

EVALUATION OF COMPOUNDS FROM OREGANO (*ORIGANUM VULGARE*) THAT INACTIVATE THE INFLUENZA VIRUS IN HOST ANIMALS

AVALIAÇÃO DOS COMPOSTOS DO ORÉGANO (*ORIGANUM VULGARE*) NA INATIVAÇÃO DO VÍRUS INFLUENZA EM HOSPEDEIROS

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ABSTRACT

The influenza is a respiratory disease considered as a zoonosis. Its transmission between humans and animals can occur through animal contact, manipulation and consumption of food from contaminated animals. Avians and swine are responsible by influenza contamination in food. The presence of compounds with antioxidant activity in food is related in the inhibition of free-radicals formation and also to inactive viruses. To evaluate the presence of influenza virus in animals and the anti-viral action from oregano (*Origanum vulgare* L.) on the influenza virus was also investigated. Samples from 34 swine and 50 ducks were collected. The hemagglutination (HA) and Gold-labeled-optimally-read-immunoassay (Gloria) tests were employed for isolation and identification of the influenza virus. Antioxidant activity in oregano extract was evaluated by β carotene / linoleic acid system and the total phenolic compounds by Folin-Ciocateau reagent. The antioxidant activity of 100 ppm of phenolic compounds from oregano extract was 89.2% and the association with BHT was observed the increase to 93.6%. Influenza positivity was observed in 10% of ducks and 53% of swine, presenting HA 04 HAU/ μ L and 12 HAU/ μ L, respectively. These titers decreased after influenza virus inactivation by contact for 1 h with 100 ppm (v/v) oregano extract. Therefore, this influenza virus could be transmitted by both workers and migratory birds. These data suggest that animals are able to generate influenza contamination of food (avian meat, eggs, swine meat) and the phenolic compounds revealed anti-viral action on influenza virus.

Keywords: Phenolic compounds. Oregano. Influenza virus. Anti-viral.

RESUMO

A doença respiratória causada pelo vírus influenza é considerada uma zoonose. Através do contato entre humanos e animais e, ainda, pelo consumo de alimentos de origem animal contaminados, pode ocorrer a transmissão viral. As aves e os suínos são os mais comuns geradores de alimentos contaminados por influenza. Os compostos fenólicos presentes em alimentos possuem ação antioxidante inibidora da formação de radicais livres e inativante viral. Este trabalho avaliou a presença de influenza nesses animais e a ação antiviral do orégano (*Origanum vulgare* L.). Amostras de 34 suínos e 50 patos foram coletadas. O isolamento e identificação do vírus influenza foram realizados através dos testes de hemaglutinação (HA) e *gold-labeled-optically-read-immunoassay* (GLORIA). A atividade antioxidante do orégano foi avaliada pelo sistema β -caroteno/ácido linoleico e os teores de fenólicos totais pelo reagente de Folin-Ciocateau. A atividade antioxidante de 100 ppm dos compostos fenólicos extraídos do orégano foi de 89,2% e na associação deste com o BHT foi observado o aumento desta atividade para 93,6%. Os patos (10%) e os suínos (53%) foram positivos aos testes de influenza, apresentando, respectivamente, 4 UHA/ μ L e 12 UHA/ μ L. Esses títulos de HA foram reduzidos após a inativação do vírus, promovida pelo contato por uma hora com 100 ppm de extrato de orégano. Esses animais são capazes de contaminar o alimento com o vírus influenza (carnes de suíno, de patos ou ovos). Os compostos fenólicos do orégano revelaram ação antiviral sobre o vírus influenza.

Palavras-chave: Compostos fenólicos. Orégano. Vírus influenza. Antiviral.

1 Introduction

Swine and ducks were considered the principal transmitters of the influenza virus to the human population in pandemics such as Spanish and Asian Flu that occurred in 1918 and 1957, both caused by influenza type A (H_1N_1) and (H_2N_2), respectively, provoking high levels of morbidity and mortality around the world.

In Northern Italy, Giannchini et al. (2006) observed that there is serological evidence of human infection with low pathogenic influenza A (H_7N_3) of turkey viruses, and also they previously had observed that this subtype of turkey influenza virus came from interspecies transmission of duck.

Wallenstein et al. (2006), developed a study in ducks caught during spring migration throughout Sweden, which led them to conclude that wild ducks could transmit the influenza infection to juvenile and other susceptible avians at the breeding sites.

