TABLE 4. Amino acid substitutions in the H proteins of the IC, SI, and Edmonston strains

Amino acid no.	Amino acid substitution		
	IC	SI[a]	Ed[b]
7	R	Q*	R
71	H	R*	H
174	A	A	T
176	A	A	T
211	S	S	G
235	G	E	E
243	G	G	R
252	H	H	Y
276	F	F	L
284	F	F	L
296	F	F	L
302	R	R	G
334	R	Q	Q
390	N	M*	I
416	N	N	D
446	T	S	S
481	N	N	Y
482	L	F*	L
484	T	T	N
546	S	G*	S
555	F	L*	F
564	I	L*	I
575	K	Q	Q
600	V	V	E

[a] Asterisks indicate amino acids unique to the SI strain.
[b] Edmonston strain; GenBank accession number K01711.

was missing in cells infected with the SI strain. The M proteins of the SI and IC-B strains were expressed in cells by the use of expression plasmids. The carboxyl termini of the M proteins were tagged with mCherry red fluorescent protein. All the MAbs detected the IC-B strain-derived M protein despite the mCherry tag (Table 3). In contrast, none of the MAbs detected the SI strain-derived M protein, although bright mCherry fluorescence was detected in these cells (Fig. 3B and Table 3). These data indicated that the antigenicity of the M protein of the SI strain was totally different from that of the M protein of the IC-B strain and that none of the MAbs recognizing antigenic sites II, III, and IV reacted with the M protein of the SI strain. Therefore, we could not reach a conclusion as to whether the M protein was expressed in cells infected with the SI strain. However, analyses using the expression plasmids demonstrated that, unlike the M protein of the IC-B strain, the M protein of the SI strain was distributed homogeneously in cells (Fig. 3B). The M protein of the IC-B strain was distributed beneath the plasma membrane and formed small dots in the cytoplasm (Fig. 3B). To elucidate the functional difference between the IC-B and SI strains with respect to the M gene, we generated a recombinant MV with a modified SI strain genome in which the M gene was replaced with the M gene of the IC-B strain. The resulting recombinant MV was designated SI/ICM-AcGFP. A growth kinetics analysis showed that, unlike SI-AcGFP, SI/ICM-AcGFP produced cell-free virus well and the cell-free virus titer of SI/ICM-AcGFP was ~1,000 times higher than that of the SI-AcGFP at 10 days p.i. (Fig. 3C). The result demonstrated that SI-M protein was less involved in the budding stage. With these data, we concluded that the SI strain does not express a functional M protein.

The SI strain exhibits limited syncytium-forming activity. Various types of cells were infected with SI-AcGFP and IC323-AcGFP. IC323-AcGFP poorly entered Vero cells ($SLAM^-$/$CD46^+$) and did not produce a syncytium (Fig. 4). On the other hand, SI-AcGFP was able to produce syncytia in Vero cells (Fig. 4). Table 4 shows the amino acid substitutions in the H protein. Among them, the S546G substitution is the one that probably contributed to the ability of SI-AcGFP to produce syncytia in Vero cells, because this mutation allows MV to use CD46 as a receptor (69). On the other hand, SI-AcGFP failed to produce syncytia in II-18 cells (ECR^+, $CD46^+$), although IC323-AcGFP replicated and produced syncytia in these cells efficiently (Fig. 4). An MAb against CD46 (M75) had a neutral effect on the SI-AcGFP infection of II-18 cells. Similar results were obtained for the infection of SLAM-positive cells (CHO/hSLAM, A549/hSLAM). SI-AcGFP produced syncytia poorly in these cells, whereas IC323-AcGFP produced syncytia very efficiently. These data demonstrate that the SI strain has limited activity in inducing syncytia in SLAM- or ECR-expressing cells, although it has acquired the ability to use CD46 as an alternative receptor. Although three neural cell lines (SK-N-SH, IMR-32, and SH-SY5Y) were infected with SI-AcGFP and IC323-AcGFP, no syncytia were observed in these cells (Fig. 4 and data not shown).

The membrane-associated protein genes (M, F, and H) determine the growth phenotype of the SI strain. The amino acid sequences of the RNP component proteins (N, P, and L pro-

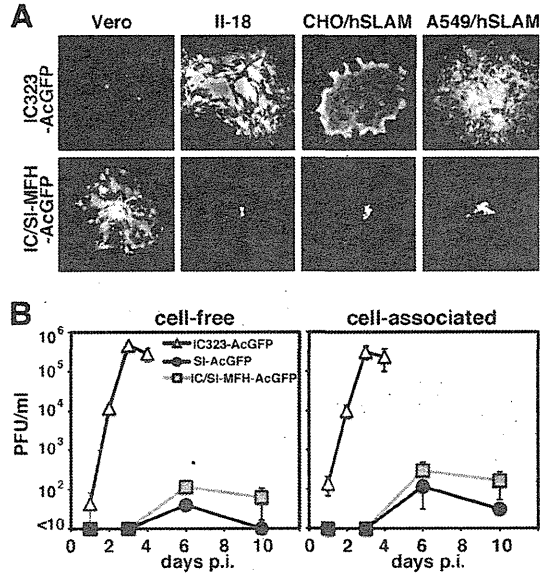

FIG. 5. Effect on viral growth of strain IC possessing the SI-MFH gene in various cell lines. (A) AcGFP fluorescence in cells infected with recombinant MVs. Vero, II-18, CHO/hSLAM, and A549/hSLAM cells were infected with IC323-AcGFP or IC323/SI-MFH-AcGFP. The cells were observed under a fluorescence microscope at 3 (II-18, CHO/hSLAM, and A549/hSLAM) and 6 (Vero) days postinfection. (B) Replication kinetics of recombinant MVs. Vero/hSLAM cells were infected with recombinant MVs at an MOI of 0.01. At various time intervals, infectious titers in culture medium (cell-free) and cells (cell-associated) were determined. Data represent the means ± standard deviations (SD) of the results of experiments performed with triplicate samples.

