한국가축위생학회지 제43권 제2호 (2020)
Korean J Vet Serv, 2020, 43(2), 107-112
ISSN 1225-6552, eISSN 2287-7630
https://doi.org/10.7853/kjvs.2020.43.2.107

Korean Journal of
Veterinary Service
Available online at http://kjves.org

< Short Communication>

Detection of foot-and-mouth disease virus (FMDV) and avian influenza virus (AIV) from animal carcass disposal sites using real-time RT-PCR

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(Received 18 May 2020; revised 22 June 2020; accepted 22 June 2020)

Abstract

Foot-and-mouth disease (FMD) and avian influenza (AI) are highly pathogenic viral disease which affects the livestock industry worldwide. Outbreak of these viruses causes great impact in the livestock industry; thus, disease infected animals were immediately disposed. Burial is the commonly used disposal method for deceased animals. However, there is potential for secondary environmental contamination, as well as the risk that infectious agents persisting in the environment due to the limited environmental controls in livestock burial sites during the decomposition of the carcasses. Therefore, this study aimed to investigate the detection of FMD and AI viruses from animal carcass disposal sites using real-time reverse transcription PCR. Soil samples of more than three years post-burial from livestock carcass disposal sites were collected and processed RNA isolation using a commercial extraction kit. The isolated RNA of the samples was used for the detection of FMDV and AIV using qRT-PCR. Based on the qPCR assay result, no viral particle was detected in the soil samples collected from the animal disposal sites. This indicates that 3 years of burial and their carcass disposal method is efficient for the control or at least reduction of spread infections in the surrounding environment.

Key words : AI, Carcass decomposition, FMD, qRT-PCR

INTRODUCTION

Foot-and-mouth disease (FMD) and avian influenza (AI) are socioeconomically important diseases that affects the livestock industry worldwide. FMD is a highly contagious disease affecting cloven-hoofed animals including cattle, sheep, goats, and pigs. The FMD virus belongs to the genus *Aphthovirus* of the family Picornaviridae (Grubman and Baxt, 2004; Guan et al, 2010). The FMD virus has seven immunologically distinct serotypes, O, A, C, Asia 1, and the South African Territories 1, 2, 3, and multiple subtypes circulating worldwide (Spackman et al, 2002; Moniwa et al, 2007). The avian influenza viruses belong to the *Influenzavirus A* genus of the *Orthomyxoviridae* family. In Korea, FMD and AI are among the diseases included in the first-class disease and specifically managed by the Infectious Disease Control and Prevention Act (KCDC, 2014).

Outbreaks of FMD and AI have severe economic consequences in the livestock industry. In response to an outbreak of highly pathogenic (HP) and low pathogenic (LP) AI, infected birds are immediately depopulated or disposed of by an in-house composting (Stephens and Spackman, 2017). An important factor in the prevention of an infectious animal disease outbreak is control and disposal of infected carcasses. To prevent the spread of disease, large number of animal mortalities are quickly disposed on-farm. Various options for dis-

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posal of infected and potentially infected animal carcasses includes burial, incineration, composting, rendering, lactic acid fermentation, alkaline hydrolysis, and anaerobic digestion (Graiver et al, 2009). Complete decomposition of carcasses may take 5 to 10 years after burial. However, by-products of decomposition such as leachate may be released for 20 years or longer (Wingfield and Palmer, 2009). In Korea, on-farm burial is the most preferred disposal method for disease infected animal carcasses (Yoon et al, 2017) while composting, rendering, and incineration are alternative methods which can also be used (Gwyther et al, 2011; Yuan et al, 2013). The Ministry of Environment (MOE) has set the environmental management guideline of carcass burial sites in Korea, to which all the AI- or FMD-infected and AI- or FMD-suspected livestock are eradicated within the farm land area where the outbreaks have occurred. Burial sites were constructed within a short period of time to prevent the spread of FMD and AI. However, these livestock carcass burial sites pose environmental implications due to its inappropriate construction and management due to lack of time, equipment, and available labors (Kim and Pramanik, 2016). Burial sites determined or suspected of leachate leaking, were excavated and redisposed by using bioaugmentation (Kim et al, 2015). Furthermore, after the burial of animal carcasses during an outbreak, there is a no-touch policy for the burial sites which lasted for three years. After 3 years, with the permission of the local government, people could use the land used for the burial sites. The three-year no-touch period is referred as the no-touch phase and the period after this as the re-use phase (Ko et al, 2017).

During a disease outbreak, the safe disposal of animal carcasses is an important environmental and health matter in both humans and livestock animals. Generally, after three years of burial, the land can be utilized again for other purposes. However, growing concern on the inactivation and degradation of viruses, as well as contaminants generated from buried animal carcasses may pose risk to public health and environment. Public concerns for potential on environmental pollution and the spread of the infectious diseases has been raised aiming at the massive infected animal carcass burials. Therefore, we investigated the virus existence in the burial sites by real-time reverse transcription polymerase reaction (qRT-PCR).

