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MOLECULAR EVIDENCE OF MAREK'S DISEASE VIRUS EVOLUTION FROM COMMERCIAL POULTRY FLOCKS IN IRAQ

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ABSTRACT

The poultry industry is facing serious threats caused by avian pathogens, which cast a shadow over its economic impact, among them Marek's Disease Virus (MDV). The threat of MDV appeared due to oncogenicity and immunosuppression, controlled successfully by vaccination and biosafety measures. A successful vaccination outcome requires identifying circulating viruses; otherwise, vaccine failure occurs. To better understand the circulating virus, 160 samples were collected from different layers of farms in Iraq. Samples were filtered initially by real-time PCR, and positive samples were used to amplify viral genes Meq, PP38, and later to study viral virulence by detection of the 132bp repeat region. PCR products of Meq and PP38 genes were sent to a commercial sequence company (Macrogene Inc./South Korea). Feedback data were analyzed by MEGA11 software; phylogenetic trees were created by Bio Edit software. The results showed a high matching rate of the two studied Meq gene samples against the reference strain, as well as with already described strains from Japan, Germany, France, and China, but varied from the previously described isolates from Iraq. Regarding PP38, the four analyzed isolates occupied two clusters: the less variable isolates (A1, A2, and A3) located adjacent to the Thailand strain, while the other involved the higher variable isolate 2P close to the Pakistan isolate. All recent local isolates were of high virulence based on 132 tandem repeat region amplification. In conclusion, the recent study provides molecular evidence for mutations with MDV virulence genes that require more attention for vaccine selection to control the disease.

Keywords: 132 tandem repeat, Marek's virus, Meq genPP38 gene, Phylogenic analysis.

INTRODUCTION

Marek's Disease Virus (MDV) is a lymphoproliferative viral disease of chickens caused by a Herpesvirus within Alphaherpesvirinae known as *Gallid herpesvirus 2* (Zhu *et al.,* 2024). The causative agent is a DNA virus that comprises a large genome that may reach 180kb for virulent strains, encoding for more than a hundred viral proteins (Tulman *et al.,* 2000). Previous studies have revealed several genes that encode vital virulence factors, including meq, vil-8, PP14, PP38, and vlip (Bertzbach *et al.,* 2020). Vaccination was used to control the significant economic impact of the disease among the infected flocks, with the emergence of higher virulent strains of the virus that claimed the selective pressure of the vaccine (Zhu *et al.,* 2024).

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MDV encodes an essential virulence factor, Meq oncoprotein, which plays a central role in the virus oncogenicity. The Meq gene involved a standard of 339 amino acids, and several isoforms were described with longer or shorter lengths (Tai *et al.,* 2017; Chacon *et al.,* 2024). In addition to its role in oncogenesis, the Meq protein is also involved in virus immunosuppression, and point mutation within this gene was associated with increased virus virulence (Chacon *et al.,* 2024).

The MDV phosphoprotein PP38 plays an important role in MDV pathogenesis, such as lymphoid tropism with cytolytic infection of B cells, but not the feather follicle epithelium cells, reactivation from latency status, indirectly maintaining tumorigenesis by blocking the cellular apoptosis mechanism, and supporting the immune suppression (Gimeno *et al.,* 2005).

Additionally, PP38 is expressed in lytic and transformed cells, although it is not involved directly in the tumorigenesis mechanism (Reddy *et al.,* 2002).

Experimental studies of (Sun *et al.,* 2022) find that pp38 supports the function of meq oncogene, as the recombinant virus that lacks both genes showed a fully attenuated strain that cannot restore its virulence, as with recombinant strains that lacked meq gene only, thus this double gene mutated virus can act as a very effective vaccine strain.

During the attenuation process, either in vitro or in vivo with wild-type virus, a specific region within the repeated regions of the viral genome (TRL/IRL) of the long segment (LR), known as the 132kb tandem repeat, will expand from two copies into more than 20-30 copies. This expansion leads to the loss of viral virulence as it will interrupt the transcription pattern of the genome, especially the virulence adjacent region of 1.8kb RNA (Niikura *et al.,* 2006). The 132 tandem repeat genome region and its expansion were described as a characteristic feature to differentiate between virulent and attenuated strains of MDV and as a diagnostic proof for MDV-1, the only serotype that carries this gene (Kalyani *et al.,* 2011).

In Iraq, Marek's disease was not studied intensively like other avian diseases of economic importance such as Avian Influenza (Khamas, 2008; Allawe and Hidayat, 2022) Infectious bronchitis (Ali and Allawe, 2023), the immunosuppressive Infectious Bursal Disease (Jumaa *et al.,* 2020), and the relative herpesvirus infection of Infectious Laryngiotrachitis (Allawe *et al.,* 2016), leaving gray areas in the genomic structure and mutation rates of MDV that reflect passively on the understanding of the viral effect on the poultry industry and the efficacy of vaccine administration on disease control and if the mutations interfere with vaccine activity.

The goal of this study was to address some gray areas in MDV field genotypes and study some of their virulence genes, simultaneously with the emergence of highly virulent field strains of MDV within local farms during the last few years, despite ongoing vaccination programs.

MATERIALS AND METHODS

Samples collection

In this study, a total of 160 tissue samples were collected from Marek's disease suspected flocks in 50 layer farms from different Iraqi governorates. Ethical approval was granted through the local committee of animal care and use at the College of Veterinary Medicine within the University of Baghdad (Number 1240 on 2024/06/27). The collected samples include liver, spleen, kidney, and feather follicles from acute cases. These samples were submitted to the laboratory, crushed, and prepared for viral nucleic acid extraction.

