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Detection of genes of RNA viruses from freshly biopsied gastric mucosa by reverse transcription polymerase chain reaction

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Abstract: The reverse transcription polymerase chain reaction (RT-PCR) was performed to detect genes of RNA viruses in the freshly biopsied gastric mucosa of seven patients with low gastric acidity. Although nucleoprotein genes of Sendai virus and hemmaglutinin genes of influenza virus A were not detected, nucleoprotein genes of influenza virus B were detected in samples from three of the seven patients. The first patient had had antrectomy and vagotomy for gastric ulcer, the second patient was receiving a histamine type 2 receptor blocker for gastritis, and the third patient was receiving a proton pump inhibitor for gastric ulcer. Virus isolation from gastric mucosa and from gargles was negative for all seven patients. These findings suggest that genes of influenza viruses may exist in the gastric mucosa of patients with low gastric acidity.

Key words: persistent infection, gastric acid, orthomyxovirus, paramyxovirus

Introduction

Viral genes in gastric tissues may be detected by polymerase chain reaction (PCR) and in situ hybridization. Genes of the Epstein-Barr virus (EBV), a DNA virus, have been detected in gastric tissues, and a causal relationship between gastric cancer and EBV has been postulated.[1–4] The detection of genes of RNA envelope viruses, however, has seldom been reported, probably because RNA is more unstable than DNA and envelope viruses are likely to lose infectivity in the gastric acid. Sendai virus is a negative sense RNA envelope virus, and persistent infection may occur in the respiratory tract. Influenza viruses are envelope viruses of spherical, bacillary, or filamentous morphology[5] and have a segmented negative sense RNA genome. Two kinds of glycoprotein spikes, hemagglutinin (HA) and neuraminidase (NA), are inserted in the envelopes of influenza viruses. Type A and type B influenza viruses can cause minor respiratory illness, gastritis,[6] fatal pneumonia, encephalitis,[7] and myocarditis. In experimental animals, the gastrointestinal tract is a possible site of influenza virus replication.[8] We performed revense transcription (RT)-PCR to detect the genes of these RNA envelope viruses from the freshly biopsied gastric mucosa of patients with low gastric acidity.

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Patients and methods

Clinical specimens

Informed consent was obtained from the patients and approval was gained from the Ethics committee. Specimens of gastric mucosa were obtained endoscopically from seven patients with gastritis or peptic ulcer disease. Five patients were receiving a histamine type 2 receptor blocker (cimetidine) and two, a proton pump inhibitor (omeprazole) (Table 1). None of the seven patients had presented with flu-like syndromes, such as fever, sore throat, myalgia, and arthralgia within 3 months prior to or at the time of the endoscopic examinaion.

Detecion of influenza virus gene from the gastric mucosa and gargles by RT-PCR

The biopsied gastric mucosa sample was homogenized and extraction of RNA was performed based on a previously described method,[9] with modifications. Ethanol-precipitated RNA was dissolved with 100 μl of

Table 1. Summary of patients' background

Patient	Age/Sex (years)	Diagnosis	Biopsy site	Medication	RT-PCR[a]
1[b]	70/F	GU	C	H2B	−/+/−
2	74/M	GI	C	H2B	−/+/−
3	78/F	GU	C	PPI	−/+/−
4	57/F	DU	C	PPI	−/−/−
5	30/F	GI	A	H2B	−/−/−
6	38/M	GI	C	H2B	−/−/−
7	56/M	GI	C	H2B	−/−/−

GU, Gastric ulcer; GI, gastritis; DU, duodenal ulcer; C, corpus of the stomach; A, antrum of the stomach; F, female; M, male; H2B, histamine type 2 receptor antagonist (cimetidine); PPI, proton pump inhibitor (omeprazole); RT-PCR, reverse transcription-polymerase chain reaction
[a] Influenza A/influenza B/Sendai virus: +, virus genes detected; −, virus genes not detected
[b] This patient underwent antrectomy and vagotomy 25 years before the study