MATERIALS AND METHODS

Study site and sampling method

Soil samples were collected from three livestock carcass burial sites located in Chungbuk-do, South Korea (>3 years post burial). Each site was sampled thrice having 1～5 m diameter distance away from each other and collected at a depth of approximately 10～20 cm from the soil surface using a soil core sampler. After which, soil columns were secured using cling wrap, transferred to 200 mL sterile containers and then transported in the laboratory. The collected samples were stored at −20℃ until analysis.

RNA extraction

To analyze total nucleic acid viruses in the samples, 1 g of soil was mixed with 2 mL of sterile distilled water and then filtered twice using a 0.2 μm pore-size Minisart syringe filter (Satorious AG, Göttingen, Germany). A total of 200 μL of mixture was used for viral DNA/RNA extraction using an the QIAamp® AllPrep PowerViral DNA/RNA kit (Qiagen, Hilden, Germany) according to the manufacturers' instruction. For positive control, an inactivated FMD vaccine formulated from A and O type (ARRIAH-VAC PLUS, Vladimir, Russia) and a LPAI strain H9N2 (1×10^{10}/mL) were used. For FMDV control sample, 1 mL of vaccine was placed in a microcentrifuge tube and centrifuged at 14,000 rpm for 20 minutes. The supernatant was discarded and the pellet was resuspended with 350 μL of buffer RLT. For AIV control sample, 1 mL of virus culture was added with 350 μL of buffer RLT. Then, RNA isolation proceeded using QIAamp RNeasy mini kit (Qiagen, Hilden, Germany) following manufacturer's instruction. The RNA concentration was estimated using an Optizen Nano Q spectrophotometer (Mecasys Co. Ltd., Korea). The samples were stored at −80℃ until analysis.

Quantification of viral RNA using real-time RT-PCR

Detection of FMD and AI virus in the soil samples were conducted by qRT-PCR. The primers and probes used in the study are indicated in Table 1. Both assays were performed using the reagents supplied from the QuantiTect Probe RT-PCR kit (Qiagen, Hilden, Germany). For FMDV, the PCR reaction mixture had a final volume of 25 μL and contained 4.8 μL of RNase-free water, 12.5 μL of 2× QuantiTect probe reverse transcription PCR master mix (Qiagen, Hilden, Germany), 2 μL of each primer, 1.0 μL of the probe, 0.2 μL of QuantiTect RT Mix, and 2.5 μL of RNA extract or RNA standard. Cycling conditions were as follows: reverse transcription for 30 min at 50℃, 15-min activation of DNA polymerase at 95℃, followed by 50 cycles at 95℃ for 10 s, and 60℃ for 1 min. For AIV, a final volume of 25 μL PCR reaction mixture contained 6.8 μL of RNase-free water, 12.5 μL of 2× QuantiTect probe reverse transcription PCR master mix (Qiagen, Hilden, Germany), 1 μL of each primer, 1.0 μL of the probe, 0.2 μL of QuantiTect RT Mix, and 2.5 μL of RNA extract or RNA standard. Cycling conditions were as follows: reverse transcription for 30 min at 50℃, 15-min activation of DNA polymerase at 95℃, followed by 40 cycles at 94℃ for 10 s, 60℃ for 30 s, and 72℃ for 10s. Thermal cycling was conducted using the Bio-Rad C1000 Touch™ Thermal Cycler; CFX96™ Real-Time System (Bio-Rad, USA). All samples were run in triplicate. Positive and negative controls were included with each qRT-PCR run.

RESULTS AND DISCUSSION

Disposal options for animal mortalities includes burial, incineration, composting, rendering, lactic acid fermentation, alkaline hydrolysis, and anaerobic digestion (Graiver et al, 2009). Among them, burial is one of the most commonly used methods to dispose of animal carcasses. However, this burial can be a source of problem such as soil and groundwater contamination (Gwyther et al, 2011). Decomposition commences immediately after death, and breakdown results from the action of proteolytic and lipolytic bacteria or enzymes (Howard et al, 2010) that are already present in body tissues and intestines or of enzymes that are otherwise derived from endogenous soil microorganisms (Dent et al, 2004). Buried carcasses decay naturally and lasts for several years, and causes rapid changes in the composition of the surrounding environmental conditions (Kim et al, 2017). The infectivity of viruses may be affected by temperature which is influenced by the suspending medium, with organic matter providing some protection against inactivation. In some cases, the virus may stay stable almost indefinitely at temperatures below freezing. Some viruses may also retain infectivity for more than one year in a simple media at a temperature of 4℃ (Geering and Lubroth, 2002). Inactivation of virus is more rapid when subjected to high temperature.