Extraction of viral nucleic acid

Viral nucleic acid was extracted manually using the highly qualified traditional kit QIAamp DNA kit from Qiagen®. The process briefly involved the mixing of 200 µL of sample suspension with an equal volume of lysis buffer, with additional support of 20 µL of proteinase K. The reaction took place in a heat block at 56 °C. After that, the reaction mix was moved into a silica column with 200 µL of absolute ethanol, and centrifuged to precipitate DNA on the silica barrier. Then, this DNA pellet was washed twice with washing buffer to get rid of any contaminated proteins and other cellular components. Finally, the DNA was rehydrated, collected in a fresh tube, and stored at -70 C till tested.

Initial detection of MDV by real-time PCR

In order to detect the viral DNA of MDV, a specific Taqman real-time PCR designed by (Baigent *et al.,* 2016) that targets a conserved region of the pp38 gene was chosen. The primers were manufactured by Alpha DNA/Canada, and involved PP38F 5'- GAG CTA ACC GGA GAG GGA GA-3', PP38R 5'- CGC ATA CCG ACT TTC GTC AA-3', and a TaqMan probe PP38 Pr 5'- CTC CCA CTG TGA CAG CC-3' with FAM as reporter and BHQ as a quencher molecule.

The primers were mixed with the reaction buffer Go Taq® Probe 1-Step RT-qPCR system manufactured by Promega. For each sample, the reaction mix was prepared with 2x buffer solution mixed with 0.4 µL of enzyme mix, 1 µL of 10 pmol forward and reverse primers, 0.5 µL of TaqMan probe, 0.05 µL of Rox passive reference dye, and 2.05 µL of nuclease-free water. The reaction buffer mix was finalized to a volume of 20 µL by adding 5 µL of viral DNA template. This mix was passed under a specific thermal condition of initial denaturation and hot start activation at 95 °C for 2 minutes, followed by 40 cycles of 95 °C for denaturation and 60 °C for annealing and extension, lasting 3, and 30 seconds, respectively. The amplification process took place using an Applied Biosystem 7500 fast thermal cycler, and the results analysis was carried out according to instrument settings.

Studying MDV virulence genes Meq, and pp38

For this step of the study, specific primers suggested by (Dunn *et al.,* 2010) against Meq gene that amplifies 1.4 kb region were chosen as follows: MEQ1.4F 5'- GAG CCA ACA AAT CCC CTG AC-3', and MEQ1.4R 5'- CTT TCG GGT CTG TGG GTG T-3' as well as a primer set that amplifies 1006bp of pp38 described by (Tian *et al.,* 2011) selected FP 5'-TCA TCT TCA ACC CAC AGC CAT CC-3', and RP 5'-TCG CTT AAT CTC CGC CTC CAA C-3'. These two genes were amplified using GoTaq®G2 Green Master Mix/Promega for this study. The master mix was ready to use at 12.5 µL/sample with 1 µL of both upstream and downstream primer at 10 pmol/µL, and 5.5 µL of nuclease-free water/ sample. After that, 5 µL of DNA template was added for each test to complete the reaction volume to 25 µL. For thermal condition, both genes have an initial denaturation hold of 2 minutes at 95 °C and 35 cycles of amplification started with a denaturation step of 1 minute at 95 °C for both genes, followed by the annealing step of 1 minute at 55 °C for Meq gene and 1 minute of 60 °C for pp38 gene, and an extension step of 2 minutes at 72 °C for both of them, finalized by a final extension hold of 10 minutes at 72 °C. The amplification process was accomplished with applied Biosystems thermal cycler

Genetic analysis of target genes

The process of sequence analysis of the two target genes (Meq and pp38). Positive PCR products were packed and sent to Macrogen, Inc. / South Korea. The feedback results were analyzed by MEGA11 software; later, phylogenetic trees were created from these data through

Bio Edit Sequence Alignment Editor software. A highly virulent Gallid Alphaherpesvirus2 described previously (Tulman *et al.,* 2000) with accession No. (NC_002229.3) was used for comparison and analysis of local strains.

Study viral virulence by Conventional PCR

The 132 bp tandem repeat region of MDV genome was selected for studying the virulence nature of the virus isolates by conventional PCR. For this purpose, the selected region was amplified with the following primer set (Tian *et al.,* 2011): 132bp Repeat F5'- ATG CGA TGA AAG TGC TAT GGA G-3' and 132bp Repeat R 5'- ATC CCT ATG AGA AAG CGC TTG A-3'. The amplification reaction used GoTaqG2 Green Master Mix from Promega at a final volume of 25 ul that composed of 12.5 µL of 2X master mix, 1 µL of both forward and reverse primers, 5.5 µL of nuclease-free water, and 5 µL of DNA template. Simultaneously, the thermal condition for reaction started with 4 minutes at 95 °C for initial denaturation, followed by 45 cycles of 1 minute at 95 °C, 1 minute at 62 °C for annealing, 2 minutes at 72 °C for extension, then an additional step at 72 °C for 10 minutes as final extension. The thermal reaction was performed with the Applied Biosystem ProFlex PCR system. The results were visualized on 1.5% agarose stained with ethidium bromide, electrophoresed with 70 Volts for 90 minutes, and the amplicons were visualized on a UV light source.

RESULTS

Initial detection of MDV virus by real-time PCR

Taqman PCR chemistry detected 28 positive samples out of the 160 tested ones, representing 12 layer farms originated from 4 Iraqi governorates (data are not shown here), according to the technique criteria, all the samples that showed a cycle threshold cycle (Ct) below 35 regarded as positive samples, highly concentrated samples with Ct between 25-30 were selected for viral genes detection.

