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Isolation of Newcastle Disease Virus Genotype VII from Native Chicken in Republic Democratic of Timor-Leste

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Abstract

Newcastle disease (ND) is a contagious disease and still a threat to the development of chicken farms in several countries including the Republic Democratic of Timor-Leste. There were reported local outbreaks every year in the country. The causative agent of ND is Avian orthoavulavirus-1 (AOAV-1) common name Newcastle disease virus (NDV). The objective of this study was to isolate the NDV currently circulating in Timor-Leste and to determine its genotype based on phylogenetic tree analysis and its virulence based on molecular analysis of the Fusion (F) gene cleavage site. In this study samples of dead chickens suspected due to ND were taken from two different sites in Timor-Leste namely Kilotons and Atabae districts. Tissue samples were collected for histopathological examination and viral isolation. Allantoic fluids were harvested and confirmation of NDV was carried out by standard methods hemagglutination test (HA) and the hemagglutination inhibition test (HI). Partial fragments of the F and HN proteins gene were amplified using NDV-specific primers in a one-step RT-PCR reaction. The PCR product was then sequenced, and the nucleotide sequences were then used for building a phylogenetic tree with other NDV strains representative of genotype I-VII that are available in the GenBank. Based on phylogenetic analysis it was found that the new isolates belonging to genotype VII with the amino acid sequence of the F gene cleavage site were a virulent type and possibly viscerotropic velogenic NDV.

KEYWORDS
Cleavage site, Fusion protein, HN protein, NDV, Timor-Leste

INTRODUCTION

Free-range chicken is one of the animal commodities that are mostly kept as backyard poultry farming in Timor-Leste. The chicken contributes to a very meaningful financial need of rural families. However, the main constraint to productivity is the high mortality rate due to the disease. Newcastle disease (ND) is suspected to be one of the deadly threats, the ND outbreak is commonly found throughout the year in the country.

The causative agent of ND is Avian orthoavulavirus-1 (AOAV-1) commonly named ND virus (NDV), the virus is belonging to the orthoavulavirus genus superfamily of Mononegavirales in the family Paramyxoviridae (Amarasinghe *et al*., 2019). This family belongs to the envelope ribonucleic acid (RNA) virus. The envelope component is an infectious part of the virus due to two proteins namely Fusion (F) and Hemagglutinin Neuraminidase (HN) that play an important role in the mechanism of viral infection (Kim *et al*., 2017).

The F protein functions to penetrate the virus into the host cell and play a role in the formation of syncytia in infected cells, while the HN protein mediates the attachment of the virus to the host cell. The HN protein is responsible for attaching virions to target cells, while F protein functions to destroy target cells and induce the incorporation of certain cell membranes and has the ability to replicate in certain cells (Kim *et al*., 2017).

Based on its virulence to chickens, the NDV is classified into 3 strains, namely: velogenic, mesogenic, and lentogenic. However, based on clinical symptoms there were 5 virus pathotypes, name-

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ly viscerotropic velogenic, neurotropic velogenic, mesogenic, lentogenic, or respiratory and asymptomatic types (OIE, 2021). NDV virulence can be predicted by molecular approach, the sequence of the F protein cleavage site of velogenic and mesogenic strains is a dibasic amino acid cleavage motif surrounding glutamine ^{112}(R/K) RQ(R/K)RF^{117}. The dibasic amino acid motif concedes for cleavage by protease(s) which are available in cells throughout the body developing a pantropic or systemic infection. The lentogenic F cleavage site is a monobasic ^{112}(G/E)(K/R)Q(G/E)RL^{117}, limited to tissues that have certain trypsin-like protease(s), exhibit mild infection limited to certain organs such as the respiratory tract and digestive tract (OIE, 2021).

ND is still endemic in many countries, and many reports regarding the current isolation of NDV from various parts of the world (Msoffe *et al.*, 2019; Moharam *et al.*, 2019; Alazawy and Al Ajeeli, 2020; Mousa *et al.*, 2020) including Indonesia (Adi *et al.*, 2019b). However, as far as the author's knowledge, there have been no reports of native Timor-Leste isolates. So the objective of this study was to isolate and determine the genotype of the Timor-Leste field isolate. Virus isolation was performed in this study from tissue samples of ND suspected chickens. Histopathological examination of the tissue sample and molecular analysis of the partial F and HN gene of the isolated virus was performed.

