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**MOLECULAR TECHNIQUES FOR DIAGNOSIS OF ANIMAL
DISEASES**

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“ Molecular Techniques for Diagnosis of Animal Infectious Diseases”

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Introduction

Viruses are non-cellular organisms which require the replication machinery of a cell to replicate; they have no metabolic activity outside the host cell (Harper, 2012). There are several classification systems for viruses; however, the most used is the Baltimore Classification in which viruses are classified according to the nature of its genome (Harper, 2012). Basically, there are two main groups of viruses, DNA viruses and RNA viruses. Among DNA viruses of veterinary importance are Herpesviridae, Adenoviridae, Poxviridae and Parvoviridae whereas the most important RNA viruses are Caliciviridae, Coronaviridae, Paramyxoviridae, Rhabdoviridae and Retroviridae (Patel & Heldens, 2009).

Multiple techniques have been developed to detect viruses that affect animals and humans. The diagnosis was mainly based in morphological and antigenic characteristics of the pathogen; in many cases these analysis require viral isolation which is a cumbersome and often insensitive procedure. The development of the molecular biology has revolutionized the detection and diagnosis of viral diseases.

Molecular diagnostic techniques allow us to use molecules (DNA, RNA, proteins, etc.) in order to identify and characterize a pathogen. Molecular techniques also allow the identification of pathogens that cannot be grown or grow slowly in culture. This is the case of de Hepatitis C virus (HCV), which cannot be culture and investigators had been able to isolate the virus RNA for cloning and perform a RT- PCR in order to detect this pathogen. Genome analysis of HCV has also been useful to synthesize artificially antigens for serological investigation of the virus (Tang *et al.*, 1997).

The Polymerase Chain Reaction (PCR) is the most important tool in molecular diagnosis. It increases the sensitivity of nucleic acid analysis because it amplifies a specific DNA segment from an initial DNA molecule (Mullis, 1990). It is used to diagnose a number of infectious agents, including viruses. Different applications of the PCR technique enable rapid and reliable diagnosis of many infectious diseases, such as Varicella Zoster Virus, Herpes Simplex Virus, Epstein-Barr Virus, Adenovirus, HIV, and Influenza (Cobo, 2012).

One of the pathogens that were first detected using molecular techniques was the Kaposi's Sarcoma Virus (KSV). KSV also known as Human Herpesvirus-8 was identified in 1994 and causes a disease similar to cancer in immunocompromised persons, especially people with AIDS. The etiology of the association of Kaposi's Sarcoma in people with AIDS was unknown until investigators used a Representational Difference Analysis (RDA) in which two genomic samples are compared in order to detect differences between them. In this analysis, a PCR is performed followed by a DNA hybridization analysis. RDA allowed discriminating KSV from Herpesvirus Saimiri and Epstein-Barr Virus (Chang *et al.*, 1994).

Another example in which molecular biology has been useful is the Human Papilloma Virus (HPV); this virus is recognized as the cause of most cervical cancers. There are number of HPV genotypes that are classified as genotypes of low and high risk of causing this type of cancer. Normally, a HPV infection is suspected using Papanicolaou Test (Pap Test), however molecular analysis is necessary for the identification of high risk genotypes (Speers, 2007). Currently, there are several molecular techniques such as PCR, Real Time PCR, Southern Blot, nucleic acid hybridization assays and Microarrays, that are used for the detection and genotyping of HPV, which lead to a more rapid diagnose and a more accurate treatment.

Molecular techniques also represent an important tool in the investigation and tracking of antibiotic resistant microorganisms like MRSA *Staphylococcus aureus* methicillin-resistant (MRSA) is a strain of bacteria that is resistant to most commonly used antibiotics in medicine (Speers, 2007). Due to the aggressiveness of the strain and its heterogeneous resistance, makes it difficult to identify MRSA in culture. In order to detect this microorganism, it is necessary an accurate and rapid diagnosis method in which a multiplex PCR of the *mecA* and *nuc* genes is performed in combination with culture of the bacteria (Louie *et al.*, 2002; Roher *et al.*, 2003).