Detection of Meq and pp38 genes

The detection process and electrophoresis of meq product on 1.5% agarose pre-stained with ethidium bromide showed amplified bands of about 1.4 kb for three samples (M1, M2, and M3) were sent for sequence analysis. The feedback results showed only a good sequence for M1 and M2, while the M3 sequence was unclear with high background noise, so it was neglected. Regarding the PP38 gene, four samples showed clear amplification bands (A1, A2, A3, and 2P) of about 1 kb, which were selected for sequence analysis.

Alignment of Meq gene

The sequence analysis of both target Meq genes M1 and M2 revealed the recovery of 1049 nt for M1 and 769 nt for M2. Upon alignment with the highly virulent reference standard gene, both of these genes showed high identity with very few SNPs except the gene terminals, which showed a little higher mutation rate, especially the M1 sequence (Figure 1) and (Figure 2). It is clearly noted that three SNPs are shared between M1 and M2, which are located at positions 5795 (G→A), 5906 (A→G), and 5965 (C→A), while the other three SNPs were unique to M1 at positions 5561, 6158, and 6178. Alternatively, M2 expresses multiple SNPs starting from position 6193 to 6318 that do not appear in the M1 sequence.

Alignment of PP38 and Meq genes

The alignment process for the four samples of the pp38 gene against the standard strain sequence revealed a highly matching sequence of the three isolates (A1, A2, and A3) with the standard sequence, and in almost all positions within the gene where single-nucleotide polymorphism (SNP) is seen. These three samples showed an identical nucleotide replacement (Figure 3 and Figure 4). The fourth gene (2P), on the other hand, expresses a higher level of

variation in both field strains and standard strains, this variation appears clearly in the second half of the sequenced gene, which appeared as mutations in the form of SNPs and insertions as a unique feature for this strain (Figure 4).

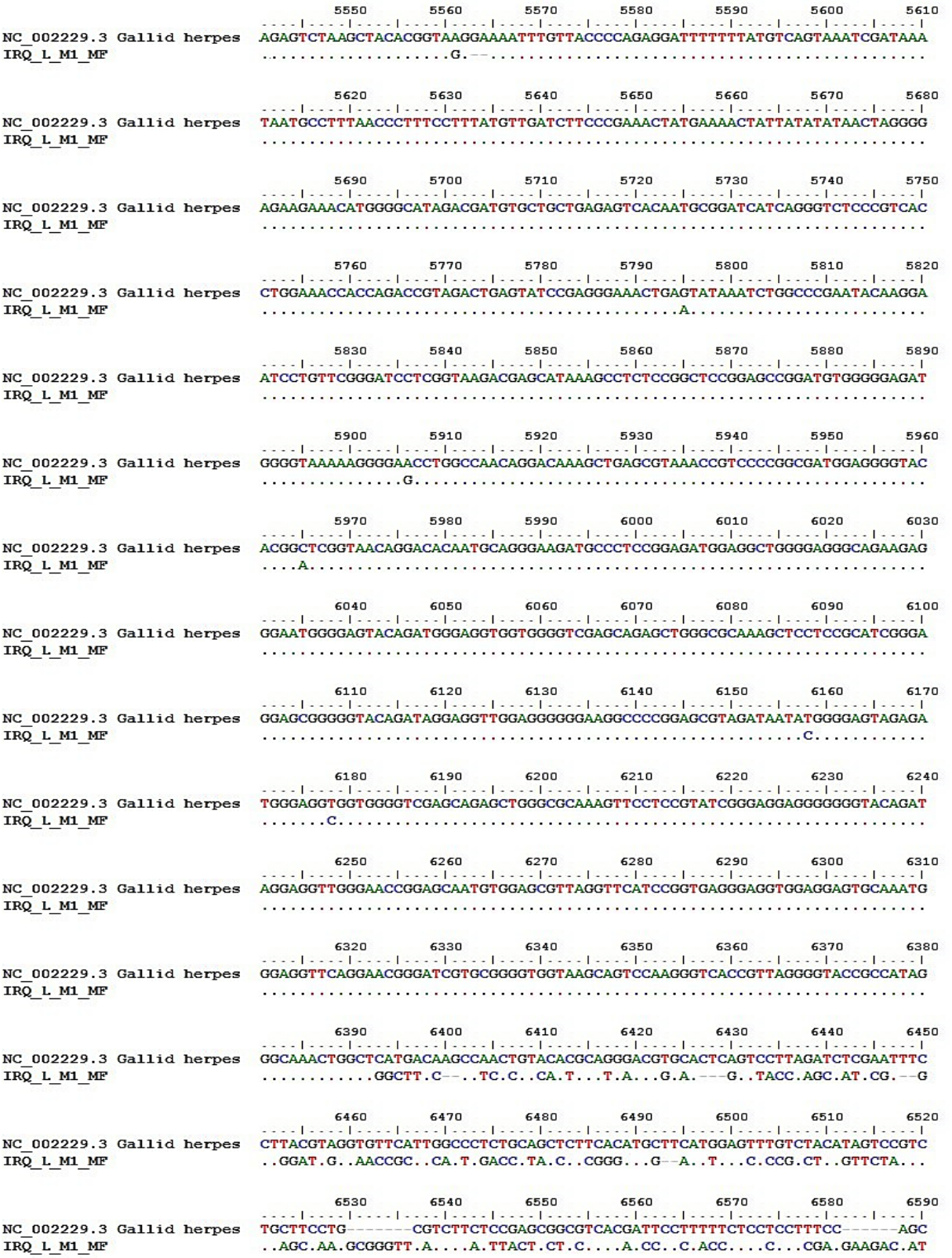


Figure 1: the alignment of the Meq gene product from the M1 sample with the control reference sequence, with a high variation rate at the sequence terminal compared with the standard strain sequence. The alignment was analyzed by MEGA 11 software.