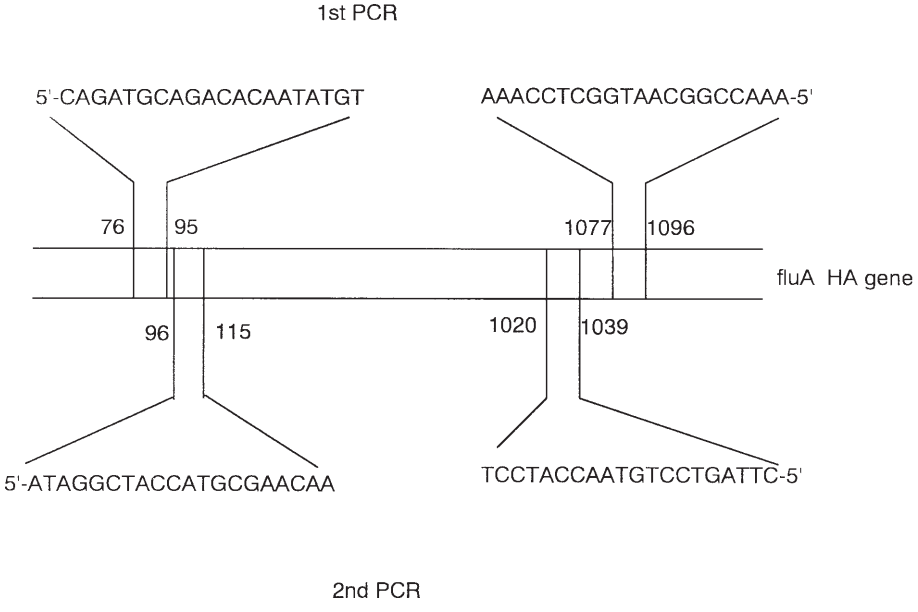

Fig. 1. Polymerase chain reaction (PCR) primers for influenza virus A hemagglutinin (*HA*) gene. The nucleotide sequences are based on previously reported data (Gene Bank accession number J02143).[10] First PCR product, 1021 bp; second PCR product, 944 bp

double-distilled water (DDW). Twenty microliters of RNA suspension was mixed with 42 μl of DDW and 15 μl of 150 mM Tris HCl, pH 8.3, before denaturing was performed at 90°C for 3 min. Eight microliters of 25 mM deoxynucleoside triphosphate (dNTP), 8 μl of 10× Mg,K solution (100 mM $MgCl_2$, 1 M KCl), 2 μl of 80 mM dithiothreitol, 3 μl of random primers (100 pmol/μl hexadeoxyribonucleotide mixture; TaKaRa, Kyoto, Japan), and 2 μl of reverse transcriptase derived from Moloney murine leukemia virus (Wako, Tokyo, Japan) were added and incubated at 42°C for 1 h. Synthesized DNA was extracted by the phenol-chlorform method.[9] After ethanol precipitation, DNA was dissolved with 50 μl of DDW. Nested PCR was performed to improve the specifity of the reaction. The primers (20 pmol/μl) for nested PCR are summarized in Figs. 1, 2, and 3.[10–12] The HA genes of influenza A/WSN (H1N1) and influenza A/Aichi/2/68 (H3N2), extracted from infected Madin-Darby Canine Kidney (MDCK) cells, were successfully amplified, using primers of type A influenza virus HA gene (data not shown). In the first PCR, the touchdown method was utilized to amplify the target squence more effectively and specifically.[13] Briefly, a mixture of 76 μl DDW, 10 μl 10× PCR buffer, 10 μl 2.5 mM dNTP, 1 μl TaKaRa Taq (TaKaRa), 1 μl sense primer, 1 μl antisense primer and 1 μl template DNA was overlayed with one drop of mineral oil. After heating at 95°C for 30 s, there were two cycles of denaturing at 94°C for 1 min, annealing at 60°C for 30 s, and elongation at 72°C for 2 min, performed on a DNA Thermal Cycler (Perkin Elmer-Cetus, Norwalk, CONN, USA). The annealing temperature was subsequently decreased

