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Comparative sequence analysis of the P-, M- and L-coding region of the measles virus CAM-70 live attenuated vaccine strain[1]

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Abstract

Measles virus is a highly contagious agent which causes a major health problem in developing countries. The viral genomic RNA is single-stranded, nonsegmented and of negative polarity. Many live attenuated vaccines for measles virus have been developed using either the prototype Edmonston strain or other locally isolated measles strains. Despite the diverse geographic origins of the vaccine viruses and the different attenuation methods used, there was remarkable sequence similarity of H, F and N genes among all vaccine strains. CAM-70 is a Japanese measles attenuated vaccine strain widely used in Brazilian children and produced by Bio-Manguinhos since 1982. Previous studies have characterized this vaccine biologically and genomically. Nevertheless, only the F, H and N genes have been sequenced. In the present study we have sequenced the remaining P, M and L genes (approximately 1.6, 1.4 and 6.5 kb, respectively) to complete the genomic characterization of CAM-70 and to assess the extent of genetic relationship between CAM-70 and other current vaccines. These genes were amplified using long-range or standard RT-PCR techniques, and the cDNA was cloned and automatically sequenced using the dideoxy chain-termination method. The sequence analysis comparing previously sequenced genotype A strains with the CAM-70 Bio-Manguinhos strain showed a low divergence among them. However, the CAM-70 strains (CAM-70 Bio-Manguinhos and a recently sequenced CAM-70 submaster seed strain) were assigned to a specific group by phylogenetic analysis using the neighbor-joining method. Information about our product at the genomic level is important for monitoring vaccination campaigns and for future studies of measles virus attenuation.

Key words
- Measles virus
- Vaccine
- Nucleotide sequence
- RNA sequencing

[1]The measles virus gene sequences reported in the present study have been deposited in GenBank Data Libraries under accession numbers AF517559, AY124779 and AF527380.

Introduction

Measles virus, a member of the *Morbillivirus* genus (Paramyxoviridae family), is the causative agent responsible for a highly contagious airborne transmissible exanthematic illness that most commonly strikes children. Due to immune suppression, further serious complications causing high morbidity and mortality have been reported around the world (1,2). The genome consists of a single negative strand RNA 15,894 nucleotides long. There are six genes ordered as 3'-leader N, P/C/V, M, F, H, L-5' (3). Measles virus are divided into 21 genotypes (4) based on the C-terminus of the N gene or H gene nucleotide sequences, which are the most variable genes. The major structural polypeptide is encoded by the N (nucleocapsid) gene. The N protein is essential for introducing the genome into a ribonucleoprotein complex that serves as template for transcription, replication and packaging into progeny virions. The P cistron specifies three polypeptides: P, C and V. The P (phosphoprotein) polypeptide is a subunit of the viral RNA polymerase. P protein also acts as a chaperone that interacts by regulating the cellular localization of N protein and probably contributes to nucleocapsid assembly. The C and V polypeptides are nonstructural proteins that are translated from P mRNA by the use of alternative reading frames; C protein is synthesized from a downstream translation start signal, whereas V protein is translated from an edited mRNA that contains an extra G residue. The M gene encodes the matrix protein that lines the inner surface of the viral envelope and participates in virion maturation. The F (fusion) and H (hemagglutinin) genes encode envelope glycoproteins that mediate cell surface recognition, membrane fusion, and virus entry. Finally, the L (large) gene encodes the multifunctional catalytic subunit of the viral RNA-dependent RNA polymerase (5,6).

The Edmonston strain of measles virus isolated in cell culture in 1954 (7) became the progenitor for many live attenuated measles vaccines such as Moraten, Schwarz, Zagreb and Edmonston B (8). Several measles strains were also used for the development of measles vaccines in other countries such as Russia, China and Japan. All these attenuated strains are used around the world and despite the differences in methods employed for attenuation of the original viruses, which involve various cell culture systems, incubation temperatures, and number of passages, remarkable nucleotide sequence similarity has been found among the strains (9).