In Brazil (2001), a seroepidemiology study of avian influenza virus (AIV) was performed through the hemagglutination inhibition (HI) technique in plasma of 225 birds from the Rio-Zoo Foundation,

Bwana Park and from small flocks of Rio de Janeiro State. It was observed that of the 225 birds, 60 (26.6%) were positive, 22 (9.8%) of them for the H_1N_1 (A/Turkey/Weybridge/79) and 28 (12.4%) for the subtype H_3N_2 (A/Duck/Hong Kong 29/76). The authors point out that influenza occurrence indicates a potent risk of transmission to both industrial poultry and humans from Rio de Janeiro city. (OLIVEIRA et al., 2001).

Considering this knowledge of influenza interspecies transmission by ducks, Webster et al. (2006), performed an experimental vaccination against HPAI (H_5N_1) intending to immunize these transmitter avians. These authors verified that after the virus challenge performed in these vaccinated ducks, all of them remained alive showing they were protected against the virus.

Study concerning the evolution of swine A (H_3N_2) influenza viruses in USA investigated isolates of the virus and showed that the internal gene complex was associated with three recent phylogenetically distinct human-like hemagglutinin (HA) molecules. (WEBBY et al., 2000).

In Europe and Asia, the human H₃ and avian H₁ influenza viruses have been isolated from pigs. Due to incorrect husbandry practices, the maintenance of this virus has been possible. (BROWN, 2000; OLSEN et al., 2000).

In Spain, studies revealed that the swine influenza virus A (H₁N₁) strain is still endemic in pig populated areas in this country. (MALDONADO et al., 2005).

The influenza illness in ducks is not apparent, because their immune system is not able to recognize the virus as an infectious agent. Although, the virus continues being sprayed by the ducks to the other hosts. (MOON et al., 2005).

In our previous study with migratory birds such as passerines, influenza virus type A was isolated. (KAWAMOTO et al., 2004).

Some natural and synthesized antioxidants have been showed great antiviral action on several viruses, including influenza virus. The phenolic compounds present in food are related to the virus inactivation, which is obtained by the antioxidant activity. The ability to delay the lipid oxidation on biological membranes, in addition to their capacity to act as a prophylatic agent as inhibitor of free radicals has motivated research into biomedicine. (MANCINI-FILHO et al., 2005; MELO et al., 2005).

Phenolic compounds from spices like oregano were demonstrated as being an efficient antioxidant activity. (MANCINI-FILHO et al., 2005). This activity also demonstrates antiviral action, considered to be an inhibitor of influenza virus fusion with the host cells, blocking the virus entry and its multiplication.

This study investigated influenza type A virus in both aquatic avians, belonging to *Anseriformes* Group like ducks, and swine that are cited to generate contaminated food, and also the inhibition of the influenza virus isolated from these animals, was evaluated by treatment with phenolic compounds from oregano extract.

2 Material and methods

Sample collection

Animal Samples collection

From the São Paulo city, SP, area, cloacal and oral-nasal samples in transport media previously described by Kawamoto et al. (2005) were collected from fifty ducks and thirty-four swine, during the period of the Autumn/Winter of 2007-2008.

Virus investigation

Hemagglutination Test (HA)

For the influenza virus investigation in both swine and duck samples, the HA test was used as follows: Hemagglutination titers were determined at room temperature in a microtiter system. Serial two-fold dilutions of virus (25 µL) in phosphate buffered pH 7.2 were mixed with 25 µL of a 0.5% suspension from rooster red blood cells. Hemagglutination titers were determined after 1 h, unless otherwise started, and are expressed as the reciprocal of the maximum dilution of virus that caused complete agglutination. (ISODA et al., 2006)

Virus identification by serology

For serology identification of the influenza in isolates obtained from duck and pig, the hemagglutination inhibition test was performed using human influenza antigens and antisera supplied by Bethesda (MA) USA as follow:

Antigen type A: A/Chile/1/83 (H₁N₁) and A/Philippine/2/82 (H₃N₂)

Anti-sera for type A: A/Chile/1/83 (H₁N₁) and A/Philippine/2/82 (H₃N₂)

Hemagglutination Inhibition Test (HI)

The pattern sera were treated with 20% kaolin in 0.01 M phosphate buffer solution (PBS), pH 7.4 in order to eliminate nonspecific antibodies. After this treatment, duplicate dilutions were carried out in series, in "V" bottom microplates. Antigens of the influenza viruses, obtained from duck

and swine samples containing 4 hemagglutinating units, were added to the wells. After a hour reaction at room temperature, 5% rooster erythrocytes were added to the wells. Reading was processed after 30 min, with the reciprocal of the last dilution, which induced hemagglutination inhibition that was considered as being the antibody titer. Sera presenting inhibitory antibody titers of 20 HIU/ μ L or superior to the antigens test were considered positive. A constant volume of 0.025 mL was used for all reagents. (ISODA et al., 2006).