Finally, the most useful tool of molecular biology today is DNA sequencing. It allows us to determine the exact nucleotide sequence of any DNA molecule of interest. There are several advantages of sequence analysis; it is used to discriminate between organisms that are closely related, screening of diseases or risk factors, etc. (Tang *et al.*, 1997). One example of the benefits of DNA sequencing is the detection of Down syndrome in pregnant women. This analysis is performed sequencing the maternal plasma DNA and identifying and quantifying specific sequences that belong to chromosome 21 (Palomaki *et al.*, 2011).

DNA sequencing has also benefited Single Nucleotide Polymorphisms (SNPs) analysis which has played an important role in the characterization of the human genome (Baird *et al.*, 2008). SNPs are the most important and abundant genetic markers, however, they are difficult to identify. The most common method used to detect these markers is genotyping them in several individuals using a number of molecular techniques such as DNA probes, PCR, DNA sequencing, microarrays and restriction enzymes (Baird *et al.*, 2008).

SNP analysis improved the genotyping of Rotaviruses, therefore, the detection of their serotypes. Rotaviruses are the first cause of diarrhea in children in developing countries. The serotype classification is made according to the outer capsid proteins, VP4 and VP7, which are rotaviruses P and G serotypes respectively (Chizhikov *et al.*, 2002). There have been described approximately 14 G serotypes (V27 protein) and 17 P serotypes (VP4 protein). These serotypes are identified with a combination of two molecular techniques, PCR and hybridization microarrays. Such combination optimized the genotyping because most of this serotypes may have random mutations (SNPs) that are detected only with the hybridization, this process generates sub-genotyping of isolates that could belong to the same genotype of the virus (Chizhikov *et al.*, 2002).

Genotyping is performed with several organisms and different pathogens, like dengue virus. This is an ssRNA virus that causes two kinds of syndromes known as dengue fever and dengue hemorrhagic fever (Johnson *et al.*, 2005). There are four serotypes of Dengue virus (DEN-1, DEN-2, DEN-3 and DEN-4), which cause infections and co-infections in humans. These serotypes can be detected using serological techniques, however, specific diagnosis is often not possible because this serotypes are closely related serologically and have cross-reactivity of their antibodies (Lanciotti *et al.*, 1992). Virus isolation is also very difficult to achieve. Today, RT-PCR, qPCR and hybridization techniques are used to detect each of the four dengue serotypes more easily and faster than the serological analysis (Johnson *et al.*, 2005; Lanciotti *et al.*, 1992).

The emergence of new infectious diseases is probably due to changes in the ecology of the pathogens, the host or both (Scharg & Weiner, 1995). Also, there are many events that could

trigger a pathogen to circulate in multiple environments, such as ecological and biomedical manipulation, urbanization, agricultural intensification, introduction of species, translocation of populations, and others (Dasak *et al.*, 2000). Most emerging diseases exist within a host, only a few of them affect exclusively any one group. Examples of diseases that overlap between hosts are canine distemper (domestic animals to wildlife), cat scratch fever (domestic animals to humans) and rabies (all three categories) (Scharg & Weiner, 1995). There is a great interest in understanding the pathogenesis, transmission and epidemiology of several families of viruses that affect wild, industrial and domestic animals, and especially those viruses that could cause a zoonotic disease (Dasak *et al.*, 2000).

Here we present the identification of two different viruses that cause an infectious disease in animals, using molecular techniques.

