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Original Article

Retrospective analysis of Marek's disease virus outbreak in 2013 on chicken farms in North Sumatra, Indonesia

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Abstract

Objective: Marek's disease is lymphoma in chicken caused by Marek's disease virus serotype 1 (MDV-1), characterized by paralysis and tumour formation. The disease has caused enormous economic loss worldwide, including in Indonesia. Unfortunately, molecular data related to MDV-1 from Indonesia is limited. Therefore, this study aimed to identify genetic characteristics of MDV-1 causing outbreaks in commercial farms in North Sumatra, Indonesia, 2013.
Methods: The amplification of Meq, pp38, gC, and gE genes of the strain MDN2013 was carried out using the PCR protocol. Fragment gene sequencing was accomplished using the Sanger method. Subsequently, the bioinformatic analyses of BLASTN and phylogenetic tree were applied to analyze the molecular characteristics of these gene sequences.
Results: The Meq, pp38, gC, and gE genes of the strain MDN2013 were successfully sequenced and submitted to the NCBI GenBank database. The Meq genes share a high identity with Asian viruses, mainly those originating from Indonesia, India, and China. The pp38, gC, and gE genes share similarities with other viruses in more diverse regions in Asia, Africa, Europe, and America. The phylogenetic tree analysis of the Meq gene demonstrated specific patterns for pathotype biodiversity where the strain MDN2013 has been categorized as virulent up to very virulent virus.
Conclusion: In conclusion, the molecular approach is an effective tool for investigating the MDV-1 biodiversity in the field, especially the Meq gene sequence analysis.

Keywords: Indonesia; Marek's; MDV-1; Meq; gC; gE; pp38

INTRODUCTION

Marek's disease is lymphoma disease in chicken caused by the infection of Marek's disease virus serotype 1 (MDV-1) known as Gallid herpesvirus 2 (GaHV-2) belonging to the Mardivirus genus of Alphaherpesvirus subfamily [1]. In addition, the two other members of Mardivirus are non-pathogenic viruses, including MDV serotype 2 (MDV-2), also known as Gallid herpesvirus 3, GaHV-2), and serotype 3 (MDV-3), also known as Meleagrid herpesvirus 1 (MeHV-1) or Herpesvirus of turkey (HVT). There are two forms of Marek's disease clinical symptoms, namely neural and visceral type [1]. The neural type includes complete or partial paralysis of the neck, wings, and limbs

caused by lesions of the vagus, brachial, and sciatic plexuses. Gross tumours in various organs such as the gonads, liver, kidney, lung, heart, spleen, and proventriculus manifest the visceral type. The tumour could also be observed in the ocular and skin. The persistent infection of MDV-1 in the farm environment is caused by extreme virus stability in feather dander as a shedding route for transmission [2, 3]. Thus, the disease always poses a threat to commercial chicken farms all over the world.

Nowadays, the vaccination strategy is known to be the most effective preventive system for Marek's disease. Despite the global number of disease outbreaks having reduced significantly over the last decades, several cases have still occurred [4,5]. The vaccination could protect chickens from the clinical manifestations; however, the field strain of viruses can still infect vaccinated chickens as a subclinical infection, resulting in persistent environmental circulation [6]. According to historical virus evolution caused by vaccine intervention, the possibility of virus mutation becoming more pathogenic is relatively high since several strains have caused outbreaks, even in vaccinated flocks [7]. Therefore, continual disease surveillance is essential to anticipate upcoming outbreaks.

Molecular characterization to analyze field strain has become essential for approaching Marek's disease surveillance [8, 9]. Many of Marek's disease gene sequences have been submitted to the NCBI GenBank database. This genetic data is valuable to develop a molecular characterization system based on bioinformatic analysis [7]. However, the molecular data of Marek's disease virus serotype 1 from Indonesia is limited, even though several outbreaks have been reported highlighting a virulent strain circulated in the area [10]. Therefore, the main objective of this study was to retrieve genetic sequence data of the retrospective sample from the MDV-1 outbreak in commercial chicken farms in North Sumatra, Indonesia, which occurred in 2013. Bioinformatic analyses were performed to identify this Indonesian strain's molecular characteristics.

MATERIALS AND METHODS

Field sample

The field sample of MDV-1 (spleen) was obtained from the disease outbreak in layer commercial farms in North Sumatra, Indonesia in 2013, encoded as MDN2013. The main clinical symptom was leg paralysis with the enlarged size of the spleen and liver with white spots appearance (Figure 1). In addition, the vaccine strain CVI988 Rispens (MERIAL SELECT, INC.) was used for the positive control.

