a positive test is received in any given "pool", each sample will then be tested individually and the infected unit(s) will be discarded. Donations in the pool whose samples are not positive for WNv can be used.

58 The use of mini-pool screening in 2003 prevented hundreds of WNv infections, but it failed to detect donations with a low level of viremia, some of which were antibody negative and infectious. The use of targeted nucleic acid amplification testing of individual donations in high-prevalence regions, was a strategy that was successfully implemented in 2004. See Busch et al., 2005.

# The WNv Genome

## *Molecular Biology*

WNv is an arthropod-borne virus ("arbovirus") that belongs to the *Flaviviridae* family, and is a member of the Japanese encephalitis virus (JEV) serogroup. All flaviviruses are antigenically related which accounts for serologic cross-reactions observed in diagnostic tests; however, members of the JE complex are more closely related. Specialized tests (eg. virus neutralization assays) are needed to differentiate the infecting flavivirus (Petersen and Roehrig, 2001).

As described by Brinton (2002), the WNv genome is a single-stranded RNA of positive polarity (mRNA sense) that is 11,029 nt in length, and contains an open reading frame (ORF) of 10,301 nt (see Appendix D, Figure 8). A type 1-cap structure ($m^7$GpppAmp) is present at the 5' end, while the 3' end terminates with $CU_{OH}$ (see Appendix D, Figure 9). Both ends contain noncoding regions (NCR), the 5' NCR is 96 nts, and the 3'NCR is 631 nts in length. Three viral structural proteins, capsid (C), membrane (prM/M), and envelope (E) are encoded within the 5' portion of the ORF, while seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) are encoded within the 3' portion.

According to descriptions from both Petersen and Roehrig (2001) and Brinton (2002), WN virions[59] are small (~50nm in diameter), spherical and enveloped. The isosahedral[60] nucleocapsid that encloses the virion RNA is ~25 nm in diameter, and is composed of multiple

59 A complete viral particle, consisting of RNA or DNA surrounded by a protein shell and constituting the infective form of a virus. The shell is called a capsid, and protects the interior core that includes the genome and other proteins. After the virion binds to the surface of a specific host cell, its DNA or RNA is injected into the host cell and viral replication occurs with eventual spread of the infection to other host cells.

60 A polyhedron having 20 faces.

copies of the C protein[61] (12-kDa). The capsid is enclosed in a host cell-derived envelope, that has been modified by the insertion of two integral membrane glycoproteins - E (53 KDa) and prM (18-20kDa). The prM protein (immature virions) are processed to pr + M protein late in the virus maturation, by the convertase enzyme furin.[62] The prM protein now cleaved to M protein (8kDa), is incorporated into the mature virion. The E-glycoprotein is the most immunologically important structural protein and is the viral hemagglutinin,[63] which mediates virus-host cell binding. Composed of three domains,[64] it elicits most of the virus neutralizing antibodies. Mutations in Domain III have been found to alter virulence, and Domain II may be involved in the binding of virus to cells.

According to Brinton (2002), the seven multifunctional nonstructural proteins make up the intracellular replication machinery of the virus. They appear to be either directly or indirectly involved in viral RNA synthesis, as viruses utilize cell proteins during replication cycles for attachment, entry, translation, transcription/replication, and assembly. NS2A, NS2B, NS4A, and NS4B are small hydrophobic[65] proteins that may facilitate the assembly of viral replication complexes, and/or their localization on cytoplasmic membranes. NS1 is a glycoprotein with 2

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61 A capsid is the protein shell of a virus, and consists of several oligomeric subunits made of protein. The capsid encloses the genetic material of the virus.

62 Furin is a protease of animal cells that is similar in structure to the bacterial protease subtilisin. It is enriched in the Golgi apparatus, where it functions to cleave other proteins into their mature/active forms. In addition to processing cellular precursor proteins, furin is also utilized by a number of pathogens.

63 Hemagglutinin (HA)is an antigenic glycoprotein found on the surface of bacteria and viruses. It is responsible for binding the virus to the cell that is being infected. The name comes from the protein's ability to cause red blood cells to clump together.

64 The ectodomain of E consists of three structural domains, DI, DII, and DIII. DI is structurally positioned between DII and DIII. The dimerization DII contains a 12-residue-long loop essential for virus–cell membrane fusion. The C-terminal DIII undergoes a major, pH-triggered, positional rearrangement essential for fusion and may also be involved in receptor binding.

65 The hydrophobic effect is an important driving force for biological structures, and responsible for protein folding, protein-protein interactions, formation of lipid bilayer membranes, nucleic acid structure, and protein-small molecule interactions.

glycosylation sites and 12 conserved cysteines,[66] which are essential for virus viability. It interacts with NS4A and plays a critical role in RNA synthesis, and may have a role in a cell activation mechanism. NS3 is a highly conserved protein and encodes a serine[67] protease that is not active, until it forms a stable complex with NS2B. The NS3 - NS2B complex is required for efficient polyprotein[68] processing, it upregulates the C-prM precursor, and promotes efficient secretion of prM-E from a vector. It also interacts with the polymerase NS5 to facilitate the coordination of helicase,[69] and polymerase[70] activities. NS5 is the largest and most highly conserved protein, and contains motifs characteristic of all RNA-dependent RNA polymerases (RdRps[71]). Stable secondary structures are located at both the 3' and 5' termini of the WNv genome RNA, and could be important for the formation of binding sites for viral and/or cellular proteins. There are several short conserved sequences with the 3' NCR, but their functional significance is not known. The 5' NCR and additional regions of the 3' NCR may be important for initiation of minus-strand RNA synthesis from the genome RNA.

Flaviviruses are transmitted between insect and vertebrate hosts during their natural life cycle, binding to an unknown cell receptor(s). As described by Brinton (2002) virions enter cells via receptor-mediated endocytosis[72] followed by low-pH fusion of the viral membrane, with the endosomal vesicle membrane[73] releasing the nucleocapsid into the cytoplasm (see Appendix D,

66 An amino acid, C3H7O2NS, derived from cystine and found in most proteins.
67 An amino acid, CH2OHCH(NH2)COOH, is a common constituent of many proteins.
68 A protein that after synthesis, is cleaved to produce several functionally distinct polypeptides (long proteins).
69 Any of various enzymes that catalyze the unwinding and separation of double-stranded DNA or RNA, during its replication.
70 An enzyme that catalyzes the formation of new DNA and RNA, from an existing strand of DNA or RNA.
71 RNA-dependent RNA Polymerase.
72 A process whereby cells absorb material such as proteins, from the outside by engulfing it with their cell membrane.
73 An endosome is a membrane-bound compartment inside cells. Many endocytotic vesicles derived from the plasma membrane, are transported to an endosome and fuse with it.

Figure 10). The genome RNA is released, and translated into a single polyprotein. The viral serine protease, NS2B-NS3, and several cell proteases cleave the polyprotein at multiple sites to generate the mature viral proteins. The RNA polymerase (RdRP), NS5, and other viral nonstructural and cell proteins copy complementary minus strands from the genomic RNA template, to serve as templates for the synthesis of new genomic RNAs. Viron assembly occurs in association with rough endoplasmic reticulum membranes. Intracellular immature virions accumulate in vesicles, and are transported through the host secretory pathway to the plasma membrane and released by exocytosis.[74]

***Phylogenetic Relationships***

The WNv can be divided genetically into two lineages on the basis of signature amino acid substitutions, or deletions in their envelope proteins (Brinton, 2002) (see Appendix E, Figure 11). Lineage 1 includes viruses from Africa north of the equator, Europe, Asia, and North America, with the Kunjin virus from Australia constituting a subtype, and is associated with the emergence of increased virulence (Burt et al., 2002). Lanciotti et al. (2002), further subdivide line 1 into three clades: WN viruses from Africa/Europe/United States/Middle East/Russia (1a); Kunjin viruses (1b); and India WN viruses (1c). Line 2 consists solely of viruses from Africa and Madagascar, and demonstrate ~ 75% nucleotide identity with lineage 1 viruses (Lanciotti et al., 2002). Originally thought only to be associated with endemic infection of low virulence in Africa as confirmed by Burt et al. (2002), strains of lineage 2 could also be pathogenic. According to Petersen and Roehrig (2001), the recent outbreaks in Europe and Asia have been most closely related to a line 1 WN virus first isolated in Romania in 1996 (ROM96) and Kenya

74 The process by which a cell directs secretory vesicles to the cell membrane. These membrane-bound vesicles contain soluble proteins to be secreted to the extracellular environment, as well as membrane proteins and lipids, that are sent to become components of the cell membrane.

in 1998, while the WN virus responsible for the 1999 US outbreak (NY99) was not. Petersen and Roehrig (2001) stated that the closest relative of the NY99 virus was Isr98 which circulated in Israel from 1997 to 2000, however only the US and Israel have reported illness and death in humans and animals caused by this Irs98/NY99 variant. The close genetic relationship between the Israel and New York WNv isolates, $\geq$ 99.7% nucleotide identity and $\geq$ 99.7% amino acid identity (only 2 nucleotide differences), suggested that the virus was imported into North America from the Middle East – very likely from Israel (Lanciotti, et al., 1999 and 2002). The means of its introduction however, will likely remain unknown.

Lanciotti et al. (2002) reported a comparison of the amino acid sequences for European WN isolates and revealed six amino acid changes consistent with the geographic origin of viruses: two in the E-protein (E-126 and E-159),[75] one in NS1,[76] one in NS3,[77] one in NS4A,[78] and one in NS4B.[79] Of the amino acid changes, only the regions at or near E-protein amino acids 126 (one of two primary Domain II – binding sites for virus neutralizing antibodies to Yellow Fever (YF) and Murray Valley Encephalitis (MVE) viruses), and 159 (associated with flavivirus attenuation) have been associated with phenotypic changes. There were no amino acid differences observed among the European and US WN viruses, within the pre-M and M proteins (Lanciotti et al., 2002). Lanciotti et al. (2002) furthered that E-glycoprotein changes resulted in the ablation of a potential E-protein glycosylation acceptor motif – NYS, believed to be important in stabilizing the E-protein conformation in low-pH environments. Modifying this motif will result in viral

75 E – 126; Ile → Thr and E – 159 (Italy 1998 and Romania 1996 only); Val → Ile.
76 NS1 – 70; Ala → Ser.
77 NS3 – 356; Thr → Ile.
78 NS4A – 85; Ala → Val.
79 NS4B – 249; Glu → Asp.

attenuation, or altering it at low pH results in mutants with changed virus-mediated cell membrane fusion characteristics (Lanciotti, et al., 2002).

According to Davis et al. (2005), there has been continued divergence of WNv as the temporal and spatial distribution of the virus has expanded. Davis et al. (2005) reported that North American WNv isolates obtained during 2003 and 2004 had accumulated a larger number of amino acid substitutions, than those obtained in 2001 and 2002. While the majority of isolates shared a conserved amino acid substitution at E159 (from V $\rightarrow$ A), the largest number of substitutions was found in the NS3 gene, with most deduced amino acid substitutions occurring in NS5. As 2002 was the year of the largest recorded epidemic of arboviral encephalitis in North America and geographic expansion by the WNv, Davis et al. (2005) suggested that these genetic changes may be significant. The nucleotide divergence may reflect the nature of the error-prone polymerase of WNv in that as the WNv replicates over time and location, mutations to the genome will continue, the consequences of which remain unknown (Davis et al. 2005). As suggested by Davis, et al. (2007), the rapid emergence of this dominant genotype across North America suggested a fitness advantage conferred by a mutation. Transmitted by *Culex pipiens* after fewer days of extrinsic incubation than needed by the prototypical strain, this may lead to an increase in transmission efficiency. The changing face of the WNv is a global phenomenon, requiring international cooperation in developing effective vaccines, and future planning using innovative strategies to introduce their use.

# Methodology

## *Research Question*

There are four licenced[80] WNv equine vaccines in use today. What approaches are researchers currently studying, with the goal of developing a safe and effective human WNv vaccine?

"Public health is a scientific and technical as well as a social and political endeavour that aims to improve the health and wellbeing of communities or populations" (Rychetnik et al., 2004, p. 538). According to Rychetnik, et al. (2004), "[e]vidence based public health can be defined as a public health endeavour in which there is an informed, explicit and judicious use of evidence that has been derived from any of a variety of science and social science research and evaluative methods" (p. 538). This definition highlights the use of a particular type of evidence that can include descriptive, taxonomic, analytic, interpretive, explanatory, and evaluative study types to inform public health decisions, with an emphasis on clear reasoning in the process of appraising and interpreting that evidence.

The narrative review is an important link between research evidence, and those decision-makers who plan future research agendas and establish clinical policy to provide optimal health care. Narrative reviews are useful to obtain a broad perspective on a topic, and often explore a range of related issues. According to Cook, et al. (1997) they are appropriate for describing the history or development of a problem and its management, and in particular are suited for cutting-

80 Fort Dodge Innovator® and Merial Recombitek® are licenced in Canada and the US. Intervet Prevenile™ and Fort Dodge West Nile Innovator DNA are licenced in the US.

edge developments like WNv vaccine research. When used in public health, they answer questions about health sector initiatives, as well as social policies that affect health (Rychetnik, et al., 2004). This project utilized a narrative systematic review of heterogeneous studies to describe the range of available evidence to identify, appraise, and synthesize WNv vaccine research.

This narrative review is a retrospective observational research study that describes and appraises previous work within a defined scope. A comprehensive search was used to find relevant studies, standards were developed to appraise study quality, and criteria were met to include studies in the review. The narrative review utilized information from 18 studies representing seven research approaches[81] (see Table 4). An effort was made to proportionately represent each research method, while acknowledging publication bias to Chimeric approach (both YF and Dengue) literature.

**Table 4. Summary statistics for "Checklist for Research Studies" instrument.**

| Column | N | Mean | Variance | Std. Dev. | Std. Err. | Median | Range | Min | Max | Q1 | Q3 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Time 1 | 18 | 87.55556 | 65.32026 | 8.082095 | 1.9049681 | 90 | 34 | 66 | 100 | 80 | 95 |
| Time 2 | 18 | 88.333336 | 52 | 7.2111025 | 1.6996732 | 90 | 29 | 71 | 100 | 85 | 95 |

The data set from "The Checklist for Research Studies" instrument was used, to determine the inclusion of studies in the narrative review. The sample of 18 was tested and there was no significant difference between the two sets of scores at time 1 and time 2, indicating the instrument reliably measured what it was designed to measure.

According to Rychetnik et al. (2004), critical appraisal criteria are checklists (standards) that are used to evaluate research evidence. The instrument "Checklist for Research Studies" (see

81 Some research approaches used more than one method. An attempt was made to classify a research study according to the primary research method used.

Appendix F) was developed to assess the strength of a study for use in the narrative review. It examined a study's population (research subjects), data collection, study design, results, and scored it for validity. To estimate the instruments reliability, its stability was evaluated using a test-retest strategy. The studies were rated using a strict criterion at time 1, and 2 weeks later at time 2 (see Appendix G). The reliability coefficient indicates that the magnitude of this test's reliability is quite high (see Table 5). As shown in Table 6, this indicates that a significant

**Table 5. Correlation matrix (Pearson).**

| Variables | Time 1 | Time 2 |
|---|---|---|
| Time 1 | **1** | **0.902** |
| Time 2 | **0.902** | **1** |

**Table 6. P-values.**

| Variables | Time 1 | Time 2 |
|---|---|---|
| Time 1 | **0** | **< 0.0001** |
| Time 2 | **< 0.0001** | **0** |

Values in bold are significantly different from 0 with a significance level alpha=0.05.

relationship exists between the two sets of scores. That difference between the means of Time 1 and Time 2 however are not significantly different from zero (see Table 7), further supporting the instruments reliability. For further statistical analysis, see Appendix H.

**Table 7. T-test for two paired samples (two-tailed test).**

| 95% confidence interval on the difference between the means (-2.514, 0.958) | |
|---|---|
| Difference | -0.778 |
| T (Observed value) | -0.945 |
| T (Critical value) | 2.110 |
| DF | 17 |
| p-value (Two-tailed) | 0.358 |
| Alpha | 0.05 |

The risk to reject the null hypothesis H0 while it is true is 35.77%.

The narrative review minimized bias by:

- clearly defining the review question
- searching the literature
- determining publication bias effect
- assessing a studies eligibility for inclusion, quality, and findings
- combining complex research in a qualitative easy to understand aggregation of information and
- placing the findings in context, and their applicability to policy and practice decisions.

The absence of effective treatment against WNv infection encourages vaccine development to prevent the infection in humans. As more literature becomes available, the challenge to keep-up-to-date with recent developments is immense. In addition, the technical aspects of published studies are often intimidating, time-consuming, and beyond the scope of many interested public health practioners. This narrative review discusses the various WNv vaccine approaches in an easy to understand format that will provide a good working knowledge of current research developments. The project contains excellent references, in which interested readers can expand their technical knowledge of each approach.

## WNv Vaccine Narrative Review

This narrative review of the WNv vaccine literature will inform those interested, of the current technology by summarizing evidence and explaining differences among approaches for the same research question. This knowledge can help plan future research agendas, establish

informed decision-making for clinical policy, and will strengthen the link between best research evidence and optimal [public] health care (Cook, et al., 1997).

The absence of effective treatment against WNv in horses encouraged vaccine research and development to prevent the infection. WN encephalitis emerged as a significant problem for the equine industry in both the US and Canada, through the deaths of valuable horses, restrictions of their movement and cancellation of equestrian events and racing (Monath, 2001). Several equine vaccines have been developed with some in use since 2001, which act as the catalyst for human research (Dauphine and Zientara, 2007).

