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Correlation of Genetic Variability with Safety of Mumps Vaccine Urabe AM9 Strain

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The Urabe AM9 strain of mumps vaccine live is known for its genetic instability and some vaccines derived from this strain were withdrawn from the market due to an excessive number of vaccine-associated parotitis and meningitis cases. To identify the molecular basis of this instability, we determined complete nucleotide sequences of several stocks of the Urabe strain used for vaccine production by different manufacturers and of two clinical isolates from cases of vaccine-associated meningitis. In contrast to previously published studies relating the $Lys_{335} \rightarrow Glu$ mutation in the viral HN gene with neurovirulence of mumps virus, we could not confirm any association of this mutation with the safety of mumps vaccine. Each of the three vaccine stocks studied had its own characteristic profile of mutations that was identified by cDNA sequencing and quantitated by mutant analysis by PCR and restriction enzyme cleavage. Determination of the mutational profile of mumps vaccine lots could allow vaccine manufacturers to characterize seed viruses and monitor the consistency of vaccine production to prevent emergence of virulent revertants.

Key Words: nucleotide polymorphism; reversion; neurovirulence; vaccine production; quality control.

INTRODUCTION

Mumps virus is a member of the Rubulavirus genus of the *Paramyxoviridae* family. It has a single-stranded negative-sense RNA genome of 15,384 nucleotides that encodes seven proteins: nucleocapsid (NP), phosphoprotein (P), matrix (M), fusion (F), small hydrophobic (SH), hemagglutinin-neuraminidase (HN), and RNA polymerase (L). Due to the inherent genetic instability of RNA viruses, mumps virus contains a heterogeneous mixture of genomic RNA molecules with slightly different nucleotide sequences (quasispecies) (Afzal *et al.,* 1992, 1993; Boriskin *et al.,* 1993; Brown *et al.,* 1996; Yates *et al.,* 1996). Virus growth both *in vitro* and *in vivo* results in selection of different subpopulations, depending on growth conditions (Boriskin *et al.,* 1992, 1993; Turner *et al.,* 1991). This affects important properties of the virus such as virulence. This genetic plasticity permitted selection of attenuated strains of mumps virus by serially passaging it in chicken embryos and cell cultures of quail or chicken fibroblasts (Buynak and Hilleman, 1966; Hilleman, 1966; Stokes, 1967). Following the introduction and success of the Jeryl Lynn vaccine strain in the United States (Hilleman, 1966), a number of other strains were developed for vaccine manufacture internationally. Some of the strains have a mixed record of efficacy and safety because of the propensity of attenuated viruses to change, often with partial regaining of virulent properties (Levenbuk *et al.,* 1979; Sugiura and Yamada, 1991). The Urabe AM9 strain, which was developed in Japan in the late 1960s, was used by a number of Japanese and European manufacturers for vaccine production. Postmarketing surveillance in Japan, Canada, and the United Kingdom revealed an unacceptably high rate of vaccine-associated meningitis (Autret *et al.,* 1996; Black *et al.,* 1997; Dourado *et al.,* 2000; McDonald *et al.,* 1989; Miller *et al.,* 1993; Nalin, 1989; Rebiere and Galy-Eyraud, 1995; Ueda *et al.,* 1995), parotitis (Brunell *et al.,* 1972), and orchitis cases (Kuczyk *et al.,* 1994), which led to a withdrawal of the product from the market in these countries (Colville *et al.,* 1994; Sawada *et al.,* 1993). However, other manufacturers still continue to use this strain for vaccine production without an excess number of adverse reactions. Although a passive adverse event reporting system can be inaccurate, still this suggests that the Urabe AM9 strain is prone to genetic variability, which may affect its safety profile.

It is clear that there are gaps in understanding the pathogenicity of the virus, hindering our ability to develop adequate laboratory tests for mumps vaccine safety (Minor, 1997). Knowledge of the molecular nature of attenuation and reversion to neurovirulence of oral poliovirus vaccine (OPV) allowed us to develop a molecular method for consistency monitoring of vaccine production by directly measuring contents of neurovirulent revertants in batches of OPV (Chumakov, 1999; Chumakov *et al.,* 1991). Elucidation of the molecular mechanism of attenuation of other live vaccines, and of pathways of their reversion to virulence, could help in development of similar molecular quality control methods. In the case of mumps vaccine live (MVL), the development of such methods has addi-

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tional importance since at present there is no validated *in vivo* or *in vitro* test for neurovirulence of the virus that could be routinely used for quality control of this vaccine (Afzal *et al.,* 1999; Maximova *et al.,* 1996; Rubin *et al.,* 1998, 1999). In addition to safety concerns, variability and plasticity of mumps virus genome may also have implications for vaccine efficacy, since some attenuated strains of the virus were found to be insufficiently immunogenic (Chamot *et al.,* 1998; Gassner *et al.,* 1995).

The issue of instability of attenuation of the Urabe AM9 strain was previously addressed in a number of studies. Partial analysis of cDNA sequences of several isolates from vaccine-associated meningitis and parotitis cases was performed and led to identification of a sequence heterogeneity in the viral HN gene (Brown *et al.,* 1996). It was suggested that the $Lys_{335} \rightarrow Glu$ mutation in the HN gene is responsible for attenuation of the wild-type virus, and reversion at this site partially restores virulence of the vaccine (Brown and Wright, 1998). The vaccine produced by different manufacturers was found to contain variable amounts of both Lys and Glu at this site, and it was hypothesized that the safety of the vaccine depends on the relative abundance of the variants. However, the conclusions were based on only partial sequencing of the viral genome, which was done after cDNA cloning. Therefore, it is possible that some of the mutations responsible for attenuation/reversion may have been missed. In this communication we report our studies of the mutational composition of different mumps virus stocks made from the Urabe strain in attempts to develop surrogate molecular methods for consistency control of this vaccine.

