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DETECTION OF INFLUENZA B VIRUS IN THROAT SWABS USING THE POLYMERASE CHAIN REACTION

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Summary. - An assay protocol based on exploiting the polymerase chain reaction (PCR) for the direct detection of influenza B virus in throat swabs is described. By the use of PCR with nested primers, it was possible to detect the virus in throat swabs. Dilution experiments showed that as little as 1 plaque forming unit of virus was sufficient for detecting the HA gene by the PCR. All throat swab samples from which influenza B virus had been isolated by conventional methods were also positive by the PCR method.

Key words: PCR; influenza B virus; throat swabs; diagnosis

Direct detection of influenza virus in respiratory secretions by immunofluorescent staining (Daisy *et al.*, 1979) or ELISA (Berg *et al.*, 1980; Coorod *et al.*, 1988) has proved inefficient, because of low sensitivity of false-positive results. The polymerase chain reaction (PCR) provides a technique which allows the nucleic acids of pathogens in any sample to be specifically amplified by up to 10^6-fold prior to attempting detection (Saiki *et al.*, 1985). The obvious potential of this new technique in the field of viral diagnosis has already resulted in its application to several virus system, especially for DNA viruses or subgenomic DNA (Kwok *et al.*, 1987; Sibata *et al.*, 1988; Jarrett *et al.*, 1990).

Recently, we have reported a PCR method for the direct detection of influenza A virus genomes in throat swabs taken from patients with influenza-like symptoms (Yamada *et al.*, 1991). In the present study, we used the PCR method for the detection of influenza B virus in throat swabs, with the application of nested primers giving a higher sensitivity than we reported previously for influenza A virus.

Two pairs of oligonucleotide primers were synthesized using an Applied Biosystems 381A synthesizer (Foster City, Calif.). The base sequences for the primers were those that were conserved between B/Great Lakes/54, B/Singapore/222/79, and B/Yamagata/16/88 (Kanegae *et al.*, 1990). For the initial round of the PCR, primers IB-1 and IB-2 which amplify 496 base pairs were used. For the second round of the PCR, primers IB-8 and IB-3 which amplify an

Table 1. Influenza B virus primer sequences (5'→3')

IB-1 : GCAAAAGCTTCAATACTCCAC
IB-2 : CGCTTTGTGGTAGCCCTCCGT

IB-3 : TTGGAACCTCAGGATCTTGCC
IB-8 : GTGGTAGCCCTCCGTCTTCTG

internal fragment of 315 base pairs were used. The nucleotide sequences of these primers are shown in Table 1.

To extract the RNA from throat swabs or chorioallantoic fluid (CAF), 50 μl of throat swab or CAF was mixed with 50 μl of 10 mmol/l Tris-HCl (pH 7.5), 1 mmol/l EDTA, 400 μl of RNA extraction buffer (4.2 mol/l guanidine thiocyanate, 25 mmol/l Tris-HCl, pH 8, and 0.5 % Sarcosyl), and 25.2 μl of 2-mercaptoethanol. Then 50 μl of 10X phenol extraction buffer (1 mol/l Tris-HCl, pH 8, 100 mmol/l EDTA, and 10 % SDS) was added.

After the addition of 500 μl of phenol to each sample, incubation was carried out at 65 °C for 30 min with occasional mixing. After extraction with phenol and chloroform twice, the RNA was precipitated in 1 ml of isopropanol and 2.5 mmol/l NH_4OAc, and then resuspended in 10 μl of distilled water. For cDNA synthesis, 9 μl of the RNA was mixed with 20 μl of the amplification buffer (50 mmol/l KCl, 10 mmol/l Tris-HCl, pH 8.4, 2.5 mmol/l $MgCl_2$, and 0.02 % gelatine), 1 mmol/l of dNTP, 2 units of RNase inhibitor (Takara), 50 pmol of oligonucleotide (primer IB-1), and 1 μl of avian myeloblastosis virus (AMV) reverse transcriptase (Life Sciences, 28 units/μl). Incubation was performed at 43 °C for 60 min. After incubation, the reaction mixture was added to 61.5 μl of distilled water, and then incubated at 95 °C for 5 min (cDNA solution).

Amplification of the cDNA of influenza B virus HA gene was performed as follows. The reaction mixture (99 μl) contained 10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 2.5 mmol/l $MgCl_2$, 0.02 % gelatine, 20 pmol each of the primers (primer IB-1, primer IB-2), 2.5 units of Taq DNA polymerase (Perkin-Elmer Cetus), and 81.5 μl of c-DNA solution. Amplification was performed by incubation at 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 3 min for 25 cycles in a DNA thermal cycler (Coy's Laboratory, U.S.A.). One-tenth of the amplified reaction mixture was applied to 1.5 % agarose gel containing 0.5 μg/ml ethidium bromide. When the first amplified product was negative for ethidium bromide staining, an aliquot (10 μl) of the reaction mixture was subject to another 25 cycles of amplification under the same conditions with freshly added dNTP, Taq polymerase, and primers (primer IB-3 and primer IB-8). After the PCR mixture was applied to an agarose gel, products of 495 or 314 base pairs could theoretically be visualized under ultravilet light following the first or second amplification procedures, respectively.