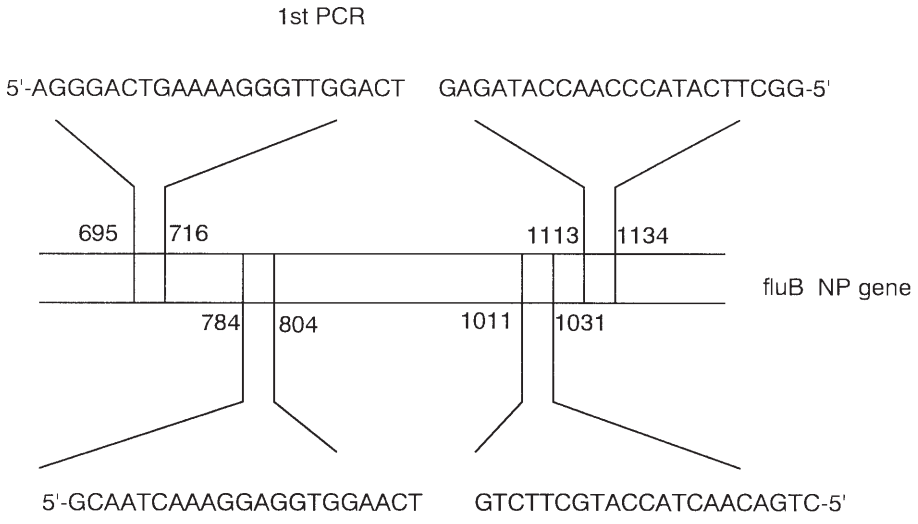

Fig. 2. PCR primers for influenza virus B nucleoprotein (*NP*) gene. The nucleotide sequences are based on previously reported data (Gene Bank accession number K01395).[11] First PCR product, 440 bp; second PCR product, 248 bp

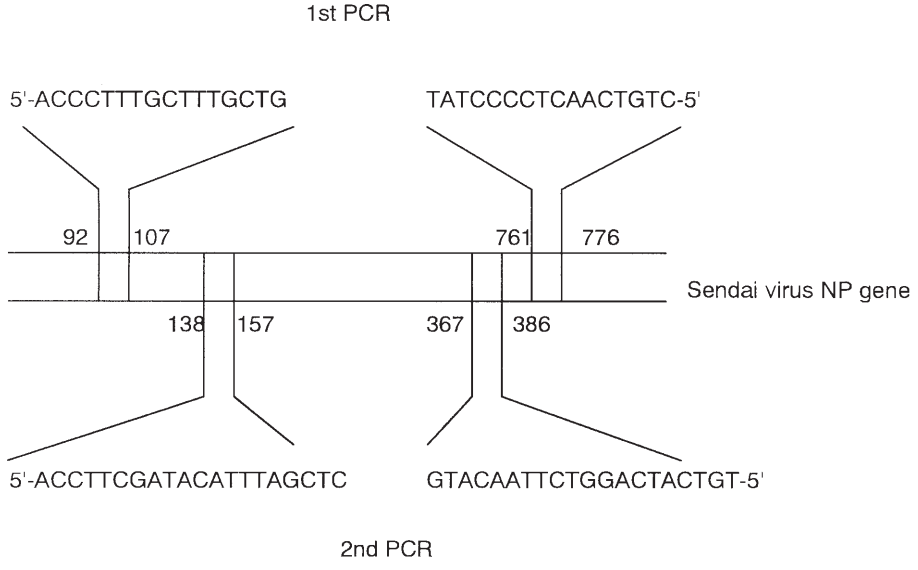

Fig. 3. PCR primers for Sendai virus nucleoprotein (*NP*) gene. The nucleotide sequences are based on previously reported data (Gene Bank accession number M30204).[12] First PCR product, 685 bp; second PCR product, 249 bp

by 1°C every other cycle and finally, 15 cycles of annealing were performed at 50°C.