RESULTS

Quantification of 7616-G → A

To study the $Glu_{335} \rightarrow Lys$ mutation in the HN gene (7616-G → A at the nucleotide level), we have used a MAPREC, mutant analysis by PCR and restriction enzyme cleavage, to directly measure contents of both 7616-G and 7616-A in virus samples (Chuma Kov *et al.,* 1991). Unlike the similar test described in Brown *et al.* (1996), which detected only 7616-A by digestion with *Mse*I restriction endonuclease (recognizing TTAA), we also digested the PCR-amplified DNA with 7616-G-specific *Hin*fI (recognizing GANTC). As a result we could directly measure both nucleotides and confirmed that the balance of their contents was close to 100%. When we tested vaccine batches produced by SmithKline Beecham (Lot Nos. MP51, MP52, and MP53) that were withdrawn from the market, we found that they contained 88.9 ± 1.9% of the "wild-type" 7616-A and only 9.2 ± 1.3% of "attenuated" 7616-G (Fig. 1). On the other hand, vaccine that is still produced by Chiron SpA (Lot Nos. PS22, PS23, PS24, PS25, and PS27) and was not associated with an excessive number of adverse reactions contained no

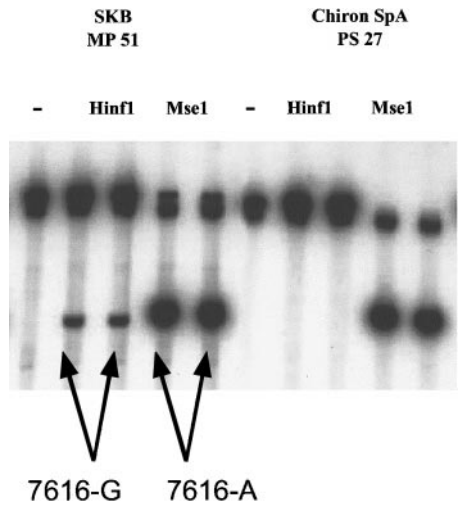

FIG. 1. MAPREC analysis of nucleotide 7616 (coding for amino acid 335 in HN protein) in mumps vaccine batches produced by SB and Chiron SpA. *Hin*fI cuts DNA with 7616-G (Glu_{335}), and *Mse*I cuts DNA with 7616-A (Lys_{335}). Digested materials were loaded on duplicate lanes. Upper band represents undigested DNA molecules, and this band is poorly resolved from another minor unidentified PCR product moving slightly ahead of it. Lower band represents DNA molecules cut with respective restriction enzymes, and their quantities reflect the contents of 7616-G and 7616-A.

detectable "attenuated" base, but had 100% of the wild-type base. This result is not consistent with the interpretation of the previously published studies that Lys_{335} is associated with higher virulence of the Urabe strain. Interestingly, the Jeryl Lynn strain, which is highly attenuated and has an excellent safety record, also contains 100% of the wild-type 7616-A, as do all other mumps virus strains that we sequenced (G. Amexis *et al.,* to be published).

Sequences of commercial Urabe AM9 substrains and of isolates from vaccine-associated meningitis cases

We determined the complete nucleotide sequences of the Urabe substrains made by Biken, SB, and Chiron SpA that appear to have different safety records. We also sequenced two isolates from cases of vaccine-associated meningitis hoping to match any mutations identical to those that may be present in the vaccine batches. Nucleotide sequences of all three vaccine sources differed from the published sequence at nucleotide 7409 (A in the published sequence but G in all stocks that we studied). This mutation may have been a result of a cloning step used to derive the previously published sequence. There were also a few nucleotide heterogeneities that distinguished the three virus stocks from one another. Some of them matched the mutations found in clinical isolates. All mutations identified in vaccine stocks and clinical isolates are listed in Table 1. However, there was no mutation that was present in both clinical isolates, suggesting that their increased virulence was a result of different reversion pathways.

TABLE 1

Nucleotide Differences and Heterogeneities in Mumps Vaccines Made from the Urabe Strain by Three Manufacturers and in Two Clinical Isolates from Patients (1004 and 1005)

Nucleotide	Gene	Published sequence[a]		Biken		Chiron SpA		SB		Clinical isolates 1004		Clinical isolates 1005	
1,880	NP	C		C		C		C		C		T	
6,385	HN	C		C		C		C		C		A	
7,409	HN	A		G		G		G		G		G	
7,573	HN	T		T		T		T		T		C	
7,616	HN	A	Lys	A	Lys	A	Lys	A/G	Lys/Glu	A	Lys	A	Lys
8,005	HN	C	Asn	C	Asn	C	Asn	A/C	Lys/Asn	C	Asn	A	Lys
8,015	HN	G	Glu	G	Glu	A/G	Lys/Glu	A/G	Lys/Glu	G	Glu	G	Glu
9,972	L	T	Phe	T	Phe	C/T	Ser/Phe	C/T	Ser/Phe	C	Ser	T	Phe
10,529	L	C	Pro	C	Pro	C	Pro	C/T	Pro/Leu	C	Pro	T	Leu
11,692	L	G	Leu	G	Leu	G	Leu	G/T	Leu/Phe	G	Leu	T	Phe
14,049	L	C		C		C		C		C		T	
15,328	UTR	G		G		G		G		A		G	

Note. Amino acids are presented only for missense mutations.

