Bulgarian Journal of Veterinary Medicine, 2014, **17**, No 3, 223–230
ISSN 1311-1477; online at http://tru.uni-sz.bg/bjvm/bjvm.htm

Original article

EVALUATION OF THE TRANSMISSION POTENTIAL AND INFECTIVITY OF H9N2 AVIAN INFLUENZA VIRUS IN DOGS FED INFECTED CHICKEN CARCASSES

S. PANAHI[1], N. MOSLEH[2] & M. ABBASZADEH HASIRI[3]

[1]Graduate from School of Veterinary Medicine; [2]Avian Disease Research Center;
[3]Department of Clinical Studies; School of Veterinary Medicine, Shiraz University, Iran

Summary

Panahi, S., N. Mosleh & M. Abbaszadeh Hasiri, 2014. Evaluation of the transmission potential and infectivity of H9N2 avian influenza virus in dogs fed infected chicken carcasses. *Bulg. J. Vet. Med.,* **17**, No 3, 223–230.

Despite the frequent reports of avian influenza A virus infections in dogs and cats, which have attracted the attention of veterinary practitioners and scientists in the respective fields of virology and epidemiology during the last decade, few reports are available regarding the natural exposure of dogs to low pathogenic viruses in field conditions. This may be important in the epidemiology of viruses in dogs on chicken farms. One of the possible causes of the interspecies transmission of viruses may be the consumption of infected carcasses by dogs. This hypothesis was examined by feeding dogs with experimentally H9N2 infected chicks. To this end, 8 dogs were selected and divided into two groups: test (n=5) and control (n=3). The dogs were fed commercial and standard diets during a 3-week adaptation period and from day 4 to 10 after being fed chicken carcasses. The test group was given H9N2 infected chicken carcasses, whereas the control group was fed on healthy ones from days 1 to 3 of the experiment. Clinical signs including depression, sneezing, coughing, nasal and ocular discharge, respiratory and heart rate changes, and rectal temperature, were monitored daily from day 0 to 10. Faeces and nasal swabs were collected on a daily basis for virus detection via RT-PCR during the experimental period. HI tests for antibody titre measurement were also performed on each day of the experimental period. The results of the study indicated that the aforementioned clinical parameters remained normal in both the test and control groups. No signs of viruses were detected in the nasal swab and faeces of dogs in both groups. HI test results showed no considerable antibody titer against the H9N2 virus in either group. In conclusion, it seemed unlikely that the consumption of H9N2 infected chicken carcasses by dogs could be a cause of infection in dogs.

Key words: dog, HI, H9N2, influenza, interspecies transmission, PCR

INTRODUCTION

Influenza A viruses, as members of the *Orthomyxoviridae* family, are highly contagious pathogens that have been isolated from a wide variety of animals (Webster *et al.*, 1992). Avian influenza A (AI) viruses currently circulating in avian species

are capable of infecting mammals, and their replication in mammals may facilitate their adaptation to humans (Hinshaw *et al.*, 1981). Since the recognition of canine influenza A virus (equine H3N8) in the United States in 2004, the interest in the potential role of dogs in the ecology of influenza A viruses has been renewed (Crawford *et al.*, 2005). Some other examples of interspecies transmission of influenza viruses include the successive intraspecies transmissions of avian H3N2 among dogs (Song *et al.,* 2008, 2009; Lee *et al.*, 2009), the infection of dogs after scavenging on H5N1 positive chicken carcasses (Song *et al.*, 2008) as well as infection with (human) H1N1 viruses (Dundon *et al.*, 2010).

The interspecies transmission of influenza A virus is a crucial feature of its ecology and epidemiology (Webster, 1998) and is a possible threat to global human health (Korteweg & Gu, 2008). Dogs which come into close contact with humans may also play a role in the interspecies transmission of influenza viruses. Antigenic and genetic analyses of H9N2 viruses isolated during the last two decades indicate that these viruses are continuously evolving and have reassorted with other avian influenza viruses to generate multiple novel genotypes (Xu *et al.*, 2004; Li *et al.*, 2005; Xu *et al.*, 2007). By 1997, H9N2 viruses had been isolated from multiple avian species throughout Asia, the Middle East, Europe and Africa (Alexander, 2000, Perk *et al.*, 2006).