Influenza A/B Rapid Test (GLORIA)

Influenza virus isolates were characterized by influenza A/B Rapid Test (Roche Laboratories), as follow: the test principle is based on the Roche diagnostics: GLORIA (gold-labeled-optimally-read-immunoassay). In this test is detected the viral nucleoprotein and viral nucleic acid that are released by lysing the influenza virus envelope with Lysis/Elution Solution. (KAWAMOTO et al., 2005).

The test uses two pairs of monoclonal antibodies to specific influenza A and other specific influenza B. Both antibody pairs are conjugated to either biotin or digoxigenin. In the presence of the viral antigen, a sandwich complex is formed, consisting of the biotin- conjugated antibody, the nucleoprotein, and the digoxigenin- conjugated antibody. When the test strip is placed in the reaction cup, the complex migrates chromatographically, solubilizing colloidal gold particles incorporated in the red pad of the strip. The colloidal gold particles bind to the digoxigenin of the complex, which is then bound by the biotin to the immobilized streptavidin on the strip (positive result line). Any excess gold particles continue to migrate to the second line (control line), which then becomes visible. This indicates the correct chromatographic migration. No cross reactivity occurs with other probable respiratory viruses or other organisms such as bacteria or fungi, as it is informed in the package insert of the Influenza A/B Rapid Test Kit.

Oregano Phenolic compounds extraction

The spice oregano was acquired at a local market in São Paulo city, Brazil. The aqueous extract was obtained from 4g samples of the spice oregano,

with 40 mL of hot water (80°C) while shaking for 10 min, followed by centrifugation at 3.000g for 20 min. After residue is removed the supernadant was completed to 40 mL with water. The amount of dry material in each extract was determined gravimetrically.

Phenolic compounds evaluation

The determination of the total phenolic compounds was accomplished, through the espectrophotometric method with Folin – Ciocateau reagent used with catechins as standard. Suitable aliquots of the fractions were taken in a test tube. Then, 0.5 mL of Folin – Ciocateau reagent and 1 mL of saturated sodium carbonate solution were added sequentially in each tube. The total volume of the system was adjusted to 10 mL with distilled water. The tubes were vortexes, placed in the dark for 60 min and the absorbance was recorded at 725 nm. The results were expressed as microgram of total phenolic compounds in catechin equivalent per mL of the extract.

Antioxidant activity determination

The antioxidant activity of aqueous extrat from oregano containing 50, 100 and 200 ppm of phenolic compounds, the synthetic antioxidant butylated hydroxi toluene (BHT) in the same concentration and the association of the aqueous extract with BHT, were determined by the completed oxidation of β carotene and linoleic acid, according methodology described by Mancini-Filho et al. (2005) and Melo et al. (2005).

A solution of β carotene was prepared by dissolving 1 mg of β carotene in 10 mL of chloroform 1.0 mL of this solution was pipetted into a round-botton flask, which contained 20 mg linoleic acid and 200 mg tween 40 emulsifier. After removal of chloroform, 50 mL of aerated distilled water with O_2/CO_2 (95/5) were added to the flask with vigorous shaking. Aliquots (5 mL) of this emulsion were transferred to the tubes with different concentrations of antioxidants (natural or synthetic). Reading at 470 nm were taken at 15 min intervals for 120 min. Antioxidant activity was subsequently expressed as an oxidation inhibition percentage, calculated in relation to the 100% oxidation that occurs in the control (without antioxidant). The BHT antioxidant

activity was determined under the same conditions as a means of comparison.

Treatment of influenza virus with oregano extract and antiviral assay

Influenza virus samples isolated from both duck and pig were treated with 100 ppm of phenolic compounds from oregano extract (v/v) at 4 °C for 1 h. After this, the samples were evaluated by the hemagglutination test. The virus inactivation level was measured by the formula for virus inhibition percentage (IP) calculation (BENENCIA; COURREGES, 1999), as follow:

$$\text{Formula IP: } 1 - \frac{[\text{HA titer of Influenza isolate treated with antioxidant}]}{\text{HA titer of Influenza isolate (Control)}} \times 100\%$$

Statistical analysis

All analyses were performed in triplicate. The data obtained were submitted to Tukey test, at the 5% significance level, using minitab statistical Window program.

