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RESEARCH ARTICLE

Estimation of Pathological and Molecular Findings in Vaccinated and Non-Vaccinated Chickens Challenged with Highly Pathogenic Avian Influenza H5N1 Virus

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ABSTRACT

The present study was performed to evaluate the findings in chickens vaccinated with various regimes and challenged with highly pathogenic avian influenza virus (H5). Forty SPF chicks were divided into four groups of 10 birds each, in which group I served as negative control. Group II was challenged with H5N1 avian influenza virus (positive control) at day 31. Group III was given inactivated vaccine at day 10, then challenged with H5N1 virus at day 31. Group IV was vaccinated with recombinant fowlpox vaccine at day old and boosted with inactivated vaccine at day 10, then challenged with HPAIV at day 31. Quantitative RRT-PCR was carried out on tracheal swabs of living birds and organs of dead birds to evaluate viral load. In addition, specimens from trachea, lungs, bursa of Fabricius, spleen and brain were collected from all birds for histopathologic, immunohistologic and electron microscopic examination. Viral RNA and antigen were demonstrated in examined organs in group II only using indirect immunoperoxidase and quantitative RRT-PCR. The pathological lesions detected were severe in group II, far less in group III and mild in groups I and IV. In conclusion, vaccination regime involving the use of two different vaccines resulted in much alleviation of the pathological alterations and conferred a better protection of chicken against highly pathogenic avian influenza than the use of one vaccine.

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INTRODUCTION

Infection of domestic poultry by avian influenza (AI) viruses produces syndromes ranging from asymptomatic infection, respiratory disorders and drop in egg production to severe, systemic disease with 100% mortality (Swayne *et al*., 2013). In natural and experimental highly pathogenic avian influenza virus infections (HPAIV), the association between the severity of lesions and the affinity of the virus for endothelial cells was demonstrated indicating that endothelial tropism plays a central role in the pathogenesis (Jones and Swayne, 2004).

In Egypt, HPAI subtype H5N1 virus was first reported in poultry in 2006 and till the meantime, it became enzootic. Even after 10 years, the virus is still deeply entrenched in which the total eradication is far from reach but also new waves of infections have been observed (Abdelwahab *et al*., 2016). Emergency vaccination was initially used to protect breeders, however, the disease spread among Egypt, and the decision was made in 2006 to vaccinate all commercial flocks (Abdelwahab *et al*., 2011). Avian influenza vaccination is still widely practiced in commercial poultry sector but with little or no post-vaccination monitoring (Kilany *et al*., 2014). From 2007 to 2011, the antigenic-variant viruses were widely embedded in commercial poultry sectors, which may be occurred due to the use of suboptimal vaccination regimes. Currently, more than 20 inactivated vaccines, derived from H5N1 or H5N2 strains, are licensed and often used in commercial poultry sector in Egypt (Abdelwhab *et al*., 2011). Studies carried on the use of inactivated H5 vaccines, under field conditions,

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have indicated their limited effectiveness and HPAI outbreaks were recorded in many vaccinated broilers, layer breeder and turkey flocks (Kilany *et al*., 2011). Recently, recombinant live vaccines based on baculovirus, Newcastle disease virus, herpesvirus of turkeys or poxvirus vectors carrying the HA of Egyptian viruses or other related H5 viruses (clade 2.2) conferred good protection (Kilany *et al*., 2014). The recombinant fowlpox-H5 avian influenza hemagglutinin vaccine can provide protection against varieties of HPAI-H5 viruses. Furthermore, frequent optimizing of hemagglutinin inserts to overcome genetic drift in the vaccine may not provide adequate field protection (Marcelin *et al*., 2012). The use of two different vaccination regimes, as inactivated vaccine and live Fowl Pox, vectored H5 vaccine, has provided a sufficient immune response against H5N1 challenge (Arafa *et al*., 2015). Eventually, the expected results of vaccination on the dynamics of infection are to reduce infection susceptibility and virus dissemination. The reduction in the amount of virus shedding is an important aid to reduce and /or eliminate the spreading of HPAI in poultry populations (Lee *et al*., 2004). Although numerous vaccines have been developed to protect against HPAI-H5N1, there are insufficient comparisons of their performance (Gambaryan *et al*., 2012).

