

Quantitative mutant analysis of viral quasispecies by chip-based matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

Georgios Amexis*, Paul Oeth†, Kenneth Abel†, Anna Ivshina*, Francois Pelloquin‡, Charles R. Cantor†, Andreas Brau†, and Konstantin Chumakov*[§]

*Center for Biologics Evaluation and Research, Food and Drug Administration, 1401 Rockville Pike, HFM 470, Rockville, MD 20852; †SEQUENOM, Inc., Genomics Department, 3595 John Hopkins Court, San Diego, CA 92121; and ‡Aventis Pasteur, 1541 Avenue Marcel Merieux, 69280 Marcy l'Etoile, France

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RNA viruses exist as quasispecies, heterogeneous and dynamic mixtures of mutants having one or more consensus sequences. An adequate description of the genomic structure of such viral populations must include the consensus sequence(s) plus a quantitative assessment of sequence heterogeneities. For example, in quality control of live attenuated viral vaccines, the presence of even small quantities of mutants or revertants may indicate incomplete or unstable attenuation that may influence vaccine safety. Previously, we demonstrated the monitoring of oral poliovirus vaccine with the use of mutant analysis by PCR and restriction enzyme cleavage (MAPREC). In this report, we investigate genetic variation in live attenuated mumps virus vaccine by using both MAPREC and a platform (DNA MassArray) based on matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Mumps vaccines prepared from the Jeryl Lynn strain typically contain at least two distinct viral substrains, JL1 and JL2, which have been characterized by full length sequencing. We report the development of assays for characterizing sequence variants in these substrains and demonstrate their use in quantitative analysis of substrains and sequence variations in mixed virus cultures and mumps vaccines. The results obtained from both the MAPREC and MALDI-TOF methods showed excellent correlation. This suggests the potential utility of MALDI-TOF for routine quality control of live viral vaccines and for assessment of genetic stability and quantitative monitoring of genetic changes in other RNA viruses of clinical interest.

Nucleotide sequence heterogeneity is such a profound feature of RNA viruses that these are often referred to as quasispecies (1, 2). Populations of RNA viruses contain a broad dynamic spectrum of mutants differing from a consensus sequence at one or more sites. This enhances the ability of RNA viruses to adapt rapidly to new growth conditions both *in vivo* and *in vitro* and to evolve while retaining vestiges of their history (3). This characteristic has importance for live attenuated viral vaccines, in which the presence or relative abundance of mutant virus variants can affect both phenotypic and pathiopathologic features (4) and the diversity of immunologic responses in vaccine recipients. For instance, neurovirulence of oral poliovirus vaccine (OPV) is often determined by the presence of a tiny fraction of mutant viral particles in an overwhelming excess of non-neurovirulent virus (5). Genetic stability is therefore a required characteristic of live attenuated viral vaccines and is of particular importance for new vaccines derived by targeted genetic manipulations and cloning.

Different strains of mumps virus, another RNA virus, are used for production of live vaccines against parotitis. Although some strains have questionable safety and efficacy, others have demonstrated an excellent long-term record (6–8). The Jeryl Lynn strain is used for production of one of the safest and most efficacious vaccines (9) and has been used for over 2 decades to control mumps parotitis in the U.S. and other countries. The

vaccine consists of two distinct substrains present at roughly a 4:1 ratio (10). Even though there are no direct experimental data to suggest that variations in the ratio between two substrains in the Jeryl Lynn vaccine can affect its safety, this possibility cannot be discounted without consideration and investigation. Substantial sequence heterogeneity of the vaccine can also contribute to its higher efficacy by providing a broader spectrum of antigenic determinants and likewise loss of this heterogeneity can diminish vaccine efficacy. At the time of vaccine development in the 1970s, consideration of molecular genetic structure was not taken into account. However, because of advancements made since then, consideration of production consistency should require strict molecular monitoring. It is therefore incumbent on vaccine manufacturers to maintain consistent ratios between the genetic components to match the composition of the product that has undergone clinical evaluation at the time of licensure.

