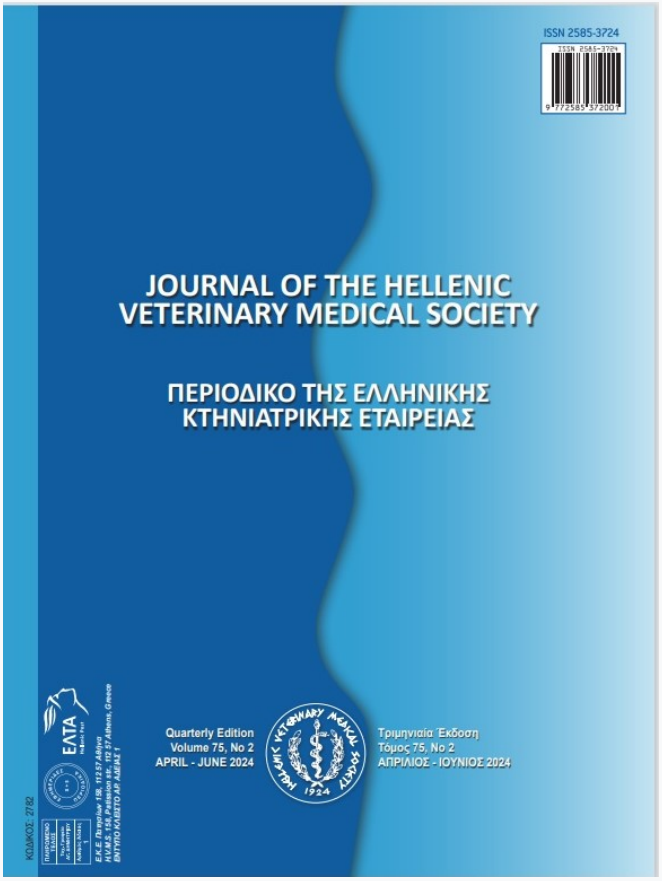
Journal of the Hellenic Veterinary Medical Society

Vol 75, No 2 (2024)



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doi: 10.12681/jhvms.35373

To cite this article:

Turan, T., Kalin, R., & Isidan, H. (2024). Investigation of the Presence of Chicken Parvovirus in Turkish Backyard Poultry Flocks: A Comparative Analysis of PCR Primer Sets . *Journal of the Hellenic Veterinary Medical Society*, *75*(2), 7611–7618. https://doi.org/10.12681/jhvms.35373

J HELLENIC VET MED SOC 2024, 75 (2): 7611-7618
ΠΕΚΕ 2024, 75 (2): 7611-7618

Research article
Ερευνητικό άρθρο

Investigation of the Presence of Chicken Parvovirus in Turkish Backyard Poultry Flocks: A Comparative Analysis of PCR Primer Sets

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ABSTRACT: Enteric syndromes, such as runting-stunting syndrome or malabsorption syndrome cause significant economic loss in industrial-scale poultry production. These syndromes are commonly thought to have a multifactorial aetiology; nevertheless, the extent of parvoviral involvement is still largely unknown. In addition, backyard flocks could be a potential reservoir for parvoviruses, in situations where fewer precautions are implemented. In this study, we aimed to investigate the potential contribution of chicken parvovirus to enteric diseases in farmyard layers owned by semi-professional producers by comparing different PCR primer sets. 404 faecal samples were collected from eight provinces during the period of January to May 2018. The presence of chicken parvovirus (ChPV) was investigated in all samples using five PCR methods, including nested and conventional PCR assays with different primer set combinations. Some samples were further sequenced, and the resulting sequences were analysed *in silico*. There was a significant variation in positivity percentages depending on the PCR method used (7.9% to 44.6%) in faecal samples from backyard flocks. Phylogenetic analysis further showed that the nucleotide identity of the Turkish strains ranged from 94.51% to 99.10 and that most of these strains fell into two distinct clusters. The nested PCR approach developed in this study could be an alternative to other conventional PCR primers due to its higher sensitivity. This is the first time that ChPV strains have been identified in Turkish poultry. overall, the results highlight the increasing health risk posed by ChPV infections in backyard poultry production and emphasize the need for biosecurity measures to prevent disease transmission.