The first pathogen identified is a DNA virus, *Gallid Herpesvirus I*, member of the Herpesviridae that causes a respiratory disease in chickens called avian infectious laryngotracheitis (ILT) (Williams *et al.*, 1992). The virus could have a period of latency causing mild symptoms or an acute phase, in which symptoms occur, such as conjunctivitis, dyspnea, sneezing, nasal exudates, oral and ocular discharges (Fuchs *et al.*, 2007; Ojkic *et al.*, 2006). The infectious laryngotracheitis virus (ILTV) has a worldwide distribution of a great economic importance in several countries. In this paper, we studied an outbreak of ILT that occurred in Ecuador in 2011. We used molecular techniques in order to identify the virus. Additionally, we characterized the strain of the virus that caused the outbreak. In previous studies, for the characterization of the ILTV, virus cultivation and isolation was required, and a subsequent PCR amplification and digestion of their products with restriction enzymes was

performed (Creelan *et al.*, 2006). Here, we describe a technique which does not require either viral cultivation nor endonuclease digestion of DNA products. We analyzed nucleotide sequences from amplicons (ICP4 and TK genes) obtained from animal samples. These genes have been used in other studies for ILTV characterization with other genes such as those encoding viral glycoproteins like the Glycoprotein G gene (*gG*), which is considered as a virulent factor of ILTV. However, the most commonly used are ICP4 and TK genes (Han & Kim, 2001). The second pathogen identified in this study was a *Morbillivirus*, known as Canine Distemper Virus (CDV). Canine Distemper Virus is one of the animal pathogens that could affect mainly dogs and several other carnivore species as dogs (primary host) causing severe respiratory disease which can lead to death (Deem *et al.*, 2000). Pinniped species are also susceptible to a CDV infection (Harder & Osterhaus, 1997). In the period of 2011-2012, an increase of pup mortality of the Galapagos sea lion, *Zalophus wollebaeki*, was registered. Since the animals showed respiratory symptoms and the fact that on the island, sea lions are in constant contact with domestic dogs in some islands, we suspect a possible infection with canine distemper. A molecular analysis was carried on to detect the presence of CDV in several tissue samples of symptomatic animals. Using a semi-nested PCR, in which a set of three primers is used to amplify a portion of the phosphoprotein gene (P) of CDV, followed by a sequence analysis of the positive samples

The outbreak of CDV in San Cristobal may be an example of an epidemiological phenomenon known as “pathogen spillover”, in which a pathogen is transmitted from its reservoir (a domesticated animal) to a sympatric new host (a wild animal) (Power & Mitchell, 2004; Dasak *et al.*, 2000). The spillover of CDV has had a major impact on other species of

carnivores, for example, it has caused the extinction of the African wild dog and black-footed ferret (Dasak *et al.*, 2000).

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Detection and characterization of infectious laryngotracheitis virus from an outbreak of respiratory disease in Ecuador

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Keywords: Infectious laryngotracheitis virus, vaccinal strain, ICP4 gene, Thymidine Kinase gene (TK), DNA sequence, single nucleotide polymorphisms (SNPs).

Abstract

Infectious laryngotracheitis (ILT) is an acute respiratory disease transmitted through respiratory secretions or fomites from naturally infected birds or the use of live vaccines. We investigated an outbreak of respiratory disease occurred in 2011 in Ecuador, a country where the disease was regarded as exotic. Viral detection was carried out by PCR (genes ICP4 and TK) and partial viral characterization was accomplished by amplicon sequencing. Our results suggested that the outbreak was caused by a field strain of ILTV.

Introduction

Avian infectious laryngotracheitis (ILT) is an avian respiratory disease caused by a virus of the family Herpesviridae, *Gallid herpesvirus I* (Williams *et al.*, 1992). The severity of the disease depends on the viral strain; highly virulent strains produce severe respiratory signs such as bloody respiratory tract discharges and high mortality whereas other strains cause a milder symptoms such as sinusitis, watery eyes, depression and low mortality (Fuchs *et al.*, 2007; Ojkic *et al.*, 2006; Hughes *et al.*, 1987). This virus can also cause lifelong persistent infections which also contribute to viral dissemination (Williams *et al.*, 1992). Although the ILTV has been described primarily in poultry, the virus circulates in other birds such as pheasants, partridges, and peafowl (Samber *et al.*, 1969).

