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Sequence analysis of fusion, small hydrophobic, and hemagglutinin-neuraminidase genes and biological characteristics of Mumps Virus obtained from patients with vaccine associated illness

Takeshi TAKAMI*, Takayuki MORI**, Masaharu NAKAGAWA**, Tetsuo NAKAYAMA**

*Department of Pediatrics, Tokyo Medical University, 6-7-1 Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan.

**The Department of Virology, The Kitasato Institute, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8642, Japan.

SUMMARY

We have investigated the relationship between biological characteristics and the nucleotide sequences of the fusion (F), small hydrophobic (SH), and hemagglutinin-neuraminidase (HN) genes of the Hoshino vaccine related strains. No nucleotide change was observed after five passages in Vero cells or after eight passages in chick-embryo cells. After five passages in HeLa cells, one nucleotide changed with amino acid (AA) substitution from Ala to Asp at the 521 AA position and this change was preserved after 18 passages in HeLa cells. Among eight clinical isolates from patients with vaccine-associated parotitis or aseptic meningitis, 6 of 8 (75%) showed nucleotide changes at different positions in those genes. Four of them showed the same changes from Ser to Ile at the 65 AA position of the HN gene. One had two AA changes from Gln to Arg at the 159 and from Met to Leu at the 366 positions in the F gene, and the other one had a nucleotide change in the 3′-end of the F non-coding region. There were no nucleotide changes of the SH gene in any of the cases. The vaccine seed strain had small plaques and all vaccine-related isolates had large plaques. However they demonstrated similar characteristics of virus growth at different temperatures, 33°C and 40°C. The results suggest that the nucleotide changes in F, SH and HN genes observed in vaccine associated clinical isolates had no direct relationship with their biological characteristics. Further analysis in the phosphoprotein (P), large protein (L), and nucleocapsid protein (N) is necessary.

INTRODUCTION

The mumps virus belongs to the family of Paramyxoviridae, genus Rubulavirus, which is a single stranded negative sense RNA virus consisting of seven component proteins, nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), small hydrophobic (SH), hemagglutinin-neuraminidase (HN) and large (L) proteins[1]. Mumps virus particles have two surface envelop proteins of F and HN which play a critical role in the process of infection. The virus infection is initiated at the cellular surface membrane with attachment of virus

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Reprint requests to : Takeshi Takami, M.D., Department of Pediatrics, Tokyo Medical University 6-7-1, Nishishinjuku, Shinjuku-ku, Tokyo, Japan 160-0023

HN protein to the virus receptors. The virus F protein induces fusion with cell membranes in association with HN protein and then the virus genome penetrates into host cells[2, 3]. Putative SH protein was found in infected cells but not in the particles. The sequence of nucleotides in the SH gene was more heterogeneous than in the other regions[4, 5]. SH protein is now considered to have an activity to promote virus assembly and budding. These three component proteins have crucial structural proteins, and mutations in these regions may affect the biological activity of the mumps virus.

Mumps virus infects glandular tissues including the salivary glands, testicles, ovaries, pancreas and the central nervous system and causes parotitis, pancreatitis, ependymitis, deafness and aseptic meningitis. In Japan, live attenuated monovalent mumps vaccines were licensed in 1981 and measles-mumps-rubella (MMR) trivalent vaccines were introduced in 1989. Although the MMR vaccine has clinical benefits, it was discontinued because of an unexpectedly high incidence of neurologic complications of aseptic meningitis[6]. Approximately one in 1000–2000 recipients developed aseptic meningitis after MMR vaccination in Japan. However, the reason for the high incidence of vaccine-associated complication after immunization of mumps vaccine is still unknown. The Hoshino vaccine strain was established through plaque purification method at 33°C by Sasaki[7]. The biological differentiation of vaccine strains from wild strains depended upon the plaque size or different virus growth at 33°C or 40°C. We previously reported that several vaccine strains isolated from patients with vaccine-associated illness produced large plaques and that vaccine related strains did not always demonstrate small plaques[8].

In order to know whether vaccine strains would mutate during virus replication in host human cells, we investigated the nucleotide sequences of the F, SH and HN protein regions among the Hoshino vaccine strains which were obtained from patients with complications after vaccination. In addition, we investigated the vaccine strains propagated in different cell lines. We also examined plaque size and virus growth to compare the biological characteristics.