This study was designed to estimate the efficacy of two vaccination programs against highly pathogenic avian influenza in Egypt.

MATERIALS AND METHODS

Birds: Day old (50-55 g), forty male Specific Pathogen Free (SPF) chicks, were purchased and housed in separate isolators (Bioflex 60-Bell laboratories) at 35±2°C, which was reduced 2°C/weekly, with a relative humidity of 60%. Birds received autoclaved pelleted diet (Cairo Poultry Comp., Egypt) and water *ad libitum.*

The virus: HPAI A-virus (A/chicken/Egypt/128S/2012 (H_5N_1), which represents the currently circulating H5N1 clade 2.2.1 viruses, was kindly provided by the AHR Institute, where the experiment was done. Each challenged bird administered intra-nasally 10^6 median EID_{50}, in 100 µl buffer. The protocol was approved by the research committee of the Institute in accordance with FELASA guidelines (2008).

Vaccines: Inactivated H5N1 Re-5 (A/duck/Anhui/1/2006-H5N1) within Clade 2.3.4) (QYH Biotech Co. Ltd, China) and Fowl pox vectored recombinant vaccine Trovac (Batch: 11091543, Merial, France) were used.

Experimental design: Forty male SPF chicks were divided into 4 groups, 10 birds each, namely: Group I (GI) neither vaccinated nor challenged and served as negative control. Group II (GII) (positive control) wasn't vaccinated and was challenged with purified HPAI virus (A/chicken/Egypt/128S/2012(H5N1) at day 31. Group III (GIII) was vaccinated once with inactivated AIV-H5N1 Re5 vaccine at day 10 (0.5mL/bird subcutaneously {s/c}). Birds of group IV (GIV) were prime vaccinated s/c with 0.2 mL of Trovac AIV-H5 at day one, then re-vaccinated with inactivated AIV-H5N1 Re5 vaccine at day 10 (0.5 mL/bird s/c). Birds of GIII and GIV were challenged at day 31 with H5N1 virus, where dead as well as sacrificed birds, were subjected to post-mortem and histopathological examination.

Histopathologic and Immunohistopathologic examination: Tissue specimens from trachea, lungs, spleen, bursa of Fabricius and brain of each bird were fixed in 10% neutral buffered formalin, processed by the paraffin embedding technique, sectioned at 5µm using microtome (Leica 2135) and stained with Haematoxylin and Eosin (H&E) (Suvarna *et al*., 2012). Histopathological changes including necrosis, hemorrhage, inflammatory cell infiltration, degenerative changes, edema and congestion were assessed based on a modified scale in which 1=none or negligible, 2=mild, 3=moderate, 4=moderate to severe and 5=severe (Jaleel *et al*., 2017). Tissue sections were microscopically examined and photographed by camera (Olympus XC30, Tokyo, Japan).

Immunohistologic examination of organs was done using diluted polyclonal primary H5 antibodies prepared in chicken (OIE Reports, 2010) and secondary antibody, anti-Chicken Horse Radish Peroxidase (KPL®, USA). Diaminobenzidine (DAB) substrate (Sigma chemical co., St. Louis, MO, USA) and hematoxylin, as counterstain, were performed according to manufacturer's instructions (Suvarna *et al*., 2012)

Transmission Electron Microscopy: One mm^3 blocks from trachea and bursa were fixed in 5% cold glutaraldehyde and processed at the Electron Microscope Unit, Assuit University. Sections were counter-stained with uranyl acetate and lead citrate and examined using TEM100CXII electron microscope and photographed using CCD digital camera (Bozzola and Russell, 1991).

Real-time reverse transcriptase-polymerase chain reaction: Quantitative real-time PCR for Matrix gene was carried out on tracheal swabs collected at the 3rd, 7th and 10th day post challenge and organs of seven birds each group. Automated viral RNA extraction from swabs and supernatant fluid of specimens were done using MagNA Pure LC Total Nucleic Acid Extraction Kit (Roche, Mannheim, Germany) following manufacturer's recommendations. RNA was eluted in a 60 µL diethyl pyro-carbonate treated water, stored at -70°C and examined.