A live attenuated vaccine (CAM-70) derived from a Japanese isolate has been used in Brazil since 1982. National vaccination campaigns achieved more than 95% coverage after the Brazilian Measles Eradication Program was established in 1992, although some epidemic outbreaks have occurred after the introduction of the program, mainly in 1997 (2,10). Nevertheless, since December 2000 no cases of measles have been reported in Brazil. Efforts towards prevention, with an intense vaccination program consisting of mass vaccination and follow-up campaigns (2,11), have contributed strongly to the achievement of this goal.

CAM-70 vaccine was developed in 1970 from the Tanabe strain in Japan by adaptation to the chorioallantoic membrane (CAM) of chick embryos (12-15). This vaccine has been produced at Bio-Manguinhos/ FIOCRUZ since the early eighties by a technology transfer from the Biken Institute, Japan.

The current worldwide trend is that vaccine strains should be very well characterized with respect to their biological and genetic properties. Thus, scientists in other countries have been using molecular biology techniques to obtain the complete genome sequences of the attenuated vaccines administered to the population (6,9,16,17). However, the genetic basis for attenuation has yet to be determined (16). The CAM-70 Bio-

Manguinhos (CAM-70 BM) vaccine has not been completely analyzed; only the N, H and F genes were sequenced (9). P and L gene sequences from CAM-70 Kitasato Submaster Seed (CAM-70 KSS) have recently been reported (16).

We report the sequence analysis of the coding region of the P, M and L genes of Brazilian measles live attenuated CAM-70 vaccine, compared to other sequenced vaccine and wild-type strains of genotype A. For this purpose, plasmidial clones with determined regions of these genes were obtained, sequenced and analyzed.

Material and Methods

Cells and viruses

Vero cells (18) obtained from the American Type Culture Collection were cultured in 199 Earle's medium supplemented with 5% fetal bovine serum and 40 µg/ml gentamicin.

The lyophilized CAM-70 vaccine (lot No. 967SB005Z 0897) was supplied in vials containing five doses (Bio-Manguinhos/ FIOCRUZ). The vaccine was obtained through three passages of FMS-7 master seed virus in primary chick embryo fibroblast cells (19) (Figure 1A and B) and was named CAM-70 BM. The vaccine virus was propagated only once in Vero cells to obtain a stock for RNA production.

RNA preparation, RT-PCR and cloning

Total RNA was extracted from CAM-70 BM-infected Vero cells by the acid guanidinium-phenol method (20). Reverse transcriptase reactions were performed using M-MLV RT (Promega Corp., Madison, WI, USA) with a specific antisense primer (Table 1) or Superscript one-step RT-PCR (Invitrogen, Carlsbad, CA, USA). For the L gene, two long PCR fragments (about 3.5 kb each) were synthesized using the Elongase

Table 1. Primers used for cDNA, PCR and sequencing (seq) in this study. The primers' position refers to the complete genome.