MATERIALS AND METHODS

Materials

This study consisted of six Hoshino vaccine strains with different passage histories. KO3-0 was a Hoshino vaccine seed strain and KO3-8CH was propagated after eight passages in chick embryo cell cultures in the same manner the procedure for vaccine production. KO3-1HL, KO3-5HL and KO3-18HL were propagated in HeLa cell cultures after the first, fifth, and 18th passage, respectively. KO3-5Vero was also propagated after the fifth passage in Vero cell cultures. Six samples (AP-1, AP-2, AP-3, AP-4, AP-5, AP-6) were obtained from nasopharyngeal swab (NPS) of patients with acute parotitis and two samples (AM-1, AM-2) were obtained from cerebrospinal fluid (CSF) of patients with aseptic meningitis within 4 weeks after vaccination with the Hoshino strain. Virus isolation was performed using Vero cells. Briefly, 0.1 ml of NPS or CSF was inoculated on monolyer of Vero cell culture of 24 well plate in duplicate. Cytopathic effects were observed within two passages in above 8 samples and they were identified as mumps virus by neutralization test with hyperimmune rabbit serum against mumps virus. Virus isolation and passage were done in 5%CO_2 atmosphere at 37°C. Clinical isolates were used within five passages. These eight clinical isolates produced large plaques and they were identified as the Hoshino vaccine strains by our method of RFLP in the HN gene[9], which was reported for the genetic differentiation of the Hoshino vaccine strain from wild strains of the mumps virus in the part of the HN gene. Seven wild strains were used for the control of biological activity.

PCR and Nucleotide sequencing

Total RNA was extracted from 200 μl of NPS, CSF and from virus cell culture fluids, as reported by Chomczynski et al [10]. The RNA pellet was resuspended in 20μl of sterile distilled water and 5μl of RNA was applied for cDNA synthesis. Genomic RNA was reverse-transcribed to cDNA with AMV reverse transcriptase (Life Sciences Inc. St Petersburg, FL, USA), using a positive sense primer located at the 3′ end of the M gene for F gene analysis and using positive sense primer located at the 3′ end of the F genes for SH and HN sequencing. PCR was done using a set of primers which were designed to produce approximately 600

nucleotides and to analyze the full span of the F, SH and HN genes. The -21M13 forward type universal primer sequence aligment was attached at the 5′ end of mumps virus specific sequence. The first three cycles were at 90°C and 56°C each for 1 min, and then 73°C for 1.5 min. They were followed by 35 cycles (at 94°C for 45 sec. at 58°C for 1 min. and at 73°C for 2 mins) with a final additional extension period of 5 mins at 73°C, using DNA thermal cycler (PCR Thermal Cycler MP, TaKaRa Co. Ltd., Tokyo, Japan), as previously reported[9]. PCR products were electrophorased through 1% agarose gel (Low melting, FMC Bioproducts Corp., Rockland, ME) and were then excised from the gel. Direct sequencing was carried out, using a Taq Dye Primer Cycle Sequencing Kit (Applied Biosystems Japan Inc., Tokyo, Japan), and was analyzed with an automated nucleotide analyzer, the 373A DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Plaque size assay

A monolayer of Vero cells was inoculated with 0.5 ml of culture fluids diluted serially by 1 : 10 in 60-mm plastic plates. An overlay of 5 ml of MEM supplemented with 2% calf serum and 0.5% agarose was added. After 7 days of incubation at 37°C in 5% CO_2, the overlay agarose was removed and the plate was incubated with rabbit antiserum to mumps virus. The plate was stained with biotinylated anti-rabbit IgG immunoglobulin, using Vectrastain Elite ABC Kit (Vector Laboratories, Inc., Burlingame, CA, USA).

Virus growth

Virus samples were inoculated in monolayer of Vero cells in 6-well plates, and incubated in 5% CO_2 at 33°C or 40°C. Culture fluids were obtained on days 1, 3, 5 and 7 of culture. Infectivity was calculated by the Reed-Muench method in 48-well plates of Vero cells culture at 37°C, depending upon the appearance of CPE.