The second PCR was performed with 76 μl DDW, 10 μl 10 × PCR buffer, 10 μl 2.5 mM dNTP, 1 μl TaKaRa Taq, 1 μl sense primer, 1 μl antisense primer, and 1 μl of the first PCR products. After heating at 95°C for 30 s, we performed 25 cycles of denaturing at 94°C for 1 min, annealing at 50°C for 30 s, and elongation at 72°C for 2 min. As positive controls, DNA templates synthesized from RNA extracted from MDCK cells infected with influenza virus A/Aichi/2/68, or influenza virus B/Lee/40, and from Vero cells infected with the Fushimi strain of Sendai virus were used.

Gargles with phosphate-buffered saline (PBS) + bovine albumin (BA) were filtered through Millipore filters and RT-PCR was performed as above.

Isolation of influenza viruses from gastric mucosa and gargles

The biopsied gastric mucosa sample was homogenized in PBS supplemented with BA (PBS + BA), filtered through a Acrodisc (Gelman Sciences, MI, USA) and directly innoculated onto preformed confluent MDCK cell monolayers. After 30 min at room temperature, an agar medium containing 4 μg/ml acetyl trypsin (Sigma)

was added,[14,15] and the dishes were incubated at 34°C for 4 days. Gargles were added to PBS + BA and serial dilutions were innoculated onto MDCK monolayers and treated as above.

Sequencing of the NP gene of type B influenza virus

DNA amplified by PCR, extracted from agarose gel with a gel extraction kit (QIATEXII; Qiagen, Hiden, Germany), was then cloned in a pCR-Script Direct SK(+) Directional Cloning Kit (Stratagene, La Jolla, CA, USA). The nucleotide sequence was determined by the dye terminator method on an automated DNA sequencer (Model 373A; Applied Biosystems, Forster City, CA, USA).

Serological examination

The patients were phlebotomized at the time of endoscopy, and antibody titers to influenza A/Yamagata/32/89 (H1N1), influenza A/Wuhan/359/95 (H3N2), and influenza B/Mie/1/93 were examined by hemagglutination inhibition (HI) tests.

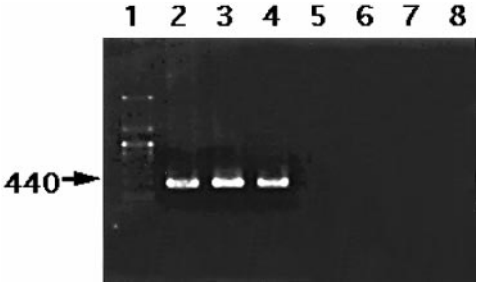

Fig. 4. First PCR of influenza virus B nucleoprotein gene. The RNA was extracted from a freshly biopsied gastric mucosal sample, and nested reverse transcription (RT)-PCR was performed as described in the text. *Lanes 1–7*, Patients 1–7; *lane 8*, molecular weight marker. The expected first PCR product was 440 bp

Results

Detection of influenza virus gene from gastric mucosa and gargles by RT-PCR

The HA gene of influenza virus A and the NP gene of Sendai virus were not detected in any patients (data not shown). However, the NP gene of influenza B was clearly detected by nested RT-PCR from the gastric mucosa in three of the seven patients, indicating the presence of the influenza virus genome in the gastric mucosa (Figs 4 and 6). No virus genes were detected in gargle fluid by RT-PCR.

Sequencing of the NP gene of type B influenza virus

Partial DNA sequences of the NP gene of type B influenza virus detected from the gastric mucosa of patient 1 were identical to those of influenza B/Lee/40, except for six point mutations (Fig. 5).

Virus isolation from gastric mucosa and gargles

Influenza virus isolation from the gastric mucosa and gargles by direct plaquing in MDCK cells was negative in all seven patients (data not shown).

Serological examination

There was no elevation of HI titers to influenza A/Yamagata/32/89 (H1N1), influenza A/Wuhan/359-95 (H3N2), and influenza B/Mie/1/93 in any of the seven patients.