***Keywords*:** Chicken parvovirus, Backyard poultry, Molecular characterization, nested PCR, Phylogenetic analysis

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Date of initial submission: 10-09-2023
Date of acceptance: 22-12-2023

INTRODUCTION

The *Parvovirinae* subfamily includes many different species that can infect vertebrate hosts. While some virus species, such as those detected in Derzsy's disease in goslings and Muscovy ducks, have inflicted significant damage on the waterfowl industry, members of the *aveparvovirus* genus are associated with enteric diseases, leading to malabsorption syndrome, reduced growth, and mortality in chicken and turkey flocks. (Kapgate *et al.*, 2018). Aveparvoviruses play a minor role in runting-stunting syndrome (RSS) in chickens and poult enteritis mortality syndrome (PEMS) in turkeys. (Devaney *et al.*, 2016; Shehata *et al.*, 2021). Chicken parvoviruses (ChPV), classified under the *Galliform aveparvovirus 1*, are recognized as a significant viral pathogen implicated in enteric disorders that cause diarrhea, anorexia, poor growth, stunting, and neurological symptoms in chickens (Marusak *et al.*, 2010; Kapgate *et al.*, 2018; Cotmore *et al.*, 2019). In general, the prognosis for the infection strictly depends on age (1-4 weeks) and the infected birds manifest a broad range of symptoms, including ruffled feathers, malabsorption, watery diarrhoea, and osteoporosis (Trampel *et al.*, 1983; Kisary, Nagy & Bitay, 1984; Kisary, 1985a).

ChPV has been reported in numerous countries, indicating a global presence of the infection (Saif *et al.*, 2020). The high prevalence of aveparvoviruses has been revealed in commercial breeders, layers, and turkeys in countries with vast poultry industries, such as China, India, Brazil, and the USA (Zsak, Strother & Day, 2009; Nuñez *et al.*, 2015; Pradeep, Reddy & Kannaki, 2020; Zhang *et al.*, 2020). On the other hand, backyard poultry operations own a rather minuscule part of the overall poultry production and are often overlooked. A recent study suggested that a significant number of poultrymen exist, especially in both urban and rural areas in Turkey (Özdemir, 2020). The cohabitation of different bird species is common in backyard poultry, allowing pathogens to cross the species barrier (Pauly *et al.*, 2019). Furthermore, the lack of biosecurity measurements in farmyards contributes to the contamination of wild bird habitats with various diseases (Ayala, Yabsley & Hernandez, 2020).

Since the first identification of chicken parvoviruses via electron microscopy, several methods have been developed to investigate ChPVs. Serological methods such as Immunofluorescence assay (IFA), immunohistochemistry (IHC), or capture-ELISA have served as detection methods for virus particles (Kisary, 1985b; Strother & Zsak, 2009; Zsak, Cha & Day, 2013). Despite Real-time PCR enabling researchers to not only detect the virus but also quantify viral load (Finkler *et al.*, 2016), the conventional PCR assay has long been considered a convenient method for investigation since it is versatile and applicable in an average laboratory. Primers amplifying the non-structural (NS) gene of ChPV are frequently preferred, as it is relatively better conserved than the structural proteins (Kapgate *et al.*, 2018). In this regard, the main goal of this study was to investigate the presence and frequency of ChPV in free-range hens using a combination of nested primer pairs, which might offer higher detection capacity among samples. We further inquired into the phylogenetic relationship of strains through the elicited sequencing data, thereby revealing the molecular characteristics of local strains for the first time in Turkey.

MATERIALS AND METHODS

Sampling and nucleic acid extractions

Sample collection for surveillance

Between January and May of 2018, the eight provinces located in the Middle East Anatolian territory of Turkey (Tokat, Sivas, Erzincan, Malatya, Elazig, Tunceli, Bingol and Diyarbakir) were subjected to this study. Sampling size was determined according to the overall population of laying chickens (TUIK, 2022) and calculated using OpenEpi version 3.01 (www.openepi.com) with the parameters as follows: Confidence level, 95%; Hypothesized frequency of outcome factor in the population, + 50%; and design effect, 1. Hence, the objective was to collect a total of 404 faecal samples from the eight provinces. For this purpose, local veterinary practitioners were asked to report any suspicion and they contributed to obtain samples from chickens with enteric disease symptoms including acute diarrhoea, pale comb and wattles, ruffled feathers, panting, as well as lethargy and cachexia. All chickens manifesting the enteric disease symptoms were over 25 weeks and were in the laying period. Sterile swabs were used for cloacal sampling from each animal, and the collected samples were promptly transported to the laboratory within a 24-hour timeframe, ensuring the maintenance of a cold chain throughout the entire process.