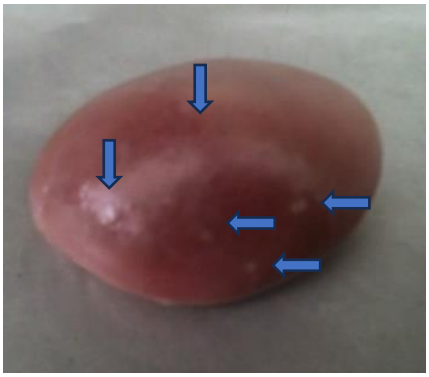

Figure 1. Splenomegaly with numerous white/grey dots indicating tumour formation as the result of MDV-1 infection in the susceptible chicken. The white/grey dots presence are pinpointed with blue arrows.

Identification of Marek's Disease Virus Serotype 1

The molecular identification of MDV-1 was performed using conventional polymerase chain reaction (PCR) and real-time polymerase chain reaction (qPCR) for four genes, including Meq, pp38, gC, and gE. The primers for the Meq gene amplification followed the previous study [10]. Meanwhile, the primers for pp38, gC, and gE were designed using Primer-BLAST software (Table 1). The genetic material of MDV-1 was extracted from the samples using the QIAamp DNA Mini Kit (QIAGEN, Germany) as per the manufacturer's instruction.

The conventional PCR was carried out using HotStarTaq®Plus Master Mix Kit (QIAGEN, Germany) in the Applied Biosystem 9700 (Thermo Fisher Scientific, USA) machine. PCR reactions were performed in a total volume of 20 µL; the PCR

Table 1. The set of primers used for the MDV-1 identification

Gene	Primer identity	Nucleotide sequence (5′-3')	Size of amplicon
meq	meqF26	CTATGCCCTACAGTCCCGCT	972 base pairs
	meqR998	GGAAACCACCAGACCGTAGA	
pp38	pp38F4	GAATTCGAAGCAGAACACGAAG	810 base pairs
	pp38R814	CGACAATGCCTGCACAGAAAG	
gC	gCF236	CAGAAACGACGGGCAAGAAC	840 base pairs
	gCR1076	GGGATAGCTGTGGCATTCGT	
gE	gEF606	CAATGTCTCGACGGCGGTTA	835 base pairs
	gER1441	AAGCGGTATAACCCGAACCC	

mixture contains 10 µL of HotStarTag®Master Mix (2X), 0.5 µL of each respective forward and reverse primer (20 µM), 2 µL of Coral load (10X), 5 µL of water and 2 µL of template DNA. The PCR temperature was set in several stages, including initial denaturation at 95°C for 5 minutes, 40 cycles of amplification (95°C for 30 seconds, 60°C for 90 seconds, 72°C for 2 minutes), and final elongation at 72°C for 10 minutes. The gene amplification was visualized by gel electrophoresis.

Subsequently, the qPCR based on SYBR Green was carried out using PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, USA) in the Thermal Cycler CFX96TM Real-Time System (BIO-RAD, USA). The PCR reactions were performed in a total volume of 10 µL; the qPCR mixture contains 5 µL PowerUp™ SYBR™ Green Master Mix (2X), 0.5 µL of each respective forward and reverse primer (20 µM), and 4 µL of template DNA. The PCR temperature consists of initial denaturation at (50°C for 2 minutes followed by 95°C for 2 minutes), 40 cycles of amplification (95°C for 15 seconds, 60°C for 15 seconds, 72°C for 15 seconds), and melting curve analysis was carried out from 60°C to 95°C with ramp temperature 0.5°C for 10 seconds.

Gene sequencing and bioinformatic analyses

Fragment gene sequencing of Meq, pp38, gC, and gE was performed with Sanger's method from the PCR products by Macrogen Services (Korea). Gene amplification for these genes was accomplished by the previous protocol, which is the conventional PCR using HotStarTaq®Plus Master Mix Kit (QIAGEN, Germany). The sequencing results were edited by BioEdit 7.2.5.0 software (https://bioedit.software.informer.com) and submitted to the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). The phylogenetic tree was constructed using MEGA 6.06 software (https://www.megasoftware.net).