[a] Urabe AM9 sequence (GenBank Accession No. AB000387).

Mutational profiles of different Urabe vaccine products

Vaccines produced by Chiron SpA and SB contained a number of sites where two nucleotides could be detected. Six such heterogeneous sites were found in SB batches (7616-G+A, 8005-C+A, 8015-G+A, 9972-T+C, 10529-C+T, and 11692-G+T) and two in Chiron SpA batches (8015-G+A and 9972-T+C), leading to a change at the amino acid level as well. It is noteworthy that a mutation at one of the heterogeneous sites (9972) was also found in one of the clinical isolates. It can be assumed that biological properties of the vaccines depend on the composition of the respective quasispecies comprising them. To determine the quantitative mutational profiles of the viral stocks, we designed MAPREC assays for these mutations and tested the commercial vaccine preparations available to us. Figures 2A and 2B show nucleotide heterogeneity at position 8005 observed in vaccine batches from SB, but not in batches from Chiron SpA. 8005-A coding for Lys_{464} in HN protein was also found in one of the clinical isolates. In contrast, Chiron SpA batches appeared homogeneous at this nucleotide and contained only 8005-C (Asn_{464}). Figure 3 shows the difference between batches of vaccines from SB and Chiron SpA at nucleotide 10,529. Batches produced by SB were heterogeneous and contained both 10529-C and 10529-T, while the Chiron SpA product contained only 10529-T. Table 2 summarizes the results of quantitative mutational analysis of both products and shows that they have distinguishing mutational profiles.

Passaging of Urabe stocks in CEF cultures

Virus growth during vaccine production can result in accumulation of some mutations and affect the final mutational profile of the product. Therefore, to assess the genetic stability of the vaccine, we performed serial passaging of Chiron SpA mumps virus stock in chicken embryo fibroblast (CEF) cells and determined contents of mutations at heterogeneous sites by MAPREC. There was no change at nucleotide 8005 at which the Chiron SpA vaccine was homogeneous but the SB product contained both A and C (Fig. 3C). The same result was obtained for nucleotide 10,529 (data not shown). Additional passaging in CEF cells resulted in a disappearance of sequence heterogeneity at nucleotide 9972, which was present in both SB and Chiron SpA vaccines (Fig. 4C). Vaccine batches produced by both companies contained 26–37% 9972-T, while after additional passaging in CEF cells no 9972-T could be detected, suggesting

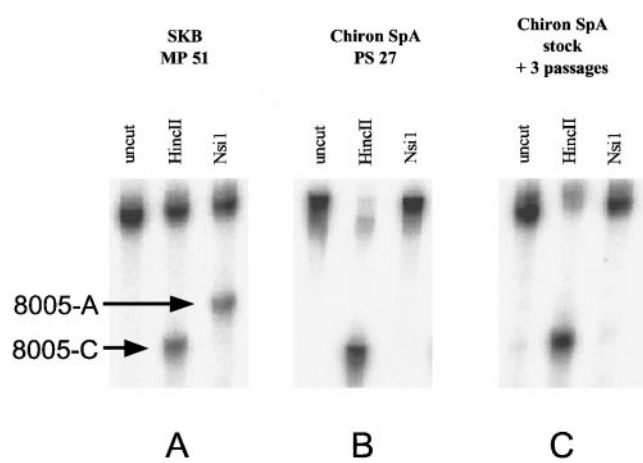

FIG. 2. MAPREC analysis of nucleotide 8005 in mumps vaccine batches produced by SB (A) and Chiron SpA (B) and in virus stock produced by growing a Chiron SpA vaccine lot in CEF cells for three additional passages (C). *Hinc*II cuts 8005-C sequences, and *Nsi*I cuts 8005-A.

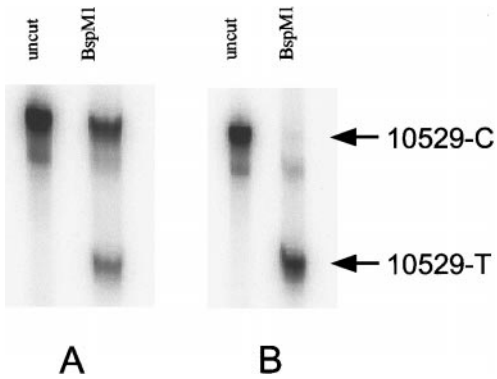

FIG. 3. MAPREC analysis of nucleotide 10,529 in mumps vaccine batches produced by SB (A) and Chiron SpA (B). *Bsp*MI cuts 10529-T, and the lower band is indicative of the amount of this base. There is no 10529-C-specific restriction enzyme, so its content is assessed by the quantity of undigested DNA (upper band).

