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Research Article

FEASIBILITY OF REAL TIME PCR OVER CELL CULTURE IN DIAGNOSIS OF INFLUENZA VIRUS INFECTION: AN EXPERIENCE OF GRADE I VIRAL DIAGNOSTIC LABORATORY OF DEVELOPING COUNTRY

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Abstract

Introduction: In spite of the discovery of viral culture technology about a century ago, its application in diagnostic labs is being used since 1970s. It served as the "gold standard" for virus detection for long. In recent years, rapid, technically less challenging, sensitive and highly specific viral identification is possible by molecular tools. Hence, the purpose of this study was to analyze the importance of real time PCR over virus culture in diagnosis of Influenza virus infections, the biggest viral challenge of present India, a developing country, so that prompt and correct diagnosis can help physicians as well as the policy makers to control the virus spread.

Objective: To study the feasibility of real time PCR vis a vis viral culture technique and evaluate the utility of these methods for laboratory diagnosis of Influenza virus infections.

Methodology: The study was conducted in Grade I Virology Diagnostic laboratory, Dept of Microbiology, KGMU, Lucknow. We used both real time RT-PCR and viral culture methods (on MDCK cell lines) for detection of Influenza virus infection and compared the effect of transport time, cost per sample and turnaround time on both the techniques.

Results & Conclusion: Real time PCR is more practical, more sensitive, quicker and cost effective. It needs less expertise and availability of reagents is better. Though viral culture proved to be highly specific and useful for wider application but the use should currently be limited to mostly research facilities. Therefore for epidemiological diagnosis purposes real time PCR detection of Influenza virus is advised.

Keywords: Conventional viral culture; Real time RT PCR; Molecular diagnosis; Transportation time; cost per sample

Introduction

Virus detection and typing are essential laboratory tools required for epidemiology of viral infections, which mainly depend on either culture or molecular methods (Hsiung *et al.,* 1994; Specter and Lancz 1992; Wiedbrauk and Johnson, 1993). John Enders and his colleagues in the late 1940s successfully grew vaccinia and polio viruses in cell cultures (Enders *et al.,* 1949; Feller *et al.,* 1940)**.** It's application in diagnostic virology could be started only in 1970s. Isolation of viruses in cell culture was considered the preferred "gold standard" method for detecting many of the common human viruses (Hsiung, 1984)**.** Other than animal or egg inoculation, it is the only method that confirms the presence of viable viruses and, theoretically, has the potential to isolate a single virus. With time non culture methods significantly impacted viral diagnosis. Molecular technologies like PCR and real-time PCR have taken a long leap. These tests are rapid, sensitive, specific, economical and require fewer skills to perform (Boivin *et al.,* 2001; Espy *et al.,* 2006)**).** There are various excellent reviews describing their importance individually (Domiati-Saad and Scheuermann 2006; Espy *et al.,* 2006; Vernet, 2004)**.** Influenza virus imposes severe threat to human health in form of epidemics and pandemics which was recently witnessed in form of pandemic H1N1. In many centers of world both the molecular as well as culture methods are being used for diagnosis and various research activities related to Influenza virus as continuous surveillance (López Roa *et al.,* 2011; Nadia *et al.,* 2003; Poddar *et al.,* 2002; Zitterkopf *et al.,* 2006). We are also detecting and studying this virus with the help of both the techniques, therefore, present study was planned to evaluate the feasibility of real time PCR vis a vis viral culture in Influenza virus detection along with more aspects to consider before concluding eg transportation conditions, manpower and space required to establish these techniques besides cost, turnaround time and sensitivity - specificity.

Materials and Methods

Study site:

The study was conducted at Grade I Viral Diagnostic laboratory. This laboratory has the capacity of diagnosing different human pathogenic viruses by serology, immunofluorescence, viral culture and molecular methods. The study protocol was approved by Ethics committee of Institute and written consent was obtained before taking samples.

For this particular study, we chose 500 consecutive samples from cases of acute respiratory infection, for detection and typing of Influenza virus. Diagnosis and typing was done by 1) culturing on MDCK (Madin Darby Canine Kidney) cell line following confirmation and subtyping by Haemagglutination / Haemagglutination inhibition (HA/HAI), 2) Real time PCR for Influenza virus A and B; A (seasonal H1N1), A (pandemic H1N1) and A (seasonal H3N2) and 3) immunofluorescence assay for detecting Influenza virus antigen on cells of patients' respiratory lining.

