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Molecular Diagnostic of Chicken Parvovirus (ChPV) Affecting Broiler Flocks in Ecuador

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■Keywords

Chicken parvovirus, enteric diseases, molecular diagnostic, PCR.

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ABSTRACT

Enteric diseases affect poultry and cause important economic losses in many countries worldwide. Avian parvovirus has been linked to enteric conditions, such as malabsorption and runting-stunting syndrome (RSS), characterized by diarrhoea, and reduced weight gain and growth retardation. In 2013 and 2016, 79 samples were collected from different organs of chickens in Ecuador that exhibited signs of diarrhea and stunting syndrome, and analysed for the presence of chicken parvovirus (ChPV). The detection method of ChPV applied was Polymerase Chain Reaction (PCR), using primers designed from the conserved region of the viral genome that encodes the non-structural protein NS1. Out of the 79 samples, 50.6% (40/79) were positive for ChPV, and their nucleotide and amino acid sequences were analysed to determine their phylogenetic relationship with the sequences reported in the United States, Canada, China, South Korea, Croatia, Poland, Hungary, and Brazil. Strong similarity of nucleotide and amino acid sequences among all analyzed sequences and between the analysed and reference sequences was demonstrated, and the phylogenetic analysis clustered all the sequences within the same group, demonstrating a strong relation between the studied strains and the reference chicken parvovirus strains.

INTRODUCTION

The intestinal health of birds is related to animal welfare and the productive capacity of animals. Enteric problems cause economic losses around the world, especially in young chickens, due to the costs of therapeutic treatments, decreased productivity and even increased morbidity and mortality. Viral diseases are characterized by the presence of diarrhoea, decreased weight gain, and increased feed conversion (Goodwin *et al*., 1993; Otto *et al*., 2006; Pantin-Jackwood *et al*., 2008; Kang *et al*., 2012). Several viruses are associated with enteric problems in chickens, such as avian coronavirus (IBV), avian reovirus (AReo), chicken astrovirus (CAstV), avian rotavirus-A (ARTv-A), fowl aviadenovirus (FAdV), and chicken parvovirus (ChPV) (Guy, 1998; Zsak *et al*., 2008; Nuñez & Ferreira, 2013), but there is limited information on the effects of individual viruses and their interactions on gut health (Pantin-Jackwood *et al*. 2008; Domanska-Blicharz *et al*., 2012; Mettifogo *et al*., 2014).

Avian parvovirus was first reported by Kisary *et al*. (1984),who found parvovirus-like virus particles that caused Derzsy's disease in geese, using electron microscopy with gut samples from chickens with Runting-Stunting Syndrome (RSS).The family *Parvoviridae* contains two subfamilies: *Parvovirinae* that infect vertebrates, and *Densovirinae* that infect invertebrates (Nuñez & Ferreira, 2013).

The chicken parvovirus (ChPV)belongs to the genus *Aveparvovirus,* which also includes the turkey parvovirus (Cotmore *et al*., 2014). The particles of ChPV are small (19-24 nm in diameter), non-enveloped, and have icosahedral symmetry. The linear genome is single-stranded DNA and it is5 kilobases long (Kisary *et al*., 1984; Cotmore & Tattersall, 1995; Domanska-Blicharz *et al*., 2012).The genome contains 3 open reading frames (ORFs), including ORF 5', which is 2085 nt long, ORF 3',which is 2028 nt long, and a small ORF that is 306 nt long, located between 5' and 3' ORFs. The 5'ORF encodes a non-structural protein, NS1, whereas the 3'ORF appears to encode the capsid proteins VP1, VP2 and VP3, whereas the function of the small ORF has not been defined yet(Day & Zsak, 2010).

ChPV is related to enteric diseases that cause diarrhoea, growth retardation and lower than average weight gain, specially in 2- to 7-year-old chicks, and it is considered to be one of the aetiological agents for RSS (Zsak *et al*., 2013). This syndrome is also called malabsorption syndrome (MAS), helicopter disease, infectious stunting syndrome and brittle bone disease (Finkler *et al*., 2016).Viral replication and pathogenic effects mainly occur in cells with high proliferative rates (Hueffer & Parrish, 2003).

The aim of this study is to determine the presence of ChPV in organs obtained from broilers in Ecuador with signs of enteric disease, using Polymerase Chain Reaction (PCR) and nucleotide sequencing procedures.

MATERIALS AND METHODS

Samples

In 2013 and 2016, 79 samples were received at the Laboratory of Avian Diseases of the University of São Paulo, Brazil, corresponding to imprints of different organs, including the thymus, spleen, trachea, lung, air sac, gut, caecal tonsil, bursa, kidney and bone marrow of broilers between 1 to 4 weeks of age reared in Ecuador. Out of those samples, 42 were obtained in 2013, and 37 in 2016. The samples were used for the molecular analysis of enteric viruses that could be affecting commercial broiler flocks, whose clinical history included enteric problems such as diarrhoea, malabsorption, and delayed growth. These birds belonged to different commercial flocks distributed in the northern region of Ecuador, and after necropsy, several imprints were collected on FTA cards (GE Healthcare, Buckinghamshire, UK) for shipment to Brazil.