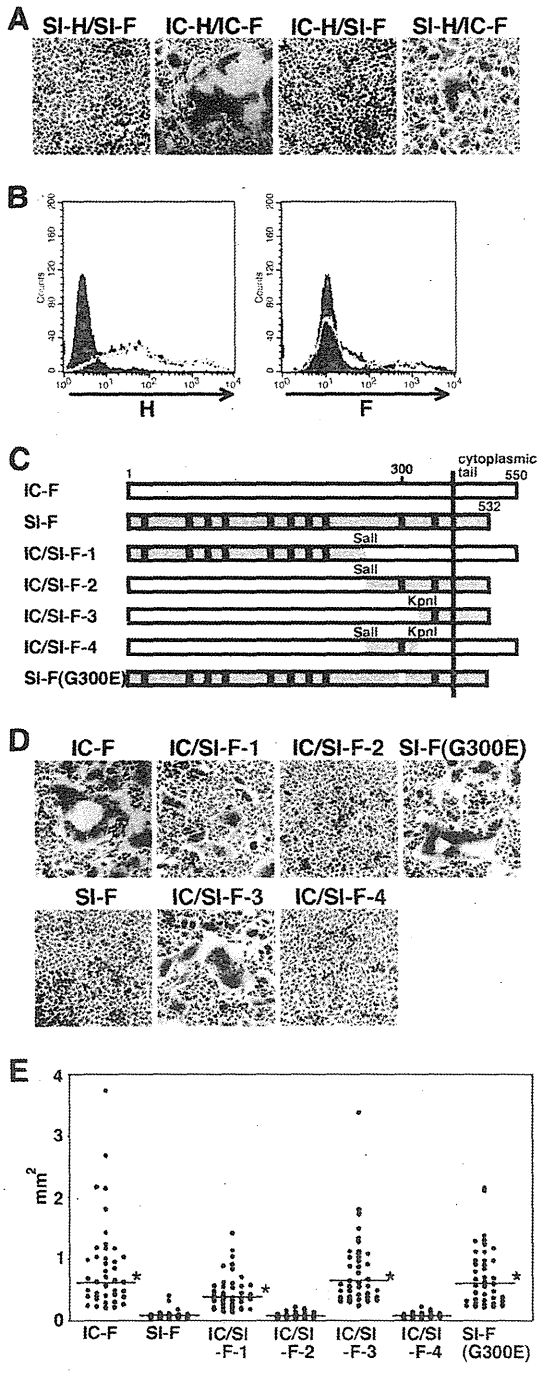

FIG. 6. Syncytium formation in cells expressing H and F proteins and identification of the amino acid residue in the F protein of the SI strain critical for reducing cell-to-cell fusion. (A) Syncytium formation in cells expressing H and F proteins of the IC-B or SI strains. CHO/hSLAM cells were transfected with a plasmid expressing the H protein of the IC-B or SI strain (IC-H or SI-H, respectively) together with a plasmid expressing the F protein of the IC-B or SI strain (IC-F or SI-F, respectively). At 24 h posttransfection, the cells were observed under a microscope after Giemsa staining. (B) Expression of the MV envelope proteins on the cells. CHO/hSLAM cells were transfected with a plasmid expressing IC-H, SI-H, IC-F, or SI-F. The cells expressing IC-H (black line) and SI-H (gray line) were stained with an anti-H protein MAb (left panel), and the cells expressing IC-F (black line) and SI-F (gray line) were stained with an anti-F protein MAb (right panel). All the cells were subsequently stained with an Alexa Fluor 488-conjugated secondary antibody. The cells without transfection were stained with an anti-H protein MAb or an anti-F protein MAb followed by an Alexa Fluor 488-conjugated secondary antibody (shaded regions). (C) Diagrams of the chimeric F proteins. There are 10 amino acid differences (shown by vertical lines) between IC-F and SI-F. The regions derived from SI-F are shaded, and those derived from IC-F are white. The restriction enzyme-replaced fragments are indicated. (D) Syncytium formation in cells expressing the chimeric or mutant F proteins. CHO/hSLAM cells were transfected with a plasmid expressing IC-H together with plasmids expressing IC-F protein, SI-F protein, chimeric F protein (IC/SI-F-1, -F-2, -F-3, or -F-4), or mutant SI-F protein (G300E). At 24 h posttransfection, the cells were observed under a phase-contrast imaging microscope after Giemsa staining. (E) Quantification of syncytium formation. CHO/hSLAM cells were transfected with IC-H-expressing plasmids and IC-F-, SI-F-, chimeric F-, or mutant F-expressing plasmids together with an mCherry-expressing plasmid. At 48 h posttransfection, areas of each syncytium with mCherry autofluorescence were measured using an Axio Observer.D1 microscope and ImageJ software. Forty syncytia were measured for each F protein. Asterisks indicate that the area of syncytia induced by IC-F, chimeric F, or mutant F was significantly larger than that induced by SI-F, based on the results of a t test ($P < 0.001$). The horizontal bars indicate the median values of the areas of syncytia.

teins) and nonstructural C and V proteins were well conserved in the SI strain (Table 1). We generated a recombinant MV possessing the IC323 genome in which the M, F, and H genes were replaced with those of the SI strain. The recombinant MV was designated IC/SI-MFH-AcGFP. The various types of cells shown in Fig. 4 were infected with IC/SI-MFH-AcGFP. IC/SI-MFH-AcGFP replicated poorly in SLAM- and ECR-positive cells and did not produce syncytia in these cells (Fig. 5A). A growth kinetics analysis of Vero/hSLAM cells, which were susceptible to all recombinant MVs, showed that IC/SI-MFH-AcGFP hardly produced cell-free viruses and exhibited a growth phenotype similar to that of SI-AcGFP (Fig. 4 and 5B). These data indicated that the membrane-associated protein-encoding genes (i.e., the M, F, and H genes) were responsible for the growth phenotype of the SI strain.

The E300G substitution in the F protein is responsible for the reduced membrane fusion activity. Previous papers have indicated that the typical changes in SSPE strains, namely, the lack of M protein expression and cytoplasmic tail truncation of the F protein, enhance the syncytium-forming activity of MV (6, 7). Indeed, other previous papers have shown high fusogenic activities of SSPE strains (1, 4, 8). Despite exhibiting the changes typical in SSPE strains, SI-AcGFP and IC/SI-MFH-AcGFP showed limited syncytium-forming activities (Fig. 4 and 5B). Using expression plasmids, the syncytium-forming activities of the H and F proteins of the SI strain were analyzed in CHO/hSLAM cells ($SLAM^+$). When the F protein of the SI strain (SI-F) was expressed together with the H protein of the SI strain (SI-H), no syncytia were detected (Fig. 6A; SI-H/SI-F). In contrast, many syncytia were observed when the F and H proteins of the IC-B strain (IC-F and IC-H, respectively) were expressed (Fig. 6A; IC-H/IC-F). Flow cytometry analyses indicated that the expression levels of SI-F and SI-H, respectively, were similar to those of IC-F and IC-H (Fig. 6B). The combination of SI-F and IC-H also showed poor syncytium-forming activity (Fig. 6A; IC-H/SI-F). On the other hand, when IC-F

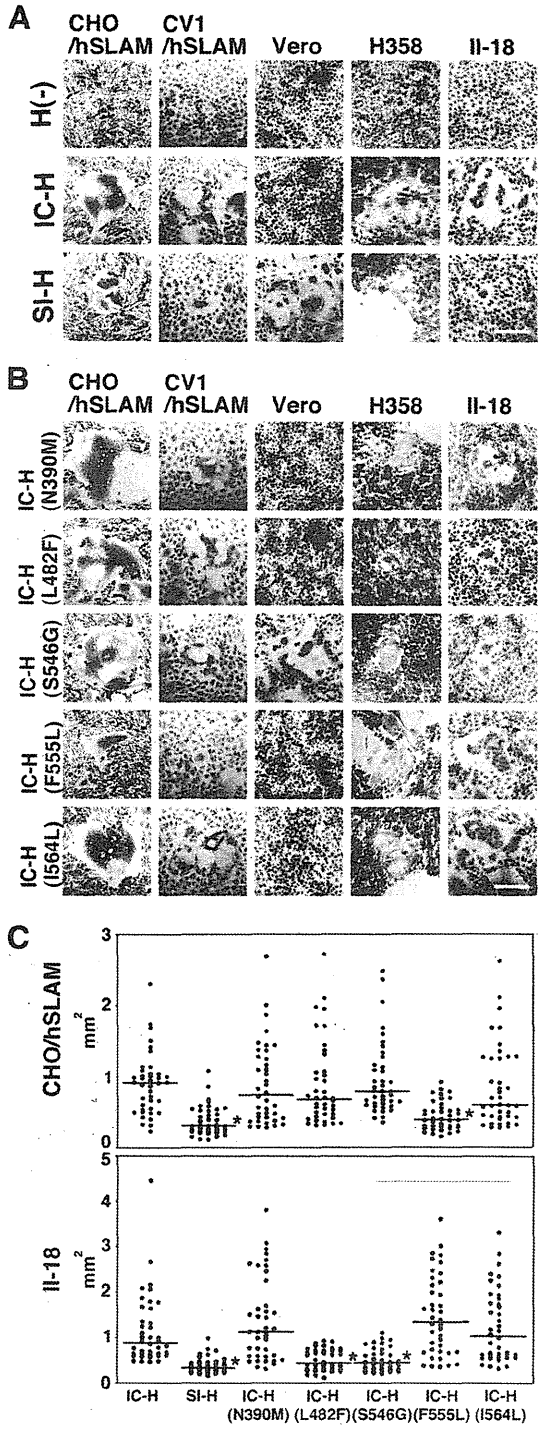

FIG. 7. Characterization of the amino acid residues in the SI-H protein that support cell-to-cell fusion in cells expressing SLAM, CD46, or ECR. (A and B) CHO/hSLAM, CV1/hSLAM, Vero, H358, and II-18 cells were transfected with plasmids expressing the H protein of the IC-B or SI strain (IC-H or SI-H, respectively) or no H protein [H(−)] (A) or mutant IC-H protein (N390M, L482F, S546G, F555L, or I564L) (B) together with a plasmid expressing the F protein of the IC-B strain. The CV1/hSLAM, H358, and II-18 cells were then incubated with an anti-CD46 MAb (M75). At 1 (CHO/hSLAM and CV1/hSLAM), 2 (Vero and II-18), or 3 (H358) days posttransfection, the cells were observed under a phase-contrast imaging microscope after Giemsa staining. Bars, 0.2 mm. (C) Quantification of syncytium formation. CHO/hSLAM and II-18 cells were transfected with IC-F-expressing plasmids and IC-H-, SI-H-, or mutant H-expressing plasmids together with an mCherry-expressing plasmid. At 48 h posttransfection, areas of each syncytium with mCherry autofluorescence were measured using an Axio Observer.D1 microscope and ImageJ software. Forty syncytia were measured for each H protein. Asterisks indicate that the area of syncytia induced by SI-H or mutant H was significantly smaller than that induced by the IC-H, based on the results of a t test ($P < 0.001$). The horizontal bars indicate the median values of the areas of syncytia.

was coexpressed with SI-H, many syncytia, albeit smaller in size than the syncytia induced by IC-F and IC-H, were detected (Fig. 6A; SI-H/IC-F). These data indicated that both the SI-F and SI-H proteins exhibited lower activities than the IC-F and IC-H proteins in inducing syncytia in CHO/hSLAM cells. To identify the mutation(s) that impaired the syncytium-forming activity of SI-F, four chimeric F proteins (IC/SI-F-1, -F-2, -F-3, and -F-4) were generated using the SI and IC-B strains (Fig. 6C). These chimeric F proteins were coexpressed with IC-H. Two chimeric F proteins, IC/SI-F-2 and IC/SI-F-4, failed to produce syncytia (Fig. 6D and E). These data showed that a region between the SalI and KpnI recognition sites (amino acid positions 271 and 324) in SI-F severely restricted its membrane fusion activity (Fig. 6C). In this region, only a single amino acid substitution, E300G, was found in comparisons of SI-F and IC-F (Fig. 6C and Table 2). A glycine residue at amino acid position 300 in SI-F was replaced with a glutamic acid. The mutant F protein [Fig. 6C; SI-F(G300E)] was expressed with IC-H. The data indicated that SI-F(G300E) caused membrane fusion as well as IC-F did ($P < 0.01$) [Fig. 6D and E; SI-F(G300E)]. These findings indicated that the SI-F protein exhibited a restricted membrane fusion activity that was mainly caused by the E300G substitution.