Partial amplification of gene matrix was done using One-Step Real-Time PCR-Kit (QIAGEN, Valencia, CA). Primers and probes targeting M gene of AIV were used (Spackman *et al*., 2002); namely, forward primer M+25: 5´-AGATGA-GTC-TTC-TAA-CCG-AGG-TCG-3´, reverse primer M-124: 5´-TGC-AAA-AAC-ATC-TTC-AAG-TCT-CTG-3´and probe M+64: 5´-FAM-TCA-GGC-CCC-CTC-AAAGCC-GA-TAMRA-3´. QRT-PCR reaction was done in Stratagene MX3005P real-time PCR machine (Fig. 4a).

RESULTS

Clinical signs and necropsy findings: Birds of the negative control didn't show any clinical signs or mortalities during the entire experimental period. On the

other hand, the non-vaccinated and challenged chickens showed 100% mortalities within 48 hours. The birds had low feed intake and showed depression, severe respiratory distress shortly before death. Post-mortem examination revealed the presence of severe congestion of comb, wattles, lungs, liver, and spleen together with variable sized hemorrhagic foci in the lungs. In GIII, one bird died 48 hours post-challenge and post-mortem examination could not be done. Remaining birds of GIII and GIV survived the experiment and showed no clinical signs.

Histopathological findings: Chickens infected only with H5N1 expressed the most severe lesions (Table 1), in which trachea showed necrosis, sloughing of the epithelial lining, mild to moderate mononuclear inflammatory cells infiltration and congestion. Necrosis of bronchial epithelium, with intraluminal exudates in the bronchi, together with hemorrhages, mild to moderate mononuclear inflammatory cells infiltration was detected in the lungs. Severe depletion of the lymphoid follicles and heterophilic cells infiltration were also found in the spleen accompanied with few hemorrhagic foci and congestion of the red pulp. In the bursa, lymphoid follicles were severely depleted, sometimes with cystic formation, in addition to hemorrhages, interfollicular edema, congestion, together with necrosis and sloughing of epithelial lining the plicae (Fig. 1a). Degenerative changes of many neurons, with necrosis of some, accompanied by mild diffuse and focal gliosis were noticed in the brain (Fig. 1d).

The pathologic changes in the organs of chickens of groups III and IV were markedly less pronounced in comparison to GII (Table 1). Those birds receiving one vaccine (GIII), showed mild tracheitis, mild diffuse interstitial mononuclear cell infiltration, congestion, and interlobular edema in the lungs. Moreover, congestion of splenic red pulp, thickening, and edema in the vascular wall and moderate depletion of the lymphoid follicles were noticed. Lymphoid depletion and mild multifocal necrosis of the epithelial lining in the bursa were also noticed (Fig. 1b), in addition to mild multifocal gliosis and perivascular lymphocytic cuffing in the brain (Fig. 1e). In group IV, twice vaccinated, lesions were confined to congestion of tracheal blood vessels. Moreover, mild mononuclear cells infiltration, scattered, few focal areas of necrosis and moderate interstitial edema were found in the lungs. In addition, mild lymphoid depletion, mild blood vessel congestion in the spleen was detected. Remarkably, there were no histopathologic changes in the brain and the bursa of Fabricius (Fig. 1c) in this group.

Indirect Immunoperoxidase: Viral antigens were demonstrated in the trachea, lungs, bursa of Fabricius and brain of the challenged positive control group. Furthermore, in the same group, viral antigen was demonstrated in the endothelial lining and tunica media of blood vessels in the lungs (Fig. 2a), spleen (Fig. 2b), epithelial lining and lymphoid elements of lymphoid follicles in bursa of Fabricius (Fig. 2c) and in the endothelial lining and degenerated neuronal cells in brain (Fig. 2d). On the other hand, in the vaccinated and challenged groups (GIII and GIV) viral antigen could not be detected in all examined organs.

