showed in the Table 3 grouped by region and province. The study area comprises the thirteen following provinces: Azuay, Cañar, Cotopaxi, Loja, Tungurahua, Santo Domingo de los Tsáchilas (Tsáchilas), Guayas, El Oro, Los Ríos, Santa Elena, Sucumbíos, Zamora Chinchipe, and the Galapagos Islands.

Experimental Design

A serologic screening test (EIISA) was conducted in order to detect the presence of seroreactive animals. Farms included in this study were selected mainly based on the size of their production as well as the presumptive diagnosis of ILT according to the presence of compatible clinical signs [5]. According to the sampling design for epidemiological surveillance of avian diseases 2012, all Ecuadorian provinces with large poultry production were included (thirteen in total). Serological samples were selected: broilers, laying hens and breeding hens, considering that previous studies (unpublished data) gave us an apparent prevalence of 19.33%. Pastaza was an exception since it holds a small number of backyard chickens.

According to the results of the serologic survey, a second survey was conducted in seven seropositive farms: chickens with acute or mild respiratory illness were tested for the presence of ILTV DNA in tracheal tissues from these seropositive farms in El Oro, Azuay, Cotopaxi and Tungurahua provinces.

Farms were visited by technicians (focal points) of the Poultry Health National Program AGROCALIDAD to meet the annual sampling of 2012 programmed into active surveillance of avian diseases.

Sampling strategy

Fourteen to fifty birds per farm were randomly sampled for the serologic screening according to the population size. Blood samples were collected and serum was extracted to detect ILTV specific antibodies by ELISA test. Furthermore, according to the results of the ELISA test, seropositive farms were selected to detect ILTV DNA by real time PCR. Fourteen chickens in each serpositive farm were slaughtered and fragments of trachea from each 2 animals were collected together in one tube for DNA extraction.

Serologic test

Biocheck® Elisa Test was performed to detect ILTV specific antibodies following the instructions of the manufacturer.

Nucleic acid extraction

The inner epithelia from each two pooled trachea were scrapped and the cells collected into 3mL of phosphate-buffered saline solution at 0.1 M, pH 7.4 (PBS) and centrifuged at 3000 x g for 20 min. The supernatant was further used for DNA extraction.

Total DNA was isolated from 200 µl of supernatant using High Pure Viral Nucleic Acid Kit (Roche) according to manufacturer's instructions. DNA was eluted in 50 µl of nuclease free water and further processed by Real Time-PCR or stored at −20°C until analysis.

Amplification procedures by real time PCR (rt-PCR)

The primers and probe targeted a 100% conserved region and amplified a 103 bp product fragment of the ILTV glycoprotein C gene [21]. The fluorogenic TaqMan probe was labeled at the 5' end with the reporter dye FAM and with the quencher dye BHQ at the 3'-end (Roche, Inc).The amplification was carried out by duplicated using a commercial PCR amplification kit, Light Cycler 480 Probe Master (Roche), according to manufacturer's instructions. Briefly, rt-PCR reaction contained 3 µl of isolated DNA with 10 µl Probe master-mixture, each ILTV specific primers at a final concentration of 0.2 µM, the probe at a final concentration of 0.2 µM, and nucleic acid-free water bring the volume to 20 µl. The amplification conditions were as follows: 10 min at 94°C and 45 cycles of 10 s at 95°C, 30s at 55°C and 4s at 72ºC. Positive control DNA was kindly supplied by the Colombian institute "ICA" (Instituto Colombiano Agropecuario)

Data analysis

The farms were classified as positive to ILTV if one or more serum samples tested positive to the ELISA test. Each tracheal pooled sample was classified as positive when the threshold cycle (Ct) value was < 45 in the rt-PCR analysis. Threshold cycle value (Ct) mean and standard deviation of each farm were calculated.

The proportion of seroreactors (animals that tested positive) was calculated in each poultry farm, as well as the proportion of positive farms in each province. The confidence intervals were estimated in WINPEPI version 11.22 [31].

III RESULTS AND DISCUSSION

Specific antibodies to ILTV were found in 424 serum samples corresponding to 48 farms located in 12 of the 13 investigated provinces. Sucumbíos was the only province without seropositive reactors, just one small flock was examined (Table 2). High proportion of seroreactors at the animal and farm-level were detected in this study: 0.194 and 0.592 respectively (Tables 2 and 3). The distribution of seroreactive farms (Fig. 1) was mainly grouped in the highland region of the country (provinces of Tungurahua, Cotopaxi and Azuay), where

a higher density of farms/km2 and birds/farm are present. However, it wasn´t observed a systematic association with different climatic region because the lowest values were obtained at Loja province that it was in highland climate. The population density of farms and animals influenced to the rates of seroreactors.