DNA Isolation

The material impregnated on the FTA cards was cut and suspended in PBS (Phosphate Buffered Solution), 0.1 M, pH 7.4, at 1:1 ratio, then macerated into 2-mLmicrotubes using a bead mill (TissueLyser LT Bead Mill, Qiagen, Hilden, Germany) for 5 minutes. The material was finally centrifuged for 30 min at 12,000 x g and at 4 °C. An aliquot of the supernatant was then collected for the extraction of DNA by the phenol/chloroform technique described by Chomczynski (1993). The extracted DNA was stored at -20 °C.

Polymerase chain reaction (PCR) for the detection of chicken parvovirus

The primers used in this reaction were those described by Zsak *et al*. (2009), PVF1 5'-TTCTAATAACGATATCACT-3' and PVR1 5'-TTTGCGCTTGCGGTGAAGTCTGGCTCG-3',corresponding to the conserved region of the non-structural NS gene, which amplify a 561-bp fragment. The PCR reaction conditions for ChPV amplification were performed as reported by Zsak *et al*. (2009), with some variations. PCR components were mixed in a DNA-free microfuge tube that included 1X reaction buffer, 1.25 mM of each deoxynucleotide triphosphate, 0.5 µM of each primer, 1.25 U of Platinum® Taq polymerase (Invitrogen® by Life Technologies, Carlsbad, CA, USA), and 2 µL of extracted DNA. Thermocycling parameters included one cycle of DNA denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, followed by final extension at 72°C for 10 min. The PCR products of all samples were run on 1.5% Agarose gel using SyBR® Safe DNA gel stain (Invitrogen™) and a 100 bp DNA Ladder (Invitrogen™) to determine band size.

DNA sequencing and nucleotide sequence analysis

The amplified product was purified using the GPX™ PCR DNA and Gel Band Purification kit (GE Healthcare, Piscataway, New Jersey, USA), according to the manufacturer's instructions. Each purified product was sequenced in the forward and reverse direction using the BigDye® Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems by Life Technologies, Carlsbad, CA, USA). Sequencing reactions were carried out in ABI 3730 DNA Analyzer (Applied Biosystems by Life Technologies). The sequences obtained were edited using the CLC Main Workbench 7.7.3 software and aligned with previous reported sequences obtained from the GenBank database belonging to Brazil,

Canada, Croatia, China, Hungary, South Korea, Poland, and the United States, using the CLUSTAL W method available in the ClustalX 2.1 software. Accession numbers of the reference sequences are detailed in the phylogenetic tree (Figure 1). The phylogenetic tree was inferred using the neighbour-joining method, with 1,000 bootstrap replicates integrated in the MEGA 7.0.18 software. The nucleotide and amino acid sequence similarity matrix was generated in the BioEdit Sequence Alignment Editor v. 7.2.5.

RESULTS

PCR

PCR products were run on 1.5% agarose gel, and the location of the DNA band of each positive sample confirmed the amplification of the 561 bp segment in 40/79 samples, out of which 17/42 corresponded to the samples received in 2013 and 23/37 to the samples received in 2016. The details of the positive samples are described in Table 1.

Table 1 – Sample identification and origin, type of bird, clinical signs, year of collection and accession number from the NCBI GenBank database.

