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Comparison between Real-Time PCR and Conventional PCR for Detection of LPAI-H5N1 in Different Water Types.

Neveen M. Rizk[1*], Ahmed Kandeil[1], Rabeh El Shesheny[1], Ahmed B. Barakat[2], Sahar A. Showman[2], Fagr Kh. Abdel-Gawad[1], and Mohamed A. Ali[1].

[1]Environmental Research Division, National Research Centre, El-Buhouth Street, Dokki, Giza 12622, Egypt.
[2] Microbiology Department, Faculty of science, Ain Shams University.

ABSTRACT

The current study aimed to compare between conventional reverse transcription PCR and real-time reverse transcription PCR for detection of LPAI-H5NL in different water types with different environmental conditions (temperature and pH). The experiment was conducted using sea water and Nile water adjusted at different temperatures 20 and 34°C and different pH 6.5, 7.0, 7.5, 8.0. The results of real-time reverse transcription PCR showed that the Low Pathogenic Avian Influenza H5Nl virus was stable for extended periods of time in different water types compared with conventional reverse transcription PCR. In general, reverse transcription PCR and real time reverse transcription PCR showed the same results for the persistence of the virus at pH=7.0 when the temperature was 20°C. By using two different techniques, the results revealed that the LPAI-H5Nl virus persisted in Nile water longer than in sea water. In conclution, real-time PCR is the most powerful tool for detection of Avian Influenza H5N1 virus in different water types.

Keywords: Comparison, Real time PCR, Conventional PCR, Different water types

Corresponding author

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INTRODUCTION

The avian influenza virus related to the *Orthomyxoviridae* family of segmented negative-sense RNA viruses that are separated into six different genera, containing influenza types *A*, *B*, *C*, *Isavirus*, *Thogotovirus*, and *Quaranjavirus* [1]. There are 18 different eleven different neuraminidase (NA) antigens (N1 to N11) and hemagglutinin (HA) antigens (H1 to H18) subtypes of influenza A, labeled according to H number (for the type of HA) and N number (for the type of NA) [2-4]. Interestigly, Egypt is ranked the worst affected country by H5N1 after Indonesia and the worst affected country in the Eastern Mediterranean region [5]. Aquatic habitats contaminated with influenza viruses may serve as a rich transmission medium. Avian influenza viruses have been isolated from waterfowl habitats when infected ducks were found [6,7] and from sediment of aquatic habitats following bird migration [7,8]. *In vitro* experiments using distilled water showed that the virus could survive in water for weeks to months and the stability showed an inverse relationship to salinity and temperature [9,10]. In water, avian influenza H5N1 virus can persist for a long time. This persistence in water might be enhanced by environmental factors such as temperature, pH and salinity [11]. A variety of molecular methods are used for the detection and identification of microorganisms. In general, molecular methods may allow the detection of microorganisms which are difficult to identify, in addition to their higher sensitivity than the other ordinary alternative methods such as microscopy, biochemical and culture ones. Problems and drawbacks of these ordinary techniques are noticed in time consuming (cultivation), providing only late and retrospective diagnosis (ELISA) [12]. Sensitive and rapid polymerase chain reaction methods include reverse transcription PCR (RT-PCR), one-step RT-PCR, real-time reverse transcription (rt RT-PCR), duplex real-time RT-PCR and multiplex real-time RT-PCR have been developed for accurate and rapid diagnosis of avian influenza virus (H5N1) because these assays can provide results within 4 to 6 hours [13-19]. So, the aim of this study to compare between traditional reverse transcription PCR and real-time reverse transcription PCR for detection of LPAI-H5N1 in different water types with different environmental conditions (pH and temperature).

MATERIAL AND METHODS

Virus

Low pathogenic avian influenza virus (LPAIV), A/chicken/Egypt /Q1995D /2010 (H5N1) [HA Genbank accession no. KC436135], was obtained from Center of Scientific Excellence for influenza viruses, National Research Centre. The pathogenicity of obtained virus was previously modified by altering the multiple basic amino acids coding sequence (RRKKR) at the cleavage site of the HA of the highly pathogenic H5N1 virus to create the non-pathogenic form monobasic sequence (R) using plasmid based reverse genetics [20].

Virus propagation

Low pathogenic avian influenza virus (LPAIV), A/chicken/Egypt /Q1995D /2010 (H5N1) was used to conduct the experiments. The virus stock was obtained after propagation in Specific Pathogen Free (SPF) 9-to-11-day-old embryonated chicken eggs [21].