Recently, we determined the complete nucleotide sequences of both major (JL1) and minor (JL2) Jeryl Lynn substrains (G.A., unpublished results) and found they differ at 414 nucleotides, or about 2.6% of the 15,384-nt genome. Currently we are investigating potential phenotypic roles of these differences and the impact they may have on vaccine safety. Interestingly, different virus replication systems and/or growth conditions used by different vaccine manufacturers resulted in differential accumulation of either the major (in cell cultures) or the minor substrain (in hen's embryonated eggs). Until the consequences of these varying ratios to vaccine efficacy and safety are better understood, as well as for the sake of molecular consistency of vaccine production, it is desirable to accurately quantify these viral subcomponents in materials collected at different stages of production (for example, seed viruses and final vaccine lots) and obtained from different manufacturers.

As stated above, the high genetic plasticity of RNA viruses makes a strong case for molecular consistency monitoring of all live vaccines. Development of new methods for rapid analysis of viral nucleic acids, with emphasis on quantitative characterization of mutation profiles, is important for improving vaccine consistency and safety. Previously, we have developed a very sensitive method called mutant analysis by PCR and restriction enzyme cleavage (MAPREC) (5). By using this technique, we showed that even a small increase in the content of certain revertants in OPV batches, from levels around 0.5% to slightly over 1%, results in vaccine lot failure in the monkey neurovirulence test and makes the batch unacceptable for use in humans

Abbreviations: MAPREC, mutant analysis by PCR and restriction enzyme cleavage; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; OPV, oral poliovirus vaccine; CEF, chicken embryo fibroblast.

[§]To whom reprint requests should be addressed. E-mail: chumakov@cber.fda.gov.

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(11–13). Excellent correlation between neurovirulence in monkeys and the relative contents of nucleotide 472-U/C mutants in the 5' noncoding region of the attenuated type 3 poliovirus genome led the World Health Organization to recommend this MAPREC assay as a routine test to control the consistency of production and the safety of OPV (14). Although effective, however, the use of PAGE and autoradiography means there are inherent limitations to this methodology in terms of speed, throughput, and cost.

Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry is now being used for analysis of nucleic acids (15–17), including genetic variations such as microsatellites, insertion/deletions, and especially single-nucleotide polymorphisms (SNPs) (18–20). The output data are a measure of an intrinsic characteristic of the DNA products being studied (molecular mass in daltons); no indirect measurement of the products is involved, as with fluorescent or radiolabel tagging. The ability to resolve oligonucleotides varying in mass by less than a single nucleotide makes MALDI-TOF mass spectrometry an excellent platform for SNP and mutant analysis. A highly automated processing platform incorporating MALDI-TOF mass spectrometry, designated DNA MassArray, has been developed (21–23). DNA MassArray incorporates region-specific amplification of genomic DNA spanning the genetic variation, capture of one PCR product strand serving as template for a variant-specific primer extension (usually one to two bases), dispensing of nanoliter quantities of extension products onto a matrix-loaded chip array, and product analysis by MALDI-TOF mass spectrometry. Spectra are interpreted automatically according to input assay design parameters to identify the variant DNAs (alleles or mutants).

DNA MassArray has great utility for accurate genotyping of individual DNA samples (24–26). Also, given that allelic frequencies are proportional to mass spectral peak areas, the technology provides accurate allele frequency estimates by using pooled DNA templates (27, 28) and is therefore well suited for quantitative mutant analysis in viral populations. To date, little has been reported on the use of MALDI-TOF for such analyses. Here we report the use of DNA MassArray for quantitative analysis of mutations in attenuated mumps virus vaccines and compare it with MAPREC, which is the World Health Organization-approved method for molecular consistency monitoring of OPV. We demonstrate the usefulness of DNA MassArray for assessing genetic stability of natural or recombinant viruses and for monitoring production consistency for live attenuated viral vaccines against parotitis.

Materials and Methods

Virus Samples. Mumps vaccine (Jeryl Lynn strain) was obtained from commercial vaccine batches produced by Merck (lot number JL2033162). Mumps virus was grown in primary cultures of chicken embryo fibroblasts (CEF) or in Vero cells (continuous cell line from African green monkey kidney) grown in Dulbecco-modified MEM with 10% embryonic bovine serum (Life Technologies, Gaithersburg, MD). Serial passaging was performed by infecting cell cultures, and the infected cell monolayers were incubated at 37°C until complete cytopathic effect occurred in 5–7 days. Homogeneous JL1 strain was plaque-purified in Vero cells.