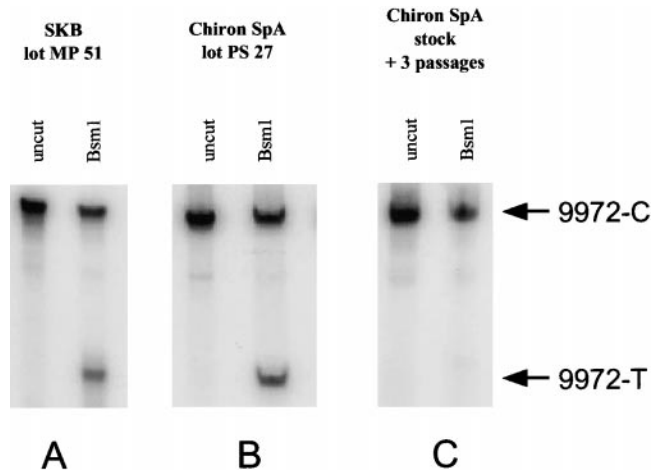

FIG. 4. MAPREC analysis of nucleotide 9972 in mumps vaccine batches produced by SB (A) and Chiron SpA (B) and in virus stock produced by growing a Chiron SpA vaccine lot in CEF cells for three additional passages (C). *Bsm*I cuts 9972-T, and the lower band is indicative of the amount of this base. There is no 9972-C-specific restriction enzyme, so its content is assessed by the quantity of undigested DNA (upper band).

that the mutant 9972-C-containing virus has a selective advantage during virus growth in these cells. Interestingly, 9972-C, coding for Ser_{512} in the L protein, could also be found in one of the clinical isolates.

DISCUSSION

Some mumps virus strains used for manufacture of live vaccines are known for their genetic instability, believed to cause increased neurovirulence and an excessive number of parotitis and aseptic meningitis cases in vaccine recipients. A number of factors, including those determined by a host, affect the level of pathogenicity of attenuated vaccine strains. However, different safety records of mumps vaccines produced from different strains strongly suggest that genetic properties of an attenuated strain play a major role. For instance, while the Jeryl Lynn strain was never found to cause excessive adverse reactions, there are a number of reports from different countries that the Urabe strain can cause an extremely high number of postvaccination meningitis cases and other complications (Autret *et al.,* 1996; Black *et al.,* 1997; Brunell *et al.,* 1972; Dourado *et al.,* 2000; Kuczyk *et al.,* 1994; McDonald *et al.,* 1989; Miller *et al.,* 1993; Nalin, 1989; Rebiere and Galy-Eyraud, 1995; Ueda *et al.,* 1995), which led to a withdrawal of some Urabe AM9-based vaccines from the market. Further evidence that the residual level of pathogenicity is controlled by viral determinants comes from observations of genetic instability of some attenuated mumps virus strains. Levenbuk *et al.* (1979) found that batches of the attenuated L3 strain with higher levels of neurovirulence in monkeys were also linked to an excessive incidence of postvaccination meningitis. Similar genetic instability is probably a property of the Urabe strain since vaccines made by different manufacturers have different safety records. Therefore studies aimed at understanding the underlying genetic differences were initiated by several laboratories a few years ago.

Since direct laboratory methods for evaluation of neuropathogenicity of live mumps vaccine are not yet validated (Rubin *et al.,* 1999), it is important to seek additional surrogate methods for evaluation of the genetic stability of the virus. In this work we tried to create a surrogate test for mumps vaccine prepared from the Urabe strain based on a molecular consistency approach similar to the one that we used for quality control of OPV (Chumakov, 1999; WHO, 1999). Two sources of information can be used to identify appropriate sentinel mutations. The first source is the mutations occurring in attenuated strains isolated from cases of vaccine-associated adverse reactions, and the second source is the

TABLE 2

MAPREC Quantification of Mutants in Mumps Vaccines Made by Chiron SpA and SB

Nucleotide variant	Restriction enzyme	Percentage		MAPREC fragment nucleotides
		Chiron SpA	SB	
7616-A	*Mse*I	96	88.9	7,581–7,668
7616-G	*Hin*fI	0	9.2	
8005-A	*Nsi*I	2	50	7,963–8,052
8005-C	*Hin*cII	90	45	
9972-C[a]	n/a	n/a	n/a	9,900–10,010
9972-T	*Bsm*I	26	37	
10529-T	*Bsp*MI	93	27	10,490–10,560
10529-C[b]	n/a	n/a	n/a	

[a] No 9972-C-specific enzyme is available.
[b] No 10529-C-specific enzyme is available.

mutations that accumulate in the viral genome upon growth under conditions of vaccine production. It is important to stress that both approaches can identify not only mutations that are the true markers of pathogenicity, but also incidental neutral mutations. While it is certainly preferable to be able to accomplish the former goal, it may not be possible without resorting to reverse genetics, which is not well developed for this virus. In addition, pathogenicity is a complex phenomenon that is likely to be determined by multiple factors, making it hard and in some cases impossible to identify a single mutation as being the key to this property. However, failure to identify the key molecular markers of pathogenicity does not mean that a sensible surrogate method for monitoring of genetic stability cannot be developed. We call this the molecular consistency approach, which is based on tracing of any shifts in mutational profiles of attenuated virus during vaccine production. This approach has shown its utility in our previous studies of OPV (Rezapkin *et al.,* 1994, 1995, 1999; Taffs *et al.,* 1995).