**1. Inactivated "killed" virus.**

Formalin-inactivated whole virion vaccines produced in mouse brain tissue or cell cultures, have been successfully developed for other flaviviruses. They are relatively simple to develop and manufacture, have a good safety record, and can be used in both humans and livestock. According to Monath (2001) for preparation of a vaccine, the virus must replicate to high titer providing sufficient antigenic mass, and critical epitopes[82] must not be degraded. WNv replicates to very high titers (often $10^{10}$ infectious particles/ml) in several cell substrates, suitable for vaccine manufacture. According to Yang, et al., (2001) inactivated vaccines are safe, because they are non-replicating and noninfectious. However, there are several disadvantages to this approach, in that inactivated vaccines require multiple doses to elicit and sustain an effective immune response (Arroyo, et al., 2001). They do not elicit a strong cellular immune response, there is a difficulty in patenting a classical inactivated vaccine, which may reduce commercial

82 Sites on the surface of an antigen molecule, to which a single antibody molecule binds. An antigen has many different epitopes, and reacts with antibodies of many different specificities.

interest, and as they require multiple doses, they are less suitable for use in an impending outbreak (Monath, 2001). In addition, there are potential production problems on a commercial scale to grow virus for economical yield, and there are hazardous containment issues for large-scale growth of non-attenuated live virus (Lieberman et al., 2007).

The Fort Dodge Animal Health (FDAH) Innovator® WNv formalin-inactivated vaccine for horses was the first candidate available in 2001, and was licensed by the USA Department of Agriculture in 2003 (Dauphine and Zientara, 2007). According to the Innovator® product label, the 1 ML single pre-loaded syringe is administered intramuscularly (i.m.) in 2 doses, 3 - 6 weeks apart with annual revaccination with 1 dose (Fort Dodge, 2005). According to Agri-Med (2007a), horses need up to 4 weeks to develop immunity, and in safety tests in 649 horses, 96.28% were free of local or systemic reactions. Each series is tested to ensure that there is no surviving virus in the vaccine. It is combined to the adjuvant MetaStim™[83] for enhanced efficiency. The vaccine was shown to be 95% effective in WNv challenge studies. The vaccine contains thimerosal, neomycin, and polymyxin B as preservatives, cannot be used in horses within 21 days of slaughter; since horses will develop WNv-neutralizing antibodies (measured by PRNT), this may affect their ability to be imported/exported (Fort Dodge, 2005).

In their study, Wolf et al. (2006) evaluated the Fort Dodge WN Innovator® equine vaccine in baboons infected with WNv. They established that the vaccine reduced peripheral viremia and induced high titer neutralizing antibodies, which suggested that it accelerated the WNv-specific adaptive immune response. However when Innovator® was evaluated by Tesh et al. (2002),

83 MetaStim™ contains easy-to-metabolize lipids that improve vaccine performance, while minimizing the potential for injection site reactions.

their data suggested that the immune response in hamsters was insufficient to completely inhibit virus replication and that some degree of virus replication occurred after challenge with wild-type WN virus. It is not clear if vaccine efficiency is host-dependent however, according to Marra et al. (2004) "[b]irds inoculated with [Innovator] vaccine...have displayed varying resistance; with some species appear[ing] to acquire protection, while others do not" (p. 399).

Two "killed" human vaccines are currently in different stages of development. Baxter/Immuno's (Austria) candidate uses a Vero cell platform[84] whose origin is African Green Monkey *(Cercopithecus aethiops)* kidney cells, developed by Yasuma and Kawakita in 1963 (Sheets, 2000). Vero cells have been used as a vaccine cell substrate for virus replication studies, and plaque assays. According to Sheets (2002), the rationale behind the use of Vero cells (rather than primary monkey kidney cells), is that the cells can be banked and well characterized which helps to avoid lot-by-lot variability, contamination of primary cultures initiated for each production run, and ethical and economic issues associated with animal use. Vero cells can be readily adapted for growth and provide consistently high yields of virus, which allows for greater purity, larger lots, and more economical vaccine production. The working cell bank (WCB) continuous cell lines have bacterial and fungal sterility, are free from mycoplasma contamination, and of extraneous or inapparent viruses (Sheets, 2000). According to Barrett (2007), Baxter's WCB (no. 133) grows WNv to very high titers with high yields that are free from egg protein, thiomersal, and antibiotic additives. In addition, the master cell bank (MCB

84 The cell line brought to the NIAID, NIH at passage level 93 was submitted to the American Type Culture Collection (ATCC) at passage level 113, and propagated to passage level 121 to establish a bank of cells. Most vaccine manufacture is performed with cells at passage levels in the 130's or 140's, after further propagation for establishment of manufacturer's master and working cell banks and culture for vaccine production.

no. 128) is tested for the absence of tumorigenicity,[85] and for identity/genetic stability. The safety of Vero cells for the production of biologicals has been demonstrated for ~ 20 years and according to Baxter Vaccines (n.d.), is an ideal platform for the production of a WNv vaccine.

Crucell (2006) has produced an alum adjuvanted vaccine that protects against the WNv Israel 1998 goose strain, using their PER.C6®[86] cell technology that overcomes limitations in production capacity, processing time, and potential safety risks associated with the use of animal derived substrates (Crucell, 2007) (see Diagram 1). Ron Bout and Frits Fallaux (United States

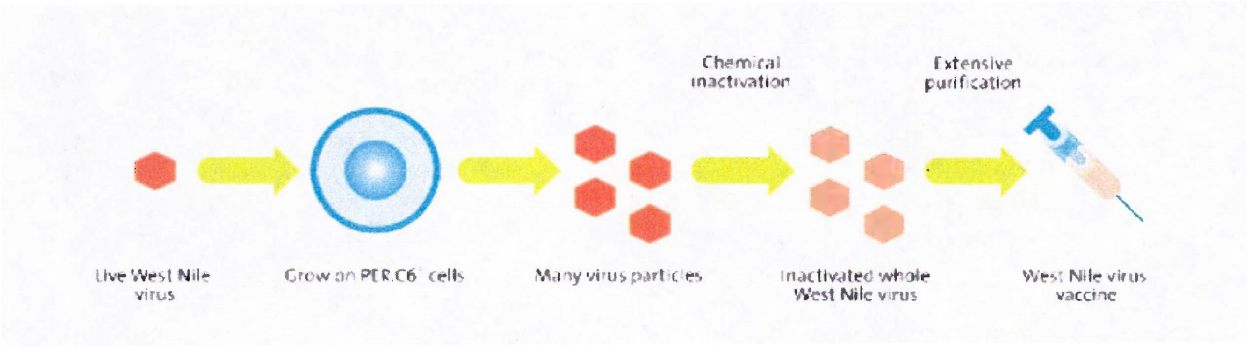


**Diagram 1. WNv Vaccine Production Process.**

Source: From "*About West Nile Virus*" Crucell Factsheet. West Nile Virus (2007), http://www.crucell.com/page/downloads/Factsheet_West_Nile_Virus_june2007.pdf. For personal use only.

of America [USA] Food and Drug Administration [FDA], 2001), created PER.C6 cells for pharmaceutical manufacturing of adenovirus vectors in 1995. Made from thawed embryonic

85 The ability of neoplastic cells growing in tissue culture, to multiply and develop into tumors when injected into animals.

86 PER.C6™ is a production cell line, based on healthy immortalized human cells. It is an attractive alternative production technology for the manufacturing of inactivated whole virus, live-attenuated, live-vector, subunit, and recombinant vaccines.

retinal cells that were transformed by a clone fragment[87] of the Adenovirus 5 genome, the cells came from tissue samples from a healthy aborted fetus[88] of 18 weeks gestation stored by Dr. Alex J. Van der Eb, Leiden University (USA FDA, 2001). According to Crucell (2006), their vaccine has been manufactured at the Netherlands Vaccine Institute (NVI) BioSafety Level 3 plant for use in clinical trials since 2005. According to PipelineReview (2007), the first safety study in humans with a whole inactivated flavivirus vaccine was completed in January 2007. The phase I randomized, double blind, placebo-controlled, dose-escalation study, tested three different dosages of the vaccine in 47 subjects. The two i.m. injections given within a three-week interval were locally and systemically well tolerated, and induced encouraging immune response.

In their study, Samina, et al. (2005) produced a formalin-inactivated vaccine in suckling mouse brains, and tested its protective immunity in commercial geese. The vaccine effectively protected geese against lethal, intracerebral (i.c.) WNv challenge, with one dose 60 - 70% effective and two doses giving 80 - 90% protection. Samina et al. (2005) reported that since 2001, when the vaccine was first used on a national scale, until the end of 2003, there were no reports of WN disease in geese. Crucell collaborated with the Israeli Kimron Veterinary Institute in the development of a WNv vaccine for geese, which produced excellent safety data and almost 100% efficacy in WNv challenge experiments (PipelineReview, 2007). In June 2004, the

87 In 1985, the cells were stored in liquid nitrogen with one of the files thawed in 1995 for the generation of the PER.C6 cells. Transfection had yielded a number of different colonies after 18 days, however, clone 6 was chosen because it gave the highest yield of viruses and also had high expression of E1A and E1B gene products. See USA FDA 2001.

88 According to Dr. Alex J. Van der Eb, the mother was healthy and the father unknown, which was the reason for the abortion.

existing WNv vaccine for geese was replaced with Crucell's PER.C6® based vaccine (Crucell, 2007).

**2. Live Attenuated.**

Evidence arising from projects to develop vaccines to prevent other flavivirus diseases has suggested that a live virus vaccine approach is preferable, as live vaccines have the potential to afford lifelong protection after a single dose (Markoff, 2007). According to Monath (2001), live viral vaccines replicate in the host and elicit an immune response similar to that mounted after natural infection. As antigenic mass of the virus expands, the antigens presented mimic those of the wild-type virus, and the cytokine environment created is similar to that induced by the pathogen itself. Intracellular replication elicits strong cytotoxic T cell responses and generation of long-term memory cells, which result in the stimulation of both humoral and cellular immunity. A single dose will result in a strong immune response, with immunity often life-long that does not require booster shots. There are, however, according to Lieberman et al. (2007), several disadvantages to using a live virus, as under-attenuation may result in disease whereas over-attenuation of the virus may abrogate vaccine efficacy. There remains the potential for reversion to wild type, or for mutation(s) to increase in virulence or decrease in efficacy, and live viral vaccines are contra-indicated for some specific populations such as the immune deficient/suppressed, pregnant, or elderly patients.

An approach by Lustig et al. produced a live attenuated WNv isolate derived from a wild type WNv strain, passed in *Aedes aegypti* mosquito cells. One dose of the attenuated virus showed 100% protection in both mice and geese using i.c. inoculation, following challenge with a homologous wild type WNv (Arroyo, et al., 2001). A potential 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 mutations may revert to the original virulent variant, once the virus infects the host (Castillo-Olivares and Wood, 2004).

**3. Infectious clone-derived.**

The development of cDNA infectious clones of WNv allows the attenuation of the virus by direct mutagenesis, by introducing mutations in regions of the genome that are known for their genetic stability such as the 3' noncoding region (NCR) (Castillo-Olivares and Wood, 2004). According to Markoff (2007), previous work has suggested that mutations in the stem-and-loop structure at the 3' terminus of the 3' NCR in WNv RNA (referred to as the "3'SL) of any flavivirus genome, could result in attenuation of virulence without a lethal effect on virus replication. Preliminary data from studies in the mouse model of the neurotropism (neurovirulence and neuroinvasiveness) of 3'SL mutant viruses selected for study, suggested that they are attenuated compared to the NY99 strain currently circulating in the US.

Markoff (2007) suggested that a candidate live WN vaccine should exhibit no lethal effect in the mouse model at any dose. Additional mutations that target the NS2B/NS3 protease genes and the envelope (E) gene will likely result in further attenuation of the virulence, and may be essential for eliciting a durable protective WN-specific immune response in humans. Using previously identified mutations in the viral protease encoded within the NS2B and NS3 gene segments, "second-site" mutations may alter virus replication without abrogating it. According to Markoff (2007), mice vary in their sensitivity to WN infection in relation to age, which makes it difficult to do WNv challenge studies. Therefore very attenuated single or double mutant WN

viruses will be evaluated in the hamster model (who do not vary in their sensitivity), for their ability to protect hamsters against WNv challenge.

Yamshchikov, et al. (2004) suggested that the development of a live-attenuated WN vaccine with potentially better capability to elicit balanced humoral and cell-mediated immune response, is hindered by the high virulence and pathogenicity of the NY99 strain. WN viruses of line 2 have not been associated with disease outbreaks therefore Yamshchikov et al. (2004) suggested that they may be more attractive for development of a live attenuated vaccine. The development of the first WN infectious clone (WN1415)[89] was designed on the basis of isolate 956D117B3[90] (WN-Nigeria or WN-Wengler), a laboratory derivative of the B956 strain (Ugandan woman), which is 6,500-fold less virulent than NY99. WN1415 (made from pWN1415, a molecular clone used to prepare synthetic RNA) was highly attenuated by an additional 50-fold; it induced a vigorous and balanced immune response and protected mice at low doses against the virulent NY99 strain challenge. The excellent growth characteristics in tissue culture, efficient protection induced by immunization with the attenuated molecular clone WN1415, and the availability of the highly stable infectious clone pWN1415 suggested that this approach might be useful in the development of veterinary and human live WN vaccines.

Borisevich et al. (2006) also suggested that the high virulence and pathogenicity of the NY99 strain make it questionable for use in the development of a live-attenuated WN vaccine. The envelope protein (E) is an important structural protein in virus-cell interactions, and is a major

89 There were silent substitutions in positions 4566 (C) and 8355 (U) of the WN1415 genome, as compared to 956D117B3. See Yamshchikov et al., 2004.

90 There were 32 point mutations, resulting in 14 amino acid changes over the genome. Most were in NS4A and NS4B, with only 3 in the structural protein region suggesting most of the changes were associated with intracellular replication with little or no effect on the virion properties. See: Yamshchikov et al., 2004.

target of host antibody responses (Shirato, et al., 2004). According to Shirato et al. (2004), the WN NY99 strain contains an *N*-linked glycosylation[91] motif (N-Y-T/S) at residues 154 - 156 of the E protein, whereas other strains lack the glycosylation site due to amino acid substitutions. Although controversial,[92] glycosylation of the E protein is a molecular determinant of neuroinvasiveness for the NY99 strains of WNv when administered in the mouse model by subcutaneous (s.c.) inoculation. In their study, Borisevich et al. (2006) assembled an infectious clone of the line 1 NY99-385 strain (Snowy Owl) and created chimeric[93] viruses, carrying reciprocal exchanges of the WN lineage 2 virus W956. The E protein of W956 (the molecular clone of parent 956D117B3, ancestor of prototype B956) is not glycosylated and carry a deletion of the entire 4 aa site. As can be seen in Diagram 2, the pCMVW956 and pCMVNY99

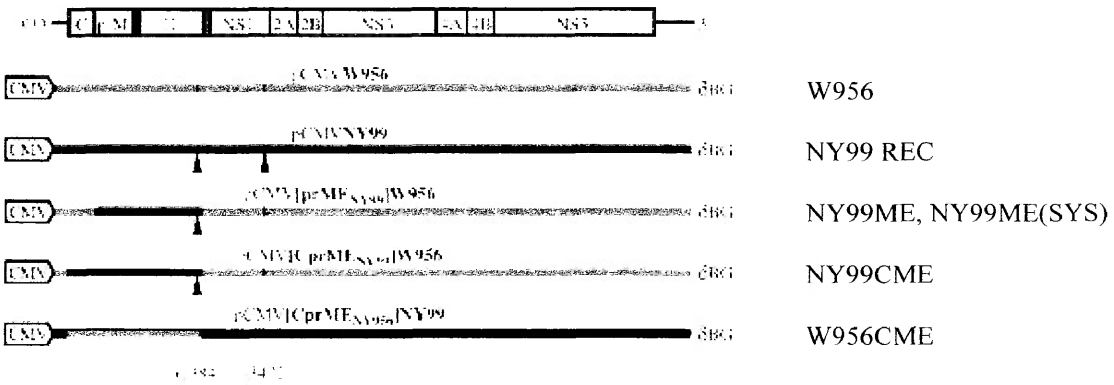


**Diagram 2. Parent and chimeric infectious DNA.**

Approximate locations of the intron present in particular constructs are marked by a filled arrowhead, genome positions of intron insertions are shown below.

Source: From "*Biological properties of chimeric West Nile viruses*" by Borisevich et al. (2006), *Virology,* 349, p. 372.Copyright © 2006 Elsevier Inc. For personal use only.

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91 Glycosylation is the process or result, of the addition of saccharides to proteins and lipids. It is an enzyme-directed site-specific process to the amide nitrogen of asparagine side chains. Polysaccharides linked at the amide nitrogen of asparagine in the protein, confer stability on some secreted glycoproteins. Glycosylation may play a role in cell-cell adhesion, a mechanism employed by cells of the immune system.

92 A glycosylated clone of the Kunjin virus (a subtype of WNv), produced 10 to 100-fold more virus in cell culture than non-glycosylated Kunjin virus. Therefore, the relationship of E protein glycosylation with WNv pathogenicity remains controversial. See Shirato et al. (2004).

93 Composed of parts that are of different origin, and are seemingly incompatible.

constructs were used to create the plasmid pCMV[prME$_{NY99}$]W956 which carried only the prM- E region of NY99 (not including its prM signal sequence) from position 466-2405 **[NY99ME]**, and its variant that encoded NY99 E in which the glycosylation site was mutated NYS→SYS **[NY99ME(SYS)]**. The next construct pCMV[CprME$_{NY99}$]W956 carried genes of all NY99 structural proteins instead of W956 **[NY99CME]**, and pCMV[CprME$_{w956}$]NY99 which transferred a fragment[94] for CprME of W956 into CMVNY99 **[W956CME]**. Neurotropism has been associated with the envelope protein therefore Borisevich et al. (2006) anticipated that the transfer of the NY99 structural proteins would result in the transfer of the highly virulent phenotype. This did not occur as both NY99ME and NY99CME that carried the structural protein genes of NY99 retained the phenotype of W956, although they reported an increased virulence.[95] The variant NY99ME(SYS) appeared as attenuated as W956 despite the presence of NY99 prM-E; however, the observed increase in virulence was eliminated.[96] The transfer of the structural protein genes from attenuated W956 into the genome of virulent NY99 in W956CME demonstrated the cytopathic phenotype of NY99, therefore the effect of E glycosylation on virulence and/or neuroinvasiveness is likely virus-specific. Borishevich et al. (2006) concluded that attenuation of highly virulent NY99 by mutagenesis of the E protein might not be adequate to yield a safe vaccine. The high immunogenicity, good growth characteristics and its highly attenuated nature makes W956 and NY99ME(SYS) suitable for continued testing in the development of WN live vaccines.

