VIROLOGY

A comparative study of immunocapture ELISA and RT-PCR for screening clinical samples from Southern Greece for human influenza virus types A and B

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An immunocapture (IC) ELISA and reverse transcriptase (RT)-PCR assays were evaluated as screening methods for the detection of influenza virus types A and B in clinical samples collected from individuals presenting with influenza-like symptoms in Southern Greece. Standard virus isolation in embryonated hens' eggs was taken as the reference method. According to the reference method, 25 (16.7%) of the 150 clinical samples examined were infected by influenza viruses – 19 type A (H3N2) and 6 type B. The sensitivity of immunocapture ELISA was 64% and that for RT-PCR was 100%. The specificity of IC ELISA was 98% and by RT-PCR 97%. The positive diagnostic value of IC ELISA was 94% and of RT-PCR 86%, whereas the negative diagnostic values for IC ELISA and PCR were 93% and 100%, respectively. These findings confirm that RT-PCR provides significantly increased sensitivity over IC ELISA and can be of value in the management of regional influenza screening surveys conducted by the national public health services.

Introduction

Influenza virus types A and B have a single-stranded segmented RNA genome of negative polarity. Because of their high rate of mutation, they exhibit increased genetic and antigenic instability resulting in frequent antigenic drift, and in the case of type A, more rarely antigenic shift [1]. Influenza viruses are the primary cause of annual influenza epidemics. They constitute the most important and widespread pathogens of the upper and lower respiratory tract in man, causing high rates of morbidity and mortality world-wide, especially among the elderly and patients suffering from chronic cardiovascular and respiratory problems [2]. To ameliorate the impact of influenza epidemics, national public health services follow a rigorous influenza surveillance and vaccination policy, espe-

cially for populations at high risk [3]. However, the efficacy of vaccination depends on the specific cocktail of protective antigenicities being used to maximise coverage by the most dominant viral genotypic variants being periodically identified. This sorting process requires regular surveys of influenza activity in samples collected from patients with flu-like symptoms, which include high fever, head and muscle aches and general fatigue [4]. However, other human pathogens of the respiratory tract, including respiratory syncytial virus (RSV), parainfluenza virus types 1, 2, 3 and 4, adenoviruses and rhinoviruses, cause similar symptoms. This multitude of agents, in addition to influenza viruses, causing similar clinical symptoms, renders primary screening of influenza-positive patients a major task. Efficient control of influenza epidemics requires continuous optimisation of all aspects of vaccine development and administration including painstaking assessment of its efficacy. Effective monitoring of viral outbreaks involves the implementation of numerous screening methods of influenza activity, including virus isolation from embryonated hens' eggs and tissue culture, immunofluorescence [5, 6], enzyme immunoassays (ELISA)

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[7, 8], reverse transcriptase (RT)-PCR [9, 10], multiplex RT-PCR [6, 11] and PCR coupled to enzyme immunoassay [12, 13]. A number of investigators have evaluated most of these methods and have assessed their sensitivities, specificities, diagnostic values and false positive or negative results, in an attempt to normalise and compare the large variety of influenza surveys conducted world-wide every year [14–18].

The present study compared the performance characteristics of immunocapture (IC) ELISA and RT-PCR as screening methods for influenza virus type A and B activities. It reports the sensitivity, specificity, diagnostic value, false positive or negative results of each method in detecting and identifying influenza infection directly from the clinical specimens and after passage to embryonated hens' eggs; virus isolation was the method of reference or 'gold standard'.

Materials and methods

Clinical specimens

The 150 nasal and pharyngeal swabs or primary samples were collected from individuals with influenza-like illness, 3–4 days following the onset of symptoms. All primary samples were collected during two World Health Organization (WHO) influenza surveillance periods, from Oct. 1996 to April 1997 and from Oct. 1997 to April 1998. Patients' ages ranged from 20 days to 65 years. Specimen collections were from out-patient facilities of hospitals and private practice physicians in Southern Greece. All samples were held in 2.5 ml of virus transport medium containing (L) bacto-tryptose 24.9 g, gelatin 5 g, streptomycin 0.5 g, penicillin 500 000 U and fungizone 50 mg, at 4°C for 2 days. The swabs were removed from the vials and the clinical specimens were centrifuged at 1500 *g* at 4°C for 15 min. The supernates were either tested immediately, or stored at –80°C.