Previous studies by Brown *et al.* (1996) pointed at a $Glu_{335} \rightarrow Lys$ mutation in the viral HN gene (nucleotide 7616 of the antigenome) as the one that was associated with increased neurovirulence. Therefore it was logical to start the search for appropriate sentinels from this mutation. To our surprise we found that some vaccine stocks not linked to excessive adverse reactions contained 100% of the wild-type nucleotide (7616-A or Lys_{335}) at this site. On the other hand, vaccine that was withdrawn from the market contained roughly 90% of the wild-type 7616-A and only about 10% of the "attenuated" 7616-G. The stocks produced at Biken, the company that was first to produce mumps vaccine from the Urabe strain, are presumably closest to the original vaccine strain and also contained 100% of 7616-A, as did both clinical isolates from vaccine-related meningitis cases. Therefore the only stock that contained a low but measurable amount of 7616-G was the one with a higher level of vaccine-associated meningitis, which, if anything, suggests that 7616-G may be the culprit rather than an attenuating nucleotide. Recently published findings (Wright *et al.,* 2000) showing that mumps virus strains with Lys_{335} are temperature sensitive and have a small-plaque phenotype are also in line with these doubts. The role of the 7616-A mutation in the pathogenicity of mumps virus was questioned by others as well (Afzal *et al.,* 1998; Mori *et al.,* 1997). Additional evidence against the role of 7616-A in neurovirulence comes from the sequencing of several substrains of Jeryl Lynn vaccine (G. Amexis, manuscript in preparation), which was never causally associated with severe adverse reactions. However, Jeryl Lynn differs from Urabe at more than 900 nucleotides, so its safety is likely to be determined by a number of other genetic loci.

The reason for the discrepancy between our conclusions and the conclusions reached by previous investigators who described the mutation (Brown *et al.,* 1996; Brown and Wright, 1998) may be due in part to the method of quantification. We always directly quantified both "mutant" and "vaccine" bases by using different restriction enzymes to ensure that the sum of both mutations is close to 100%. In contrast, in the previously published study only digestion with 7616-A specific enzyme was used. Incomplete restriction enzyme digestion would result in overestimation of the attenuated 7616-G. Thus it is important to positively identify both nucleotide variants rather than simply assume that undigested DNA represents an alternative base at the site. The conclusion that we could reach was that even though some Urabe stocks are heterogeneous at this site, others are clearly homogeneous and this property does not seem to correlate with vaccine performance in the field. Instead, this mutation is likely to be only a neutral marker of a particular virus substrain that was selected by passaging in Vero cells. Regardless of the biological role of this mutation or absence thereof, it does not appear to be a suitable marker for monitoring the consistency of the genetic composition of the Urabe vaccine production.

Since there were several manufacturers that used the Urabe strain and the safety records of the products were different, we determined complete nucleotide sequences of batches of mumps vaccine from different sources. Sequencing showed that they all had characteristic patterns of mutations that could be used to distinguish between them. Quantitative measurement (MAPREC) of the mutations that we identified in commercial vaccine batches produced by different manufacturers showed that the products can be easily distinguished from one another and that individual vaccine lots had very similar mutational profiles.

Comparison of nucleotide sequences of vaccine batches with the sequences of two isolates from cases of vaccine-associated meningitis showed that some of the mutations detected in vaccine batches as nucleotide heterogeneities were selected in clinical isolates and completely replaced bases normally present in vaccine virus. No consistent pattern of mutations could be identified after analysis of just two isolates. More sequence information could help reveal important virulence determinants. However, it is possible that there may be many different pathways leading to reversion of the attenuated phenotype, and there will be little or no consistent pattern of mutations, making sequence information practically useless for predicting virus properties. Although we could not identify molecular markers of mumps virus virulence and although the phenotypic manifestation of the mutations identified in this study is unknown, the sequence information can be used for monitoring vaccine consistency by focusing on mutations that occur and are selected during vaccine production. After establishing a profile of mutations typical of the product with a proven safety record, monitoring the profile to ensure its

TABLE 3

Primers for Amplification of Mumps Virus Genome Segments Used for Nucleotide Sequence Determination

Name	5′-	Primer sequence	-3′
S1.0	1	ACCAAGGGGA AAATGGAGAT GGGATGTTGG	30
A2.5	2,550	GGGTTGCACC ACTCAAAGAC CCGGA	2,526
S2.2	2,210	CCCAUCAUAG UCUCAUCCUC CGAAG	2,234
S4.3	4,351	GGAGTCCATT CAGGAAGTCT GCCTCAATGA	4,380
A4.5	4,550	TTCATGATTA CTGATCAATT CTATT	4,526
A6.5	6,550	AGCTTGTTCT GGCTTGATTT TTTCT	6,526
S6.3	6,375	TGTGGTGCGA CATGCAGCAC TGTACCAGAG	6,404
A8.5	8,526	CATCTGGCTC CAAATCATTT GGTAACTGGC	8,550
S8.2	8,175	CCCTTAATAA TCTTAAAGTA CTAGCC	8,200
S9.5	9,446	GAGGAGGCAA ACAAGGTTCT TCTGGACTTG	9,475
A10.0	10,050	CCACTGGTGA CATACTCAAG CTCCT	10,026
S11.0	10,946	ATATACAGTA AGAGGGTGTT TTACAAAGGT	10,975
A12.0	12,012	CTGCTGCAAT GCTCACATTC ATCTGACCCA	11,983
S12.5	12,446	ACAGATTCTA ACCTGATTTA CCAGCAAGTT	12,475
A15.4	15,385	GACCAAGGGG AGAAAGTAAA ATCAATTTTT TCTTAAAATG	15,346

consistency can serve as a surrogate test for the stability of the genetic composition of viral quasispecies regardless of the biological role of the mutations involved. Strict adherence to the certified seeds and monitoring of mutational profiles may ensure adequate safety of the vaccine.

