Acta virologica **58**: 287 – 291, 2014 doi:10.4149/av_2014_03_287

Wild and attenuated vaccine RS-12 strains of mumps virus exhibit differences in amino acid sequences of their proteins

B. ALIREZAIE[1], R. SHAHBAZI[1], S. S. SAFAVIEH[2], A. MOHAMMADI[1]

[1]Department of Human Viral Vaccines, Razi Vaccine and Serum Research Institute (RVSRI), Beheshti Blv, P.O. Box 31975/148, Karaj, Alborz, Iran; [2]Department of Biotechnology, Razi Vaccine and Serum Research Institute (RVSRI), Beheshti Blv, P.O. Box 31975/148, Karaj, Alborz, Iran

Received November 11, 2013; accepted August 1, 2014

Summary. – Attenuated mumps virus (MuV) RS-12 strain-based vaccine is one of several effective vaccines available in the prevention of mumps. Since previous studies have unveiled only about one-third of the attenuated vaccine RS-12 strain genome sequence, the rest of sequence and molecular basis for attenuation remained unsolved. Therefore, in this study, the full-length genome sequences of wild and attenuated RS-12 strains were determined and compared. The comparison revealed nucleotide substitutions at 9 positions leading to amino acid substitutions at 6 positions in P, V, I, M, and L proteins, while the remaining substitutions were silent. This result indicates that the observed mutations in P, V, I, M, and L proteins of MuV might be responsible for the attenuation of the RS-12 vaccine strain.

Keywords: mumps virus; vaccine; attenuation; mutation

Introduction

Mumps is a contagious disease caused by the mumps virus, a member of the *Rubulavirus* genus. The main sign of the disease is a non-suppurative painful swelling of one or both parotid glands, which largely recovers without any sequelae. Despite the fact that parotitis and inapparent infections are more common than sever form, in some cases other complications including orchitis, oophoritis, pancreatitis, meningitis, encephalitis, myocarditis, pericarditis, nephritis, and arthritis might develop. Mumps is classified as a vaccine-preventable disease. Mumps was in the past one of the most widespread diseases in the world; however, extensive use of less virulent viruses as a prophylactic device has dramatically reduced the rate of this disease in most parts of the world (Betáková *et al*., 2013). Attempts to develop live avirulent vaccines have previously met with success; for example, attenuated vaccine strain RS-12 is one of several effective mumps vaccinal strains derived from wild-type isolate. This strain was isolated in 1986 in Iran and was serially propagated in MRC-5 cells (Sassani *et al.,* 1991). As a consequence of consecutive passages, the wild RS-12 strain gradually evolved to display the properties of an attenuated strain. A previous study based on marker test and experimental infection of monkeys disclosed that attenuated RS-12 vaccine strain displayed phenotypically, clinically and histopathologically distinc features from its parental isolate (Sassani *et al.,* 1991). Previous clinical trials also have showed that this vaccinal strain is efficacious and safe (Sassani *et al.,* 1991; Esna-ashari *et al*., 2002; Feiterna-Sperling *et al*., 2005). A standard seed lot system for this strain has recently been established and subsidiary clinical trials are ongoing (Shahkarami *et al*., 2012). Although this isolate has been partially characterized, a molecular basis for attenuation remains to be determined. Phylogenetic analysis based on the entire SH gene determined that the wild RS-12 strain pertained to the genotype H and was located within the

E-mail: b.alirezaie@rvsri.ac.ir; phone: +98-263-4570038.

Abbreviations: F = fusion; HN = hemagglutinin-neuraminidase; L = large polymerase; M = matrix; MuV = mumps virus; N = nucleoprotein; P = phosphoprotein; SH = small hydrophobic

European strains that compose an entirely separate cluster from the Far Eastern isolates (Alirezaie *et al*., 2008).

In this study, we attempted to determine the sequence of the complete genome of MuV, but mainly to compare the sequences of wild and attenuated vaccine RS-12 strains, and to reveal the nucleotide and amino acid changes, which had occurred during serial passaging and resulted in attenuation.

Materials and Methods

Virus. The supernatants of the wild RS-12 strain (second passage) and attenuated vaccine RS-12 strain (19th passage), which had been stored below -70°C, were thawed and used for sequence analysis.