RESULTS

Hoshino vaccine strains

Table 1 shows the nucleotide changes among the Hoshino vaccine strains which were propagated through different cell passages. No nucleotide change was demonstrated among KO3-8CH strain (eight passages in chick embryo cell culture), KO3-5Vero (five passages in Vero cell cultures), and KO3-1HL (first passage in HeLa cell) in comparison with seed strain (KO3-0). KO3-5HL (after five passages in HeLa cells) had a nucleotide change with amino acid substitution from Ala to Asp at the amino acid position of 521 of HN gene. This change was preserved after 18 passages in HeLa cells.

Table 1 Changes of the nucleotide sequences of Hoshino vaccine strain

Strain	Change
KO3-8CH	—
KO3-5Vero	—
KO3-1HL	—
KO3-5HL	HN521 (Ala ⇨ Asp)
KO3-18HL	HN521 (Ala ⇨ Asp)

Clinical isolates

Table 2 shows the changes of nucleotide sequences of clinical isolates from patients with complications after vaccination in comparison with the seed strain (KO3-0). Strains AP-1 and AM-2 had the same sequence in the F, SH, and HN regions as the seed strain (KO3-0). Strain AP-2 had a nucleotide change at the 3′ end of F non-coding from AAA<u>CTAAATT</u> to AAT<u>CTAAATT</u>. The underlined sequence of CTAAATT is the intergenic sequence between the F and SH genes. Strains AP-3, AP-4, AP-5, and AP-6 had a common amino acid change from Ser to Ile (HN65) in the coding region. Strain AM-1 had two amino acid substitutions from Gln to Arg (F159) and from Met to Leu (F366).

Plaque size test

The results of the plaque size test are shown in Figure 1. KO3-0 produced small plaques (< 1.0 mm of diameter) and KO3-8CH produced mainly small plaques mixed with medium plaques. Otherwise KO3-1HL, KO3-18HL and clinical isolates (AM-1, AM-2, AP-1, AP-2) had large plaques, which were larger than 2 mm at diameter. The remaining AP-3, AP-4, AP-5, and AP-6 showed also large plaques (data not shown).

Virus growth

The virus growth was examined and the results of the virus growth of the vaccine related strains of AP-6 is shown in panel Vac. Those of one wild strain are also shown in the panel Wild in Figure 2. The vaccine related strains (the seed strain and all the clinical iso-

Table 2 Changes of the nucleotide sequences of post-vaccination clinical isolates

AP-1 (Parotitis)	—
AP-2 (Parotitis)	F-AAACTAAATT-SH ⇨ F-AATCTAAATT-SH
AP-3 (Parotitis)	HN65(Ser⇨Ile)
AP-4 (Parotitis)	HN65(Ser⇨Ile)
AP-5 (Parotitis)	HN65(Ser⇨Ile)
AP-6 (Parotitis)	HN65(Ser⇨Ile)
AM-2 (Meningitis)	—
AM-1 (Meningitis)	F159(Gln⇨Arg), F366(Met⇨Leu)

lates) grew more efficiently at 33°C culture than at 40°C. Six of seven wild strains grew faster at 40°C than at 33°C, but one strain demonstrated similar characteristics to the vaccine related strains. Vaccine related strains had the same temperature-sensitive (*ts*) characteristics as the vaccine seed strain.

DISCUSSION

Measles-mumps-rubella (MMR) trivalent vaccine containing the live attenuated Jeryl Lynn strain of mumps virus is given to children in many developed countries. The incidence of complications in the central nervous system, following the Urabe strain vaccines was higher than that observed after vaccination with Jeryl Lynn strain[11]. Forsey et all[12] demonstrated neurologic complications after MMR vaccine, caused by Urabe vaccine strains, by using PCR and nucleotide sequencing in parts of the F region. There were several reports on the discrimination of vaccine strains from wild strains of mumps virus[13~15]. There is a great clinical significance in distinguishing whether the complications were caused by vaccine strains or by concomitant infection with circulating wild strains. We developed a simple method to distinguish the Hoshino vaccine strain from wild strains by restriction fragment length polymorphism (RFLP) in the HN gene[9]. MMR vaccines containing the Hoshino strain was introduced but discontinued for the same reason of neurologic complications in 1993. Since