Case Selection

Consecutive 500 patients presenting as ILI (Influenza like illness, defined by CDC as sudden onset of fever more than 100° F with cough and/or sore throat in absence of other infection) (CDC Flu Activity & Surveillance) were enrolled after taking informed consent for detection and typing of Influenza virus.

***Samples collection and processing*:**

Throat swabs and Nasal swabs were collected in 3.5ml of Viral Transport Media (VTM) (Hanks' balanced salt solution with 1 mg/ml Gentamicin and 2µg/ml Amphotericin B and 2% Bovine serum albumin) and transported to the virology laboratory on ice and processed on the same day. Specimens were vortexed at 500 rpm for 10 seconds to remove cells which remained adherent to swabs and aliquotted into three parts. One part was used for virus isolation in conventional cell culture on MDCK cell line, one part was used for real time RT PCR, and one was used for immunofluorescense

Culture on MDCK cell lines:

Influenza viruses were isolated using MDCK cell line, maintained in Earle's Minimal Essential Medium (Sigma, USA) supplemented with 10% fetal calf serum (Gibco.), 0.03% L-Glutamine (Bio world), 200 IU/ml penicillin (Calbiochem, USA), and 0.2 mg/ml streptomycin (Calbiochem, USA). The monolayer of cell line was prepared onto T-25 flask (Corning Incorporation, NY, USA) at 35^0C in presence of 5% CO_2. The processed samples were inoculated (300µl) in duplicate once >80% of monolayer was ready. The inoculums was allowed to adsorb for 1 hour at 37^0C, followed by washing with 5 ml of PBS and addition of 5 ml of serum free virus growth medium with 2µg/ml TPCK Trypsin (Sigma, USA) to each flask. The flasks were again incubated at 35^0C with 5% CO_2. Cell culture was observed daily for cytopathic effect (CPE) for 7 days. Cells were harvested either when 3+ or 4+ CPE (1+ = 25% of total cells showing CPE, 2+ = 50%, 3+ = 75%, 4+ = 100%) was observed or on 7th day, whichever was earlier. If no CPE was observed in 1st passage, 2nd passage was done. Inoculum in 2nd passage was 500 µl of fluid from T-25 flask of 1st passage. Presence of influenza virus was confirmed by haemagglutination assay (HA) using 0.75% guinea pig red blood cells. When virus titer was 1:8 then it was subjected to HAI for further subtyping. Control antigens and antisera of Influenza A (seasonal H1N1), (seasonal H3N2), (pandemic H1N1), and Influenza B (Yamagata and Victoria) were provided by CDC (WHO, 2002).

Real time PCR

RNA was extracted according to manufacturer details using HIGH PURE total viral nucleic acid kit (ROCHE). Influenza Virus A and B were detected and Influenza A virus was subtyped by one step real time PCR method using Centers for Disease Control and Prevention (CDC) (WHO, 2009) and WHO protocols (Potdar *et al.,* 2010; WHO, 2002) on ABI 7500 (Applied Biosystem) real time PCR machine. Reactions were performed in duplicate in 25 µL final volume reaction containing 5 µL of purified nucleic acid template, 12.5 µL of 2x RT PCR buffer, 25 x RT PCR enzyme mix (AgPath One-Step Quantitative RT-PCR mix, Invitrogen, Carlsbad, CA, USA), 0.5 µL of 40 mM primers, both forward and reverse (final concentration at 800 nM) and 0.5 µL of 10 mM probe (final concentration at 200 nM). Thermocycling reaction conditions were: reverse transcription at 45ºC for 10 min; Taq activation and denaturation at 95ºC for 10 min, followed by 40 PCR amplification cycles including one step at 95ºC for 15 s and one step at 55ºC for 45 s (hybridization-elongation).

Immunofluorescense

Immunofluorescence was used as "the reference method" for this particular study to compare efficiency of real time PCR and conventional cell culture.The specimen was centrifuged at 500 rpm for 10 min and smears were prepared from deposit. Smears were air dried and fixed with acetone and were stained using commercially available antibodies (supplied by Abcam); primary antibodies were goat polyclonal antibodies to Influenza A and mouse monoclonal antibodies to Influenza B, and their respective secondary antibodies were used. One drop of primary antibodies was added on the respective smears. After incubating the slide in humidified chamber at 37ºC for 30 minutes the slide was washed by wash buffer three times, followed by one drop of ready to use FITC conjugated secondary antibodies was added on all spots and kept in the humidified chamber at 37ºC for 20 minutes. Slides were washed, mounted with buffered glycerol and seen under fluorescent microscope (Garcia and Isenberg 2007).