Unfortunately, H9N2 infection persists in many poultry farms in Iran (Nili & Asasi, 2003; Mosleh *et al.*, 2009), and some farmers own dogs that are usually fed with dead chickens. In a recent study, Abbaszadeh *et al.* (2012) discovered a high serologic prevalence of influenza infections in a population of Iranian dogs. Moreover, Amirsalehy *et al.* (2010) experimentally demonstrated that H9N2 could infect dogs intranasally. However, there is no report in the literature about the natural exposure of dogs to H9N2 viruses. If dogs can indeed be infected by H9N2 in field conditions, they may be to a great extent responsible for the epidemiology of viruses among dogs or in chicken farms.

To evaluate the replication and transmission of the avian H9N2 virus in dogs, experiments were carried out to determine whether feeding dogs with H9N2 infected chicken carcasses could lead to significant infections in the dogs and whether this virus could be shed.

MATERIALS AND METHODS

H9N2 AI virus

The virus used for this study, that is, A/chicken/Iran/772/1998 (H9N2), was obtained from Razi Serum and Vaccine Research Institute, Tehran, Iran. The virus was propagated in 10-day-old embryonated chicken eggs and stored at a constant temperature of –70 °C. The AI virus was titrated to determine the 50% Egg Infectious Dose (EID_{50}) using the method of Reed & Muench (1937).

Virus inoculation to chickens

Forty one-day-old commercial broiler chickens were purchased and randomly allocated to 2 groups with equal numbers of chickens (n=20 each). The chickens had unrestricted access to food and water and were reared under biosecurity conditions. Prior to challenge (21 day of age), all birds were serologically tested against AI using the haemagglutination inhibition (HI) method. All 20 were negative for antibodies to H9N2 influenza virus antigens. On the 22nd day, the chickens in the

second group were inoculated through the nares with 10^8 EID_{50}/bird H9N2 AI virus, while those in the first group (the control group) received normal saline. On days 7, 14 and 20 post-inoculation (PI), five chickens from each groups were randomly selected and used for serum sample collection. Oropharyngeal and cloacal swabs were collected from each chicken on the 3rd and 5th days post-challenge (pc) to evaluate H9N2 influenza virus infection by using RT-PCR. Chicken carcasses (whole body) were used to feed dogs on days 5 to 7 PI.

Experimental design

The experiment was conducted in compliance with the Animal Welfare Act (7 U.S.C. 2131 et seq.) and USDA Regulations and Policies. Eight healthy 1- to 2-year-old dogs were purchased and housed in different rooms under biosecurity conditions. The dogs were fed commercial and standard diets during a 3-week adaptation period. Blood samples were collected from all animals and serum tested to determine the presence of antibodies against H9N2 virus using the HI assay. In addition, nasal and faecal swabs from the dogs were used to evaluate H9N2 influenza virus infection via RT-PCR. All dogs tested negative to influenza H9 antigen in the HI and RT-PCR assays. Subsequently, the eight dogs were randomly divided into test (n=5) and control (n=3) groups. For three consecutive days, the test group was fed with H9N2 infected chicken carcasses, whereas the control group was given healthy ones during the same period. From day 4 to 10, all the dogs of both groups were fed with commercial and standard diets. The animals were monitored daily (8:00 AM to 2:00 PM) from day 0 to 10 in search of overt clinical signs of disease, including the presence of depression, sneezing, coughing, nasal and ocular discharge (serous, mucous, purulent, haemorrhagic or serosanguineous), changes in respiratory rate, fluctuations in heart rate, rectal temperature, and appetite condition (food consumption according to product table considered normal but less than that assumed as a decrease in appetite). Moreover, faecal and nasal swabs were collected daily for virus isolation during this period. HI tests were also performed on a daily basis (from day 0 to 10) and day 15 collected from dogs in order to measure antibody titre.

Preparation of dog sera

In the next stage of the experiment, serum samples collected from the dogs were treated at a constant temperature of 56 °C for 30 min to remove potential inhibitors. Subsequently, 150 µL of serum was mixed with 50 µL of 1% chicken red blood cells and incubated at room temperature for 30 min. The samples were then centrifuged at 1500 rpm for 10 min and used for HI assay.