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94 The *Bg*/II – *Mfe*I fragment. See Borisevich et al., 2006.

95 An about 100-fold increase in virulence was observed, based on values of $LD_{50}$ (the lethal dose that kills 50% of the animals tested). See Borisevich et al. (2006).

96 Elimination of the glycosylation site in NY99$_{ME}$ by the (NYS→SYS) mutation completely eliminated the observed ~ 100-fold increase in virulence. See Borisevich et al. (2006).

**4. Live recombinant vector, poxvirus.**

A vector is any agent that acts as a carrier or transporter, and vectors are flexible means of vaccine delivery (Schlom and Abrams, 2003). With several major categories of vectors now in use,[97] viral vectors are generally safe, have low toxicity, are stable, and can have cell type specificity.[98] The advantages of a vector-based vaccine are that the entire antigen gene, parts of the gene or multiple genes can be inserted into some types of vectors. The relative cost for this type of vaccine production is low when compared with other approaches, and many vectors have the ability to infect professional APCs,[99] so that the antigens they express can be processed[100] (Schlom and Abrams, 2003).

The development of a live recombinant vector vaccine is highly regulated, with specific documentation of the vectors and recombinant viruses used in their development (Poulet et al. 2007). According to Despres et al. (2005), a live attenuated strain of measles virus (MV) is safe, and induces life-long immunity (after 1 or 2 low-dose inoculations). Easily produced on a large scale it has a stable genome, replicates in the cytoplasm, and has no reversion to pathogenicity, which makes it an attractive candidate vaccination vector. The secreted form of the WNv E (sE) glycoprotein mediates viral attachment and entry by membrane fusion,[101] and it has been shown to elicit protective immunity against WNv infection. In their study, Despres et al. (2005)

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97 Viral vectors include replication competent -vaccinia, adenovirus, RNA viruses (replicons); replication defective - avipox (fowlpox, canarypox), MVA, adenovirus; bacterial vector – listeria, salmonella, BCG; and DNA vectors. See Schlom and Abrams, 2003.

98 The viral receptor can be modified to target the virus to a specific kind of cell.

99 Antigen presenting cells (APCs) fall into two categories: professional or non-professional. The term professional is often limited, to those specialized cells that can prime T cells (i.e., activate a T cell that has not been exposed to antigen).These cells generally express MHC class II as well as MHC class I molecules, and can stimulate $CD4^+$ (helper) cells as well as $CD8^+$ (cytotoxic) T cells.

100 Professional APCs internalize antigen (either by phagocytosis or endocytosis), and then display a fragment of the antigen, bound to a class II MHC molecule on their membrane to T cells.

101 Membrane fusion involves the merger of two phospholipid bilayers in an aqueous environment. In viral fusion, proteins are responsible for merging the viral with the host cell membrane during infection. These proteins undergo spontaneous and dramatic conformational changes upon activation.

developed a Schwarz strain recombinant MV vector by inserting IS-98-ST1 cDNA coding for $sE_{WNV}$, into the Schwarz MV genome[102] (MVschw- $sE_{WNV}$) (see Diagram 3). The live attenuated MV expressing the additional viral gene proved stable, induced high titers of WNv-specific IgG antibodies, and protected mice against WNv encephalitis. The long-term occurrence of anti-WNv-specific IgG antibodies is essential for protection against WNv, and Despres et al. (2005) suggested that MVschw- $sE_{WNV}$ has the potential to elicit long-term immunity against both MV and WNv in children and adolescents.

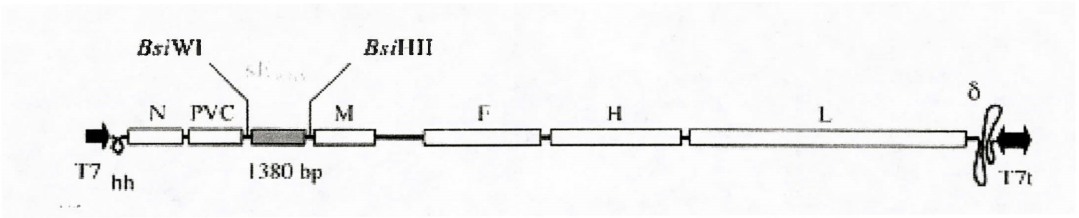


**Diagram 3. Schematic diagram of MVschw- $sE_{WNV}$**

Source: From "Live Measles Vaccine Expressing the Secreted Form of the West Nile Virus Envelope Glycoprotein Protects against West Nile Virus Encephalitis" by Despres et al., 2005, *The Journal of Infectious Diseases,* 191, p. 210. Copyright ©2004, Infectious Diseases Society of America. For personal use only.

The use of poxvirus vector started with the development of a rabies vaccine for wildlife[103] (Poulet, et al., 2007). According to Carithers (2004), poxviruses are large, double-stranded DNA viruses that are the only DNA viruses that replicate in the cytoplasm of the host cell rather than in the nucleus, therefore replication cannot occur in mammalian cells.[104] Canarypox is a member of the *Avipox* virus genus of the *Orthopox* virus family and is highly host specific, only replicating and causing disease in avian species. Originally isolated from a single pox lesion on an infected canary it was attenuated in chicken embryo fibroblasts and designated KANAPOX®,

102 A cDNA plasmid containing a truncated WNv E protein sequence was introduced between the P and M genes in MV genome, between the $B_{SI}W1$ and $B_{SS}HII$ sites of the additional transcription unit at position 2. See Despres, et al., 2005.

103 Merial, USA RABORAL V-RG®.

104 ALVAC and ALVAC-derived recombinants do not replicate in non-avian cell lines, including monkey, mouse, cat, and human. See Carithers, 2004.

which was further subjected to purification before a single plaque isolate was selected, propagated, and designated ALVAC. Canarypox recombinant vaccines contain only a small portion of the genetic material of a pathogen and not the complete pathogenic organism, therefore it is impossible for the vaccine to produce the disease. According to Poulet et al. (2007), once the sequence of the immunogen is known, synthetic genes can be made and inserted into the ALVAC genome, which allows for rapid generation of new constructs for emerging diseases. The ALVAC vector can express engineered foreign genes that result in the synthesis of proteins, which when presented to the immune system mimic natural infection and induce both antibody and cell-mediated immunity.

According to Thompson (2004), Merials RECOMBITEK® equine WNv vaccine utilizes recombinant DNA technology to isolate the portions of the WNv genome that code for the specific proteins (M and E) necessary to stimulate protective immunity against WNv. It has been safety tested in 713 horses (including 231 foals 2 - 4 months of age), and was found to provide early protection after a single dose. After two doses, it was proven 90 - 100%[105] effective following a challenge using live WNv-infected mosquitoes. Recommended annually, RECOMBITEK licensed in 2004 contains a 1 ML lyophilized recombinant canarypox vectored vaccine with a sterile liquid diluent containing gentamicin.[106] The second i.m. dose is given 4 - 6 weeks later, and vaccination is not recommended within 21 days prior to slaughter. According to Carithers (2004), vaccination with one canarypox recombinant vaccine does not interfere with

105 In a 2-dose immunity study, 9/10 horses were fully protected following a WNv-infected mosquito challenge. In another study, the vaccine demonstrated 100% efficacy in live challenge 2 weeks after the 2-doses series. A single dose study documented 8/9 horses were protected at 26 days post-vaccination, including 6 with an absence of neutralizing antibody indicating that cell-mediated immunity played a role.

106 As reported by Dauphin and Zientara (2007), RECOMBITEK® does not contain a Carbopol adjuvant according to the MSDS product label.

the immune response to the same recombinant vaccine or another recombinant vaccine. In addition, a single dose of RECOMBITEK can be given to horses previously vaccinated with Innovator® with excellent anamnestic, virus-neutralizing immune response (Merial, 2004).

The rate of WNv infection in cats and dogs in endemic areas is high, however infection rarely leads to severe clinical disease or mortality (Karaca, et al., 2005). Karaca et al. (2005) evaluated the safety and efficacy of ALVAC-WNV[107] a recombinant canarypox virus encoding the prM and E genes derived from a NY99 isolate of WNv, in cats and dogs. As with horse studies, a live mosquito challenge was performed, by allowing NY99-4132 strain (American crow) infected *Aedes albopictus* mosquitoes to feed on them. The ALVAC-WNv vaccine did not cause any adverse reactions regardless of dose, induced virus-neutralizing antibodies, and conferred protection against viremia. Although Karaca et al. (2005) suggested that it is unlikely that vaccinating cats and dogs will have an impact on WNv transmission within a community, ongoing safety and efficacy trials (including humans) suggest that other applications will be developed shortly for this approach (Poulet et al., 2007).

**5. Chimeric viruses (17D, DEN4).**

A great deal has been written about chimeras, that have the WNv surface antigen genes pre-membrane (prM) and envelope (E) inserted into the genetic "backbone" of a known attenuated flavivirus genome, from which the native prM and E gene segments have been deleted (Markoff, 2007). Infectious clone technology replaces the structural proteins of the vector virus which

107 Laboratory designation vCP2017, it was diluted in a phosphate buffer solution (PBS) prior to use. See Karaca et al. (2005).

results in a virion that has the protein coat of WNv, and contains all antigenic determinants for neutralizing antibodies and many epitopes for cytotoxic T lymphocytes (Monath, 2001).

The availability of the Acambis ChimeriVax ™ vaccine technology for the delivery of flavivirus protective antigens has contributed to the rapid development of a vaccine candidate against WNv (Arroyo, et al., 2004). A safe, live attenuated virus vaccine against YF (17D) was developed in 1936. After isolation of the Asibi and French strains of YF in 1927, the Rockefeller Foundation in New York developed the attenuated vaccine by serial passage of the Asibi strain in embryonated chicken eggs (National Network, 2005). According to Monath, et al. (2002), a single dose will induce neutralizing antibody response that is probably life-long,[108] and is an ideal live vector for the development of a new WNv vaccine.

The YF vaccine provides rapid and durable humoral and cell-mediated immune responses that closely mimic those directed against the wild-type virus (Arroyo et al., 2001). According to Monath (2001), ChimeriVax ™ WN uses the genes that encode the nucleocapsid (C) protein, nonstructural proteins (NS), and the untranslated terminal regions (UTR) responsible for replication of the YF 17D virus. The chimeric virus replicates in the host like YF 17D but immunizes specifically against WNv, because the prM-E genes from the WNv are inserted into the YF infectious clone. The cDNA transcribes to sense RNA, and the RNA is transfected into Vero cells to generate progeny virus (see Diagram 4). According to Arroyo et al. (2001), with positive-strand viruses there is a high rate of genetic variation caused by viral replication by polymerases, and these mutations involved in adaptation to different host cells can lead to

108 Revaccination is recommended every 10 years.

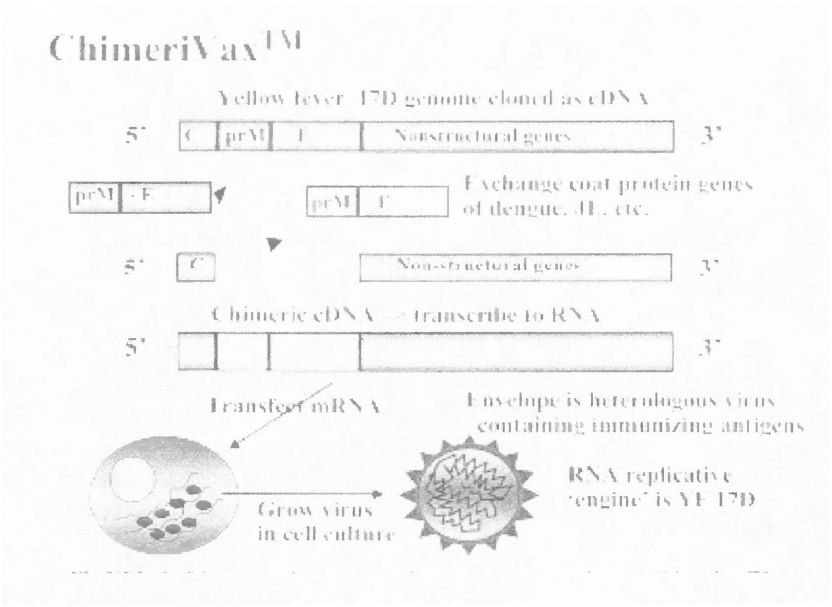


**Diagram 4. Construction of ChimeriVax™ vaccine using an infectious clone of YF17D**

Source: From "Prospects for Development of a Vaccine against the West Nile Virus" by Thomas Monath 2001, *Annals New York Academy of Sciences,* 951, 1, p. 6. No copyright listed. For personal use only.

unexpected surprises. In addition, replacing the structural genes of YF 17D with those of other flaviviruses might alter tropism, with the potential of replication in unanticipated tissues. The WNv prM and E genes used in the chimeric were from the wild type virulent WN NY99-383 (flamingo), and the construct was found to be significantly less neurovirulent than the YF 17D vaccine, which indicated that YF genes and the NS UTR had a dominant effect on the expression of neurovirulence (Monath, 2001).

In their study Langevin, et al. (2003), evaluated a ChimeriVax-WN candidate created from the WNv NY99-4132 (American crow) and YF 17D in fish crows (*Corvus ossifragus*). Fish crows only developed antibodies to the vaccine when inoculated with a high dose (20,000

PFU)[109] or a high booster dose (100,000 PFU). Chickens inoculated with YF 17D did not develop specific neutralizing antibodies, which indicated an inability of the virus to replicate and stimulate an immune response. The ChimeriVax-WN vaccine failed to induce protective immunity to WNv in chickens and fish crows, which indicated that it would not likely amplify in avian hosts or establish a natural transmission cycle.

The basis of attenuation lays within the genome sequence of RNA viruses, therefore sequence changes affect the phenotype by altering the function of the gene products, and/or the secondary structure integrity of the RNA molecules (Arroyo et al., 2001). According to Monath (2001), there are advantages in not introducing mutations in the WN prM-E genes since any changes must be retained through manufacturing, and after replication in hosts. However, altering the WNv E gene by site-directed mutagenesis at specific determinants is associated with altered virus tropism and changes in virulence (Arroyo, et al., 2001). In their study Arroyo et al. (2004) in designing a safer, further attenuated WN vaccine changed amino acids in the envelope protein that are established as genetic determinates of virulence.[110] Mutagenesis of the WN residues, E107, E316, and E440 (see Diagram 5) caused further attenuation of the YF/WNv chimera which was designated ChimeriVax-$WN_{02}$ and selected for clinical studies. The candidate was not neuroinvasive compared to the WN NY99 virus, and had reduced neurovirulence compared to YF 17D vaccine virus. Further attenuation was conferred by a mutation at E107 (L→F). The mutation was thought to reduce virulence, by altering the function of the fusion peptide in the

109 Plaque forming unit. A plaque is a clear area which results from the lysis of bacteria. Each plaque arises from a single infectious (bacterio)phage, intracellular parasites, that multiply inside bacteria by making use of some or all of the host biosynthetic machinery (i.e., viruses that infect bacteria.). The infectious particle that gives rise to a plaque is called a PFU.

110 For ChimeriVax-JE.

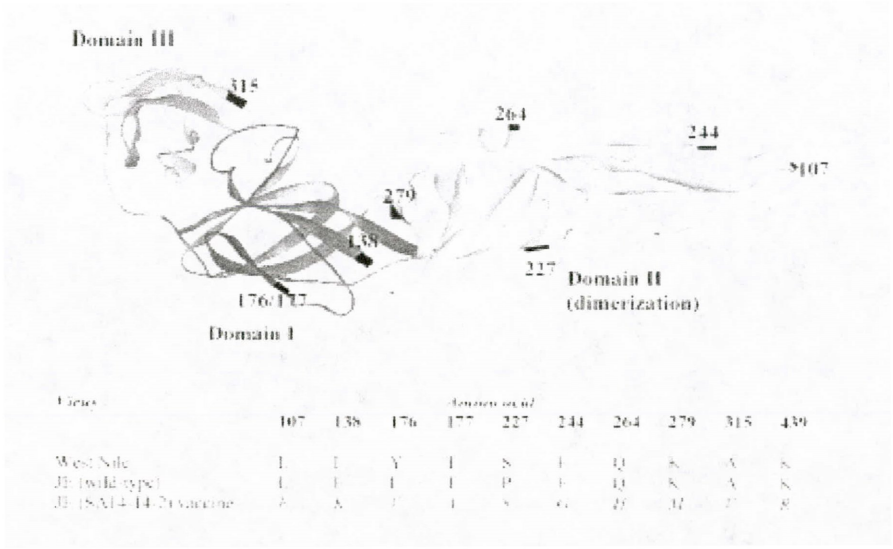


**Diagram 5. Sequence differences in the E glycoprotein of Japanese encephalitis vaccine, wild-type JE and WNv.**

The locations of the amino acid differences in the crystal structure of the E protein. It is hypothesized that insertion of mutations at the same locations in WNv will attenuate neurovirulence.