Primer name/position	Sequence (5'-3')	Use
M13 F (-20)	gTA AAA CgA Cgg CCA g	seq
M13 R (-20)	CAg gAA ACA gCT ATg AC	seq
P 1807 S	ATg gCA gAA gAg CAg gCA CgC	PCR/seq
P 3351 A	ggC Agg TAA gTT gAg CTg TAg gTg CTT CAT	cDNA/PCR
P 2206 S	ggC CTT gAT ggT gAT AgC ACC CTC	seq
P 2240 A	TCT CCT CCT gAg Agg gTg CT	seq
P 2601 S	Agg TgC ACC TgC ggg gAA TG	seq
P 2631 A	ACA CTC ggg gAC ATT CCC Cg	seq
P 3001 S	ggC AgA gAT TCA ggC CgA gC	seq
P 3035 A	AgA ACT TCg gCC AgT gCT Cg	seq
M 4463 A	ATT gCT ggg CAC TAC ggT CTA	cDNA/PCR
M 3433 S	CCA CAA TgA CAg AgA CCT AC	seq
M 3459 A	CgA AgT CgT Agg TCT CTg TC	seq
M 3916 S	gAg CAT CAC CCg TCT TTC gg	seq
M 3900 A	ACC TCT gCg ggg TAT CgA gC	seq
L 9161 S	CAT CAg gCA TAC CCA CTA gT	PCR/seq
L 12631 A	AgC TCT CgC CAg CTg CAC Tg	PCR/seq
L 12475 S	CCA CAA AAg gCT TgA TTC gAg CC	PCR/seq
L 15877 A	ACC AgA CAA AgC Tgg gAA	cDNA/PCR/seq
L 9568 S	CgC TgT ACT CCA AAg TCA	seq
L 9556 A	CAC ggA TCT TCC TCg TTg AC	seq
L 9983 S	gAg CTT CTA ggA AgA gTC Ag	seq
L 10050 A	CgA gTg CAg ggA AgA AAC CAT	seq
L 10533 S	gAg CAg TgC gTT gAT AAC Tgg	seq
L 10583 A	gCA gCC AAA TTT CAC TCC Ag	seq
L 10996 S	CCA CAC TCT AgC TgT CTC A	seq
L 11011 A	CAg CTA gAg TgT ggA gTg CC	seq
L 11491 S	CCT ATC TAT ACC Tgg CTg CT	seq
L 11531 A	ATC CTT ACT CCg CTC TCA	seq
L 11991 S	CTC CTC ACA AAC AAC GAC CT	seq
L 11995 A	gAg gAg ggg TAT gAC TAC AT	seq
L 12961 S	gTA CTC ATg ggC TTA Cgg Tg	seq
L 12992 A	ATC ATC ATC ACC gTA AgC CC	seq
L 13462 S	CAA ggC TAT ACA CCC AgA gCC	seq
L 13466 A	CCT TgT TgC ATC TCT gTC	seq
L 13957 S	CCT ACC TCg ACC TgT TgT T	seq
L 13978 A	TTC AAC AAC Agg TCg Agg TA	seq
L 14451 S	CCC ATT TCA ggg ggC AAT CT	seq
L 14474 A	ggC gAg ATT gCC CCC TGA AA	seq
L 14839 S	TAT CCC TAC CTC TAg TgT ggg g	seq
L 14956 A	CCC Agg AgC AgA gCC ATC gA	seq
L 15117 S	CTC AAg gCT AAC CGG CTA A	seq
L 15470 S	gAT gTT CCA CgC TTA CCC Cg	seq
L 15472 A	ACg ggg TAA gCg Tgg AAC A	seq

S = sense; A = antisense.

Enzyme Mix (Invitrogen). All reaction conditions followed manufacturer recommendations. The amplification products were submitted to 0.8% agarose gel electrophoresis in 90 mM Tris-borate, 2 mM EDTA buffer and visualized by ethidium bromide staining in order to confirm that fragments were of the correct size (21). The PCR products were introduced into pCR2.1 using the TOPO TA cloning kit (Invitrogen) and recombinant plasmid DNA was purified using the Maxiprep CONCERT kit (Invitrogen).

Sequencing and analysis

Nucleotide sequences were determined by the dideoxy chain-termination method with dye-labeled terminators and T7 DNA polymerase (Applied Biosystems, Foster City, CA, USA) according to manufacturer instructions, followed by analysis with an ABI Prism 3100 automated sequence apparatus (Applied Biosystems). Three plasmidial clones for each gene or cloned portion (L gene) were sequenced to resolve compression or other ambiguities. Nucleotide sequences were aligned with the CLUSTAL X program version 1.8 (22) and the phylogram was constructed with the Phylogeny Inference Package, version 3.57c (J. Felsenstein and University of Washington, 1995, downloaded from the address http://evolution.genetics.washington.edu/phylip.html). The neighbor-joining method was applied and previously published nucleotide sequences available at GenBank under accession numbers AF266286-266290, U03665, AF462047, AF462051 and AF128250-128252 (6,9,16,17) were used for comparison.

Results

CAM-70 passage history

The CAM-70 measles live attenuated vaccine was developed from the Tanabe isolate of measles virus by adaptation to the CAM of chick embryos by Ueda and co-workers in 1970 (13,15,23). The data shown in Figure 1A illustrate the passage history of this vaccine at Bio-Manguinhos since 1982 when it was received as a master seed (FMS-7) from the Biken Institute.

