Global Advanced Research Journal of Agricultural Science (ISSN: 2315-5094) Vol. 7(3) pp. 071-079, March, 2018 Issue.
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Full Length Research Paper

Evolutive phylogenetic based on amino acids sequence surround the fusion protein cleavage site gene of Newcastle disease virus from field samples of surveillance program and vaccine strains in Brazil

Maria Angela Orsi[1,2*], Luciana Helena Antoniassi da Silva[1], Clarice Weis Arns [1] and Tânia Rosária Pereira Freitas[3]

[1] Animal Virology Laboratory, Institute of Biology, University of Campinas, UNICAMP, Campinas;
[2] Animal and Plant Health National Laboratory of São Paulo, LANAGRO-SP, Campinas, Brazil;
[3]Animal and Plant Health National Laboratory of Minas Gerais, LANAGRO-MG, 33600-000 Pedro Leopoldo, MG, Brazil.

Accepted 02 March, 2018

The AAvV-1 official surveillance provided an opportunity for the survey of infections in poultry for internal consume and to exportation, applying molecular methods. In this work, the RT-PCR and direct sequencing of partial fusion protein gene from AAvV-1isolates from poultry production of different Brazilian geographic areas as from live vaccines applied in each region were performed. The deduced amino acid sequences were inferred and phylogenetically compared with those from strains that were previously reported in other geographic regions. Since genetic studies on the sampling from the Brazilian surveillance program (BSP) from have not been described, we first established in this paper the comparative phylogenetic among field samples and vaccine strains. Our results showed that all of the Brazilian isolates collected from 2002 to 2005 (n = 39) belongs to the nonvirulent AAvV-1. The substitution of amino acid at position 116 from arginine (R) to alanine (A) (112G-K-Q↓G-A-L117) and at position 114 from glutamine (Q) to threonine (T) (112GR-T↓G-R-L117) in two virus BR-1568 and BR-6225 were observed. Several field isolates were highly similar to the live vaccine strains or strains derived from La Sota or Ulster-type from vaccines currently marketed. The circulation of AAvV-1 strains with different ICPI induced by vaccine strains highlights the expanding of the surveillance program is quite necessary, for knowing the characteristics of the virus in the nature, and consequently, to avoid needless risks.

Keywords: Newcastle disease virus, AAvV-1, molecular survey, Brazil

INTRODUCTION

Newcastle disease (ND) is a devastating infectious disease of wild and domestic avian and considered one the major cause of economic losses to producers, with significant social consequence (Alexander, 2003). In the last decades, Brazilian poultry production for internal consumption and exportation had a considerable approach in technology,

*Corresponding Author's Email: giulia96@yahoo.com

especially in South, Southeast and Midwest region where the major poultry farms are found (Ferraz and Felício, 2010). Those improvements in conjoint with the official active surveillance and the contingency programs allowed Brazilian poultry production reaching the status-free of the virulent Newcastle disease (Ferraz and Felício, 2010; Brasil, 2013). The Newcastle disease virus NDV (NDV) is a synonymous of Avian Paramyxovirus serotype 1 (APMV-1), member of Avulavirus genus, from Paramyxoviridae family in the order Mononegavirales (Alexander, 2003). The Avulavirus genus shelters thirteen species of Avian Paramyxovirus identified as APMV-2 to APMV-13 which have renamed in 2016 to AAvV (1-13), by the International Committee on Taxonomy of Viruses (http://talk.ictvonline.org/taxonomy). AAvV-1 virion is enveloped, containing a single-stranded, negative sense, non-segmented, RNA genome of approximately 15kb in length. The AAvV-1 genome encodes for six major proteins: nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), the RNA dependent RNA polymerase (L), and also for a seventh protein (V) that is produced by a frame shift within the P coding region (Miller et al., 2009). The live lentogenic vaccines are the main preventive action against AAvV -1 of commercial poultry breeding. The vaccine virus strains La Sota, B1, V4, VG-GA or Ulster 2C are the most applied in active immunization (Orsi et al., 2001). Until now, the AAvV-1 laboratorial diagnosis is performed by conventional serological techniques. The virus isolation and the Intra-Cerebral Pathogenicity Index (ICPI) are the most important methods to determine the AAvV-1 virulence of field isolates (Brasil, 2013). Since then, it was postulated that nucleotide sequence analysis may allow the differentiation of highly related isolates and thus may be a promising epidemiological tool (Alexander et al., 1999; Liu et al., 2007). The molecular AAvV-1 characterization methods to improving the surveillance programs should be included. Here, the reverse-transcriptase polymerase chain reaction (RT-PCR), following the nucleotides sequencing and deduction of coded amino acids to diagnosis of AAvV-1, were applied. The sampling included isolates from healthy broiler chickens from distinct geographic regions, during the period of 2002 to 2005, and live AAvV-1 vaccine strains from the same regions were characterized by a partial sequencing of the F protein gene for pathotype prediction and by the phylogenetic relationships among them.