Influenza virus propagation and typing

All primary samples and negative controls were inoculated into the amniotic (150 μ l) and allantoic (150 μ l) cavities of 8-day-old embryonated hens' eggs and incubated at 35°C for 3 days. Propagated samples were the primary samples that had been passed in embryonated hens' eggs. After incubation, amniotic and allantoic fluids were harvested and viral titres were determined by the haemagglutination (HA) test with chicken red blood cells 0.5%, according to standard WHO protocols. All isolates were typed by the haemagglutination-inhibition (HI) method as type A, subtype H1N1 and H3N2, or type B with antisera and guidelines provided by WHO for the surveillance of influenza.

Immunocapture ELISA

The primary samples were screened by IC ELISA, and typed as influenza type A or B with monoclonal antibodies (MAbs) raised against the respective viral nucleoproteins. ELISA microplates (Greiner Laboratories, Germany) were coated (100 μ l/well) with solutions of polyclonal antibodies raised in rabbits against influenza type A or B specific antigens (kindly provided by Professor S. van der Werf, Institut Pasteur, Paris, France), which had been diluted 1 in 6000 in 0.05 M carbonate-bicarbonate buffer (pH 9.6). Polyclonal antibodies were adsorbed to plastic surface at 4°C overnight. After each step of the IC ELISA, plates were washed five times with phosphate-buffered saline (PBS) containing Tween-20 0.05%. Non-specific binding was eliminated by the addition of 100 μ l of saturation buffer containing gelatin (Merck) 0.5% in 0.05 M carbonate-bicarbonate buffer (pH 9.6) to each well, followed by incubation at 37°C for 1 h. Each ELISA microplate contained duplicate positive control wells consisting of 100 μ l of allantoic fluids infected with influenza type A or B of known HA titre diluted in PBS-bovine serum albumin (BSA) 1% containing, Tween-20 0.1%, and duplicate wells containing 100 μ l of uninfected allantoic fluids as negative controls. Unknown samples were added in duplicate at 100 μ l/well and incubated at 37°C for 1 h. Anti-A or anti-B influenza mouse MAbs (WHO Influenza Reference Center, CDC, Atlanta, USA), diluted 1 in 1250 in PBS containing BSA 1% and Tween-20 0.1% were added (100 μ l) to the wells. The plates were incubated for 30 min at 37°C and 100 μ l of biotinylated anti-mouse antibodies (BIOSYS, France) diluted 1 in 4000 in PBS containing BSA 1% and Tween-20 0.1% were added to each well. After incubation at 37°C for 30 min, 100 μ l of horseradish peroxidase-avidin D conjugate (Vector Laboratories, CA) diluted 1 in 5000 in PBS containing BSA 1% and Tween-20 0.1% were added to each well. The plates were incubated at 37°C for 10 min. Finally, 100 μ l of substrate solution containing 10 mg *o*-phenylene diaminehydrochloride in 25 ml of 0.1 M citrate-phosphate buffer (pH 5) and 0.03% H₂O₂ were added immediately before use to each well and the plates were incubated in the dark at room temperature for 30 min, at which time the reaction was stopped by the addition of 3 N HCl 100 μ l/well. The absorbance was measured at 492 nm and 630 nm in a Dynatech ELISA microplate reader MR 5000 (Dynatech Laboratories, VA, USA). The positive cut-off was adjusted to 0.3 OD units, as all the negative controls gave an OD value < 0.3 units. All samples with OD values above the positive cut-off were considered positive and those with OD values under this cut-off were considered negative.

Viral RNA extraction and cDNA synthesis

Viral RNA was extracted from 250- μ l portions of either the primary samples or the infected allantoic or

amniotic fluids, or both, with TRIzol (Gibco BRL, Life Technologies, NY, USA), according to the manufacturer's recommendations. Type A (H1N1) and (H3N2) and type B isolates previously identified by the WHO Influenza Reference Center in London served as positive controls; while uninfected allantoic fluids served as negative controls. For cDNA synthesis, 5 μ l of extracted viral RNA were incubated at 70°C for 5 min with 2.5 μ l of random hexanucleotide primers (40 μ g/ μ l) (New England Biolabs, MA, USA) and RNasin (Promega, WI, USA) 20 U/ μ l, quenched on ice and then 5 μ l of 5 $\times$ reaction buffer (Promega), M-MLV reverse transcriptase (Promega) 100 U/tube, 5 μ l of 10 mM solution of the four deoxynucleotide triphosphates (Promega) and sterile H₂O (Sigma) were added to give a 25- μ l final volume. The reactions were incubated at 37°C for 1 h and at 95°C for 5 min in a Techne Progene thermal cycler. The reaction product was stored at -20°C.