RNA extraction. Viral RNAs were extracted from supernatant of infected tissue culture fluids using a High Pure Viral Nucleic Acid kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions.

RT-PCR. Based on the alignment of all available MuV full-length genomes from the GenBank database, genus-specific primers for relatively conserved regions were designed using Oligo® software (version 5.0 [National Biosciences]). The primers used for PCR amplifications and/or sequencing are listed in t 1.

Extracted RNAs were reverse-transcribed into cDNA using Expand Reverse Transcriptase (Roche Applied Science, Mannheim, Germany) at 42°C for 45 min. The reaction mixture contained 1X buffer, 10 mmol/l DTT, 1 mmol/l dNTPs, 20 U RNase inhibitor, 50 U expand reverse transcriptase, 50 μmol/l random hexamers, and 1 μg RNA. In order to achieve full-length genome sequencing, PCR fragments with overlaps were generated. The name and sequences of designed primers are shown in Table 1. All PCRs were run under the same conditions using *Taq* DNA polymerase in 50 μl of reaction mixture comprising 1X PCR buffer (10 mmol/l tris-HCl, 50 mmol/l KCl), 5 μl cDNA, 1.5 mmol/l $MgCl_2$, 0.5 μmol/l each primer, and 1 U SmantTaq DNA polymerase (CinnaGen, Tehran, Iran). Cycling was carried out using a Mastercycler thermocycler (Eppendorf) with the following cycle program: Initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 46°C for 1 min and 72°C for 2 min, followed by a final extension of 15 min at 72°C. With the purpose of decreasing the impact of possible nucleotide substitutions introduced by *Taq* DNA polymerase, high concentrations of template and primers were used. Moreover, each amplicon was amplified in five different tubes and subsequently pooled.

All nucleotide variations leading to amino acid substitutions were confirmed by the direct sequencing of the amplicons that were amplified using *Pwo* DNA polymerase (Roche Applied Science, Mannheim, Germany). To this end, the conditions for each primer pair (annealing temperatures, $MgSO_4$ concentrations, and extension times) were optimized separately (Table 1). Other parameters including cDNA template (5 μl), dNTPs (200 μmol/l), primers (0.5 μmol/l), buffer (1X), and *Pwo* DNA polymerase (2.5 U), were the same for all primer pairs.

Sequencing and sequence analysis. Sequencing was carried out directly on the pooled purified PCR product by Eurifins MWG, Ebersberg, Germany. The DNAMAN software package (version 4.13) was used for multiple DNA sequence alignment (MSA), gene assembly, protein translation, and protein alignment.

Results and Discussion

Although the accurate *Pwo* DNA polymerase enzyme possesses proofreading activity and its fidelity is about 10-fold higher than *Taq* DNA polymerase, *Pwo* has relatively low specificity, especially when single stranded DNA is used as a template (Steffen *et al*., 1999; McPherson and Møller, 2006). Therefore, three precautionary measures were carried out in order to ensure the authenticity of the PCR products: (i) the optimal reaction conditions for each primer pair were determined individually to reduce non-specific amplification and PCR artifacts; (ii) the same products amplified with *Taq* DNA polymerase were included in each run as a positive control; (iii) high concentrations of first strand cDNAs were used.

The complete genome sequences of wild and attenuated vaccine strains RS-12 have been deposited in GenBank under Acc. Nos JQ388690 and JQ388691, respectively.

Multiple sequence alignment (MSA) revealed nucleotide substitutions at 9 positions leading to amino acid substitutions at 6 positions in P, V, I, M, and L proteins, while the remaining substitutions were silent (Table 2).