Hemagglutination inhibition (HI) assay

The anti-viral antibody titres in the sera collected from the dogs and chickens were evaluated according to the HI assay guidelines outlined by the WHO Animal Influenza Training Manual (Anonymous, 2002). The HI assays were performed using homologous viruses.

RT-PCR

Virus RNA was extracted from faecal and nasal samples of the dogs as well as oropharyngeal and cloacal swabs of the chickens using an RNA extraction solution (RNX, Cinnagen Co., Tehran, Iran). The extraction process was conducted in accordance with the manufacturer's instructions. Viral RNA was extracted from

200 µL of the supernatants of 10% faecal suspensions.

The cDNA was synthesised via an AccuPowder®RT PreMix kit (BioNeer Corporation, South Korea) according to the manufacturer's instruction. The primers were specific to the H9 protein gene (Lee *et al.*, 2001). Five µL of total RNA and 20 pmol of each H9-specific primer were used for the preparation of cDNA. The AccuPower PCR PreMix kit was used to perform PCR so as to amplify a 488-bp fragment of the H9 protein gene of AI virus in a 20 µL reaction mixture containing 5 µL of cDNA and 10 pmol of each primer. The reaction mixture was then subjected to 35 cycles at temperatures of 94 °C, 53 °C and 72 °C for 1 min each, followed by a final extension at 72 °C for 10 min (Tajmanesh *et al.*, 2006). The reaction was fixed at 4 °C until further use. Finally, the PCR products were separated in 1% agarose gel and visualised under ultraviolet light.

RESULTS

On day 4th after inoculation, most chickens showed depression and respiratory signs. On day 5, two chickens died, with the necropsy revealing tracheal congestion and casts in their syrinxes. The control chickens remained healthy during the study. All birds in the control group were negative for virus, while viral shedding in the faecal and oropharyngeal samples of the challenged birds was shown by RT-PCR. As shown on Fig. 1, antibody titre elevation against H9N2 virus, was observed from day 7 PI and reached 6 (log 2) on 20th day PI in the challenged group which also indicated chickens infection. Continuous reduction in maternal antibody titres was observed in control group throughout the study.

All dogs used in the study had normal respiratory rate, heart rates, and rectal temperatures before, during and after the consumption of infected chickens, with no significant changes being observed in these parameters. Moreover, there were no signs of coughing, sneezing, depression, or decreased appetite in the dogs of the control and test groups.

The mean ± standard deviation of the dogs' antibody titre before the experiment was 4.1 ± 0.54. The HI test results for the test and control groups conducted during the study are shown on Fig. 2. There was no significant difference between the two groups. All samples from both the control and test groups of dogs were void of virus

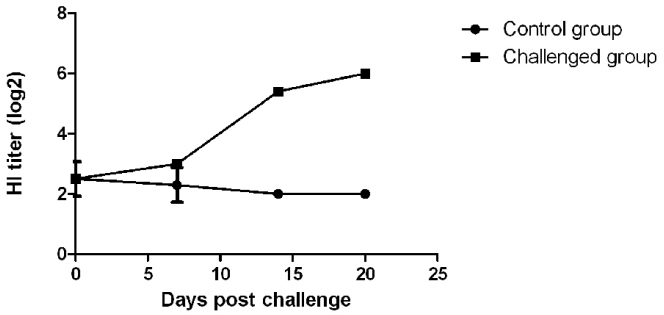

Fig. 1. HI antibody titres (mean±SD) in chickens at 0, 7, 14 and 20 days after H9N2 inoculation days in control and challenged groups.

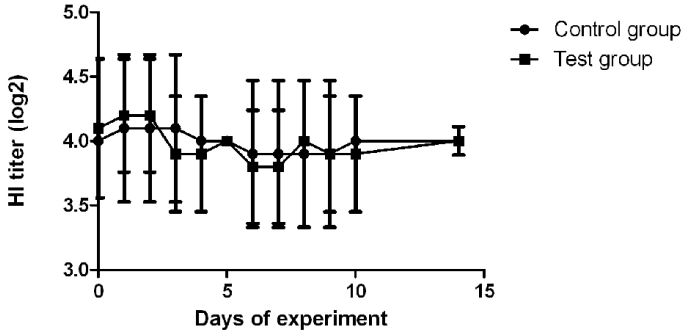

Fig. 2. HI antibody titres (mean±SD) from day 0 to 10 (on a daily basis) and day 15 after feeding dogs with H9N2 infected chicken carcasses.