RESULTS

Gene identification of Marek's Disease Virus Serotype-1

The Meq, pp38, gC, and gE genes of MDV-1 strain CVI988 Rispens and MDN2013 were successfully amplified using the respective PCR protocols in conventional and real-time platforms (Figure 2). The amplification of the Meq gene showed the most significant outcome in the conventional platform. The amplicon size from samples are 972 base pair as expected; the vaccine strain showed two bands with slightly different sizes of amplicon, On the other hand, the field sample only showed a single band close to the S-Meq isoform. However, the qPCR platform based on SYBR provides similar vaccine and field strain results. The only difference occurred on the cycle threshold (CT) level, where the vaccine strain had a lower value than the field strain. In addition, the melting curve analysis of the Meq gene showed no difference in about 87.5°C for both vaccine and field strains.

The amplification of the pp38, gC, and gE genes using the respective PCR protocols showed similar outcomes between the vaccine and field strains. The size of amplicons and their disassociation temperature for pp38, gC, and gE genes are in about 810 base pairs (89°C), 840 base pairs (82°C), and 835 base pairs (80.5°C), respectively. In addition, the gene amplifications of the vaccine strain had a brighter band in the conventional PCR and lower CT value in the real-time PCR than the field strain.

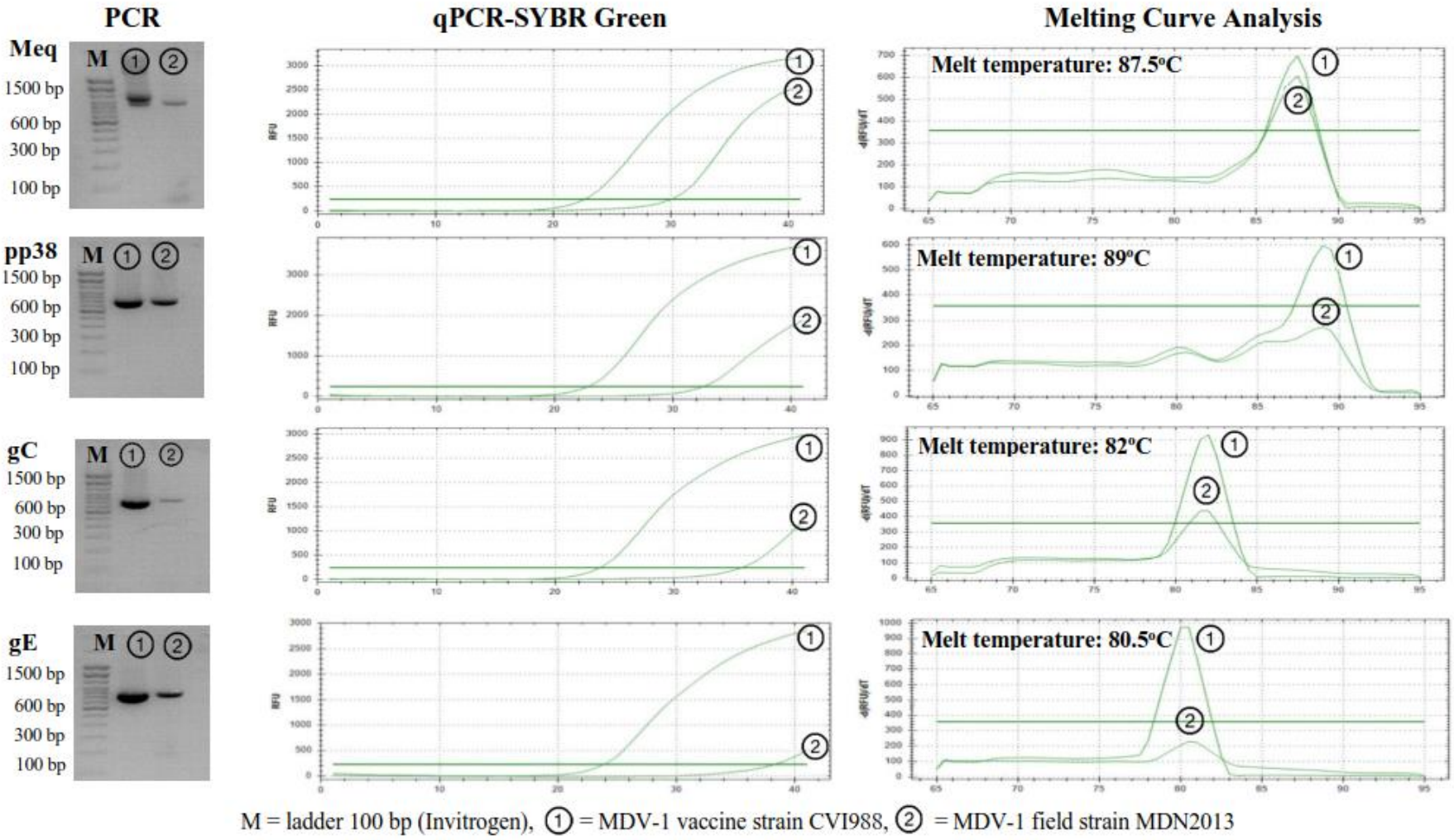

Figure 2. The meq, pp38, gC, and gE gene amplification for the MDV-1 identification using the conventional and real-time PCR as well as the melting curve analyses on strain CVI988 Rispens and MDN2013.