MATERIAL AND METHODS

***Poultry Sampling*:** 4,236 pools of tracheal and cloacae swabs collected from 122,835 birds of 8,189 flocks were submitted to viral isolation. The swabs pools came from previously tested by ELISA, being 3,036 positive and 1,200 negative samples. All samples were harvested during active surveillance, regardless of clinical symptoms. The official survey was divided in three periods first (1st), second (2nd) and third (3rd) study, respectively. In the 1st study, the AAvV-1 was screened the Southeast, South and Midwest regions from 2002 to 2003 (Orsi, et al, 2010a). The 2nd study was run on from December 2003 to March 2005 on the same regions of the first period (Orsi et al., 2010b). The 3rd study, the virus screened in flocks, not directed to poultry meat export was coming from North, Northeast and Southeast regions during 2004 until 2005, as is showed in the table 1. All samples were shipped on dry ice to Laboratório de Sanidade Aviária (Avian Disease Laboratory – ADL) of LANAGRO/SP, for conventional assays. The AAvV-1 strains, biologics and all reference samples were kindly provided by ADL. The molecular assays were performed in Laboratório de Virologia Animal (Animal Virology Laboratory) of Universidade Estadual de Campinas (UNICAMP).

AAvV-1 isolation and live-vaccine virus: the **t**racheal and cloacae swabs samples were clarified by centrifugation, at 1000g for about 10 minutes, and then, 0.2 ml of supernatant fluids were inoculated into each of five 9-11 days old specific-pathogen-free (SPF) embryonated chicken eggs. Then, the allantoic fluid was harvested at four-five days post inoculation (PI). Hemagglutination Activity (HA) was carried out on each of the three passages with allantoic fluid from individual eggs before the samples be considered negative. The Hemagglutination Inhibition (HI) using a panel of Paramyxovirus reference antisera from AAvV-1 to AAvV-9 was applied for the virus identification. The Intra-Cerebral Pathogenicity Index (ICPI) to determine the AAvV-1 virulence was carried out in one-day-old SPF chicks, daily examined during eight days. Vaccine: Eleven samples of commercial lyophilized vaccines either manufactured in Brazil or imported have been produced with seeds of AAvV -1 strains B1, La Sota (n=6), Ulster 2C, Clone 30, VG-GA and C2 were titrated by successive dilution to find the minimal dose for adequate infection in chick embryos.