Figure 2: the alignment of the Meq gene product from M2 sample with the control reference sequence, the high matching is very clear except in the last two lines were the mutations as SNP appeared, alignment analysis was created by MEGA11 software.

Phylogenetic analysis

The phylogenetic analysis of the two Meq genes M1 and M2 explains a high relationship to the virulent standard sequence (NC 002229.3) as well as to a group of previously described strains from Germany, France, China, and Japan. On the other hand, the recently analyzed strains did not cluster with the previously described genotypes from Iraq, Iran, and Türkiye (Figures 5) and (Figure 6).

The phylogenetic analysis of pp38 sequenced samples showed that these samples are split into two clusters. The first one included samples A1, A2, and A3, which are situated close to sequenced samples from Thailand, India, and Egypt. The second cluster involved sample 2P, which shares a high identity with a sample isolated from Pakistan (Figure 7).

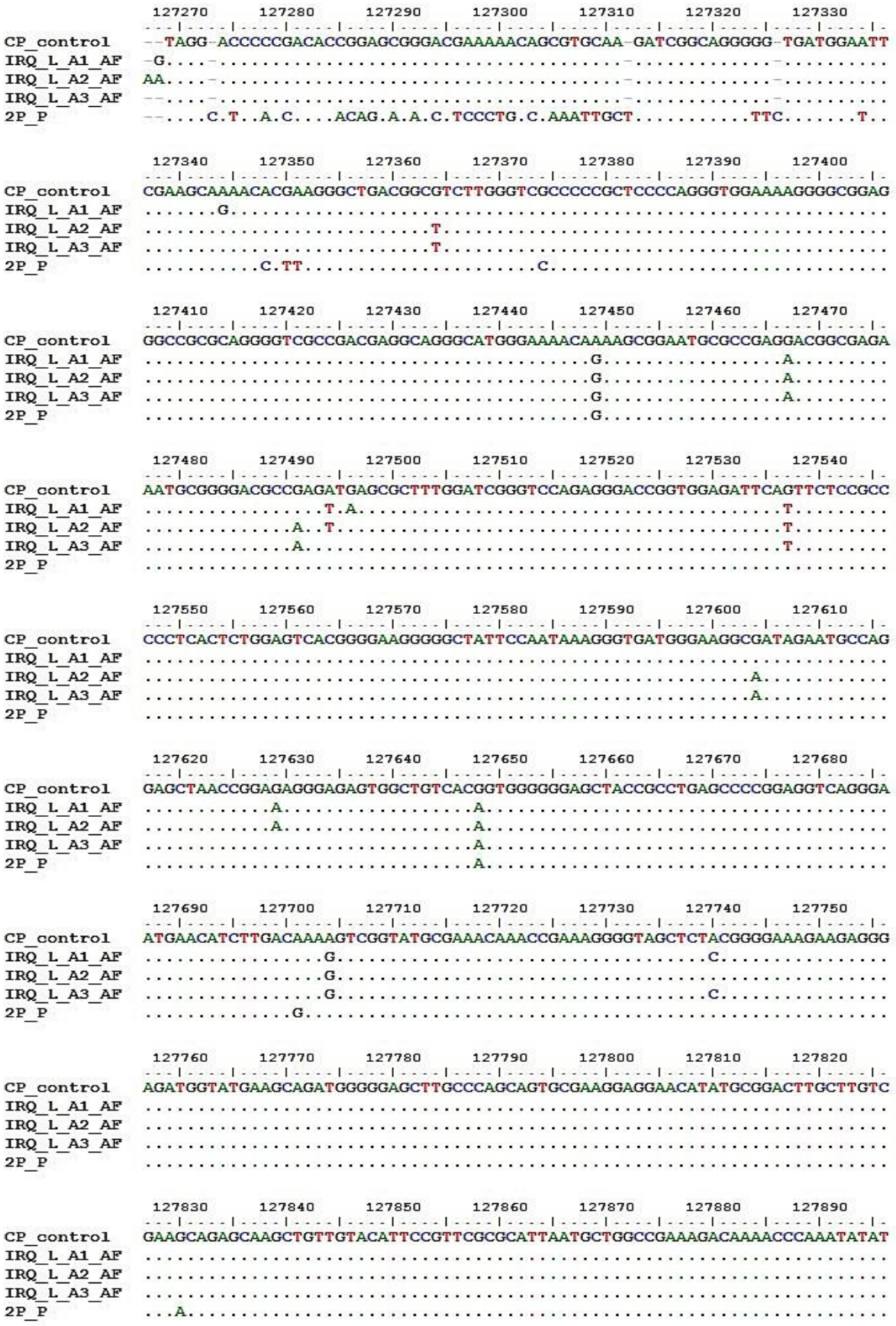


Figure 3: the alignment analysis of the pp38 gene with many SNPs that shared almost between tested samples although high matching is seen between tested samples and standard control.