MAPREC Assay. Quantitative mutant analysis was performed as described (11, 29) and as illustrated in Fig. 1A. Briefly, viral genomic RNA was isolated from batches of vaccine or infected Vero cell cultures by phenol/SDS extraction and reverse-transcribed into cDNA with random dN6 primers by using SuperScriptII RT (Life Technologies). A region containing a mutation of interest was PCR-amplified by using a partially mismatched primer, which created a restriction site affected by

the mutation. The other primer carried a 5'-end biotin tag so that an aliquot of the product could be used in DNA MassArray. PCR amplification was performed with a 10-fold excess of one primer to generate predominantly single-stranded DNA. Radiolabeled double-stranded PCR products were produced by one-step primer-extension reaction with a primer labeled by T4-polynucleotide kinase and [³²P]-γATP and were digested with the appropriate restriction endonuclease(s). The products were analyzed by electrophoresis in 10% native polyacrylamide gels followed by detection (30-min exposure) by using a Storm 860 Fluorimager (Molecular Dynamics) and IMAGEQUANT software (Molecular Dynamics). Appropriate PCR primers and restriction enzymes used for MAPREC analysis were identified by using RESTMAC custom software and are listed in Table 1 *Upper*.

Viral Genome Sequencing. Nucleotide sequences of overlapping PCR products of about 3–4 kb in length (30) were determined by using an ABI Prism 310 Genetic Analyzer and d-Rhodamine DNA sequencing kits (Applied Biosystems). Sequences were assembled with AUTOASSEMBLER and FACTURA software (Applied Biosystems) and analyzed by using MACVECTOR (Oxford Molecular, Beaverton, OR).

Mutant Quantitation by DNA MassArray (MALDI-TOF). Reactions using DNA MassArray were performed as previously described (28) and as illustrated in Fig. 1B. PCR products were generated by using the same primers as for MAPREC, including one biotinylated PCR primer. Twenty-microliter aliquots of PCR products [and subsequent MassExtend (SEQUENOM, San Diego, CA) products] were mixed with 15 μl of streptavidin-coated magnetic beads (Dynal, Great Neck, NY) resuspended in 10 μl of 3× binding buffer (30 mM Tris/3 mM EDTA/3 M NaCl, pH 7.5) and incubated at room temperature for 20 min. The beads were then collected with a magnetic platform and the PCR mixture removed by using a modified Beckman MultiMek 96-head robotic pipettor (SpectroPREP). Double-stranded PCR products were denatured by the addition of 50 μl 0.1 N NaOH for 5 minutes at room temperature and washed three times with 10 mM Tris-HCl, pH 8.0. MassExtend reactions containing 10 pmol of extension primer (Integrated DNA Technologies, Coralville, IA), a nucleotide mix, and heat-stable DNA polymerase, were performed by using a universal temperature program (80°C for 30 seconds, followed by 3 cycles of 40°C for 15 seconds and 60°C for 1 min). Products were washed two times with 10 mM Tris-HCl, pH 8.0, and denatured at 60°C for 5 min in 50 μM NH₄OH. Approximately 7 nl of the extension product was then dispensed onto a SpectroCHIP (SEQUENOM) array prespotted with a matrix of 3-hydroxypicolinic acid by using a SpectroJET (SEQUENOM) piezoelectric nanoliter pump (23). Mass spectrometric data were generated by using a modified Bruker (Billerica, MA) Biflex III MALDI-TOF mass spectrometer (SpectroREADER). Table 1 *Lower* shows MassExtend primer sequences used and the expected product masses for each viral nucleotide position assayed.