In the present study, we investigated if viral particles will be detected in soil samples from livestock burial sites. Molecular diagnostic techniques like, polymerase chain reaction aids in the rapid and accurate detection of diseases caused by pathogens. The most commonly used diagnostic method to detect viral RNA during an animal disease outbreak is real-time reverse transcription polymerase chain reaction (qRT-PCR) (Hwang et al,

Table 1. Primers and probes for detecting FMD and AI virus genes using qRT-PCR

Primer	Sequence (5'→3')	Target gene	Reference
FMDV-F	ACTGGGTTTTACAAACCTGTGA	3D polymerase (3D)	(Callahan et al, 2002)
FMDV-R	GCGAGTCCTGCCACGGA		
FMDV-P	FAM-TCCTTTGCACGCCGTGGGAC-TAMRA		
M+25	AGATGAGTCTTCTAACCGAGGTCG	Matrix (M)	(Spackman et al, 2002)
M-124	TGCAAAAACATCTTCAAGTCTCTG		
M+64	FAM-TCAGGCCCCCTCAAAGCCGA- TAMRA		

2015). Ten-fold serial dilutions were performed for the positive control standard for qPCR assay. The amplification plot and standard curve established are shown in Fig. 1 and 2. The results of the qRT-PCR assay were assessed by the quantification cycle (Cq) value. The negative Cq value for samples and control sample considered Cq of ≥41.0 and ≥37.0 and was selected as the positive/negative cut-off Cq values for FMDV and AIV, respectively.

Based on the results of qRT-PCR assay, viral RNA was not detected in post-burial soils from livestock burial sites (Table 2). This indicates that virus was not retained in the burial site after several years of burial. The undetectable level of viral RNA in the soil suggests sufficient degradation of the viral nucleic acids structure. This study is important for disease control purposes since some viruses may remain their infectivity which might post a public health concern. A study on the survival of the FMD virus, reported that the virus survival depended on internal location of dead body at which the virus was detected (Gale, 2002). It was reported that FMD virus may survive at 4°C for approximately 2 months on wool, 2～3 months in bovine feces or slurry, and reportedly more than 6 months in the soil surface under snow (Kim and Kim, 2012). The FMD virus is sensitive to both acid and alkaline conditions and it is most stable at pH 7.4～7.6; however, all strains are rapidly inactivated at pH <4 and pH >11 (Geering and Lubroth, 2002). Likewise, the survival of AIV is influenced by environmental factors including temperature, pH, and the presence of heavy metals (Stallknecht et al, 1990). In our study, the pH values of post-burial soils from different location ranged from 5.16～7.80. This phenomenon may be due to the high pH buffering effect of the type of soil (Wong et al, 2008), and possible decreased microbial activity in waterlogged soil (Carter et al, 2010). Reports also showed that AI virus can remain active in a dead animal body up to a few months de-

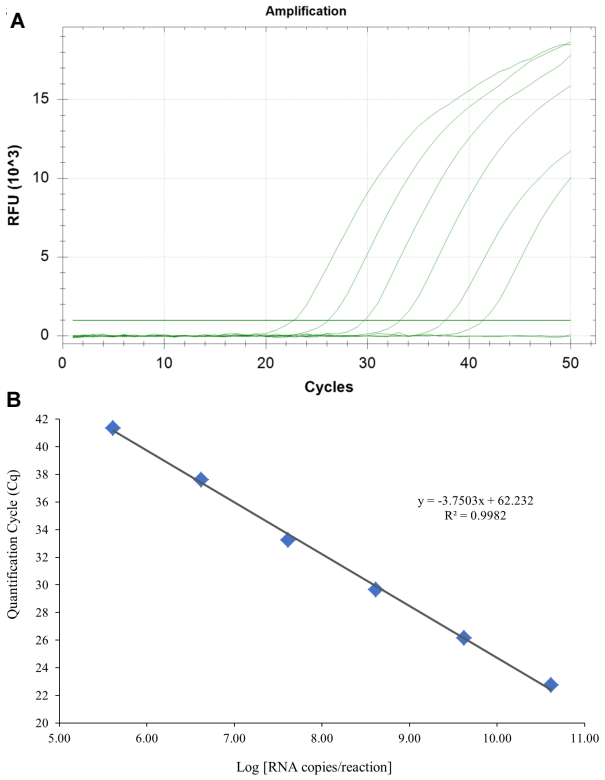

Fig. 1. Amplification plot (A) and standard curve (B) of the qRT-PCR assay. Serial 10-fold dilutions of FMDV (from 10^{10} to 10^{5} copies/reaction) RNA standard were plotted against the quantification cycle (Cq). The coefficient of determination (R^2) and the equation of the regression curve (y) calculated.