Source: From "Prospects for Development of a Vaccine against the West Nile Virus" by Thomas Monath 2001, *Annals New York Academy of Sciences,* 951, 1, p. 10. No copyright listed. For personal use only.

natural cycle of the virus replication. Residue 316 (A→V) may play a role in WNv cell entry, and E440 (K→R) may be involved in anchoring the E protein during its translation in the endoplasmic reticulum, therefore these mutations may alter the natural association of the E protein with prM. The safety of ChimeriVax-$WN_{02}$ was evaluated in the mouse model, rhesus monkeys, and cynomolgous macaques inoculated by the i.c. route. The ChimeriVax-$WN_{02}$ vaccine rapidly elicited a neutralizing antibody response, and provided protection against aggressive i.c. challenge with WN NY99 virus.

In their study Monath et al. (2006), reported on further testing of the ChimeriVax-$WN_{02}$ in monkeys, as well as the results of the Acambis phase I randomized, double blind placebo-controlled study in healthy male and female adults 18 - 40 years completed in May 2005. Of

interest was the higher viremias seen previously and in this study in monkeys inoculated with ChimeriVax-$WN_{02}$, which was associated with early replication of the virus in skin (inoculation site) and lymph nodes. However, in the clinical trial, ChimeriVax-$WN_{02}$ was well tolerated with nearly all subjects experiencing the expected low viremia, higher at the lower 3.0 $\log_{10}$ PFU dose than the 5.0 $\log_{10}$ PFU dose. This paradoxical response may have been due to a lower innate and delayed adaptive immune response to the lower dose. After a single dose, all subjects developed high titers of WN-specific neutralizing antibodies, and CD4+, and CD8+ T-cell responses against WN virus within 14 - 28 days. Of vaccinated subjects tested at 12 months, 97.2% were seropositive and retained high titers of neutralizing antibodies, which indicated that the vaccine could be useful in preventing illness and limiting outbreaks of WNv infection. In December 2005, ChimeriVax-$WN_{02}$ began a two-part randomized, double blind, placebo controlled phase II safety, tolerability, and immunogenicity trial in healthy adults >18 years. The first part of the study compared a single s.c. injection of 2 doses of vaccine with an inactive control, to determine the optimum vaccine dose.[111] The second stage of the trial is currently recruiting healthy adults aged 41 years and over, to assess the safety, tolerability, and immunogenicity of the optimum dose compared to placebo, for completion in January 2009 (ClinicalTrials.gov, 2007).

In September 2006, Intervet Inc. (licenced by Acambis in 2003) launched PreveNile™, a single 1 ML dose lyophilized YF-WN chimera equine vaccine, with sterile diluent containing gentamicin. In a safety study of 919 horses (229 horses 4 months or younger, 302 pregnant mares including 17 in 1st trimester, 11 in 2nd and 274 in 3rd), 100% of horses were protected from

111 In the first part of the clinical trial, Acambis evaluated a single dose of ChimeriVax-West Nile in 112 healthy adults aged 18 to 40 years. The primary immunogenicity endpoint was seroconversion rate (the percentage of subjects who generated neutralizing antibodies at a titre of at least 1:10), in which, over 97% of subjects seroconverted 28 days after a single vaccination. See Acambis (n.d.).

fever and viremia, and 95% were protected from clinical signs of disease. PreveNile™ could be given as early as at 5 months of age, and was >99% reaction-free. As with the other equine vaccines, it is not to be given to horses within 21 days of slaughter, revaccination is annual, and horses previously vaccinated with another product require only 1 dose (Agri-Med, 2007b).

Like WNv, Dengue is a flavivirus with the same ten genes coding 3 structural proteins and 7 nonstructural proteins. Transmitted by *Stegomyia aegypti* (formerly *Aedes aegypti*) mosquitoes, it is caused by any of the four viral serotypes designated DEN1-DEN4 (Dussart, et al., 2006). Pletnev et al. (2002) constructed a WN/DEN4 chimera (clone 18), in which the structural prM and E protein genes of WN NY99-35262 strain (flamingo) were substituted for the corresponding genes of the dengue virus type 4 (DEN4) (Caribbean strain 814669) (see Diagram 6). In addition, the WN/DEN4-3'Δ30 chimera was generated with a 30 nucleotide deletion in

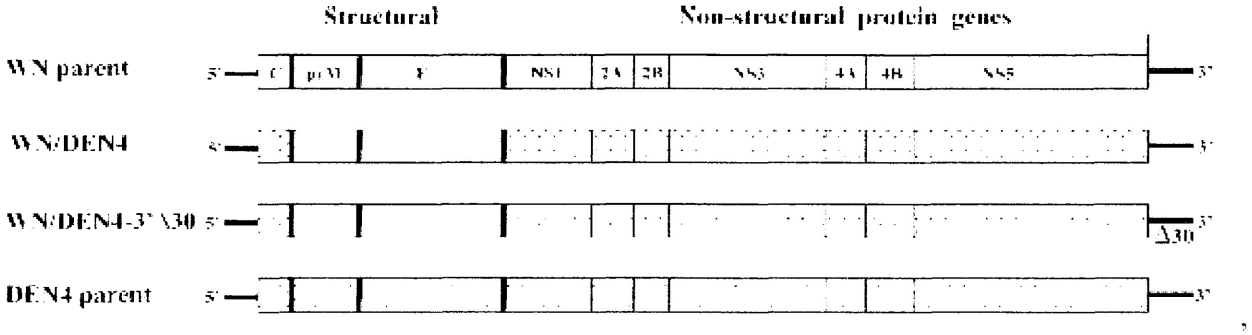


**Diagram 6. Structure of full-length genome of parental WN, DEN4 and their chimeric viruses.**

The 5'- and 3'- termini and the shaded regions are from DEN4, the unshaded are from WN. The vertical solid lines represent hydrophobic domains in the polyprotein, and Δ30 the position of a 30-nucleotide deletion in the 3' noncoding region of the DEN4 genome between nucleotides 10478 and 10507.

Source: From "Molecularly engineered live-attenuated chimeric west nile/dengue virus vaccines protect rhesus monkeys from west nile virus" by Pletnev, et al. 2003, *Virology,* 314, p. 191. Copyright ©2003 Elsevier Inc. For personal use only.

the 3' noncoding region (clone 1), that had been previously shown to render DEN4 safe but still immunogenic. The genetically stable 30-nucleotide deletion mutation has been proven to attenuate dengue virus in mice, monkeys, mosquitoes, and humans (Pletnev, et al., 2003). Analysis of the plasmid DNAs revealed four differences in nucleotide sequence, with the unmodified chimera slightly more immunogenic than the mutant one.[112] The unmodified WN/DEN4 chimera was ~28,571 times less neurovirulent, than its WN and DEN4 parents. Both chimeras were immunogenic in mice, and the chimera with the 30-nucleotide deletion was also found to be significantly less neurovirulent. Although dose-dependant, both stimulated a moderate-to-high level of serum neutralizing antibodies against WN and 90 - 100% protection against WNv challenge (Pletnev et al., 2002).

Pletnev et al. (2003) furthered their studies to rhesus monkeys and found that the WN/DEN4 candidate caused reduced viremia during infection, which demonstrated that chimerization itself resulted in attenuation. A high titer of neutralizing antibodies was induced, and monkeys were resistant to infection with WN challenge virus. It was proposed that the phenotype would be stable following replication in vivo, which suggested that many nucleotide changes in the WN/DEN4 genome would have to occur to restore virulence. The Δ30 mutation independently attenuated the chimera in monkeys and despite its high level of attenuation the WN/DeN4-3'Δ30 chimera induced a moderate titer of serum neutralizing antibodies. It also prevented viremia with WN challenge, which makes it a promising live-attenuated virus vaccine for humans.

112 Three differences produced amino acid substitutions in WN preM ($Ile_6 \rightarrow Thr$ and $Ile1_{46} \rightarrow Val$) and E ($Thr_{282} \rightarrow Ala$), and variability in the DEN4 NS4B ($Leu_{112} \rightarrow Ser$). See Pletnev, et al. (2002).

According to Pletnev et al. (2003), DEN4 is a desirable backbone for a chimera as primary infection in humans rarely results in severe liver or central nervous system disease, as opposed to other flaviviruses such as YF or JE. The DEN4 virus also has a large "menu of mutations," that include genetically stable deletion mutations that can attenuate it (Pletnev, et al., 2003). According to Whitehead (NIH), the vaccine candidates show a decreased rate of dissemination in mosquitoes, and although they are not efficacious in geese, they are in horses. The WN/DEN4 chimera was better than WN/DEN4Δ30 in equines, but both induced better immune responses than the FDAH inactivated Innovator® vaccine (WHO, 2004). A phase I study of the safety and immunogenicity of the WN/DEN4-3'Δ30 chimeric virus vaccine has been recruiting 8 - 50 year old patients since May 2006. The randomized, double blind, placebo control parallel assignment study is sponsored by the NIAID. With an expected enrollment of 84 healthy adults, the study will last 180 days in which participants in 3 cohorts will receive escalating doses of WN/DEN4-3'Δ30 or placebo (ClinicalTrials.gov, 2006 May).

Markoff (2007) suggested that although the preliminary results arising from the testing of these two chimeric vaccines have been encouraging, they only expose the vaccinee to two of [at least] ten WN genes and to none of the seven NS gene products. As exposure to the NS proteins may be essential for eliciting a durable protective WN-specific immune response in humans, there is the possibility that the YF 17D or DEN4 chimeric WN vaccines will not prove satisfactory.

**6. Subunit (E. coli, Baculovirus).**

Subunit vaccines use only parts of an organism, to stimulate an immune response. The gene or genes that encode appropriate subunits are isolated from the genome of the infectious agent.

The genetic material is placed into bacteria or yeast host cells, which then produce large quantities of subunit molecules by transcribing and translating the inserted foreign DNA. These subunit molecules are encoded by genetic material from the infectious agent, rather than from the host, so they do not cause the disease. These "foreign" molecules can then be isolated, purified, and used as a vaccine (Acambis, n.d.). According to Ledizet et al. (2005), a recombinant subunit vaccine uses a single highly purified antigen, which reduces the potential for cross-reactivity with host proteins. Higher antigen doses may be used to trigger a stronger immune response directed towards the protective E protein epitopes, which may be necessary to evoke protective responses in the elderly and immunocompromised populations. A disadvantage to subunit vaccines is that they require multiple doses to elicit and sustain an effective immune response, which make them less suitable for use in an impending outbreak, and they do not elicit a strong cellular immune response (Monath, 2001).

The envelope (E) protein is the outer surface protein of flaviviruses that mediates viral entry into host cells, and most protective antibodies against flaviviruses are directed against it (Ledizet, et al., 2005). According to Wang, et al. (2001), it has been suggested that the WN premembrane protein is necessary for immunity, either for the genesis of a protective response against the (pre) membrane or by stabilizing the E protein. In their study Wang et al. (2001), created a bacterially-expressed truncated form of the WNv E protein and demonstrated that recombinant E protein vaccination afforded full protective immunity. E protein antibodies were sufficient for partial immunity in mice and the initial 406aa (80%) of the E protein generated a protective immune response (Wang et al., 2001).

The gene that encoded the WNv E protein in the Wang et al. (2001) study was cloned as cDNA from isolate NY99-2741 (mosquito) (see Diagram 7). The recombinant maltose binding protein[113] (MBP)-E antigen consisted of amino acids 1 - 406 fused to the distal carboxyl terminus (C-terminus) of MBP, expressed in *Drosophila* S2[114] cells in the drosophila expression system. According to Ledizet et al. (2005), insect cells carry out various post-translational modifications including glycosylation,[115] which can result in an aberrant tertiary structure of the expressed protein, due to inadequate disulfide bond formation[116] and/or improper glycosylation. However, the secretion pathway in insect cells is similar to mammalian cells; therefore, antigens produced in these cells may resemble their mammalian native counterparts. The C-terminal

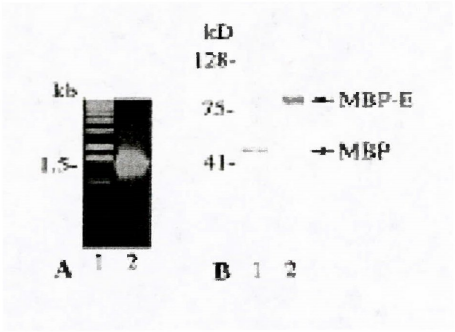


**Diagram 7. WNv E Protein.**

Patients with WNv infection develop Abs to the E proteins. **A.** Amplification of the gene encoding the E protein from WNv isolate 2741 (*lane 2*). *Lane 1*, Molecular mass markers. **B.** Purified recombinant WNv E protein (*lane 2*), expressed as a fusion protein with MBP. The 82-kDa recombinant fusion protein is designated MBP-E. *Lane 1*, Recombinant MBP.

Source: From "Immunization of mice against west nile virus with recombinant envelope protein" by Wang et al. 2001, *The Journal of Immunology,* 167, p. 5275. Copyright ©2001 The American Association of Immunologists. For personal use only.

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113 MBP is a part of the maltose/maltodextrin system of *Eschericia coli* bacteria, which is responsible for the uptake and efficient catabolism of maltodextrins. It is a complex regulatory and transport system involving many proteins and protein complexes.

114 The S2 cell line was derived from a primary culture of late stage (20-24 hours old), Drosophila melanogaster embryos. This versatile cell line grows rapidly at room temperature without CO2, and is easily adapted to suspension culture. It is available frozen in both serum-containing (Schneider's Drosophila Medium) or serum-free medium (Drosophila SFM).

115 Glycosylation is the process or result of the addition of saccharides to proteins and lipids.

116 Disulfide bonds play an important role in the folding and stability of some proteins, usually those secreted to the extracellular medium.

portion of the WN E protein contains a hydrophobic region, which may interfere with production and secretion of the recombinant protein in the absence of the prM protein. A truncated version of the E protein designated rWNV-$E_T$[117] lacked this region, and with two cloned non-native residues added to the *N*-terminus[118] it was not heavily glycosylated. rWNV-$E_T$ retained epitopes present in native WNv envelope protein; however, a possibility remained that it lacked some protective epitopes. According to Wang et al. (2001), mice immunized and boosted at days 14 and 28, developed a strong humoral response to the recombinant E protein and were fully protected from i.p. WNv challenge at a dose of $10^1$ PFU. The protective effect of vaccination, however, could be overcome by increasing the viral dose to $10^6$ PFU, which suggested the efficacy of protection might be dependent upon dose and the route of viral inoculation. Ledizet et al. (2005) immunized mice (boosted i.p. at day 28), and horses (boosted i.m. at day 25) with rWNV-$E_T$ absorbed to Alhydrogel adjuvant (Accurate).[119] Mice were protected against a lethal WNv challenge, and although horses were not challenged, a strong antibody response directed against the viral envelope protein was evident with antibodies still detectable 6 months after immunization. In previously vaccinated horses, a single booster injection administered 1 year after the initial immunization was sufficient to elicit circulating antibodies, and appeared as effective as FDAH Innovator®. The ability of rWNV-$E_T$ to induce a protective immune response in mice and horses indicated that the antigen might be suitable for further development as both equine and human vaccine.

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117 rWNV-ET does not contain inappropriate intermolecular disulfide bonds. See Ledizet et al., 2005.

118 After the recombinant protein is expressed and extracted from E. coli, the *N*-terminal extension can be used to purify the protein, and subsequently removed from the *N*-terminus to generate a nearly natural *N*-terminus sequence on the final product.

119 Aluminium hydroxide is included as an adjuvant for induction of a good antibody (Th2) response, however it has little capacity to stimulate cellular (Th1) immune responses.

A proprietary method of expression to produce recombinant envelope proteins from flaviviruses has been developed by Hawaii Biotech. In addition to the WNv envelope protein, a non-structural protein (NS1) has been included in their formulation to enhance the ability of the vaccine to elicit a cell-mediated immune response, along with the humoral component of immunity (Lieberman, et al., 2007). According to Lieberman et al. (2007), NS proteins are not present in mature virions, but are a necessary part of the enzymatic system for replication. Peptide epitopes processed from these proteins are displayed on the surface of infected antigen-presenting cells (in association with MHC class I or II molecules) and may be recognized by CD8+ or CD4+ T lymphocytes. When activated cytotoxic T cells are capable of eliminating virus-infected cells, this cellular immune response may contribute to the overall protective efficacy of a subunit vaccine. In addition, NS1 may elicit a humoral protective immune response involving the complement[120] fixing activity of antibodies to the protein. Lieberman et al. (2007) produced the WNV envelope (E) protein and non-structural (NS1) proteins in the *Drosophila* S2 expression system, which was efficient in terms of the quality and quantity of antigen produced. For the expression of a carboxy-truncated WNv envelope protein (80E), a synthetic gene encoded the prM protein and 80% of the E protein,[121] and was designated pMttWNprM80E. For the expression of a full-length WNv NS1 protein,[122] a gene fragment that represented nucleotides 2470 - 3525 and contained 352 amino acid residues, was constructed and designated pMttWNNS1. Mice vaccinated twice s.c. at 4 week intervals with the viral antigens that

120 The complement system is a biochemical cascade, which helps clear pathogens from an organism. It consists of a number of small proteins found in the blood, which work together to kill target cells by disrupting the target cell's plasma membrane. It belongs to the innate immune system, however it can be recruited and brought into action by the adaptive immune system.

121 From WNV NY99. The C-terminal truncation of the E protein at amino acid 401 eliminated the transmembrane domain to allow the E protein to be secreted into the medium. See Lieberman et al., 2007.

122 From WN NY99 flamingo.

included the adjuvant ISCOMATRIX,[123] elicited a humoral response displaying high titers of antibodies to both 80E and NS1 antigens. A cell-mediated immune response characterized by antigen-stimulated lymphocyte proliferation and cytokine production, represented a balanced Th1/Th2 immune response.