***RNA extraction and sequencing*:** AAvV -1 vaccine and the earliest allantoic fluids infected with field samples were inoculated once in the embryonated eggs prior to use in molecular studies. In both assays, the total RNA was extracted from 0.2 ml fluid using the High Pure Viral Nucleic extraction kit (Roche Diagnostic TM, Manheim, Germany). After run the RT-PCR, the cDNA was synthesized using High Capacity cDNA kit (Applied Biosystems TM, Foster City, CA). The forward primer MSF 1 (5'-GACCGCTGACCACGAGGTTA-3') and reverse primer 2 (5'-AGTCGGAGGATGTTGGCAGC-3') previously reported (Aldous et al., 2003). Each cDNA sample was analyzed by electrophoresis through 1% agarose gel in buffer at 100 volts, for 1 hour, which was ethidium bromide stained and photographed on a UV transiluminator. PCR products size marker (1kb Plus DNA ladder - Invitrogen) ran on the first lane. The negative control was no infected allantoic fluid, as showed in the figure 1.

Table 1: – Relationship among serum positivity, number of birds and number of isolation

Study	Number of birds	Flocks (n)	ELISA seropositive Flocks %	Number of Flocks swabs used isolation		NDV Isolates (n)	Reference
				ELISA+	ELISA –		
1st study	23,745	1,583	619 (39.1)	619	24	77	Orsi et al. 2010a
2nd study	81,825	5,455	1,571 (28.8)	1,571	1,176	15	Orsi et al 2010b
3rd study	17,265	1,151	846 (73,5)	846	-----	12	-------
Total	122,835	8,189		3,036	1,200	104	

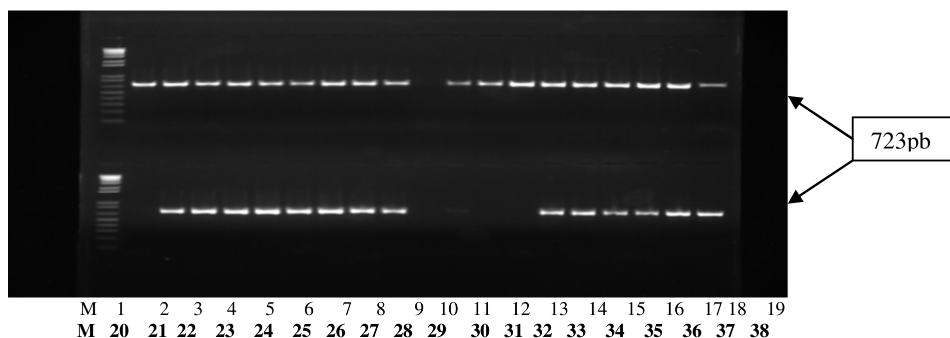

Figure 1. Analysis of RT-PCR products of different NDV isolates by gel electrophoresis
M-Molecular weight marker; (1) 728, (2) 732, (3) 733, (4) 734, (5) 766, (6) 941, (7) 943, (8) 944, (9) 945, (10) allantoic liquid (Neg), (11) 982, (12) 1052, (13) 1103, (14) 1220, (15) 1321, (16) 1374, (17) 1524, (18) 1612, (19) 4961, (20) White 1, (21) 4980, (22) 5100, (23) 5818, (24) 5958, (25) 6424, (26) 7550, (27) 8486, (28) 8487, (29) allantoic liquid (Neg), (30) CER1 (31) CER2, (32)-White, (33)-45/Vaccine Clone 30, (34) Standard Ulster, (35) 47/06 Vaccine La Sota, (36) 61/Vaccine C2, (37) 65/Vaccine B1, (38) 71/Vaccine VG-GA.

Nucleotide Sequence and Phylogenetics: PCR products were sequenced three times each, both in forward and reverse directions, with fluorescence dideoxynucleotide terminators sequencer using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems TM, Foster City, CA). Assembly and editing of sequencing data was performed using the Bio Edit software, version 7.0.5.2 . The sequence alignments were performed using the Clustal W software, version 1.83 using full alignment and a number of 2,000 total replications on the bootstrap, in order to ensure a higher level of confidence to our analysis. The phylogenetic comparing the fusion protein using neighbor joining method of MEGA version 4 software packages (Kimura, 1980; Saitou and Nei, 1987; Tamura et al. 2007). The AAvV-1 F-gene nucleotide sequences reference was retrieved from the GenBank database (http://wwwncbi.nlm.n/ih.gov/enbank/index.html). Circulating AAvV-1 sequences from USA, Taiwan/China, China, Slovakia, Barbados, Russia, Northern Ireland, Argentina, United Kingdom, Hungary and South Africa, were included (Figure 2).