Outbreaks of ILTV could originate from direct contact with secretions (or fomites) from acutely or persistently infected animals with either field or vaccinal strains (Shehata *et al.*, 2013; Han *et al.*, 2002; García & Riblet, 2001; Cover, 1996; Keller *et al.*, 1992; Hughes *et al.*,

1991; Guy *et al.*, 1991). Detection of the source of viral strains could be crucial for disease control (Chacón *et al.*, 2010; Neff *et al.*, 2008; Oldoni & García, 2007; Creelan *et al.*, 2006; Ojkic *et al.*, 2006). Efforts to discriminate vaccine strains from field isolates have focused in nucleotide differences causing restriction length fragment polymorphism (RLFP) in the ICP4 gene (Oldoni & García, 2007; Creelan *et al.*, 2006; Graham *et al.*, 2000).

Due to their potential infectious nature, live vaccines are often prohibited in countries (such as Ecuador) where ILTV is considered exotic. In Ecuador, despite the local regulations live vaccines are often smuggled and may be causing outbreaks of respiratory disease. In this study we used a simple approach consisting of PCR and amplicon sequencing to detect and characterize ILTV in an outbreak of respiratory disease occurred in 2011 in Ecuador.

Materials and Methods

Sampling

In 2011, 219 tracheal samples from acutely ill animals were obtained from 26 poultry farms (16 broiler and 10 laying hens operations); 9 farms were located in the Ecuadorian Coast and the rest were located in the Sierra region. All the samples came from animals that exhibited dyspnea, conjunctivitis, sneezing and nasal and ocular discharges. Samples were transported on ice in viral transport medium Remel M4RT and preserved at -20°C until analyzed.

DNA extraction: A modified CTAB method was used for viral DNA isolation from samples. Tracheal epithelial cells were obtained using a sterile Papanicolaou brush; cells were suspended in 500 µl of PBS 1X buffer. Cell lysis was performed by adding 700 µl of CTAB solution (Doyle & Doyle, 1987). The solution was then treated with an equal volume of

chloroform:isoamylalcohol (24:1). DNA was precipitated in sodium acetate 3M and washed in ethanol 70% and finally eluted in 50 µl of TE buffer (10 mM Tris-HCl, 5 mM EDTA, pH 8.0).

Amplification and sequencing of ICP4 and TK genes: To assess the presence of ILTV in tracheal samples, a 222 pb fragment corresponding to the ICP4 gene was amplified using PCR primers described by Creelan et al., 2006 (ICP4f 5'-CTCTTCCTCCTCTTCCTCAT-3' and ICP4rev 5'-GTTACTGACTGAACCGACCC-3'); A 649 pb fragment corresponding to the Thymidine Kinase gene (TK) was amplified as previously described (Han & Kim, 2001) using the primers TKIPf 5'-CTTAGCGGAACCTATGCAAG-3' and TKIPrev 5'-TAGCGTCTGGTCGATTGAAG-3'. Amplicons sequences were compared to those of field and vaccinal viruses using BLAST and ClustalX (MEGA version 5.0) (Tamura *et al.*, 2011). All samples were tested for the beta-actin gene amplification which was used as an internal control.

Results

We amplified ILTV DNA sequences (ICP4 and TK genes) from 80% (n=8) of farms housing laying hens and none from broiler farms. The viral nucleotide sequences from the Ecuadorian samples had two unique single nucleotide polymorphisms (SNPs) and 2 unique insertions when compared with ILTV sequences in the GenBank (Figure 1). The TK sequences were identical to other TK sequences in the GenBank. These results suggest that ILTV was one of the causes of respiratory sickness observed in Ecuadorian laying hens in 2011. Differences in nucleotide sequences of amplicons suggested that the viruses causing the 2011 outbreak did not correspond to any previously sequenced ILTV.