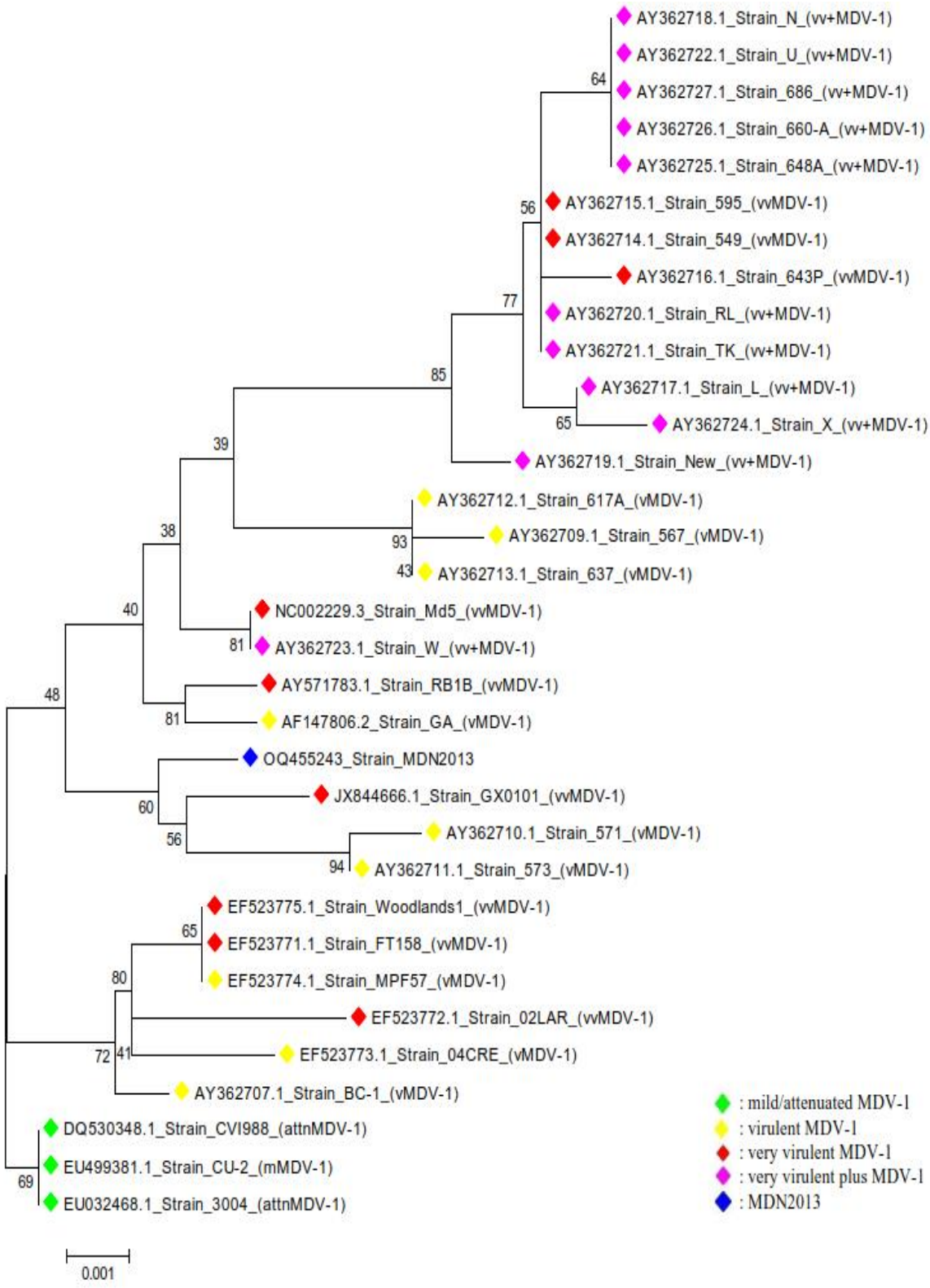

Figure 3. Phylogenetic tree analysis based on the Meq gene of the MDV-1 characterized isolates for pathotypes was designed by MEGA 6.06 using neighbor-joining analysis (1000 bootstraps) with the Kimura 2 parameter.