Phenogram analysis

The phenograms obtained based on the

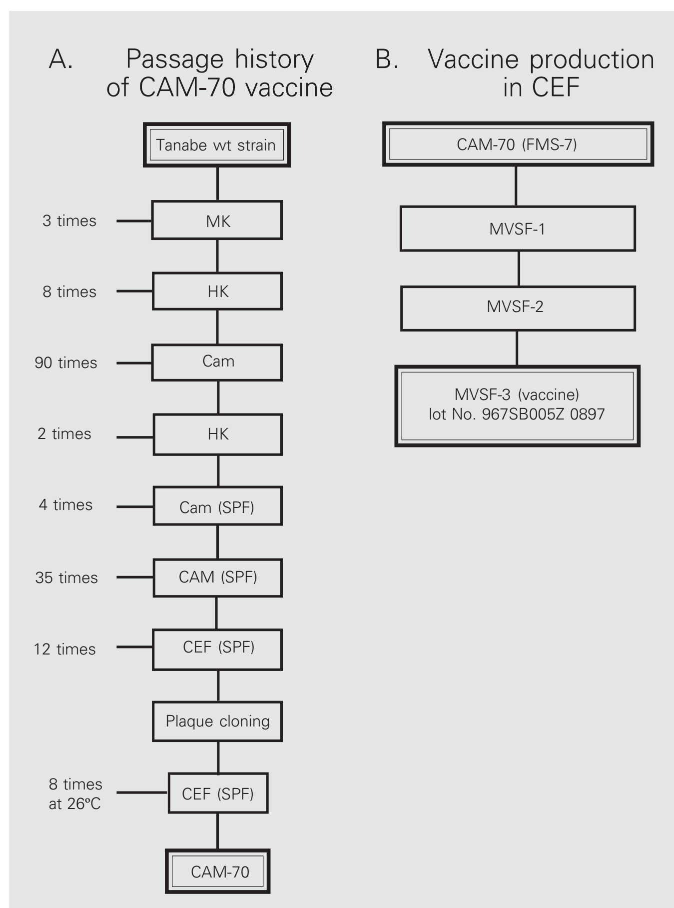

Figure 1. *A*, Passage history of the Tanabe wild-type (wt) strain, the progenitor of the CAM-70 BM vaccine (CAM-70), with the number of passages for each cell type. MK = monkey kidney; HK = human kidney; Cam = amniotic membrane; CAM = chorioallantoic membrane; SPF = specific pathogen free (avian leucosis); CEF = chick embryo fibroblasts. The temperature of incubation was 37ºC, except when indicated. *B*, Vaccine production scheme showing the number of passages of the FMS-7 master seed in CEF.

combined P, M and L nucleotide sequences are presented in Figure 2A-C.

All Edmonston-derived vaccine strains were assigned to one main group and CAM-70 strains to a single group. It was also shown that the CAM-70 BM vaccine was very similar to CAM-70 KSS (16) presenting a similarity of 99 and 100% in P and L genes, respectively.

Despite their similar origin, the non-Edmonston-derived vaccines Changchun-47 and Shanghai-191 were closely similar to the Edmonston wild type but not to the CAM-70 BM vaccine.

P gene nucleotide sequence analysis

Among the gene nucleotide sequences analyzed in this study as well as the strains selected for nucleotide sequence comparison, the P gene was the most divergent, presenting 12 nucleotide substitutions shared by both the CAM-70 BM vaccine and CAM-70 KSS strains. Two exclusive mutations were found only in the CAM-70 BM vaccine. Eighty-five percent of these nucleotide substitutions resulted in predicted amino acid changes (Table 2A). These changes were not found in Edmonston- or non-Edmonston-derived strains (16).

M gene nucleotide sequence analysis

Exclusive point mutations were found in the CAM-70 BM vaccine nucleotide sequence, located at genome positions 3968, 4111 and 4118 (Table 2B), two of which represented silent changes. The exception was for mutation at position 4111, which predicted replacement of arginine with methionine at amino acid position 225.