Experimental procedure

Two types of water (sea and surface) were separately divided to 200 ml aliquots in 500 ml sterile flasks which equipped with aerators. The aliquots were inoculated with influenza A virus (A/chicken/Egypt /Q1995D /2010 (H5N1)) with concentration 10^8 EID_{50}/ml and 2 ml of antibiotic-antimitotic mixture were added under a bio-safety level-2 laminar flow cabinet. The pH of the aliquots of the two different water types in the flasks were adjusted at 6.5, 7.0, 7.5, 8.0 by using 1N solutions of NaOH and HCl, then exposed to temperatures 20°C and 34°C which represent mean winter and summer temperatures in Egypt. Sampling started at the inoculation day which represents zero time then at weekly basis and tested for the presence of H5N1 virus by RT-PCR and real time RT-PCR through 5 weeks as a trial period. This experiment was repeated two times with the same conditions. Viral RNA was extracted from 140 µl of collected water samples by using a QIAamp viral RNA mini kit (QIAGEN, USA) according to the manufacturer's protocol in a class II bio-safety cabinet. The extracted RNA was aliquoted and kept at -80°C. To detect influenza A, extracted RNA was subjected to RT-PCR to amplify 244 bp of the M gene of influenza A viruses according to a WHO protocol [21]. Real time reverse transcription PCR (rt RT-PCR) targeting the M gene was performed on all RNA extracted from each water samples according to **Kayali *et al.*** [22].

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RESULTS

By using rt RT-PCR, the persistence of the virus in Nile water was the same (4 weeks) at pH 6.5, 7.0 and 7.5, followed by 3 weeks at pH 8.0 when the temperature condition was 20°C, while by using RT-PCR the virus persist for 4 week at pH 7.0 followed by 3 weeks and 1 week at pH 7.5 and 6.5, respectively. The virus was not detected in Nile water at pH 8.0 when the temperature condition was 20°C by using RT-PCR (Table 1, figure 1).

Table 1: Comparison between real-time RT-PCR and conventional RT-PCR for detection of LPAI-H5N1 in different water types at different pH values and temperature 20°C.

pH	Detection method	Water type	Zero time	1st week	2nd week	3rd week	4th week	5th week
6.5	RT-PCR	Nile	+	+	-	-	-	-
		Sea	+	-	-	-	-	-
	rt RT-PCR	Nile	+	+	+	+	+	-
		Sea	+	+	+	+	-	-
7	RT-PCR	Nile	+	+	+	+	+	-
		Sea	+	+	-	-	-	-
	rt RT-PCR	Nile	+	+	+	+	+	-
		Sea	+	+	+	+	+	-
7.5	RT-PCR	Nile	+	+	+	+	-	-
		Sea	+	-	-	-	-	-
	rt RT-PCR	Nile	+	+	+	+	+	-
		Sea	+	+	+	+	+	-
8	RT-PCR	Nile	-	-	-	-	-	-
		Sea	-	-	-	-	-	-
	rt RT-PCR	Nile	+	+	+	+	-	-
		Sea	+	+	+	+	-	-

(+) The virus detected (-) The virus not detected

Table 2: Comparison between real-time RT-PCR and conventional RT-PCR for detection of LPAI-H5N1 in different water types at different pH values and temperature 34°C.

pH	Detection method	Water type	Zero time	1st week	2nd week	3rd week	4th week	5th week
6.5	RT-PCR	Nile	+	-	-	-	-	-
		Sea	-	-	-	-	-	-
	rt RT-PCR	Nile	+	+	+	+	-	-
		Sea	+	+	+	+	-	-
7	RT PCR	Nile	+	+	-	-	-	-
		Sea	+	-	-	-	-	-
	rt RT-PCR	Nile	+	+	+	+	+	-
		Sea	+	+	+	+	+	
7.5	RT PCR	Nile	+	-	-	-	-	-
		Sea	+	-	-	-	-	-
	rt RT-PCR	Nile	+	+	+	+	-	-
		Sea	+	+	+	-	-	-
8	RT-PCR	Nile	-	-	-	-	-	-
		Sea	-	-	-	-	-	-
	rt RT-PCR	Nile	+	+	+	+	-	-
		Sea	+	+	+	-	-	-

(+) The virus detected (-) The virus not detected

The results of rt RT-PCR showed that the persistence of the virus in sea water was the same (4 weeks) at pH 7.0 and 7.5, followed by 3 weeks for both of pH 6.5 and pH 8.0 when the temperature condition was 20°C, while by using RT-PCR the virus persist for one week at pH 7.0, and did not detected by the end of the first week at pH 6.5, pH 7.5 and pH 8.0 (Table 1).