According to Lieberman et al. (2007), the inclusion of NS1 in a vaccine may provide further benefit by generating additional antibodies and/or T cells, capable of protective activities. In another study, the partial protection of hamsters against WNv encephalitis was obtained by vaccination with a formulation that contained NS1 as the only immunogen (Lieberman et al., 2007). With the 2006 Hawaii Biotech announcement that human clinical trials for safety are set to start shortly on ~50 patients (McAvoy, 2006), this WNv recombinant subunit-based vaccine may offer an important alternative approach.

Virus-like particles (VLPs) have been generated in insect and mammalian cell expression systems for many viruses,[124] and they appear to be an effective way to deliver antigens to vaccinate against viral infections. They consist of viral protein(s) derived from the structural proteins of a virus, and morphologically resemble the virus from which they were derived but lack viral nucleic acid. VLPs are capable of activating cells involved in both innate and adaptive immunity and therefore generate both humoral and cell-mediated immunity (Warfield et al., 2003). Qiao et al. (2004) generated WNv-like particles (WNV-LPs) containing the WNv structural proteins prME (nt 335-2427) and CprME (nt1-2636) from HNY99 (human brain), by

123 A saponin-based adjuvant containing Quil A and cholesterol components from CSL Ltd., Parkville, Melbourne, Victoria, Australia.
124 Including rotaviruses, parvoviruses, human papillomavirus, and HIV.

the use of the Bac-to-Bac baculovirus expression system.[125] The recombinant baculovirus designated bvWNVprME contained the coding sequence for PrM and E, and bvWNVCpreME contained core prM and E, which directed the production of polymorphic WNV-LPs in insect cells (see Diagram 8). In their study, Qiao et al. (2004) found that all mice immunized with prME-LPs with or without the adjuvant AS01B[126] developed anti-E antibodies after the fourth immunization, while the adjuvant enhanced both the anti-E antibody and anti-M antibody responses significantly. CprME-LPs induced a weaker antibody response to the M and E proteins and while prME-LP immunized mice had detectable titers of neutralizing antibodies, CprME-LP immunized did not. After WNv challenge, no morbidity or mortality was found in mice immunized with prME-LP while 67% mortality was observed in the CprME-LP group.

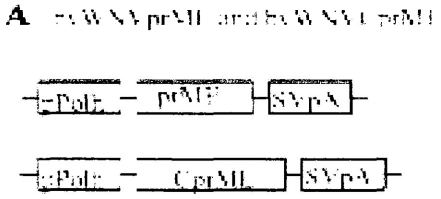

**Diagram 8. Segments of the WNV genome in the recombinant baculovirus expression vector.**

The bvWNVprME construct (top) contains the coding sequences for prM and E. The bvWNVCprME construct (bottom) contains the coding sequences for core, prM and E. pPolh = baculovirus polyhedron promoter; SV40pA = simian virus 40 polyadenylation sequence.

Source: From "Induction of sterilizing immunity against west Nile virus (WNV), by immunization with WNV-like particles produced in insect cells" by Qiao, et al. 2004, *Journal Infectious Diseases,* 190, p. 2106. Copyright ©2004 Infectious Diseases Society of America. For personal use only.

125 The Bac-to-Bac® expression cassette of the pFastBac™ vector recombines with the parent bacmid in DH10Bac™ E. Coli Competent Cells to form an expression bacmid, which is then transfected into insect cells for production of recombinant baculovirus particles.

126 Contains monophosphoryl lipid A and Qs21, provided by GlaxoSmithKline.

The presence of high titers of anti-E antibodies before challenge correlated with protective immunity. After challenge all mice had further increases in titers of anti-E, consistent with the presence of an anamnestic response directed toward the VLP's, of which the E protein was the major immunogenic component. None of the mice had circulating infectious virus or viral RNA when immunized with prME-LP plus AS01B, therefore viral replication was completely inhibited. While sterilizing immunity might be achieved in mice immunized with prME-LPs, it was not apparent why the CprME particles different only in the addition of core protein were less immunogenic. VLP-based vaccines are noninfectious and are easily controlled for quality and safety, which suggests that this approach may be promising for the development of an effective WNv vaccine.

**7. DNA/RNA.**

Unencapsulated ("naked") DNA plasmids started to be explored as a vaccine technology in the early 1990s, using an *E. coli* origin plasmid vector (Vasconcelos, 2006). DNA vaccines use the genetic code of an organism, to induce an immune response by delivering DNA constructs that encode specific immunogens directly into the host. Expression cassettes[127] transfect[128] host cells to become the in vivo protein source for the production of antigen, which results in immune response. By directly introducing DNA into the host, cells are instructed to produce the antigenic protein, which mimics viral replication. Snippets of viral DNA are used to produce a strip of DNA with the genetic code for proteins that are to be inserted into a loop of nonviral

127 Made up of one or more genes and the sequences controlling their expression, comprised of a promoter sequence, an open reading frame, and a 3' untranslated region that contains a polyadenylation site. The cassette is part of vector DNA used for cloning and transformation, that directs the cell's machinery to make RNA and protein.

128 Infection of a cell with purified viral nucleic acid, resulting in subsequent replication of the virus in the cell.

DNA called a plasmid. The plasmid and the foreign DNA are cut by restriction endonuclease,[129] producing intermediates with sticky and complementary ends. The two intermediates recombine by base pairing, and are stitched by the action of DNA ligase.[130] A new plasmid containing the foreign DNA as an insert is obtained, and introduced into bacterial cells to produce copies (Yang et al., 2001).

According to Yang et al. (2001), DNA vaccines are safe because they are non-replicating and noninfectious, with virtually no risk of reversion of the DNA to the disease-causing pathogen. They are affordable as production is simple and relatively inexpensive, and can induce long-lasting immune responses (Ishikawa et al., 2007). In addition, DNA vaccines offer a quick turnaround time and are less vulnerable to temperature changes, both assets in emerging epidemics and vaccination in the developing world (CDC, 2005 July). According to Monath (2001), DNA vaccines have a complex intellectual property landscape, and regulatory concerns due to the absence of preceding licensed products. The vaccine delivery is inefficient requiring the use of special injection devices (gene gun, electroporation[131]) and multiple doses. In addition to the slow onset of immunity, poor immunogenicity will be achieved without priming and boosting with different formulations. DNA vaccines are [currently] unproven for any infectious disease, and there is the issue of potential immunopathology due to the induction of an autoimmune reaction to the DNA over the long term (Lieberman, et al., 2007).

---

129 Restriction enzyme surrounds the DNA molecule at the point it seeks*(sequence GAATTC)*. It cuts one strand of the DNA double helix at one point and the second strand at a different, complementary point (between the G and the A base). The separated pieces have single stranded "sticky-ends," which allow the complementary pieces to combine.

130 The mechanism of DNA ligase is to form covalent phosphodiester bonds between 3' hydroxyl ends of one nucleotide, with the 5' phosphate end of another.

131 A method used to facilitate delivery of plasmid DNA into cells *in vitro,* that has been adapted for use in live animals, whereby an electrical field is created in tissues near the vaccine injection site. It is believed that transient discontinuities of cellular membranes enable entry of DNA directly into cells.

In their study Yang et al. (2001) constructed a DNA vaccine cassette that encoded for the WNv capsid (WNVCp) protein from isolate NY99, cloned into pCWNVCp (see Diagram 9). The antigen was chosen as a starting point to build a multicomponent vaccine for WNv that would consist of the structural genes (capsid, membrane, and envelope). The i.m. immunization of mice at days 0 and 14 with the WNVCp plasmid vaccine resulted in the induction of antigen-specific antibody and T cell-mediated immune response.[132] In addition, the infiltration of immune cells in the muscle at the injection site[133] suggested that the Cp vaccine could be an important component of a multiantigen vaccine for WNv.

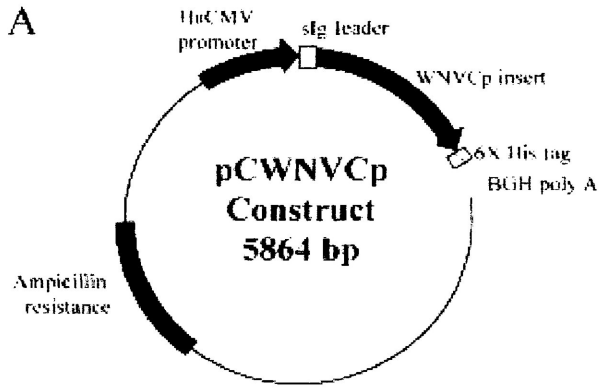


**Diagram 9. Construction of pCDNA3.1-V5/His plasmid pCWNVCp.**

Complete coding sequence of WNv capsid WNVCp was cloned into pCWNVCp. HuCMV = human cytomegalovirus; sIg leader = primer that encodes the human immunoglobulin secretory leader signals; 6 x His tag = for the exclusive purification of proteins in Escherichia coli; BGH poly A (bovine growth hormone polyadenylation) = a specialized termination sequence for protein expression; Ampicillin = plasmids carry drug resistance genes, only the bacteria containing the drug resistance plasmid will be able to grow.

Source: From "Induction of potent Th1-type immune responses from a novel DNA vaccine for west nile virus New York isolate (WNV-NY1999)" by Yang, et al. 2001, *Journal Infectious Diseases,* 184, p. 810. Copyright ©2001 Infectious Diseases Society of America. For personal use only.

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132 Strong and persistent IgG responses in serum, T-cell-mediated responses (significant expression of Th1-type cytokines IFN-γ and IL2) and elaboration of chemokines (MIP-1β and RANTES) in mice immunized with pCWNVCp.

133 CD4 and CD8 T cells and macrophages in mice immunized with p-CWNVCp.

Most of the gene-engineering flavivirus vaccines have been developed using the prM and E genes, as E protein plays important roles in receptor binding, fusion to the cell membrane and possesses most of the neutralizing epitopes. Although DNA vaccines are less effective at inducing humoral immunity, cells expressing prM and E secrete subviral extracellular particles (EPs) which are excellent immunogens (Ishikawa et al., 2007). The uptake of DNA plasmids by cells upon injection is very inefficient. According to Ulmer et al. (2006), only a small portion of the injected material is internalized by cells, to result in successful transfection. The types of immune responses induced by DNA vaccines therefore depend on the administration route. Studies using DNA vaccines that express EPs show that i.m. innoculations by a normal needle/syringe injection induce only Th1-dominat responses. Needle-free jet injections induce mixed Th1/Th2 responses, therefore administration of a protein-based vaccine using an injector can enhance the immunogenicity of the vaccine (Ishikawa et al., 2007). In addition, i.m. injection of plasmids immediately followed by electroporation increases antigen production by facilitating plasmid DNA delivery directly into cells (Ulmer et al., 2006). According to Inovio Biomedical Corporation, electroporation uses extremely short electrical field pulses to temporarily open cell membranes so therapeutic DNA can enter target cells. Once inside the cells this DNA then elicits the production of proteins to stimulate an immune response or provide therapeutic gene expression (Business Wire, 2006). The increase in immunogenicity makes a decrease in vaccine dose possible, which is critical for safety, time, and cost effectiveness (Ishikawa, et al., 2007). In their study, Ishikawa et al. (2007) constructed a DNA vaccine designated pcWNME from the WN NY99-6922 strain (mosquitoes). Mice were immunized twice i.m. by a spring-powered needle-free jet injector and challenged with WNv. Results

indicated that pcWNME induced neutralizing antibodies, and protected mice from lethal challenge at a low dose (0.1μg) when co-administrated by needle-free jet injector.

The study by Turell et al. (2003) tested a DNA plasmid designated pCBWN that coded for the WNv prM and E glycoproteins from NY99-397 strain (American crow) in fish crows. While all fish crows vaccinated i.m. with the DNA vaccine survived WNv challenge, a single dose did not elicit complete protection or sterile immunity in this species known to be highly susceptible to lethal WNv infection.

Hall et al. (2003) used the genetically stable Australian Kunjin (KUN) virus antigenically closely related to the WNv Sarafend strain and genetically related to the Ugandan strain, to generate a plasmid DNA. Created from KUN virus (MRM61C, 1960 Australia mosquito) and the WNv NY99-4132 strain (crow brain), it was designated pKUN1 and coded for the attenuated (by NS1 protein mutation), but infectious full-length KUN RNA.[134] Hall et al. (2003) demonstrated that mice immunized i.m (without coelectrotransfer[135]) twice had protective immunity against challenge with both the wild type KUN virus and the pathogenic NY99 strain. A significant advantage of a DNA vaccine expressing the full-length genome is that combined humoral responses to the NS1 protein and cell-mediated responses to NS23 epitopes may contribute to more efficient viral clearance and protection.

The successful development of a DNA vaccine for horses and salmon[136] could enhance the development of a human vaccine (Ulmer, et al., 2006). FDAH developed the world's first licenced (July 2005) WNv DNA vaccine for horses (CDC, 2005). West Nile – Innovator DNA

134 The KUN genome in the pKUN1 plasmid, is derived from the full-length cDNA clone FSDX. The sequence of the cDNA clones contain a Pro→Leu substitution at amino acid 250 in the NS1 gene.
135 Local application of electric pulses after DNA injection.
136 Licensed in 2005, it protects salmon against infectious hematopoietic necrosis virus (IHNV).

with thimerosal preservative is given i.m. in two 2 ML doses 2 - 4 weeks apart with one dose annual revaccination, and is not to be given to horses within 21 days of slaughter. According to the label, the duration of immunity has not been established, and revaccination is advisable when horses are faced with an outbreak or other conditions, which might make exposure to WNv likely (Drugs.com, 2007). According to FDAH, there has always been a problem with maternal antibodies from colostrum interfering with the foal's ability to mount an immune response using traditional vaccines. Maternal antibodies target proteins of the pathogen, and since DNA is not a protein, there are no antigens for the maternal antibodies to block. According to FDAH, "[o]nce in the foal's cells, the DNA directs them to make immunogens, which teach the immune system to recognize the pathogen and destroy it....when given early in the course of disease, a DNA vaccine can act therapeutically to reduce disease signs and severity...(West and Smith Thomas, 2005, p. 4). According to a CDC press release, horses vaccinated with a DNA vaccine can be differentiated from those that have been naturally infected, which may prove to be important in the importing/exporting of horses (CDC 2005, July). As the DNA vaccine contains a plasmid that encodes for structural proteins (PrM and E), a test to detect antibodies to the non-structural proteins which are normally produced in a naturally infected host, would differentiate between DNA vaccinates and wild-exposure (J. Tuttle, personal communication, October 3, 2007).[137] Although not listed on the product label, it was stated by a veterinary consultant at FDAH: "[t]his is a naked DNA vaccine – not recombined with anything....[i]t's just the DNA of the virus and an adjuvant..."[138] (West and Smith Thomas, 2005, p. 4). According to Ulmer et al. (2006), in the case of plasmid DNA vaccines, the immunostimulatory CpG motifs which trigger innate immune responses and are present in bacterial plasmids are important for effectiveness.

137 It was not disclosed if this "test" has been developed, or is currently in use.

138 According to Dr. J. Tuttle DVM Fort Dodge Professional Services, the DNA vaccine is adjuvanted with Metastim, a proprietary, dual-phase metabolizable stimulant. (Personal communication, October 3, 2007).

Adjuvant-active molecules such as cytokines, chemokines, and co-stimulatory molecules expressed from or mixed with the plasmid improve efficacy. In addition, using DNA plasmids that encode ligands[139] for NKG2D, a stimulatory receptor found on natural killer cells and activated CD8+ T cells can also increase effectiveness. For the application of DNA-based vaccines in humans, it has become apparent that novel adjuvants are needed. Vical Inc. announced in May 2007 the development of its patented Vaxfectin™ adjuvant that in animal studies with rabbits using DNA vaccines[140] delivered by needle-free injection yielded significantly higher antibody responses. With initial human testing[141] anticipated to begin in the second half of 2007, the dose sparing and immunogenicity-enhancing capabilities of the adjuvant are making it the preferred choice for Vical's new development projects (PR Newswire, 2007).

The NIAID with the Vaccine Research Centre (VRC) and Vical Inc. in March 2005 tested a WNv recombinant DNA plasmid vaccine in 15 healthy adults aged 18 - 50 years. The 32-week phase I study designated VRC-WNVDNA017-00 evaluated the safety, tolerability, and immune response to the vaccine that encodes the WNv prM and E genes.[142] The 3-dose 1 ML injections administered on days 0, 28, and 56[143] were given using the Biojector 2000 Needle-Free Injection Management System (ClinicalTrials.gov, 2005). The biojector forces the DNA plasmid into muscle cells where the inner machinery of the myocytes "read" the DNA, and converts it into the two WNv proteins. Recognizing that the proteins are foreign, they are displayed on the cell surface to alert the helper T cells for the production of antibodies to block the WNv from gaining

139 A ligand is an effector molecule binding to a site on a target protein.
140 Encoding cytomegalovirus (CMV) glycoprotein B (gB).
141 Using a DNA vaccine for influenza.
142 Although not specifically stated in the clinical trial information, the vaccine being tested used prM and E from NY99-397 strain (American crow) developed by the CDC's Jeff Chang DVM that was tested in mice, horses and American crows.
143 +/- 7 days, at least 21 days between injections.

entry into the cells, while the T cells kill infected cells outright (NIAID, 2005). The vaccine was safe, well-tolerated, and produced neutralizing antibody WNv-specific responses (in some subjects after 2 doses) in all 11 volunteers who returned for follow-up[144] (PipelineReview, 2006). In March 2006, a second 32-week phase I study started to test the safety, tolerability and immunogenicity of the WNv recombinant DNA plasmid vaccine designated VRC-WNVDNA020-00-VP in healthy adults aged 18 - 65 years. With an expected participation of 30 subjects enrolled in two groups of 15 each, aged 18 - 50 and 51 - 65 years, the same protocol will be followed with a long-term follow-up at week 52 (ClinicalTrials.gov, 2006 March). In June 2007, Vical Inc. was awarded a grant from the NIAID for further development of a DNA vaccine manufacturing process with the potential to produce several million doses of vaccines in a matter of days. The RapidResponse™ DNA vaccine manufacturing platform is a chemical synthesis enzyme reaction, that does not use any type of cell-based process. Instead, it uses polymerase chain reaction (PCR) to make small segments of DNA called linear expression cassettes that include only the DNA sequences essential for the vaccine, instead of a plasmid, which includes DNA sequences needed by the bacteria used in the fermentation process. The platform has the potential to cut the cost of production and to increase the speed of producing vaccines, and it can be used in a wide variety of pathogens (Levine, 2007).