Analysis methods

Results obtained by cell culture, real time PCR and immunofluorescence were analysed under following heads:

Positivity was depicted as percentage of positive samples out of total samples tested. **Sensitivity** and **specificity**, were analyzed using Baysian analysis (Table 2). **Effect of Transport Time on positivity of samples:** Time taken in transportation of samples and its effect on positivity of samples was analyzed in four groups; < 4 hours, 4 – 8 hours, 8 – 12 hours and > 12 hours. **Positivity ratio** was calculated by ratio of no. of specimen positive by real time PCR to the no. of specimen positive by conventional viral culture. **Turn Around Time (TAT) in days:** was calculated from sample receiving to report issuing of each sample. Samples were categorized according to different TAT and then average was taken. Time taken in each step was considered for TAT calculation. **Direct Cost calculation per sample:** was done. Indirect cost e.g. cost of instruments, manpower etc was not calculated.

Results

In this study 500 consecutive samples, presenting as Influenza Like Illness were studied and Influenza virus was detected by Viral culture, Real time PCR and Immunofluorescence. Total 26/500 (5.2% positivity) samples were positive for Influenza Virus by viral culture, 104/500 (20.8% positivity) by real time PCR and 98/500 (19.6% positivity) by Immunofluorescence method. Diagnostic efficiency of techniques was tested taking Immunofluorescense as "the reference test" **(Table 1).** Viral culture showed lower sensitivity of 26.5% while real time PCR had higher sensitivity of 98%. Conventional viral culture was proven to be highly specific (100%).

Positivity is greatly affected by transportation time of samples to the laboratory in case of viral culture method. Positivity ratio remained around 3.7:1 up to 8 hours. In case of delay of more than 8 hours, positivity ratio started rising (5.6:1 in >8-12 hours) and culture positivity was nil if delay was more than 12 hours, while molecular positivity was also affected with delay (23.3% in <4 hours to 13.8% in >12 hours) but not in that proportion leading to increase in positivity ratio **(Table 2).** Turnaround time was about 5 times higher for cell culture (11.57 days) in comparison to real time PCR (2.66 days) **(Table 3).** Cost per sample for both the methods is compared in **Table 4** which shows cell culture method to be the costlier

Table 1: Evaluation of real time PCR and Viral culture* for Influenza virus detection

	Real time PCR positives	Real time PCR negatives	Culture positives	Culture negatives	Total
IF positive	96	2	26	72	98
IF negative	8	394	0	402	402
TOTAL	104	396	26	474	**N=500**

**Immunofluorescence (IF) used as "the reference test" (N=500).*
Real Time PCR: Sensitivity = 98%, Specificity = = 98%
Viral culture: Sensitivity = 26.5%, Specificity = 100%

Table 2: Effect of Transport Time on positivity of samples using real time PCR and Viral culture

Time Taken in Transport	Total Samples	Positives (% Positivity)		Positivity Ratio (Real time PCR vs Conventional Viral Culture)
		Real Time PCR	Viral Culture	
< 4 hrs	180	42 (23.3)	13 (7.2)	3.2:1
>4 – 8 hrs	171	37 (21.6)	10 (5.8)	3.7:1
>8 – 12 hrs	91	17 (18.7)	3 (3.3)	5.6:1
>12 hrs	58	8 (13.8)	0 (0.0)	8:0
TOTAL	**500**	**104**	**26**	

Discussion

The present study was conducted with the aim of contributing more evidences in analyzing "the preferred method" between viral culture and real time PCR for influenza virus diagnosis particularly for epidemiological purposes especially in developing countries. Diagnostic evaluation of any technique can be determined by calculating sensitivity, specificity, positive and negative predictive value. Real time PCR showed higher sensitivity and comparable specificity than conventional Viral culture. Viral culture was highly specific but very less sensitive in comparison to real time PCR. There are several studies supporting this finding (Atmar *et al.,* 1996; Espy *et al.,* 2006; Ruest *et al.,* 2003; Zitterkopf *et al.,* 2006)**.** Positivity and Positivity ratio were quite high in real time PCR in relation to conventional Viral culture method. It was about 3.5 times higher in molecular techniques. Therefore chances of diagnosing viral infection with the help of real time PCR are higher than that of viral cultur