According to the surveyed farms, ILT distribution pattern also highlighted areas or regions where the disease was detected but several neighbor farms resulted unaffected (Fig.1). Considering the transmissibility of ILTV, undamaged non-infected farms could have a proper biosecurity or a low contact rate with infected farms.

Table 2.- Proportions of seroreactor animals to avian infectious lanryngotracheitis virus determined by ELISA in different provinces of Ecuador

Natural Region	Province	Animals		Proportion seroreactor	Confidence Interval 95%	
		Positive	Tested		Inf Limit	Sup Limit
Highland	Azuay	6	100	0,060	0,022	0,127
	Cañar	58	200	0,290	0,228	0,359
	Cotopaxi	56	100	0,560	0,457	0,660
	Loja	5	121	0,041	0,014	0,095
	Tungurahua	193	475	0,406	0,362	0,452
Coastal Lowlands	Santo Domingo de los Tsáchilas	4	82	0,049	0,013	0,120
	Guayas	81	282	0,287	0,235	0,344
	El Oro	6	302	0,020	0,007	0,043
	Los Ríos	3	25	0,120	0,026	0,312
	Santa Elena	3	180	0,017	0,004	0,048
Amazonic	Sucumbíos	0	5	0,000	0,000	0,522
	Zamora Chinchipe	1	46	0,022	0,001	0,115
Island	Galápagos	8	272	0,029	0,013	0,057
Overall		424	2190	0,194	0,177	0,211

Figure 1.- Ecuador map showing the geographic distribution in which the serum samples were collected. Negative farms to ELISA were signed by yellow tacks and positive farms with red dots. For easier visualization Galapagos Islands are arbitrarily situated at the right bottom corn.

However, the structure of the poultry trade was not studied in the current investigation.

The results of this study suggests either an active or a wide ILTV circulation among poultry premises in several provinces. It is worth to note that this study included the main poultry producing areas which are distributed in thirteen from the twenty-four provinces of the country. Analyzing the proportion of seroreactor birds in each province (Table2), we can see wide-ranging values except at the Tungurahua province where more animals were included in the study. The proportion of positive farms is high in several provinces (Table 3) suggesting that the efficiency of ILTV transmission among poultry premises was high in the main poultry production areas of the country. Tungurahua and Cotopaxi were the provinces with the highest poultry densities, they were coincidently those with the highest proportion of positive reactors to ILTV. Thus, as the poultry subpopulation in a farm is larger, the direct contact rates between animals is facilitated [32, 33].

Regarding to poultry categories, the highest proportions of positive farms were found in laying hens (24/30) and breeders (6/7) while in broiler farms seemed less affected (13/53). Cover [34] observed that all birds are susceptible to infection, but clinical disease is more frequent in adults or in layer hens; our results agree with this statement because the higher proportions of positive farms were found in laying hens and poultry breeding flocks. This could be explained because both categories have a longer productive life span compared to broilers; therefore, the ITLV latent infection with reactivation of infection is long lasting in these animals.

The rt-PCR assay amplified ILTV DNA from 59.18% of the total 29 nucleic acid samples purified from epithelial scrapings of trachea. These positive samples were found in six of the seven egg layer farms tested by rt-PCR (Table 4) which previously were seroreactive by ELISA test and the Ct values mean were between 23.77 and 37.7. Typical amplification curves were obtained in all farms as it is shown by rt-PCR analysis of samples from farm 2 in Latacunga, Cotopaxi province (Fig 2). The Ct values obtained indicate high levels of ILTV DNA in the samples and they are in the range considered

Table 3.- Proportions of positive farms to avian infectious lanryngotracheitis virus determined by ELISA in different provinces of Ecuador

Natural Region	Province	Farms		Proportion positive farms	Confidence Interval 95%	
		Positive	Tested		Inf Limit	Sup Limit
Highland	Azuay	3	4	0,750	0,194	0,994
	Cañar	3	5	0,600	0,147	0,947
	Cotopaxi	3	4	0,750	0,194	0,994
	Loja	1	5	0,200	0,005	0,717
	Tungurahua	16	19	0,842	0,604	0,966
Coastal Lowlands	Santo Domingo de los Tsáchilas	2	4	0,500	0,068	0,933
	Guayas	9	11	0,818	0,482	0,977
	El Oro	3	13	0,231	0,050	0,538
	Los Ríos	1	1	1,000	0,025	1,000
	Santa Elena	3	7	0,429	0,099	0,816
Amazonic	Sucumbíos	0	1	0,000	0,000	0,975
	Zamora Chinchipe	1	2	0,500	0,013	0,988
Island	Galápagos	3	16	0,188	0,041	0,457
Overall		48	92	0,522	0,415	0,627

Table 4.- Presence of ILTV- DNA in pooled tracheal samples of chickens from seroreactive farms by real time PCR.