S546G, L482F, and F555L substitutions affected the fusion-helper function of the H protein. To analyze the fusion-helper function of SI-H in different cell types, the protein was expressed in CHO/hSLAM ($SLAM^+$), CV1/hSLAM ($SLAM^+$, $CD46^+$), Vero ($CD46^+$), H358 (ECR^+, $CD46^+$), and II-18 (ECR^+, $CD46^+$) cells together with IC-F. CD46-dependent infection was blocked by an anti-CD46 antibody (M75) when CV1/hSLAM, H358, and II-18 cells were used for the assessment of SLAM- and ECR-dependent infection. IC-H was used as a control. When IC-F was expressed alone, no syncytia were observed in either cell line [Fig. 7A; H(−)]. As reported previously, IC-H supported cell-to-cell fusion efficiently in SLAM-positive (CHO/hSLAM) and ECR-positive (H358 and II-18) cells but not in Vero cells (Fig. 7A; IC-H) (45, 49, 59). SI-H exhibited a fusion-helper function in Vero cells (Fig. 7A; SI-H), probably because of the S546G substitution. However, SI-H supported cell-to-cell fusion less efficiently than IC-H in CHO/hSLAM, CV1/hSLAM, H358, and II-18 cells (Fig. 7A; SI-H). To identify the substitution(s) responsible for the altered fusion-helper function of SI-H, five substitutions were individually introduced into IC-H and the mutated proteins were expressed in cells together with IC-F. The five selected substitutions were N390M, L482F, S546G, F555L, and 564L, since these substitutions were unique to the SI strain and

located in the receptor-binding globular head domain (Table 4). As expected, IC-H with S546G, but not the other mutant H proteins, supported cell-to-cell fusion in Vero cells (Fig. 7B). Instead, IC-H with S546G showed a reduced fusion-helper function in H358 and II-18 cells (Fig. 7B). No significant changes were observed in CHO/hSLAM and CV1/hSLAM cells after the introduction of the S546G substitution (Fig. 7B). Similarly, IC-H with L482F showed a reduced fusion-helper function in H358 and II-18 cells but showed activities similar to those seen with IC-H in CHO/hSLAM and CV1/hSLAM cells (Fig. 7B). Quantified and statistical analyses of cell-to-cell fusion in II-18 cells indicated that the areas of syncytia produced by IC-H(S546G) and IC-H(L482F) were significantly smaller than those produced by IC-H ($P < 0.01$) (Fig. 7C). None of the N390M, F555L, and I564L substitutions significantly affected the fusion-helper function in H358 and II-18 cells (Fig. 7B and C). These findings suggested that the L482F and S546G substitutions compromised the ability of the H protein to interact with ECR. It was also noted that the H protein with F555L showed a reduction in the fusion-helper function in CHO/hSLAM and CV1/hSLAM cells (Fig. 7B and C).

DISCUSSION

SLAM is expressed on cells of the immune system and functions as the principal receptor for MV infection (69). However, this molecule probably plays a minor role in MV growth in the CNS, because neural cells in the brain do not express SLAM (28). Indeed, the ability of the SI strain to use SLAM was compromised by the F555L substitution. We and another group recently demonstrated that certain epithelial cells that form tight junctions are highly susceptible to MV infection (25, 50, 59). These data demonstrated the existence of ECR on some epithelial cells (25, 50, 59). ECR probably contributes to the efficient transmission of MV from a patient to other individuals (53), but its roles in persistent infection of the brain with MV remain to be elucidated. ECR is a candidate for an MV receptor in the brain. However, our data indicated that the SI strain had mutated via the S546G and L482F substitutions to use ECR inefficiently. With these data, the idea that ECR functions as a receptor for MV in the brain seemed unconvincing. Instead, the SI-H protein had adapted to use CD46 via the S546G substitution. Woelk et al. identified several positive-selection amino acid sites in the SSPE strain (67), but S546G was absent from the list. It is possible that the S546G substitution was introduced into the SI strain genome during the propagation in Vero cells but not in the brain, since the SI strain was isolated using Vero cells (29). Vero cells are 100 to 1,000 times less sensitive than SLAM-positive B95a cells for the isolation of wt MV strains (22, 34), and wt MV strains readily adapt to use CD46 after several passages in Vero cells (69). However, Ogura et al. (34) indicated that Vero cells were more sensitive than B95a cells for the isolation of SSPE strains. Although their data demonstrated that SSPE strains show cell specificities different from those of wt MV strains, some SSPE strains were shown not to use CD46 as a receptor (47). Nevertheless, it is still possible that the acquisition of the ability to use CD46 contributes to the growth of some SSPE-derived strains in the brain, since various SSPE strains may employ different strategies to acquire the ability to spread in the brain. The SI strain used only CD46 efficiently. Much evidence obtained using CD46-transgenic mice has shown the contributions of CD46 in establishing MV infection of the brain. Analyses using human brain samples also showed that CD46 is a candidate molecule that contributes to the growth of some SSPE strains in the brain (5, 28, 33).

Analyses using animal models have demonstrated that MV uses a transsynaptic route to spread between neurons (24, 27, 35, 40). The data indicated that receptors for the H protein are not required for the transsynaptic transmission (27, 70). It has been suggested that the F protein causes microfusion between neurons without the support of the H protein (27, 70). Ayata et al. (1) demonstrated that the F proteins of some SSPE strains contribute to the exhibition of neurovirulence in animals by showing a hyperfusion activity. Cattaneo et al. (4, 8) also demonstrated that the F proteins of SSPE strains exhibit higher levels of fusion activities than the standard F protein. These data suggest an important role for the F protein in the propagation of SSPE strains in the brain. However, our data indicated that the F protein of the SI strain showed limited membrane fusion activity because of the E300G substitution. It is unlikely that the F protein of the SI strain had acquired the E300G substitution during the propagation in cultured cells, since viruses usually acquire mutations that confer better fitness. Consequently, our data suggest that a high level of membrane fusion activity of the F protein was not a prerequisite for this SSPE strain to spread in the brain. Watanabe et al. (65) suggested that a reduction in cell-to-cell fusion mediated by amino acid changes in the F protein contributes to the persistence of MV in the brain. Their observations are consistent with our data for the SI strain. Thus, the data obtained in the present study provide a clear example of an SSPE-derived strain that exhibits limited fusion activity.

In the present study, we also established a reverse genetics system for the SI strain. Although we previously reported very efficient reverse genetics systems for MV, as shown using recombinant vaccinia viruses encoding T7 RNA polymerase (VV-T7) (30, 55, 56), they were not applicable for rescue of the SI strain from cloned cDNAs. When the previous systems were used (30, 56), infectious cycles of rSI-AcGFP were efficiently initiated in CHO/hSLAM cells by the use of the full-length genome plasmid (data not shown). However, since rSI-AcGFP did not produce cell-free virus particles and replicated poorly, it was impossible to isolate rSI-AcGFP from VV-T7. We tried to use a VV-T7-free system reported by Radecke et al. (39), but neither syncytia nor AcGFP fluorescence was detected. Therefore, a new, efficient VV-T7-free system was required for the rescue of rSI-AcGFP from cloned cDNAs. We are convinced that this new system used for the SI strain would be applicable for other SSPE strains. The success in establishing a reverse genetics system for an SSPE strain is a significant step toward the elucidation of the molecular bases and pathogenesis of SSPE.

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SUPPLEMENT ARTICLE

Global Distribution of Measles Genotypes and Measles Molecular Epidemiology

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A critical component of laboratory surveillance for measles is the genetic characterization of circulating wild-type viruses. The World Health Organization (WHO) Measles and Rubella Laboratory Network (LabNet), provides for standardized testing in 183 countries and supports genetic characterization of currently circulating strains of measles viruses. The goal of this report is to describe the lessons learned from nearly 20 years of virologic surveillance for measles, to describe the global databases for measles sequences, and to provide regional updates about measles genotypes detected by recent surveillance activities. Virologic surveillance for measles is now well established in all of the WHO regions, and most countries have conducted at least some baseline surveillance. The WHO Global Genotype Database contains >7000 genotype reports, and the Measles Nucleotide Surveillance (MeaNS) contains >4000 entries. This sequence information has proven to be extremely useful for tracking global transmission patterns and for documenting the interruption of transmission in some countries. The future challenges will be to develop quality control programs for molecular methods and to continue to expand virologic surveillance activities in all regions.