Nested PCR

Influenza viruses were typed as A or B by nested PCR with primers that amplified a highly conserved region of the internal viral matrix protein gene [19]. Type A viruses were further subtyped with HA1, HA2 and HA3 as well as NA1 and NA2 specific primers that hybridise to conserved regions of the haemagglutinin and neuraminidase genes of influenza, respectively [19]. The sensitivity and specificity of the primer pairs in the nested PCR have been reported [19]. Nested PCR took place in two consecutive amplifications. The first amplification reaction contained 5 μ l of 10 $\times$ reaction buffer (Minotech, Crete, Greece), 4 μ l of a 10 mM solution of the four deoxynucleotide triphosphates (Promega), 2 units of Taq DNA Polymerase (Minotech), 2 μ l of 25 mM MgCl₂, 4 μ l of cDNA, 200 pmoles of the outer primers and sterile H₂O to 50 μ l total volume. The reactions were amplified in a Techne Progene Thermal Cycler for 25 cycles of denaturation at 94°C for 10 s, annealing at 50°C for 10 s and elongation at 72°C for 30 s. Two μ l of the first PCR amplification were added to a second PCR reaction containing the inner pair of primers and amplified for 40 cycles under the same cycling conditions. Amplification products were analysed by agarose (Gibco

BRL, Ultra Pure agarose, electrophoresis grade) 2% gel electrophoresis in 1 $\times$ TBE buffer, stained with ethidium bromide 1 μ g/ml and visualised with a UV transilluminator (Foto/Phoresis, Fotodyne, WI, USA).

Statistical analysis

The SPSS statistical package was used to analyse the data generated in this study. Differences between the two screening methods were determined by the χ^2 test.

Results

Tables 1–5 summarise the study findings. More specifically, screening 150 primary samples which had been propagated in embryonated hens' eggs by the HI method, revealed that 25 (16.7%) were infected by influenza viruses, 19 (76%) with type A (H3N2) and 6 (24%) with type B strains. Screening the same primary samples by IC ELISA revealed 17 (11.3%) cases infected with influenza virus, 13 (76.4%) of which were type A and 4 (30.7%) type B. Eight additional samples were found to be influenza positive, six with type A and two with type B strains, by IC ELISA after three passages of the primary samples in embryonated hens' eggs. Of the 25 samples positive by IC ELISA, 24 (96%) were positive for influenza infection by the reference method. One primary sample that was positive by IC ELISA did not yield a positive result after three passages in eggs. Thus, IC ELISA underestimated influenza positive primary samples, relative to the reference method. It follows from the results that propagated samples contained a higher concentration of

Table 1. Influenza virus detection by virus isolation, IC ELISA and RT-PCR

Method	Number (%) of samples (n = 150)	
	Positive	Negative
Virus isolation	25 (16.7)	125 (83.3)
IC ELISA on primary samples	17 (11.3)	133 (88.7)
IC ELISA on propagated samples	25 (16.7)	125 (83.3)
RT-PCR on primary samples	29 (19.3)	121 (80.7)
RT-PCR on propagated samples	29 (19.3)	121 (80.7)

Table 2. Detection of influenza virus in primary samples by virus isolation, IC ELISA and RT-PCR

Method		Number of samples	Number (%) with equivalent result by		
			Virus isolation	IC ELISA	RT-PCR
Virus isolation	positive	25	...	16 (64)	25 (100)
	negative	125	...	125 (100)	121 (96.8)
IC ELISA	positive	17	16 (64)	...	17 (100)
	negative	133	125 (94)	...	121 (91)
RT-PCR	positive	29	25 (86.2)	17 (58.6)	...
	negative	121	121 (100)	121 (100)	...

Table 3. Year 1 influenza identifications by virus isolation, IC ELISA and RT-PCR

Method	Number (%) of samples (n = 70)	
	Positive	Negative
Virus isolation	11 (15.7)	59 (84.3)
IC ELISA on primary samples	7 (10)	63 (90)
IC ELISA on propagated samples	11 (15.7)	59 (84.3)
RT-PCR on primary samples	13 (18.6)	57 (81.4)
RT-PCR on propagated samples	13 (18.6)	57 (81.4)

Table 4. Year 2 influenza identifications by virus isolation, IC ELISA and RT-PCR