MATERIALS AND METHODS

Ethical Approval

This study was approved by the Animal Ethics Committee of the Veterinary Medicine Faculty, Udayana University (Reference number: B/85/UN14.2.9/PT.01.04/2019).

Chicken Suspected Newcastle Disease and Tissues Sample

Chickens suspected of having ND were taken from backyard flocks at two different districts in Timor-Leste, i.e., Kilotons and Atabae districts. The choice of sampling location was based on the survey on the availability of dead chickens suspected of having ND in the period from October to November 2020. Four chicken cadavers were taken from each place. By eliminating all possible lesions that are not caused by ND that may interfere with the result then one chicken without worms was selected for further work from each place. These two roosters suspected of having ND, 10 to 14 months old were selected based on the characteristics of clinical findings before the death such as severe greenish diarrhea, depression, respiratory distress, eye swelling, and no worm found during necropsy. Infected tissues such as the brain, trachea, lung, proventriculus, intestine, and spleen were collected for histopathological examination and viral isolation. Tissue samples for histopathological examination immerse in 10% neutral-buffered formaldehyde. Those for viral isolation were placed in ice gels, transported to the laboratory then kept frozen at −40°C before shipping on dry ice to the Virology Laboratory Disease Investigation Center at Denpasar Bali-Indonesia.

Histopathological Examination

The fixed tissue was further processed in a tissue processor starting from dehydration, clearing, and impregnation then the paraffin-embedded tissue was manually sectioned with a microtome to obtain 4-5 µm-thick paraffin sections, and the dewaxed sections were stained with hematoxylin and eosin (HE).

Virus Isolation

The virus was isolated and propagated in embryonated chicken eggs (ECEs) 9 to 11 day-old seronegative (SN) for ND. Pooled tissues of the brain, trachea intestine, lung, and spleen were then crushed and the supernatant was inoculated via the allantoic cavity route of the ECEs. The ECEs were incubated at 37°C for 48-72 h. All ECEs with dead and dying embryos at more than 24 h post-inoculation (p.i.) were removed and chilled for 4 h at 4°C. Allantoic fluid was harvested and the hemagglutination (HA) test is carried out to detect the presence of virus capable to agglutinate red blood cells followed by serological NDV testing with hemagglutination inhibition (HI) test with anti-NDV antiserum using conventional microtiter methods as described by OIE (2021). Isolated viruses were aliquoted and kept at −80°C until further use.

Isolation of Viral RNA and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

The viral RNA was extracted from allantoic fluid with Trizol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Briefly allantoic fluid mix with Trizol in a ratio of 1:3. The mixture was then allowed to stand at room temperature for 5 min, then 200 µl chloroform was added. After being vortexed, the mixture is allowed to stand for 15 min then followed by a centrifuge for 15 min at 14,000 rpm and the aqueous phase was collected. The aqueous phase was then added 500 µl of isopropyl alcohol and returned to allow for 10 minutes. The mixture was centrifuged at 12,000 rpm for 10 minutes and the supernatant was thrown away. The pellets were then washed with 1,000 µl of 70% alcohol, and centrifuge at 7,500 rpm. Finally, the supernatant was removed, RNA dried, and suspended in distilled water which is free from the RNAse enzyme (diethylpyrocarbonate treated water). The extracted RNA was then used as a template for RT-PCR amplification.

One-step RT-PCR was performed using SuperScript™ III One-Step RT-PCR system with Platinum® Taq DNA Polymerase (Invitrogen). The major steps carried out in RT-PCR for DNA amplification included the synthesis of the complementary DNA (cDNA) by using the reverse transcription enzyme (50°C for 60 min). Then was followed by an initial denaturation (95°C for 7 min), denaturation (94°C at 45 s), annealing (52°C for 45 s), and extension (72°C for 1 min). The cycle repeated 39 cycles and a single cycle of final extension at 72°C for 5 min. For the positive and negative control, the viral RNA was extracted from a commercial live ND vaccine (Medivac ND LaSota, Medion, Bandung, Indonesia) and distilled water, respectively. The RT-PCR reaction was set up in a total volume of 10 µL per reaction comprised R-mix (dNTP, $MgSO_4$, and buffer) 5 µL, 10 pmole concentration of each primer (forward primer and reverse primer) each 0.6 µL, Tag DNA Polymerase 0.25 µl, distilled water 2.55 µl, and 1 µL RNA template.