A critical component of laboratory surveillance for measles is the genetic characterization of circulating wild-type viruses to provide support for molecular epidemiologic studies [1, 2]. The World Health Organization (WHO) Measles and Rubella Laboratory Network (LabNet) provides for standardized testing and reporting, with laboratories in 183 countries. The primary function of LabNet is to provide laboratory confirmation of suspected cases of measles and rubella [3, 4]. LabNet also supports genetic characterization of currently circulating strains of measles virus and is responsible for standardization of the nomenclature and laboratory procedures that are used for genetic characterization of wild-type measles and rubella

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viruses [5–9]. This standardization has allowed sharing of virologic surveillance data among laboratories and permitted efficient communication of this data throughout the measles control programs [10]. For molecular epidemiologic purposes, the genotype designations are considered the operational taxonomic unit, whereas related genotypes are grouped by clades. The WHO currently recognizes 8 clades, designated A, B, C, D, E, F, G, and H. Within these clades, there are 23 recognized genotypes, designated A, B1, B2, B3, C1, C2, D1, D2, D3, D4, D5, D6, D7, D8, D9, D10, E, F, G1, G2, G3, H1, and H2, and 1 provisional genotype, d11. Viruses with related sequences within some of the genotypes (eg, B3 and H1) are referred to as clusters. The WHO recommends that the 450 nucleotides coding for the COOH-terminal 150 amino acids of the nucleoprotein (N - 450) are the minimum amount of sequence data required for assigning a measles genotype [8, 11–14].

Virologic surveillance for measles was initiated in response to the global measles resurgence that occurred in the late 1980s [15]. These activities have increased significantly during the past 10 years because of the expansion of LabNet. The goal of this report is to briefly describe the lessons learned from nearly 20 years of virologic surveillance for measles, to describe the global databases for measles sequences, and to provide brief regional updates regarding circulating measles genotypes detected by surveillance activities, with a focus on the past 3 years.

LESSONS LEARNED FROM MOLECULAR EPIDEMIOLOGY OF MEASLES VIRUS

The combination of molecular epidemiology and standard case classification and reporting provides a very sensitive means to describe the transmission pathways of measles. Virologic surveillance is especially beneficial when it is possible to monitor the viral genotypes in a particular country or region over time, because this information has helped to document the interruption of transmission of endemic measles [1, 2, 16–19]. Evidence of the absence of an endemic genotype(s) is one of the criteria for verifying measles elimination in a country or region (endemic genotype(s) are defined as a genotype or genotypes associated with endemic transmission of measles).

The virologic surveillance data has shown that vaccination programs can reduce the number of co-circulating chains of transmission and eventually interrupt measles transmission. However, viruses are continually being introduced from external sources, and if the number of susceptible individuals increases, sustained transmission of the newly introduced viral genotype is possible. This results in what appears as a rapid change in the endemic genotype [20–23].

All measles vaccines belong to genotype A [24], which is a genotype that is not associated with documented endemic transmission in any part of the world. Because serologic methods cannot distinguish between a vaccine-induced antibody response and antibodies derived from natural disease, molecular characterization of viral isolates provides the only method to differentiate between natural infection and vaccine-induced adverse events. In elimination settings, rapid confirmation of these vaccine reactions will be needed to ensure that a public health response is not initiated needlessly.

Another lesson learned is that, if large measles outbreaks are occurring anywhere in the world, the viruses are soon detected almost everywhere. Measles transmission can occur anywhere, and molecular techniques are often the only method for identifying the source of an outbreak or isolated case when standard case reporting fails to identify a source. Exposures can occur in airports or other areas frequented by international travelers, such as amusement parks, conferences, and sporting events. In 2005, sequence information was used to link cases that occurred in the Netherlands to an exposure in an airport in the United States [25], whereas in 2007, sequence data were used to link cases that occurred in Texas and Michigan to an imported case at an international youth sporting event in Pennsylvania [26, 27]. Of course, molecular studies can only confirm independent sources of infection if different genotypes or clearly distinct lineages are detected (lineage is defined as a group of viruses with identical or nearly identical N-450 sequence that suggest that they represent a single chain of transmission). However, if viruses from the same lineage are detected in nonlinked cases in a particular country, the molecular data alone may not be able to differentiate between continuous circulation of virus and multiple introductions from the same source. This limitation can be addressed by analysis of the epidemiologic data. In addition, expanding the size of the region of the measles genome used for sequence comparison may allow more-precise definition of lineages, and this method is currently being evaluated by LabNet [28].

MEASLES DATABASES

Because of the importance of molecular surveillance activities for measles viruses, it is now even more necessary to be able to compare sequence information, in addition to the genotype information. Genotypes contain multiple distinct lineages. Therefore, comparing sequences is the most sensitive means to identify and map transmission chains. However, to make real-time monitoring of measles transmission chains possible, sequence and genotype information must be reported to centralized databases in a timely manner, and this information must be available to members of LabNet. Until recently, the public access database, GenBank (http://www.ncbi.nlm.gov), was the only repository for measles sequence information. Unfortunately, the release of information through GenBank is often delayed, and the entries are not curated and often lack important epidemiologic information. More recently, a number of different systems or databases have become available to collect and disseminate measles genotypic information.

However, the information collected varies widely as to whether it is aggregate or individual data, whether it includes genotype information only, and whether the sequence information is available (Table 1). The first global database available for measles genotypes was developed at WHO Headquarters in Geneva, Switzerland. Timely reporting of genotype information to the WHO database is a performance indicator for accreditation of the Regional Reference Laboratories. The WHO database for measles contained 7600 entries from 124 countries as of 1 June 2010. Although sequence data are not reported to this database, the GenBank accession number, if available, is listed. Contact details of the submitting laboratory are provided in the event that further information is needed. A similar database has been developed for rubella viruses, which contained >600 entries from 39 countries at the same point in time.

The MeaNS (Measles Nucleotide Surveillance; http://www.who-measles.org) database is a joint project between the Health Protection Agency (London, UK) and the WHO. Currently, the database collects sequence information from the complete sequence of the measles hemagglutinin (H) gene, the complete sequence of the nucleoprotein (N) gene, or the sequence of the COOH-terminal 450 nucleotides of the N gene (N-450). Additional information, including epidemiological information on the patient, is also collected. There must be sufficient information provided to create a standardized name for the sequence, as recommended by the WHO [11]. Sequence data are entered into the database either by individual contributors or by a weekly search of sequences submitted to GenBank. The data are quality checked and organized, first automatically by the database application and then manually by a curator. In addition, the deposited sequences are assigned a genotype and a cluster identifying number by matching against WHO reference sequences and the unique sequence clusters in the database, respectively. All individual sequences can be assigned for "Public viewing" or "Private." If the latter is chosen, the sequence information is only available to the administrators or those working in the WHO regional or global offices.

Dynamic reports and graphical charts can be created on any user-selected fields in the MeaNS database (eg, genotype or sequence variation in a geographical location or time period). Relevant data can be uploaded to GenBank using a specially created interface, and all submitted sequence names and genotypes are submitted weekly to the WHO. Bioinformatics tools in MeaNS allow one to find identical or similar sequences, assign a genotype, display phylogenetic trees, and to temporally and spatially track measles transmission chains. Access to the database is by registration on the website. Currently, only sequences in ASCII format can be submitted, but future developments are planned to enable uploading of sequence trace files and quality-checking mechanisms to be undertaken. It is anticipated that a similar database for rubella will be developed to enable similar tracking of rubella sequences.

To date (1 June 2010), there are 4751 sequences entered into the database from 4403 different samples. Of these, >4200 sequences are from N-450, and ~480 are full-length H gene. Thirty-four percent of the samples submitted belong to genotype D4, which reflects the recent outbreaks in different parts of the world caused by this genotype. The number of sequences submitted from different countries varies tremendously, with 39% of all the sequences in the database from the United Kingdom reflecting their use of oral fluid samples for routine measles surveillance, which can be used for both case confirmation and molecular epidemiology.

GLOBAL DISTRIBUTION OF MEASLES GENOTYPES

LabNet support for virologic surveillance is now well established in all WHO regions. Although virologic surveillance in some areas is still not adequate, a global picture has emerged (Figure 1, Tables 2–7) [2, 14]. Figure 1 is based on submissions to the WHO genotype database, whereas more-specific information for each region is presented in Tables 2–7. Some tables contain information that has not yet been reported to the WHO database. Note that the lack of an entry in the column describing endemic genotypes in Table 2 and Tables 4–7 is not meant to imply that the country has achieved measles elimination.

In general, 3 patterns of measles genotype distribution have been described. In countries that still have endemic transmission of measles, the majority of cases are caused by one or several endemic genotypes that are distributed geographically. In these cases, multiple co-circulating lineages within the endemic

Table 1. Databases That Support Molecular Epidemiology of Measles Virus

Database			Epidemiological information				Sequence	
Name	Public/private	Link	WHO name[a]	Clinical	Travel	Epi link	Genotype	Sequence
CISID	WHO	http://data.euro.who.int/cisid/	N	Y	Y	Y	N	N
WHO-LabNet	WHO-LabNet	http://workspace.who.int/sites/genotype	Y	N	Y	Y	Y	N
MeaNS	Public/private	http://www.who-measles.org	Y	Y	Y	Y	Y	Y

NOTE. WHO, World Health Organization.

[a] Standardized name as recommended by WHO.