Data were imported into SEQUENOM's SPECTROTYPYR database (28), which automatically identified variant-specific products according to input assay design features and also quantitated their relative abundances. The software uses a set of digital filters optimized for mass spectra of DNA. Products from each MassExtend reaction were spotted and laser scanned in quadruplicate to improve the precision of the frequency estimates obtained. For each spectrum acquired, peak areas corresponding to expected MassExtend products were automatically normalized relative to baseline and integrated. Within a given spectrum, frequencies of variant products were calculated as the proportion of either peak area relative to the total area for both expected peaks. Frequency estimates obtained for the four measurements

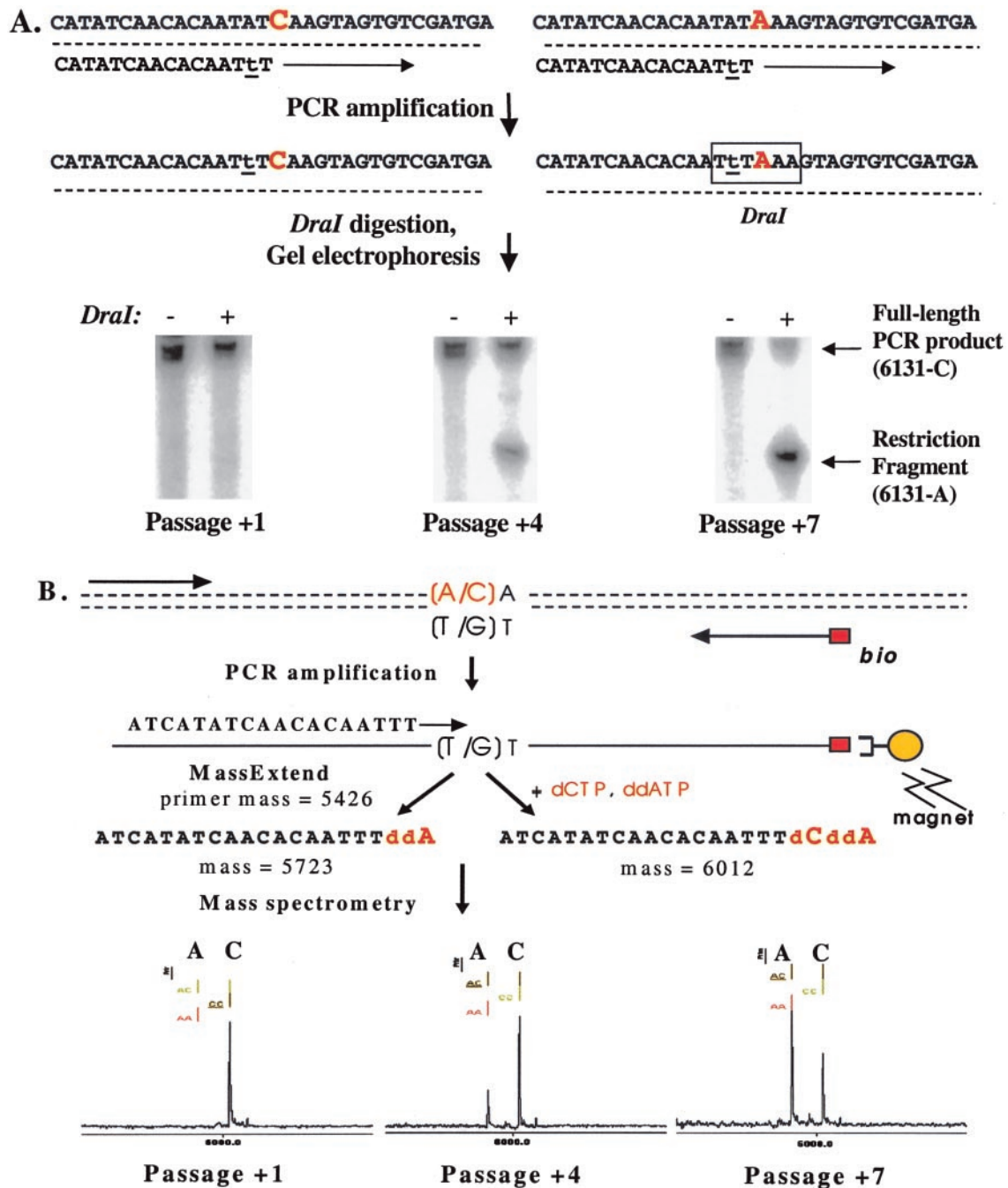


Fig. 1. (A) Assay for MAPREC analysis of nucleotide 6131-C/A variants. Sequence surrounding the variant site is PCR amplified by using viral sequence-specific primers. One primer introduces a base mismatch near its 3' end (underlined), so that amplification of the sequence containing the mismatch and the 6131-A variant creates a site for the restriction endonuclease *DraI* (TTAAA). Restriction digest products are resolved by gel electrophoresis and their relative levels quantified by using a PhosphorImager. (B) Assay for MassArray analysis of nucleotide 6131-C/A variants. Sequence containing the variant site is PCR amplified by using the same primers as for MAPREC. One primer carries a 5'-biotin tag, enabling subsequent product capture by using streptavidin-coated magnetic beads in the presence of a magnet. After denaturation, the captured strand is template for the MassExtend reaction, including a primer binding immediately adjacent to the variant site, DNA polymerase, and a nucleotide mix containing deoxyCTP and dideoxyATP. Differential incorporation of ddATP causes chain termination and generates products of different masses (primer masses shown in daltons). Variant DNA products are resolved by using chip-based mass spectrometry and their relative levels automatically quantified by using SPECTROTYPE software. For both methodologies, representative data from passage 4 are shown.