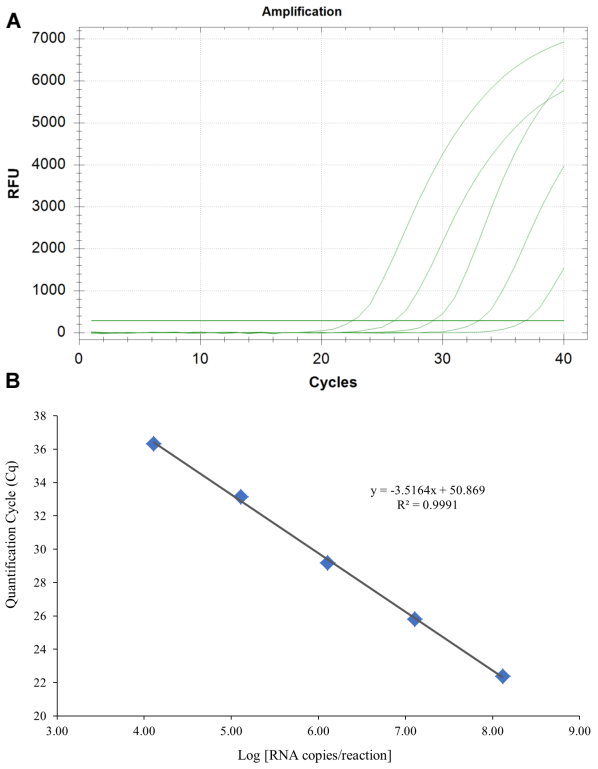

Fig. 2. Amplification plot (A) and standard curve (B) of the qRT-PCR assay. Serial 10-fold dilutions of AIV (from 10^{10} to 10^{6} copies/reaction) RNA standard were plotted against the quantification cycle (Cq). The coefficient of determination (R^2) and the equation of the regression curve (y) calculated.

Table 2. Detection of FMD and AI viral RNA in post-burial soil samples from carcass burial sites using real-time RT-PCR

Parameter	Livestock	Soil pH	qRT-PCR	Post-burial intervals (year)
Site 1-a	Swine, goat and bovine	7.80	-	>8 (Jan 2011～Apr 2019)
Site 1-b	Swine, goat and bovine	6.82	-	>8 (Jan 2011～Apr 2019)
Site 1-c	Swine, goat and bovine	5.56	-	>8 (Jan 2011～Apr 2019)
Site 2-a	Chicken	7.80	-	>3 (Nov 2016～)
Site 2-b	Chicken	6.82	-	>3 (Nov 2016～)
Site 2-c	Chicken	5.56	-	>3 (Nov 2016～)
Site 3-a	Chicken	5.24	-	>7 (Jan 2011～Dec 2018)
Site 3-b	Chicken	5.16	-	>7 (Jan 2011～Dec 2018)
Site 3-c	Chicken	5.18	-	>7 (Jan 2011～Dec 2018)

(-) represents viral RNA beyond detectable level.
NA: not applicable.

pending on the infected location (Yamamoto et al, 2010).

In most strains of FMD virus, the virus can be inactivated at 56℃ for 30 minutes. In addition, direct sunlight has little effect on the virus. Environmental inactivation is related more to the effects of desiccation (< 60% relative humidity) and temperature (Geering and Lubroth, 2002). Furthermore, studies showed that at ambient temperatures close to 20℃, the FMD virus survived for 10 days in the skin tissue that contained the highest initial concentration of viral RNA (Guan et al, 2010). The inactivation of the virus at this temperature is due to the decrease of pH during postmortem biochemical changes in tissues (Panina et al, 1989) and/or microbial activity during tissue decomposition (Vass, 2001). The degradation of the FMD viral RNA occurred more slowly at ambient temperatures than in compost, and the findings are consistent with observations on degradation of avian influenza at ambient temperatures and in compost (Guan et al, 2009).

Based on the results, viral RNA was not detected from the soil burial samples. This indicates that the viral nucleic acid structure was destroyed or inactivated on during carcass decomposition. The three-year period of burial was sufficient to eliminate the viral particles which suggests that the disposal method for disease infected animals was efficient for the control or at least reduction of spread of FMD and AI infection. However, other experimental conditions such as temperature, salinity, pH and moisture can affect the survivability of infectious agents. However, consistent monitoring is essential to verify safety of disposal against spread of disease and to prevent environment contamination.

ACKNOWLEDGEMENTS

This work was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture and Forestry (IPET) through Animal Disease Management Technology Development Program, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA) (118099-03).

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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