RESULTS

The titration of AAvV-1 vaccine strains by successive dilution ranged from $10^{5.50}$ to $10^{6.70}$ EID_{50} pointed to the efficacy of vaccine applied. The ICPI mean score per bird per observation over the 8-day period ranged from 0 to 0.66, in different states and regions. The ICPI varied from 0.0 to 0.50, in 89.74% of isolates from vaccinated birds

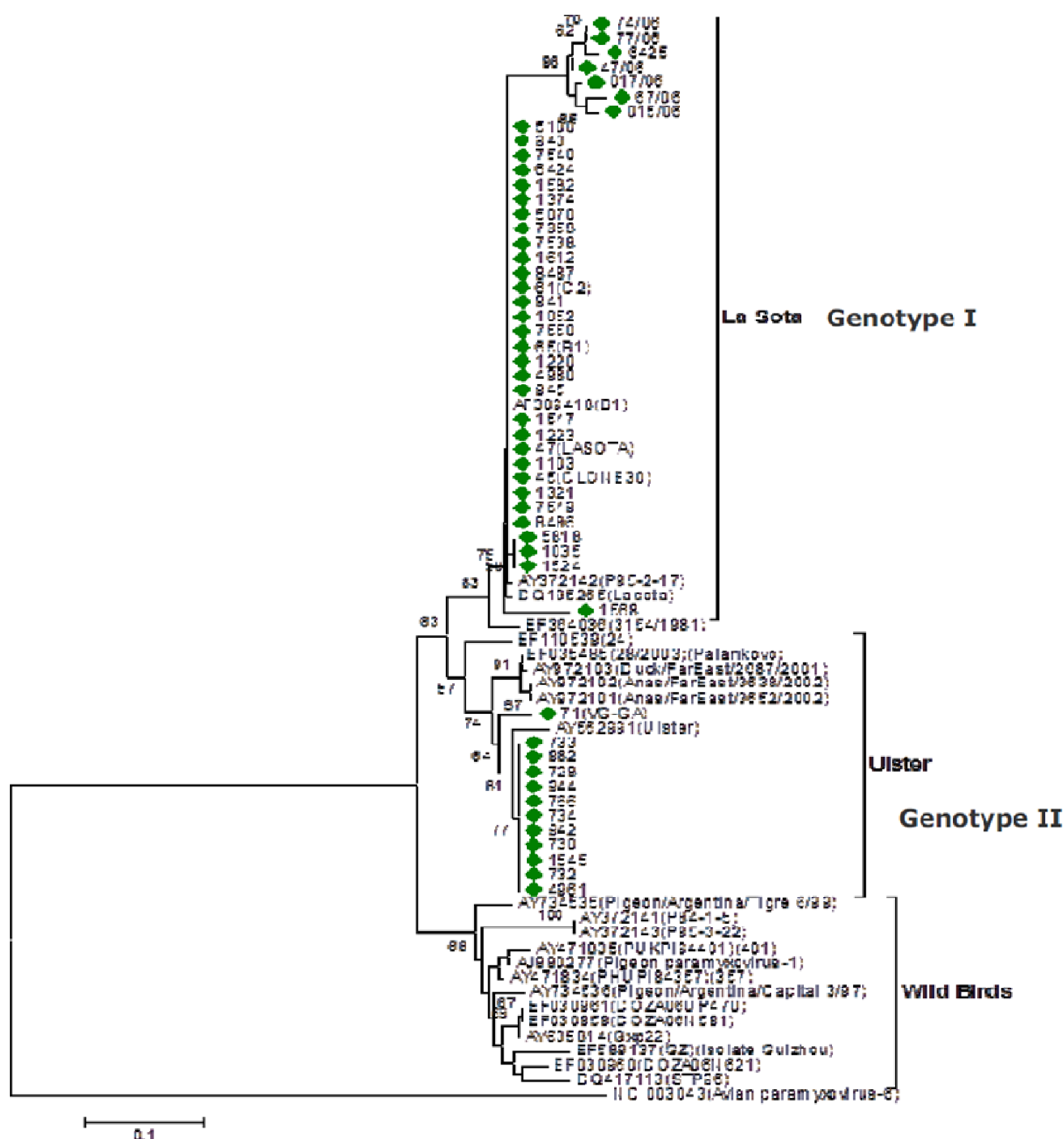

Figure 2. Phylogenetic tree of Brazilian isolates and their relationship to class II NDV viruses. Phylogenetic analysis based on the partial nucleotide sequences of Brazilian isolates of genotypes I and II the fusion gene of isolates representing NDV class II. For the parameters used to measures genetic relationship was the GenBank accession number are between square bracket. The isolates characterized in the present study are assigned with a green lozenge; the tree was roosted using a NDV sequence.

with Ulster 2 C, V4 Queensland, B1 and La Sota vaccine. In the regions where vaccination status indicated the use of B1 (n=3) or the vaccination status was not informed (n=1), the ICPI varying from 0.51 to 0.66 (n=4). The sera prevalence of AAvV -1 strains from commercial poultry farms, figured out in 37.07% positive and 62.93% negative flocks. One-hundred-four samples of tracheal and cloacae swabs from *Gallus gallus*, collected in slaughterhouses, were reagent positive