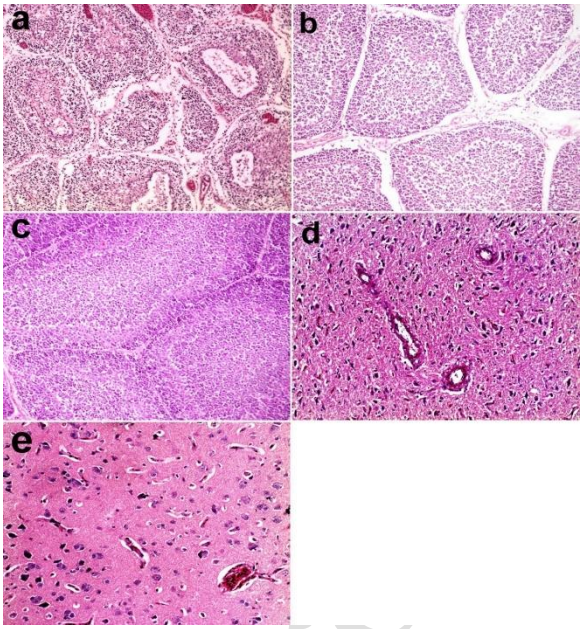

Fig. 1 (a-c): bursa of Fabricius, (a): marked lymphoid depletion with cystic formation, GII. (b): mild to moderate lymphoid depletion and interfollicular edema, GIII. (c) No lymphoid depletion, GIV (X200). Figures (d-e): Brain, (d): neuronal degeneration and neuronophagia in brain stem, (GII). (e): necrosis and degenerative changes in neurons, cerebrum, GIII (X400). Chicken, (H&E stain).

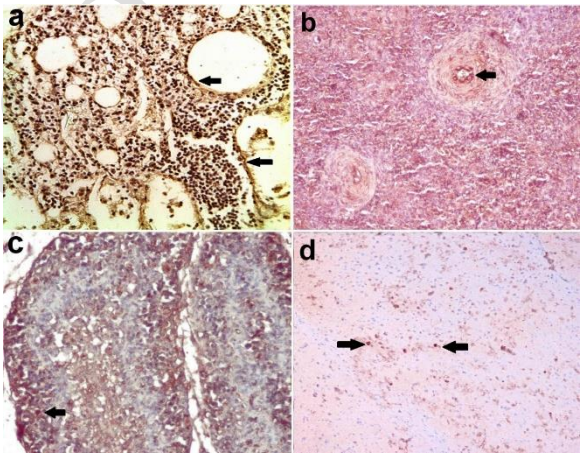

Fig. 2 (a-d): Avian Influenza in chicken (GII). (a): brown granular positive staining of viral antigen, pneumocytes and endothelial lining of blood vessels in Lung (X200). (b): in endothelial lining and tunica media of blood vessels, spleen (X400). (c): in lymphocytes of lymphoid follicles, bursa (X200). (d): in neurons and endothelial cells, brain (X100). Immunoperoxidase stain.

Transmission electron microscope findings: Ultrastructural examination revealed a large number of viral particles in the trachea (Fig. 3a) and lymphoid follicles of the bursa which was accompanied with variable figures of nuclear damage in GII (Fig. 3c). In GIII, few numbers of orthomyxovirus-like particles were detected in the cytoplasm of tracheal epithelium and bursal lymphocytes (Fig. 3b, d) accompanied with nuclear chromatin disruption, however, no viral particles were found in group IV.

Quantification of matrix gene of AIV using quantitative RT PCR: Viral RNA was detected in examined organs of positive control chickens, in which high virus load was observed in lungs and bursa and low

load in the brain using qRT-PCR (Fig. 4). In GIII and GIV, tracheal swabs from live birds at 3, 7 and 10 days post challenge were negative for AIV-RNA by real-time RT-PCR. In addition, the viral RNA was not detected in all organs of chickens of both groups except in one dead bird in group III after challenge, which was quantified as 1.7×10^5.

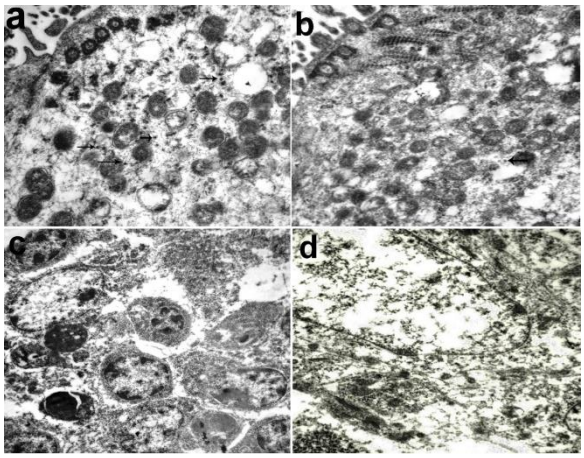

Fig. (3a-3b): Avian Influenza, Electron micrograph of trachea, (3a): large numbers of virus particles in epithelium GII. (3b): few virus particles in epithelium, GIII (X19000). Figures (3c-3d): Avian Influenza, bursa (3c): viral particles, nuclear degeneration, and hyaline bodies in lymphocytes, GII (X5800). (3d): Few viral-like particles and nuclear chromatolysis in lymphocytes, GIII (X19000). TEM.