3 Results and discussion

From the results obtained by the hemagglutination test, it was verified that duck samples presented a percentage of the hemagglutinating activity at the titers of 2 HAU/ μ L (6%), 4 HAU/ μ L (2%) and 16 HAU/ μ L (2%), totalling to a medium titer of the 4 HAU/ μ L among the duck samples. Referring to the swine, there were more high levels of the hemagglutinating activity at all titers of: 2 HAU/ μ L (11.76%), 4 HAU/ μ L (14.70%), 6 HAU/ μ L (2.94%), 8 HAU/ μ L (11.76%), 16 HAU/ μ L (5.88%), 32 HAU/ μ L (8.82%), with a medium titer of the 12 HAU/ μ L. The data showed in Figure 1 demonstrated the differences between pig and duck ($p < 0.05$), and the influenza virus could be transmitted by both workers and migratory birds.

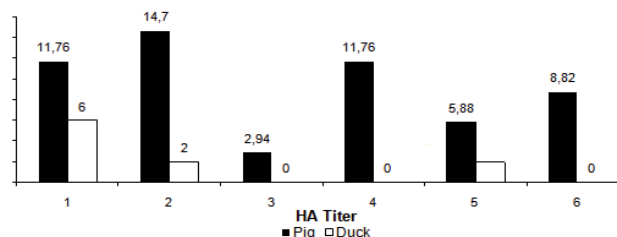


Figure 1 – Positivity of Influenza hemagglutinant activity in duck and pig samples.

By the GLORIA test, the influenza virus was detected in 18 (53%) pigs and 5 (10%) ducks (Table 1) confirming the results obtained by hemagglutinating activity measured (Figure 2).by the hemagglutination inhibition test, using patterns of anti-sera to influenza virus (Bethesda, MA, USA), it was verified that 80 % and 60 % of the pig samples were recognized by the antibodies to the subtypes A (H_1N_1) and A (H_3N_2), respectively.

Table 1 - Detection of influenza virus in samples collected from pigs and ducks, by GLORIA test.

Samples	Total	Positive samples	Percentage
Pig	34	18	53
Ducks	50	5	10

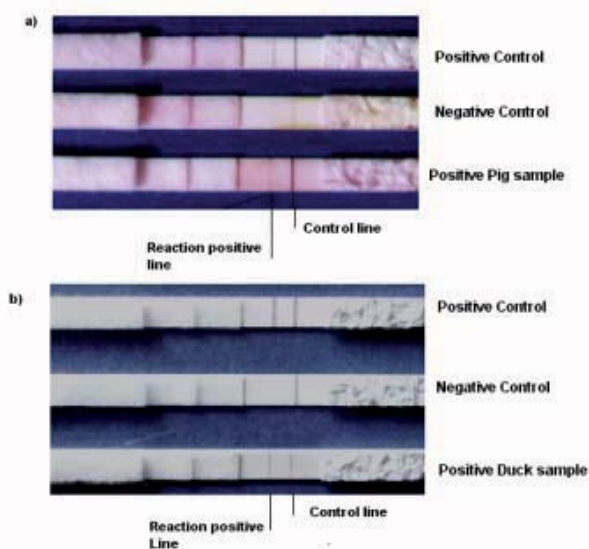


Figure 2 – Influenza vírus detected chromatographically in pig and duck samples by GLORIA Test.

The patterns of antigen to the same subtypes A (H_1N_1) and A (H_3N_2) were included to the HI test as positive controls (Table 2).

In the duck samples the influenza virus was recognized in 50% and 10% by the antibodies of the sera related to subtypes A(H_1N_1) and A(H_3N_2), respectively (Table 2).

Table 2 - Identification of the influenza virus in pigs and ducks, by HI test

Pattern Sera	Duck samples (%)	Pig samples (%)
A(H_1N_1)	50	80
A(H_3N_2)	10	60

Figure 3 demonstrates the oxidation inhibition activity of phenolic compounds from oregano. The phenolics from oregano (100 ppm) demonstrated 89.2% inhibition of oxidation, whilst BHT (200 ppm) presented 95% inhibition. The mixture of phenolics from oregano with BHT (1:1) resulted in an increase to 93.6% (100 ppm) in inhibition, demonstrating the synergism between natural and synthetic antioxidants in oxidation inhibition ($p < 0.05$)

