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# Genetic changes that affect the virulence of measles virus in a rhesus macaque model

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**Abstract**

To identify genetic changes that lead to the attenuation of measles virus (MV), a strain of MV that is pathogenic in rhesus macaques was adapted to grow in Vero cells, Vero/hSLAM cells and, to simulate the process used to derive live attenuated vaccines, in primary chicken embryo fibroblasts (CEF). Comparison of the complete genomic sequences of the pathogenic wild-type (Davis87-wt) and four cell culture-adapted strains derived from it showed complete conservation of sequence in the Vero/hSLAM-passaged virus. Viruses adapted to Vero cells and CEF had predicted amino acid changes in the nucleocapsid protein, phosphoprotein, V protein, C protein, matrix protein, and the cytoplasmic tail of the hemagglutinin protein. All four cell culture-adapted strains, including the Vero/hSLAM cell-passaged virus, were able to productively infect Vero cells, but the peak viral titers differed. The Vero cell-adapted strains were unable to replicate in Chinese Hamster Ovary cells expressing CD46, indicating that they had not adapted to use the CD46 receptor. The Vero/hSLAM cell-passaged virus retained pathogenicity in rhesus macaques as measured by the appearance of a skin rash while the Vero cell-adapted and CEF-adapted strains had lost the ability to cause a rash. There were no significant differences in viral titers in peripheral blood mononuclear cells among monkeys infected with any of the viral stocks tested. These results identify a limited number of genetic changes in the genome of MV that lead to attenuation *in vivo*.


*Keywords:* Measles; Cell culture adaptation; Pathogenesis; Rhesus; Attenuation

## Introduction

The widespread use of live attenuated measles vaccines has resulted in a dramatic reduction in the global morbidity and mortality caused by measles infections (Anonymous, 2006; Wolfson et al., 2007). The live-attenuated measles vaccines that are in use today have outstanding safety and efficacy profiles; however, the genetic basis for the attenuation of measles virus (MV) vaccine strains is unknown. Many of the current vaccine strains were derived from the prototype Edmonston isolate, while others were derived from unrelated wild-type viruses (Rota et al., 1994). All of the vaccines were developed well in advance of modern molecular biologic techniques and the genetic characterization of vaccine viruses has been a relatively recent activity. The WHO currently recognizes 23 genotypes of MV (WHO, 2005), and all of the vaccine strains are members of genotype A. Comparison of the sequences of the complete genomes of all of the Edmonston-derived vaccine strains with the sequence of the Edmonston wt virus (Ed-wt) (Parks et al., 2001a,b) identified a limited number of nucleotide substitutions in the vaccine strains relative to Ed-wt. However, the stock of Ed-wt had been passaged 13 times in cell culture prior to the sequence analysis (Rota et al., 1994). Since the original clinical specimen for Ed-wt is not available for sequence analysis, a direct comparison of the genomic sequences of the wild-type virus and vaccine strains is not possible.

There have been no reports of measles outbreaks associated with genotype A in the last two decades, but genotype A viruses have been associated with a small number of sporadic cases (Riddell et al., 2005). Because many of these events could be

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due to laboratory contamination or unrecognized vaccine reactions, the identification of genomic markers for attenuation of MV would facilitate surveillance for wild-type MVs. In addition, this genetic information could be used to monitor the safety and stability of new vaccine lots and would contribute to the development of improved vaccines for MV.

MV is a member of the genus *Morbillivirus* of the family *Paramyxoviridae.* Its monopartite, single-stranded, negative-sense RNA genome contains six genes, which encode eight proteins (Griffin, 2001). The noncoding regions of the termini contain the promoters for transcription and replication and the encapsidation signals. The nucleoprotein (N) encapsidates the viral genome and binds the polymerase complex. The P gene encodes three proteins, the phosphoprotein (P) and the C and V proteins. C is translated from an overlapping reading frame while V shares an amino terminal domain of 231 amino acids with P, but has a unique carboxyl terminus as a result of RNA editing (Cattaneo et al., 1989). P is a necessary component of the polymerase complex and acts as a scaffolding protein in nucleocapsid assembly. The C and V proteins regulate polymerase activity (Bankamp et al., 2005; Reutter et al., 2001; Tober et al., 1998; Witko et al., 2006) and act as antagonists of the innate immune response (Shaffer et al., 2003; Takeuchi et al., 2003). The matrix protein (M) plays a role in viral assembly and in the transport of viral glycoproteins to the apical membrane of polarized cells (Naim et al., 2000). The fusion (F) and hemagglutinin (H) glycoproteins are expressed on the surface of infected cells and of the virion, where the F protein promotes fusion with adjacent membranes. The H protein binds to specific receptors on the host cell and is a required co-factor for fusion. The Large protein (L) is the catalytic subunit of the polymerase complex.