were averaged, and standard errors were determined for mean values from multiple experiments.

Results

Studies of the genetic stability of the Jeryl Lynn strain of mumps virus are complicated by the fact that vaccines consist of a mixture of two substrains (10). In addition, passaging of the

mixed JL strain in cell cultures and *in vivo* can result in the preferential replication and accumulation of one of the subcomponents of the mixture, as well as the emergence and selection of new mutations in either of the substrains. To reduce this complexity, we began by studying the latter process only, by using only JL1 substrain plaque-purified in Vero cells and then passaged in cell cultures of CEFs (CEF is the substrate used for

Table 1. Oligonucleotides Used in MAPREC and MassArray Assays

MAPREC					
Nucleotide variant	Primer name	PCR primer sequences	Amplicon, bp	Restriction enzymes	Fragment detected, bp
877-A/G	p5-874	848-TTCGAACTCCAGTTGGCGAGACGGCAG-874	60	<i>HinfI</i>	31
	*pA-879	908-CCACCATTGCATAGTATCTGTTGATAGGA-879			
6131-A/C	*p5 (<i>Dral</i>)-6130	6095-GAATCAACTTCAAACAAAATCATATCAACACAATT-6130	76	<i>Dral</i>	40
	*p5 (<i>EcoRV</i>)-6129	6095-GAATCAACTTCAAACAAAATCATATCAACACGATA-6129			
	pA-6135	6171-AATCTAAGATTAGTACCTGATGAGATCATCGACACTA-6135			
11293-T/C	*p5-11291	11245-GTGTCTATTACCATCTCAATTGGGGGCTTAACTTCTTTCATGGA-11291	102	<i>HinfI</i>	52
	pA-11299	11347-CACATCAGCAATTGCAGACACTAGTGGATCTCCTATGTTTCTATTA AAC-11299			
12997-C/T	p5-12983	12946-GGAACCTTCATACCAAATGTATTACCTAAGAATTCAAG-12983	101	<i>HinfI</i>	49
	pA-13003	13047-GTTATGGCCATTCTCGGTATCTCTCAAGGTCATATACACATAG-13003			
13832-A/G	p5-13817	13779-GACCACTAGGTTTGTTCATCCACTCATGGTATAAAACAA-13817	103	<i>EcoRI</i>	50
	*pA-13834	13882-CTCTGCCAAGTATAGATGGGCCACCGTCAGATATCTTCATATGGCTAATAAGAA-13834			
DNA MassArray					
Nucleotide variant	Primer name	EXTEND primer sequences	Primer mass, Da	Variant-1 product mass	Variant-2 product mass
877-A/G	877-E-S	CAGTTGGCGAGACGGCAGGG	6248	6545 (+A)	6866 (+GT)
6131-A/C	6131-E-S- <i>Dral</i>	ATCATATCAACACAATTT	5426	5723 (+A)	6012 (+CA)
11293-T/C	11293-E-S	TAAACTTCTTTCATGGAG	5793	6066 (+C)	6370 (+TC)
12997-C/T	12997-E-S	TCAAGGCATACAAGGAAT	5541	5814 (+C)	6118 (+TC)
13832-A/G	13832-E-S	TATAAAACAATCAGTGTTTT	6114	6411 (+A)	6741 (+GA)