Recognition of nucleotide differences between the genome of the attenuated vaccine strain RS-12 and its parental wild isolate would be an indispensable step toward determining the basis of strain attenuation through consecutive passages in MRC-5 cells. Multiple sequence alignments showed that the two genomes were identical in length. Moreover, noncoding regions, including the leader, trailer, transcription start and stop codons, intergenic regions as well as ORFs for the N, F and SH proteins, were identical between the two genomes, yet the genomes differed by 9 nucleotides in ORFs for the P, V, I, M, and L proteins. Amino acid variation detected in the P gene included a change at the position 2417 of the genome. The N-terminal portion of the P gene ORF also encodes the N-terminal regions of the V and I proteins. As a consequence, the V and I proteins contain the same amino acid substitution. Variation in the sequence of this region close to the insertion site has been reported previously in association with the accuracy of the insertion of non-templated G in the gene transcripts and subsequently the accuracy of expression of these proteins in different mumps strains (Elliott *et al*, 1990). Analysis of the M protein revealed one predicted amino acid variation. Substitution in matrix proteins could contribute to the attenuation by affecting the efficiency of virus maturation. MSA also showed two predicted amino

Table 1. Primers and reaction conditions used for amplification and sequencing of individual genes of MuV RS-12 strains

Gene / protein	Amplicon size (bp)	Primer name[a]	Sequence (5′-3′)	PCR conditions using *Pwo* DNA polymerase		
				$MgSO_4$ concentration (mmol/l)	Annealing temperature (°C)	Extension time (S)
N	1160	MuN-1+	ACCAAGGGGAAAATGGAGAT	2.5	46	60
		MuN-1160-	CTCCCATAGCATAACTGAA			
	1217	MuN-812+	ATCAGGAAAAGTCTTGTTG	2.5	46	60
		MuN-2028-	CCTGTCTCAATTAAATCACC			
P	1823	MuP-1452+	TGGAGGAATCAGATGACG	1	47	90
		MuP-3274-	GAGGAATTTTGATCTGTG			
M	1403	MuM-2720+	GGTGACCCAAATAAAGAATG	2.5	46	60
		MuM-4122-	TGTGACCGCCTGCATGGA			
	1529	MuM-3684+	CAAATATCTGCGGACCAT	2.5	46	60
		MuM-5212-	CTTGTATACTAATCGGTGAC			
F	985	MuF-4363+	GGAAGTCTGCCTCAATGA	2.	46	45
		MuF-5347-	GCATCTCATCTAGCAGAAC			
	1105	MuF-5149+	GAATTAACAACAGTGTTTCAG	2.5	46	60
		MuF-6253-	GTCACGAGACGTTACGAC			
SH	1164	MuSH-5656+	GCACTGGATGGAACAATT	2.5	46	60
		MuSH-6819-	GACAACTGATTGCTCAAG			
HN	1213	MuHN-6236+	GTCGTAACGTCTCGTGAC	2.5	46	60
		MuHN-7449-	GTAAGTTTCTGGGTAGGTG			
	1488	MuHN-7202+	GTAATTAATGCCAACTGC	2.5	46	60
		MuHN-8689-	GATCCTTGCAATGAGTTC			
L	1266	MuL-8323+	CCAAATTCTACCTGTGCT	2.5	46	60
		MuL-9588-	TCTCGGACCTTGGATGCT			
	953	MuL-9341+	CAATTACAGTATGGTGATCC	2.5	46	45
		MuL-10293-	CTGTGCTCTGATATAATACC			
	1289	MuL-10064+	GACCCTGAATTTTGTGCAT	2.5	46	60
		MuL-11352-	CGTTTCACATCAGCAATTG			
	1256	MuL-10995+	TAAAGAACGTGAGCAAGATG	2	47	60
		MuL-12250-	TAACACGAGTGGAAGCTAAC			
	1550	MuL-12164+	GTATCACTTAAATCAGCACTC	2.5	52	60
		MuL-13713-	CTTGGGAGAGAGTATTTC			
	2185	MuL-13200+	CCAACATTTCGCAAGGTA	2.5	46	90
		MuL-15384-	ACCAAGGGGAGAAAGTAAA			

[a]The numbers correspond to the nucleotide position of the 5′ ends of the synthetic primers relative to the genome.