Province/City/Village	N^O positive	Examined Positive (%)	Ct value Mean	Standard Deviation
El Oro/ Piñas/ San Roque	0	0	-	-
215 Pichincha / Quito / Machachi	5	71.43	24.27	3.82
216 Azuay / Cuenca / Sayausy	2	28.57	37.62	1.06
Cotopaxi /Salcedo/Matrix	7	100	25.53	4.77
217 Cotopaxi / Latacunaga/ AlaquezB	6	85.71	28.99	6.42
220 Cotopaxi /Latacun ga/ AlaquezP	7	100	23.77	6.35
BM1304027 TUNGURAHUA/ Ambatos/ Atahualpa	2	28.57	32.45	5.55
Total	29	59.18		

Table 5.- Presence of ILTV- DNA in tracheal samples of chickens from seroreactive farms by real time PCR.

Province/Village	Total tested	N^O positive	Examined Positive (%)	Range of Ct value
El Oro/ Piñas	7	0	0	<45
Tungurahua/ Pillaro	7	7	100	20.54-37.12
Azuay/Cuenca	7	3	42.95	36.87-37.7
Cotopaxi/Salcedo	7	7	100	19.28-32.6
Cotopaxi/Latacunga1	7	6	85.71	18.06-32.55
Cotopaxi/Latacunga2	7	7	100	18.99-33.43
Total	42	30	71.42	

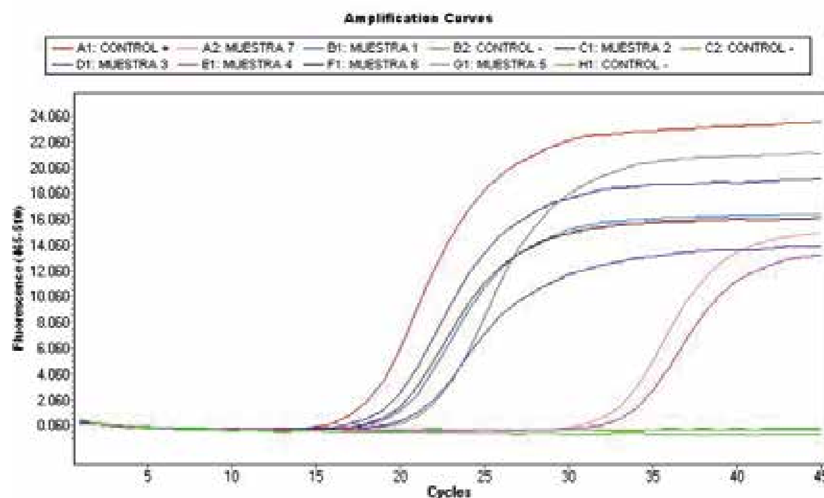

Figure.- 2 Amplification curve of Real Time PCR analysis of the tracheal samples from one of the representative tested farms (Cotopaxi/Latacunga2)

positive elsewhere (Table 5). [24].

The positive results by the ELISA test and the confirmation by rt-PCR were consistent with the ILT characteristic symptoms [5] such as tracheitis, watery eyes, and dyspnea observed in the screened farms before or during the serologic survey. Acute clinical signs were not observed in all animals during the sampling process. However, the tracheal tissues showed hemorrhages and mucoid exudate in all positive animals to the rt PCR suggesting mild or subclinical infection. Clinical signs usually appear between 6 and 12 days post infection (PI) but the rt-PCR technique can detect DNA virus already on the third day PI [5]. Consequently, it is possible that some samples were collected in the early stages of infection before to the appearance of clinical signs.