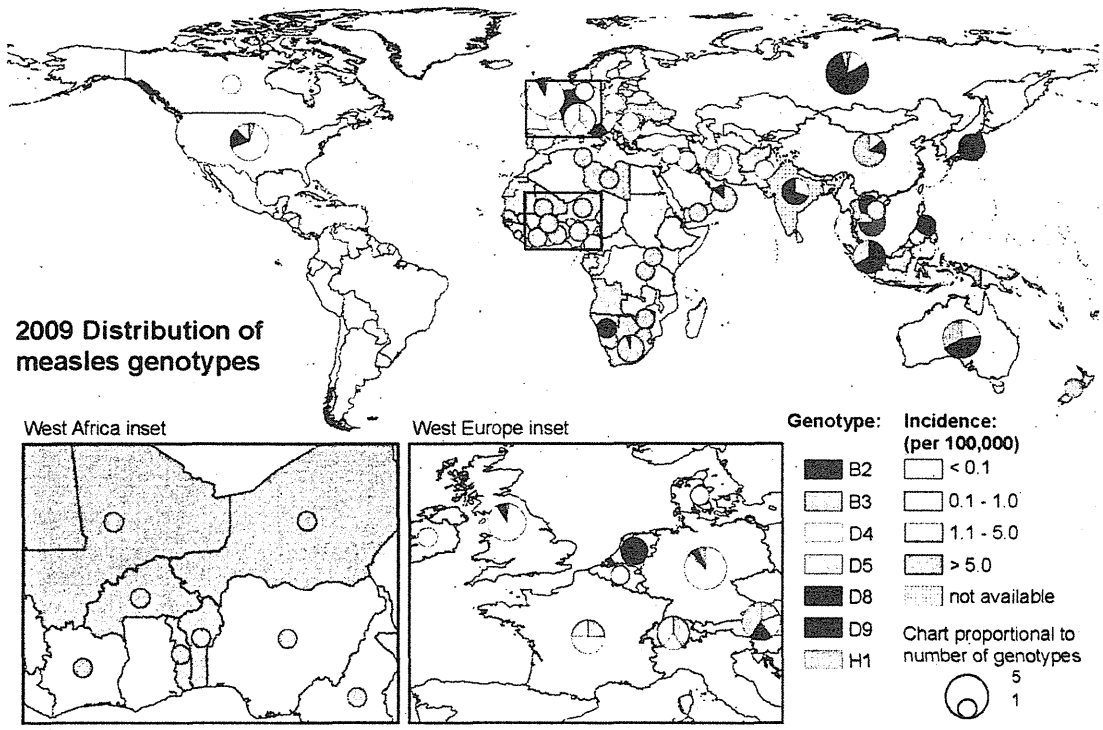

Figure 1. Global distribution of measles genotypes and measles incidence in 2009. Colored circles indicate measles genotypes reported to the World Health Organization (WHO) Database for the year 2009, and the size of the circles is proportional to the number of genotypes reported for the indicated areas (see insert of figure). For more specific information for each region, see Tables 2–7. Countries are shaded in gray to indicate measles incidence rates (see insert in figure). Two areas, Western Africa and Eastern Europe, are also shown as inserts to provide more resolution. The boundaries and names shown and the designations used on this map do not imply the expression of any opinion whatsoever on the part of the WHO concerning the legal status of any country, territory, city, or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. Dotted lines on maps represent approximate border lines for which there may not yet be full agreement.

genotype or genotypes are present. In countries that have eliminated measles, the small numbers of cases are caused by a number of different genotypes that reflect various sources of imported virus and suggest the lack of sustained transmission of an endemic genotype or genotypes. The third pattern occurs in countries or regions that have had very good measles control but are experiencing an increase in the numbers of susceptible individuals because of failure to maintain high vaccination coverage rates. In this situation, reintroduction of measles usually results in outbreaks that are associated with a single genotype of virus with nearly identical sequences. In countries approaching measles elimination, introduction of measles can result in small-to-moderate outbreaks that can contribute to establishing and maintaining international chains of transmission.

MEASLES VIROLOGIC SURVEILLANCE IN THE WHO AFRICAN REGION

Virologic surveillance has improved substantially in the WHO African Region (AFR). During 2007–2009, viral genotype information was obtained from 21 countries (Table 2). The most frequently detected genotype was genotype B3, which represented 197 (89%) of 220 of the sequences reported to the WHO Global Sequence database. In 2010, genotype B3 was also found to be circulating in Malawi, Liberia, and Mauritania. Genotype B3 is clearly the endemic genotype in most of the African continent with the exception of the Northern African countries in the Eastern Mediterranean Region. Genotype B3 has been divided into 2 clusters [29]. Genotype B3, cluster 1, viruses have previously been isolated from Cameroon, Ghana, and Nigeria and as far east as Kenya and Tanzania, suggesting that genotype B3 viruses are widely distributed throughout Africa [29, 30]. The circulation of genotype B3 cluster 2 viruses appears to be more limited to Western Africa [21, 31]. Genotype B3 has been associated with frequent importations from African countries into other parts of the world [25]. Genotypes D2, D4, and D10 had been the most frequently detected genotypes in the southern and eastern parts of the African continent [32–35], although more recent outbreaks in Kenya, Uganda, Burundi, and Tanzania have been caused by genotype B3 viruses [25]. Genotypes D4 and D10, which had been circulating in eastern Africa, have not been detected in that region in the past 3 years, and D10 has not been detected anywhere since 2005 [36].

Genotype B2 was considered inactive because, until recently, no representative viruses had been isolated since 1984. However, genotype B2 viruses were detected in Angola and South Africa during 2002–2003, primarily in association with cases and importation from Angola and in the Central African Republic [37, 38], and in 2004, there was an abrupt shift from genotype B3 to genotype B2 during an outbreak in Kinshasa (Democratic Repubic of the Congo) [39]. During 2007–2009, 21 genotype B2 sequences were reported from the Democratic Republic of the Congo, Zambia, and Angola (Table 2). A single case of genotype B2 was found in Namibia in 2010, indicating that the genotype is still circulating. Two viruses in genotype D8 were detected in South Africa, and these likely represented importations of virus from India, where genotype D8 is endemic (S. Smit, unpublished data).

MEASLES VIROLOGIC SURVEILLANCE IN THE AMERICAS

The Measles and Rubella Laboratory Network for the Region of the Americas consists of 21 National Laboratories, 124 subnational laboratories, 2 Regional Reference Laboratories, and 1 Global Specialized Laboratory. Endemic transmission has been eliminated in many areas of the world, including the all of the countries in the Western Hemisphere, Region of the Americas (AMR) [16, 40]. Analysis of viruses isolated from measles cases and outbreaks in the Americas indicates that there is no ongoing transmission of an endemic genotype or genotypes. Rather, the diversity of genotypes detected in the past 15 years is indicative of multiple, imported sources of virus [17, 40]. Five different genotypes were associated with imported cases in the AMR from 2007 through 2009 (Table 3). Some were associated with isolated cases, whereas others were responsible for relatively small outbreaks [41–44]. Most recently, in 2010, an outbreak occurred in Canada that was due to importation from travelers and athletes attending the 2010 Winter Olympic Games. Genotype H1 and 2 distinct strains of D8 were detected (National Microbiology Laboratory, unpublished data).

Table 2. Measles Genotypes Detected in the African Region, 2007–2009

Country	Genotypes associated with endemic transmission	Genotypes associated with importation
Benin	B3	
DR Congo	B3, B2	
Chad	B3	
Zambia	B2, B3	
Angola	B2, B3	
Central African Republic	B3	
Cameroon	B3	
Niger	B3	
South Africa	B3	D8
Equatorial Guinea	B3	
Burkina Faso	B3	
Ethiopia	B3	
Nigeria	B3	
Burundi	B3	
Uganda	B3	
Togo	B3	
Mali	B3	
Cote d'Ivoire	B3	
Zimbabwe	B3	
Lesotho	B3	
Senegal	B3	

MEASLES VIROLOGIC SURVEILLANCE IN THE WHO EASTERN MEDITERRANEAN REGION

The WHO Regional Office for the East Mediterranean Region (EMR) has established a regional goal to eliminate measles by 2010 with laboratory support of surveillance as an essential component of the program. All 22 countries in the region have established National Measles and Rubella Laboratories.

In 2002, very few countries in the Region had initiated virologic surveillance for measles [45]. However, starting in 2007, the countries in the EMR have made remarkable progress in identifying circulating measles virus as a result of the increased capacity of the laboratory network for virus detection and genotyping [46–48].

Seventeen (77%) of the 22 countries in the EMR have identified measles genotypes between 2007 and 2009, of which 16 have reported measles genotypes to the WHO Genotype Database (Table 4). The 166 sequences reported included genotypes B3, D4, D5, D8, and H1. The most frequently detected genotype is D4, which was detected in 12 of the 17 countries and represented 53% of the sequenced genotypes. Genotype D4 has been associated with ongoing endemic transmission in the EMR and has been associated with major outbreaks in Syria, Egypt, Iraq, and Iran, despite reported high vaccination coverage in these countries. However, in some countries, such as Morocco,

Table 3. Measles Genotypes Detected in the Region of the Americas, 2007–2009

Country	Genotypes associated with imported cases
Canada	D4, D5, D8
Peru	D4
United States	B3, D4, D5, D8, H1
Argentina	D8
Jamaica	D4
Venezuela	B3
Chile	D4

Oman, and Bahrain, genotype D4 was only found in limited numbers of cases after importation from other countries that were probably within the region. Genotype B3 caused outbreaks in Libya in 2009 and was imported to Tunisia. An outbreak associated with genotype B3 occurred in Yemen and was imported to Oman. Genotype B3 was detected in 6 countries and was the second most common genotype in the region, comprising of 29% of the reported genotypes.