Number of positive samples	Sample Identification	Type of Sample	Bird	Clinical signs		Year of collection	GenBank Accession number
				Diarrhea	Stunting		
1	EC 513-14	Spleen	Broiler	Yes	Yes	2013	KY649239
2	EC 513-16	Lung	Broiler	Yes	Yes	2013	KY649240
3	EC 513-17	Trachea	Broiler	Yes	Yes	2013	KY649241
4	EC 513-18	Kidney	Broiler	Yes	Yes	2013	KY649242
5	EC 513-19	Timus	Broiler	Yes	Yes	2013	KY649243
6	EC 513-22	Air sac	Broiler	Yes	Yes	2013	KY649244
7	EC 513-23	Trachea	Broiler	Yes	Yes	2013	KY649245
8	EC 513-24	Timus	Broiler	Yes	Yes	2013	KY649246
9	EC 513-25	Bone marrow	Broiler	Yes	Yes	2013	KY649247
10	EC 513-26	Spleen	Broiler	Yes	Yes	2013	KY649248
11	EC 513-29	Trachea	Broiler	Yes	Yes	2013	KY649249
12	EC 513-30	Trachea	Broiler	Yes	Yes	2013	KY649250
13	EC 513-32	Trachea	Broiler	Yes	Yes	2013	KY649251
14	EC 513-33	Cecal tonsils	Broiler	Yes	Yes	2013	KY649252
15	EC 513-34	Gut	Broiler	Yes	Yes	2013	KY649253
16	EC 513-37	Cecal tonsils	Broiler	Yes	Yes	2013	KY649254
17	EC 513-38	Cecal tonsils	Broiler	Yes	Yes	2013	KY649255
18	EC 722-3	Trachea	Broiler	Yes	Yes	2016	KY649256
19	EC 722-15	Trachea	Broiler	Yes	Yes	2016	KY649257
20	EC 722-17	Kidney	Broiler	Yes	Yes	2016	KY649258
21	EC 722-18	Bursa	Broiler	Yes	Yes	2016	KY649259
22	EC 722-19	Bursa	Broiler	Yes	Yes	2016	KY649260
23	EC 722-20	Bursa	Broiler	Yes	Yes	2016	KY649261
24	EC 722-21	Bursa	Broiler	Yes	Yes	2016	KY649262
25	EC 722-22	Bursa	Broiler	Yes	Yes	2016	KY649263
26	EC 722-23	Bursa	Broiler	Yes	Yes	2016	KY649264
27	EC 722-24	Bursa	Broiler	Yes	Yes	2016	KY649265
28	EC 722-25	Bursa	Broiler	Yes	Yes	2016	KY649266
29	EC 722-26	Bursa	Broiler	Yes	Yes	2016	KY649267
30	EC 722-27	Bursa	Broiler	Yes	Yes	2016	KY649268
31	EC 722-28	Bursa	Broiler	Yes	Yes	2016	KY649269
32	EC 722-29	Bursa	Broiler	Yes	Yes	2016	KY649270
33	EC 722-30	Bursa	Broiler	Yes	Yes	2016	KY649271
34	EC 722-31	Bursa	Broiler	Yes	Yes	2016	KY649272
35	EC 722-32	Bursa	Broiler	Yes	Yes	2016	KY649273
36	EC 722-33	Bursa	Broiler	Yes	Yes	2016	KY649274
37	EC 722-34	Bursa	Broiler	Yes	Yes	2016	KY649275
38	EC 722-35	Bursa	Broiler	Yes	Yes	2016	KY649276
39	EC 722-36	Bursa	Broiler	Yes	Yes	2016	KY649277
40	EC 722-37	Bursa	Broiler	Yes	Yes	2016	KY649278

Table 2 – Matrix of similarity for nucleotide and amino acid sequences. To the left, nucleotide sequences, and to the top, amino acid sequences obtained in the study, compared with the reference sequences obtained from GenBank. EC=Ecuador, BR=Brazil (21), CA=Canada (22), HR=Croatia (23), HU=Hungary (24), PL=Poland (26 and 27), CH=China (29), US=United States (28), KR=South Korea (25). To the left, nucleotide sequences, and to the top, amino acid sequences obtained in the study, compared with the reference sequences obtained from GenBank. EC=Ecuador, BR=Brazil, CA=Canada, HR=Croatia, HU=Hungary, PL=Poland, CH=China, US=United States, KR=South Korea. (Part 1)