DNA Extraction

Faecal samples were diluted 1:10 with 1 M phos-

phate buffered saline with Antibiotic-Antimycotic solution (Gibco, USA) and centrifuged for 10 min at 3,500 rpm to remove large cellular debris. After the centrifugation, supernatants were submitted to a nucleic acid extraction procedure using a GF-1 Viral Nucleic Acid Extraction Kit (Vivantis Technologies, Malaysia) according to the manufacturer's instructions. Eluted DNA extracts were stored at −80ºC until use.

PCR screening of samples

Conventional PCR assay was implemented to the samples using different primer sets. A primer pair previously described by Zsak et al., (2009) targeting 561 bp partial sequence of the NS1 gene was used for detecting viral genome (Zsak *et al.*, 2009). Alternatively, a nested primer set (ChPVF/R and ChPVFn/Rn) was further designed. For this purpose, available sequence data of NS1 genes were retrieved from GenBank (https://www.ncbi.nlm.nih.gov/nucleotide/) and aligned using Geneious software (Kearse *et al.*, 2012). For primer designing, primer3 v2.3.7 plugin (https://primer3.org) was utilized in the same software. The details of these primer set are given in Table 1.

All DNA samples were analysed using five different PCR methods, named methods 1-5. Method 1 followed the exact same protocol as previously used by Zsak and co-workers (Zsak et al., 2009), but with an extended number of PCR cycles (40 cycles in total). Methods 2 and 3 were conventional PCR amplifications using two different primer sets: ChPVF/ChPVR and ChPVFn/ChPVRn, respectively. In method 2, the PCR parameters were as follows: an initial denaturation at 95°C for 2 minutes, followed by 40 cycles of denaturation at 94°C for 45 seconds, annealing at 57°C for 45 seconds, and extension at 72°C for 60 seconds, with a final extension step at 72°C for 10 minutes. Method 3 had a shorter protocol, consisting of an initial denaturation at 95°C for 2 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 57°C for 30 seconds, and extension at 72°C for 45 seconds, with a final extension step at 72°C for 10 minutes. Method 4 utilized a nested PCR approach, where PVF1/PVR1 and ChPVFn/ChPVRn primer sets were used for the first and second rounds of amplification, respectively. The PCR conditions and cycles (40 cycles) used in methods 1 and 3 were applied for the first and second rounds, respectively. Finally, method 5 also employed a nested PCR, using the primer sets (ChPVF/ChPVR and ChPVFn/ChPVRn) specifically designed for this study. Method 5 involved the sequential application of methods 2 and 3, each with 40 cycles of PCR.

All PCR mixtures were prepared as a 50 µl final volume, except for the second reactions of nested PCRs. A mixture contained ~ 100 ng template, 5 µL of 10× PCR buffer, 10 mM of dNTP, 10 pmol/µL of each set of sense/antisense primers, and 5 U of Taq DNA polymerase (Vivantis, Germany). For the second reaction of nested PCRs, the quantity of mixtures was reduced to 25 µl and 1 µl of aliquots from the first step PCR was added sequentially. All amplicons were run by electrophoresis in 1,5% agarose gels and stained with ethidium bromide (0.5 µg/mL). DNA bands were determined under the UV light and only bright bands with the correct sizes were considered positive.

Sequencing and *in silico* analyses

Five positive samples from method 1 and two samples from method 2 were randomly selected for sequencing. Positive samples of DNA amplicons were cut from the agarose gel and purified using a commercial PCR Clean-Up System (Promega, Madison, WI) according to the manufacturer's instructions. Bidirectional sequencing was performed twice using BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an automated sequencer (ABI 3100; Applied Biosystems, Foster City, CA). Raw data with high coverage (>90%) obtained from forward and reverse reads were assembled for each sample and deposited in GenBank (Accession nos: MN717246 - MN717252).