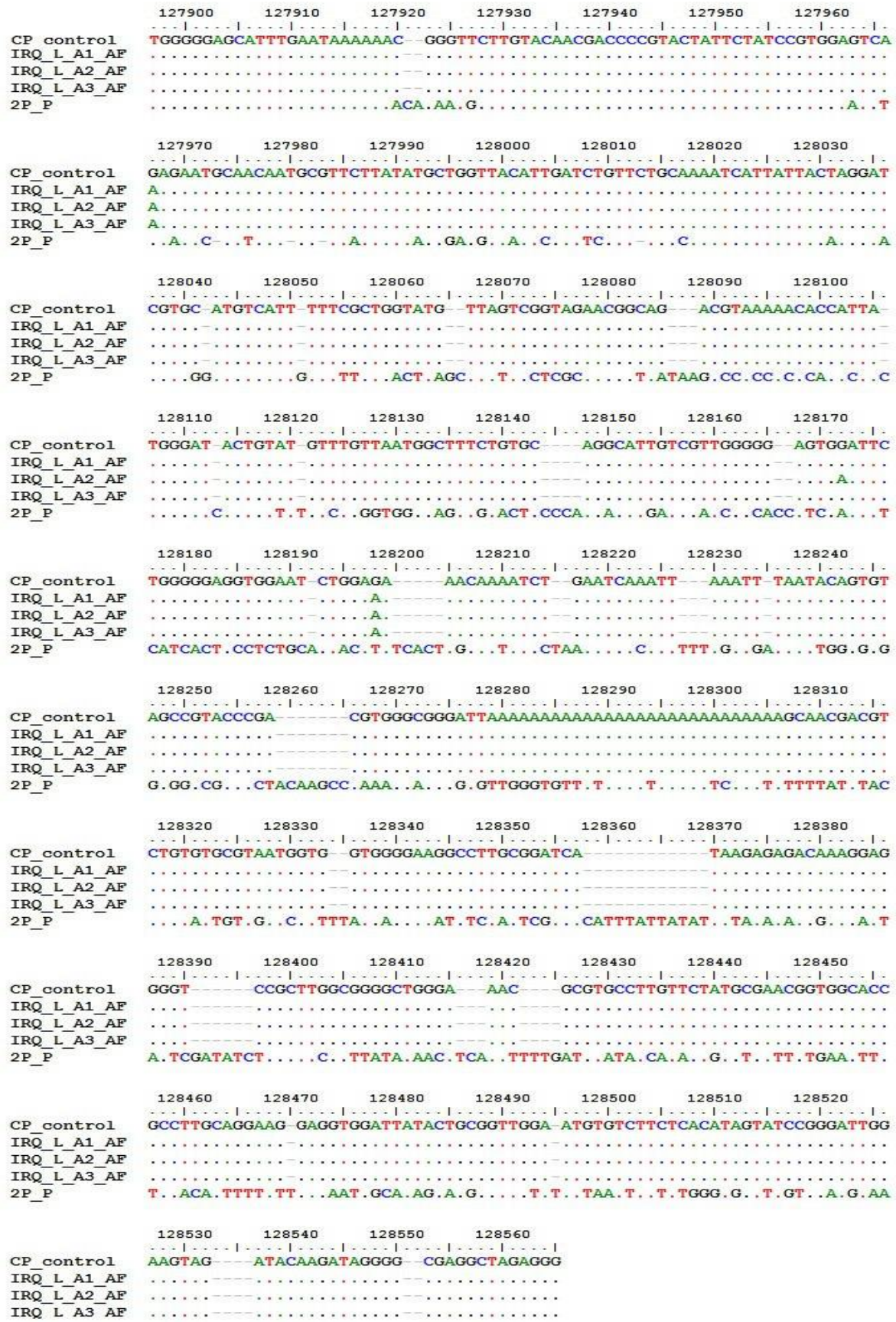
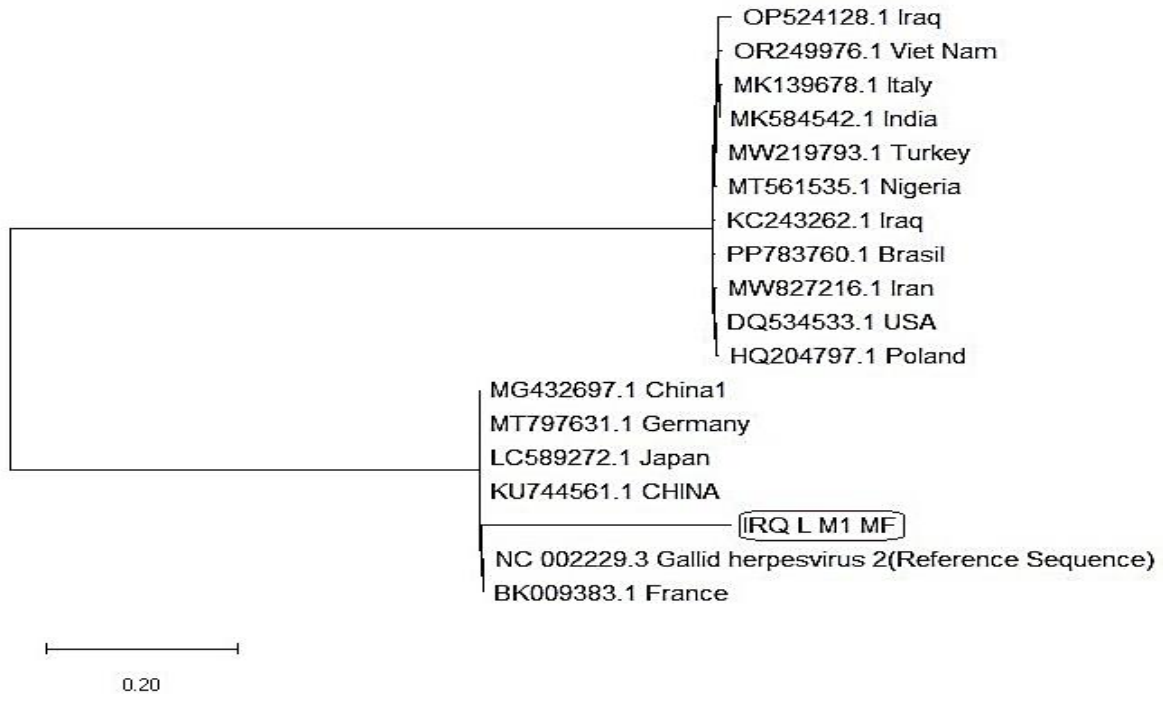


Figure 4: the second part of pp38 gene alignment, in which sample 2P showed several insertions that give it some specificity among other tested samples as well as the control sample.