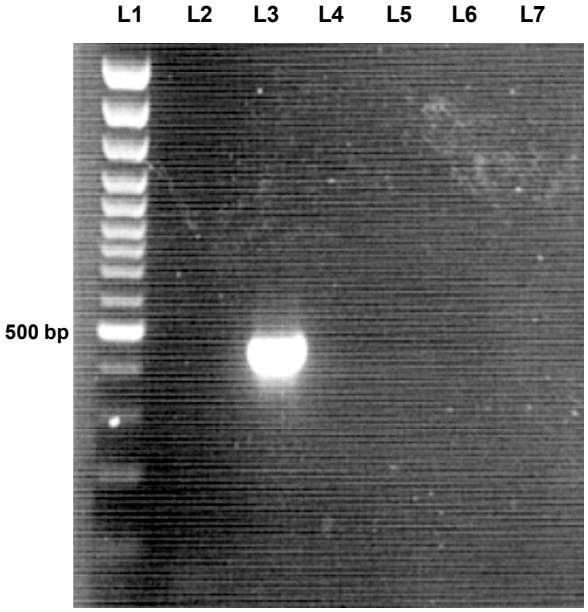

Fig. 3. Results of the PCR assay in samples collected from dogs, amplifying a 488-bp segment of H9 gene of AI virus. Lane 1: DNA marker (100-bp), lane 2: negative control, lane 3: positive control (RNA of the challenged AI virus), lanes 4 to 7: negative samples.

during the study as confirmed by RT-PCR (Fig. 3).

DISCUSSION

Since 2004, there have been several reports of trans-species transmission of influenza A virus (FLUAV) infections in dogs. Dogs have been infected with equine influenza H3N8 (Crawford *et al.*, 2005), avian influenza H3N2 (Song *et al.*, 2008; 2009) and H5N1 (Song *et al.*, 2008). In this study, the transmission potential and infectivity of H9N2 avian

influenza virus in dogs fed with infected chicken carcasses was investigated.

Amirsalehi *et al.* (2010) induced avian H9N2 infection by means of intranasal inoculation in dogs. They showed that avian H9N2 influenza virus which has been isolated from outbreaks in broiler farms can infect dogs. Furthermore, affected animals expel the virus in faeces and nasal discharges. To the best of the researchers' knowledge, there is no report about the infection of dogs with H9N2 viruses resulting from the consumption of infected carcasses in field conditions. In 2012, 209 serum specimens from dogs in Iran were screened, with no canine serum samples showing evidence of exposure to the 5 different influenza A viruses, including H9N2 (unpublished data). As previously stated in the results section, no virus was detected in the samples obtained from the test group dogs in our study, an observation which is in contrast to the results obtained by Amirsalehi *et al.* (2010). It should be mentioned that in the research of Amirsalehi *et al.* (2010), the animals were intranasally inoculated with 2 mL of (H9N2) avian influenza virus with a titer of $10^{6.5}$ (EID_{50}). Consequently, it is possible that swallowed viruses could cause positive samples in PCR tests of excrement. However, the virus was detected in the nasal swabs of dogs; furthermore, the inoculated group of animals which had come into contact with contaminated surfaces became seroconverted (Amirsalehi *et al.*, 2010), so other explanations may exist for this phenomenon.