ChPV isolates and reference strains		Numbers corresponding to the access number of ChPV from Ecuador isolates in comparison with reference strains																					Reference strains obtained in the GenBank data base								
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	
1	EC_513-14	-	1,000	1,000	0,969	1,000	1,000	1,000	0,992	1,000	1,000	1,000	0,984	1,000	1,000	1,000	1,000	0,992	0,992	0,931	0,931	0,992	0,946	1,000	0,992	0,962	0,984	0,992	0,984	0,992
2	EC_513-16	0,987	-	1,000	0,969	1,000	1,000	1,000	0,992	1,000	1,000	1,000	0,984	1,000	1,000	1,000	1,000	0,992	0,992	0,931	0,931	0,992	0,946	1,000	0,992	0,962	0,984	0,992	0,984	0,992
3	EC_513-17	1,000	0,987	-	0,969	1,000	1,000	1,000	0,992	1,000	1,000	1,000	0,984	1,000	1,000	1,000	1,000	0,992	0,992	0,931	0,931	0,992	0,946	1,000	0,992	0,962	0,984	0,992	0,984	0,992
4	EC_513-18	0,954	0,957	0,954	-	0,969	0,969	0,969	0,962	0,969	0,969	0,969	0,984	0,969	0,969	0,969	0,969	0,977	0,962	0,924	0,924	0,962	0,946	0,969	0,977	0,931	0,984	0,962	0,984	0,977
5	EC_513-19	0,979	0,992	0,979	0,959	-	1,000	1,000	0,992	1,000	1,000	1,000	0,984	1,000	1,000	1,000	1,000	0,992	0,992	0,931	0,931	0,992	0,946	1,000	0,992	0,962	0,984	0,992	0,984	0,992
6	EC_513-22	1,000	0,987	1,000	0,954	0,979	-	1,000	0,992	1,000	1,000	1,000	0,984	1,000	1,000	1,000	1,000	0,992	0,992	0,931	0,931	0,992	0,946	1,000	0,992	0,962	0,984	0,992	0,984	0,992
7	EC_513-23	0,997	0,984	0,997	0,957	0,977	0,997	-	0,992	1,000	1,000	1,000	0,984	1,000	1,000	1,000	1,000	0,992	0,992	0,931	0,931	0,992	0,946	1,000	0,992	0,962	0,984	0,992	0,984	0,992
8	EC_513-24	0,989	0,977	0,989	0,954	0,969	0,989	0,992	-	0,992	0,992	0,992	0,977	0,992	0,992	0,992	0,992	0,984	1,000	0,931	0,931	1,000	0,939	0,992	0,984	0,954	0,977	0,984	0,977	0,984
9	EC_513-25	0,997	0,984	0,997	0,957	0,977	0,997	1,000	0,992	-	1,000	1,000	0,984	1,000	1,000	1,000	1,000	0,992	0,992	0,931	0,931	0,992	0,946	1,000	0,992	0,962	0,984	0,992	0,984	0,992
10	EC_513-26	0,997	0,984	0,997	0,957	0,977	0,997	1,000	0,992	1,000	-	1,000	0,984	1,000	1,000	1,000	1,000	0,992	0,992	0,931	0,931	0,992	0,946	1,000	0,992	0,962	0,984	0,992	0,984	0,992
11	EC_513-29	0,974	0,977	0,974	0,969	0,979	0,974	0,977	0,974	0,977	0,977	-	0,984	1,000	1,000	1,000	1,000	0,992	0,992	0,931	0,931	0,992	0,946	1,000	0,992	0,962	0,984	0,992	0,984	0,992
12	EC_513-30	0,964	0,977	0,964	0,979	0,979	0,964	0,967	0,964	0,967	0,967	0,974	-	0,984	0,984	0,984	0,984	0,992	0,977	0,931	0,931	0,977	0,962	0,984	0,992	0,946	1,000	0,977	1,000	0,992
13	EC_513-32	0,962	0,974	0,962	0,959	0,982	0,962	0,964	0,962	0,964	0,964	0,979	0,977	-	1,000	1,000	1,000	0,992	0,992	0,931	0,931	0,992	0,946	1,000	0,992	0,962	0,984	0,992	0,984	0,992
14	EC_513-33	0,992	0,979	0,992	0,952	0,972	0,992	0,989	0,987	0,989	0,989	0,982	0,962	0,964	-	1,000	1,000	0,992	0,992	0,931	0,931	0,992	0,946	1,000	0,992	0,962	0,984	0,992	0,984	0,992