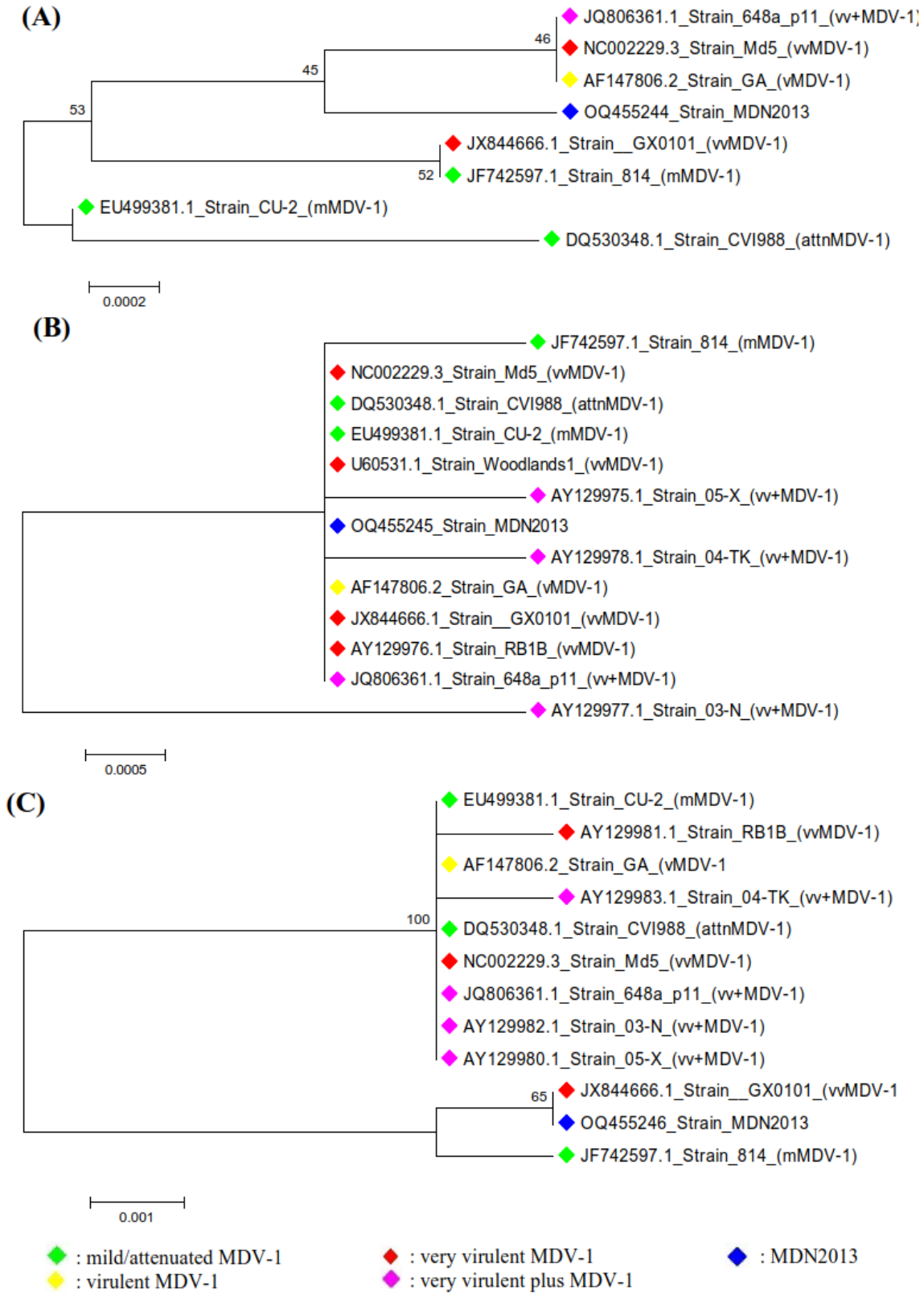

Figure 4. Phylogenetic tree analysis based on the pp38 gene (A), the gC gene (B), and the gE gene (C) of the MDV-1 characterized isolates for pathotyping was designed by MEGA 6.06 using neighbor-joining analysis (1000 bootstraps) with Kimura 2 parameter.