(Figure 5) the phylogenetic analysis of the M1 sample which showed a close relationship with Chinese, Japanese, and German strains as well as a high matching with the French and the virulent strain used as a reference control. The phylogenetic tree was created with Bio Edit Sequence Alignment Editor software.

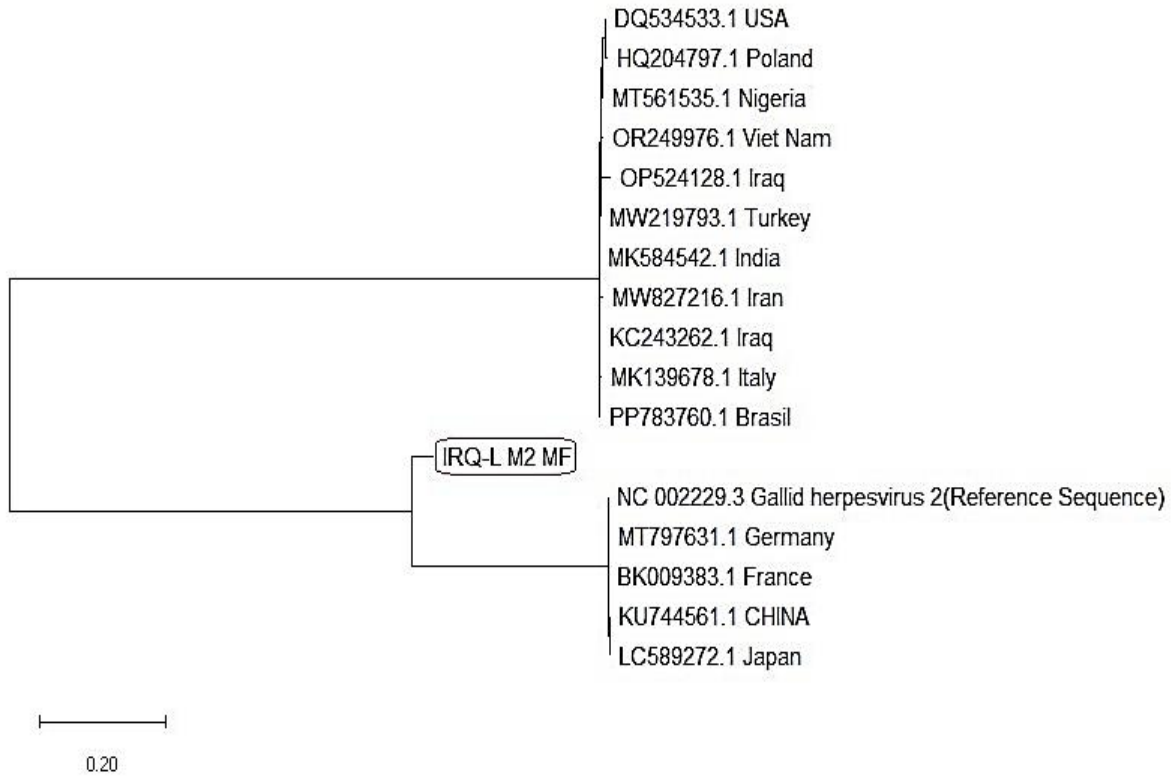
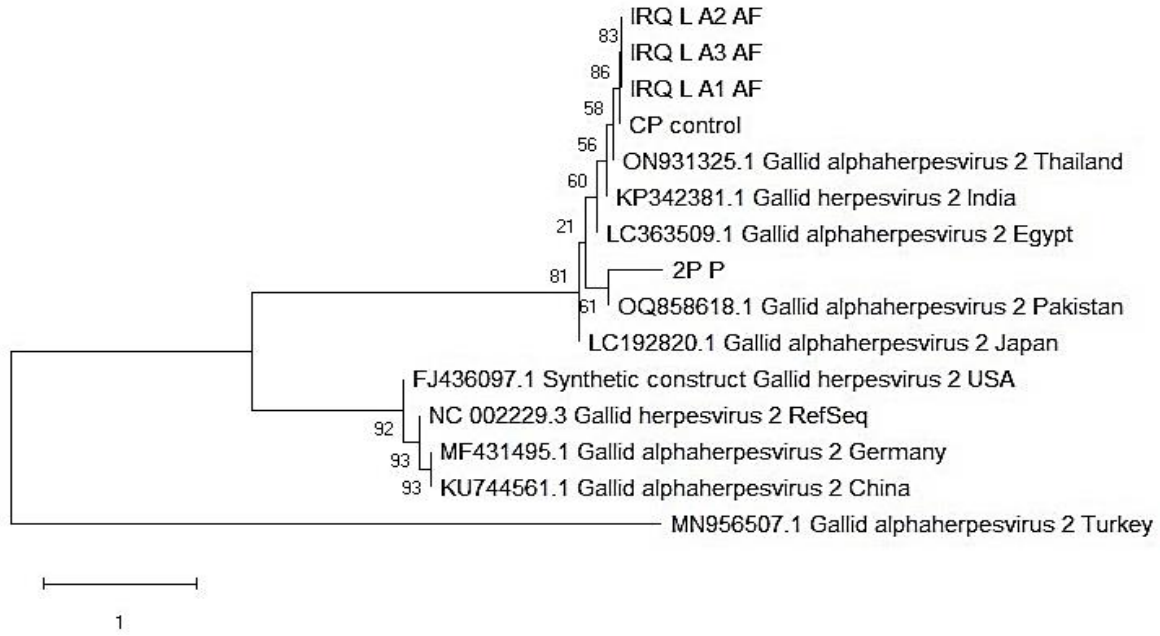


Figure 6: the phylogenetic analysis of M2 sample. The phylogenetic tree was created with Bio Edit Sequence Alignment Editor software.



