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Type-specific identification of influenza viruses A, B and C by the polymerase chain reaction

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Summary

The aim of this study was to develop a polymerase chain reaction for specific detection of influenza A, B, and C RNA genomes. Three primer sets were selected from conserved regions of the genome coding for the non-structural proteins and were tested on 61 influenza A (22 H1N1, 9 H2N2, and 30 H3N2), 11 influenza B, and three influenza C isolates. Specific amplified products were obtained with all these strains after electrophoresis on a 2% agarose gel. The specificity of the reaction was increased by hybridization with oligonucleotide probes. When nucleic acids from a variety of micro-organisms from the respiratory tract were subjected to the PCR with these primers, no specific amplified products were generated. The sensitivity of the technique was found to be at the subpicogram level. The RNA-PCR was applied to 21 clinical specimens from patients with a culture/IF proven influenza infection. Six influenza A positive patients and 13 influenza B positive patients could be confirmed in the RNA-PCR. In two cases, influenza B positive IF specimens were found negative by the PCR. No virus could be isolated on eggs or tissue culture from these samples. RNA-PCR is a specific and sensitive technique for the detection of influenza virus genomes.

Influenza; Non-structural; PCR; Clinical specimen; Diagnosis

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Introduction

Influenza viruses contain a segmented, single stranded (−) RNA genome. The genus is divided in three types, A, B, and C, based on specific epitopes on the internal nucleoprotein and the matrix protein. Human influenza A virus strains are further classified in the subtypes H1N1, H3N2 and the presently not circulating H2N2 subtype, based on the antigenic differences of the hemagglutinin (H) and the neuraminidase (N) surface proteins.

Laboratory diagnosis of infections with influenza virus is mainly performed by culturing a respiratory sample, nasopharyngeal aspirates or swabs, for 16–24 h, with, subsequently, an immunofluorescent analysis of the propagated virus or by direct immunofluorescence (Espy et al., 1986; Stokes et al., 1988). Recently, the polymerase chain reaction (PCR) has been used for detection of numerous pathogens and the technique appeared to be very sensitive and specific (Wright and Wynford-Thomas, 1990). Amplification of influenza RNA genome segments by RNA-PCR has also been carried out. The technique was used for obtaining, rapidly, nucleotide sequence information (Katz et al., 1990; Robertson et al., 1990; Ludwig et al., 1991) and a nested PCR was used for typing and subtyping of cultured influenza strains (Zhang and Evans, 1991). In addition, some applications to clinical specimens are described. Influenza A hemagglutinin can be amplified from nasopharyngeal lavages (Bressoud et al., 1990) and throat swabs (Yamada et al., 1991). However, the genome segments coding for the H and N surface glycoproteins show a great variability in their RNA nucleotide sequences and are, therefore, not suitable for the development of a type specific PCR for influenza A. Other segments of the influenza A genome are much more conserved in their nucleotide sequences. In influenza B and C strains the segmented genomes contain conserved segments as well (Air and Laver, 1990; Lamb, 1989).

In the present study, the polymerase chain reaction was used for development of a specific RNA-PCR for influenza virus types, which may be used for diagnostic purposes. The specificity and sensitivity of the selected primer sets were evaluated using influenza strains of all (sub)types and a variety of other respiratory micro-organisms. In addition, the RNA-PCR was used for clinical specimens.

Material and Methods

Virus strains and clinical specimens

The influenza A and B strains used were collected at the National Influenza Center, Erasmus University, Rotterdam, The Netherlands. The isolates had been propagated on embryonated hen's eggs and allantoic fluids were stored after freeze drying. The influenza C strains (in allantoic fluid) were kindly provided by Dr. U. Desselberger, East Birmingham Hospital, Birmingham,

UK. Sixty-one influenza A isolates (22 H1N1, 9 H2N2, and 30 H3N2) with all recent reference strains, 11 influenza B, and three influenza C isolates were analyzed. All isolates of bacterial and viral respiratory tract organisms were obtained from the clinical bacteriology (Prof. M. Michel) and clinical virology departments from the University Hospital, Rotterdam, except for the isolates of rhinoviruses and enteroviruses, which were kindly provided by Dr. A. Dumas, Municipal Health Services, Rotterdam and Dr. W. Melchers, University Hospital, Nijmegen, respectively.