In Cotopaxi and Tungurahua provinces, the presence of ILTV infections was confirmed by rt-PCR, regardless the samples for ILTV DNA detection were taken a month after the serologic sampling. It is well documented the latency of ILTV infections, after recovery from the acute phase, the virus shedding initially ceased, then intermittent, low level shedding of virus could be detected by PCR or viral isolation in trachea and trigeminal ganglion [34, 35,36] considering the high sensitivity of the rt-PCR [8, 3,26]. Furthermore, the ILTV infection could be cycling within the large poultry population of these farms, as well located in poultry densely populated areas, where ILTV infections use to be recurrent [38].

Failure to confirm the presence of ILTV infection by rt-PCR in El Oro province (Table 4) could be explained by the sanitary and control measures implemented in this farm after the positive serologic results. This farm was selected to undergo the rt-PCR due to its large number of commercial animals although only 3/25 animals were seroreactors to the ELISA test. All the other farms tested by rt-PCR yield specific amplification curve in at least two of the seven tracheal pools. The Ct values obtained indicate moderate levels of ILTV DNA in the samples and they are in the range considered positive elsewhere.

Clinical ILT affecting poultry in Ecuador was informed to the OIE in 2012 [29].In a similar period, results of positive chickens to a serological ELISA conducted for monitoring purposes were published [39]. These authors did not observe clinical signs of ILT although the evaluation is limited to the Floreana Island in the Galapagos archipelago. ILTV infections on poultry were not reported in Ecuador before 2011, but Ayala et al (2014) obtained positive samples for the presence of ILTV. The origin of the outbreaks and infections by ILTV remains to be elucidated in Ecuador although there are several possible sources:

a) Given that some ILT outbreaks themselves may be entirely mild [5, 8], they could be consequently misdiagnosed before 2012[29].

b) ILTV vaccination was officially banned in the country until 2012. Therefore most of the poultry population become immunologically naive in which the virus incursion could induce not only the chronic or mild form of ILT [5, 11]. The recognized role of ILT vaccines causing outbreaks [10, 12, 13,17,15] could be fairly ruled out in Ecuador, at least as the main cause of the observed outbreaks.

c) ILTV could be introduced in Ecuador from neighboring countries like Colombia and Peru where the disease have been clinically demonstrated or existed limited to one or more zones [40].

Further molecular PCR analysis in association with restriction fragment length polymorphism analysis and gene sequencing [2, 13] would help to clarify the main sources of ILTV circulating in Ecuador, as well as aiming to complement further disease control strategies.

IV CONCLUSIONS

The serologic survey and the confirmatory molecular diagnostic suggested evidence of a fairly presence of ILT infection in the main poultry farms of Ecuador. The current work highlights the need to apply a control program of ILT based on vaccination and standard biosecurity measures in the poultry farms of Ecuador.

Aknowledgement
The authors would like to thank Diego Vizcaino and Luis Ramos by promoting research, and the “Agencia Ecuatoriana de Aseguramiento de la Calidad del Agro (AGROCALIDAD)” from the Ministry of Agriculture, Livestock and Fisheries of Ecuador (MAGAP) and Prometeo Project of SENESCYT (“Secretaría de Educación Superior, Ciencia, Tecnología e Innovación de la República del Ecuador”) for the financial support to carry out this research. Finally, we would like to thank Dr. Nelson Cabrera for the management of this project.

References
[1] A.M.Q King, M.J. Adams, E.B. Carstens, E.J. Lefkowitz (2012) “Ninth Report of the International Committee on Taxonomy of Viruses”. Elsevier Academic Press, San Diego, pp. 1344.

[2] J.L. Chacón, M.Y. Mizuma, A.J.P. Ferreira (2010) “Characterization by restriction fragment length polymorphism and sequence analysis of field and vaccine strains of infectious laryngotracheitis virus involved in severe outbreaks”, *Avian Pathol*. 39, 425-33.

[3] S.C Ou, J.J. Giambrone (2012) “Infectious laryngotracheitis virus in chickens”, *World J Virol.* 1, 142-149.

[4] J. Humberd, M. García, S.M. Ribler, R.S. Resurrección, T.P. Brown (2002) ”Detection of Infectious Laryngotracheitis Virus in formalin-fixed, paraffin-embedded tissues by Nested polymerase chain reaction”, *Avian Dis.* 46, 64-74.

[5]J. S. Guy, M. Garcia (2008) “Laryngotracheitis” In: Diseases of poultry, 12th ed. Y. M. Saif, A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan, D. E. Swayne, eds. Iowa State University Press, Ames, IA. pp. 137-152.

[6] I. Dolka, R. Sapierzyński, W. Bielecki, E. Malicka, A. Żbikowski, P. Szeleszczuk (2012) “Histopathology in diagnosis of broiler chicken and layer diseases--review of cases1999-2010”, *Pol J Vet Sci*. 15, 773-779.