Discussion

Our study provides evidence of ILTV infection in Ecuador; this is the first report of the disease in this country. The analysis of nucleotide sequences obtained in this research suggested that the outbreak was not caused by vaccinal strain or any other previously described ILTV. We speculate that the virus may have entered Ecuador from neighboring countries where the disease has been reported previously (Alvarado *et al.*, 2013).

Our results suggest that PCR amplification and amplicon sequencing is a simple and inexpensive method to detect and characterize ILTVs. Additionally, nucleotide sequences provide a portable record which may allow monitoring of viral strains causing disease in different parts of the world.

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Figures

	*2233
Ecuador	C T C T T C C T C C C T T T C C T C A T T C T C G C T G
12/D/02/BCK	
USA T C
193435/2007 T C
Anhui-2011-2 T C
V1-99 T C
81658 T C
CSW-1 T C
63140/C/08/BR T C
USDA T C
402/A/06/BR	
ICP4 T C
CEO low	
passage T C
Vaccine	
Laryngo T C
Vaccine LT	
Blen T C

Figure 1. Nucleic acid sequence of ICP4 gene from field and vaccine strains. All DNA sequences were obtained from GenBank except for the Ecuadorian one. Letters indicate single nucleotide substitutions.

***Sequence numbers correspond to a previously published ILTV sequence (Zhao et al., 2013).**

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Ecuador	A	T	G	T	C	G	C	G	C	T	A	G	A	G	G	C	C	A	C	T	T	C	T	G	G	C	G	A		
12/D/02/BC																														
K USA
193435/2007
Anhui-2011-																														
2
V1-99
81658
CSW-1
63140/C/08/																														
BR
USDA
402/A/06/B																														
R ICP4
CEO low																														
passage
Vaccine																														
Laryngo
Vaccine LT																														
Blen

Figure 1 (Cont...). Nucleic acid sequence of ICP4 gene from field and vaccine strains.

All DNA sequences were obtained from GenBank except for the Ecuadorian one. Letters indicate single nucleotide substitutions.

***Sequence numbers correspond to a previously published ILTV sequence (Zhao et al., 2013).**

Detection of Canine Distemper Virus from an outbreak of a respiratory disease in Galapagos Sea Lion (*Zalophus wollebaeki*) in 2011-2012

Abstract

Canine Distemper Virus (CDV) is a paramyxovirus that causes a systemic disease in several species of carnivores including sea lions. We investigated an outbreak of a respiratory disease in Galapagos Sea Lion (*Zalophus wollebaeki*) in 2011-2012 of San Cristobal Island. Detection of CDV was conducted by partial amplification of the phosphoprotein (P) gene of the virus. Sequence analysis of amplicons suggested that the outbreak was caused by a CDV strain.

Keywords: Canine Distemper Virus, Morbillivirus, phosphoprotein (P) gene.

Introduction

Canine distemper is the second most important disease affecting dogs worldwide, after rabies (Deem *et al.*, 2000). It is caused by the canine distemper virus (CDV), an enveloped single stranded RNA virus, member of the genus *Morbillivirus*, family Paramyxoviridae. This genus includes Rinderpest virus (RPV), Measels (MV) and peste des Petits ruminants virus (PPRV) (Deem *et al.*, 2000; Harder & Osterhaus, 1997). Canine distemper is highly contagious; its transmission is mainly through aerosols from respiratory and other body exudates (Deem *et al.*, 2000; Greene & Appel, 1990). The spread of the virus is similar in all

mammal species which often results in a systematic viremia. Within the first 24h of infection, the virus reaches the respiratory tract; between 2-6 day postinfection, the virus infects lymph organs such as spleen. Between days 9-14, depending on the animal immune system, the virus could reach the central nervous system through blood (Greene & Appel, 1990).