The Edmonston-derived vaccine strains shared with the CAM-70 BM vaccine the same nucleotide silent changes at positions 3627 (Moraten, Rubeovax and Schwarz) and 4292 (Zagreb). Two exclusive coding changes predicted replacement of glycine with serine and of tyrosine with aspartic acid

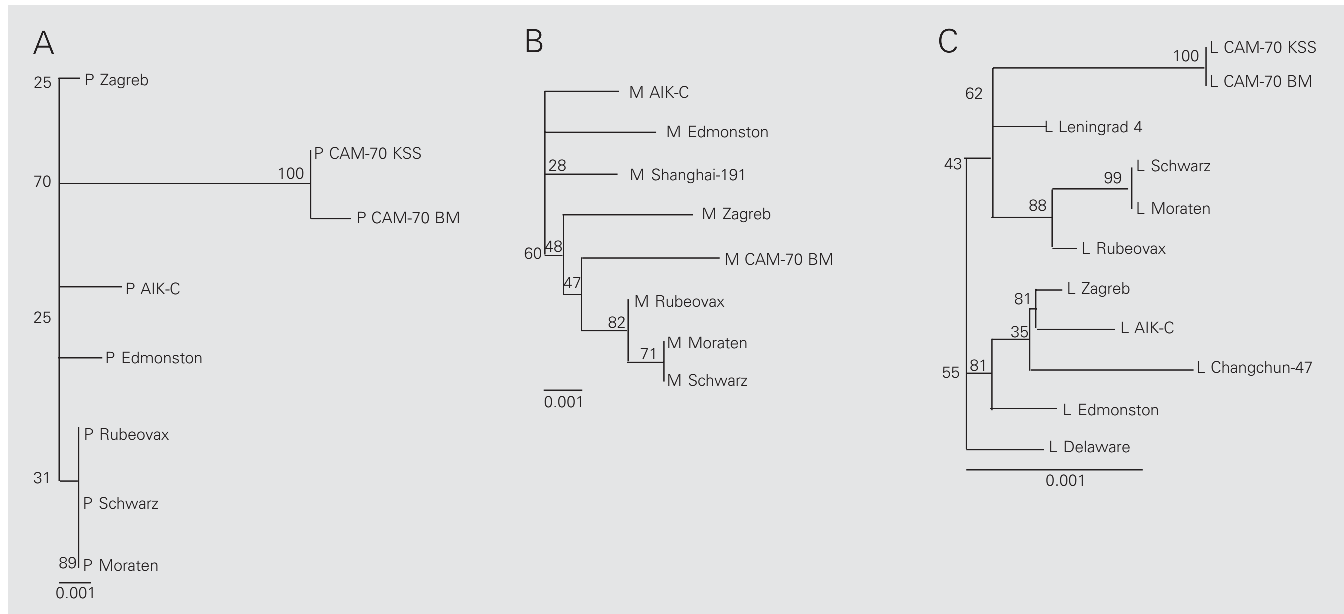

Figure 2. *A-C*, Unrooted phylograms based on the comparison of phosphoprotein (P), matrix (M) and large (L) gene sequences of available genotype A measles virus, respectively. The sequence alignment was performed using the CLUSTAL X program and the trees were drawn using the programs PUZZLE32, SEQBOOT and DNADIST in the PHYLIP Package. The phylograms were obtained using the TREEVIEW program. The scale indicates 0.1% nucleotide divergence.

in Shanghai-191, a non-Edmonston-derived strain (9). These changes were not found in the CAM-70 BM vaccine nucleotide sequence.

Table 2. Amino acid alteration of phosphoprotein (A), matrix (B) and large (C) proteins compared to other genotype A strains.

A. Phosphoprotein

Amino acid position Virus strain	98	99	110	121	130	138	165	266	285	412	419
Edmonston	R	N	Y	K	M	D	I	G	W	K	L
AIK-C	R	N	Y	K	M	D	I	G	W	K	L
CAM-70 BM	G	D	C	E	V	G	V	A	R	R[1]	I
CAM-70 KSS[2]	G	D	C	E	V	G	V	A	R	K	I
Moraten	R	N	Y	K	M	D	I	G	W	K	L
Rubeovax	R	N	Y	K	M	D	I	G	W	K	L
Schwarz	R	N	Y	K	M	D	I	G	W	K	L
Zagreb	R	N	Y	K	M	D	I	G	W	K	L

B. Matrix

Amino acid position Virus strain	177	225	227
Edmonston	M	R	K
AIK-C	M	R	K
CAM-70 BM	I	M	N
Shanghai-191	M	R	K
Moraten	M	R	K
Rubeovax	M	R	K
Schwarz	M	R	K
Zagreb	M	R	K