15	EC_513-34	0,992	0,979	0,992	0,952	0,972	0,992	0,989	0,987	0,989	0,989	0,982	0,962	0,964	1,000	-	1,000	0,992	0,992	0,931	0,931	0,992	0,946	1,000	0,992	0,962	0,984	0,992	0,984	0,992
16	EC_513-37	0,977	0,989	0,977	0,957	0,992	0,977	0,974	0,967	0,974	0,974	0,987	0,977	0,979	0,979	0,979	-	0,992	0,992	0,931	0,931	0,992	0,946	1,000	0,992	0,962	0,984	0,992	0,984	0,992
17	EC_513-38	0,974	0,987	0,974	0,969	0,989	0,974	0,977	0,969	0,977	0,977	0,979	0,989	0,977	0,967	0,967	0,987	-	0,984	0,924	0,924	0,984	0,954	0,992	0,984	0,954	0,992	0,984	0,992	0,984
18	EC_722-3	0,989	0,977	0,989	0,954	0,969	0,989	0,992	0,994	0,992	0,992	0,979	0,964	0,967	0,992	0,992	0,972	0,969	-	0,931	0,931	1,000	0,939	0,992	0,984	0,954	0,977	0,984	0,977	0,984
19	EC_722-15	0,904	0,917	0,904	0,896	0,917	0,904	0,902	0,904	0,902	0,902	0,912	0,914	0,924	0,907	0,907	0,917	0,909	0,909	-	1,000	0,931	0,893	0,931	0,939	0,893	0,931	0,931	0,931	0,939
20	EC_722-17	0,904	0,917	0,904	0,896	0,917	0,904	0,902	0,904	0,902	0,902	0,912	0,914	0,924	0,907	0,907	0,917	0,909	0,909	1,000	-	0,931	0,893	0,931	0,939	0,893	0,931	0,931	0,931	0,939
21	JX861894	0,954	0,967	0,954	0,952	0,974	0,954	0,957	0,959	0,957	0,957	0,972	0,969	0,992	0,957	0,957	0,972	0,969	0,964	0,922	0,922	-	0,939	0,992	0,984	0,954	0,977	0,984	0,977	0,984
22	JF267316	0,912	0,924	0,912	0,932	0,929	0,912	0,914	0,912	0,914	0,914	0,927	0,942	0,937	0,914	0,914	0,929	0,932	0,917	0,879	0,879	0,929	-	0,946	0,954	0,984	0,962	0,939	0,962	0,954
23	JF428870	0,962	0,974	0,962	0,959	0,982	0,962	0,964	0,962	0,964	0,964	0,979	0,977	0,994	0,964	0,964	0,979	0,977	0,967	0,919	0,919	0,992	0,932	-	0,992	0,962	0,984	0,992	0,984	0,992
24	GQ281296	0,954	0,967	0,954	0,962	0,974	0,954	0,957	0,954	0,957	0,957	0,972	0,979	0,987	0,957	0,957	0,972	0,969	0,959	0,919	0,919	0,984	0,939	0,987	-	0,954	0,992	0,984	0,992	1,000
25	KC593420	0,924	0,937	0,924	0,922	0,944	0,924	0,927	0,924	0,927	0,927	0,942	0,939	0,962	0,927	0,927	0,942	0,939	0,929	0,886	0,886	0,959	0,968	0,962	0,954	-	0,946	0,954	0,946	0,954
26	JQ178301	0,952	0,964	0,952	0,964	0,969	0,952	0,954	0,952	0,954	0,954	0,969	0,982	0,982	0,954	0,954	0,969	0,972	0,957	0,922	0,922	0,974	0,949	0,977	0,984	0,944	-	0,977	1,000	0,992
27	JQ178303	0,952	0,964	0,952	0,944	0,972	0,952	0,954	0,947	0,954	0,954	0,964	0,962	0,979	0,949	0,949	0,969	0,967	0,952	0,922	0,922	0,972	0,927	0,974	0,972	0,942	0,972	-	0,977	0,984
28	GQ260159	0,944	0,957	0,944	0,967	0,962	0,944	0,947	0,944	0,947	0,947	0,967	0,974	0,969	0,952	0,952	0,967	0,964	0,949	0,904	0,904	0,962	0,942	0,969	0,977	0,932	0,977	0,954	-	0,992
29	KU523900	0,957	0,969	0,957	0,962	0,977	0,957	0,959	0,957	0,959	0,959	0,967	0,982	0,974	0,954	0,954	0,969	0,977	0,957	0,907	0,907	0,972	0,927	0,979	0,977	0,942	0,967	0,959	0,969	-