MEASLES VIROLOGIC SURVEILLANCE IN THE WHO EUROPEAN REGION

The WHO European Region (EUR) had adopted the target to eliminate measles and rubella and to prevent congenital rubella infection by 2015. Fifty-two of the 53 countries in the region have established National Measles and Rubella Laboratories or have access to a National Laboratory in another Member State. The EUR is very heterogeneous with respect to national strategies for measles surveillance and elimination. This is reflected in the patterns of measles transmission reported from 2007 through 2009 and in the amount of sequence information available from the different countries.

Measles cases were reported from 47 of 53 countries from 2007 through 2009, 6 countries reported zero measles cases, and 1 country did not report. Genotypes were reported from 26 countries in EUR (Table 5).

From 2007 through 2009, >2000 measles sequences were determined. Genotypes B3, D4, D5, D6, D8, D9, H1, and A (vaccine associated) were found. Transmission of the genotypes C2 and D6, which were previously endemic in some parts of Europe, has apparently been interrupted [20, 23], with no detection of C2 viruses in the EUR from 2007 through 2009 and detection of D6 only until 2007 [49]. Genotypes D4 and D5 were both associated with large numbers of cases and evidence of endemic transmission. D5 was introduced into Switzerland, presumably from Thailand, by the end of 2006. From the subsequent outbreak [50], the virus spread to Germany, Austria, Belgium, France, Norway, Denmark, and other countries [51, 52] and circulated in the EUR for at least 2.5 years. A D4 strain was introduced into the UK in spring 2007 (source unknown), spread initially within the traveler community and then within the Orthodox Jewish community before entering the more general population [53]. The outbreak continued until autumn 2009, spreading to many other countries both inside and outside the EUR. In addition, a background of other D4 variants was observed. One D4 variant was transmitted from Germany to Bulgaria and initiated an outbreak in 2009, which was ongoing in 2010 [54]. The epidemic of >24,000 cases in Bulgaria occurred after several years without endemic measles circulation and was limited mostly to the ethnic group of the Roma.

Table 4. Measles Genotypes Detected in the Eastern Mediterranean Region, 2007–2009

Country	Genotypes associated with endemic transmission	Genotypes associated with importation
Afghanistan	D4	
Bahrain	D4	D4
*Djibouti	B3	
Egypt	D4	
Iran	D4	H1
Iraq	D4	
Jordan	D4	
Kuwait	B3	D5, D8
Libya	B3	
Morocco	D8	D4
Oman	D8	B3, D4, D5
Pakistan	D4	
Qatar	D4	
Sudan	B3, D4	
Syria	D4	
Tunisia		B3
Yemen	B3	

With respect to measles elimination, the EUR gives a heterogeneous picture. Several countries in Eastern and Northern Europe reported few measles cases and have reached the elimination stage. All cases were associated with importations, and the pattern of measles genotypes detected is consistent with the elimination of indigenous measles. The Russian Federation and most Newly Independent States (NIS) countries have made remarkable progress towards elimination [49], whereas many countries in Western Europe still report a high incidence of measles. Some countries even have evidence of renewed endemic transmission after a period of low number of reported cases. Factors that contribute to the failure to reach elimination in the latter countries are low vaccination coverage in some age groups, partially because there are no mandatory vaccination programs; the presence of individuals who refuse vaccination even if offered; and the absence of effective strategies to provide vaccination to travelers and other hard-to-reach groups [55].

MEASLES VIROLOGIC SURVEILLANCE IN THE WHO SOUTHEAST ASIAN REGION

From 2007 to 2009, the majority of the 98 viral genotype reports sent to the WHO Global Measles Database from the Southeast Asian region (SEAR) were from India (Table 6). This is because of the rapid and successful expansion of LabNet activities in India, which is a country with widespread endemic circulation of measles virus. Previously, genotypes D4 and D8 have been isolated in India and Nepal [9, 56–59], and genotype D4 and D8 viruses have also been detected in measles cases imported into the United States from India [17, 40]. Genotype D7 was detected in a few sporadic cases in India [60]. During 2007–2009, all of the genotypes reported from India were D4 or D8. During this

Table 5. Measles Genotypes Detected in the European Region, 2007–2009

Country	Genotypes associated with endemic transmission	Genotypes associated with importation
Austria		D4, D5, D8, H1, B3
Belarus		D5
Belgium		D4, D5, D9, D8
Bosnia and Herzegovina		D4
Bulgaria	D4	D4, H1
Croatia		D4
Denmark		B3, D4, D5, D9, H1
France		B3, D4, D5, D8, D9, H1
Germany		B3, D4, D5, D8, D9, H1
Israel		D4, D9
Kazakhstan	D6	
Kyrgyzstan		D4, D6
Netherlands		B3, D4, D5, D8, D9
Norway		D5
Poland		D4, D6
Portugal		D4
Republic of Moldova		D6
Romania		D4, D5
Russian Federation	D6	B3, D4, D5, D6, D8, D9, H1
Serbia		D4, D9
Spain		D4, D5, D9
Switzerland	D5	B3, D4
The former Yugoslav Republic of Macedonia		D4
Turkey		D4
Ukraine	D6	
United Kingdom of Great Britain and Northern Ireland	D4	B3, D4, D5, D8, D9
Uzbekistan	D6	

period, genotypes D5 and D9 were detected in Thailand, and genotypes D4 and D8 were detected in Nepal. In 2007, genotype H1 was detected in the Democratic People's Republic of Korea.

Virologic surveillance prior to 2007 also indicated that genotypes G2, G3, and D9 appeared to be the endemic genotypes in Indonesia and East Timor [61]. Genotype G2 has been detected in Thailand in the early 2000s, in addition to genotype D5. Genotype D5 was detected from a small outbreak in the Maldives in 2005, and genotypes D5 and D9 were detected in Myanmar.

Table 6. Measles Genotypes Detected in the Southeast Asian Region, 2007–2009

Country	Genotypes associated with endemic transmission	Genotypes associated with importation
India	D4, D8	
Bangladesh	D4, D8	
Maldives	D5	
Myanmar	D5, D9, d11[a]	
Thailand	D5, D9, G2	
Indonesia	G2, G3, D9	
Nepal	D4, D8	
DRPK	H1	

NOTE. [a] Detected in China

MEASLES VIROLOGIC SURVEILLANCE IN THE WHO WESTERN PACIFIC REGION

With the target to eliminate measles by 2012, all of the countries in the Western Pacific Region (WPR) are conducting case-based surveillance for measles, and virologic surveillance is well established in the region. Some countries (eg, Australia) have a pattern of viral genotypes that is consistent with elimination of endemic virus (Table 7). From 2007 through 2009, countries in WPR submitted genotype information from 1127 cases to the WHO Global Measles Database. Of these, 990 were genotype H1, and 820 of these reports were from China (Table 7). Therefore, genotype H1 continues to be the indigenous strain in China. The Chinese genotype H1 sequences have been divided into 3 clusters, H1a, H1b, and H1c [62], and all of the recent sequences were members of cluster H1a [63, 64]. The H1

Table 7. Measles Genotypes Detected in the Western Pacific Region, 2007–2009

Country	Genotypes associated with endemic transmission	Genotypes associated with importation
Australia		D4, D8, H1, D9, D5
Cambodia	D9	H1
China	H1	D4, D9, d11
China, Hong Kong SAR	H1	H1, D9
China, Macao SAR		D9, H1
Japan	D5, H1	D4, D8, D9
Lao People's Democratic Republic	H1	D9
Malaysia	D9, G3	
New Zealand		B3, H1, D4
Philippines	D9, G3	
Republic of Korea	H1	B3, D5
Singapore	D9	D4, D5, D8, D9, H1
Vietnam	H1	

genotype is also endemic in Viet Nam and caused outbreaks in 2009. In addition to the indigenous viruses in China, genotypes D4 and D9 were detected in association with imported cases in 2009 [65]. A new genotype, designated as provisional genotype d11, was detected in 2009 in viruses that were imported into China from Myanmar [66].

Elsewhere in WPR, genotypes H1, D9, G3, and D5 were associated with endemic transmission in several countries, and genotypes D4, D5, D8, D9, H1, and B3 were associated with imported cases in the region (Table 7). Genotype D3 had been associated with endemic transmission in the Philippines, but recent viral isolates are genotypes D9 and G3. Genotype D3 has not been detected in the WPR or in any other region since the mid-2000s. During 2007–2009, 13 (87%) of the 15 countries in the WPR (excluding the 20 Pacific Island countries) have identified measles genotypes.