(Figure 7) the phylogenetic analysis of pp38 showed the local strains located into two clusters, the first one with A1, A2, and A3 that are highly matched with strains from Thailand, India, and Egypt, while the second cluster involved only one sample 2P that shows highly match with Pakistan strain, Phylogenetic tree was created with Bio Edit Sequence Alignment Editor software.

Result of Study Viral Virulence by Conventional PCR

The result of this experiment showed that samples expressed a single amplification band with a molecular weight of about 320bp, compared to the control, which showed multiple bands with different molecular weights (Figure 8).

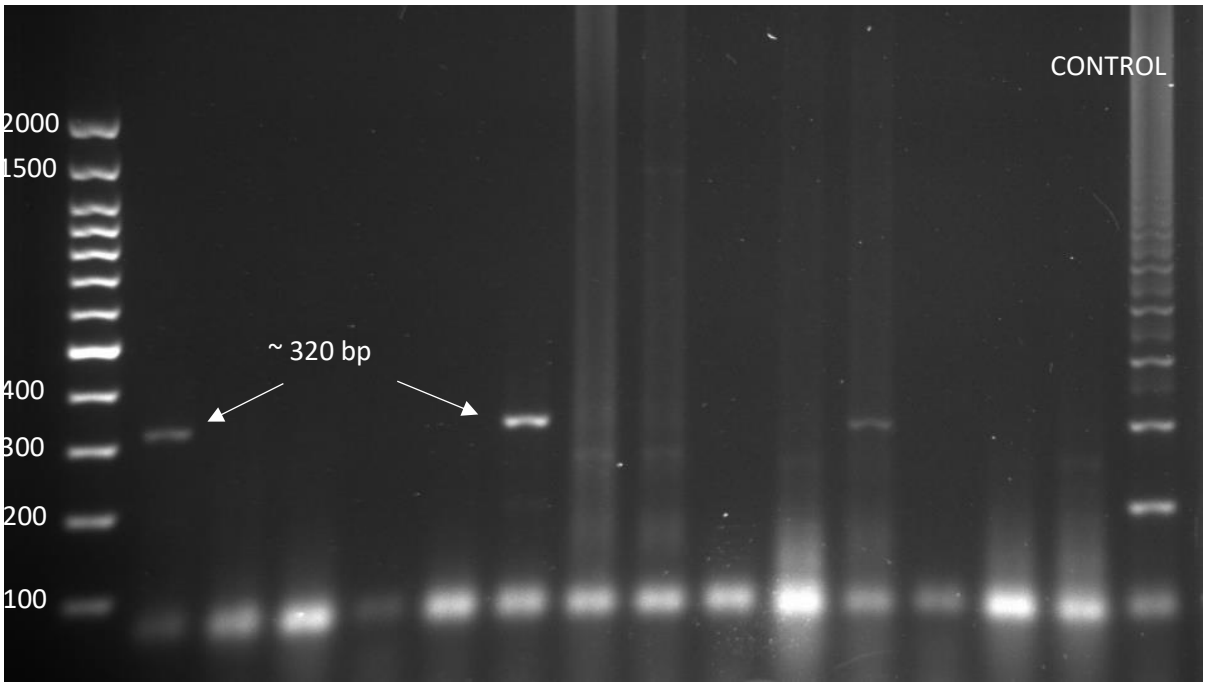


Figure 8: the viral 132bp tandem repeat region electrophoresis at 1% agarose, 90 volts for 1 hour stained with ethidium bromide. Virulent Viral strains showed only one amplification band of about 320bp, in comparison with attenuated or low virulent strains that showed multiple amplification bands.

DISCUSSION

Vaccination and the adjacent biosafety management system are considered an effective method to control and restrict avian viral infections (Davison and Nair 2005). Sometimes, especially with avian herpes viruses, live attenuated vaccines can revert and enhance viral pathogenicity into higher virulence (Allawe *et al.,* 2016; Trimpert *et al.,* 2017; Ali *et al.,* 2023).

In this study, the sequence analysis of the oncogene Meq for the two samples M1 and M2 revealed a high match with the virulent Gallid herpesvirus reference strain (NC002229) described previously (Tulman *et al.,* 2000). However, there were several point mutations represented as single-nucleotide polymorphisms in both samples, as well as two insertions in the M1 sample but not in M2 concentrated at the end terminal sequence. Those mutations are an indicator for enhanced MDV pathogenicity and virulence, which agrees with (Liu *et al.,* 2023), and this feature was seen clearly with the clinical disease and raised mortality among birds, in which the MDV overcame the vaccine barrier and showed an aggressive disease manifestation. The phylogenetic analysis of Meq gene cluster M1 and M2 in proximity with the standard strain, as well as other virulent strains from Japan, France, Germany, and China, ensures the virulent nature of the current circulating isolates from Iraq. It was interesting to notice that the current sequence of the meq gene differs from other sequences from Iraq of the same gene published earlier, like KC243262.1 (Wajid *et al.,* 2013), and OP524128.1 published by (Alkubaisy and Hameed, 2023), and this supports the theory of continuous circulation of MDV in Iraqi poultry farms and reflects the importance of this study in following up the changes in the virus genome.