Table 2. Nucleotide and amino acid substitutions in wild RS-12 strain due to its attenuation into vaccine strain

Gene/protein	Nucleotide substitution	Codon change	Amino acid substitution
P	A2417G	ACG→A/GCG	T147A or none
V	A2417G	ACG→A/GCG	T147A or none
I	A2417G	ACG→A/GCG	T147A or none
M	T3774A	TGT→T/AGT	C171S or none
L	T9373C	ATT→ATT/C	None
	A11515T	ACA→ACA/T	None
	T12106A	ATT→ATA	None
	C12111A/C	TCT→TA/CT	S1225Y or none
	A12977G/A	ATT→G/ATT	I1514V or none

acid changes in the L protein. P and L proteins play a critical role in the replication of the virus. The RNA dependent RNA polymerase (RdRp) of paramyxoviruses, which contains a minimum of a homotetramer of P and a single L protein, transcibes the genome into 5′ capped and 3′ polyadenylated viral mRNAs. It is also involved in the synthesis of the exact complementary copy of the genome (antigenome), as well as the genome. Therefore, the amino acid variations in P and L proteins may modulate the enzymatic activity of the polymerase complex, although, this suggestion needs to be examined experimentally. Further studies based on reverse genetics are required to investigate the relationship between these diversities and the molecular mechanism of attenuation and/or virulence. In spite of the fact that genetic heterogeneity in other mumps vaccine strains has also been observed (Boriskin *et al*., 1992; Amexis *et al*., 2002; Sauder *et al*., 2006; Kosutic-Gulija *et al*., 2008; Tillieux *et al*., 2009), it might be necessary to prepare a cloned attenuated vaccine RS-12 virus as a genetically homogenous seed.

There is currently limited data available on sequencing of the full-length genomes of attenuated MuV vaccine strains. Although it was recently indicated that N/M proteins are involved in viral pathogenicity/attenuation of MuV (Sauder *et al*., 2011), these patterns have not been observed in all strains (Lemon *et al*., 2007; Malik *et al*., 2009; Liang *et al*., 2010; Xu *et al*., 2012). As a result, no unique differentiation marker has been found between parental wild and attenuated passages of different vaccine strains. The presented results indicate that the regulation of P-L polymerase complex activities (transcription/replication) and/or matrix protein, and/or accessory V/I proteins activities may play a role in the diminution of pathogenicity of the vaccine strain RS-12.

Acknowledgements. The authors thank Dr. Zohreh-Azita Sadigh, Razi Institute, for constructive comments on preparing the manuscript. This work was supported by the grant No. 2-18-18-91119 from the Iran Agricultural Research, Education and Extension Organization (AREEO).

References

Alirezaie B, Aghaiypour K, Shafyi A (2008): Genetic characterization of RS-12 (S-12), an Iranian isolate of mumps virus, by sequence analysis and comparative genomics of F, SH, and HN genes. J. Med. Virol. 80, 702–710. http://dx.doi.org/10.1002/jmv.21087

Amexis G, Rubin S, Chizhikov V, Pelloquin F, Carbone K, Chumakov K (2002): Sequence diversity of Jeryl Lynn strain of mumps virus: quantitative mutant analysis for vaccine quality control. Virology 300, 171–179. http://dx.doi.org/10.1006/viro.2002.1499

Betáková T, Svetlíková D, Gocník M (2013): Overview of measles and mumps vaccine: origin, present, and future of vaccine production. Acta Virol. 57, 91–96. http://dx.doi.org/10.4149/av_2013_02_91

Boriskin YS, Yamada A, Kaptsova TI, Skvortsova OI, Sinitsyna OA, Takeuchi K, Tanabayashi K, Sugiura A (1992): Genetic evidence for variant selection in the course of dilute passaging of mumps vaccine virus. Res. Virol. 143, 279–283. http://dx.doi.org/10.1016/S0923-2516(06)80116-0

Elliott GD, Yeo RP, Afzal MA, Simpson EJ, Curran JA, Rima BK (1990): Strain-variable editing during transcription of the P gene of mumps virus may lead to the generation of non-structural proteins NS1 (V) and NS2. J. Gen. Virol.71, 1555–1560. http://dx.doi.org/10.1099/0022-1317-71-7-1555

Esna-ashari F, Mirchamsy H, Shafyi A, Shams shahrabadi M, Sabiry GM, Sassani A, Hossein Zade T (2002): Humoral immune response to mumps (RS-12 strain) vaccine of children 1-7 years old. Arch. Razi Ins. 54, 77–84.