Table 1: Primer sequences and lengths of PCR amplification products.

Primer	Oligonucleotide sequences (5'-3')	Position*	Size (bp)	References
PVF1	TTCTAATAACGATATCACTCAAGTTTC	1841-1867	561	Zsak et al., (2009)
PVR1	TTTGCGCTTGCGGTGAAGTCTGGCTCG	2375-2401		
ChPVF	GCCATCTCAACAGTTCATGCAG	1878-1899	510	This study
ChPVR	GAAGTCTGGCTCGTCWGGWAAT	2366-2387		
ChPVFn	TCCGGDTGGACMAGAAAGCCMT	2157-2178	229	This study
ChPVRn	AGTCTGGCTCGTCWGGWAATCC	2364-2385		

* Positions of primers were determined according to reference strain (NC_024452.1)

The 510 to 558 bp sequencing data from seven samples obtained from method 1 and method 2 were included in multiple sequence and phylogenetic analyses with other available sequences retrieved from GenBank. For this purpose, 218 submissions were downloaded from the database and aligned using MUSCLE v.3.8.425 algorithm. Then, JModelTest was applied to determine the best fit model for phylogeny. An initial phylogenetic tree was constructed by the maximum likelihood statistical method with the Kimura-2 substitution model and bootstrapped 100 times using Geneious Prime software (Kearse et al., 2012). This encompassing tree was used to determine the clusters, in which Turkish strains were located. Red-crowned crane parvoviruses (KY312546 and KY312547) and Pileated finch aveparvovirus (MG745672) were used for outgroup comparisons. Finally, these clusters were extracted and therefore were filtered the most relevant strains into the phylogenetic analysis.

To detect potential recombinant fragments in Turkish strains, nine algorithms (RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan, PhylPro, LARD, and 3Seq) in the RDP4 software were applied with their default parameters (P-value cutoff = 0.05) to aligned sequences simultaneously (Martin *et al.*, 2015). Sequences whose recombination event was detected by at least five algorithms were considered recombinant strains.

Statistical Analysis

Statistical analysis was conducted using IBM SPSS Statistics 23. McNemar test was performed to determine the statistical significance of variations in PCR methods and viral dispersion at the sample and flock levels (Mc, 1947). Statistical significance was assigned to values with $p < 0.05$.

RESULTS

Prevalence of chicken parvovirus based on the methodology.

The prevalence of the chicken parvoviruses within samples was highly variable depending on the methods used. Of the 404 samples, 9,9% were found to be positive using method 1, whereas method 2 had 7,9% positivity. Method 3 detected the highest positivity, which accounted for 22,8% of samples (92 out of 404) between these three conventional PCR tests. As expected, the detection sensitivity of the nested PCR experiments was relatively higher, but significantly divergent depending on the primer set used. Method 4 identified 24,8% positivity, while method 5 presented the highest percentage, 44,6% (180 out of 404) (see Table 2).

Multiple sequence and Phylogenetic analyses

Multiple sequence analysis was conducted based on the pairwise comparison of nucleotide and predicted amino acid sequences. For this purpose, the genetic data between 480/490th and 659/663rd residues of the NS1 protein depending on the primer sets were successfully elicited. Thus, 170 - 186 residues in length deduced amino acid sequences were subjected in this study. Residue-by-residue comparison analysis revealed that ChPV/TUR/45 carried a unique mutation, D522G, while N510K and W511R were detected in ChPV/TUR/49. Amino acid variations through the Turkish strains were shown in Figure 1. On the other hand, potential recombinants in the Turkish strains were investigated based on whole available sequencing data using RDP4 software (Martin *et al.*, 2015); however, no recombination event was observed between Turkish strains and available genomic data (data not shown).