The specificity of the primers was examined using different types of influenza viruses. One-tenth diluted CAFs of the viruses were used as the starting materials. When A/PR/8/34 (H1N1), A/USSR/92/77 (H1N1), A/Yamagata/120/86 (H1N1), A/Okuda/57 (H2N2), A/Sichuan/2/88 (H3N2), and A/Osaka/126/90 (H3N2) viruses were used, no amplified bands were detected by agarose gel electrophoresis (Table 2). On the other hand, when 3 strains isolated during 1979–1990 were examined, visible bands corresponding to 495 base pairs were detected on the agarose gel. Base sequence analysis of 50 nucleotides of the 495 base band was performed by the dideoxy chain termination method after elution of the DNA from the gel silica slurry (Takara Shuzo Co., Ltd., Kyoto, Japan), and this band was confirmed to be the HA gene of influenza B virus (data not shown).

To estimate the sensitivity of detection of the influenza B virus HA gene by PCR the method, we diluted CAFs of B/USSR/100/83 ($2x10^7$ PFU/ml) virus

Table 2. Specificity of the primers used to detect the influenza B virus HA sequence

Virus strain	Specific band	
Influenza A (H2N2)	A/Okuda/57	-[1]
Influenza A (H1N1)	A/PR/8/34	-
	A/USSR/92/77	-
	A/England/333/80	-
	A/Yamagata/120/86	-
Influenza A (H3N2)	A/Kumamoto/22/76	-
	A/Sichuan/2/88	-
	A/Osaka/126/90	-
Influenza B	B/Singapore/222/79	+[2]
	B/USSR/100/83	+
	B/Osaka/238/90	+

[1] No visible band was detected by ethidium bromide staining after agarose gel electrophoresis.
[2] A 496 base pair band was visible after the first PCR round.

and isolated viral RNA from each diluted sample. Amplified cDNA was detected by gel analysis of the first PCR preparation when a 50 μl of aliquot a $1 : 10^3$ CAF dilution was used (Fig. 1-(1)). On the other hand, amplified cDNA was detected after the second PCR procedure when a $1 : 10^6$ CAF dilution was used (Fig. 1-(2)). On the basis of our dilution studies, the PCR method appears to be able to detect the influenza virus HA gene at infective titres, 20 PFU/ml (1 PFU/50 μl). It has been reported that the throat swabs obtained from influenza-like patients contain infective titres of 10^5 $TCID_{50}$/ml (Dolin *et al.*, 1976).

The sensitivity of the PCR method in detecting the influenza virus HA gene in 14 specimens obtained from influenza-like patients was compared with that of viral isolation using MDCK cells or embryonated chicken eggs. All clinical samples were collected by the Osaka Prefectural Institute for Public Health from patients with influenza-like illnesses in the winter of 1989/90 (epidemic of B/Yamagata/16/88-like virus in Japan). Samples were stored at -20 °C. The results obtained when the assay protocol was used to screen the 14 throat swabs are shown in Table 3, with 9 of the 14 being positive for influenza B virus. A typical example of the results obtained with this method is shown in Fig. 2. Specimens from which influenza B virus was isolated by the conventional methods were also PCR-positive. We did not find any specimens which were positive by the PCR method, but were negative by viral isolation. These results showed that the sensitivity of the PCR method for diagnosing influenza virus infection is similar to that of isolation of the virus from throat swabs.

The use of the PCR for the amplification of influenza virus DNA in throat swabs offers several advantages over the standard isolation techniques. Only a very minute specimen (50 μl) is required for this analysis. Also, the amplification reaction and detection procedures can be performed in 24 hr, compared

with the time of more than 2–4 days required for tissue culture or embryonated egg isolation of the virus from patiens. In addition, infective virus is not necessary for a positive result by the PCR method. Many of the throat swabs used in this experiment had been stored for over 1 year at -20 °C after the procedure of virus isolation and had undergone repeated freezing and thawing. Despite these storage conditions, viral RNA could still act as an adequate cDNA template. In contrast, Havlíčková *et al.* (1990) reported that the rate of molecular hybridization using a ^{32}P-labelled DNA probe decreased in specimens which had undergone repeated thawing and prolonged storage at -20 °C. The discrepancy with our results seems to come from differences of the sensitivity of the systems used.

