Virus culture and real-time RT-PCR in identifying influenza viruses from influenza-like illness cases in Indonesia 2007-2008

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Abstrak

Latar belakang: *Adanya perbedaan hasil antara kultur virus dengan real-time polymerase chain reaction (RT-PCR) yang digunakan dalam surveilans* influenza-like illness *(ILI) menunjukkan perlunya mengevaluasi hasil kultur virus yang didapatkan dengan hasil RT-PCR sebagai pembanding.Tujuan penelitian ini adalah untuk mengevaluasi apakah kultur virus masih dapat diandalkan untuk studi surveilans ILI.*

Metode: *Usap hidung dan usap tenggorok didapatkan dari 20 sentinel ILI di Indonesia selama tanun 2007-2008. Identifikasi kultur virus dilakukan dengan menggunakan metode hemaglutinasi dan hemaglutinasi inhibisi. RT-PCR menggunakan primer yang bersifat spesifik untuk influensa A (A/H1N1, A/H3N2 and A/H5N1) dan influensa B. Primer disediakan oleh Center for Disease Control and Prevention, USA. Hasil positif kultur virus dibandingkan dengan hasil RT-PCR berdasarkan persentase kesamaan hasil.*

Hasil: *Sebanyak 112 spesimen dari 4277 spesimen kasus ILI didapatkan hasil positif influenza dengan metode kultur. Kesamaan hasil positif influenza kultur virus dibandingkan dengan real-time RT-PCR adalah 69.6%. Pada penelitian ini juga ditemukan bahwa 30,4 % (n=112) hasil real-time RT-PCR yang ditemukan positif influenza tidak dapat dideteksi oleh metode kultur.*

Kesimpulan: *Metode kultur masih relevan untuk surveilans ILI meskipun hasil positif Influenza dari kultur virus lebih sedikit dari pada hasil positif Influenza yang terdeteksi dengan metode PCR.* ***(Health Science Indones 2011;2:92-5)***

Kata kunci: *influenza-Like Illness, RT-PCR, influenza, kultur.*

Abstract

Introduction: From the influenza-like illness (ILI) surveillance in Indonesia, we learned that there was disagreement between virus culture and reverse trancriptase polymerase chain reaction (RT-PCR). This implies the need to evaluate whether virus culture is still a relevant method to be used in ILI surveillance.

Methods: The ILI specimens obtained from 20 ILI sentinels in Indonesia in 2007-2008. Real-time RT-PCR using primers were specific for influenza A (A/H1N1, A/H3N2 and A/H5N1) and Influenza B. The sequence of these primers was provided by the CDC, Atlanta. Virus culture identification was conducted with hemagglutination and hemagglutination inhibition methods. We evaluated the percentage of concordance between positive culture results vs its RT-PCR results.

Results: A number of 112 influenza positive in culture method from 4277 ILI specimens were compared with real-time RT-PCR result. There was 69.6% of virus culture result was in concordant with real-time RT-PCR result. We also found that 30.4% of positive result using real-time RT-PCR were not detectable by virus culture.

Conclusion: Virus culture was still essential and considerably efficient to support real-time RT-PCR detection in ILI cases in Indonesia although the positive Influenza results by virus culture less than RT-PCR. ***(Health Science Indones 2011;2:92-5)***

Key words: influenza-like illness, RT-PCR, influenza, culture

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Influenza-Like Illness (ILI) is a disease that shows influenza symptoms, such as cough, runny or stuffy nose, sore throat, headache or body aches, but not an influenza disease.[1] The surveillance of ILI cases is important because it detects influenza virus in patient. Since most people suffered with influenza symptoms is not diagnosed or even seeks treatment, ILI surveillance has to be maintained with reliable detection method, so that it will screen not only influenza cases, but also, other disease with similar symptoms.[2,3] It could also detect whether there was antigenic drift or shift.[4]

The National Institute of Health Research and Development, Ministry of Health (NIHRD), Indonesia, has served as the national referral laboratory in Indonesia for influenza. Since 1999, NIHRD has conducted ILI surveillance as one of its main research activity.