Morbilliviruses are epizootic viruses which not only affect terrestrial carnivores but also marine species such as dolphins and seals (Deem *et al.*, 2000; Harder & Osterhaus, 1997). Pinnipeds could be affected by at least two types of distemper viruses. In 1988, phocine distemper was described from an outbreak that killed more than 17 000 harbour seals (*Phoca vitulina*) (Osterhaus & Vedder, 1988). The Phocine Distemper Virus (PDV) caused symptoms similar to canine distemper; also, serological analysis from orphan seals that survived to the outbreak of 1988 showed canine distemper virus (CDV)-neutralizing antibodies (Osterhaus & Vedder, 1988) and sequence analysis of the two viruses (phocine and canine) confirmed that the two viruses are closely related (Mamaev *et al.*, 1996; Barret *et al.*, 1991). Canine distemper virus can also affect pinnipeds, and to this date several reports have been made (Kennedy *et al.*, 2000; Visser *et al.*, 1993).

In the period of 2011-2012, an increase in pup mortality of the Galapagos sea lion (*Zalophus worllebaeki*) was registered in San Cristobal Island. The Galapagos sea lions are in contact with humans and their pets in many islands. So we decided to investigate CDV as a potential cause of this disease.

Materials and Methods

Sampling

Since 2008 sea lions have been regularly surveyed in San Cristobal Island as part of a project led by Judith Dekinger, PhD., in which the high pup mortality of Galapagos Sea Lions (*Zalophus wollebaeki*) and potential links to domestic dogs was investigated. During each survey, the status of each colony was analyzed, and any sign of problems with members of a colony was reported. In the period 2011-2012, an outbreak of pup mortality of sea lions was recorded. A total of 80 pups were necropsied according to Geracy and Lounsbory (1993) procedure, and tissue samples from kidney, brain, liver, lung, placenta, heart, stomach, trachea, thymus, blood and muscle were taken. All samples were collected according to parameters established by The Galapagos National Park. Samples were analyzed for several pathogens, including *Morbillivirus*. Out of 80 samples, 48 were obtained from animals with an apparent respiratory infection, showing mainly oculo-nasal discharges as described by Jensen *et al.*, 2010 for animals with phocine distemper. Tissue samples were maintained at -20 °C.

Virus RNA isolation and PCR amplification

According to the symptoms that the 48 pups sampled presented, a molecular analysis was performed in order to detect a Morbillivirus, specifically Canine Distemper Virus (CDV).

RNA was extracted using the RNeasy Kit (Quiagen) according to manufacturer's instructions. Mechanical lysis was performed by placing approximately 30 mg tissue in 600 ul

of lysis buffer. The tissue (lung, kidney, liver or blood) was homogenized using a sterile pestle. Once homogenized, we continued with the RNA extraction protocol and stored it at -80 °C.

Phosphoprotein (P) gene specific primers CDV1: 5'-AACTGCAGAGTCTTCCCATC-3'; CDV2: 5'-GGCGAAGATTATTCCGAAGG-3'; CDV3: 5'-AATGCTTCATCTAACTGGGG-3') (Stanton *et al.*, 2002) were used to investigate the presence of CDV in sea lion samples. For retro transcriptase PCR we used SuperScript III One-Step RT-PCR System with Platinum Taq kit (Invitrogen, Life Technologies) and the amplification of a 149 pb fragment was considered positive. Amplicons sequences were compared to NCBI reference sequences using BLAST and MEGA version 5.0 (Tamura *et al.*, 2011). All samples were tested for beta-actin gene amplification as an internal control.

Results and discussion

A total of 48 tissue samples from symptomatic animals in San Cristobal Island were analyzed in order to detect CDV. Six samples were positive for RT -PCR and amplicon DNA sequences corresponded to CDV phosphoprotein, not to phocine distemper (PDV) as was expected (Figure 1). Ecuadorian sequences shared 99% of homology with canine distemper isolates from other parts of the world (Radtanakantikanon *et al.*, 2013; Lan *et al.*, 2006).