SUMMARY AND FUTURE CHALLENGES

As several regions move toward measles elimination goals, adequate virologic surveillance will become an essential component of the surveillance systems that will be needed to verify that the elimination targets have been reached. The information presented in this report has briefly documented the tremendous expansion of global virologic surveillance for measles through the actions of LabNet. The laboratory methods to perform genetic analysis of wild-type measles strains are firmly established in all regions, and all of the WHO Regional Reference Laboratories have the capacity for virus isolation, reverse-transcription polymerase chain reaction (RT-PCR), and sequencing. An efficient mechanism has been established for timely reporting of sequence information. All of the LabNet laboratories are now using a single cell line, Vero/hSLAM [67], for isolation of both measles and rubella viruses. It is particularly encouraging that some countries were able to detect very small numbers of imported cases despite widespread circulation of an endemic genotype.

Although viral isolates were not obtained from all countries during the 3-year time period presented in this report, most countries with endemic measles have conducted some baseline virologic surveillance. One of the challenges for LabNet will be to continue to expand virologic surveillance activities, especially in those countries where no or only sporadic virologic surveillance has occurred. Periodic training is essential to ensure that laboratory staff are proficient in the methods for cell culture as well as RT-PCR and sequencing, if applicable. LabNet laboratories are now actively developing quality control and quality assurance protocols for the molecular techniques so that this part of laboratory surveillance will be held to the same high standards that are currently in place for serologic testing.

The other major challenge for virologic surveillance is obtaining adequate samples from representative cases and outbreaks. This is particularly challenging in areas that lack the infrastructure for sample collection, storage, and shipment. The use of alternative sample collection methods, such as oral fluids and blood dried onto filter paper, should help in this regard [68].

Molecular surveillance in many countries and in the EUR, in particular, has shown that the endemic genotype or genotypes can change relatively quickly. Therefore, constant monitoring of cases and outbreaks is necessary.

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Nectin4 Is an Epithelial Cell Receptor for Canine Distemper Virus and Involved in Neurovirulence

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Nectin4 Is an Epithelial Cell Receptor for Canine Distemper Virus and Involved in Neurovirulence

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Canine distemper virus (CDV) uses signaling lymphocyte activation molecule (SLAM), expressed on immune cells, as a receptor. However, epithelial and neural cells are also affected by CDV *in vivo*. Wild-type CDV strains showed efficient replication with syncytia in Vero cells expressing dog nectin4, and the infection was blocked by an anti-nectin4 antibody. In dogs with distemper, CDV antigen was preferentially detected in nectin4-positive neurons and epithelial cells, suggesting that nectin4 is an epithelial cell receptor for CDV and also involved in its neurovirulence.

Distemper is a severe infectious disease that mainly affects dogs and other canids (5). The causative agent is canine distemper virus (CDV), which is closely related to measles virus (MV) (23). CDV belongs to the genus *Morbillivirus* in the family *Paramyxoviridae* and possesses a single-stranded negative-sense RNA genome encoding six structural and two nonstructural proteins (23). Two surface glycoproteins, H and F, play key roles in virus entry. The H protein is responsible for the receptor binding, and the F protein mediates membrane fusion. Signaling lymphocyte activation molecule (SLAM) expressed on cells of the immune system is a receptor for CDV (16). SLAM serves as a common receptor for morbilliviruses (1). Using SLAM as a receptor, CDV primarily replicates in lymphocytes and macrophages in the respiratory tract and then disseminates throughout the body (22). However, SLAM-negative cells in epithelia and the central nervous system (CNS) are also affected by CDV *in vivo* (2, 21). Recently, nectin4 was identified as an epithelial cell receptor for MV (13, 14). In humans, nectin4 is expressed mainly in the placenta and, to lesser extents, in the tonsil, oral mucosa, trachea, esophagus, nasopharynx, prostate, lung, and stomach (13, 14, 20). Although MV also exhibits neurovirulence and causes a persistent infection of the CNS, subacute sclerosing panencephalitis (SSPE), neither SLAM nor nectin4 was detected in neural cells of the human CNS (13, 14, 20). The frequency of SSPE is 1/5,000 to 1/100,000 in reported cases of acute measles (3, 19). In contrast, acute infection of animals with CDV is often accompanied by severe neurological manifestations, which are rarely seen in patients with acute measles (2, 21). The aim of the present study was to elucidate the roles for nectin4 in CDV pathogenesis, including its neurovirulence.

Six wild-type CDV strains (Ac96I, 007Lm, Th12, M24Cr, 55L, and 82Con) isolated from dogs with distemper by using Vero.DogSLAMtag cells were employed in the present study. Some of these strains were reported previously (7, 8, 10). Within 2 days after infection, they all induced syncytia in Vero cells constitutively expressing dog nectin4 (Vero/dNectin4), but not in the parental Vero cells (Fig. 1A, B, and C). The formation of syncytia was completely blocked by 20 μg/ml of a goat anti-human nectin4 polyclonal antibody (R&D Systems) and clearly reduced by 10 μg/ml of the antibody (Fig. 1D). Production of infectious virus particles was inhibited by the anti-nectin4 antibody in a dose-dependent manner (Fig. 1E). Although CDV replicated poorly in Vero cells, it replicated efficiently in Vero/dNectin4 cells (Fig. 1F), as observed in Vero.DogSLAMtag cells. CDV produced plaques in Vero.DogSLAMtag and Vero/dNectin4 cells, but not in the parental Vero cells, although PFU were reduced by ~3-fold in Vero/dNectin4 cells compared to Vero.DogSLAMtag cells (Fig. 1G). The size of plaques was also smaller in Vero/dNectin4 cells than in Vero.DogSLAMtag cells (Fig. 1G). These findings indicate that dog nectin4 functions as a CDV receptor, similar to the case with MV (13, 14).

Seven dogs with distemper were necropsied, and tissues were subjected to histopathological analyses. Hematoxylin and eosin staining of the tissue samples revealed pathognomonic changes with CDV infection, including lymphoid depletion, catarrhal enteritis, bronchointerstitial pneumonia, and nonsuppurative encephalitis (data not shown) (9). Eosinophilic intracytoplasmic and intranuclear inclusion bodies were observed in the brain, lymphoid organs, and lung (data not shown). Immunohistochemical double staining for CDV antigen and nectin4 was conducted in two ways. In the first method, CDV antigen was stained pink by Fast red II and nectin4 was stained brown by diaminobenzidine (Fig. 2). In the second method, CDV antigen and nectin4 were labeled with the red fluorescent probe Alexa Fluor 594 and green fluorescent probe Alexa Fluor 488 (Fig. 3). Nectin4 was expressed in all epithelia of the lung, kidney, intestine, and urinary bladder (Fig. 2A to D and 3A to C and data not shown). CDV antigen was detected in accordance with some of the nectin4-positive epithelial cells (Fig. 2A to D and 3A to C and data not shown). Impor-

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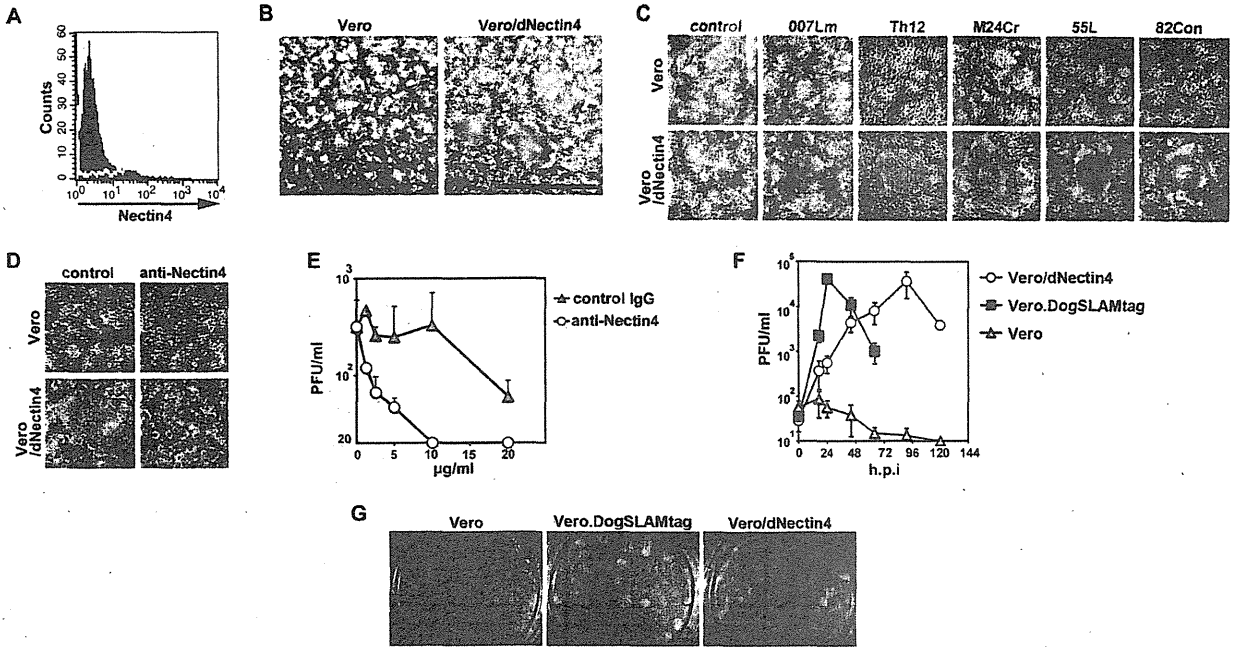