Reverse trancriptase (RT-PCR) is a method for molecular detection recommended by the United States- Centers for Disease Control and Prevention (CDC) to identify influenza virus infection in ILI cases.[5] The principle of this method is similar to *Polymerase Chain Reaction* (PCR), which is to amplify the influenza virus RNA genome using a pair of oligonucleotide primers in order to generate copies of a certain DNA sequence, however, in RT-PCR, the RNA strand has to be reverse-transcribed into cDNA (complement DNA) first using the reverse transcriptase enzyme. RT-PCR was considered more sensitive than by culture and ELISA.[6-8]

RT-PCR provides a specific and sensitive method for detection of influenza viruses A and B and discriminates between virus subtypes;[8-10] a considerable tool for influenza surveillance. Although, in the other hand, this technique requires a high level of skill and complex laboratory infrastructure, takes several hours to perform and is considered not suitable for lower level of expertise.[9]

The virus culture is considered a sensitive and useful technique for diagnosis of influenza virus.[3] Specimens usually used in this detection methods are nasal, throat, and rectal swabs.[11] Influenza virus which may be contained within these specimens, when the specimens added to the cell line, will adhere directly to the cell and will infect other cells. After the designated time, the isolation from the cell line and the identification of the virus could be done. This isolated virus could be identified by serological method as type A or B with hemaglutination inhibition test. Although detection of

influenza virus using virus culture requires time more than PCR up to 2 weeks, it was believed to be useful as alternative detection method which is necessary to avoid false negative results.[7] Cell culture also has been suggested to be performed to obtain early and late in the season influenza virus isolates which in turn is important to make sure that suitable vaccine strains will be available for the following year.[12]

In this study, our institute used two different methods, namely Real Time RT-PCR and Virus Culture. There were several researches that also aimed to compare result between different influenza detection methods in Europe and America, but most of them were focused only to see which one had the highest sensitivity or specificity.[5,6] They also used different detection object, such as Influenza A Virus and Respiratory Syncytial Virus.[5,6] Meanwhile, this research was directed to evaluate whether virus culture method was still relevant to be applied in ILI surveillance.

The objective of this result was to evaluate the percentage of concordance between RT-PCR and virus culture. This study was not aimed to determine which was the best detection method between those two methods, but to evaluate whether virus culture was still reliable as an alternative test for RT-PCR.

METHODS

Nasal and throat swabs were collected throughout Indonesia from 20 ILI sentinels who operated as primary health services in Indonesia. Viral culture is done by using 112 positive results from 4277 specimens obtained during 2007-2008. Each specimen was screened by RT-PCR prior to virus culture. Virus culture was done in biosafety cabinet class (BSC) IIA within BSL2 Influenza Laboratory, Center for Biomedical and Pharmaceutical Research and Development, NIHRD, Jakarta.

The molecular method used in this study was Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR) utilizing Thermal Cycler IQ5 (Biorad, USA). QiAmp RNA viral mini Kit (Qiagen, Germany) was used to extract the specimens according to manufacturer's instruction. Five sets of primers and probes which are syntesized by Invitrogen and Sigma (USA) were treated to the specimens to detect Influenza A, B, A/H1N1, A/H3N2. The reagent was one step qRT-PCR Superscript III with Platinum Taq Polymerase

(Invitrogen, USA). The amplification procedure were as follows: 5 µl extracted RNA of each specimen was treated by 50^0 C within 30 minutes (reverse transcriptase activation), then 95^0 C for 2 minutes (taq inhibitor activation). Forty five PCR cycles were applied in this procedure. The denaturation step was done at 95^0 C for 5 seconds, subsequently, and the annealing step was performed on 55^0 C in 30 seconds.