Table 3: Turn Around Time for viral culture and real time PCR

Techniques	Total no of samples	Time taken (days)	Average Time taken (days)
Real time PCR (N=500)			
1. Sample collection and processing	476	1 day	1.01 days
	20	1.5 days	
	4	0.5 days	
2. RNA extraction and PCR	412	1 day	1.1 days
	56	2 days	
	15	1.5 days	
	17	0.5 days	
3. Subtyping	462	0.5 day	0.55 days
	26	1.0 days	
	12	1.5 days	
TURN AROUND TIME			**2.66 DAYS**
Viral Culture (N=500)			
1. Sample collection and processing	472	1 day	1.04 days
	12	1.5 days	
	16	2 days	
2. Growth of virus (From inoculation of sample to appearance of CPE)	154	5 days	9.03 days
	32	6 days	
	13	7 days	
	104	10 days	
	56	11 days	
	83	12 days	
	58	14 days	
3. Isolate confirmation (HA)	401	1 day	0.9 days
	99	0.5 days	
4. Subtyping (HAI) (n = 26)	21	0.5 day	0.6 days
	5	1 day	
TURN AROUND TIME			**11.57 DAYS**

Table 4: Cost requirement for Viral culture and PCR

TECHNIQUES	MATERIALS**	COST PER SAMPLE
Real time PCR	Reagents for sample collection, transport and processing	INR 95/-
	RNA extraction	INR 194/-
	Amplification	INR 516/-
	Subtyping	INR 480/-
	TOTAL COST PER SAMPLE	**INR 1285/- (~26$)**
Viral Culture *	Reagents for sample collection, transport and processing	INR 51/-
	Reagents of virus growth	INR 659/-
	Isolates confirmation	INR 362/-
	Subtyping	INR 495/-
	TOTAL COST PER SAMPLE	**INR 1567/- (~30$)**

*Cost of cell line establishment, maintenance, preservation and contamination prevention is not calculated.
** Total cost required for 500 real time PCRs and 500 viral cultures were taken into account to calculate per sample cost.
# Roughly 1 $ is equal to 50 INR (Indian currency)

Modern clinical virology relies on rapid virus detection for timely infection control and antiviral therapy. Technique with less turnaround Time (TAT) is the most useful and desirable (Leland and Ginocchio, 2007). Comparative depiction of TAT in our study clearly showed significantly high TAT in viral culture technique which renders it less useful for diagnosis. Virus isolation is inevitably a slower process as it involves replication in cultures. Ideally, if an appropriate turnaround time can be offered, molecular detection should be the gold standard at all times for diagnosis purpose. Cost per test is another concern in low income countries. In our study cost of viral culture was much higher than real time PCR in spite of the fact that hidden costs like cost of cell line maintenance, cost of contamination control, cost of cell line preservation etc was not included in calculations

Viral culture facilities for diagnostic purposes are available in very few centers in developing world. Viral culture mandates use of animals (guinea pig RBCs were used in our study), which is another limitation as maintaining an animal house is not always possible and is expensive. Guidelines and techniques of culture are described in many literatures but each step of culture required in house standardization, reason may be that viability of virus depends upon several environmental conditions which show inter laboratory variation. Transportation of samples is a crucial step in any technique as preservation of virus is directly related to how early the samples are transported to the laboratory for the tests (Leland and Ginocchio, 2007). Time taken in transportation was studied and the result was analyzed in this perspective. Results by molecular techniques (conventional or real time RT PCR) showed slight decrease in positivity with increase in transport time, while positivity in case of conventional culture declined sharply as time taken in transportation increased so much so that virus could not be retrieved in the samples which were transported after 12 hours of collection in spite of strict cold chain maintenance. Since viral culture requires viable virus to be grown and detected and as time passes, viability of virus outside living cells decreases, confidence of detecting virus by this method ought to decrease after certain period.

Effective control measures and prevention of viral infections relies on the rapid and specific identification of the causative organism. The early recognition of an infectious agent allows policy makers to make sound preventive plans. Rapid molecular diagnostic tools and detection methods are definitely superior in the clinical virology laboratory to enhance the identification of viral pathogens and to assist physicians as well as epidemiologists in the diagnosis and management of a variety of viral diseases. However, viral culture method can be laborious, time-consuming, and may lack sensitivity, thereby prolonging or denying definitive diagnosis and subsequent treatment of the patient. Undoubtedly, the role of virus isolation is most significant in providing epidemiological data, in the diagnosis of new or unexpected infection, and in yielding infectious virus for further study. Viral culture is expensive and requires more technologist time and skill, the increased accuracy of the results justifies the extra resources required, particularly for research; therefore its use should be restricted to research virology.

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Conflict of interest statement: None

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