Method	Number (%) of samples (n = 80)	
	Positive	Negative
Virus isolation	14 (17.5)	66 (82.5)
IC ELISA on primary samples	10 (12.5)	70 (87.5)
IC ELISA on propagated samples	14 (17.5)	66 (82.5)
RT-PCR on primary samples	16 (20)	64 (80)
RT-PCR on propagated samples	16 (20)	64 (80)

virus particles that were detected by IC ELISA, increasing the number of overall positive results found by that method. Screening the same 150 clinical specimens before and after propagation in embryonated hens' eggs by RT-PCR revealed 29 (19.3%) infected by influenza viruses, 22 (75.8%) with type A subtype H3N2 and 7 (24%) with type B strains. All samples that were influenza positive by virus isolation were also positive by RT-PCR. There was 100% correlation in the type and subtype of influenza-positive samples determined by RT-PCR and virus isolation. RT-PCR detected four additional positive samples in the primary samples, three type A (H3N2) and one type B, compared with virus isolation. These PCR-positive samples were negative after three passages in eggs. One of the three A (H3N2) samples which was positive by PCR was found to be positive by IC ELISA on the primary samples. These findings are summarised in Tables 1 and 2. Tables 3 and 4 show the number of positive and negative influenza detections by the three methods for the first and second year of surveillance, respectively. Table 5 compares the sensitivities and specificities as well as the diagnostic values and false

positive and negative results in IC ELISA and RT-PCR with primary or propagated samples. There was a statistically significant difference ($p < 0.001$) between IC ELISA (64%) and RT-PCR (100%) in the sensitivity of influenza detection in the primary samples.

Discussion

Most of the methods frequently used to screen for influenza infection, such as ELISA and immunofluorescence (IF), require propagated clinical material for sensitive, specific and reliable viral detection. Recently developed RT-PCR methods offer increased sensitivity for direct influenza virus detection, typing and subtyping in primary clinical specimens [6, 10, 18]; thus obviating the need to propagate the virus in embryonated hens' eggs or tissue culture. RT-PCR has been shown to have increased sensitivity over ELISA and IF for influenza detection in clinical samples [6, 18]; this facilitates early administration of antiviral treatment and has a beneficial role in the overall management of influenza epidemics [17]. A weakness of RT-PCR is the ease with which minor contamination may yield false-positive results, a possibility that is even more pronounced with nested PCR methods.

In the present study, RT-PCR detected four more influenza-positive samples than virus isolation in eggs. This discrepancy of RT-PCR relative to the reference method may have derived from the potential presence of inactive viruses in the clinical sample, which are usually undetectable by virus isolation methods. This loss of virus viability is possibly due to prolonged storage of samples under non-ideal temperature conditions before arrival at the laboratory or the presence of partially inactivated virus in the clinical sample, or both [17, 19]. Compared with RT-PCR, IC ELISA had lower sensitivity for the detection of influenza infection directly from primary clinical samples [17, 18]. IC ELISA detected 17 influenza viruses in primary samples (as compared with 29 by RT-PCR), and 25 in propagated samples, 24 of which were identified as influenza virus-positive by the reference virus isolation method. This increase in IC ELISA sensitivity following viral propagation demonstrates the dependence of the method on the viral titre. IC ELISA detected one influenza-positive sample, which was also positive by

Table 5. Sensitivity, specificity, diagnostic value and false positive and negative values for IC ELISA and RT-PCR

Method	Sensitivity (%)	Specificity (%)	Diagnostic value (%)		False	
			positive	negative	positive (%)	negative (%)
IC ELISA on primary samples	64	98.4	94.1	92.5	5.9	7.5
IC ELISA on propagated samples	100	100	100	100	0	0
RT-PCR on primary samples	100	96.8	86.2	100	13.8	0
RT-PCR on propagated samples	100	96.8	86.2	100	13.8	0

Sensitivity: true positives the sum of influenza positive samples according to the reference method. Specificity: true negatives the sum of influenza negative samples according to the reference method. Positive diagnostic value: true positives/true positives + false positives. Negative diagnostic value: true negatives/true negatives + false negatives.

RT-PCR but was negative after propagation in eggs, suggesting the presence of inactive virus in the sample. It is concluded that, when used as a screening method for influenza detection, RT-PCR offers a significant advantage over IC ELISA because of its superior sensitivity and its ability to detect influenza infection even when viral inactivation has taken place. However, viral isolation in eggs or tissue culture and subsequent strain identification is pivotal in the yearly surveillance of influenza, as it enables antigenic analysis of the isolated virus and contributes to the yearly recommendation of the WHO for the influenza vaccine.

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