NDV specific primer used for amplification of the F gen amplified a 356 bp F gene sequence encoding the segment fusion protein that includes the cleavage site comprised of forward: 5′-GCAGCTGCAGGGATTGTGGT-3′ and reverse 5′-TCTTTGAGCAGGAGGATGTTG-3′ (Nanthakumar *et al.*, 2000). Meanwhile for amplifying the partial HN gene was used primer comprised of forward: 5′-CAGAGATCACTCATTCAT-3′ and reverse: 5′-GCCTAAGGATGTTGACACCT-3′ yielding 520 bp product (Adi *et al.*, 2019b). The amplicon was then gel electrophoresis in 2% agarose with TAE buffer (40 mM Tris-HCl, 40 mM acetic acid, and 1 mM ethylene-diamine-tetra-acetic acid (EDTA) and stained with 500 µg/L ethidium bromide visualized under UV light as previously done by Adi *et al.* (2019b).

Sequencing and Phylogenetic Analysis

PCR products are sent to the DNA sequencing service company (PT. Genetika Science, Tangerang, Indonesia) to be purified and to be sequenced from two directions (front and back). To identify the genotype of the newly studied sequences, the phylogenetic tree was built using the sequences of those reference strains available in the GenBank. The sequences were aligned using ClustalW and analyzed in MEGA7 (Kumar *et al*., 2016) with other sequences representing NDV genotype I-VII of Class II and one isolate from class I as an outgroup.

Nucleotide Sequence Accession Numbers

Partial nucleotide sequences of F and HN genes from two isolates, namely Timor-Leste-2 (TL-2) and Timor-Leste-7 (TL-7) obtained in this study were submitted to the GenBank and available under accession numbers: MW703440 and MW703442 for F gen of TL-2 and TL-7. MW703441 and MW703443 for the HN gene of TL-2 and TL-7.

RESULTS

In this study, sample selection was carried out based on a survey report of chicken death in chicken flocks with clinical symptoms of ND from two districts Kilotons and Atabae districts. We focused our study on observing the pathological features and molecular characteristics of the two new isolated viruses.

The necropsy revealed inflammation of the trachea, hemorrhages of the lungs, petechial hemorrhages in the mucosa of the proventriculus, hemorrhagic lesions in the intestine, and necrosis of the spleen. Histopathological examinations of the tissues sample of the ND case from Kilotons and Atabae both confirmed the gross lesion (Table 1). Based on microscopic examination hemorrhage and necrosis of parenchymal cells are typically found in the lung, intestine, and spleen (Figs. 1a-c). In the brain was found congestion and vasculitis in the case of Timor-Leste-2. Both did not show perivascular cuffing in their brain.

Using supernatant of pooled tissues that were inoculated via the allantoic cavity route of 10-days ECEs, it was found that the embryos were inactive within 48 h post-infection with the HA titer log 2^6-log 2^7 and HI titer log 2^7-log 2^8 for Timor-Leste-2 and Timor-Leste-7 isolates, respectively. After the amplification using one-step RT-PCR with NDV specific primer for F gene and HN gene revealed 356 and 520 bp products respectively (Fig. 2). The phylogenetic tree was built using the F gene of new isolated and the selected strains representing Genotype I-VII of Class II showed that the isolated viruses, i.e., Timor-Leste-2/2020 (GenBank accession no. MW703440) and Timor-Leste-7/2020 (GenBank accession no. MW703442) belonged to the genotype VII (Fig. 3a) very close to Indonesian isolate with genetic distance

Table 1. Microscopic lesion of the tissue examined from chicken case

Sample tissue	Lesions	Timor-Leste-2	Timor-Leste-7
Brain	Congestion	√	√
	Vasculitis	√	None
	Perivascular cuffing	None	None
Trachea	Tracheitis hemorrhagic	√	√
Lung	Necrosis	√	√
	Hemorrhage	√	√
	Lymphocytic cells infiltration	√	√
Proventriculus	Necrosis	√	√
	Hemorrhage	√	√
Intestine	Necrosis	√	√
	Hemorrhage	√	√
Spleen	Hemorrhage	√	√
	Lymphoid Depletion	√	√