to AAvV-1 by HA and HI test. The virus was successfully isolated from all ELISA positive samples from which, thirty-nine samples were selected to genomic amplification and sequencing. The correlation between the selected samples showed that nineteen samples (48.71%) came from Southeastern, with ICPI ranged from 0 to 0.61 of which, seventeen samples (43,58%) came from vaccinated chicken and being fifteen (38,46%) vaccinated with B1 vaccine. The remaining two, one unvaccinated (no-vaccinated = NV) and one that

Table 2: Newcastle disease virus isolates in Broiler chickens used in this study

NDV strain*	Region of Brazil	ICPI	Vaccine used
BR-728	Southeastern	0.05	B1
BR-730, BR-732, BR-733, BR-734	Southeastern	0, 0, 0.06, 0	B1
BR-766, BR-982	Midwestern	0, 0.08	WI
BR-942, BR-944, BR-1374,BR-1612, BR-941	Southern	0, 0.12, 0.14, 0.15, 0..22	WI
BR-943, BR-6424, BR-1103, BR-945	Southern	0.30,0.36,0.48, 0.66	WI
BR-1035, BR-1321, BR-1547, BR-1052	Southeastern	0.14, 0.48, 0.48, 0.58	B1
BR-1220, BR-1223	Southeastern	0.50, 0.48	+
BR-1545, BR-1568, BR-4961	Southeastern	0.10, 0.15, 0	B1
BR-1524, BR-1582, BR-6425	Southeastern	0.61, 0.60, 0.47	B1
BR-4980	Midwestern	0.10	-
BR-5070	Southeastern	0.15	-
BR-5100	Southeastern	0.02	WI
BR-5818	Southern	0.04	-
BR-7538, BR-8487, BR-7549, BR-7550	Northeastern	0.20, 0.21, 0.25, 0.37	B1
BR-7540, BR-8486, BR-7958	Northeastern	0.06, 0.11, 0.14	B1