The clinical specimens were obtained from the PAMM Foundation, Eindhoven (Dr. R. Diepersloot), the University Hospital, Rotterdam, and the Sophia Pediatric Hospital, Rotterdam. These samples had been found positive for influenza by the culture/IF method, i.e., immunofluorescence with monoclonal antibodies (Imagen, Novo Nordisc) after culturing the sample for 16–24 h. The specimens, 11 nasopharyngeal aspirates and 10 swabs, had been stored at −70°C in Dulbecco's minimal essential medium (Flow Laboratories, Irvine, UK) containing antibiotics and 1 vol of sucrose for 3 months at most. After mixing well, 50μl of this solution was used for PCR analysis.

Nucleic acid extractions

Freeze-dried isolates were resuspended in water and then propagated by growing them for 5 days on Madin-Darby canine kidney cells (Madin and Darby, 1958). Processing of the cultured influenza strains was carried out at 20 min intervals in a laminar flow cabinet (Clean Air, Woerden, The Netherlands). After mixing vigorously the culture flask, 1 ml of solution was centrifuged at 15000 × *g* for 10 min. RNA was extracted by a modification of the method described by Chomzcynski and Sacchi, (1987). After washing with phosphate buffered saline (PBS), the pellet was resuspended in 0.4 ml of guanidinium thiocyanate (GTC) solution which contained 4 M of GTC, 25 mM sodium citrate, pH 7, 0.5% sarcosyl and 0.1 M β-mercaptoethanol. Then 0.3 ml phenol (ultra-pure) and 0.3 ml phenol-chloroform (phenol/chloroform/isoamylalcohol 25:24:1) were added. After shaking gently, the mixture was centrifuged at 12000 × *g* for 20 min. With this GTC-phenol-chloroform (GPC) extraction the nucleic acids are recovered in the aqueous phase. The aqueous phase was transferred to a new tube and was again extracted with phenol, phenol-chloroform and chloroform, respectively. The nucleic acids were recovered by ethanol precipitation (Sambrook et al., 1989).

cDNA reaction

For RNA-PCR, an in-vitro DNA amplification was carried out on cDNA generated from the influenza RNA genome. Preparation of cDNA was carried out with approx. 50 ng of RNA in a 25 μl volume containing 50 mM Tris-hydrochloride, pH 8.3; 75 mM KCl; 3 mM $MgCl_2$; 10 mM DTT; 0.5 mM (each) dATP, dCTP, dGTP, and dTTP; 10 pmol of cDNA-primer; 20 U

RNAsin (Promega, Leiden, The Netherlands); and 100 U MMLV reverse transcriptase (Gibco-BRL Breda, The Netherlands). First, the target RNA was mixed with the cDNA primer and placed at 80°C. After 2 min the solution was placed on ice and completed with the rest of the cDNA reaction mix. Incubation was at 42°C for 45 min. Finally, the reaction mixture was placed at 95°C for 3 min and kept on ice until the PCR was carried out. The cDNA reaction and the PCR were carried out using a strict protocol, with precautions to prevent contamination (Kwok and Higuchi, 1989). Distilled water and MDCK RNA were included as controls.

Polymerase chain reaction

PCR was carried out using the 25 μl reaction mixture from the cDNA reaction. PCR mix was made up to a volume of 100 μl, which made a reaction mix containing 50 mM Tris-hydrochloride, pH 9; 50 mM KCl; 1.5 mM $MgCl_2$; 0.01% gelatin; 0.1% Triton-X-100; 0.2 mM (each) dATP, dCTP, dGTP, dTTP; 20 pmol of cDNA primer and 20 pmol of the reverse primer (Table 3); and 2 U Taq DNA polymerase (Promega, Leiden, The Netherlands). Three drops of mineral oil were added to prevent evaporation. The amplification was carried out in 40 cycles in a PCR processor (Biomed, Ditfurth, Germany). Each cycle consisted of denaturation at 92°C for 1 min, annealing of the primers at 50°C for 2 min, and chain elongation at 74°C for 2 min.

Analysis of the amplified products

After the last cycle of amplification, 20 μl of the amplified products was analyzed by electrophoresis on a 2% agarose gel. The gel was then soaked for 20 min in a denaturation solution (0.5 N NaOH/1.5 M NaCl), neutralized for 15 min in 3 M NaAc, pH 4.8. Subsequently, the amplified products were transferred to a nylon membrane (Hybond N+, Amersham, Bucks, UK) by diffusion blotting in 10 × SSC (150 mM sodium citrate and 1.5 M NaCl) for at least 3 h (Sambrook et al., 1989).