The key factor in influenza infection is the prevalence of sialic acid molecules in the virus-specific receptors (Ning *et al.*, 2012). Receptors containing sialic acids with an α-2,3-linkage to the penultimate galactose (SAα-2, 3-gal) are widely believed to be the receptors for the AI viruses, while human viruses prefer receptors that contain an α-2,6-linkage (SAα-2, 6-gal) (Suzuki, 2005; Stevens *et al.*, 2006). In birds (unlike humans), influenza virus could replicate in the GI (gastrointestinal) tract. Ning *et al.* (2012) showed that a dog's respiratory tract (trachea and bronchus) is strongly positive for α-2,3-sialic acid-linked receptors, and some of these cells demonstrated weak-to-intermediate staining for α-2,6-sialic acid-linked influenza virus receptors. Surprisingly, in the GI tract (aside from the colon, caecum and rectum), most endothelial cells of the mucosa and glands in the lamina propria did not indicate influenza virus receptors. Based on the distribution of influenza virus receptors in the respiratory and GI tracts, it could be assumed that the occurrence and development of influenza virus infection in canines may be easier via the former compared to the latter. This can account for the differences between the results of the present study (where dogs were fed with infected chicken carcasses) and that carried out by Amirsalehi *et al.* (2010) (where intranasal inoculation of H9N2 virus to dogs was performed). In the present research, the absence of noticeable clinical signs and considerable antibody titer against H9N2 virus in HI tests, as well as the nonexistence of virus in the nasal swabs and faeces of dogs in the test group, may be due to the lack of α-2,3-sialic acid linked receptors in major parts of the GI tract of dogs. Yet, according to Amirsalehi *et al.* (2010), the presence of α-2,3-sialic acid-linked influenza virus receptors and some α-2,6-sialic acid-linked influenza virus receptors in the respiratory tract might have been responsible for the infections and positive nasal swabs. On the other hand, some influenza virus receptors are present in the sub-

mucosa layer and lamina propria of the mucosa of the GI tract of dogs (Ning *et al.*, 2012); therefore, it is possible that viraemia (after intranasal inoculation) has resulted in the infection of the GI tract. This may be the reason for the positive samples in the PCR test of faeces in the study of Amirsalehi *et al.* (2010) and the negative results of the present one.

Low-grade fever and signs like nasal discharge, coughing and sneezing are common clinical symptoms of influenza infection in dogs (Payungporn *et al.*, 2008). Similar to this study, Amirsalehi *et al.* (2010) found no significant relation between fever, heat rate, respiratory rate, or change in appetite and induced infection with H9N2. Holt *et al.* (2010) found no relation between dogs seropositive for H3N8 and respiratory discharges, coughing or body temperature.

Based on the present study, it appears that the consumption of H9N2 infected chicken carcasses by dogs cannot be considered as a means infection in dogs; in other words, feeding dogs with dead chickens in poultry farms may not pose a risk of transmission of H9N2 virus, although other factors, such as virus dosage or duration of exposure, could prove potential sources of infection and thus warrant further research.

REFERENCES

Abbaszadeh, M., S. Nazifi, E. Mohsenifard & M. Ansari-Lari, 2012. Serologic prevalence of antibodies against avian origin influenza virus in dogs referred to the Veterinary Clinic at Shiraz University. *Comparative Clinical Pathology*, **21**, 1127–1130.

Alexander, D. J., 2000 A review of avian influenza in different bird species. *Veterinary Microbiology*, **74**, 3–13.

Amirsalehy, H., H. Nili & A. Mohammadi, 2010. Experimental infection of dogs with avian influenza (H9N2) virus. In: *Proceedings of the 3rd International Oxford Influenza Conference Influenza. Zoonotic Influenza & Human Health*, Oxford, England, pp. 21–23.

Anonymous, 2002. WHO Manual on Animal Influenza Diagnosis and Surveillance. WHO, Geneva, pp. 15–40.

Crawford, P. C., E. J. Dubovi, W. L. Castleman, I. Stephenson, E. P. J. Gibbs, L. Chen, C. Smith, R. Hill, P. Ferro, J. Pompey, R. Bright, M. J. Medina, C.M. Johnson, C. W. Olsen, N. J. Cox, A. Klimov, J. Katz & R. Donis, 2005. Transmission of equine influenza virus to dogs. *Science*, **310**, 482–485.

Dundon, W. G., P. De Benedictis, E. Viale & I. Capua, 2010. Serologic evidence of pandemic (H1N1) 2009 infection in dogs, Italy. *Emerging Infectious Diseases*, **16**, 2019–2020.

Hinshaw, V. S., R. G. Webster, B. C. Easterday & J. Bean, 1981. Replication of avian influenza A viruses in mammals. *Infection and Immunity*, **34**, 354–361.