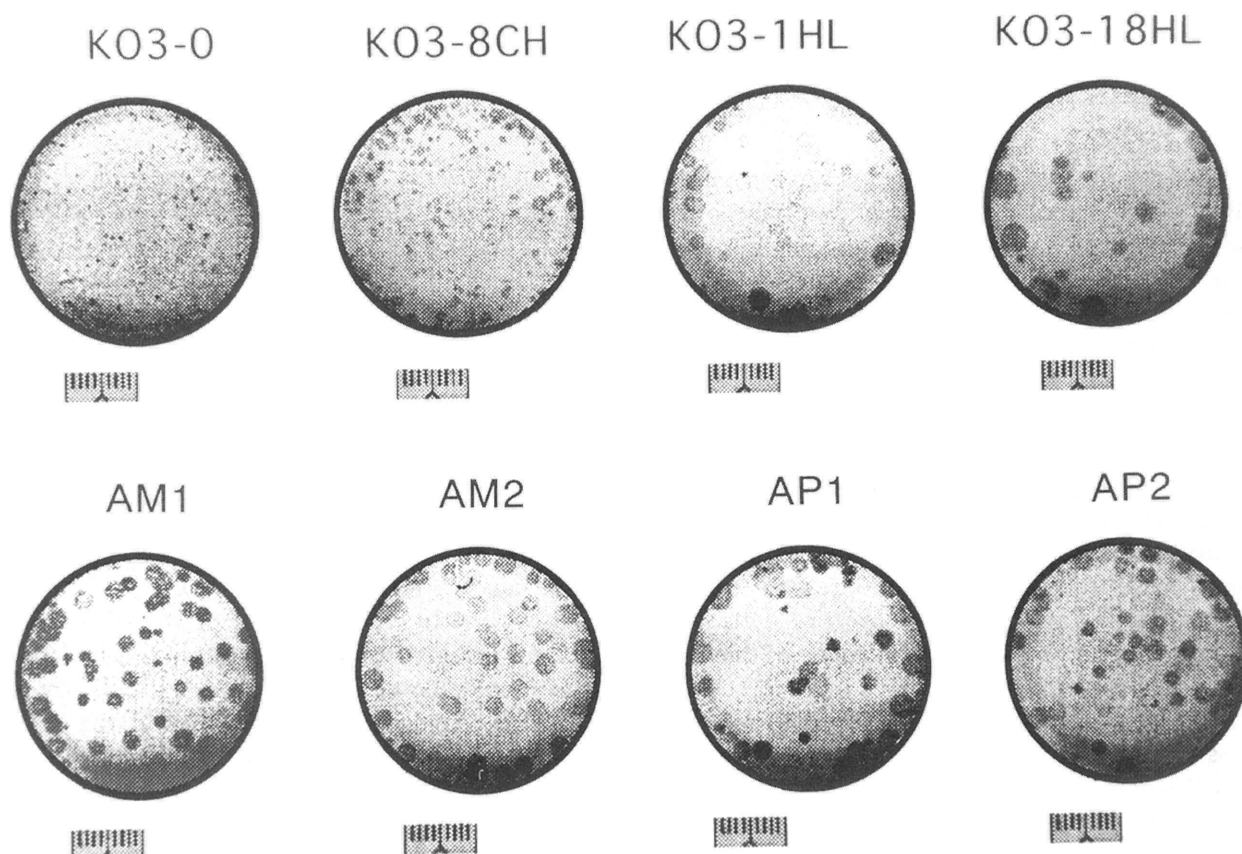

Fig. 1 The results of plaque size test. Small plaques are smaller than 1.0 mm in diameter. Large plaques are larger than 2 mm in diameter. Actual measurements are gruen in the figures.