Discussion

We performed RT-PCR of freshly biopsied gastric mucosal samples to avoid degradation of viral RNAs. Although the HA gene of influenza virus A and the

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                740         750         760         770         780
          TCAGTACTTT TGCCGGAAGC ACACTACCCA GAAGATCAGG TACAACTGGT
                                                    (C)
      790         800         810         820         830         840
GTTGCAATCA AAGGAGGTGG AACTTTAGTG GATGAAGCCA TCCGATTTAT AGGAAGAGCA
                                    (CA)        (T)
      850         860         870         880         890         900
ATGGCAGACA GAGGGCTACT GAGAGACATC AAGGCCAAGA CGGCCTATGA AAAGATTCTT
                                                (A)
      910         920         930         940         950         960
CTGAATCTGA AAAACAAGTG CTCTGCGCCG CAACAAAAGG CTCTAGTTGA TCAAGTGATC
                              (C)
      970         980         990        1000
GGAAGTAGGA ACCCAGGGAT TGCAGACATA GAAGACCTAA
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Fig. 5. Partial sequencing of the nucleoprotein (NP) gene of type B influenza virus detected from the gastric mucosa of patient 1. The NP gene of the reference strain (influenza virus B/Lee/40), positive sense, is shown. Nucleotide sequences of type B influenza virus detected from the gastric mucosa of patient 1 which differ from the reference strain are shown in *parentheses*

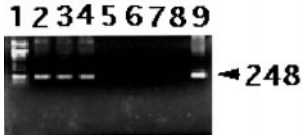

Fig. 6. Second PCR of influenza virus B nucleoprotein gene. The second PCR was performed with the first products as templates. *Lanes 1–7*, Patients 1–7; *lane 8*, molecular weight marker; *lane 9*, Madin-Darby Canine Kidney (MDCK) cells infected with influenza virus B/Lee/40. The expected second PCR product was 248 bp

NP gene of Sendai virus were not detected, the NP gene of influenza B was clearly detected in three of the seven patients we tested. Influenza virus isolation and RT-PCR from the gargles were negative for all seven patients, rulng out the possibilly of contamination of gastric biopsy specimens with viruses in the respiratory tract. Virus isolation from the gastric mucosa and serum antibody titers to influenza virus were also negative, suggesting that few influenza virus particles were produced, or that virus genes were replicating without forming particles. In cultured cells, influenza virus genes can persist without virus particle formation.[16] Type A influenza viruses infect humans, horses, birds, swine, and seals.[17] Reassortment of genes can cause an antigenic shift, leading to an influenza virus infection pandemic.[18] Type B influenza viruses infect only humans[19] and the reservoir in the interepidemic period has yet to be identified. The results of our experiments may reflect the possible persistence of virus genes in gastric mucosa, in which the genes are amplified to a extent that they are not diluted by the turnover of gastric epithelial cells.

To determine whether the gastric mucosal infection with influenza viruses is acute or persistent, we will perform gastric biopsies 3–6 months after the first examination. It is interesting to note that all three patients with influenza B-positive gastric mucosa had low gastric acid. The first patient had received antrectomy and vagotomy for gastric ulcer, the second patient was receiving cimetidine for superficial gastritis, and the third, omeprazole for gastric ulcer. Influenza B viruses have envelopes, composed of a lipid bilayer inseted with glycoprotein spikes, and these are thought to be degraded by gastric acid. Although only three of our seven patients with low gastric acidity had gastric influenza virus infection, low gastric acidity may be a predisposing factor for influenza virus infection of the stomach. It is important to study the detection rate of influenza B virus infection in control subjects who have not received acid suppression therapy. Our finding that virus isolation from the throat and gastric mucosa was negative suggests that influenza virus genes may be present in the gastric mucosa of patients with low gastric acidity.

Although virus induction from gastric mucosa in which influenza genes exist has yet to be proved, it does seem that gastric mucosa may be a site of type B influenza virus infection. Influenza viruses cause lysis and apoptosis of infected cells.[20] Gastrointestinal symptoms are more common manifestations of influenza B than of influenza A virus infection.[21] To elucidate the clinical significance of the presence of influenza B virus in the gastric mucosa, we will perform gastric biopsies in more patients with various types of gastric disorders.

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