Two cellular receptors for MV have been characterized to date, CD46 (Membrane Cofactor Protein) (Dorig et al., 1993; Naniche et al., 1993) and signaling lymphocyte activation molecule (SLAM) (Tatsuo et al., 2000). SLAM serves as a receptor for all wild-type isolates of MV as well as cell culture-adapted strains but is only expressed on a subset of cells of the immune system (Erlenhoefer et al., 2001; Hsu et al., 2001; Ono et al., 2001). CD46 is expressed on all nucleated human cells, including the epithelial surfaces of the respiratory tract, where MV infections are thought to initiate (Liszewski et al., 1991). However, CD46 can serve as a high affinity receptor only for cell culture-adapted, especially Vero cell-adapted MV strains (Buckland and Wild, 1997). Vero cells (African green monkey kidney cells) express a CD46 homologue, as do the cells of other Old World monkeys (Hsu et al., 1997), including rhesus macaques. However, Vero cells do not express SLAM. The sensitivity of isolation of MV from clinical samples has been greatly improved because of the recent development of Vero/hSLAM cells (Ono et al., 2001). Chicken embryo fibroblasts (CEF), which were used to generate many of the currently used measles vaccines (Rota et al., 1994), do not express either of the two known receptors for MV (Yanagi et al., 2002). Past attempts to characterize nucleotide changes induced by adaptation to cell culture have focused on Vero cells, while little is known about adaptation of MV to avian cells.

Rhesus macaques are an excellent animal model for measles infections, since they reproduce most of the pathologic and immunologic aspects of the human disease (El Mubarak et al., 2007; McChesney et al., 1997). An outbreak of measles in a rhesus monkey colony led to the isolation of Davis87-wt, a wild-type MV that retained pathogenicity in monkeys through exclusive passaging in lymphoid tissue or peripheral blood mononuclear cells of rhesus macaques (McChesney et al., 1997).

In this study, we have adapted Davis87-wt to growth in three different cell lines, including avian cells, and identified nucleotide changes that resulted from these adaptations. The cell culture-adapted virus stocks were examined for their growth characteristics in cell culture and for pathogenicity in rhesus macaques.

## Results

### *Development of cell culture-adapted virus stocks*

Prior to this work, the Davis87-wt strain was propagated exclusively in rhesus macaques or rhesus lymphoid cells (McChesney et al., 1997). Davis87-wt was passaged nine times in Vero/hSLAM cells to generate the D-V/S stock as described in Materials and methods. Syncytia, the characteristic cytopathic effect (CPE) of infection with MV, were visible from the first passage. Adaptation of Davis87-wt to Vero cells followed a similar protocol, but a greater starting titer (1000 pfu/25 $cm^2$ flask instead of 25 pfu/25 $cm^2$ flask) was used, followed by several blind passages. CPE was first visible at passage five. Adaptation to Vero cells was carried out twice to generate two viral stocks, D-VI and D-VII. Two attempts were made to directly infect primary chicken embryo fibroblasts (CEF) with Davis87-wt, using 1250 pfu/25 $cm^2$ flask as an inoculum. The cells were passaged up to nine times without visible CPE. No MV could be detected by indirect immunofluorescence or by RT-PCR (data not shown). To establish the CEF-adapted virus stock, D-CEF, cells were infected at an MOI of 1 with D-V/S and passaged as described for the Vero cell cultures. Table 1 summarizes the passage history and the titers of the final stocks. It is important to note that the viruses were not plaque purified. The high titers suggest that the viruses are well adapted to grow in their respective cell lines.

### *Sequence analysis*

The complete genomic sequences of all four cell culture-adapted strains and Davis87-wt were determined. Sequence

Table 1
Passage history and viral titers of cell culture-adapted virus stocks used in this study

| Virus stock | Passaged on | Source for infection | Number of passages | Titer on Vero/hSLAM cells |
|---|---|---|---|---|
| D-V/S | Vero/hSLAM | Davis87-wt | 9 | $2.1\times10^7$ pfu/ml |
| D-VI | Vero | Davis87-wt | 19 | $9.2\times10^7$ pfu/ml |
| D-VII | Vero | Davis87-wt | 12 | $2.9\times10^7$ pfu/ml |
| D-CEF | CEF | D-V/S | 21 | $4.0\times10^7$ pfu/ml |