In conclusion, application of PCR-based detection to the influenza B system

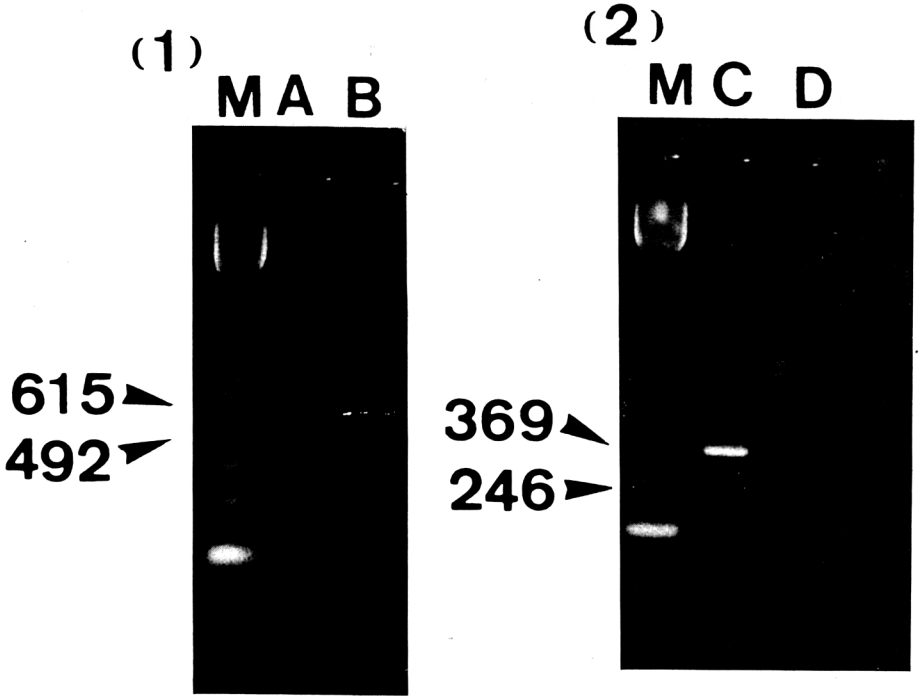

Fig. 1

Sensitivity of HA gene detection by the PCR method

B/USSR/100/83 ($2x10^7$ PFU/ml) was diluted in TE buffer (10 mmol/l Tris-HCl, pH 7.5, and 1 mmol/l EDTA) and viral RNA was extracted as described in the text. Amplified DNA was detected by agarose gel electrophoresis and staining with ethidium bromide following the 1st PCR (1) and the 2nd PCR (2). Lane M, ladder of DNA size markers (BRL): lane A, ($1x10^3$ PFU/50 μl): lane B and lane C (1 PFU/50 μl): lane D, ($1x10^{-3}$ PFU/50 μl).

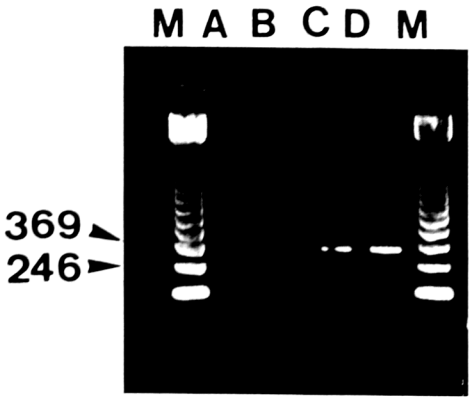

Fig. 2
Typical results from sreening throat swabs using the PCR method
The PCR assay was carried out as described in *Materials and Methods.* Track M, ladder of DNA size markers (BRL); track A, positive control B/USSR/100/83; tracks B, C, D specimens from throat swabs. From B and C, influenza B virus was isolated, while influenza A (H3N2) virus was isolated from D using MDCK cells.

should allow very large increases in sensitivity and automation. It may also give us new information about diseases to which influenza B virus is thought to be related, such as Reye's syndrome.

Table 3. Comparison of detection of influenza B virus in throat swabs by the PCR method and viral isolation using conventional methods

Sample No.	Detection of HA gene[1] by the PCR method	Viral isolation with[2] MDCK	egg
1	1	1	1
2	2	1	n.d.
3	2	2	n.d.
4	2	1	n.d.
5	2	2	n.d.
6	2	2	1
7	2	2	2
8	2	2	2
9	2	2	3
10	-	-	-
11	-	-	-
12	-	-	-
13	-	-	-
14	-	-	n.d.

[1] Numbers 1 and 2 indicate detection of the HA gene product by the first or second PCR, respectively; - indicates a negative result after the second PCR.
[2] Numbers 1, 2 and 3 indicate the passage numbers at which the virus was isolated; - indicates no isolation after the third passage; n.d., not done.

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