vaccine production). The complete viral genome sequence was determined for the plaque-purified clone and also for viral suspensions collected at each of 10 sequential passages in CEF. Sequence comparisons showed that the initial seed was remarkably stable, with very few detectable mutations during the course of passaging (data not shown). Among those identified, only a C to A mutation at nucleotide 6,131 consistently accumulated in CEF cells. This mutation introduces a stop codon at amino acid position 529 of the fusion (F) gene, with the expected result of truncating the encoded protein 11 amino acids before its normal C terminus. Any possible role this mutation may play in viral replication remains unknown, but its consistent accumulation suggests that it can be used as a gauge of the passage history of a viral stock.

To initially compare the two methodologies, we focused on the 6131-C/A variants and quantified their levels at each passage by using both MAPREC and MALDI-TOF mass spectrometry (DNA MassArray). The assays developed for 6131-C/A by using each method are illustrated in Fig. 1, and primer sequences for all assays are shown in Table 1. Both methods used the same PCR primers to amplify the region containing the variant but then differed in their respective enzymology and detection schemes for distinguishing between the variants. Results from all passages are shown in Fig. 2. The 6131-A mutation could not be detected by either method in the initial JL1 seed or first two passages, suggesting this particular site was relatively stable under normal growth conditions during early passaging. However, MAPREC showed that the 6131-A mutant accumulated during subsequent passages, peaked at ~59% of total in passage 7, then appeared to decrease slightly by the last analyzed passage. It is unclear why the frequency of the A-strain drops in these last two passages. Consistent frequency estimates for 6131-A at all passages were also obtained by using MassArray. This was seen both for PCR reactions performed independently and for PCR products from a single reaction divided for analysis by both methods. A strong correlation was observed in the frequency estimates for this sequence variant obtained from both MAPREC and MassArray (Fig. 2B).

Assays were also developed for sequence variants distinguishing between the JL1 and JL2 substrains, to permit quantifying their relative levels in mixed cultures or mumps virus vaccines.

Although there is no definitive information about differences in biological properties of the substrains, it is desirable to ensure that production batches have a consistent genetic composition.

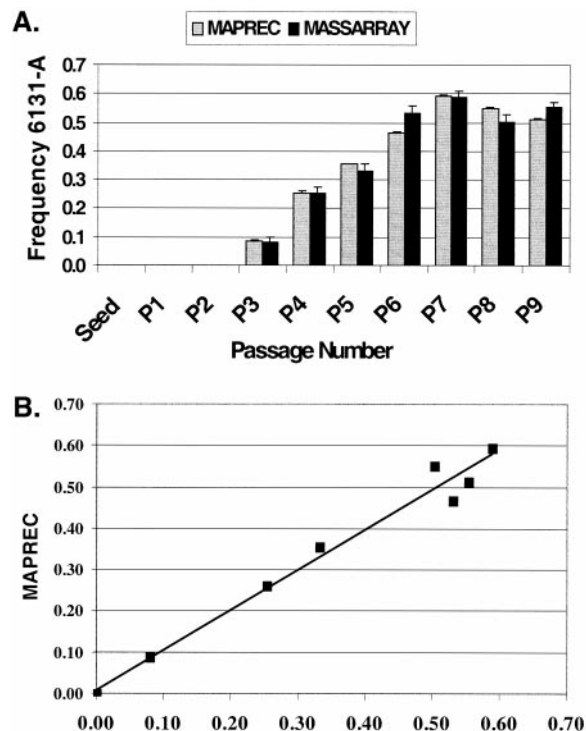


Fig. 2. Characterization of mutant 6131-A content in serial passages of cloned JL1 stock. Representative data for several passages by using either MAPREC or MassArray. (A) Comparison of 6131-A frequency estimates in the initial seed and in nine serial passages by using both methods. 6131-A was undetectable by either method in the initial seed and first two passages. Error bars reflect standard deviation determined from three MAPREC experiments and from four MassArray experiments. (B) Correlation between frequency estimates for 6131-A obtained from both methods for all passages (initial seed and passages 1 and 2 had observed values of 0).