Strains =B1, - not vaccinated; WI-without information; +vaccinated

provided information on the vaccination status (without information=WI). Three (7.69%) samples came from Midwestern region with ICPI ranged from 0 to 0.1 being two samples WI and NV. Ten (25.64%) came from the Southern region with the ICPI ranged from 0 to 0.66. In exception of one sample NV, the others were WI. Seven poultry samples (17.94%) from the Northeastern with ICPI ranging from 0.06 to 0.37, all samples (100%) came from B1-vaccinated poultry (Table 2-supplement). The virus RNA was extracted and RT-PCR was running satisfactory produced the AAvV-1 F-gene cDNA amplified and analyzed by electrophoresis and ethidium bromide staining. The cDNA fragments generated PCR products with 723 bp (base pairs) from infected and vaccine samples, numbered from 1 to 36 identified by comparison with the molecular weight marker ladder (line M – Figure 1). The negative controls from lanes 10 and 29 did not showed bands fragments. In addition, no contaminant nucleic acid was observed (Figure 1).

Phylogenetic analysis of partial F gene sequences

The selection criterion for genetic analysis of the samples was concerning in the pathogenicity of the virus samples and the geographic distribution. Thirty-nine AAvV-1 positive isolates which presented different ICPI values and representative of the different Brazilian geographic regions were selected. The classification and inclusion into the

Table 3: Cleavage site and genotype of the NDV isolates in Brazil

NDV strain	Cleavage site	Genotype
BR-728, BR-730, BR-732, BR-733	GKQ↓GRL	I
BR-734, BR-766, BR-942, BR-944	GKQ↓GRL	I
BR-941, BR-943, BR-945, BR-1035	GRQ↓GRL	II
BR-982, BR-1545	GKQ↓GRL	I
BR-1052, BR-1103, BR-1220, BR-1223	GRQ↓GRL	II
BR-1321, BR-1374, BR-1524, BR-1547	GRQ↓GRL	II
BR-1568	GKQ↓GAL	II
BR-1582, BR-1612, BR-5070, BR-5100	GRQ↓GRL	II
BR-4961	GKQ↓GRL	I
BR-4980	GRQ↓GRL	II
BR-5818, BR-6424	GRQ↓GRL	II
BR-6425	GRT↓GRL	II
BR-7538, BR-7540, BR-7549, BR-7550	GRQ↓GRL	II
BR-7958, BR-8486, BR-8487	GRQ↓GRL	II

evolutionary correlated groups was performed PCR products sequencing looking for identifying of nucleotides and deduction of amino acids adjacent to the proteolytic cleavage site within the F protein gene (Gould et al, 2001; Aldous et al., 2003). The analysis about inferred evolutionary relationships among the virus isolated and a set of virus have allowed to build a phylogenetic tree is presented in Figure 2. The cleavage-site motifs of the NDV strains were listed (Table 3-supplement). Among the cleavage-site motifs of low virulence and avirulent strains, the 112GRQ↓GRL117 motifs was present in most of the isolates. Twenty-six isolates showed the amino acid sequence 112G-R-Q-G-R-L117 at the C-terminus of the F2 protein, and eleven isolates exhibited a 112G-K-Q-G-R-L117 motif. The isolated sample numbered 1568 showed a substitution the amino acid of 116 arginine (R) to alanine (A) 112G-K-Q-G-A-L117 and the sample numbered 6425 isolate 112G-R-T-G-R-L117, exhibited at position 114 from glutamine (Q) to threonine (T). Among these clusters, 28 NDV isolates belongs the VVvV-1 genotype II or the La Sota-like (Figure 2), and 11 belongs to AAvV-1 genotype I. The Brazilian isolates belong to one cluster II genotype II, which includes a virus of the pigeon from Taiwan/China and, the Brazilian isolates that belong to cluster I genotype I include only an isolate of chickens from Northern Ireland, designated as Ulster/67. The BR-6425 and the BR-1568 isolates were related to the La Sota-like and La Sota strains from China and the domestic fowl in Slovakia, respectively.