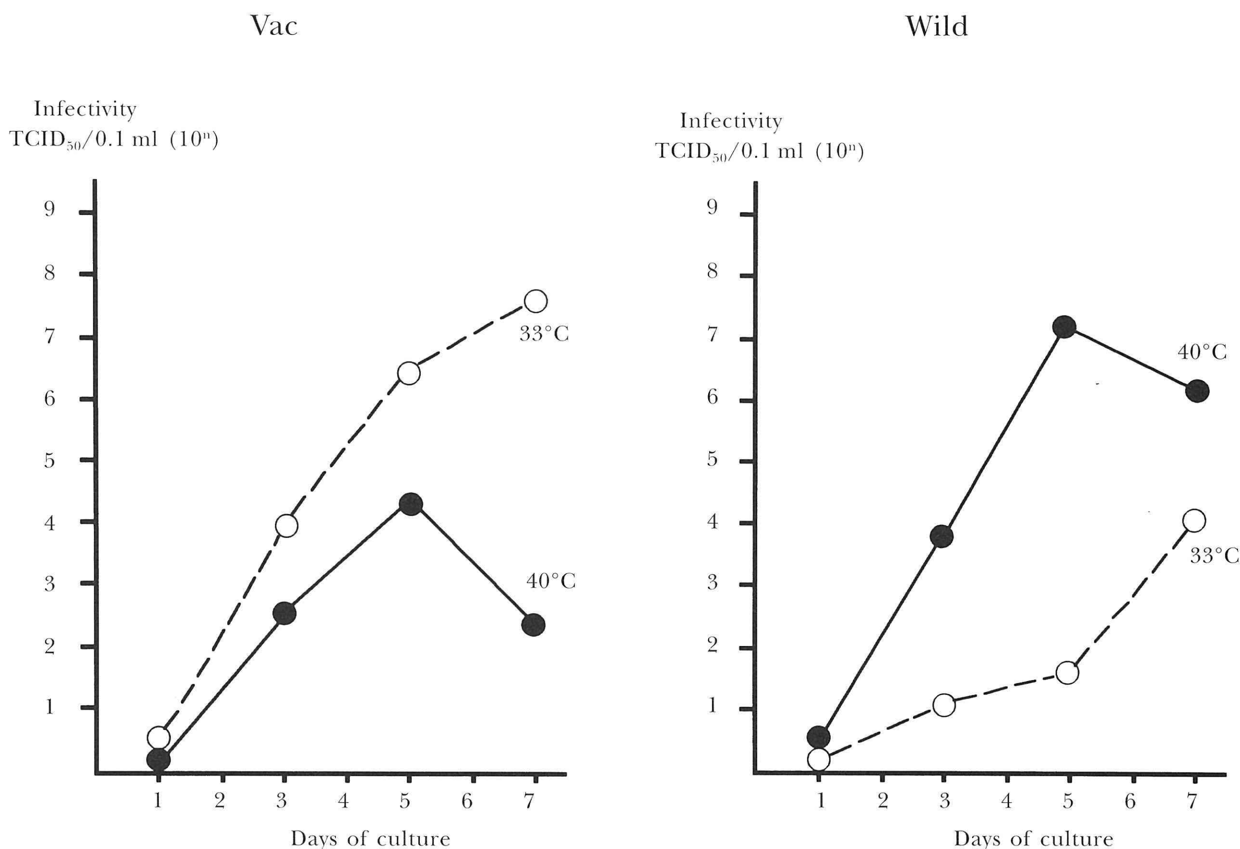

Fig. 2 The results of virus growth. The left side graph shows the infectivity of the vaccine related strain of AP-6 strain. The right side graph shows the infectivity of one wild strain. The experiment was done in triplicate and the mean infectivity titers are shown.

1993, mumps monovalent vaccines have been used in Japan.

In this study we have confirmed the nucleotide changes of the Hoshino strains, which were propagated in HeLa cell cultures different from vaccine manufacturing procedure until the 18 th passage. Mutations at different positions were observed in six strains isolated from patients with complications after immunization with the Hoshino strain, but not in the other two isolated strains. There may be some possibility that these changes took place during the isolation process in Vero cells. But this possibility is unlikely because of lack of mutation through five passages in Vero cells in the F, SH, and HN genes. We suppose that these nucleotide changes in clinical isolates were induced during the virus replication of the vaccine strain in recipients. Alternatively vaccine seed strains might have a mixed population like the Jeryl Lynn strain[16].