In the past three decades, new morbillivirus have been discovered in marine mammals including PDV, which affects pinnipeds. On the other hand, it is known that canine distemper virus, affects several families such as Canidae, Felidae, Hyaenidae, Mustelidae, Procyonidae, Ursidae, Viverridae and Otariidae (Deem *et al.*, 2000; Visser *et al.*, 1993). Sequence analysis of the viruses that have affected pinniped species demonstrated that PDV is closely related to

CDV (Barret *et al.*, 1991), suggesting that PDV could have evolved from CDV (Mamaev *et al.*, 1996; Barret *et al.*, 1991).

Our study may corroborate the notion that CDV can infect pinnipeds including the Galapagos sea lion, *Zalophus wollebaeki*. These results suggested that the high mortality of Galapagos sea lion in San Cristobal Island was caused by a CDV strain not PDV strain, as we could expect.

Human pets, such as dogs and cats, are in frequent contact with sea lions in San Cristobal and this fact could become one of the most important threats to these animals. Sea lions health is clearly affected, the main reason could be that the pathogens from pets are crossing the land-sea barrier and are now colonizing wild animals. Wildlife populations are considered a link in the transmission and emergence of pathogens (Daszak *et al.*, 2001), so this could be the case of CDV in Galapagos.

The recent appearance of CDV in the Galapagos Islands is an important fact that requires the respective measures in order to prevent a new outbreak and the rapid spread of the virus to other islands of the archipelago, which could have devastating consequences.

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Figures

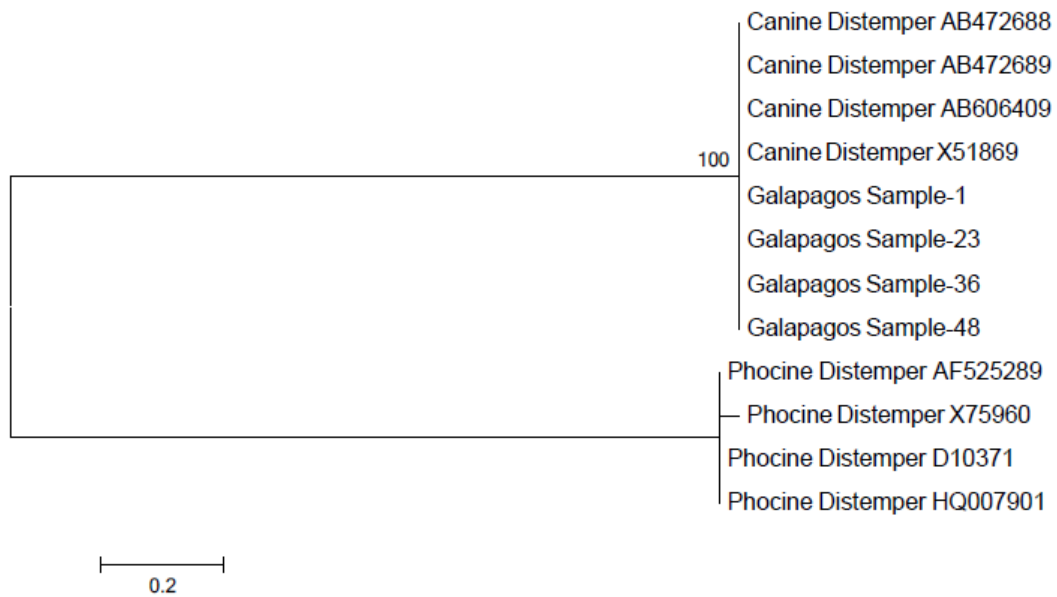


Figure 1. Phylogenetical analysis of Ecuadorian isolates (Galapagos Sample-1, Galapagos Sample-23, Galapagos Sample-36 and Galapagos Sample-48) phosphoprotein protein (P) gene, using reference sequences from Genbank.