Feiterna-Sperling C, Brönnimann R, Tischer A, Stettler P, Durrer P, Gaedicke G (2005): Open randomized trial comparing the immunogenicity and safety of a new measles-mumps-rubella vaccine and a licensed vaccine in 12- to 24-month-old children. Pediatr. Infect. Dis. J. 24, 1083–1088. http://dx.doi.org/10.1097/01.inf.0000183746.92218.f9

Kosutic-Gulija T, Forcic D, Šantak M, Ramljak A, Mateljak-Lukacevic S, Mazuran R (2008): Genetic heterogeneity of L-Zagreb mumps virus vaccine strain. Virol. J. 5, 1–8. http://dx.doi.org/10.1186/1743-422X-5-79

Lemon K, Rima BK, McQuaid S, Allen IV, Duprex WP (2007): The F gene of rodent brain-adapted mumps virus is a major determinant of neurovirulence. J. Virol. 81, 8293–8302. http://dx.doi.org/10.1128/JVI.00266-07

Liang Y, Ma S, Liu L, Zhao H, Wang L, Jiang L, Xie Z, Dong C, Li Q (2010): Identification and development of a promising novel mumps vaccine candidate strain. Microbes. Infect. 12, 1178–1187. http://dx.doi.org/10.1016/j.micinf.2010.08.004

Malik TH, Wolbert C, Nerret L, Sauder C, Rubin S (2009): Single amino acid changes in the mumps virus haemagglutinin-neuraminidase and polymerase proteins are associated with neuroattenuation. J. Gen. Virol. 90, 1741–1747. http://dx.doi.org/10.1099/vir.0.009449-0

McPherson MJ, Møller SG (2006): PCR. Taylor & Francis Group, New York, pp. 23–62.

Sassani A, Mirchamsy H, Shafyi A, Ahourai P, Razavi J, Gholami R, Mohammadi A, Ezzi A, Rahmani M, Fateh G, Paravandi T (1991): Development of a new live attenuated mumps virus vaccine in human diploid cells. Biologicals 19, 203–211. http://dx.doi.org/10.1016/1045-1056(91)90036-J

Sauder CJ, Vandenburgh KM, Iskow RC, Malik T, Carbone KM, Rubin SA (2006): Changes in mumps virus neurovirulence phenotype associated with quasispecies heterogeneity. Virology 350, 48–57. http://dx.doi.org/10.1016/j.virol.2006.01.035

Sauder CJ, Zhang CX, Ngo L, Werner K, Lemon K, Duprex WP, Malik T, Carbone K, Rubin SA (2011): Gene-specific contributions to mumps virus neurovirulence and neuroattenuation. J. Virol. 85, 7059–7069. http://dx.doi.org/10.1128/JVI.00245-11

Shahkarami MK, Azad TM, Aghaiypour K, Shafyi A, Taqavian M, Mohammadi A (2012): Establishment of a standard seed lot system of an Iranian Mumps virus strain; RS-12, for mass production of mumps and MMR vaccines. Iran. Red Crescent Med. J. 14, 508–514.

Steffen C, Ankenbauer W, Bauer-Hofmann R, Bye S, Eisel D, Frey B, Keesey J, Rosenstraus M, Schäferkordt S, SchlichtHJ (1999): Roche Molecular Biochemicals: PCR Applications Manual. Auflage, Roche Diagnostics GmbH, Mannheim, pp. 9–50.

Tillieux SL, Halsey WS, Sathe GM, Vassilev V (2009): Comparative analysis of the complete nucleotide sequences of measles, mumps, and rubella strain genomes contained in Priorix-Tetra and ProQuad live attenuated combined vaccines. Vaccine 27, 2265–2273. http://dx.doi.org/10.1016/j.vaccine.2009.01.112

Xu P, Luthra P, Li Z, Fuentes S, D'Andrea JA, Wu J, Rubin S, Rota PA, He B (2012): The V protein of mumps virus plays a critical role in pathogenesis. J. Virol. 86, 1768–1776. http://dx.doi.org/10.1128/JVI.06019-11