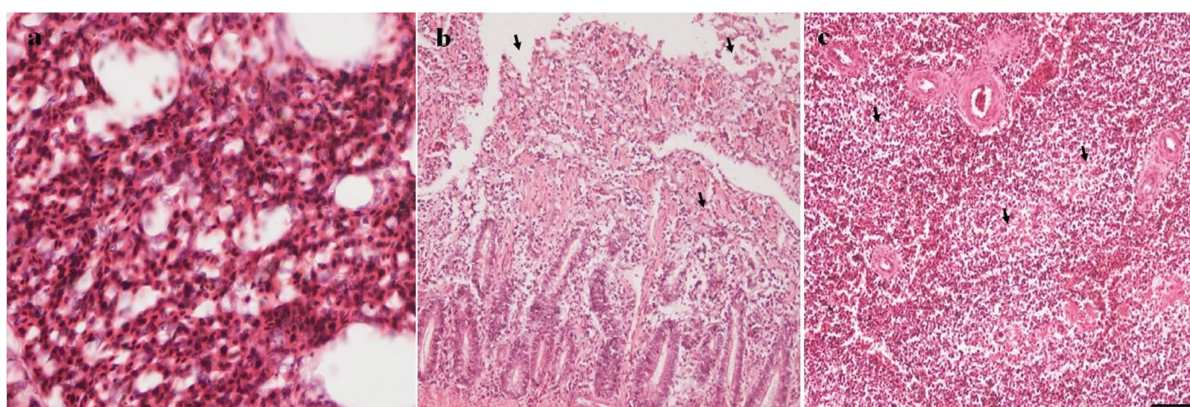

Fig. 1. Prominent histopathological features observed in several tissues were hemorrhage and necrosis (arrow). (a) The lung of chicken with hemorrhage (bar=50 µM). (b) Intestine with hemorrhage and necrosis (bar=100 µM). (c) Spleen with depletion of the lymphoid follicle of white pulp (arrow) and hemorrhage (bar=100 µM).

4%. Amino acid analysis of the F protein cleavage sites revealed that the two newly NDV isolates have dibasic amino acid surrounding glutamine (RRQKRF), typical sequences of the virulent type (Fig. 3b).

DISCUSSION

In this study phylogenetic tree based on HN was also performed. It was found that the HN sequences Timor-Leste-2/2020 HN and Timor-Leste-7/2020 share monophyletics branch with isolates from Indonesia, Tabanan-1/ARP/2017 (Fig. 3c) with a genetic distance 2%. Prominent pathological lesions of both samples were hemorrhage and necrosis, especially in the respiratory and digestive tract as well as the spleen. Although virulent NDV is pantropic as reported previously by Mousa *et al.* (2020), lesions due to viscerotropic velogenic virus are more dominant in the gastrointestinal tract with extensive inflammatory and necrotic features in the spleen, thymus, bursa of Fabricius, and gut-associated lymphoid tissues (GALT) (Kai *et al.*, 2015; Mariappan *et al.*, 2018; Kabiraj *et al.*, 2020). Necrosis of lymphoid cells in the spleen, as well as gut-associated lymphoid tissue (GALT), is possibly due to viral replication (Eze *et al.*, 2014). In this study, the thymus and bursa of Fabricius are not available due to the age of the chickens, however, depletion of lymphoid cells in the spleen (Fig. 1c) is found.

Some reports suggest that infection with genotype VII virus exhibits severe lesions in the spleen (Perozo *et al.*, 2012; Adi *et al.*, 2019a). It was reported that matrix (M), F, and HN genes were associated with the severity of lesions in the spleen (Kai *et al.*, 2015). The two Timor-Leste isolates are very close to Tabanan-1/ARP/2017 isolate which also causes severe lesions in the spleen (Adi *et al.*, 2019a). The more rapid progress in the field of nucleotide sequencing techniques, the greater the number of APMV-1 viruses in the online database. Long or short base sequences all