The advent of new technological tools for direct and accurate mutant quantification led to the possibility of quality control of live viral vaccines. Recently MAPREC was recommended by the World Health Organization as a routine *in vitro* test for lot release of OPV (WHO, 1999). The results presented in this paper demonstrate that a similar approach can be used for other live viral vaccines.

MATERIALS AND METHODS

Vaccine bulks and clinical isolates

Bulk materials of MVL made from the Urabe AM9 strain from two manufacturers, Chiron SpA (lots PS22–PS27) and SmithKline Beecham (SB Lot Nos. MP51, MP52, and MP53), were provided by the manufacturers, and two cell culture isolates from cases of postvaccination meningitis (strains 87 1004 and 87 1005) were kindly provided by Dr. Laszlo Palkonyay (Canadian Bureau of Biologics, Ottawa, Ontario, Canada). Biken Lot MU-9901 was kindly provided by Dr. Akihisa Takamizawa of the Kanonji Institute Research Foundation for Microbial Diseases of Osaka University Kanonji, Kagawa, Japan. All of the samples were used directly for PCR amplification and sequencing without further passaging in cell cultures.

RNA extraction

RNA extraction was performed using the phenol/SDS method. SDS was added to a final concentration of 1%, and the virus samples were extracted twice with water-saturated phenol. The samples were precipitated with 2 vol of isopropanol overnight at −70°C.

RT reaction

Reverse transcription was performed with random hexamer primers (New England Biolabs, NEB) in a 50-μl volume with 600 Units of SuperScript II (Gibco BRL). The final RT mix was preincubated for 5 min at room temperature to allow primer binding and then incubated for 45 min at 40°C. After heat inactivation of the RT (4 min at 95°C), 2–4 μl of the generated cDNA was used as a template for PCR amplification.

cDNA amplification by PCR

Eight different primer pairs (S1/A2.5, S2.2/A4.5, S4.3/A6.5, S6.3/A8.5, S8.2/A10, S9.5/AS12, S11/A13, and S12.5/A15.4), designated Sx.x for sense and Ax.x for antisense, with x.x denoting location (in kilobases) within the mumps virus genome, were used to generate overlapping 1.6- to 3-kb-long PCR fragments spanning the entire mumps virus genome. Amplification was performed in a thermocycler (Perkin–Elmer 480) with 20 pmol of each primer starting with initial denaturation for 1 min at 94°C, followed by 35 cycles consisting of 10 s at 94°C, 40 s at 60°C, and 4 min at 68°C, and a final extension step (20 min at 72°C). Purification of the PCR fragments was performed with the Qiagen PCR purification kit. Primer sequences are given in Table 3.

DNA sequencing

A total of 69 primers were used for PCR amplification and DNA sequencing of Chiron batches designated PS22, PS23, PS24, PS25, and PS27, SB batches MP51 and MP52, and Biken batch MU-9901. The primers were

TABLE 4

Sequences of Primers Used for MAPREC Tests

Nucleotide	Sense primer	Antisense primer	Restriction endonucleases specific for	
7,616	ATGGGGGTGT CTTGCCCAAT AGTACACTCG	CCTGAACATG GATTATATGG ATTgACAGGC CGGA	7616-A *Mse*I	7616-G *Hin*fI
8,005	ATATTCATTC ACTCGTCCTG GTTCAGtCA	GCAGTTGGGC ACACATTTTC ACCAaTGC	8005-A *Nsi*I	8005-C *Hin*cII
9,972	TGATGCGAAT CGGCCTCTAC CACAAgCA	TCTAGAAAAT TCAACAGTAG CCGTCGGTT	9972-T *Bsm*I	n/a
10,529	ACATTGAATT CAATGTATGG TATACCC	CGCATTAGCC TTAAATGTAT CCATTCAAgC A	10529-T *Bsp*MI	n/a

Note. Lowercase letters denote nucleotides changed to create restriction sites.

synthesized based on the published sequence of the Urabe AM9 strain (Mori *et al.,* 1997), GenBank Accession No. AB000387. DNA sequencing was performed with an Applied Biosystems Prism 310 Genetic Analyzer using dRhodamine and BigDye terminators for fluorescence detection. Then 150–250 ng of each PCR fragment and 3.2 pmol of a sequencing primer were used in 20-μl reactions to generate 600–700 bases of readable overlapping information from both strands. The generated sequences were edited with the Factura software package and aligned with Auto Assembler software (both from Applied Biosystems). The sequences were deposited with GenBank under Accession Nos. AF314558, AF314559, AF314560, AF314561, and AF314562.

Mutant quantification by MAPREC

The principal and general MAPREC procedures were described previously (Chumakov *et al.,* 1991; Lu *et al.,* 1993). MAPREC is based on PCR amplification with a mutagenic primer that creates a restriction endonuclease site composed of both primer and template sequences. This site is toggled by a mutation of interest and can be used for detection and quantification of the mutation. Briefly, the procedure involves PCR amplification of single-stranded DNA (produced by using a 10-fold excess of the sense primer), which is then radiolabeled by second-strand DNA synthesis with 5′-^{32}P-labeled antisense primer and *Taq* DNA polymerase. This dsDNA is digested separately with restriction endonucleases specific for both nucleotide variants. Restriction digests are separated by polyacrylamide gel electrophoresis, and radioactive DNA bands are quantitated using a Storm 860 PhosphoImager (Molecular Dynamics). PCR primer sequences used for MAPREC are listed in Table 4.