(Pre)hybridization was carried out at 37°C in 5 × SSC (75 mM sodium citrate and 750 mM NaCl), 5 × Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), 75 mM EDTA, and 0.1 mg denatured herring sperm DNA per ml. Hybridization was performed over night using 10^6 counts per ml of a γ^{32}P-labeled oligonucleotide probe, directed against internal sequences of the amplified products. The blots were washed in 2 × SSC-0.1% SDS for 15 min, twice at 37°C and once at 55°C. Autoradiography was performed on Fuji RX 100 X-ray films.

Plaque assay

MDCK cells were grown to 10^7 cells/ml in 6-well, 35 mm clusters (Costar, Cambridge, USA). The monolayers were rinsed twice with phosphate buffered

saline (PBS, pH 7.4). The virus stocks were diluted in infection medium i.e., minimum essential medium with Hepes (Flow, Irvine, UK) with 20 mM L-glutamine, 0.225% sodium bicarbonate and 6 μg/ml trypsin. The monolayers were infected with 2 ml of the virus dilutions at 35°C in 5% CO_2 for 1 h. The monolayer was rinsed twice with PBS and subsequently 2 ml of overlay medium was added, i.e. minimum essential medium with glutamine (Life Technologies, Paisley, UK) and supplemented with 20 mM Hepes, 0.225% sodium bicarbonate, 6 μg/ml trypsin and 0.7% agarose. After incubation at 35°C in 5% CO_2 for 2–3 days, the monolayer was stained with 0.01% Neutral Red (Flow, Irvine, UK) in PBS.

Results

Selection of the primers

Comparisons (Queen and Korn, 1984) between the known sequences from homologous segments of influenza A and B viruses revealed that genus-specific primers could not be selected. However, type specific primers were easily found. The nucleotide sequence of segment 8 of influenza A viruses, coding for the non-structural (NS) proteins, was found to be very well conserved (Buonagurio et al., 1986a). This segment was used to select type-specific influenza A primers for the PCR (Table 1). Primers for influenza B and C were also selected from the sequences of the genome segments coding for the NS proteins (Table 1), which are segment 8 and 7, respectively (Yamashita et al., 1988; Buonagurio et al., 1986b).

TABLE 1

Sequences of the primers and probes for the detection of influenza A, B and C
The nucleotide numbers are according to the references. The length of the amplified products is indicated in the last column.

Primer	Nucleotide	Sequence	bp
A		(Buonagurio et al., 1986a)	
cDNA	467–486	AAGGGCTTTCACCGAAGAGG	
rev	637–656	CCCATTCTCATTACTGCTTC	190
probe	564–593	GTCCTCATCGGAGGACTTGAATGGAATGAT	
B		(Yamashita et al., 1988)	
cDNA	746–755	ATGGCCATCGGATCCTCAAC	
rev	967–986	TGTCAGCTATTATGGAGCTG	241
probe	820–849	CCAATTTGGTCAAGAGCACCGATTATCACC	
C		(Buonagurio et al., 1986b)	
cDNA	312–331	GCTCCAAGCAACATAGCACC	
rev	967–986	TCGGTAGCCATAACGAATCC	326
probe	820–849	ATCCTTCACGTATGAGATTGAGCTATGCAT	

Specificity of the primers

Twenty-two influenza A/H1N1, nine influenza A/H2N2, 30 influenza A/H3N2, 11 influenza B, and three influenza C strains were tested by the RNA-PCR method, using the selected primer sets. The products were analyzed by electrophoresis on a 2% agarose gel. All influenza A strains generated a specific amplified product of 190 bp. The primers selected for influenza B produced amplified products of 241 bp in an RNA-PCR on RNA of the 11 influenza B strains (Fig. 1). With the influenza C primers specific 326 bp products are generated with influenza C virus RNA. For an optimal specificity and sensitivity of the PCR, all results were confirmed by southern blot hybridization with an oligonucleotide probe, directed against the internal part of the amplified sequences (Fig. 1).