Table 2 – Matrix of similarity for nucleotide and amino acid sequences. To the left, nucleotide sequences, and to the top, amino acid sequences obtained in the study, compared with the reference sequences obtained from GenBank. EC=Ecuador, BR=Brazil (21), CA=Canada (22), HR=Croatia (23), HU=Hungary (24), PL=Poland (26 and 27), CH=China (29), US=United States (28), KR=South Korea (25). To the left, nucleotide sequences, and to the top, amino acid sequences obtained in the study, compared with the reference sequences obtained from GenBank. EC=Ecuador, BR=Brazil, CA=Canada, HR=Croatia, HU=Hungary, PL=Poland, CH=China, US=United States, KR=South Korea. (Part 2)

ChPV isolates and reference strains		Numbers corresponding to the access number of ChPV from Ecuador isolates in comparison with reference strains																		Reference strains obtained in the GenBank data base									
	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	21	22	23	24	25	26	27	28	29	
30	EC_722-18	-	1,000	0,984	0,984	0,984	0,977	0,977	0,969	0,969	0,962	1,000	1,000	0,984	1,000	1,000	0,984	0,984	0,946	0,916	0,916	0,992	0,946	1,000	0,992	0,962	0,984	0,992	0,984	0,992
31	EC_722-19	1,000	-	0,984	0,984	0,984	0,977	0,977	0,969	0,969	0,962	1,000	1,000	0,984	1,000	1,000	0,984	0,984	0,946	0,916	0,916	0,992	0,946	1,000	0,992	0,962	0,984	0,992	0,984	0,992
32	EC_722-20	0,987	0,987	-	1,000	0,984	0,962	0,992	0,984	0,984	0,977	0,984	0,984	0,984	0,984	0,984	1,000	1,000	0,946	0,916	0,916	0,977	0,962	0,984	0,992	0,946	1,000	0,977	1,000	0,992
33	EC_722-21	0,984	0,984	0,997	-	0,984	0,962	0,992	0,984	0,984	0,977	0,984	0,984	0,984	0,984	0,984	1,000	1,000	0,946	0,916	0,916	0,977	0,962	0,984	0,992	0,946	1,000	0,977	1,000	0,992
34	EC_722-22	0,979	0,979	0,972	0,969	-	0,977	0,977	0,977	0,977	0,962	0,984	0,984	1,000	0,984	0,984	0,984	0,984	0,946	0,916	0,916	0,977	0,946	0,984	0,977	0,946	0,984	0,977	0,984	0,977
35	EC_722-23	0,972	0,972	0,959	0,957	0,969	-	0,969	0,954	0,954	0,954	0,977	0,977	0,977	0,977	0,977	0,962	0,962	0,962	0,931	0,931	0,969	0,924	0,977	0,969	0,939	0,962	0,984	0,962	0,969
36	EC_722-24	0,974	0,974	0,982	0,979	0,969	0,957	-	0,977	0,977	0,984	0,977	0,977	0,977	0,977	0,977	0,992	0,992	0,946	0,916	0,916	0,969	0,954	0,977	0,984	0,939	0,992	0,984	0,992	0,984
37	EC_722-25	0,964	0,964	0,972	0,969	0,959	0,944	0,964	-	1,000	0,962	0,969	0,969	0,977	0,969	0,969	0,984	0,984	0,939	0,909	0,909	0,962	0,946	0,969	0,977	0,931	0,984	0,962	0,984	0,977
38	EC_722-26	0,964	0,964	0,972	0,969	0,959	0,944	0,964	1,000	-	0,962	0,969	0,969	0,977	0,969	0,969	0,984	0,984	0,939	0,909	0,909	0,962	0,946	0,969	0,977	0,931	0,984	0,962	0,984	0,977
39	EC_722-27	0,952	0,952	0,959	0,957	0,952	0,949	0,977	0,957	0,957	-	0,962	0,962	0,962	0,962	0,962	0,977	0,977	0,931	0,901	0,901	0,954	0,939	0,962	0,969	0,924	0,977	0,969	0,977	0,969
40	EC_722-28	1,000	1,000	0,987	0,984	0,979	0,972	0,974	0,964	0,964	0,952	-	1,000	0,984	1,000	1,000	0,984	0,984	0,946	0,916	0,916	0,992	0,946	1,000	0,992	0,962	0,984	0,992	0,984	0,992
41	EC_722-29	0,982	0,982	0,969	0,967	0,962	0,959	0,957	0,957	0,957	0,939	0,982	-	0,984	1,000	1,000	0,984	0,984	0,946	0,916	0,916	0,992	0,946	1,000	0,992	0,962	0,984	0,992	0,984	0,992