[7] N. Timurkaan, F. Yilmaz, H. Bulut, H. Ozer, Y. Bolat (2003) “Pathological and immunohistochemical findings in broilers inoculated with a low virulent strain of Infectious laryngotraqueitis virus”, *J.Vet. Sci.* 4, 175-180.

[8] OIE (2008) “Avian Infectious Laryngotracheitis” in “Manual of Diagnostic Tests and Vaccines for Terrestrial Animals”, Chapter 2.3.3, Last accessed Feb. 2015, available online at http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/A_index.htm.

[9] T.J Bagust, M.A. Johnson (2008) “Avian Infectious Laryngotracheitis: virus host interactions in relation to prospects for eradication”, *Avian Pathol.* 24, 373-391

[10] L. Dufour-Zavala (2008) “Epizootiology of infectious laryngotracheitis and presentation of an industry control program”. *Avian Dis.* 52, 1-7.

[11] M.J. Coppo, C.A. Hartley, J.M. Devlin (2013) “Immune responses to infectious laryngotracheitis virus” *Dev Comp Immunol.* 41, 454-62.

[12] I. Davidson, S. Nagar, I. Ribshtein, I. Shkoda, S. Perk, M. García (2009) “Detection of wild- and vaccine-type avian infectious laryngotracheitis virus in clinical samples and feather shafts of commercial chickens”, *Avian Dis.* 53, 618-23.

[13] A. Moreno, A. Piccirillo, A. Mondin, E. Morandini, L. Gavazzi, P. Cordioli (2010) ”Epidemic of infectious laryngotracheitis in Italy: characterization of virus isolates by PCR-restriction fragment length polymorphism and sequence analysis”, *Avian Dis*. 54, 1172-77.

[14] H.P Blacker, N.C. Kirkpatrick, A. Rubite, D. O'Rourke, A.H. Noormo hammadi (2011) “Epidemiology of recent outbreaks of infectious laryngotracheitis in poultry in Australia”, *Aust. Vet. J.* 89, 89-94.

[15] J.M. Madsen, N.G. Zimmermann, J. Timmons, N.L. Tablante (2013) “Prevalence and differentiation of diseases in Maryland backyard flocks”, *Avian Dis*. 57, 587-94.

[16] S.W. Lee, J.M. Devlin, J.F. Markham, A.H. Noormohammadi, G.F. Browning, N.P. Ficorilli, C.A. Hartley, P.F. Markham (2013) “Phylogenetic and molecular epidemiological studies reveal evidence of multiple past recombination events between infectious laryngotracheitis viruses”. *PLoS One,* 8, e55121.

[17] S.W Lee, P.F. Markham, M.J. Coppo, A.R. Legione, J.F. Markham, A.H. Noormohammadi, G. F. Browning, N. Ficorilli, C.A. Hartley and J.M. Devlin (2012) “Attenuated vaccines can recombine to form virulent field viruses”, *Science.* 337, 188.

[18] K.R. Menendez, M. García, S. Spatz, N.L. Tablante (2014) “Molecular epidemiology of infectious laryngotracheitis (ILT): a review”, *Avian Pathol.* 43, 108-117.

[19] A. Mashchenko, S.M. Riblet, G. Zavala, M. García (2013) “In ovo vaccination of commercial broilers with a glycoprotein J gene-deleted strain of infectious laryngotracheitis virus”. *Avian Dis.* 57, 523-31.

[20] B. Bauer, J.E. Lohr, E.F. Kaleta (1999) “Comparison of commercial ELISA test kits from Australia and the USA with the serum neutralization test in cell cultures for the detection of antibodies to the infectious laryngotracheitis virus of chickens”, *Avian Pathol.* 28, 65–72.

[21] S.A. Callison, S.M. Riblet, I. Oldoni, S. Sun, G. Zavala, S. William, R.S. Resurreccion, E. Spackman, M. Garcia (2007) “Development and validation of a real-time Taqman PCR assay for the detection and quantification of infectious laryngotracheitis virus in poultry”, *J. Virol. Meth.* 139, 31-38.

[22] J.L Chacón, A.J.P. Ferreira (2008) “Development and validation of nested-PCR for the diagnosis of clinical and subclinical infectious laryngotracheitis”. *J. Virol. Meth.* 151,188-193.