These specimens were cultured to identify the type and subtype of influenza virus. The cell line used in this culture was *Madin Darby Canine Kidney* (MDCK) cell (NAMRU II). Specimen was transported using Hank's solution (GIBCO/Invitrogen, USA) with 0.2% penicillin (Invitrogen, USA), 0.2% streptomycin (Invitrogen, USA) and 0.4% phenol red (Sigma, USA) in it. Cell culture was inoculated and passaged in two series to allow propagation of the virus. The identification of virus culture was held using Hemagglutination and Hemagglutination Inhibition assays based on World Health Organization guideline in influenza virus culture and characterization.[10] The reference antigen and antiserum A/H1N1, A/H3N3, B/Malaysia, B/Shanghai were supplied by WHO along with positive control, negative control, and *Receptor Destroying Enzyme* (RDE) Denka Seiken Co., Ltd. in influenza virus culture and characterization package.

We collected data of virus culture which reported positive results during ILI surveillance 2007-2008. Based on these results, we compared them with their RT-PCR result. The concordance percentage was meant to give information how many positive result obtained from virus culture was actually have the same results with RT-PCR. The percentage was obtained by dividing the number of positive influenza in virus with number of positive result in cultureRT-PCR.[12]

RESULTS

The comparative analysis between culture virus and RT-PCR of 112 positive influenza by RT-PCR were 70 (62.5%) Influenza B Viruses (IBV), seven out of 112 (6,1%) were Influenza A Virus (IAV) by both methods. The discordance results could be found that four out of 112 (3.6%) was identified as IAV by RT-PCR but negative by culture, 27.7 % was positive IBV by RT-PCR but negative by culture.

Furthermore, from 112 specimens positive result based on RT-PCR, there were only 78 positive influenza

specimens according to culture. The concordant percentage of virus culture to RT-PCR was relatively high (69.6%) and also for influenza A/H1N1 (7 of 10), A/H3N2 (1 of 2) and B (70 0f 101), whereas the percentage numbers were not less than 50 %.

Table 1 showed that there were negative PCR results identified as positive IAV (A/H1N1 and H3N2) or IBV by culture method. In total, there was 30.4% of negative result by virus culture which could be identified by RT-PCR as positive influenza.

Table 1. The result difference between RT-PCR and virus culture

Culture	RT-PCR		
	A/H1N1	A/H3N2	Influenza B virus
A/H1N1	7	0	0
A/H3N2	0	1	0
Influenza B virus	0	0	70
Negative	3	1	31
TOTAL	10	2	101

DISCUSSION

There was a concordance between the virus culture and RT-PCR result as there were matched positive results between virus culture and RT-PCR. The significance of the concordance between these results could be quantified to see the efficiency of virus culture against RT-PCR. Therefore, the percentage number of type and subtype of influenza virus from RT-PCR against virus culture was calculated by dividing the number of positive influenza in virus culture with number of positive result in RT-PCR.

We understand that RT-PCR method is an expensive method compare to culture. However the results from this study showed that virus culture was still considered as a reliable detection method since the percentage numbers of the consented results, mostly, were around 60%. This would mean most of the RT-PCR's results were also could be identified with virus culture although the virus culture is a time consuming method.

Negative results by virus culture shown on table 1 can be detected by RT-PCR. There were several reasons to explain about these phenomena i.e. the quality of the specimen either due to sampling method, lack of the rapid transportation since Indonesia is an archipelago

country, or the nature of the specimen itself. These factors might cause low concentration of viral RNA or RNA degradation and lead to negative result in RT-PCR.[5,7,13] Nevertheless, this event could be evidence for the capability of virus culture in propagating the viral load so that they could be detected by virus culture method.

In conclusion, virus culture was still essential and considerably efficient to support real-time RT-PCR detection in ILI cases in Indonesia although the positive Influenza results by virus culture less than RT-PCR.

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