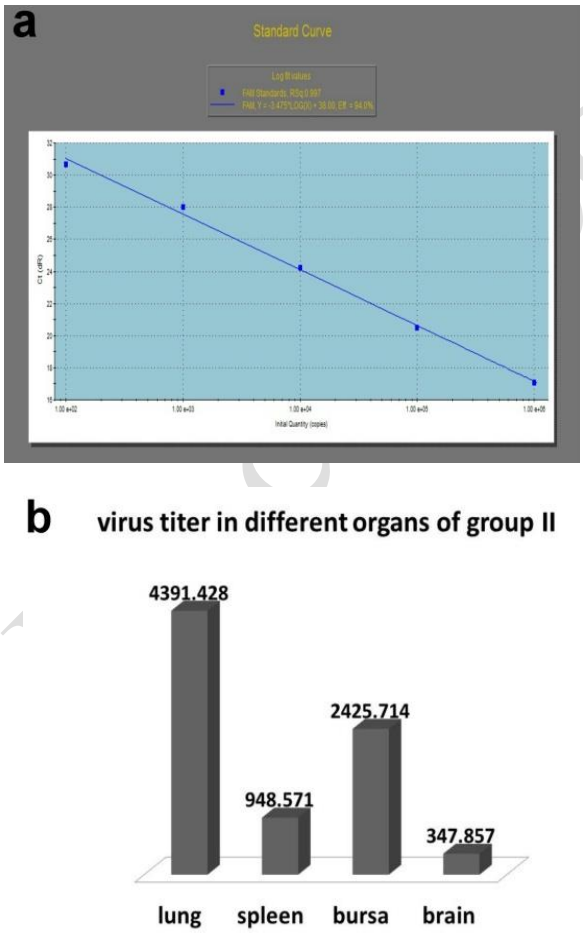

Fig. 4: (a) Standard curve used for viral quantification using QRT-PCR. (b) Avian influenza virus quantification in different organs of chickens in GII (positive control group) using quantitative RT-PCR.

DISCUSSION

Influenza-A viruses are worldwide one of most important pathogens in human and veterinary health. Infection with AI virus can be devastating disease causing enormous economic losses of poultry industry nationwide. AI virus can cause a range of disease symptoms from subclinical infection to highly virulent with 100% mortality (OIE Reports, 2010). In developing countries, vaccination might be a suitable alternative to pre-emptive culling and confinement during avian influenza outbreaks (Swayne *et al*., 2013).

Pathologic lesions were present in nervous, circulatory, respiratory, integumentary, musculoskeletal, gastrointestinal, and reproductive systems (Akanbi and Taiwo, 2014). High mortality and severe clinical signs were observed in group II infected with HPAI, in our experiment. Infection of the vascular endothelium by the HPAI virus led to rapid systemic dissemination and per-acute death by asphyxiation, in addition to hemodynamic changes like disseminated intravascular thrombosis (Brown *et al*., 1992). Similar findings were noticed also in challenged group with the Egyptian H5N1 strain without vaccination. In contrast, signs of clinical disease and death caused by HPAI challenge were generally very mild in both vaccinated groups. Vaccination protects against mortality and clinical signs, reduces virus shedding and increase resistance to infection (Swayne *et al*., 2013). The degree of protection afforded by the vaccine is based mainly on elicited immune response (Marcelin *et al*., 2012). Multiple organs are infected with HPAI virus and rapid replication and high virus load are correlated with disease pathogenicity in chickens (Suzuki *et al*., 2010).