[23] J.J. Ou, S.C. Giambrone, K.S. Macklin (2012) “Comparison of a TaqMan real-time polymerase chain reaction assay with a loop-mediated isothermal amplification assay for detection of Gallidherpesvirus 1”, *J. Vet. Diagn. Invest.* 21, 138-141.

[24] T.V. Dormitorio , J.J. Giambrone, K.S. Macklin (2013) “Detection and Isolation of Infectious Laryngotracheitis Virus on a Broiler Farm After a Disease Outbreak”, *Avian Dis.* 57, 803-807.

[25] L.G. Wang, J. Ma, C.Y. Xue, W. Wang, C. Guo, F. Chen, J.P. Qin, N.H. Huang, Y.Z. Bi, Y.C. Cao (2013) “Dynamic distribution and tissue tropism of infectious laryngotracheitis virus in experimentally infected chickens”, *Arch. Virol.* 158, 659-66.

[26] Y. Zhao, C. Kong, X. Cui, H. Cui, X. Shi, X. Zhang, S. Hu, Y. Wang (2013) "Detection of Infectious Laryngotracheitis Virus by Real-Time PCR in Naturally and Experimentally Infected Chickens", *PLoS One* 8, e67598.
[27] AGROCALIDAD (2012) "RESOLUCION No.0085". Mayo 20012.
[28] S. Ayala, M. Revelo, V. Barragán, J. Chiriboga, A. Torres, I. Santiana, G. Trueba. (2014) "Detection and Characterization of Infectious Laryngotracheitis Virus from an Outbreak of Respiratory Disease in Ecuador", *Intern J Appl Res Vet Med.* 12 (3), 245-248.
[29] OIE (2012) "Informe de notificación inmediata Referencia del informe: Ref OIE: 12139, Fecha del informe: 20/07/2012 , País: Ecuador". Acceso en línea. http://web.oie.int/wahis/reports/es_imm_0000012139_20120720_183415.pdf.
[30] FAOSTAT (2014) "Food and Agriculture Organization of the United Nations". Acceso en línea http://faostat.fao.org/site/339/default.aspx.
[31] J.H. Abramson, WINPEPI updated: computer programs for epidemiologists, and their teaching potential. (2011) Epidemiologic Perspectives & Innovations. 8,1.
[32] M. Begon, M. Bennett, R.G. Bowers, N.P. French, S.M. Hazel, J.A. Turner (2002) "Clarification of transmission terms in host–microparasite models: numbers, densities and areas", *Epidemiol. Infect.* 129, 147-153.
[33] M.J. Keeling, R. Pejman (2008) "Introduction to simple epidemic models density - dependent transmission. In: Modeling infectious disease in humans and animals" Ed, Princenton University Press 41 New Jersey 08450, pp.15-52.
[34] M.S. Cover (1996) "The early history of Infectious laryngotracheitis", *Avian Dis.* 40, 494-500.
[35] T.J Bagust, B.W. Calnek, K.J. Fahey (1986) "Gallid-1 Herpesvirus Infection in the chicken. 3. Reinvestigation of the pathogenesis of Infectious Laryngotracheitis in acute and early post-acute respiratory diseases", *Avian Dis.* 30, 179-190.
[36] R.A. Williams, M. Bennet, J.M. Bradbury, R.M. Gaskell, R.C. Jones, F.T.W. Jordan (1992) "Demonstration of sites of latency of infectious laryngotracheitis virus using the polymerase chain reaction", *J. Gen.Virol.* 73, 2415-2430.
[37] A. Rodríguez-Avila, I. Oldoni, S. Riblet, M. García (2007) "Replication and transmission of live attenuated infectious laryngotracheitis virus (ILTV) vaccines", *Avian Dis.* 51, 905–911.
[38] R.P Chin, M. García, C. Corsiglia, S. Riblet, R. Crespo, H.L. Shivaprasad, A. Rodríguez-Avila, P.R. Woolcock, M. França (2009) "Intervention strategies for laryngotracheitis: impact of extended downtime and enhanced biosecurity auditing", *Avian Dis.* 53, 574-577.
[39] S.L. Deem, M.B. Cruz, J.M. Higashiguchi, P.G. Parker (2012) "Diseases of poultry and endemic birds in Galapagos: implications for the reintroduction of native species", *Animal Conservation* 15, 73–82.
[40] WAHID (2014) "World Animal Health Information Database Interface, Country information", Last accessed February 2014, available online at http://www.oie.int/wahis_2/public/wahid.php/Countryinformation/Animalsituation.