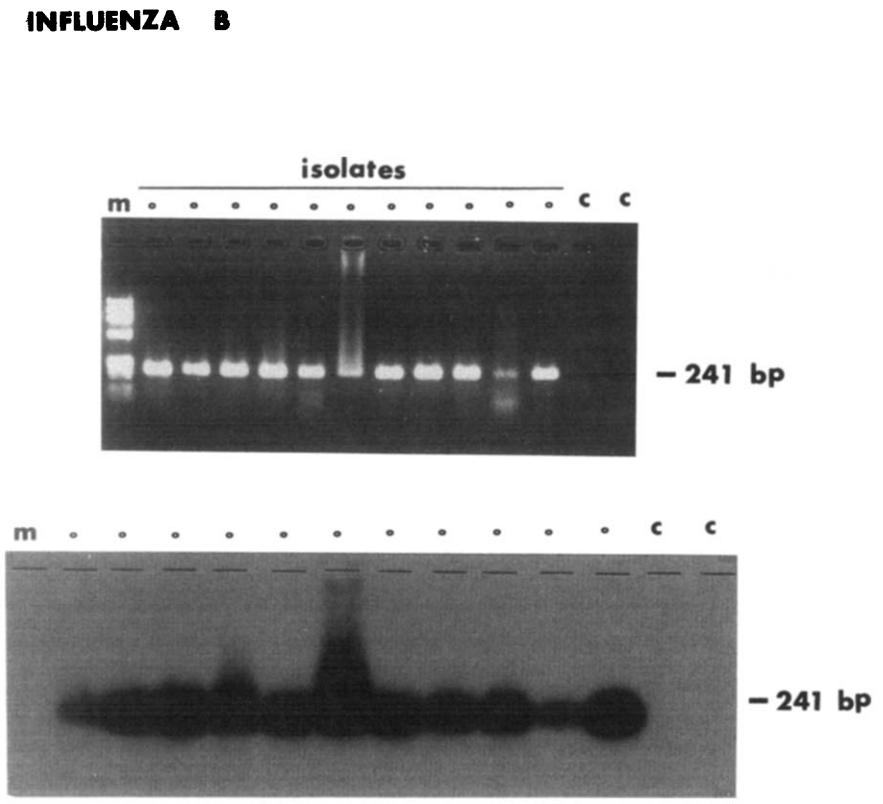

Fig. 1. RNA-PCR on 11 influenza B strains. The PCR products are electrophoresed on 2% agarose and subsequently analyzed by Southern blot hybridization. Specific 241-bp amplified products are generated with RNA from all strains. m: marker (PhiX174 RF DNA/*Hae*III), c: MDCK RNA isolates.

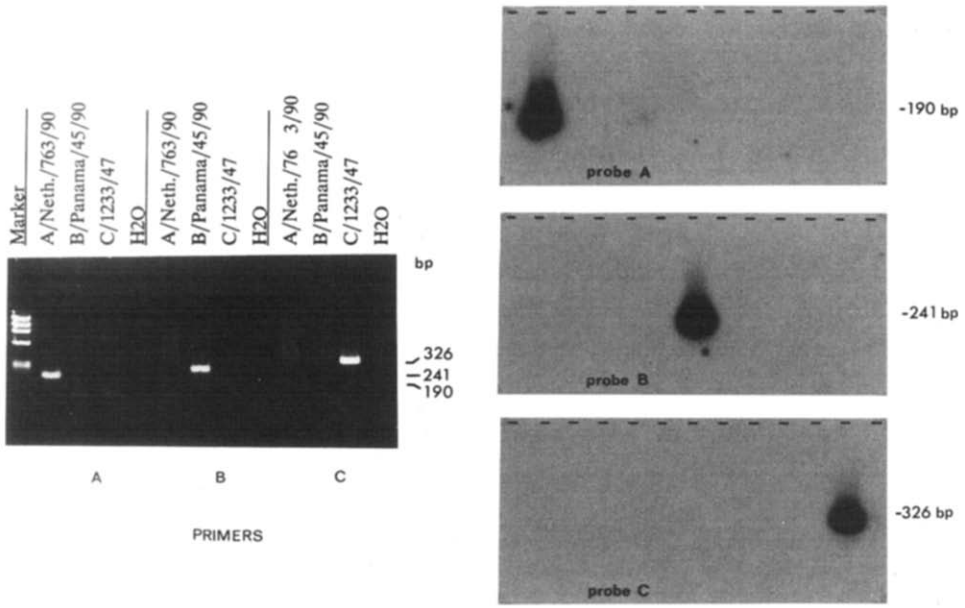

Fig. 2. RNA-PCR with primers for influenza virus A, B, and C on RNA from influenza A/Netherlands/763/90, influenza B/Panama/45/90, and influenza C/1233/47. Analysis of the PCR products on 2% agarose is shown on the left side, whereas the Southern blot hybridization, with, subsequently, probe A, B, and C, is shown at the right side. Between the hybridizations the blot was dehybridized in 0.4 N NaOH.