C. Large proteins

Amino acid position Virus strain	205	214	235	723	1091
Edmonston	I	S	I	Y	R
AIK-C	I	S	I	Y	R
CAM-70 BM	V	N	V	S	K
CAM-70 KSS[2]	V	N	V	S	K
Moraten	I	S	I	Y	R
Rubeovax	I	S	I	Y	R
Schwarz	I	S	I	Y	R
Zagreb	I	S	I	Y	R
Changchun-47	I	S	I	Y	R
Delaware	I	S	I	Y	R
Leningrad 4	I	S	I	Y	R

The one-letter amino acid abbreviation is used. The gray boxes refer to substitutions found in both CAM-70 strains.
[1]Exclusive amino acid substitution in the CAM-70 BM (Bio-Manguinhos) vaccine.
[2]CAM-70 KSS = Kitasato Submaster Seed.

Analysis of the L gene nucleotide sequence

The L gene nucleotide sequences of the CAM-70 BM and CAM-70 KSS vaccines were similar and nearly identical to the other measles strains used for nucleotide comparison. Exclusive nucleotide substitutions in CAM-70 strains were found at genome positions 9847, 9874, 9936, 11401 and 12505, predicting amino acid changes (Table 2C). Silent changes exclusively occurring in CAM-70 strains were located at positions 12506, 13247 and 14066. None of the changes found in nucleotide sequences of Edmonston- or non-Edmonston-derived strains (9) were shared by CAM-70 strains.

Discussion

Genetic analyses comparing H-, F- and N-coding genes have classified all measles vaccine strains currently used, including CAM-70 BM, as genotype A. The same CAM-70 BM vaccine lot analyzed by Rota and co-workers (9) was used in our study of the remaining genes (P, L and M). It was clearly demonstrated by phenograms obtained in previous analyses and corroborated by our study that CAM-70 BM was the most divergent member of genotype A (9).

The CAM-70 BM vaccine was derived from most geographically distant wild-type measles virus among the group of vaccines but this fact is not sufficient to explain its genetic divergence since Changchun-47 and Shanghai-191 vaccine strains, despite their distant origin, were genetically similar to Edmonston wild type.

Measles virus is a monotypic virus considered to be extraordinarily stable in terms of serology and immune response. However, the lack of proofreading of viral RNA-dependent RNA polymerase favors the accumulation of mutations and could explain the relatively high variability of the CAM-70 BM vaccine.

The adaptation to specific host cells is

probably correlated with nucleotide alterations (24) or changes in the RNA molecule "quasispecies" (25). It was demonstrated that measles virus isolated from B95a cells was fully pathogenic in *Cynomolgus* sp monkeys and had lost pathogenicity after adaptation to Vero cells (26). Amino acid changes in the P/C/V, H and L genes were identified during the adaptation process (27). Recently, the fusion induction ability of AIK-C vaccine was investigated (28). Transient expression with plasmids containing F and H protein regions from the AIK-C vaccine and Edmonston wild type at amino acid position 278 of the F gene demonstrated that leucine was responsible for reduced fusion capability and that phenylalanine was responsible for extensive cell fusion related to syncytium formation (28). Different plaque-forming variants have been isolated from wild-type virus or vaccine strains, including CAM-70 BM (29). Strains that do not form plaques but instead lead to the formation of clumps (foci) of infected cells have also been described (30). The high number of passages of CAM-70 BM in the amniotic cavity of chick embryos (Figure 1A) may have contributed to the increased number of nucleotide substitutions. Since vaccine strains have unique passage histories, probably each strain may have acquired a specific set of attenuating mutations at different positions in the genome (16).

Despite nucleotide similarity between the CAM-70 KSS and CAM-70 BM vaccine, two exclusive mutations localized on the P gene were found. The difference between these strains may be due to the number of passages. CAM-70 BM was passed three times in chick embryo fibroblasts from FMS-7 during vaccine production while CAM-70 KSS was passed only once in chick embryo fibroblasts (16).