Table 2. The blast-n of MDN2013 against the MDV-1 genome database

Gene	Acc. No.	The GenBank NCBI Database		
		% Identity	Number of Isolates	Region of Origin (Year of Isolation)
meq	OQ455243	100	1	Indonesia (2014)
		99.89	2	India (2012), China (-)
		99.78	58	India (2013, 2014), China (2006, 2007, 2008, 2011, 2014), Japan (2004, 2014), Thailand (2013, 2015, 2016, 2018, 2019, 2020, 2021), Germany (2019), Poland (2000)
		99.77	1	China (-)
		99.68	38	China (-, 2001, 2007, 2008, 2009, 2011, 2011, 2012, 2017, 2020), Japan (1980, 2004, 2005), Tunisia (2016), Italia (2017), Poland (-), Brazil (2019, 2020)
pp38	OQ455244	100	1	India (2018)
		99.87	60	India (2017, 2018), China (1974, 1986, 2001, 2007, 2011, 2014), Thailand (2013, 2015, 2016, 2019, 2020, 2021), Japan (-), Germany (-), USA (-), synthetic construct (-)
		99.74	39	India (2018), Turkey (2019), Tunisia (2016), Egypt (2015), Hungary (1970, 2000), Israel (1992), Thailand (2015, 2016, 2017, 2018, 2019, 2020, 2021), Nigeria (2017), China (2018)
gC	OQ455245	100	57	Tunisia (2016), China (2001, 2007, 2008, 2011, 2012, 2014), Poland (2000, 2010), Hungary (1970), Israel (1992), Egypt (2011), Germany (2019), Japan (2014), USA (-), Holland (-), Australia (-), synthetic construct (-)
		99.88	5	China (1974, 1986), USA (-)
		99.50	1	USA (-)
		98.75	1	India (2009)
gE	OQ455246	100	15	China (-, 2001, 2004, 2011, 2012, 2014, 2015), Poland (2010), synthetic construct (-)
		99.88	8	India (2012), China (-, 1995, 2005, 2007)
		99.75	7	Israel (1992), China (-, 1974, 2014), Japan (2014)
		99.63	2	Hungary (2000), China (1995)
		99.50	2	China (-)
		99.01	38	China (-, 2007, 2008, 2011), Hungary (1970), Germany (2019), USA (-), synthetic construct (-), Holland (-)
		98.88	4	China (-), USA (-)

(-) = data is not available

Gene characterization of Marek's Disease Virus Serotype-1

The gene sequences of MDV-1 strain MDN2013 were submitted to the NCBI GenBank database, and the accession numbers were granted, i.e., Meq (acc. no. OQ455243), pp38 (acc. no. OQ455244), gC (acc. no. OQ455245), and gE (acc. no. OQ455246). The

BLASTN analyses of these genes are presented in Table 2. As a result, the Meq gene of the MDN2013 demonstrated 100% identity with the MDV-1 isolated from Sukabumi, Indonesia, in 2014. The Meq gene of the MDN2013 also shares high similarity with the viruses isolated from India and China. Overall, the Meq gene of the MDN2013 shows higher similarity with the viruses from Asia than other regions such as Europe, Africa, and America. A slightly similar result occurred to the pp38 gene that shares the highest similarity with a virus from India. Its pp38 gene also shares high similarity with other viruses from Asia, Europe, Africa, America, and even synthetic constructs. On the other hand, the gC and gE of the MDN2013 share similarities with many isolates from diverse regions such as Asia, Europe, Africa, and America.