**Summary.**

Each of these research approaches have advantages and disadvantages related to research history, safety, immune response, delivery, or vaccine applicability. Inactivated "killed" virus vaccines have a proven research record, and are safe for a variety of species. The approach

144 15 subjects were enrolled, 12 completed the 3-dose series.

however, requires multiple delivery that does not elicit a strong cellular immune response, and there are important safety concerns associated with large-scale production. Live attenuated vaccines also have a detailed research history, and while they use minimal delivery they produce an excellent and often life-long immune response. Safety issues associated with live virus, and limitations for certain populations are major disadvantages for this approach. Attenuation by direct and second site mutations improve the safety profile in infectious clones, and combined with a solid immune response and stable growth characteristics they are applicable for manufacture. The limited research history into the effects of glycosylation and mutagenesis of the WNv E protein however, is a distinct limitation in this approach. Live recombinant vectored and poxvirus vaccines have a worthy research history, are safe, use minimal delivery to produce a sound immune response, and are applicable to manufacture. However a considerable restriction is that most poxviruses are host specific, and antigenically similar. The use of a canarypox vector is particularly unacceptable for use in avian species, some of which are highly susceptible to the WNv and are the subjects of major WNv research. The chimeric approaches using YF or DEN4 have detailed research histories, along with favourable immune responses achieved with minimal delivery. While there are safety concerns related to the replacement of structural genes and potential mutations, a significant obstacle in this approach is that they do not work well in avian species. Subunit approaches have a good safety profile and are capable of eliciting a solid immune response. Without the use of adjuvants however, they do not elicit a strong cellular response and with multiple delivery that is dose and route dependent, they may be less suitable for use in an impending outbreak. DNA/RNA vaccines are safe and their relatively simple, inexpensive, and quick manufacture, make them particularly applicable for emerging epidemics in a variety of locales. DNA/RNA vaccines have a sparse and evolving research

history, that when combined with a multiple and inefficient delivery that produces a slow onset of immunity represents serious disadvantages to this approach. Designing a vaccine depends on several factors, however as the literature grows for each of these approaches new knowledge will bring the research closer to the ideal vaccine.

## Preparedness planning for a vaccine

As new vaccines come to market, they face challenges from policy makers, healthcare providers and the public. Pharmaceutical companies, public health advocates, healthcare providers, and health educators need to understand these diverse audiences and respond appropriately to the needs of each. Public health education is often difficult and lengthy, and misinformation and miscommunication are frequent when a new technology or approach is introduced (Sherris, et al. 2006).

Preparedness planning before the release of a new vaccine in the absence of an epidemic will provide the most effective method to coordinate efforts to avoid contradictory messages, confusion, and misunderstanding. Using the diffusion of innovations theory, which utilizes research-based strategies will effectively convey the need and manage expectations of a novel WNv vaccine before its release. Health policymakers are [generally] not experts in WNv disease or vaccination issues, therefore preplanning will produce information that is easily understood, and can be presented in a way that facilitates comparisons with existing vaccine programs. The planning of proposed interventions for health outcomes and cost-benefits can provide data on anticipated burden of disease, vaccine effectiveness, coverage of the target population and time

required to measurable disease reduction that are relevant to public health decision-making (Sherris, et al, 2006).

It is important to consider public health preparations for the launch of a future vaccine now; as this will ensure that those preparations will be in effect to help deal with [emergency] situations should they arise later. Health officials and scientists do not know when/if the next WNv epidemic will take place, however as the US Assistant Secretary for Health (AST) Dr. John Agwunobi[145] has stated: "[w]e cannot prevent...[but] we can prepare"(Spotswood, 2006, n.p.). This preparation includes the process of communicating not only with health policymakers, but the pharmaceutical industry, key global agencies who are influential to country-level decision making, and healthcare providers who are considered to be the primary and most trusted source of health and vaccine information by the public (Sherris, et al., 2006). There is much to build on from previous new vaccine introduction programs, however, preparedness planning for a not-yet-developed vaccine will benefit from using a framework with established strategies. Anticipated information needs and concerns can be used now to inform and guide effective communication, that will be essential for the rapid adoption of a potential human WNv vaccine.

145 John O. Agwunobi MD, MBA, MPH was confirmed by the U.S. Senate on December 17, 2005 to be Assistant Secretary for Health (ASH), U.S. Department of Health and Human Services, and an Admiral in the U.S. Public Health Service Commissioned Corps. As the ASH, Admiral Agwunobi serves as the Secretary's primary advisor on matters involving the nation's public health and science. He also oversees the U.S. Public Health Service and its Commissioned Corps for the Secretary.

## Conclusion

Despite advances in medicine and public health, infectious diseases continue to exact a substantial toll of morbidity and mortality throughout the world. These threats are not static, therefore as Dr. A. S. Fauci,[146] the Director of the NIAID has said, we must be prepared to respond quickly and effectively to new microbes as they emerge and to familiar pathogens that re-emerge with new properties or in unusual settings. Emerging and re-emerging public health threats such as WNv, pose a perpetual challenge. The task is not only to continue WNv research and the building of a strong foundation of basic and applied science to counter its threat, but also to be able to respond appropriately when a new vaccine is developed (Fauci, 2007). While it is unknown when/if a WNv vaccine will be licensed for human use, research is rapidly progressing using several promising approaches that have been outlined in this project. It is also unknown when/if a WNv epidemic will develop in Canada or another nation, but widespread disease could have significant implications for a countries economy, national security, the healthcare system, and the basic functioning of society. While preparing for either a potential epidemic, or the successful launch of a new vaccine is tremendously challenging, just merely having a plan does not necessarily mean that we are prepared. Today's preparedness planning needs to include strategies for tomorrow, that anticipate the day the WN virus knowledge goes from the lab to the public and changes from research to vaccination.

146 In 1984, Dr. Fauci became Director of NIAID, where he oversees an extensive research portfolio of basic and applied research to prevent, diagnose, and treat infectious diseases. He serves as one of the key advisors to the White House and Department of Health and Human Services on global AIDS issues, and on initiatives to bolster medical and public health preparedness against emerging infectious disease threats.

# Appendix A

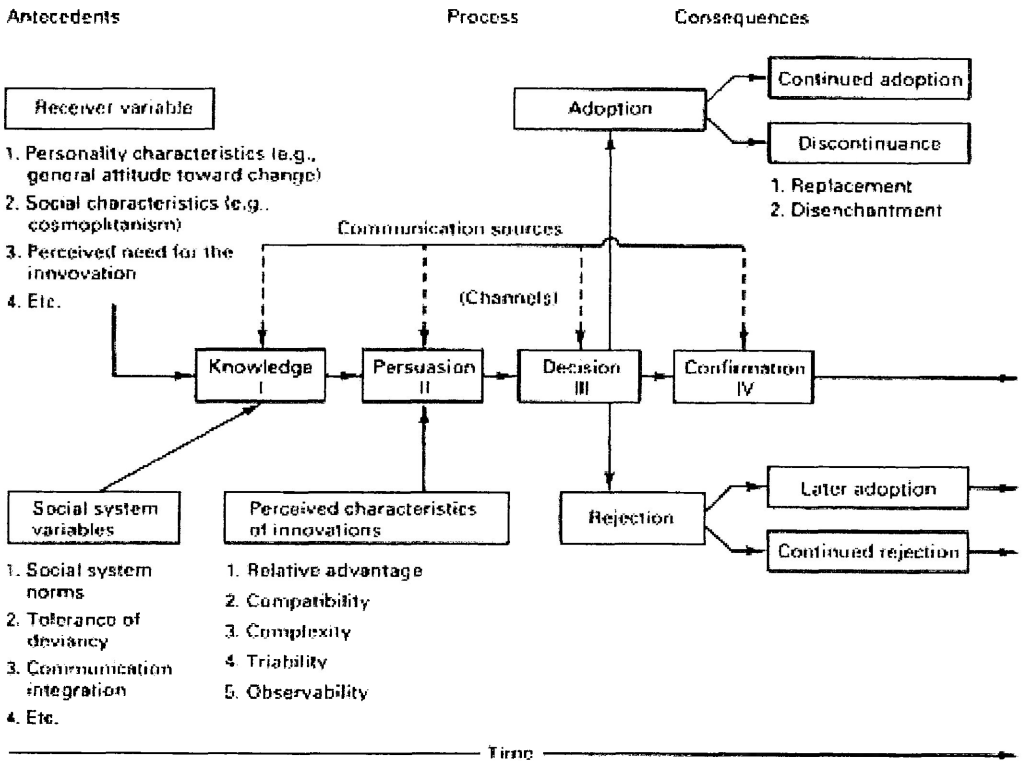



**Figure 1. Diffusion of Innovation Model.**

Source: From "*Diffusion of Innovations Theory*" by E. M. Rogers, 1995. Copyright ©2004 University of Twente, The Netherlands, http://www.tcw.utwente.nl/theorieenoverzicht/Theory%20clusters/Communication %20and %20Information%20Technology/Diffusion_of_Innovations_Theory.doc/. For personal use only.

# Appendix B

## Infectious Diseases and the Immune System

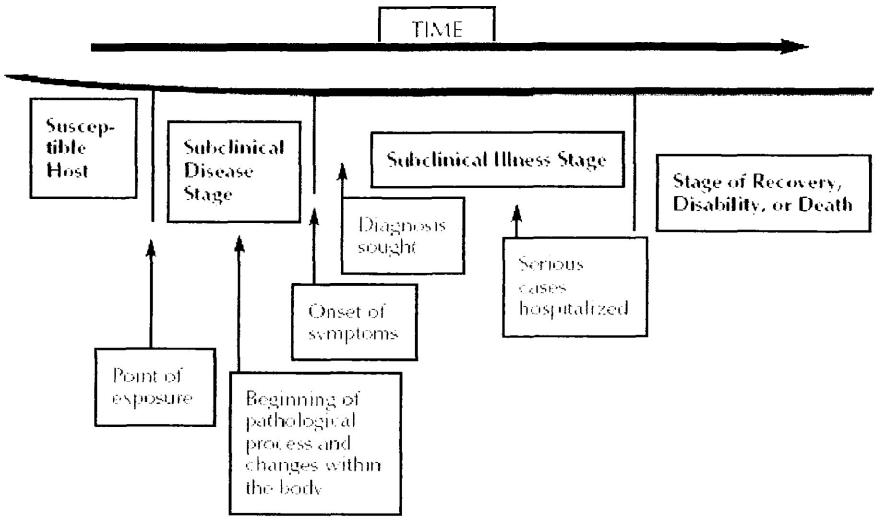



**Figure 2. Natural course of a communicable disease.**

Source: From "*An Introduction to Epidemiology*" (3rd ed.) by T. C. Timmreck, p. 31. Copyright ©2007, Jones and Bartlett Publishers. For personal use only.

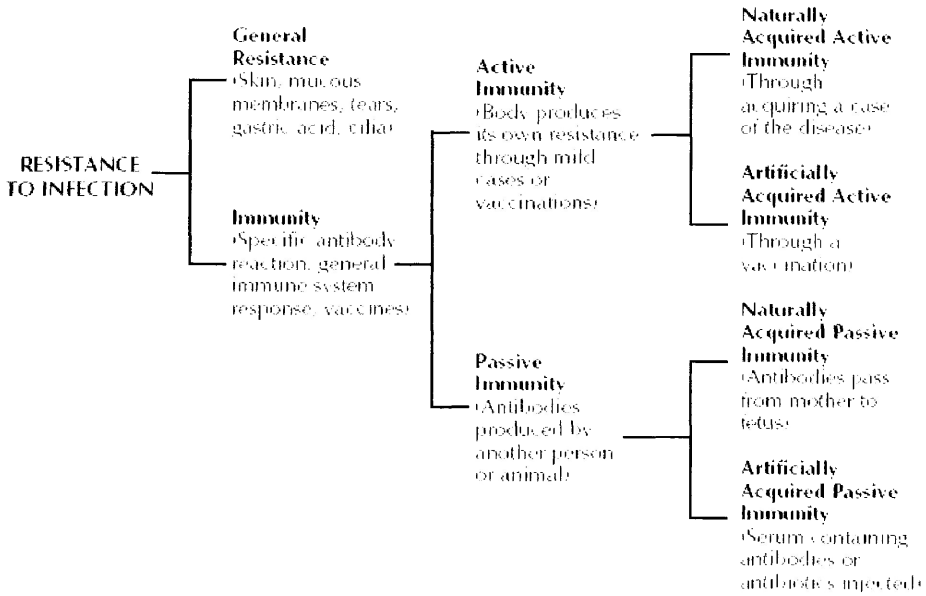



**Figure 3. How the human body resists infections.**

Source: From "*An Introduction to Epidemiology*" (3rd ed.) by T. C. Timmreck, p. 31. Copyright ©2007, Jones and Bartlett Publishers. For personal use only.

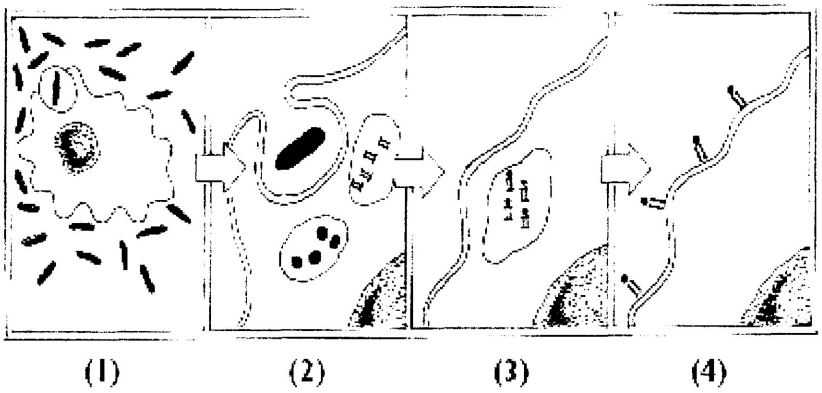



**Figure 4.**
(1) A macrophage in the presence of an infectious agent.
(2) The macrophage engulfs and breaks down the infectious agent into small fragments.
(3) The fragments bind to MHC Class II molecules that are produced by the macrophage.
(4) Complexes of antigen fragments and MHC Class II molecules are transported to the macrophage surface.

Source: From "*NPI Reference Guide On Vaccines And Vaccine Safety*" National Immunization Program, p. 6. Copyright ©2007, Centers for Disease Control and Prevention. For personal use only.

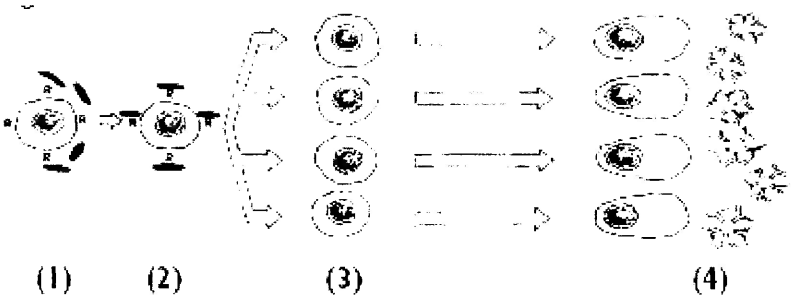



**Figure 5.**
(1) B cell in the presence of an infectious agent.
(2) Receptors on the B cell adhere to the infectious agent.
(3) The now activated B cell divides to produce many virtually identical copies of itself.
(4) The B cells mature into plasma cells that release antibodies that can adhere to the infectious agent leading to its destruction.

Source: From "*NPI Reference Guide On Vaccines And Vaccine Safety*" National Immunization Program, p. 7. Copyright ©2007, Centers for Disease Control and Prevention. For personal use only.

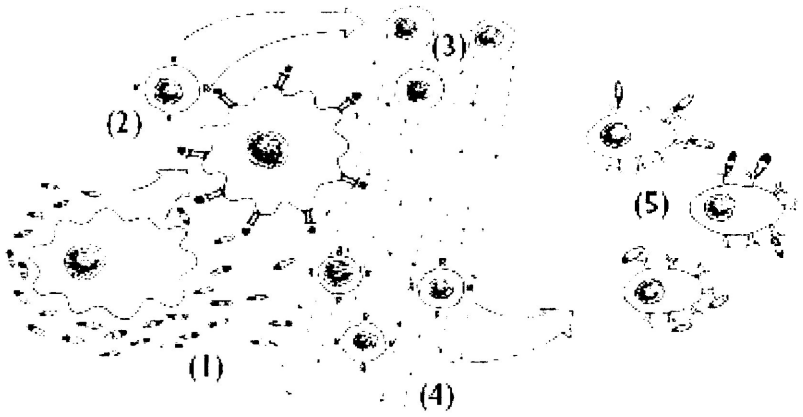



**Figure 6.**
(1) Macrophages, B cells and T cells are attracted to the site of an infection.
(2) The macrophage engulfs the agent and presents fragments to helper T cells.
(3) Activated helper T cells release cytokines that promote B cell activity.
(4) Different B cells recognize different parts of the infectious agent.
(5) Each B cell matures into an antibody releasing plasma cell.

Source: From "*NPI Reference Guide On Vaccines And Vaccine Safety*" National Immunization Program, p. 7. Copyright ©2007, Centers for Disease Control and Prevention. For personal use only.