The most divergent sequences were in the P gene with mutations predicting 11 amino acid replacements along the P protein. The multifunctional polypeptide P protein is a component of the coiled ribonucleoprotein core which forms the polymerase complex and is implicated in viral RNA encapsidation. It is also responsible for regulating the cellular localization of N protein (3,5,31). Thus, nucleotide mutations may affect the chaperone function of P protein and could be associated with the attenuation process of CAM-70 BM. However, we did not find amino acid replacements in NH_2 or COOH portions that could be associated with specific functions of the P protein (32). The CAM-70 BM vaccine P gene-predicted amino acid changes are located along the protein (amino acids 98-419) and nucleotide mutations, up to now, have not been related to loss or increase of infectivity. The original descriptions of the comparison of canine distemper virus with measles virus had already indicated that certain areas of the genome of morbilliviruses have greater plasticity than others. Although all six genes of measles virus appear to vary, though to different extents, the highest degree of variation is in the C-terminus of the N protein and in the H and P proteins. Therefore, vaccine-specific changes in nucleotide sequences, so far as these can be assessed, do not affect the functionality of the P protein, but reflect genetic variation (33).

We have analyzed the mutations found in the M protein of the CAM-70 BM vaccine by comparison with other vaccines. Most data on nucleotide sequencing for the M gene are available for the close relationship between the numerous mutations in this gene and subacute sclerosing panencephalitis (SSPE) (34,35). The nucleotide changes found in the CAM-70 BM vaccine were not related to published SSPE-derived strain sequences.

The M protein is highly basic and serves as a link between the nucleocapsid and the surface glycoproteins. It was suggested that the interaction with the acidic nucleoprotein in the nucleocapsid could be via salt bridges (36). Three exclusive mutations predicting amino acid changes were found in the M

protein of the CAM-70 BM vaccine. These amino acid substitutions moderately alter the charge of protein M and since this protein plays an important role in virus maturation we raised the question if they could be implicated in less productive viral infections (5).

The L gene of CAM-70 vaccine strains proved to be the most conserved gene analyzed in this study. The L protein is of critical importance for transcription and replication and its extreme sensitivity to mutation must be emphasized (16,37).

Sequence alignments of *Morbillivirus* L protein have revealed two highly variable regions termed hinges. The first (H1) extends from residues 607 to 650 and the second (H2) is between residues 1695 to 1717 (37). We found amino acid substitutions in CAM-70 strains at positions out of these variable regions. However, drastic amino acid substitutions were not found. This observation is in agreement with previous studies in which no variation was observed in genotype A measles virus L proteins although this was the most variable area of the non-genotype A measles virus L proteins (17,38).

The L protein of measles virus is highly homologous to the polymerases of other negative strand viruses. Poch and co-workers (39) reported highly conserved central blocks among five L proteins of unsegmented negative strand RNA virus. We found a single-amino acid substitution of arginine for lysine at position 1091 in the highly conserved block of CAM-70 strains. However, this substitution did not reflect on the hydrophilicity of the amino acid.

Specific variations have been detected by nucleotide sequencing of ten genotype A measles virus L genes. None of the CAM-70 strain mutations predicting amino acid substitutions were shared with those strains. On the other hand, two exclusive amino acid substitutions in the L gene of a non-genotype A measles virus isolate from Gambia were shared with CAM-70 strains. At position 725, we found a serine where the Gambia isolate had a cysteine and at position 1091 both the Gambia isolate and CAM 70 strains had a lysine (38).

A "vaccine strain signature" based on the Edmonston wild-type progenitor strain was proposed by Parks and co-workers (6). The CAM-70 vaccine strains cannot be considered in this proposition since they are the most divergent among the vaccine strains. Without sequence information for the progenitor Tanabe wild-type strain we can only speculate whether nucleotide differences between CAM-70 BM and other vaccines are due to cell passage history or to different origins. The intention of the present study was to acquire genetic information about the vaccine produced at our Institute. Further studies are being conducted in order to relate the mutations found in our vaccine to protein expression and to address the role of these proteins in the attenuation process. The study of molecular characteristics of vaccine strains is of utmost importance for monitoring vaccine administration in the field. Comparative sequence analysis provides tools for assessing the impact of mass vaccination campaigns on the interruption of virus circulation and on measles control.

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