42	EC_722-30	0,987	0,987	0,984	0,982	0,987	0,969	0,977	0,969	0,969	0,957	0,987	0,969	-	0,984	0,984	0,984	0,984	0,946	0,916	0,916	0,977	0,946	0,984	0,977	0,946	0,984	0,977	0,984	0,977
43	EC_722-31	1,000	1,000	0,987	0,984	0,979	0,972	0,974	0,964	0,964	0,952	1,000	0,982	0,987	-	1,000	0,984	0,984	0,946	0,916	0,916	0,992	0,946	1,000	0,992	0,962	0,984	0,992	0,984	0,992
44	EC_722-32	0,989	0,989	0,977	0,974	0,969	0,967	0,964	0,959	0,959	0,947	0,989	0,992	0,977	0,989	-	0,984	0,984	0,946	0,916	0,916	0,992	0,946	1,000	0,992	0,962	0,984	0,992	0,984	0,992
45	EC_722-33	0,984	0,984	0,992	0,994	0,969	0,957	0,979	0,964	0,964	0,957	0,984	0,967	0,977	0,984	0,974	-	1,000	0,946	0,916	0,916	0,977	0,962	0,984	0,992	0,946	1,000	0,977	1,000	0,992
46	EC_722-34	0,987	0,987	0,994	0,992	0,977	0,959	0,987	0,977	0,977	0,964	0,987	0,969	0,989	0,987	0,977	0,987	-	0,946	0,916	0,916	0,977	0,962	0,984	0,992	0,946	1,000	0,977	1,000	0,992
47	EC_722-35	0,929	0,929	0,924	0,922	0,929	0,957	0,922	0,912	0,912	0,914	0,929	0,917	0,929	0,929	0,924	0,919	0,924	-	0,962	0,962	0,946	0,909	0,946	0,954	0,909	0,946	0,946	0,946	0,954
48	EC_722-36	0,919	0,919	0,914	0,912	0,917	0,947	0,912	0,899	0,899	0,904	0,919	0,907	0,919	0,919	0,914	0,909	0,914	0,979	-	1,000	0,916	0,878	0,916	0,924	0,878	0,916	0,916	0,916	0,924
49	EC_722-37	0,919	0,919	0,914	0,912	0,917	0,947	0,912	0,899	0,899	0,904	0,919	0,907	0,919	0,919	0,914	0,909	0,914	0,979	1,000	-	0,916	0,878	0,916	0,924	0,878	0,916	0,916	0,916	0,924
21	JX861894	0,972	0,972	0,959	0,957	0,977	0,967	0,957	0,954	0,954	0,947	0,972	0,954	0,972	0,972	0,962	0,957	0,964	0,927	0,919	0,919	-	0,939	0,992	0,984	0,954	0,977	0,984	0,977	0,984
22	JF267316	0,929	0,929	0,932	0,929	0,934	0,927	0,929	0,929	0,929	0,927	0,929	0,912	0,939	0,929	0,919	0,924	0,937	0,891	0,881	0,881	0,929	-	0,946	0,954	0,984	0,962	0,939	0,962	0,954
23	JF428870	0,979	0,979	0,967	0,964	0,979	0,969	0,959	0,962	0,962	0,949	0,979	0,962	0,979	0,979	0,969	0,964	0,972	0,929	0,917	0,917	0,992	0,932	-	0,992	0,962	0,984	0,992	0,984	0,992
24	GQ281296	0,972	0,972	0,969	0,972	0,977	0,967	0,967	0,964	0,964	0,957	0,972	0,954	0,977	0,972	0,962	0,967	0,974	0,934	0,922	0,922	0,984	0,939	0,987	-	0,954	0,992	0,984	0,992	1,000
25	KC593420	0,942	0,942	0,929	0,927	0,947	0,937	0,927	0,924	0,924	0,917	0,942	0,924	0,942	0,942	0,932	0,927	0,934	0,896	0,884	0,884	0,959	0,968	0,962	0,954	-	0,946	0,954	0,946	0,954
26	JQ178301	0,969	0,969	0,972	0,969	0,979	0,967	0,969	0,967	0,967	0,962	0,969	0,952	0,979	0,969	0,959	0,964	0,977	0,934	0,922	0,922	0,974	0,949	0,977	0,984	0,944	-	0,977	1,000	0,992
27	JQ178303	0,969	0,969	0,957	0,954	0,974	0,974	0,964	0,947	0,947	0,952	0,969	0,952	0,969	0,969	0,959	0,954	0,962	0,934	0,922	0,922	0,972	0,927	0,974	0,972	0,942	0,972	-	0,977	0,984
28	GQ260159	0,962	0,962	0,964	0,967	0,967	0,949	0,967	0,964	0,964	0,964	0,962	0,944	0,972	0,962	0,952	0,967	0,969	0,917	0,904	0,904	0,962	0,942	0,969	0,977	0,932	0,977	0,954	-	0,992
29	KU523900	0,974	0,974	0,972	0,969	0,964	0,959	0,969	0,964	0,964	0,959	0,974	0,957	0,969	0,974	0,964	0,974	0,977	0,922	0,912	0,912	0,972	0,927	0,979	0,977	0,942	0,967	0,959	0,969	-