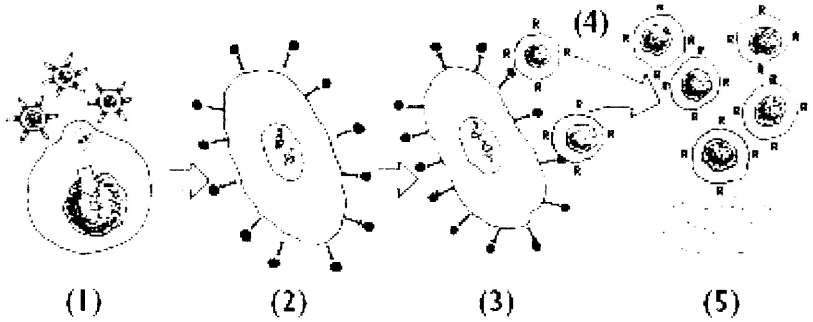



**Figure 7.**
(1) A virus infects a cell.
(2) Fragments of the virus bind to MHC Class I molecules produced by the cell.
(3) The virus antigen fragments are presented to cytotoxic T cells.
(4) Activated cytotoxic T cells divide to produce many virtually identical copies of themselves.
(5) Activated cytotoxic T cells destroy other virus-infected cells.

Source: From "*NPI Reference Guide On Vaccines And Vaccine Safety*" National Immunization Program, p. 7. Copyright ©2007, Centers for Disease Control and Prevention. For personal use only.

# Appendix C

## Vaccine Evaluation and Regulation

**Table 2. Stages of Clinical Vaccine Assessment and Detectable Adverse Events.**

| Phase | Number of subjects | Key study objectives |
|---|---|---|
| I | 10-<100 | • Immunogenicity<br>• Local/systemic reactions |
| II | 50-500 | • Optimal dose/schedule in target population(s)<br>• Ongoing safety assessment |
| III | 300-30,000 | • Immunogenicity/efficacy in target population(s)<br>• Ongoing safety assessment |
| **Regulatory authorization for vaccine marketing** | | |
| IV | Varies with study objectives (100 to many thousands) | • Immunogenicity/efficacy in not yet studied populations<br>• Possible interactions with other vaccines<br>• Expanded safety assessment |
| Post-marketing passive or active surveillance | General population | • "Real world" effectiveness<br>• Rare or unexpected adverse events ("signals") |

Source: From "*Canadian Immunization Guide*" (7th ed.) Public Health Agency of Canada, p. 60. Copyright © 2006, Public Works and Government Services Canada. For personal use only.

**Table 3. Description of Terms Used for the Frequency of Adverse Events Following Immunization.**

| Related adjective | Detectable range* |
|---|---|
| Very common | > 1/10 |
| Common | > 1/100 and < 1/10 |
| Uncommon | > 1/1000 and < 1/100 |
| Rare | > 1/10,000 and < 1/1,000 |
| Very rare | < 1/10,000 |

* The units for the detectable range may vary depending on how the data were derived and may be doses of vaccine administered, number of subjects immunized or doses of vaccine distributed.

Source: From "*Canadian Immunization Guide*" (7th ed.) Public Health Agency of Canada, p. 61. Copyright 2006, Public Works and Government Services Canada. For personal use only.

# Appendix D

## WNv Genome

→cap
MSKKPGGPGKSRAVNMLKRGMPRVLSLIGLKRAMLSLIDGKGPIRFVLALLAFFRFTAIAPTRAVLDRWRGVNKQTA
→prM
MKHLLSFKKELGTLTSAINRRSSKQKKRGGKTGIAVMIGLIASVGAVTLSNFQGKVMMTVNATDVTDVITIPTAAGK
→M
NLCIVRAMDVGYMCDDTITYECPVLSAGNDPEDIDCWCTKSAVYVRYGRCTKTRHSRRSRRSLTVQTHGESTLANKK
→E
GAWMDSTKATRYLVKTESWILRNPGYALVAAVIGWMLGSNTMQRVVFVVLLLLVAPAYSFNCLGMSNRDFLEGVSGA
TWVDLVLEGDSCVTIMSKDKPTIDVKMMNMEAANLAEVRSYCYLATVSDLSTKAACPTMGEAHNDKRADPAFVCRQG
VVDRGWGNGCGLFGKGSIDTCAKFACSTKAIGRTILKENIKYEVAIFVHGPTTVESHGNYSTQVGATQAGRLSITPA
APSYTLKLGEYGEVTVDCEPRSGIDTNAYYVMTVGTKTFLVHREWFMDLNLPWSSAGSTVWRNRETLMEFEEPHATK
QSVIALGSQEGALHQALAGAIPVEFSSNTVKLTSGHLKCRVKMEKLQLKGTTYGVCSKAFKFLGTPADTGHGTVVLE
LQYTGTDGPCKVPISSVASLNDLTPVGRLVTVNPFVSVATANAKVLIELEPPFGDSYIVVGRGEQQINHHWHKSGSS
IGKAFTTTLKGAQRLAALGDTAWDFGSVGGVFTSVGKAVHQVFGGAFRSLFGGMSWITQGLLGALLLWMGINARDRS
→NS1
IALTFLAVGGVLLFLSVNVHADTGCAIDISRQELRCGSGVFIHNDVEAWMDRYKYYPETPQGLAKIIQKAHKEGVCG
LRSVSRLEHQMWEAVKDELNTLLKENGVDLSVVVEKQEGMYKSAPKRLTATTEKLEIGWKAWGKSILFAPELANNTF
VVDGPETKECPTQNRAWNSLEVEDFGFGLTSTRMFLKVRESNTTECDSKIIGTAVKNNLAIHSDLSYWIESRLNDTW
KLERAVLGEVKSCTWPETHTLWGDGILESDLIIPVTLAGPRSNHNRRPGYKTQNQGPWDEGRVEIDFDYCPGTTVTL
→NS2A
SESCGHRGPATRTTTESGKLITDWCCRSCTLPPLRYQTDSGCWYGMEIRPQRHDEKTLVQSQVNAYNADMIDPFQLG
LLVVFLATQEVLRKRWTAKISMPAILIALLVLVFGGITYTDVLRYVILVGAAFAESNSGGDVVHLALMATFKIQPVF
MVASFLKARWTNQENILLMLAAVFFQMAYHDARQILLWEIPDVLNSLAVAWMILRAITFTTTSNVVVPLLALLTPGL
→NS2B
RCLNLDVYRILLLMVGIGSLIREKRSAAAKKKGASLLCLALASTGLFNPMILAAGLIACDPNRKRGWPATEVMTAVG
LMFAIVGGLAELDIDSMAIPMTIAGLMFAAFVISGKSTDMWIERTADISWESDAEITGSSERVDVRLDDDGNFQLMN
→NS3
DPGAPWKIWMLRMVCLAISAYTPWAILPSVVGFWITLQYTKRGGVLWDTPSPKEYKKGDTTTGVYRIMTRGLLGSYQ
AGAGVMVEGVFHTLWHTTKGAALMSGEGRLDPYWGSVKEDRLCYGGPWKLQHKWNGQDEVQMIVVEPGKNVKNVQTK
PGVFKTPEGEIGAVTLDFPTGTSGSPIVDKNGDVIGLYGNGVIMPNGSYISAIVQGERMDEPIPAGFEPEMLRKKQI
TVLDLHPGAGKTRRILPQIIKEAINRRLRTAVLAPTRVVAAEMAEALRGLPIRYQTSAVPREHNGNEIVDVMCHATL
THRLMSPHRVPNYNLFVMDEAHFTDPASIAARGYISTKVELGEAAAIFMTATPPGTSDPFPESNSPISDLQTEIPDR

AWNSGYEWITEYTGKTVWFVPSVKMGNEIALCLQRAGKKVVQLNRKSYETEYPKCKNDDWDFVITTDISEMGANFKA

SRVIDSRKSVKPTIITEGEGRVILGEPSAVTAASAAQRRGRIGRNPSQVGDEYCYGGHTNEDDSNFAHWTEARIMLD

NINMPNGLIAQFYQPEREKVYTMDGEYRLRGEERKNFLELLRTADLPVWLAYKVAAAGVSYHDRRWCFDGPRTNTIL

→**NS4A**

EDNNEVEVITKLGERKILRPRWIDARVYSDHQALKAFKDFASGKRSQIGLIEVLGKMPEHFMGKTWEALDTMYVVAT

AEKGGRAHRMALEELPDALQTIALIALLSVMTMGVFFLLMQRKGIGKIGLGGAVLGVATFFCWMAEVPGTKIAGMLL

→**NS4B**

LSLLLMIVLIPEPEKQRSQTDNQLAVFLICVMTLVSAVAANEMGWLDKTKSDISSLFGQRIEVKENFSMGEFLLDLR

PATAWSLYAVTTAVLTPLLKHLITSDYINTSLTSINVQASALFTLARGFPFVDVGVSALLLAAGCWGQVTLTVTVTA

ATLLFCHYAYMVPGWQAEAMRSAQRRTAAGIMKNAVVDGIVATDVPELERTTPIMQKKVGQIMLILVSLAAVVVNPS

→**NS5**

VKTVREAGILITAAAVTLWENGASSVWNATTAIGLCHIMRGGWLSCLSITWTLIKNMEKPGLKRGGAKGRTLGEVWK

ERLNQMTKEEFTRYRKEAIIEVDRSAAKHARKEGNVTGGHPVSRGTAKLRWLVERRFLEPVGKVIDLGCGRGGWCYY

MATQKRVQEVRGYTKGGPGHEEPQLVQSYGWNIVTMKSGVDVFYRPSECCDTLLCDIGESSSSAEVEEHRTIRVLEM

VEDWLHRGPREFCVKVLCPYMPKVIEKMELLQRRYGGGLVRNPLSRNSTHEMYWVSRASGNVVHSVNMTSQVLLGRM

EKRTWKGPQYEEDVNLGSGTRAVGKPLLNSDTSKIKNRIERLRREYSSTWHHDENHPYRTWNYHGSYDVKPTGSASS

LVNGVVRLLSKPWDTITNVTTMAMTDTTPFGQQRVFKEKVDTKAPEPPEGVKYVLNETTNWLWAFLAREKRPRMCSR

EEFIRKVNSNAALGAMFEEQNQWRSAREAVEDPKFWEMVDEEREAHLRGECHTCIYNMMGKREKKPGEFGKAKGSR

AIWFMWLGARFLEFEALGFLNEDHWLGRKNSGGGVEGLGLQKLGYILREVGTRPGGKIYADDTAGWDTRITRADLEN

EAKVLELLDGEHRRLARAIIELTYRHKVVKVMRPAADGRTVMDVISREDQRGSGQVVTYALNTFTNLAVQLVRMMEG

EGVIGPDDVEKLTKGKGPKVRTWLFENGEERLSRMAVSGDDCVVKPLDDRFATSLHFLNAMSKVRKDIQEWKPSTGW

YDWQQVPFCSNHFTELIMKDGRTLVVPCRGQDELVGRARISPGAGWNVRDTACLAKSYAQMWLLLYFHRRDLRLMAN

AICSAVPVNWVPTGRTTWSIHAGGEWMTTEDMLEVWNRVWIEENEWMEDKTPVEKWSDVPYSGKREDIWCGSLIGTR

ARATWAENIQVAINQVRAIIGDEKYVDYMSSLKRYEDTTLVEDTVL

**Figure 8. WN99.**

Deduced amino acid sequence of the polyprotein of WNv WN-Ny99 (395-99). The start of each protein is marked by an arrow. Abbreviations for protein names: cap = nucleocapsid; prM = premembrane protein; M = viral membrane protein; E = viral envelope glycoprotein; NS1 to NS5 = viral nonstructural proteins. The E-glycoprotein glycosylation motif (NYS) is underlined in bold. Single-letter abbreviations for amino acid residues are: A = Ala; C = Cys; D = Asp; E = Glu; F = Phe; G = Gly; H = His; I = Ile; K = Lys; L = Leu; M = Met; N = Asn; P = Pro; Q = Gln; R = Arg; S = Ser; T = Thr; V = Val; W = Trp; and Y = Tyr.

Adapted from: "DQ211652 West Nile Virus Strain NY99, complete genome. "National Centre for Biotechnology Information. National Institutes of Health, Genbank Database DQ211652, 2007. Copyright US National Library of Medicine, http://www.ncbi.nlm.nkh.gov/entrez/viewer.fcgi?db =

nuccore&id=7716600.
And "Origin of the west nile virus responsible for an outbreak of encephalitis in the Northeastern United States." by R.S. Lanciotti, et al., 1999, *Science,* 286, p. 2334. Copyright ©1999, *American Association for the Advancement of Science.* For personal use only.

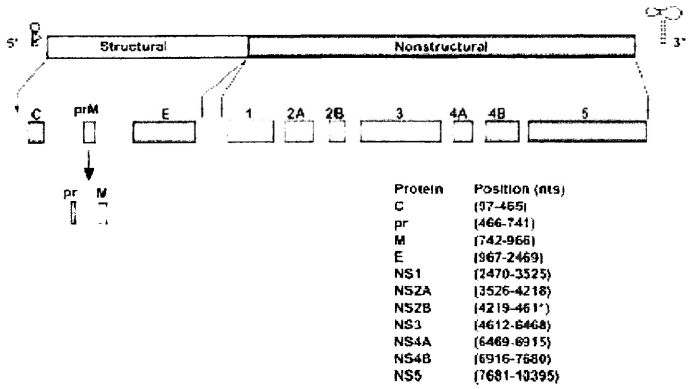



**Figure 9. The WNV Genome.**
The positions of each of the viral proteins in the nucleotide sequence of the genome of WNV, strain EG101.

Source: From "The Molecular Biology of West Nile Virus: A New Invader of the Western Hemisphere" by Margo A. Brinton, 2002, *Annual Review Microbiology,* 56, p. 374. Copyright ©2002, *Annual Reviews.* For personal use only.

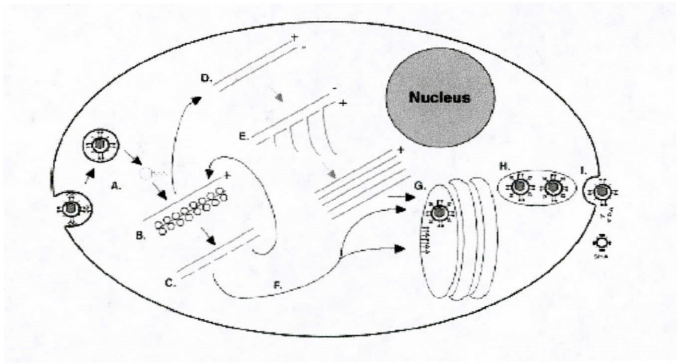



**Figure 10. The WNV Replication Cycle.**

(A) Attachment and entry of the virion.
(B) Un-coating and translation of the virion RNA.
(C) Proteolytic processing of the polyprotein.
(D) Synthesis of the minus-strand RNA from the virion RNA.
(E) Synthesis of nascent genome RNA from the minus-strand RNA.
(F) Transport of structural proteins to cytoplasmic vesicle membranes.
(G) Encapsidation of nascent genome RNA and budding nascent virions. SHA, slowing sedimenting. hemagglutinin, a subviral particle that is also sometimes released.

Source: From "The Molecular Biology of West Nile Virus: A New Invader of the Western Hemisphere" by Margo A. Brinton, 2002, *Annual Review Microbiology,* 56, p. 376. Copyright ©2002, *Annual Reviews.* For personal use only.

# Appendix E

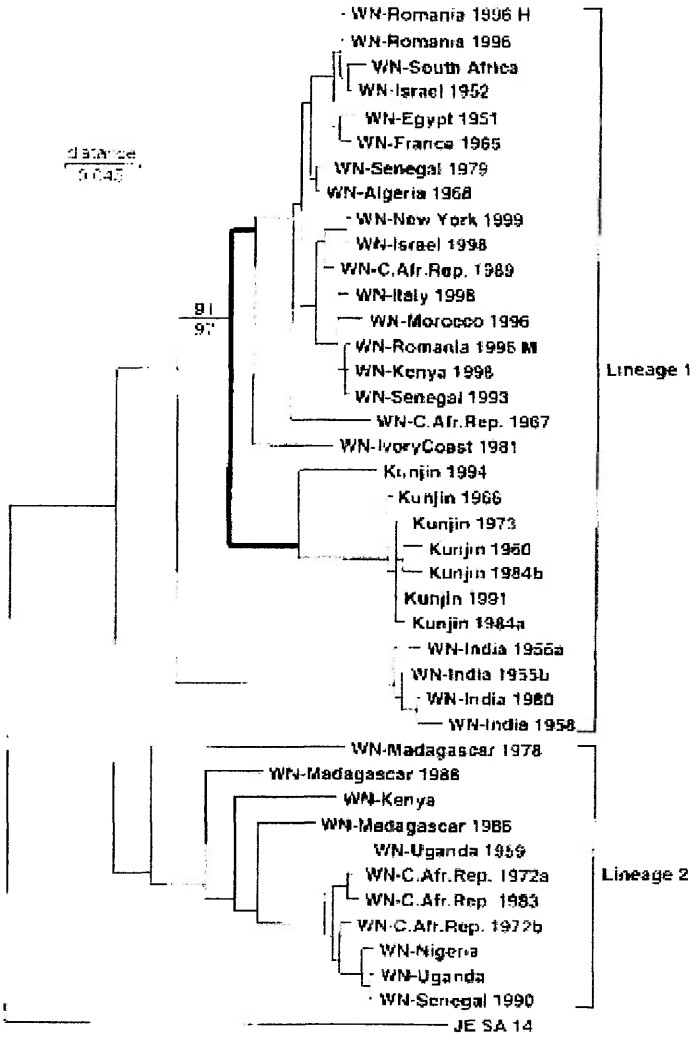



**Figure 11. Phylogenic Tree.**

Phylogenetic tree based on E-glycoprotein nucleic acid sequence data (255 base pairs). The tree was constructed with the program MEGA.