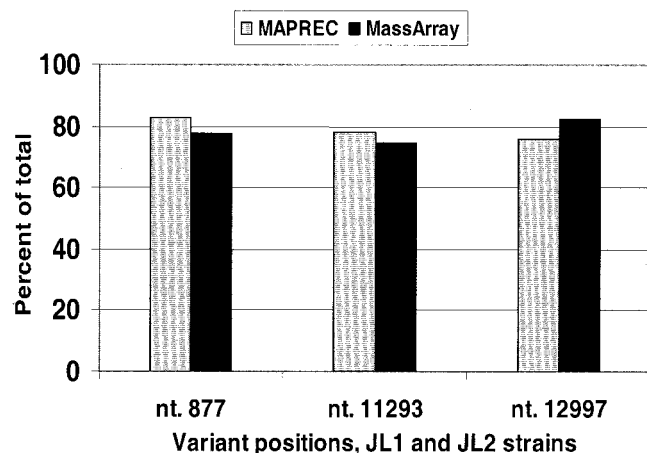


Fig. 3. Determination of substrain JL1 and JL2 contents in a commercial mumps virus vaccine. The relative levels of JL1-specific variants at three different positions in the viral genome were measured by using both MAPREC and MassArray. Estimates were obtained from one PCR experiment.

We have determined the complete nucleotide sequences of both substrains, which differ at 414 nucleotides (G.A., unpublished work). Three of these nucleotide positions (nucleotides 877, 11,293, and 12,997) were selected for assay development. Any of the other variant positions could have also been chosen. Each of these positions was observed to be genetically stable during the prior serial passaging of the JL1 substrain alone, determined by MAPREC quantification at each passage. These assays were then used to determine the substrain levels in a commercially available mumps virus vaccine (Merck) containing a mixture of JL1 and JL2 at an expected 4:1 ratio, respectively. Frequency estimates for the JL1-specific variants were similar for all three nucleotide positions by using both MAPREC and MassArray (Fig. 3). The average frequencies determined from both methods were ≈ 79 , 76, and 78% for nucleotides 877, 11,293, and 12,997, respectively.

In the last set of experiments, one of these positions was selected for characterizing the effect of passaging in embryonated chicken eggs (ECE) on the JL1/JL2 ratio. Propagation of mixed Jeryl Lynn virus in ECE typically leads to a significant accumulation of the minor JL2 component (unpublished observations). Shown in Fig. 4, both MAPREC and MassArray assays for nucleotide 877 again yielded similar frequency estimates for the respective sequence variants at each passage tested. Beginning at an initial master seed level of 20%, the relative proportion of the JL2-specific variant increased rapidly in the mixed viral population to $\approx 95\%$ of total in the final production level passage. Thus both methods confirmed the expected trend among mixed JL substrains by using this replication system.

Discussion

We report the development of assays for characterizing sequence heterogeneity in mixtures of Jeryl Lynn substrains of mumps virus. It is expected these assays will be useful for routine monitoring of live attenuated mumps virus vaccines as a test of genetic stability and to ensure consistency of its production. For example, for vaccines produced from a homogeneous plaque-purified JL1 strain, it might be advantageous to routinely monitor for the C to A mutation at nucleotide 6,131, a position that was seen to be relatively unstable during passaging of JL1 alone. This particular mutation might be expected to result in a functional change in the encoded fusion (F) protein, although the consequence of this altered protein in terms of vaccine efficacy and safety is not fully understood. Nonetheless, detec-