The cross reactivity of the influenza primers was also tested. Fig. 2 shows the results from amplifications with the three primer sets on RNA from the three different influenza types. As shown, specific signals were obtained only with the homologous type of virus. The oligonucleotide probes used for confirmation of the results in southern blot hybridization were also type-specific.

The specificity of the three primer sets was tested further on isolates of a variety of respiratory micro-organisms (Table 2). Viral isolates were propagated on cultured cells and bacterial strains were grown on agar plates. Extraction of DNA/RNA from these organisms was by GPC extraction, as described. An aliquot of 5–10 ng of the extracted nucleic acids was subjected to the RNA-PCR. Specific amplification products were not detected.

Sensitivity

The sensitivity of the RNA-PCR for influenza genomes was established in two ways. Purified influenza virus was obtained by sucrose-gradient centrifugation. The purified fraction was pelleted and resuspended in guanidinium thiocyanate. The RNA was extracted, as described (GPC), and the amount of RNA was determined by ultraviolet spectrophotometry at 260

TABLE 2

Respiratory micro-organisms used for testing the specificity of the influenza primers

Viral	Bacterial and others
Parainfluenza 1, 2 and 3	*Haemophilus influenzae*
RSV	*Streptococcus pneumoniae*
Adenovirus	*Staphylococcus aureus*
Rhinovirus	*Branhamella catharralis*
Coxsackie A	*Bordetella pertussis*
ECHO	*Klebsiella pneumoniae*
Enterovirus	*Legionella pneumophilia*
Coronavirus	*Mycobacterium* sp.
CMV	*Corynebacterium diphteria*
EBV	*Mycoplasma pneumoniae*
HSV 1	*Escherichia coli*
Measles virus	*Chlamydia trachomatis*
	Chlamydia pneumoniae
	Chlamydia psittaci
	Enterobacter sp.
	Candida albicans

nm (Sambrook et al., 1989). Dilution series of these RNA solutions were subjected to the PCR. Dilutions containing as little as 10–100 fg of RNA were found positive in the RNA-PCR.

Another way to establish the sensitivity (Yamada et al., 1991) was carried out by comparing the RNA-PCR with the plaque test assay, using 10-fold serial dilutions of two strains of each influenza virus type. In Fig. 3 the results for two B strains are shown, but they were in the same order of magnitude for the influenza A strains. All plaque forming dilutions and the first dilution which did not raise any plaques were found positive in the RNA-PCR. A plaque test for the influenza C strains was not carried out.

Clinical specimens

Twenty-one clinical specimens from the 1990/1991 season, which were found positive in the culture/IF method were subjected to the RNA-PCR. Nucleic acids were extracted from 50 μl of the clinical specimens in transport medium and an equivalent of 12.5 μl was used for each reaction. Two reactions were carried out for each specimen, one with influenza A primers and one with influenza B primers. Typical results from nine specimens are shown in Fig. 4. From 19 samples the culture/IF results were confirmed by the RNA-PCR. Six influenza A isolates (typed as A/Singapore/6/86 like) and 13 influenza B isolates (2 B/Beijing/ 1/87 and 1i B/Panama/45/90 like) were identified. Two samples were influenza B positive in culture/IF but with PCR influenza RNA was not detected. In addition, influenza virus could not be propagated from these specimens on eggs or MDCK cells.