Additionally, sequence analysis of the PP38 gene was also studied in four isolates. The results returned with a high match of three isolates (A1, A2, and A3) with the standard virulent reference strain (NC002229), even with the presence of some point mutations that were shared between the three recent isolates but differ from the standard reference strain. Alternatively, the isolate sequence 2P showed initially high matching rates with the standard reference strain along the first 650 bp. After that many mutations appeared in the form of SNPs or insertions that drive this gene into a unique sequence from both the standard strain as well as the recent local isolates, which may reflect a specific genetic characterization for the Iraqi strains and explain the higher virulent nature of the current isolates (Reddy *et al.,* 2002; Gimeno *et al.,* 2005; Schat *et al.,* 2013). The alignment results were described more clearly by the phylogenetic analysis, where the less variable strains (A1, A2, and A3) occupied a cluster adjacent to the Thailand strain, while the 2P isolate was located in a separate cluster with another strain from Pakistan, suggesting a possible introduction of a novel genotype. All the local isolates did not show a close relationship with the standard reference strain (NC002229), which may indicate a specific genotype of pp38 for the local strains. Unfortunately, there were no previously registered pp38 gene sequences from Iraqi strains at the gene bank to compare with, which may imply this study records the first analysis of this gene.

The detection of the 132kb tandem repeat region was used as an alternative method to confirm the viral serotype, and its virulence, and to overcome the shortage of inability to perform a pathogenicity study on recent local strains of MDV. The results showed a single band of about 320bp for the local strains tested samples is evidence of the high virulence viral strain, compared with multiple bands of different molecular weights for the control attenuated vaccine strain, which agreed with the previous

results described by (Niikura *et al.,* 2006; Alkubaisy and Hameed 2023).

These results confirm the genomic analysis findings of the meq and pp38 viral genes, that these current isolates are virulent strains of the MDV-1 virus (Zahid 2008; Torres *et al.,* 2019; Alkubaisy and Hameed 2023).

Molecular analysis of genes like meq or tandem 132 repeat region was described as an alternative method for the detection and determination of MDV pathogenesis by several researchers (Woźniakowski *et al.,* 2010; Cheng *et al.,* 2024)

The localization of sequenced genes (Meq and pp38) in close proximity to the virulent strain, which was used as a control or standard for the phylogenetic analysis, is additional evidence for the high virulence of the studied strains and these results are accepted (Woźniakowski *et al.,* 2010).

The limitation of this study is represented by the low number of sequenced samples due to the limited number of collected samples and the inability to perform an in vivo pathogenicity study to detect the role of the new mutations in viral virulence, as well as limited genotyping studies, which restrict the alignment of recent isolates with previous ones to understand the mutation rate over time.

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Conflict of interest

There is no conflict of interest for this article.

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ادلة جزيئية على تطور فيروس مرض الميرك في قطعان الدواجن التجارية في العراق

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الخلاصة

تواجه صناعة الدواجن تهديدات جدية من العوامل الممرضة ومن ضمنها فيروس مرض الميرك والتي تلقي بظلالها على الجانب الاقتصادي للصناعة. يلاحظ التأثير المرضي لفيروس الميرك من خلال قابليته على توليد الأورام وكذلك الاثباط المناعي، ويمكن الحد من هذه التأثيرات عبر التطعيم الوقائي وإجراءات الامن الحيوي. يتطلب نجاح التطعيمات توصيف الفيروسات الحقلية لتفادي فشلها. لأجل فهم أفضل لطبيعة الفيروسات الحقلية، جمعت ١٦٠ عينة من حقول الدواجن البياضة في العراق. تمت غربلة العينات بتقنية real-time PCR، ثم انتخبت العينات الموجبة لغرض تضخيم الجينات المستهدفة Meq وPP38. درست ضراوة الفيروس عبر دراسة منطقة التكرار الجيني 132bp. ارسلت نواتج التضخيم للجينات Meq وPP38 الى جهة خارجية (ماكروجين، كوريا) لتحليل السلسلة الوراثية. عولجت نتائج التحليل ببرنامج Mega11، وصممت شجرة الانساب ببرنامج Bio Edit software. أظهرت النتائج تماثل جيني عالي ما بين العينيتين المنتخبتين لدراسة جين Meq والعينة القياسية، ولوحظ التماثل ايضاً مع عينات موصوفة مسبقاً من اليابان، المانيا، فرنسا، الصين، وبالمقابل تغايرت العينات المدروسة حديثاً مع العينات المدروسة سابقاً في العراق. عند دراسة الجين PP38 لوحظ ان العينات الأربع المنتخبة للدراسة قد اتخذت فرعين مختلفين في الشجرة الوراثية، أولهما مثل العينات ذات التغاير الأقل (A1,A2,A3) والتي اتخذت موقعاً مقارباً للعزلة الفيروسية من تايلاند مقارنة مع الفرع الثاني الأكثر تغايراً والذي مثلته العزلة الفيروسية ٢P حيث احتلت موقعا مقارباً للعزلة الباكستانية. أظهرت الدراسة ان جميع العزولات الحديثة لفيروس الميرك هي من النوع عالي الضراوة اعتماداً على تحليل منطقة التكرار الجيني. خلصت الدراسة الحالية الى اثبات وجود الطفرات ضمن بعض الجينات المسؤولة عن الضراوة مما يتطلب ابداء المزيد من الاهتمام في انتخاب العتر اللقاحية لبرامج السيطرة.