Genetic distance analysis showed that nucleotide identity between Turkish strains varied between 89.02% and 99.10%. Phylogenetically, isolate ChPV/TUR/22 (MN717251) and isolate ChPV/TUR/33 (MN717246) had 98.82% identity and fell into a single clade (clade 1) in the phylogenetic tree with a good bootstrap value (% 87). Rest of the isolates, ChPV/TUR/36 (MN717247), ChPV/TUR/45 (MN717248), ChPV/TUR/49 (MN717249), ChPV/TUR/74 (MN717252) and ChPV/TUR/84

Table 2: Number of ChPV positive flocks and samples detected by each PCR method.

	Method 1	Method 2	Method 3	Method 4	Method 5	X^2
Sample (n=404)	40 (% 9.9)^{a}	32 (% 7.9)^{a}	92 (% 22.8)^{b}	100 (% 24.8)^{bc}	180 (% 44.6)^{cd}	202.95

Method 1: PCR method with primer pair of Zsak et al (2009). **Method 2:** PCR method with primer pair ChPVF and ChPVR. **Method 3:** PCR method with primer pair ChPVFn and ChPVRn. **Method 4 (nested PCR):** Amplicons gathered by PVF1 and PVR1 primer pair submitted to second PCR by ChPVFn and ChPVRn primer pair. **Method 5 (nested PCR):** Amplicons gathered by ChPVF/ ChPVR primer pair submitted to second PCR by ChPVFn and ChPVRn primer pair. $^{a-d}$: within a row, different superscript letters indicate statistically significant differences between compared methods ($p<0.001$).