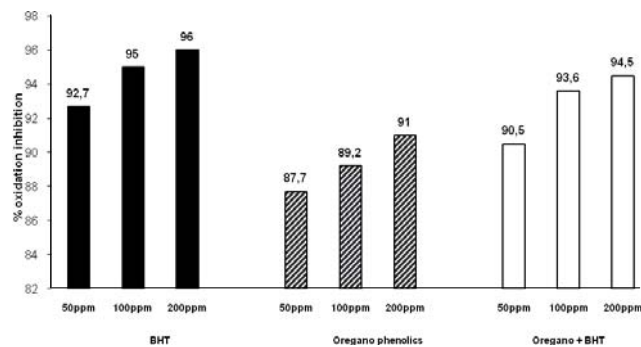


Figure 3 - Percentages of the oxidation inhibition activity of orégano (*Origanum vulgare* L.), BHT and its associations.

(MANCINI-FILHO et al., 2005). The concentration of 200 ppm in both the natural and synthetic antioxidants demonstrated the greatest inhibition of lipid oxidation (Figure 3).

Although the antioxidant activity was inferior to BHT, the whole oregano aqueous extract presented a considerable potential as oxidation inhibition. This extract can be ingested without limitation, according to the Food and Nutrition Board of the National Academy of Science.

In aqueous coriander extract, Melo et al. (2005) observed that the antioxidant activity of this is related to phenolic compounds composition, their combined effects even a common substances separately.

Therefore, considering the high antioxidant level of the oregano extract, the concentration at 100 ppm was employed to treatment of both duck and pig isolates. Through this treatment was observed that influenza virus detected in these treated samples demonstrated reduction or total elimination of the viral HA titers (Table 3 and 4).

Table 3 - Viral inhibition action of 100 ppm the oregano extract (*Origanum vulgare*, L) on influenza isolated from pigs..

Virus x Antioxidant contact (h)	Influenza HAU/ μ L	Virus Inactivation percentage
0	12	None
1	0	100

$$IP = 1 - \left[\frac{(\text{HA titer of pig Influenza isolate treated with antioxidant})}{\text{HA titer of pig influenza isolate (Control)}} \right] \times 100\%$$

Table 4 - Viral inhibition action of 100 ppm the oregano extract (*Origanum vulgare*, L.) on influenza isolated from ducks.

Virus x Antioxidant contact (h)	Influenza HAU/ μ L	Virus Inactivation percentage
0	4	None
1	0	100

$$IP = 1 - \left[\frac{\text{HA titer of duck Influenza isolate treated with antioxidant}}{\text{HA titer of duck influenza isolate (Control)}} \right] \times 100\%$$

These results are in concordance with the study in ducks developed in Sweden by Wallenstein et al. (2006).

Webster et al. (2006), observed that after birds vaccination against influenza HPAI (H_5N_1) that the remained alive were protected against influenza.

In the Spain Maldonado et al. (2005) observed that swine influenza A (H_1N_1) still endemic in pig population.

The Table 5 showed that the oregano extract (100 ppm) reduced in 87.5% the unit hemagglutinating (HAU) of the influenza virus A (H_1N_1).

4 Conclusions

Table 5 – Viral inhibition action of 100 ppm the oregano extract (*Origanum vulgare* L) on influenza A(H_1N_1).

Virus x Antioxidant contact (h)	Influenza HAU/ μ L	Virus Inactivation percentage
0	128	None
1	16	87.5

$$IP = 1 - \left[\frac{\text{HA titer of Influenza A(H}_1\text{N}_1\text{) treated with antioxidant}}{\text{HA titer of influenza A(H}_1\text{N}_1\text{) (Control)}} \right] \times 100\%$$

The results obtained from this study revealed that evaluated duck and pig isolates presented hemagglutination activity, which was considered as being an influenza viruses, with base on this virus related pattern antisera recognition. That leads to conclude that these contaminated ducks and swine were able to generate influenza contamination of food (avian meat, eggs, swine meat).

It was observed also that these influenza virus isolates were inactivated after treatment with the spi-

ce oregano extract. The antiviral action was attributed to the phenolic compounds with high antioxidant activity present in this spice.

Therefore, besides appropriate food cooking conditions, such as the high temperature, the oregano demonstrated to be an alternative complement, in order to avoid the human consumption of contaminated food.