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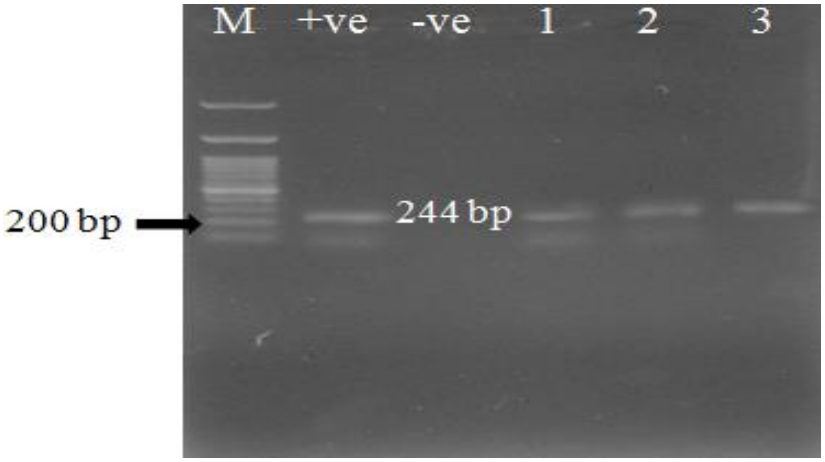

Figure 1: RT-PCR amplified product at 244 bp of M gene of LPAI H5N1 isolated from water samples. Lane 1: marker; Lane 2: positive control; Lane 3: negative control; Lane 4, 5 and 6: positive water samples.

By using RT-PCR the virus persist for one week in Nile water at pH 7.0, and did not detected by the end of the first week at pH 6.5, pH 7.5 and pH 8.0 when the temperature condition was 34^{o}C, while the persistence of the virus was the same (3 weeks) at pH 6.5, pH 7.5 and pH 8.0 by using rt RT-PCR. The viral RNA was stable for 4 weeks in Nile water at 34^{o}C at pH 7.0 by rt RT-PCR (Table 2).

By using rt RT-PCR, the highest persistence of the virus in sea water was 4 weeks at pH 7.0, followed by 3 weeks at pH 6.5 and 2 weeks for each of 7.5 and 8.0 when the temperature condition was 34^{o}C. On the other hand, RT-PCR results showed no detection of the virus in sea water by the end of the first week at pH 6.5, 7.0, 7.5 and 8.0 when the temperature was 34^{o}C. In general, the results revealed that the LPAI-H5Nl virus persist in Nile water more than in sea water by using two different techniques (Table 2).

DISCUSSION

The results obtained from real time RT-PCR for the detection of LPAI H5N1 in different water types were better than that obtained from conventional RT-PCR at the same experiment conditions (temperatures and pH values). The real-time PCR has efficiently to detect and quantify with high sensitivity and specificity, suspicious pathogens and even microorganisms that are not detected or difficultly identified after traditional methods [23]. The advantages of Real-time PCR over standard RT-PCR include speed and the reduced chance of cross contamination among samples because no post-amplification sample handling is necessary. Moreover, the labeled probe used to detect the PCR product with real-time PCR methods is target and specific, also providing an additional level of confirmation that the PCR product is the expected target, when compared to standard Reverse Transcriptase PCR [24]. Other researchers described a sensitive and specific real-time RT-PCR method for the detection of influenza A subtype H5 and for monitoring virus loads. Using serial dilutions of influenza A H5N1, the real-time RT-PCR assay reproducibly determined the lowest detection limit to be approximately 5 x 10^{-2} 50% egg infective doses (EID_{50}). In contrast, the minimum detection limit was approximately 3 EID_{50} in conventional RT-PCR with WHO primers, and 10 EID_{50} in antigen-capture ELISA [23]. In Australia, **Mackay (2004)** [25] concluded that quantitative real-time PCR has been proven before to be more reliable, sensitive, robust and faster and have more accurate quantification power than traditional endpoint PCR. Other researchers in Australia, reported some advantages of real-time PCR method such as simplicity (no electrophoresis required to verify the product) and sensitivity (able to identify from DNA equivalent to one cell) [26]. Prolonged persistence and infectivity of the virus in different types of water at different temperatures might provide an evidence for the possibility of re-infection of poultry and human who might use or deal with this contaminated water for different purposes [27-30]**.** By using rt RT PCR, the persistence of the virus in Nile water was the same (4 weeks) at pH 6.5, 7.0 and 7.5, followed by 3 weeks at pH 8.0 when the temperature condition was 20^{o}C, while at 34^{o}C, the persistence of the virus in the same water type was the same (3 weeks) at pH 6.5, pH 7.5 and pH 8.0 by using rt RT PCR. Moreover, the viral RNA was stable for 4 weeks in Nile water at 34^{o}C at pH 7.0 by rt RT PCR. This special emphasis on the effect of temperature on the persistence of virus in water was depending on the fact that the temperature is the most important predictor of virus persistence in water [31]**.** Other researchers concluded that persistence of AIV H5N1 is inversely

proportional to temperature [32]. The HPAI-H5Nl was found to persist for extended periods of time in water. Such persistence depends on physical and environmental factors such as pH, temperature, salinity and bacterial load [33].

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