Brown et al[17] showed that post-vaccination isolates produced a coding change with an amino acid change from Glu^{335} (GAA) to Lys^{335} (AAA) caused by one point substitution of G to A at the 1081 in the HN region. They also examined the virus growth, but the growth difference in Vero cells could not be explained solely by the nucleotide substitution at 1081 HN genome position. Mori et al[18] reported that the Urabe strain has a mixed population and this change is not related to the pathogenicity of complications. Our sequencing data also shows no common nucleotide changes in the F, SH, and HN region among vaccine related strains. Although the four isolates from vaccine associated cases with parotitis (AP-3, AP-4, AP-5, AP-6) showed the same change of nucleotide sequence at the 65th amino acid in the HN region, the change at this position was not observed in other strains (AP-1, AP-2, AM-1, AM-2). Furthermore, the sequencing results of clinical isolates from Hoshino vaccine associated cases with compli-

cation have no common AA change, compared with Japanese wild strains since 1976[19]. Therefore the mutation might occur at random.

Current mumps vaccine strains were attenuated empirically by laboratory passage in chick embryonic cells from wild strain virus. The Hoshino vaccine strain was propagated by plaque purification at 33°C, selecting a small plaque[7]. Therefore, vaccine related strains are believed to make small plaques and to have the same temperature sensitivity (*ts*). But we still have no knowledge about the mechanism of attenuation of the vaccine strains genetically. We already showed that clinical isolates from vaccine associated cases with a small plaque size were identified as vaccine strains by sequencing in the part of the P genes, but some with large plaque sizes were also verified as vaccine strains[8]. These results showed that mumps vaccine strains might acquire different biologic activities in vivo. In this study, clinical samples and some of the vaccine strains propagated in cell cultures had large plaques. However, they maintained similar characteristics of virus growth at 33 and 40°C to the vaccine seed strain. We supposed the strains with large plaques would show rapid growth rates, but this was not the case. Further research will be required to identify the contributing factors to plaque formation such as functional protein genes, e, g, P and L proteins.

We confirmed the mutations in F, SH, and HN regions among the Hoshino vaccine strains obtained from vaccine associated illness. However, these mutations were not consistently observed and we suppose random mutations. Amino acid substitution from Ser to Ile at the HN 65 position was observed commonly in 4 strains. However these mutations were not directly related to the different plaque sizes. Furthermore 2 strains, which had no mutations, also demonstrated large plaques and all isolates showed the same virus growth pattern. We examined only F, SH, and HN regions in this study, and more sequence data on different regions are required for the understanding of the pathogenesis of vaccine-associated illness.

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ワクチン接種副反応例より得られたムンプスウイルスにおける F，SH，HN 遺伝子の塩基配列と生物学的活性の検討

高見　剛[1]　森　孝之[2]　中川正治[2]
中山哲夫[2]

[1]東京医科大学小児科学教室，[2]北里研究所ウイルス１室

要旨：ムンプス星野ワクチン株接種後に副反応を発症した症例から得られた臨床分離株と，ワクチン製造以外の細胞で継代したワクチンウイルス株について，遺伝子の変異と生物学的活性とを比較検討した．鶏胎児胚細胞と Vero 細胞で５代継代した株では核酸の変異は認められなかったが，HeLa 細胞で５代継代培養したものでは，HN 領域の 521 番目にアラニンからアスパラギン酸へのアミノ酸レベルでの変異が認められ，18 代まで保存されていた．耳下腺炎および無菌性髄膜炎を発症した８株の臨床分離株のうち６株（75%）で核酸の変異を認め，うち４株では HN 領域の 65 番目にセリンからイソロイシンへの共通変異を認めた．また，１株で F 領域の 159 番目でグルタミンからアルギニンへ，366 番目でメチオニンからロイシンへの変異が認められ，他の１株で F3' 非翻訳領域の１ヶ所に核酸の変異を認めた．SH 領域では遺伝子の変異は認められなかった．星野ワクチンシード株はスモールプラックを示し，臨床分離株はすべてラージプラックを示したが，33°C と 40°C の異なった温度下でのウイルス増殖パターンはワクチンシード株と臨床分離株は同一の特徴を示した．今回の結果より，F, SH, HN 領域でムンプスウイルスワクチンが変異していることが確認できたが，生物学的活性との関連は認められなかった．今後は更に，P, L および N 領域での検討が必要と思われた．

〈キーワード〉ムンプスウイルス，PCR，副反応，塩基配列，プラックサイズテスト