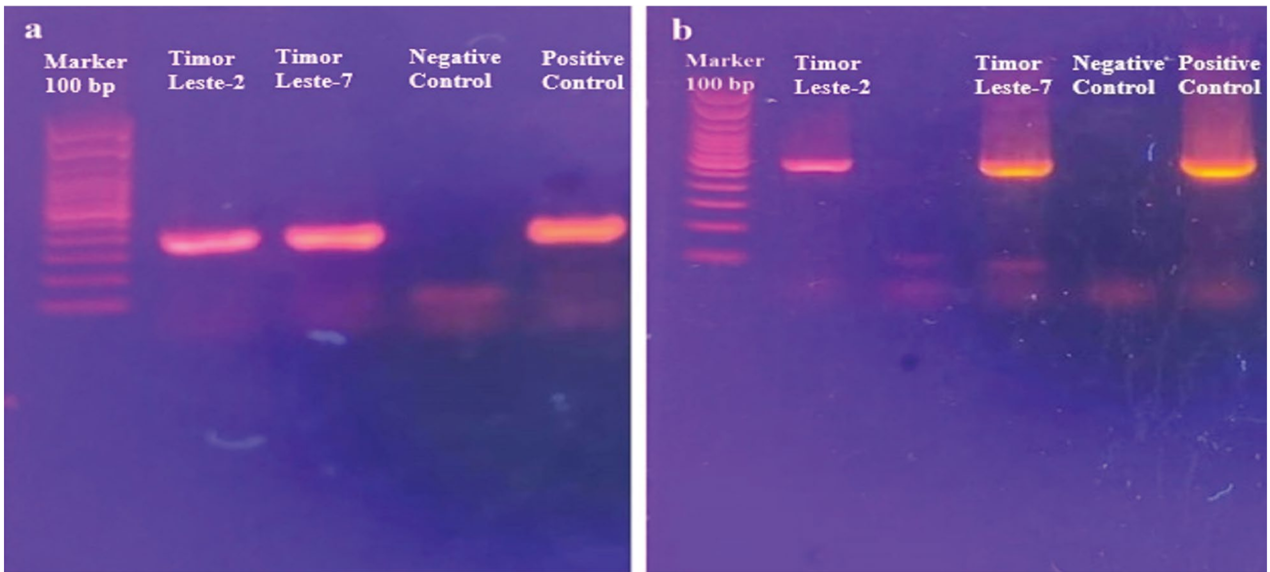

Fig. 2. Agarose gel electrophoresis of amplification targeted of 256 bp PCR products of NDV Timor-Leste-2 and Timor-Leste-7 generate using NDV specific primer for partial F gene (a) and 550 bp PCR product generated using the primer for partial HN gene (b).