Histopathologic lesions observed in respiratory organs of non-vaccinated and challenged birds were typical changes of HPAI similar to other authors (Akanbi and Taiwo, 2014). Necrosis of bronchial epithelium, with mucous exudates in bronchial lumen, in addition to severe diffuse hemorrhages with numerous interstitial mononuclear inflammatory cells infiltration and edema in lungs, were also reported (Brown *et al*., 1992). HPAI-H5N1 had a strong tropism to the cardiovascular system, principally vascular endothelium, resulting in necrosis, with subsequent hemorrhages and edema (Jones and Swayne, 2004). Severity of these lesions, however, has been decreased in the two vaccinated groups. Previous studies of natural and experimental HPAIV infections have demonstrated the association between severity of lesions and the affinity of the virus to endothelial cells, indicating that endothelial tropism has a central role in pathogenesis (Jones and Swayne, 2004).

Severe lymphoid depletion in spleen and bursa is considered a characteristic feature of HPAI that renders birds vulnerable to secondary bacterial infections which could be attributed to the direct effect of the virus, causing lymphocytic necrosis (Khanna *et al*., 2001). In our study, the severity of lymphoid depletion in spleen and bursa varied between groups; namely, least lymphoid depletion was noticed in GIV followed by group GIII, respectively, with no/mild depletion in the bursa. HPAI was known to infect CNS and induce microscopic changes (Guan *et al*., 2002) and such mild lesions were seen in GIII. Characteristically, brain lesions were absent in GIV, which might be due to the prime-boost vaccination strategy, that limits viral spread.

Table 1: Lesion score in organs and number of affected birds/group

Organ	Negative control (GI)		Positive control (GII)		R5 vaccinated (GIII)		R5+Trovac vaccinated (GIV)	
	Lesion score	Number of birds	Lesion score	Number of birds affected	Lesion score	Number of birds affected	Lesion score	Number of birds affected
Trachea	1	10/10	5	9/10	2	7/9	2	6/10
Lung	1	10/10	5	10/10	3	6/9	2	5/10
Spleen	1	10/10	5	9/10	4	6/9	3	6/10
Live	1	10/10	5	9/10	3	4/9	2	4/10
Kidneys	1	10/10	5	9/10	4	4/9	2	4/10
Bursa	1	10/10	5	8/10	4	6/9	2	5/10
Brain	1	10/10	5	9/10	4	6/9	2	5/10

(Lesions score: 1=none, 2=minimal, 3=mild, 4=moderate, 5=severe).

H5N1 antigen was detected in lungs, brain, pancreas and capillary endothelium of organs (Brown *et al*., 1992; Cavicchioli *et al*., 2015). In the present study, viral antigen was found in the lungs, brain, and bursa of group II, which confirms viral replication that induces the histopathological lesions. Although mild histopathological lesions were recorded in GIII and GIV, viral antigen was absent in these organs, which may have been attributed to neutralization of the virus via the antibodies produced by vaccination. Several studies have emphasized on the presence of viral antigen in capillary endothelium similar to our findings that demonstrated viral antigen in endothelial lining. Therefore, HPAI-virus is believed to have a strong affinity for vascular endothelium and perivascular tissue, which in turn aids viral dissemination and systemic infection (Korteweg and Gu, 2008).

Although chickens were vaccinated in GIII and GIV, few viral particles were detected by electron microscope in the trachea. It was found that the virus was still capable of replication in clinically healthy vaccinated birds (Swayne *et al*., 2013). Never less, few viral particles were detected in trachea and bursa only in GIII, denoting that two vaccination regimes have limited viral dissemination and replication, in comparison to the use of one vaccine only.

The prime-boost strategy has proved its efficacy inducing an optimal immunity against H5N1 and minimal viral replication after challenge in duck (Steensels *et al*., 2009). An effective protection against lethal challenge of HPAI-virus was noticed in chickens (Wujie *et al*., 2008). Birds treated with two vaccines showed no clinical signs and only mild histopathologic lesions. In addition, the absence of viral antigen using immune-histochemical staining and viral particles via electron microscopic examination indicated the absence of viral shedding from organs.

Conclusions: Consequently, it could be concluded that the use of two vaccination regime was effective and provided an adequate protection against HPAI H5N1 clade 2.2.1 circulating in Egypt.

Authors contribution: AB designed the study. AA and FA executed the experiment and analyzed the RT-PCR samples. AH and MK analyzed the data and wrote the manuscript. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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