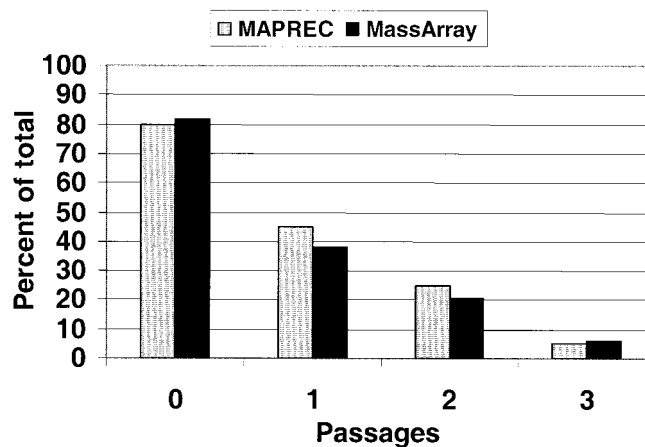


Fig. 4. Determination of the JL1/JL2 ratio in serial passages of mixed Jeryl Lynn virus in chicken embryos. The proportion of the JL1-specific variant at nucleotide 877 was measured by using both MAPREC and MassArray. Passage zero represents conventional Jeryl Lynn vaccine (Merck lot 1187E), which was grown for the specified number of passages in embryonated chicken eggs. Estimates were obtained from one PCR experiment.

tion of accumulating 6131-A in a vaccine batch might indicate it was produced under conditions favoring selection for this mutant and thus perhaps signals a production inconsistency. Measurement of the relative abundance of JL1 and JL2 substrains may have even more importance, because we have found a change in growth conditions (e.g., use of embryonated chicken eggs instead of CEF as the production substrate) can change the direction of variant selection and result in almost complete disappearance of one of the components of the vaccine within a few passages. Such a dramatic change in genetic composition of the vaccine can warrant new clinical trials unless it can be shown that the new product is consistent with the previously evaluated one. Such molecular profiling of the vaccine is critical if there has been a change in production conditions and can also be used to assess robustness of the manufacturing process if regularly used to screen every new batch of the vaccine.

Each of the compared methodologies for quantifying sequence variations was able to clearly discriminate between the highly similar JL substrains and to reveal clear trends in a dynamic setting of sequence complexity. In this report, both MAPREC and MALDI-TOF (DNA MassArray) produced similar frequency estimates for all sequence variants assayed, indicating the latter can also be used for accurate quantitation of substrain levels and mutations in mumps virus genomes and vaccines, and likely other mixed viral cultures. MAPREC is an already established method that has been approved for routine monitoring of oral poliovirus vaccine. MAPREC has been shown to be both sensitive and accurate for mutant quantitation. However, its application is limited because it is a relatively time-consuming procedure that cannot be easily scaled up either for processing of multiple samples or analysis of multiple genetic markers. In addition, in some cases, we observed a nonlinear response caused by nucleotide misincorporation during PCR resulting in incomplete restriction digestion (i.e., the sum of measured JL1 and JL2 levels can occasionally be less than 100%). Our experience with more than 100 different MAPREC assays shows that no PCR DNA can be completely digested, and that the best results are within a 96–99% range of digest efficiency. In contrast, DNA MassArray does not use restriction endonucleases but instead directly measures an inherent physical property of the variant DNA products, i.e., molecular mass. No artificial restriction site introduction is required, nor is fluorescence or radio labeling of primers required for detection.

DNA MassArray also offers the opportunity for higher screening throughput for accumulating mutations or substrain imbalances in mixed viral strains or vaccines. It provides measurements of the presence or absence of variants and their relative frequencies within only 1–2 seconds. Rather than being gel-based, it is a highly automated system that works with samples in chip-based high-density arrays. By using MassArray, hundreds to thousands of reactions could be analyzed for the presence of mutations across the viral genome within 1 8-hour day after completion of PCR. Alternatively, it could be used to survey far greater numbers of vaccine batches produced in parallel, or many more passages, to identify the most genetically stable production phases. Assay design for MassArray is also simple and flexible and has been automated to allow the designing of vast numbers of assays to be run by using a universal set of reaction conditions.

The ability of MassArray to reliably detect accumulating subcomponents from a variety of source materials, including plaque-purified clones, commercial vaccines, and master and working seeds, clearly demonstrates the potential of this application. In addition, quantitative analysis of mutants by MassArray could be used for monitoring genetic stability of viruses during clinical trials of live vaccines against various diseases for epidemiological surveillance of new virus isolates and for screening of emerging drug-resistant viral strains in the course of antiviral therapies used to control HIV, hepatitis C, influenza, and any other viruses and natural isolates, which in many cases are also mixed or quasispecies rather than unique sequences.

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