The phylogenetic tree of the meq gene is displayed in Figure 3, whereas the analyses for pp38, gC, and gE are displayed in Figure 4. Interestingly, the phylogenetic tree analysis of the Meq gene of the MDV-1 with characterized pathotypes (mild, virulent, very virulent, and very virulent plus) showed an interesting phenomenon where most viruses with similar pathotypes are clustered in the same group, even though several combinations occurred. Subsequently, the MDN2013 is clustered in the same group with strain GX0101 (very virulent) from China and strains 571 and 573 (virulent) from The United States of America. Unfortunately, the genetic database of pp38, gC, and gE genes for the characterized virus was still limited, and therefore the phylogenetic tree analysis can only be constructed for a few numbers of isolates. Thus, the phylogenetic tree analyses from three amplified genes resulted in no specific pattern for pathotype characteristics.

DISCUSSION

As a member of the Mardivirus genus from the Alphaherpesvirinae subfamily, the genome MDV-1 comprises a linear double-stranded DNA with a size of about 125-241 kilobase pair. For example, the total genome of the very virulent MDV-1 strain Md5 is 177,874 base pairs, which encode about 103 proteins [11]. Several genes are essential for virulence, including the oncoprotein Meq, oncogenicity-associated phosphoproteins pp38 and pp24, a lipase homolog, a CxC chemokine, etc [11, 12]. These genes could be utilized as markers to determine the biodiversity of the MDV-1.

This study focused on the Meq, pp38, gC, and gE genes. The Meq protein is an essential leucine zipper protein (bZIP) that plays an essential determinant in many biological processes such as transformation in tumorigenesis, apoptosis inhibition, and modulation of cellular and viral gene expression [13]. The pp38 protein is also important in the transformation process in tumorigenesis [14]. Subsequently, the gC and gE are membrane proteins, which have functions in cell attachment and cell-to-cell spreading, respectively [15]. These glycoproteins may also play a role in the immunological neutralization related to complement factors. Therefore, the PCR tests developed within this study can identify these four genes; however, these tests cannot distinguish between the field and vaccine strain. The urgency of diagnostic tools with the ability to differentiate these strains has arisen because of the massive application of life vaccines in commercial chicken farms [16]. The gene amplifications showed no specific differentiation between field and vaccine strain, except for the Meq gene displayed the presence of two isoforms of a regular size (S-Meq) and one long isoform (L-Meq) in the vaccine strain [17].

On the other hand, the strain MDN2013 has an S-Meq isoform suggesting higher transactivation activity that improved its function [18]. Since the field strain only showed a single presence of S-Meq, this difference could be explored more for developing diagnostic tools that can differentiate between vaccine and field strain. Nonetheless, this approach seems quite vague since several field strains also bear L-Meq in their genome [19].

Studies have shown that gene sequencing is a simpler method for identifying vaccine and field strains, especially when examining the Meq and pp38 genes instead of the gC and gE genes. After analyzing the sequences of the meq and pp38 genes, it was discovered that

the MDN2013 strain, which caused a disease outbreak in North Sumatra's layer commercial farms in 2013, had a strong genetic connection with viruses from Indonesia, India, and China, particularly those in the Asian region. Furthermore, the advanced bioinformatic analysis using the phylogenetic tree of the Meq gene could be used to predict phenotypic characteristics of the virus such as pathotype. Since the MDN2013 is predicted as a virulent or very virulent virus, the vaccination program using strain CVI988 Rispens as a gold standard should be able to protect the chicken from the field strain viruses [6]. However, the chicken still suffered from the disease with clinical manifestations including paralysis with tumor formation in many organs. The plausible possibility is vaccination failure due to variant MDV strains with known virulence and unexpected vaccine resistance [20]. The MDV-1 vaccine is also fragile that needs to be stored in liquid nitrogen. Therefore, cold chain management significantly influences the quality of the vaccine quality. Revaccination may induce higher immunity with better protection against field strain viruses [21]. The immune response of vaccines can be also interfered with by immunosuppression conditions such as viral infections, poor quality of feed, toxin contamination, etc. Therefore, robust and straightforward diagnostic tests should be developed for monitoring and evaluating the successfulness of the MDV-1 vaccine [22-23].

CONCLUSION

In the context of Marek's disease virus serotype 1 biodiversity monitoring, molecular methods prove to be a highly efficient alternative to traditional disease surveillance methods such as virus isolation and animal challenge tests. Among the available molecular characterization options, the Meq gene stands out as the most appropriate choice.

CONFLICT OF INTEREST

All authors declare that there has been no conflict of interest with any parties, individuals, organizations, or companies in this study.

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