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REFERENCES

Afzal, M. A., Marsden, S. A., Hull, R. M., Pipkin, P. A., Bentley, M. L., and Minor, P. D. (1999). Evaluation of the neurovirulence test for mumps vaccines. *Biologicals* **27**(1), 43–49.

Afzal, M. A., Pickford, A. R., Forsey, T., Heath, A. B., and Minor, P. D. (1993). The Jeryl Lynn vaccine strain of mumps virus is a mixture of two distinct isolates [Published erratum appears in *J. Gen. Virol.* 1994, Aug, **75**(Pt. 8), 2139]. *J. Gen. Virol.* **74**(Pt. 5), 917–920.

Afzal, M. A., Pickford, A. R., Forsey, T., and Minor, P. D. (1992). Heterogeneous mumps vaccine [Letter]. *Lancet* **340**(8825), 980–981.

Afzal, M. A., Yates, P. J., and Minor, P. D. (1998). Nucleotide sequence at position 1081 of the hemagglutinin-neuraminidase gene in the mumps Urabe vaccine strain [Letter; comment]. *J. Infect. Dis.* **177**(1), 265–266.

Autret, E., Jonville-Bera, A. P., Galy-Eyraud, C., and Hessel, L. (1996). [Aseptic meningitis after mumps vaccination]. *Therapie* **51**(6), 681–683.

Black, S., Shinefield, H., Ray, P., Lewis, E., Chen, R., Glasser, J., Hadler, S., Hardy, J., Rhodes, P., Swint, E., Davis, R., Thompson, R., Mullooly, J., Marcy, M., Vadheim, C., Ward, J., Rastogi, S., and Wise, R. (1997). Risk of hospitalization because of aseptic meningitis after measles–mumps–rubella vaccination in one- to two-year-old children: An analysis of the Vaccine Safety Datalink (VSD) Project. *Pediatr. Infect. Dis. J.* **16**(5), 500–503.

Boriskin, Y. S., Kaptsova, T. I., and Booth, J. C. (1993). Mumps virus variants in heterogeneous mumps vaccine [Letter]. *Lancet* **341**(8840), 318–319.

Boriskin, Y. S., Yamada, A., Kaptsova, T. I., Skvortsova, O. I., Sinitsyna, O. A., Takeuchi, K., Tanabayashi, K., and Sugiura, A. (1992). Genetic evidence for variant selection in the course of dilute passaging of mumps vaccine virus. *Res. Virol.* **143**(4), 279–283.

Brown, E. G., Dimock, K., and Wright, K. E. (1996). The Urabe AM9 mumps vaccine is a mixture of viruses differing at amino acid 335 of the hemagglutinin-neuraminidase gene with one form associated with disease. *J. Infect. Dis.* **174**(3), 619–622.

Brown, E. G., and Wright, K. E. (1998). Genetic studies on a mumps vaccine strain associated with meningitis. *Rev. Med. Virol.* **8**(3), 129–142.

Brunell, P. A., Brickman, A., Steinberg, S., and Allen, E. (1972). Parotitis in children who had previously received mumps vaccine. *Pediatrics* **50**(3), 441–444.

Buynak, E. B., and Hilleman, M. R. (1966). Live attenuated mumps virus vaccine. 1. Vaccine development. *Proc. Soc. Exp. Biol. Med.* **123**(3), 768–775.

Chamot, E., Toscani, L., Egger, P., Germann, D., and Bourquin, C. (1998). Estimation of the efficacy of three strains of mumps vaccines during an epidemic of mumps in the Geneva canton (Switzerland). *Rev. Epidemiol. Sante Publique* **46**(2), 100–107.

Chumakov, K. M. (1999). Molecular consistency monitoring of oral poliovirus vaccine and other live viral vaccines. *Dev. Biol. Stand.* **100,** 61–74.

Chumakov, K. M., Powers, L. B., Noonan, K. E., Roninson, I. B., and Levenbook, I. S. (1991). Correlation between amount of virus with altered nucleotide sequence and the monkey test for acceptability of oral poliovirus vaccine. *Proc. Natl. Acad. Sci. USA* **88**(1), 199–203.

Colville, A., Pugh, S., and Miller, E. (1994). Withdrawal of a mumps vaccine [Letter; comment]. *Eur. J. Pediatr.* **153**(6), 467–468.

Dourado, I., Cunha, S., Teixeira, M. G., Farrington, C. P., Melo, A., Lucena, R., and Barreto, M. L. (2000). Outbreak of aseptic meningitis associated with mass vaccination with a Urabe-containing measles–mumps–rubella vaccine: Implications for immunization programs. *Am. J. Epidemiol.* **151**(5), 524–530.

Gassner, M., Hess, U., and Berger, R. (1995). [Mumps: Efficacy of booster immunization]. *Schweiz. Med. Wochenschr.* **125**(51–52), 2506–2510.

Hilleman, M. R. (1966). Advances in control of viral infections by nonspecific measures and by vaccines, with special reference to live mumps and rubella virus vaccines. *Clin. Pharmacol. Ther.* **7**(6), 752–762.

Kuczyk, M. A., Denil, J., Thon, W. F., Djamilian, M., Truss, M., Schlick, R., and Jonas, U. (1994). Orchitis following mumps vaccination in an adult. *Urol. Int.* **53,** 179–180.

Levenbuk, I. S., Nikolayeva, M. A., Chigirinsky, A. E., Ralf, N. M., Kozlov, V. G., Vardanyan, N. V., Sliopushkina, V. G., Kolomiets, O. L., Rukhamina, M. L., and Grigoryeva, L. V. (1979). On the morphological evaluation of the neurovirulence safety of attenuated mumps virus strains in monkeys. *J. Biol. Stand.* **7**(1), 9–19.