DISCUSSION AND CONCLUSIONS

Investments in biosafety and monitoring of production continuously are indispensable to guarantee the quality of Brazilian poultry products (Brasil, 2013). Consequently, to invest the molecular methods to allow the accurate characterization AAvV-1 strains are also indispensable (Samal et al., 2011). Since genetic studies on the sampling from the Brazilian surveillance program (BSP) from have not been described, we first established, in this paper, the comparative phylogenetic among field samples and vaccine strains. Orsi et al. (2010a) based in prevalence studies showed nine Brazilian states were free of ND and the AAvV-1 strains isolated were classified as avirulent. But, the genetic analyses of AAvV-1 strains were not done. So, first of all, the AAvV-1 molecular surveyed based on partial F gene nucleotide amplified by RT-PCR and directly sequenced was performed. It was considered suitable, safety and feasibility to extensive use to attend the BSP demands and conjoined with the OIE normative (OIE, 2015). Albeit, Leeuw et al. (2005) have showed the haemagglutinin–neuraminidase (HN) protein also contributed to virulence definition, the deduction of amino

acids surrounded the fusion protein coding region (F gene) is considered a powerful tool for AAvV-1 virulence determination (Collins et al. 1993) and the epidemiological relationships among the AAvV-1 strains (Chambers et al, 1986; Mase et al, 2002). The virus survey, from 2002 to 2005, showed the status free of those areas. Afterwards, the AAvV-1 surveillance conducted, from 2004 to 2005, from geographic areas not involved in chicken meat for export found field isolates samples with ICPI with values above 0.37. Mase et al. (2002) studying the phylogeny of the AAvV-1 isolates, from 1930 until 2001, from Japan, found multiples genotypes emphasized that the NDV surveillance in wild birds is mandatory to the AAvV-1 epidemiology understanding. It was corroborated by Kim et al. (2007) whom affirmed it must concerning to the risk that wild birds infected with AAvV-1 may represent in transmitting the virus to poultry mainly due to the lack of knowledge of the biological characteristics and the phylogenetic proximity of the viral strains that infect these wild birds. Since the circulation of avirulent or low virulence or else, apathogenic AAvV-1 strains which presented quite different ICPI from vaccine strains was evidenced the need to amplify the AAvV-1 molecular survey looking for the factors that contribute to AAvV-1 endemicity. As affirmed Wajid et al. (2017) whom had isolated over and over again (2011–2016) a virulent AAvV-1 (sub-genotype VIIi) from commercial chickens and from multiple non-poultry avian species in Pakistan. Highlighting the expanding, the surveillance program applying molecular epidemiology toll as RT-PCR followed by phylogenetic analysis is quite necessary, for knowing the characteristics of the virus in the nature and consequently to avoid needless risks.

In our present study the results of the F cleavage activation sites of these virus isolates were GKQ↓GR, GRQ↓GR, GKQ↓GA and GRT↓GR, which lack dibasic amino acids at positions 112 and 115 with a substitution of an L rather than an F, at residue 117. This result indicates that all of the broiler chicken isolates were avirulent or have low virulence (OIE, 2015). The cleavage site of the F protein of avirulent or low virulence strains do not contain these multiple basic amino acids and is recognized by extracellular trypsin-like proteases, which are found in a limited number of tissues, predominantly in the respiratory and intestinal tracts. Thus, viral activation requires trypsin–like proteases that are not present in neuronal tissue (Rout and Samal, 2008). It is also demonstrated that all of the isolated viruses two of the six genetic lineages as proposed by Aldous et al. (2003) belonged to class II. Among them, 72% belonged to the AAvV-1 genotype II or the La Sota-like genotype (n=28) and 28% belonged to genotype I or the Ulster-like genotype (n=11). However, Whemann et al. (1999) showed that viruses collected in Hungary were only La Sota like, and a Canadian collection predominantly contained the B-1 genotype. Consistent with the results obtained by Whemann et al., (1999), our study showed that the field isolates of the different regions were clone derivatives of the vaccine strains used in mass vaccination. This result was also reinforced by a study conducted by Alexander et al. (1997), who reported that 18% of all isolates received by the Reference Laboratory of Newcastle disease from OIE in England (Weybridge), were identified as La Sota or B1. Here, only the avirulent/non-pathogenic viruses of genotype II were isolated, however, Miller et al. (2009) has reported that there are additional representatives of low virulence viruses and virulent viruses in this genotype. Van Borm et al. (2016) studying the apathogenic AAvV-1 strains have been isolated from captive Owl swab, the phylogenetic analysis of the complete sequence classified the isolate within AAvV-1 class II genotype II (Vaccinal NDV strain). However the pathogenic AAvV-1 (SJM) was isolated in the 1970s in Brazil and classified as the member of class II, genotype V (Fernandes et al, 2014). The data obtained in this study are consistent with the data of previous and support the ideas that viruses were isolated in healthy broiler chickens. In the analysis to identify pathogenic AAvV-1 samples the results obtained by application of this molecular method were similar to those obtained by conventional methods (ICPI), Tan et al. (2008) found in three cases of NDV lentogenic motif 112G-R-Q-G-R302 L117 in their fusion protein cleavage sites. However, both intra-cerebral pathogenicity and intravenous pathogenicity indexes showed that these AAvV-1 isolates were virulent.