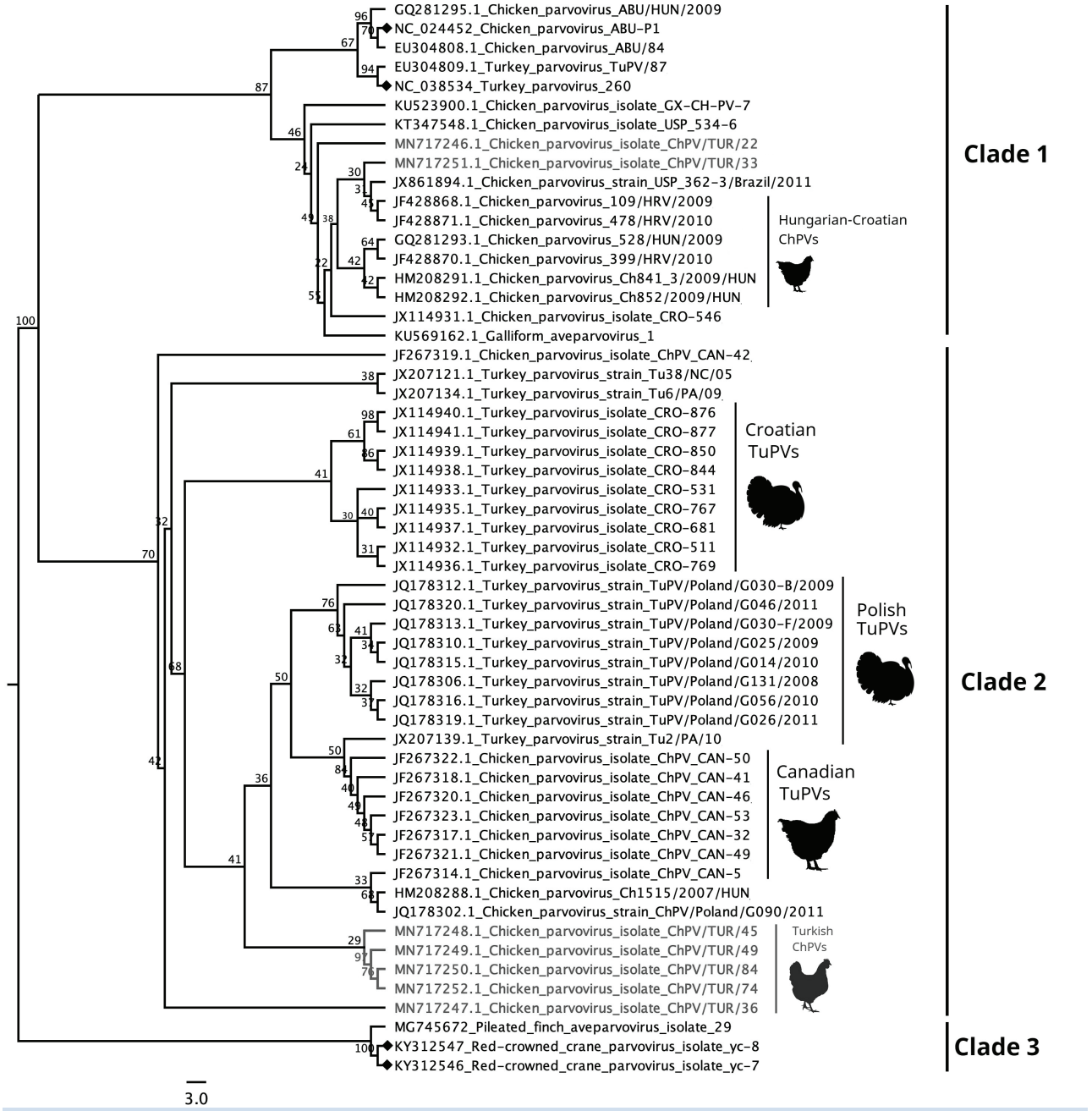

Figure 1. Phylogeny based on partial genomic sequence of NS1. The tree was constructed using maximum likelihood statistical method with Kimura-2 substitution model and bootstrapped 100 times. Red fonts represent strains detected in this study.

(MN717250) shared identity between 94.51% and 99.10% and grouped into a subclade in the clade 2, which was supported by good bootstrap value (70%). Notably, clade 2 included several strains isolated from chickens and turkeys which divaricated within the clade with significant bootstrap values (Figure 2). Turkish ChPV group in clade 2 exhibited the highest nucleotide identity to Croatian TuPVs (90.02% - 95.55%), while ChPV/TUR/36 were to the strain ChPV/Poland/G090/2011 (JQ178302.1). The ChPV/TUR/22 and ChPV/TUR/33 isolates showed the closest relation to isolate USP 507-15P (MK358350) between 96.76% and 97.76%.

DISCUSSION

Maintaining the healthy gastro-intestinal system plays a pivotal role in efficient weight increment, which is, indeed, crucial for the sustainable poultry meat industry. Enteric syndromes such as runting and stunting syndrome (RSS) or poult enteritis and mortal-

ity syndrome (PEMS) frequently lead to poor growth performance in poultry (Devaney *et al.*, 2016). The outcomes of the advanced metagenomic approaches have been achieved a consensus that runting-stunting syndrome (RSS) is a multifactorial syndrome (Devaney *et al.*, 2016; Kim *et al.*, 2020). Numerous viral agents, such as IBV-like coronaviruses (Hauck *et al.*, 2016), rotaviruses (Otto *et al.*, 2006), astroviruses (Kang *et al.*, 2018) and parvoviruses (Zsak *et al.*, 2013) believed to be so far the aetiological agents of the RSS so far. A chicken parvovirus strain (ABU-P1) leading to RSS in chickens was also confirmed by experimental infections in chicken embryonated eggs (N. Nuñez *et al.*, 2020). Furthermore, commercial poultry are usually vaccinated against common diseases including Newcastle disease, infectious bursal disease, Marek's disease, infectious bronchitis, infectious laryngotracheitis and fowl pox. Since these diseases can also infect backyard poultry, a routine vaccination program is not applied by owners. Backyard poultry can appear healthy and clean but can carry many microorganisms especially viral agents. Thus, the major focus of this study centred on the determination of ChPV prevalence in the backyard flocks in Turkey.