The same results were obtained by heating 25 μl of the clinical sample for 7 min at 95°C, cooling it on ice for 5 min, and subsequently using 5 μl of the

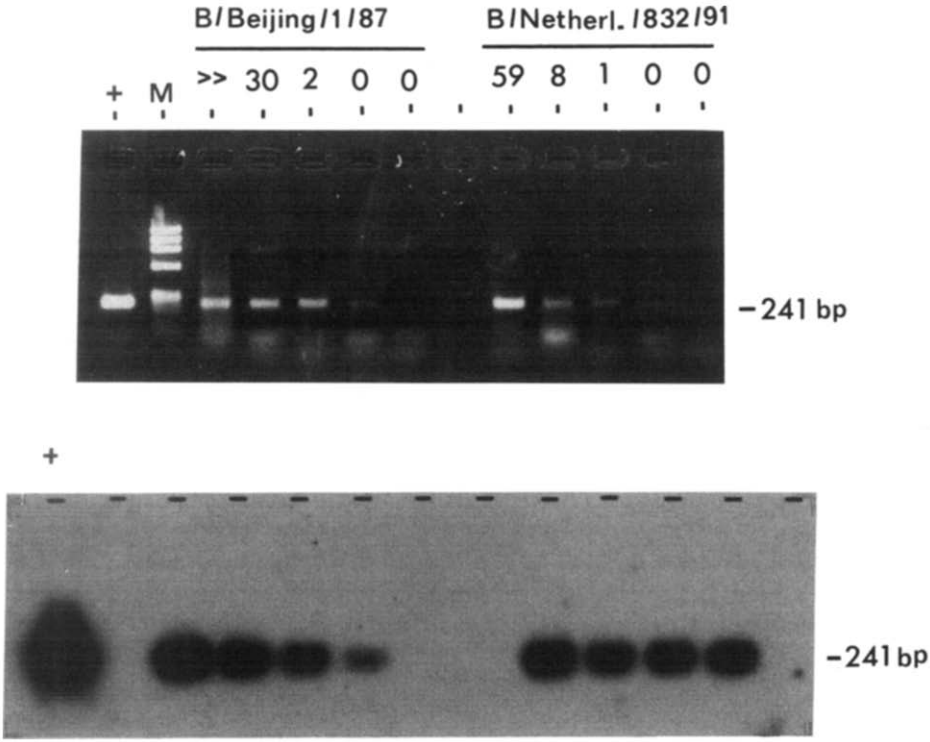

Fig. 3. Ten-fold serial dilutions of influenza B/Beijing/1/87 and B/Netherlands/832/91 subjected to RNA-PCR with the type B primers. The dilutions also were used in a plaque test assay and yielded the number of plaques indicated. +, B/Beijing/1/87 RNA; M, marker (PhiX1174 RF DNA/*Hae*III); > >, no separate plaques.

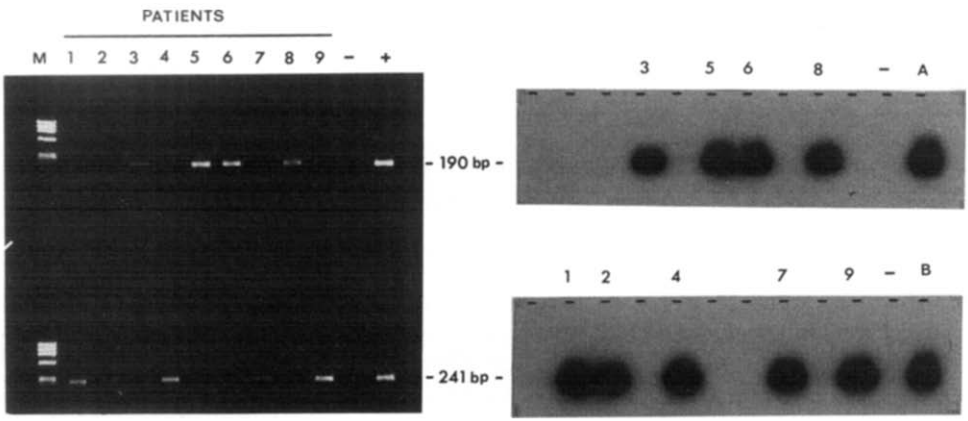

Fig. 4. RNA isolations from clinical specimens of nine patients, subjected to the RNA-PCR with influenza A and B primers. Positive signals are seen on the gel and are confirmed after Southern blot hybridization with oligonucleotide probes. M, marker (PhiX1174 RF DNA/*Hae*III); −, H_2O; +, positive control (purified RNA).

denatured sample directly in the cDNA reaction (data not shown). The laborious extraction of nucleic acids can, therefore, be omitted.