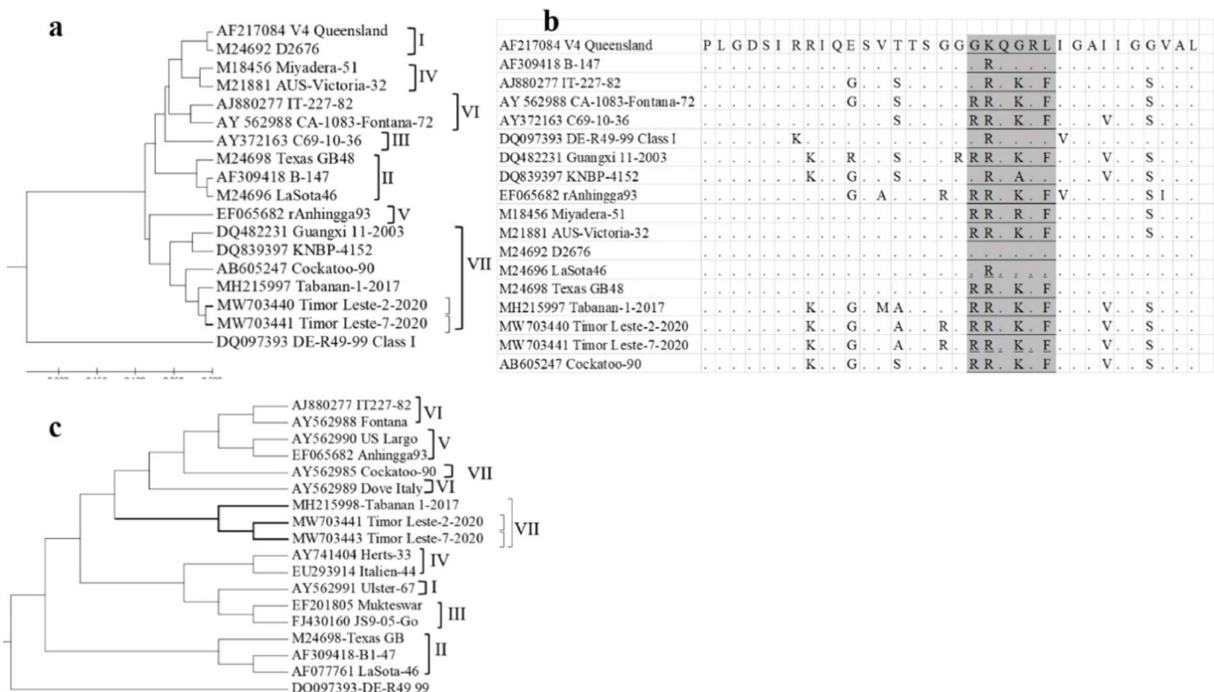

Fig. 3. (a) Phylogenetic tree was built using partial F gene (232 bp) of Class II representing genotype I-VII and Class I as an outgroup. New isolated sequences are indicated. The evolutionary history was inferred using the unweighted pair group method with arithmetic mean (UPGMA) within MEGA7 (Kumar *et al.*, 2016) (b) Alignment of amino acid sequence covering F protein cleavage site (box-shadow). Dots indicate the same amino acids as above. The new isolates have cleavage site motif RRQKRF (c) Phylogenetic tree build based on partial HN gene (507 bp) the isolates share monophyletics branch with Indonesian isolate.

contribute to phylogenetic analysis and this information is invaluable in assessing global epidemiology and local distribution from ND (Diel *et al*., 2012; Dimitrov *et al*., 2019). In this study, two short fragment genomes of F and HN protein from newly isolated NDV isolate from Timor-Leste were analyzed.

Phylogenetic analysis using the F gene sequence was used to determine the virus genotype (OIE, 2021). Virulence of the virus is molecularly determined by the motif of the amino acids at the F gene cleavage site, virulent NDV for chickens has the sequence amino acid motives: ^{112}R/K-R-Q/K/R-K/R-R-F^{117} (OIE, 2021). These two new isolated isolates also showed amino acid sequence in the fusion cleavage sites (Fig. 3b) ^{112}R-R-Q-K-R-F^{117} typical to the virulent type. Based on the phylogenetic analysis of the F gene it was revealed that the two newly isolated Timor-Leste isolates belong to genotype VII, together with strains isolated from Tabanan-Bali Indonesia with a genetic distance of 4%. NDV genotype VII is the most dominant genotype spread throughout the world, including in Indonesia (Xiao *et al*., 2012). All viruses belonging to genotype VII are virulent viruses that are common as a causative agents of ND outbreaks worldwide (Xue *et al*., 2017). Whether genotype VII solely genotype present in Timor-Leste is required to be assessed further. Although for genotyping purposes, only F gene sequence analysis is required, in this study the HN gene is also analyzed, considering that this gene also play important role in viral pathogenicity. Based on phylogenetic analysis of the HN gene it was found the Timor-Leste isolate is closely related (genetic distance 2%) to the Indonesian isolate from Bali. The common vaccine used in Timor-Leste is V4 (genotype I) live vaccine, the genetic distance between the Timor-Leste-2 and Timor-Leste-7 to the V4 vaccine was found 16%. With those vaccines, strains belong to genotype II LaSota and B1 also 16%. Based on genetic distance analysis, the choice of vaccine is (using live vaccine of Genotype I) correct, it may be necessary to evaluate the application in the field to prevent ND outbreaks in this country. Besides that, it is necessary to evaluate the virus that circulates in the field periodically.

CONCLUSION

This work is the first endeavor to isolate and analyze molecularly the genotype of NDV field strain from the Republic Democratic of Timor-Leste. The results showed that the virus currently circulating in Timor-Leste is genotype VII with the F gen cleavage site motif ^{112}RRQKRF^{117} typically of the virulent type.

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CONFLICT OF INTEREST

The author declares that no competing interests exist.

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