Lu, Z., Douthitt, M. P., Taffs, R. E., Ran, Y., Norwood, L. P., and Chumakov, K. M. (1993). Quantitative aspects of the mutant analysis by PCR and restriction enzyme cleavage (MAPREC). *PCR Methods Appl.* **3**(3), 176–180.

Maximova, O., Dragunsky, E., Taffs, R., Snoy, P., Cogan, J., Marsden, S., and Levenbook, I. (1996). Monkey neurovirulence test for live mumps vaccine. *Biologicals* **24**(3), 223–224.

McDonald, J. C., Moore, D. L., and Quennec, P. (1989). Clinical and epidemiologic features of mumps meningoencephalitis and possible vaccine-related disease. *Pediatr. Infect. Dis. J.* **8**(11), 751–755.

Miller, E., Goldacre, M., Pugh, S., Colville, A., Farrington, P., Flower, A., Nash, J., MacFarlane, L., and Tettmar, R. (1993). Risk of aseptic meningitis after measles, mumps, and rubella vaccine in UK children [See comments]. *Lancet* **341**(8851), 979–982.

Minor, P. D. (1997). Laboratory tests for mumps vaccines. *Biologicals* **25**(1), 35–40.

Mori, C., Tooriyama, T., Imagawa, T., and Yamanishi, K. (1997). Nucleotide sequence at position 1081 of the hemagglutinin-neuraminidase gene in the mumps virus Urabe vaccine strain [Letter; comment] [see comments]. *J. Infect. Dis.* **175**(6), 1548–1549.

Nalin, D. R. (1989). Mumps vaccine complications: Which strain? [Letter; comment] [see comments]. *Lancet* **2**(8676), 1396.

Rebiere, I., and Galy-Eyraud, C. (1995). Estimation of the risk of aseptic meningitis associated with mumps vaccination, France, 1991–1993. *Int. J. Epidemiol.* **24**(6), 1223–1227.

Rezapkin, G. V., Chumakov, K. M., Lu, Z., Ran, Y., Dragunsky, E. M., and Levenbook, I. S. (1994). Microevolution of Sabin 1 strain *in vitro* and genetic stability of oral poliovirus vaccine. *Virology* **202,** 370–378.

Rezapkin, G. V., Fan, L., Asher, D. M., Fibi, M. R., Dragunsky, E. M., and Chumakov, K. M. (1999). Mutations in Sabin 2 strain of poliovirus and stability of attenuated phenotype. *Virology* **258,** 152–160.

Rezapkin, G. V., Norwood, L. P., Taffs, R. E., Dragunsky, E. M., Levenbook, I. S., and Chumakov, K. M. (1995). Microevolution of type 3 Sabin strain of poliovirus in cell cultures and its implications for oral poliovirus vaccine quality control. *Virology* **211,** 377–384.

Rubin, S. A., Pletnikov, M., and Carbone, K. M. (1998). Comparison of the neurovirulence of a vaccine and a wild-type mumps virus strain in the developing rat brain. *J. Virol.* **72**(10), 8037–8042.

Rubin, S. A., Snoy, P. J., Wright, K. E., Brown, E. G., Reeve, P., Beeler, J. A., and Carbone, K. M. (1999). The mumps virus neurovirulence safety test in Rhesus monkeys: A comparison of mumps virus strains. *J. Infect. Dis.* **180**(2), 521–525.

Sawada, H., Yano, S., Oka, Y., and Togashi, T. (1993). Transmission of Urabe mumps vaccine between siblings [Letter]. *Lancet* **342**(8867), 371.

Stokes, J., Jr. (1967). The case for mumps vaccine [Letter]. *N. Engl. J. Med.* **277**(7), 376.

Sugiura, A., and Yamada, A. (1991). Aseptic meningitis as a complication of mumps vaccination. *Pediatr. Infect. Dis. J.* **10**(3), 209–213.

Taffs, R. E., Chumakov, K. M., Rezapkin, G. V., Lu, Z., Douthitt, M., Dragunsky, E. M., and Levenbook, I. S. (1995). Genetic stability and mutant selection in Sabin 2 strain of oral poliovirus vaccine grown under different cell culture conditions. *Virology* **209,** 366–373.

Turner, P. C., Forsey, T., and Minor, P. D. (1991). Comparison of the nucleotide sequence of the SH gene and flanking regions of mumps vaccine virus (Urabe strain) grown on different substrates and isolated from vaccines. *J. Gen. Virol.* **72**(Pt. 2), 435–437.

Ueda, K., Miyazaki, C., Hidaka, Y., Okada, K., Kushuhara, K., and Kadoya, R. (1995). Aseptic meningitis caused by measles–mumps–rubella vaccine in Japan. *Lancet* **346,** 701–702.

World Health Organization (1999). "Recommendations for Poliomyelitis Vaccine (Oral)." WHO, Paris.

Wright, K. E., Dimock, K., and Brown, E. G. (2000). Biological characteristics of genetic variants of Urabe AM9 mumps vaccine virus. *Virus Res.* **67**(1), 49–57.

Yates, P. J., Afzal, M. A., and Minor, P. D. (1996). Antigenic and genetic variation of the HN protein of mumps virus strains. *J. Gen. Virol.* **77**(Pt. 10), 2491–2497.