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References

- BENENCIA, F.; COURREGES, H.C. Antiviral of sandalwood oil against herpes simplex –1 and 2. **Phytomedicine**, v.2, p.119-123,1999.
- BROWN, I.H. The epidemiology and evolution of influenza viruses in pigs. **Veterinary Microbiology**, v.74, p.29-46, 2000.
- GIANNECCHINI, S.; CAMPITELLI, L.; CALZONETTI, L.; DE MARCO, M.A.; AZZI, A.; DONATELLI, I. Comparison of in vitro replication features of H_7N_3 influenza viruses from wild ducks and turkeys: potential implications for interspecies transmission. **Journal of General Virology**, v.87, p.171-175, 2006.
- ISODA, N.; SAKODA, Y.; KISHIDA, N.; BAI, G.R.; MATSUDA, K.; UMEMURA, T.; KIDA, H. Pathogenicity of a highly pathogenic avian influenza virus, A/chicken/Yamaguchi/04 (H_5N_1) in different species of birds and mammals. **Archives of Virology**, v.151, p.1267-1279, 2006.
- KAWAMOTO, A.H.N.; MANCINI, D.A.P.; PEREIRA, L.E.; CIANCIARULLO, A.M.; CRUZ, A.S.; DIAS, A.L.F.; MENDONÇA, R.M.Z.; PINTO, J.R.; DURIGON, E.L. Investigation of influenza in migrating birds, the primordial reservoir and transmitters of influenza in Brazil. **Brazilian Journal of Microbiology**, v.36, p.84-89, 2005.
- MALDONADO, J.; VAN REETH, K.; RIERA, P.; SITJA, M.; SAUBI, N.; ESPINA, E.; ARTIGAS, C. Evidence of the concurrent circulation of H_1N_2 , H_1N_1 and H_3N_2 influenza A

viruses in densely populated pig areas in Spain. **Veterinary Journal**, v.172, n.2, p.377-381, 2006.

MANCINI-FILHO, J.; TORRES, R.P.; PINTO, J.R.; MENDONÇA, R.M.Z.; MANCINI, D.A.P. Synergistic effects of synthetic and natural spice antioxidant on antiviral action against parainfluenza virus. **Bollettino Chimico Farmaceutico**, v. 44, p. 85-89, 2005.

MELO, E.A.; MANCINI-FILHO, J.; GUERRE, N.B. – Characterization of antioxidant compounds in aqueous coriander extract (*Coriandrum sativum* L.). **Lebensmittel-Wissenschaft und Technologie**, v.38, p. 15-19, 2005.

MOON, D.A.; VENIAMIN, S.M.; PARKS-DELY, J.A.; MARGOR, K.E. The MHC of Duck (*Anas platyrhynchos*) contains five differentially expressed Class I Gene. **The Journal of Immunology**, v.175, n.10, p.6702-6712, 2005.

OLIVEIRA JR, J.G.; BELLUCI, M.S.P.; VIANNA, J.S.M.; MAZUR, C.; ANDRADE, C.M.; FEDULLO, L..PL.; PRIZ, C.; LOUREIRO, B.O. Serological survey on influenza virus in domestic and wild birds from Rio de Janeiro State, Brazil. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, v. 53, p. 299-302, 2001.

OLSEN, C.W.; CAREY, S.; HINSHAW, F.; KARASIN, A.L. Virologic and serologic surveillance for human, swine, and avian influenza virus infections among pigs in North-Central United States. **Archives of Virology**, v. 145, p.1399-1419, 2000.

WALLENSTEIN, A.; MUNSTER, V.J.; KARLSSON, M.; LUNDKVIST, A.; BRYTTING, M.; STERVANDER, M.; OSTERHAUS, A.D.M.E.; FOUCHIER, R.A.M.; OLSEN, B. High prevalence of influenza A virus in ducks caught during spring migration through Sweden. **Vaccine**, v.24, p.6734-6735, 2006.

WEBBY, R.J.; SWENSON, S.L.; KRAUSS, S.L.; GERRISH, P.J.; GOYAL, S.M.; WEBSTER, R.G. Evolution of swine H₃N₂ influenza viruses in United States. **The Journal of Virology**, v.7, p.8243-8251, 2000.

WEBSTER, R.G.; WEBBY, R.; HOFFMANN, E.; RODENBERG, J.; KUMAR, M.; CHU, H.J.; SEILER, P.; KRAUSS, S.; SONGSERM, T. The immunogenicity and efficacy against H₅N₁ challenge of reverse genetics-derived H₅N₃ influenza vaccine in ducks and chickens. **Virology**, v.35, p. 303-311 2006.