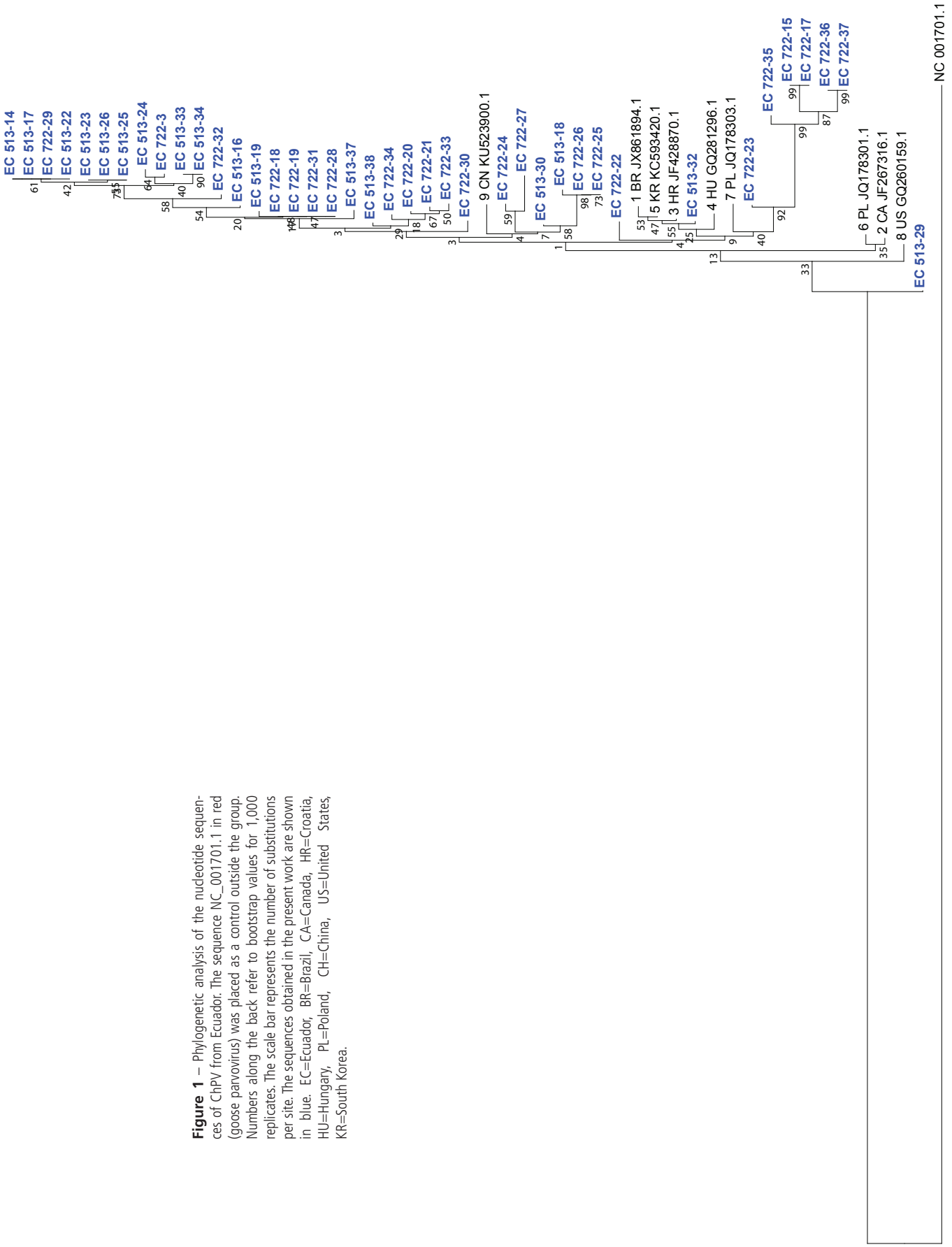

Figure 1 – Phylogenetic analysis of the nucleotide sequences of ChPV from Ecuador. The sequence NC_001701.1 in red (goose parvovirus) was placed as a control outside the group. Numbers along the back refer to bootstrap values for 1,000 replicates. The scale bar represents the number of substitutions per site. The sequences obtained in the present work are shown in blue. EC=Ecuador, BR=Brazil, CA=Canada, HR=Croatia, HU=Hungary, PL=Poland, CH=China, US=United States, KR=South Korea.

DNA sequencing and phylogenetic analysis

It was possible to sequence all positive results, obtaining a total of 40 sequences from different organs: 20 from bursae, seven in tracheas, three in caecal tonsils, two in spleens, kidneys, and thymuses, one in each of the following organs: air sac, bone marrow, intestine, and lung. The details of all positive samples, including GenBank accession numbers, are given in Table 1. The 40 sequenced fragments were analysed with a size of 398 nucleotides, showing a high percentage of similarity among nucleotides (NT)(89.6% - 100%) and amino acids (AA)(90.1% - 100%). Furthermore, there was a high percentage of similarity between sequences from Brazil (91.9% - 99.2% NT and 91.6% - 100% AA), Canada (87.9% - 94.2% NT and 87.8% - 96.2% AA), the United States (90.4% - 97.4% NT and 91.6% - 100% AA), Croatia (91.7% - 99.4% NT and 91.6% - 100% AA), Poland (92.2% - 98.2% NT and 91.6% - 100% AA), China (90.7% - 98.2% NT and 92.4% - 99.2% AA), South Korea (88.4% - 96.2% NT and 87.8% - 96.2% AA) and Hungary (91.9% - 98.7% NT and 92.4% - 99.2% AA). The similarity matrix is detailed in Table 2.

In the phylogenetic analysis, all sequences were clustered in the same group, demonstrating that the sequences obtained in this study are related to the reference sequences originating from North America, Brazil, Europe and Asia, as shown in Figure 1.

DISCUSSION

The primary aetiology of RSS or MAS in chickens is still unknown, although several viruses have been identified in birds with RSS, and ChPV being found in many of these disorders (Goodwin *et al*., 1993; Pantin-Jackwood *et al*., 2008; Domanska-Blicharz *et al*., 2012; Devaney *et al*., 2016). ChPV has a worldwide distribution, and it has been associated with enteric diseases in many other countries (Kisary *et al*., 1984; Decaesstecker *et al*., 1986; Goodwin *et al*., 1990; Zsak *et al*., 2008,2009;Bidin *et al*., 2011; Domanska-Blicharz *et al*., 2012;Tarasiuk *et al*., 2012;Nuñez *et al*., 2016). Experimentally, ChPV produces intestinal alterations such as diarrhoea, reduced weight gain and growth retardation (Zsak *et al*., 2013). In the present study, we searched for the presence of ChPV in different imprints of organs fixed in FTA cards collected from birds with enteric problems, such as diarrhoea and stunting. The results showed the presence of ChPV in 50.6% of the collected samples, demonstrating that the virus is not only related to enteric organs but also to organs of other systems, such as respiratory (trachea, lungs, and air sacs), immune (thymus, bursa, bone marrow and spleen), and urinary (kidney) organ, as previously demonstrated in the experimental studies of Zsak *et al*. (2013) and Domanska-Blicharz *et al*. (2012).

The parvovirus infections found in this study corresponded to young chickens, confirming previously published data on the occurrence of the virus in young animals (Palade *et al*., 2011; Domanska-Blicharz *et al*., 2012), which may indicate the occurrence of vertical infection in poultry farms in Ecuador.

In this study, we confirmed that the PCR protocol used for the amplification of a genome segment encoding the non-structural protein (NS1) in the 5'ORF region (Zsak *et al*., 2009) allowed for the identification of ChPV by the amplification of a 561-bp DNA fragment. Furthermore, we found a high percentage of similarity between the obtained nucleotide and amino acid sequences and others described and submitted to the GenBank from North America, Brazil, Europe and Asia. All samples used in this study derived from broilers affected with enteric disease, and therefore, it was not possible to determine the presence of ChPV in birds with no signs of enteric disease to corroborate the prevalence of natural infections of ChPV in healthy broiler flocks in the USA found by Zsak *et al*. (2008).

In conclusion, we confirmed the circulation of ChPV in poultry farms located in the northern region of Ecuador, providing the first molecular report of the virus in this country, which is possibly related to the enteric diseases described above. However, the exact role of the virus in enteropathiesis not fully understood, and thus, further pathological and epidemiological studies are needed to determine the real pathogenicity and prevalence of this pathogen in Ecuador, and to develop vaccines in the future to prevent the vertical and horizontal transmission of ChPV.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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