In Brazil, avian flocks are regularly inoculated with attenuated vaccines (avirulent) to control AAvV-1 being the strains La Sota, B1, Clone 30, Ulster VG-GA, C2 and V4 strains the most commonly used. However, we founded the presence and circulation of AAvV-1 viruses with the same genotype but of a different pathogenicity index, from the vaccines used in country. This finding indicates that both vaccinated and unvaccinated birds can be infected with non-pathogenic virus replication, similar to the vaccine virus. The outbreaks that occurred in Ireland, in 1990, (Alexander et al., 1992) and in Australia, from 1998 through 2000, (Gould et al. 2001) were each the result of low virulence viruses mutating to high virulence. In Ireland, the low virulence viruses were endemic in coastal wildlife populations, and in Australia, low virulence viruses initially circulated in poultry (Lancaster; Alexander, 1975). In this former research showed that only two AAvV-1 sequences presented amino acid change but these substitutions did not change protein. Most genomic changes in non-segmented RNA viruses are a result of recombination or the intrinsic error rate of the polymerase (it generates a large number of genetic variants that are called quasi-species). The presence of selection pressures at specific amino acids within proteins is also recognized as an adaptive evolution (Lancaster and Alexander, 1975). Recombination infrequently results in non-segmented negative strand RNA viruses (Spann et al, 2003).

To conclude, the present investigation provides important information about genotypes of AAvV-1 strains present in

commercial heaths birds in Brazil. The importance of surveillance programs supporting was the demonstration of all isolated viruses were of low virulence in 2002 to 2005 survey. Therefore, there was not a virulent virus in commercial birds during the study period, and isolated viruses were found to be genetically identical or similar to the commercial vaccines used in Brazil. Low virulence viruses have the advantage of allowing the host to live for longer periods, thus increasing the chances for viral replication (Lancaster and Alexander, 1975). The study of AAvV-1 genetic diversity is fundamental to improving secure and biosafety measures so, the expanding of AAvV-1 survey is necessary and becomes essential to strengthening of official poultry defense and health prevention services. Since, avirulent AAvV-1 can infect peridomicilary and wild avian, the surveillance of AAvV-1 infection should be expanding to improve the official programs to control and eradicated the AAvV-1 infections.

ACKNOWLEDGEMENTS

The authors gratefully the Brazilian Ministry of Agriculture, Livestock and Food Supply and to the Avian Disease Laboratory staff (LANAGRO/SP) in special Jaira Alves dos Santos for her technical assistance. We would like to thank the Animal Virology Laboratory – Unicamp, to make possible this study. This Work was supported by grants from Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP). Finally, we sincerely thank to Etna Lima Macário (FCM- Unicamp), for collaborating with the English review.

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