This is the first study of chicken parvoviruses detected in poultry in Turkey. Faecal samples obtained from flocks were tested for the presence of parvovirus, and the results showed that parvoviruses are widely distributed in chicken flocks in the inspected provinces of the country. The PCR assays identified different levels of positivity, ranging from 7.9% to 44.6%, depending on the primer sets utilized. In agreement with our study, Zsak et al., (2009) demonstrated a high level of prevalence (77% and 78% positivity in chickens and turkeys, respectively) in commercial flocks in the USA (Zsak et al., 2009). PCR assay optimized in this study (Method 5, nested PCR) detected 44.6 % (180 samples) positivity and the difference between results was significant ($p< 0.001$). In addition, Zsak et al., (2009) have designed NS gene specific primers according to their 6 parvovirus sequences. In the present study, 94 chicken and 97 turkey DNA sequences from GenBank were utilized, and overlapping primers for all sequences were synthesized (Zsak *et al.*, 2009). The most common causes of amplification failure are the presence of PCR inhibitors and a low viral load in the samples (Schrader *et al.*, 2012). Nested PCR methods are extensively used to increase the specificity of DNA amplification. Differences in results may arise from these cases. Furthermore, the ChPVNFn-ChPVNRn primer set, with a 229 bp amplicon size, interpreted the NS gene better than the PVF1-PVR1 and ChPVNF-ChPVNR primer sets. The ChPVNFn-ChPVNRn primer set determined approximately two-fold more samples, in contrast with the other primer sets when applied without nested PCR (Table 2). Taken together, the nested primer sets designed for this study have a superior detection capacity compared to the rest of the primers used in the conventional PCR method.

While ChPV/TUR/22 and ChPV/TUR/33 belonged to clade 1 (with 98.82 percent identity), ChPV/TUR/36, ChPV/TUR/45, ChPV/TUR/49, ChPV/TUR/74, and ChPV/TUR/84 belonged to clade 2 (with varied identities ranging from 94.51% to 99.10%). Turkish strains that fell into the same clades were obtained from the same area, namely Central Anatolia (clade 1) and Eastern Anatolia (clade 2). In addition to chicken strains, clade 2 also comprised viruses isolated from turkeys that diverged within the clade with substantial bootstrap values. The nucleotide analysis of the ChPV sequences showed that the Turkish strain shares a high similarity with the other ChPV strains worldwide. Turkish ChPV group in clade 2 exhibited the highest nucleotide identity to Croatian TuPVs (90.02% - 95.55%), while ChPV/TUR/36 were closest in relation to the strain ChPV/Poland/G090/2011 (JQ178302.1). The ChPV/TUR/22 and ChPV/TUR/33 isolates showed the closest relation to isolate USP 507-15P (MK358350) between 96.76% and 97.76%. Many studies conducted in different countries represented that ChPV strains from around the world have high similarities and they demonstrated different clusters (Zsak *et al.*, 2009; Palade *et al.*, 2011; Domanska-Blicharz *et al.*, 2012; Pauly *et al.*, 2019). Overall, we surmised that some of Turkish strains might have distinctive genomic markers reflecting the geographic location.

One of the shortcomings in the present study was the retrieval of limited amino acid data from the partial sequence of NS1. Nonetheless, we obtained sequences with lengths ranging from 170 to 186 amino acids, which are partially included in the C-terminus of the helicase domain, functioning as a translocator distorting the double-stranded portion of DNA or RNA (Hickman & Dyda, 2005). Our multiple sequence analysis revealed that ChPV/TUR/49 had N510K and W511R substitutions in the C domain. Members of the SF3 helicase superfamily have four well-conserved motifs, which are defined as the Walk-

er A (GPATTGKT), B (VIWWEE), B' (16 aa variable region), and C (includes a constant asparagine residue) motifs (James et al., 2003; Xie et al., 2023). The "NW" motif is identical through the strains isolated from either chicken or turkeys, whereas N510K mutations existed only in two Red-crowned crane parvovirus isolates (yc-7 and yc-8) previously reported (Wang et al., 2019). However, no recombination event was found between these sequences. Taken together, conducting comparative structural analysis on the aveparvoviral NS1 protein would be a valuable practice to gain further insights into the molecular diversity of the protein.

In conclusion, this is the first report on the molecular characterization of ChPV, which is circulating in Turkish free-range chicken flocks and is linked to enteric illnesses. However, further extensive research involving commercial chicken flocks is necessary to establish the presence and significance of ChPV in gastrointestinal disorders. The nested PCR approach developed in this study can be considered as an alternative to other PCR assays. The genetic investigation of the ChPV strains circulating in Turkey revealed a significant degree of resemblance to other ChPV viruses found in other countries. This finding might aid in developing control and prevention strategies for the disease.

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