Discussion

PCR analysis of influenza virus genomes was carried out by several groups. Direct sequencing of the PCR products generated from the hemagglutinin genome segment provides information about genetic drift and evolutionary relationships (Katz et al., 1990; Rajamukar et al., 1990; Robertson et al., 1990; Ludwig et al., 1991). Apart from epidemiological studies, PCR can also be used for subtyping influenza isolates or for diagnostic purposes (Bressoud et al., 1990; Yamada et al., 1991; Zhang and Evans, 1991).

In this study the specificity and sensitivity of the PCR for detection of influenza genomes was determined. By comparison of the nucleotide sequences three type-specific primer sets were selected which could detect influenza A, B or C genomes, respectively (Table 1). The specificity of the primer sets was tested on a number of influenza isolates, representing a selection from influenza A and B strains from 1934–1990, including the relevant reference strains. Specific amplified products were generated from all isolates. Only three human influenza C isolates were tested but, in addition, three isolates from pigs generated the specific 326 bp amplified products as well, which shows the conservation of the selected sequences. Because of several passages through eggs and/or MDCK cells the strains used in this study will be somewhat different from the original strains. However, this will not affect the specificity, because of the selection of primers from strongly conserved regions.

The three primer sets were type-specific. Cross reactivity with the other influenza types was not found and, in addition, specific signals were not obtained with DNA or RNA from isolates of a great variety of other respiratory micro-organisms. These results show that a positive signal in the influenza RNA-PCR actually reflects the presence of influenza RNA.

The sensitivity of the technique was found to be at the sub-picogram level. Another determination of the sensitivity was carried out by the plaque test. Dilutions that did not form plaques, were still positive by the RNA-PCR. It should be noted that a plaque in a plaque test assay is the result of one infectious particle. This does not mean that only one influenza particle was present, which explains the positivity of these non plaque-forming dilutions.

Using this type-specific RNA-PCR, additional epidemiological information was not obtained from the detected influenza strain. A further differentiation between the currently circulating influenza A subtypes (H1N1 and H3N2) can be made by an additional PCR, which discriminates between the H1 and H3 subtypes (Yamada et al., 1991). However, for diagnostic purposes, this is less relevant, as only the pathogen that can be detected in the clinical specimen is of importance and will have implications for therapy. In addition, new subtypes, in which the hemaglutinin or neuraminidase segment has been changed, can be

detected as well because the primers are selected from conserved sequences of the influenza genomes.

The application of the RNA-PCR for clinical specimens was carried out with 21 culture/IF positive samples from the 1990/1991 season. Negative samples could not be used for a comparison of the sensitivity, as negative samples had not been preserved. In the RNA-PCR, six were found to be positive for influenza A, 13 for influenza B. Two other samples were found negative in the PCR, although they were influenza B positive in the culture/IF assay. However, virus could not be propagated from these samples on MDCK cells or embryonated hen's eggs. Thus, no viable virus nor viral RNA were found in these specimens. After retesting one of the samples with IF, the positivity could not be confirmed. From the other sample no more material was available for retesting. Unfortunately, no serologic data were available from these patients.

If the technique is applied properly, sensitivity and specificity are the major advantages of the PCR for diagnostic purposes. A nested PCR (Zhang and Evans, 1991), which greatly increases the risk of cross-contamination of the samples, should therefore, be avoided for diagnostic purposes. An additional advantage may be that a sample may be examined simultaneously for more than one pathogen. For the respiratory tract, PCR assays for other respiratory organisms such as *Mycoplasma pneumoniae* (Bernet et al., 1989) and *Chlamydia* sp. (Claas et al., 1991) are already available. Application of the technique for routine laboratory diagnosis will include the replacement of radioactive by non-radioactive detection methods.

The RNA-PCR can also be used for detection of influenza genomes directly in tissue. Oseasohn et al. (1959) reported myocarditis in one third of fatal influenza cases, and occasionally the virus was isolated from the myocardium (Engblom et al., 1983). The etiological role of the virus in myocarditis or other influenza-associated mortalities (Tillet et al., 1983; Sprenger et al., 1990) can be examined with RNA-PCR.

Technically, the type-specific influenza RNA-PCR is suited for application for routine diagnostic procedures. However, an elaborate comparison between the culture/IF and RNA-PCR will be performed in the 1991/1992, season in order to establish the diagnostic value of this new technique.

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