FIG 1 Infection of Vero/dNectin4 cells with CDV. (A) Vero/dNectin4 (gray empty profile) and parental Vero (filled black profile) cells were stained with a goat anti-human nectin4 polyclonal antibody (R&D Systems), followed by staining with Alexa Fluor 488-conjugated anti-goat IgG. (B) Vero/dNectin4 (right panel) and parental Vero (left panel) cells were infected with the Ac96I strain. At 48 h postinfection, the cells were observed under a phase-contrast microscope. Bar, 1 mm. (C) Vero/dNectin4 (lower panels) and parental Vero (upper panels) cells were infected with wild-type strains of CDV (007Lm, Th12, M24Cr, 55L, or 82Con) or left uninfected (control). At 48 h postinfection, the cells were observed under a phase-contrast microscope. (D) Vero/dNectin4 (lower panels) and parental Vero (upper panels) cells were infected with the wild-type Ac96I CDV strain in the presence (anti-Nectin4) or absence (control) of the goat anti-human nectin4 polyclonal antibody. At 48 h postinfection, the cells were observed under a phase-contrast microscope. (E) Vero/dNectin4 cells pretreated with increasing concentrations of anti-Nectin4 antibody or control IgG were infected with 1,000 PFU of strain Ac96I and cultured with the same concentrations of the antibody or control IgG. At 48 h postinfection, the virus titers of the supernatants were determined in plaque assays. (F) Vero/dNectin4, Vero.DogSLAMtag, and parental Vero cells were infected with the wild-type Ac96I CDV strain at a multiplicity of infection of 0.05. At various time intervals, the virus titers were determined in plaque assays. (F) Vero/dNectin4 (right panel), Vero.DogSLAMtag (middle panel), and the parental Vero (left panel) cells in 12 well-cluster plates were infected with the wild-type Th12 strain and overlaid with medium containing 1% agarose. At 7 days postinfection, the plaques were observed under a stereoscope.

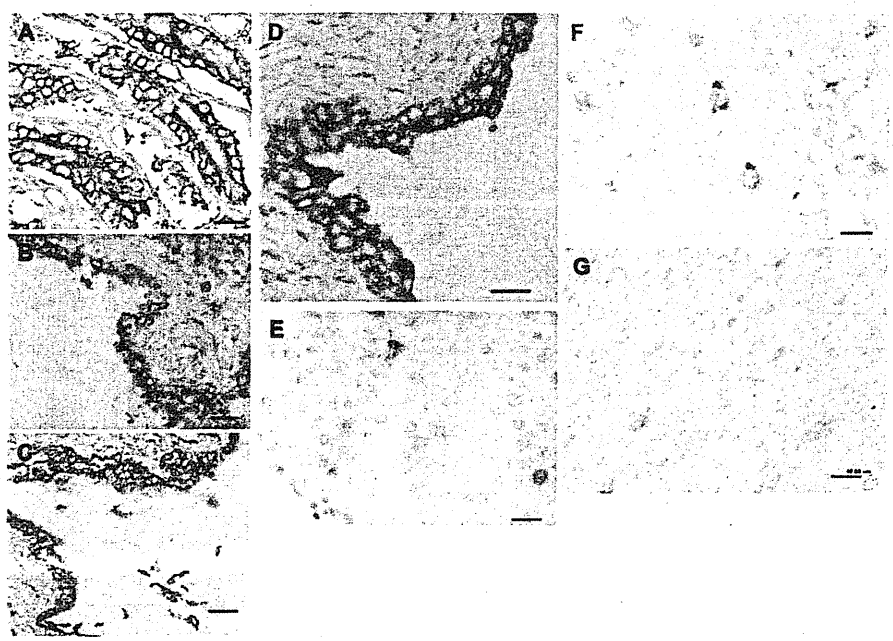

FIG 2 Immunohistochemical double staining for CDV antigen and nectin4. Tissue sections of 2-μm thickness were deparaffinized, rehydrated, and subjected to heat-induced antigen retrieval before incubation with antibodies. Subsequently, blocking of endogenous peroxidase was performed. The tissue sections were then incubated with a mouse monoclonal anti-CDV antibody (Adtec, Japan) and a goat anti-nectin4 polyclonal antibody (R&D Systems). Using an EnVision kit (Dako) and 3′,3-diaminobenzidine (Sigma), CDV antigen and nectin4 were visualized (pink and brown staining, respectively), according to the manufacturer's protocol. (A) Intestine; (B) lung; (C) renal pelvis; (D) urinary bladder; (E) cerebellum; (F) cerebrum; (G) midbrain. Bars, 20 μm (A, B, D, E, and F) or 40 μm (C and G).

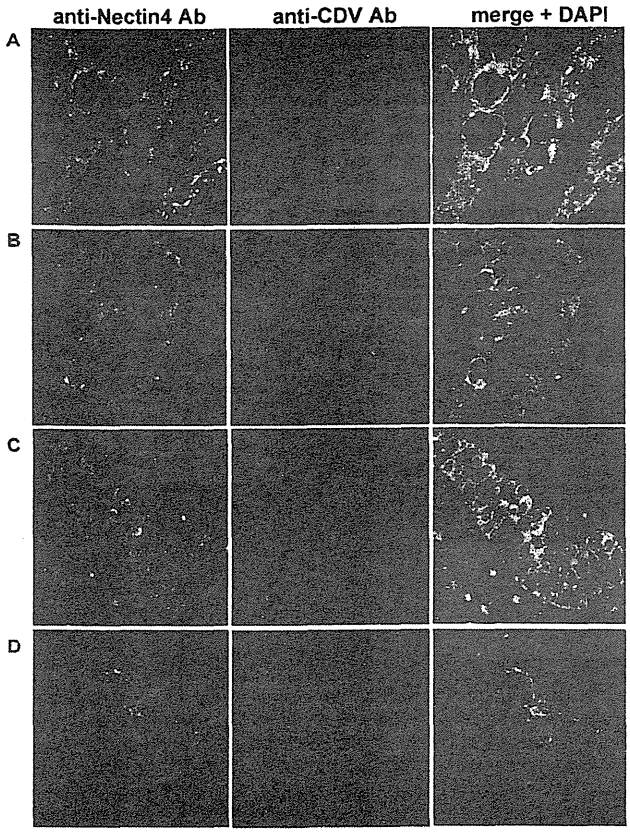

FIG 3 Immunofluorescence double staining for CDV antigen and nectin4. Tissue sections of 2-μm thickness were deparaffinized, rehydrated, and subjected to heat-induced antigen retrieval before incubation with antibodies. CDV antigen was stained with a mouse anti-CDV monoclonal antibody (Adtec, Japan) and Alexa Fluor 594-conjugated secondary (red) antibody. Nectin4 was stained with a goat anti-nectin4 polyclonal antibody (R&D Systems) and Alexa Fluor 488-conjugated secondary antibody (green). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; blue). (A) Intestine; (B and C) lung; (D) brain.

tantly, and in contrast to the reports for humans, nectin4 was detected in the brain of dogs, and CDV antigen was preferentially detected in the nectin4-positive neurons (Fig. 2E to G and 3D). These findings suggest that nectin4 may contribute to infection of the CNS with CDV.

Although SLAM is not expressed in epithelial and neural cells, CDV antigen is often detected at high levels in the epithelia of various organs and the CNS of dogs with distemper (21). Several candidate molecules that support SLAM-independent CDV infection have previously been reported. Fujita et al. (6) reported that a heparin-like molecule supports the entry of CDV into cells of the human embryonic kidney 293 cell line. CD9 has also been described to support cell-to-cell fusion by CDV (12, 15, 17). A recent study further showed that chicken embryo fibroblasts and monkey kidney Vero cells express unidentified receptor molecules for CDV with molecular masses of 57 and 42 kDa, respectively (4). However, that study did not provide a solid conclusion for the *in vivo* epithelial and neural receptors of CDV (4, 6, 12, 15, 17). For MV, a breakthrough came with the identification of nectin4 as an epithelial receptor for the virus, implying an efficient mechanism for MV transmission, although the mechanisms for MV infection of neural cells remained unclear (13, 14). In the present study, we investigated CDV as a model of morbillivirus infection of the CNS, since the virus often causes severe infection of the CNS in infected animals (21). Our data demonstrated that dog canine nectin4 functions as a CDV receptor and is expressed in neural cells of the brain as well as the epithelia of various organs. In the brain, nectin4-positive neurons were preferentially targeted by CDV. Therefore, CDV infection of the CNS could be partly explained by the knowledge that dog canine nectin4 acts as a receptor for CDV. Like SLAM, nectin4 seems to be a common receptor for morbilliviruses, because critical residues of the H protein required for nectin4 binding are highly conserved among morbilliviruses (11, 18). Thus, the findings in the present study contribute to our understanding of the pathogenesis of CDV, and possibly other morbilliviruses.

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