Source: From "Origin of the West Nile Virus Responsible for an Outbreak of Encephalitis in the Northeastern United States" by Lanciotti, R. S., et al., 1999, *Science*, 286, p. 2335.Copyright ©1999, American Association for the Advancement of Science. For personal use only.

# Appendix F

| Checklist for Research Studies | |
|---|---|
| **Study Identification** (author, title, year of publication , journal, pages) | |
| **Study Topic:** | **Study Subjects:**<br>**WNv Strain:** |

| | | YES | NO | UNCLEAR | N/A |
|---|---|---|---|---|---|
| **SECTION A**<br>**Population** | Are inclusion/exclusion criteria definitively outlined? | | | | |
| | Is the sample size large enough for precise estimates? | | | | |
| | Are the origins (eg. lab) of non-human subjects described? | | | | |
| | If a comparative study:<br>Were subjects randomized into groups?<br>Were the groups comparable at baseline?<br>If not, was incomparability addressed by the authors? | | | | |
| | If a randomized controlled trial (RCT):<br>Was the allocation of subjects to intervention and control groups truly randomized and blinded?<br>Were all the subjects in the study accounted for at its conclusion? | | | | |
| | | | | | |
| SECTION B<br>Data Collection | Are data collection methods clearly described? | | | | |
| | Are the materials used in the study clearly described? | | | | |
| | Are the tables and figures used in the publication, clear and relevant to the study? | | | | |
| | If the study is sponsored by a specific company or agency, is this disclosed? | | | | |
| | Did the study identify and make an effort to minimize bias? | | | | |
| | Is the WNv strain/source used in the study identified? | | | | |
| | | | | | |
| **SECTION C**<br>**Study Design** | Did the study have a clearly defined research question? | | | | |
| | Were the study objectives or report results clearly stated? | | | | |
| | Is the literature search sufficiently rigourous? | | | | |
| | Is the research methodology stated at a level of detail that would allow its replication? | | | | |
| | Was ethics approval obtained? | | | | |

| Checklist for Research Studies | |
|---|---|
| **Study Identification** (author, title, year of publication , journal, pages) | |
| **Study Topic:** | **Study Subjects:**<br>**WNv Strain:** |

**SECTION D**

**Results**

| | YES | NO | UNCLEAR | N/A |
|---|---|---|---|---|
| Are the outcomes clearly stated and discussed? | | | | |
| Are the results precisely outlined (p-value, CI)? | | | | |
| Are confounding variables accounted for? | | | | |
| Is any subset analysis a minor, rather than a major focus of the article? | | | | |
| Are suggestions provided for further research? | | | | |
| Are unexpected results discussed? | | | | |

ADDITIONAL COMMENTS

**VALIDITY**

**Calculation for section validity: (Y+N+U=T)**
If Y/T <75% or if N+U/T >25% then you can conclude that The section identifies significant omissions and that the Study's validity is questionable. It is important to look at The overall validity, as well as section validity.

**Calculation for overall validity: (Y+N+U = T)**
If Y/T $\geq$ 75% or if N=U/T $\leq$ 25% then you can conclude that the study is valid.

**SECTION A VALIDITY CALCULATION:**
**SECTION B VALIDITY CALCULATION:**
**SECTION C VALIDITY CALCULATION:**
**SECTION D VALIDITY CALCULATION:**

## OVERALL VALIDITY CALCULATION: _____%_____

Adapted from: "EBLIP Critical Appraisal Checklist" by Lindsay Glynn, MLIS, Memorial University of Newfoundland, http://www.newcastle.edu.au/service/library/gosford/ebl/toolkit/docs/EBL%20Critical%20Appraisal20Checklist.pdf. For personal use only.

# Appendix G

## Checklist Standards

**Section A:**
**Are inclusion/exclusion criteria definitively outlined?**
YES: the study stated the subject type (eg. mice, humans, monkeys), age, sex etc.
NO: did not state any information about the subjects
UNCLEAR: the statements about subjects were confusing or contradictory
N/A: no subjects were used in the study.

**Is the sample size large enough for precise estimates?**
YES: the study used samples $\geq$ to 10 subjects
NO: the study did not state the sample size
UNCLEAR: the study used $\leq$ 5 subjects
N/A: the study stated it did not use a sample of subjects.

**Are the origins (eg. lab) of non-human subjects described?**
YES: the study stated where the subjects used were obtained from
NO: the study did not state where the subjects used were obtained from
UNCLEAR: the statements about subject's origins were confusing or contradictory
N/A: no study subjects were used in the study.

**If a comparative study:**
**Were subjects randomized into groups?**
YES: this was stated in the study
NO: this was not stated in the study
UNCLEAR: statements made in the study were confusing or contradictory
N/A: the study was not a comparative study.

**Were the groups comparable at baseline?**
YES: the was stated in the study
NO: this was not stated in the study
UNCLEAR: the statements made in the study were confusing or contradictory
N/A: the study was not a comparative study.

**If not, was incomparability addressed by the authors?**
YES: this was discussed in the study
NO: this was not discussed in the study
UNCLEAR: the statements made in the study were confusing or contradictory
N/A: this was not a comparative study.

**If a randomized controlled trial (RCT):**
**Was the allocation of subjects to intervention and control groups truly randomized and blinded?**
YES: the study stated subjects were both randomized and blinded
NO: the study did not state the subjects were both randomized and blinded
UNCLEAR: the study stated subjects were either randomized or blinded, but not both
N/A: the study was not a RCT.

**Were all the subjects in the study accounted for at its conclusion?**
YES: the study accounted for all subjects
NO: the study did not account for all subjects
UNCLEAR: the statements regarding subjects were confusing or contradictory
N/A: the study was not a RCT.

**Section B:**

**Are data collection methods clearly described?**

YES: the study detailed the data collection method
NO: the study did not detail the data collection method
UNCLEAR: statements made regarding the data collection method were confusing or contradictory
N/A: the study did not collect data in the study.

**Are the materials used in the study clearly described?**

YES: the study described the materials used
NO: the study did not describe materials used
UNCLEAR: the statements made regarding material used in study were confusing or contradictory
N/A: there were no materials used in the study.

**Are the tables and figures used in the publication, clear and relevant to the study?**

YES: tables and figures were included and explained the study topic
NO: tables and figures were included but did not explain the study topic
UNCLEAR: tables and figures were included but were confusing or contradictory
N/A: there were no tables or figures included in the study.

**If the study is sponsored by a specific company or agency, is this disclosed?**

YES: the study had a disclosure statement for sponsors, grants, conflicts etc.
NO: the study was sponsored but had no disclosure statement
UNCLEAR: the study may or may not have been sponsored, information was confusing or contradictory
N/A: there was no evidence the study was sponsored by any company or agency.

**Did the study identify and make an effort to minimize bias?**

YES: the study screened study subjects for previous WNv (flavivirus) exposure
NO: the study did not state subjects were screened for previous WNv (flavivirus) exposure
UNCLEAR: statements regarding WNv (flavivirus) screening were confusing or contradictory
N/A: previous WNv (flavivirus) exposure was not important to the study.

**Is the WNv strain/source used in the study identified?**

YES: the WNv study isolate used in the study, its source and/or provider was stated
NO: the WNv study isolate used in the study, its source and/or provider was not stated
UNCLEAR: the WNv isolate used in the study, its source and/or provider information was confusing or contradictory
N/A: there was no WNv isolate used in the study.

**Section C:**

**Did the study have a clearly defined research question?**

YES: the purpose of the study was stated
NO: no purpose for the study was stated
UNCLEAR: the purpose for the study was confusing or contradictory
N/A: the study design required no research question.

**Were the study objectives or report results clearly stated?**

YES: the study stated objectives and/or reported results
NO: the study did not state any objectives and/or report results
UNCLEAR: the objectives or report results were confusing or contradictory
N/A: the study design required no objectives or report results.

**Is the literature search sufficiently rigourous?**

YES: the study used $\geq$ 10 peer reviewed articles, and unpublished or personal results in study
NO: the study used $\leq$ 10 peer reviewed articles, and unpublished or personal results in study
UNCLEAR: the study used information from undetermined sources
N/A: the study design required no literature review.

**Is the research methodology stated at a level of detail that would allow its replication?**
YES: the study provided detailed methods and materials information for replication
NO: the study did not provide methods and/or materials information
UNCLEAR: the study provided methods and/or materials information that was confusing or contradictory
N/A: the study design required no methods or materials.

**Was ethics approval obtained?**
YES: the study stated IRB approval, investigational new drug, and/or compliance to use of laboratory animals
NO: the study made no statements of IRB approval, investigational new drug, and/or compliance to use of laboratory animal
UNCLEAR: statements provided in the study were confusing or contradictory
N/A: the study did not use living subjects.

**Section D:**
**Are the outcomes clearly stated and discussed?**
YES: the study included a discussion section in which the outcomes were discussed
NO: the study did not include a discussion section
UNCLEAR: the study included a discussion section, but the statements were confusing or contradictory
N/A: the study design did not require a discussion section.

**Are the results precisely outlined (p-value, CI)?**
YES: the study included statistical tests with p-values, CI's etc. for stated results
NO: the study did not include statistical tests with p-values, CI's etc. for stated results
UNCLEAR: statistical tests used in the study were partially reported, confusing, or contradictory
N/A: the study design did not use statistical tests.

**Are confounding variables accounted for?**
YES: the study used commercially prepared medium, expression, and/or purification materials (eg. Vero cells, WNv isolates, proteins etc.)
NO: the study did not state the use of commercially prepared medium, expression, and/or purification materials (eg. Vero cells, WNv isolates, proteins etc.)
UNCLEAR: the statements regarding materials were confusing or contradictory
N/A: the study did not require the use of any materials.

**Is any subset analysis a minor, rather than a major focus of the article?**
YES: a subset analysis was described in $\leq 3$ paragraphs
NO: a subset analysis was described in $\geq 5$ paragraphs
UNCLEAR: subset analysis descriptions were confusing or contradictory
N/A: no subset analysis was provided in the study.

**Are suggestions provided for further research?**
YES: the study stated specific areas for future research
NO: the study did not make any statement regarding future research
UNCLEAR: the study statements regarding future research were confusing or contradictory
N/A: the study design did not require suggestions for future research.

**Are unexpected results discussed?**
YES: the study stated and discussed unexpected findings
NO: the study stated unexpected findings, but they are not discussed
UNCLEAR: the study may have stated unexpected findings, but discussion was confusing or contradictory
N/A: the study did not state unexpected findings.

# Appendix H

## Methodological Statistics

### Overall Validity Calculations for Studies

| Study Identification | Type | Time 1 | Time 2 |
|---|---|---|---|
| 1. Wolf | Inactivated | 86% | 85% |
| 2. Samina | Inactivated | 66% | 71% |
| 3. Ishikawa | Inactivated & DNA | 80% | 85% |
| 4. Karaca | Canarypox | 80% | 80% |
| 5. Yamshchikov | Live Attenuated | 100% | 90% |
| 6. Borisevich | Live Attenuated | 90% | 90% |
| 7. Despres | Live Attenuated | 90% | 90% |
| 8. Pletnev/St. Claire | Chimeric, Dengue | 89% | 89% |
| 9. Pletnev/Putnak | Chimeric, Dengue | 90% | 94% |
| 10. Langevin | Chimeric, YF | 95% | 95% |
| 11. Monath | Chimeric, YF | 95% | 95% |
| 12. Arroyo | Chimeric, YF | 95% | 95% |
| 13. Wang | Subunit, E Coli | 85% | 85% |
| 14. Lieberman | Subunit, E Coli | 90% | 95% |
| 15. Qiao | Subunit, Baculovirus | 95% | 100% |
| 16. Hall | DNA | 80% | 81% |
| 17. Yang | DNA | 80% | 80% |
| 18. Turell | DNA | 90% | 90% |

### Covariance

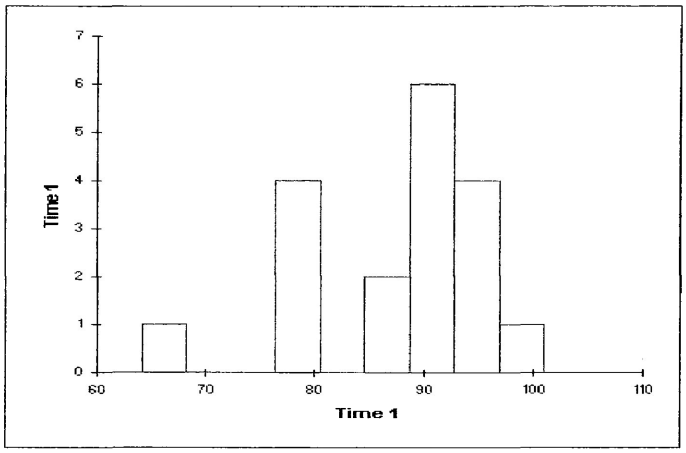
**Covariance between Time 1 and Time 2 is:** 52.568626

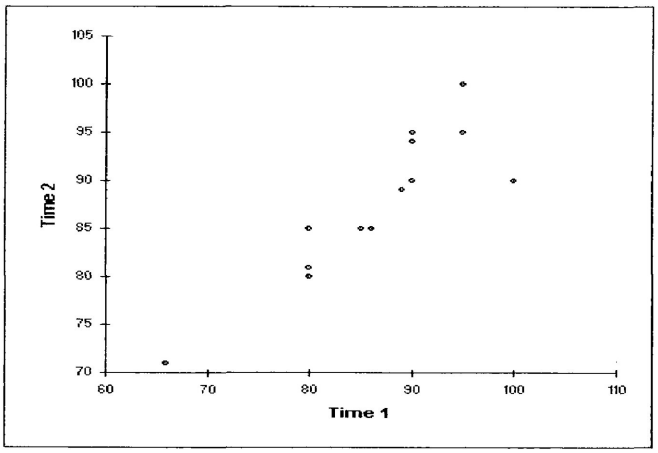
**Frequency Table**

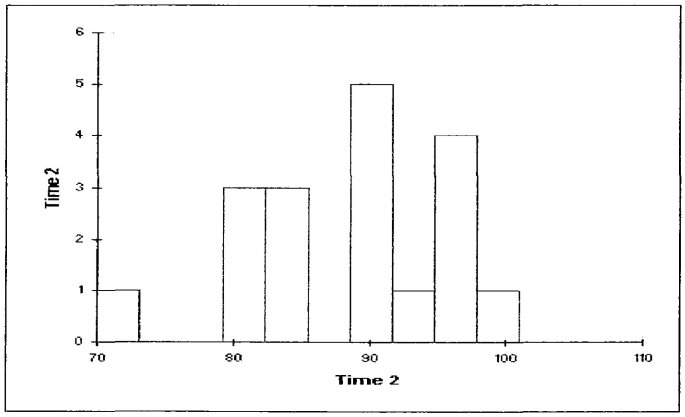
**Frequency table results Time 1 & 2:**

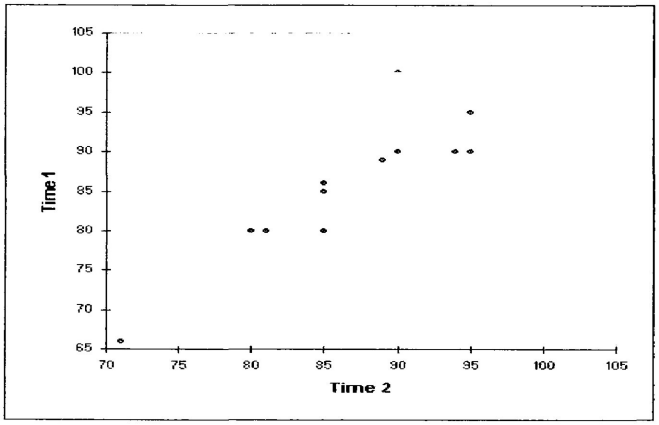
| Time 1 | Frequency | Relative Frequency | Time 2 | Frequency | Relative Frequency |
|---|---|---|---|---|---|
| **66** | 1 | 0.055555556 | **71** | 1 | 0.055555556 |
| **80** | 4 | 0.22222222 | **80** | 2 | 0.11111111 |
| **85** | 1 | 0.055555556 | **81** | 1 | 0.055555556 |
| **86** | 1 | 0.055555556 | **85** | 3 | 0.16666667 |
| **89** | 1 | 0.055555556 | **89** | 1 | 0.055555556 |
| **90** | 5 | 0.2777778 | **90** | 4 | 0.22222222 |
| **95** | 4 | 0.22222222 | **94** | 1 | 0.055555556 |
| **100** | 1 | 0.055555556 | **95** | 4 | 0.22222222 |
| | | | **100** | 1 | 0.055555556 |

**Histograms and Scatter Plots Time 1 & 2:**




**Two Sample Z-test results:**
($\square_1$ - mean of Time 1, $\square_2$ - mean of Time 2)

| Difference | $n_1$ | $n_2$ | Sample Mean | Std. Err. | Z-Stat | P-value |
|---|---|---|---|---|---|---|
| $\square_1 - \square_2$ | 18 | 18 | -0.7777778 | 2.5529966 | -0.30465287 | 0.7606 |

**Fisher's F-test (two-tailed test):**

| 95% confidence interval on the ratio of variances (0.470, 3.358) | |
|---|---|
| Ratio | 1.256 |
| F (Observed value) | 1.256 |
| F (Critical value) | 2.673 |
| DF1 | 17 |
| DF2 | 17 |
| p-value (Two-tailed) | 0.644 |
| alpha | 0.05 |

The ratio between the variances is not significantly different from 1.

Statistics by Xcel Stat Version 2007.6, and StatCrunch Data Analysis on the Web, http://www.statcrunch.com.

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