Holt, D., M. Moyer & D. C. Brown, 2010. Serologic prevalence of antibodies against canine influenza virus (H3N8) in dogs in a metropolitan animal shelter. *Journal of the American Veterinary Medical Association*, **237**, 71–73.

Korteweg, C & J. Gu, 2008. Pathology, molecular biology, and pathogenesis of avian influenza A (H5N1) infection in humans. *The American Journal of Pathology*, **172**, 1155–1170.

Lee, C., D. Song, B. Kang, D. Kang, J. Yoo, K. Jung, G. Na, K. Lee, B. Park & J. Oh, 2009. A serological survey of avian origin canine H3N2 influenza virus in dogs in Korea. *Veterinary Microbiology*, **137**, 359–362.

Lee, M. S., P. C. Chang, J. H. Shien, M C. Cheng & H. K. Shien, 2001. Identification and subtype of avian influenza viruses by

reverse transcription-PCR. *Journal of Virological Methods*, **97**, 13–22.

Li, C., K. Yu, G. Tian, D. Yu, L. Liu, B. Jing, J. Ping & H. Chen, 2005. Evolution of H9N2 influenza viruses from domestic poultry in Mainland China. *Virology*, **340**, 70–83.

Mosleh, N., H. Dadras & A. Mohammadi, 2009. Evaluation of H9N2 avian influenza virus dissemination in various organs of experimentally infected broiler chickens using RT-PCR. *Iranian Journal of Veterinary Research*, **10**, 12–19.

Nili, H & K. Asasi, 2003. Avian influenza (H9N2) outbreak in Iran. *Avian Diseases*, **47**, 828–831.

Ning, Z. Y., X. T. Wu, Y. F. Cheng, W.B. Qi, Y.F. An, H. Wang, G. Zhang & S. Li, 2012. Tissue distribution of sialic acid-linked influenza virus receptors in beagle dogs. *Journal of Veterinary Science*, **13**, 219–222.

Payungporn, S., P. Crawford, & T. Kouo, 2008. Influenza A virus (H3N8) in dogs with respiratory disease, Florida. *Emerging Infectious Diseases*, **14**, 6.

Perk, S., A. Panshin, E. Shihmanter, I. Gissin, S. Pokamunski, M. Pirak & M. Lipkind, 2006. Ecology and molecular epidemiology of H9N2 avian influenza viruses isolated in Israel during 2000-2004 epizootic. *Developments in Biologicals (Basel)*, **124**, 201–209.

Reed, L. & H. Muench, 1938. A simple method of estimating fifty percent endpoint. *American Journal of Hygiene*, **27**, 493–497.

Song, D., B. Kang, C. Lee, K. Jung, G. Ha, D. Kang, S. Park, B. Park & J. Oh, 2008. Transmission of avian influenza virus (H3N2) to dogs. *Emerging Infectious Diseases*, **14**, 741–746.

Song, D., C. Lee, B. Kang, K. Jung, T. Oh, H. Kim, B. Park & J. Oh, 2009. Experimental infection of dogs with avian-origin canine influenza A virus (H3N2). *Emerging Infectious Diseases*, **15**, 56–58.

Stevens, J., O. Blixt, T. M. Tumpey, J. K. Taubenberger, J. C. Paulson & I. A. Wilson, 2006. Structure and receptor specificity of the hemagglutinin from an H5N1 influenza virus. *Science*, **312**, 404–410.

Suzuki, Y., 2005. Sialobiology of influenza: molecular mechanism of host range variation of influenza viruses. *Biological Pharmaceutical Bulletin*, **28**, 399–408.

Tajmanesh, S., R. Toroghi, R. Momayez & S. A. Pourbakhsh, 2006. Establishment of RT-PCR for detection of avian influenza virus (H9N2) in field cases compared to virus isolation method. *Archives of Razi Institute*, **61**, 111–116.

Webster, RG. 1998. Influenza: An emerging disease. *Emerging Infectious Diseases*, **4**, 436–441

Xu, C., W. Fan, R. Wei & H. Zhao, 2004. Isolation and identification of swine influenza recombinant A/Swine/Shandong/1/2003(H9N2) virus. *Microbes and Infection*, **6**, 919–925.

Xu, K. M., K. S. Li, G. J. Smith, J. W. Li, H. Tai, J.X. Zhang, R.G. Webster, J. S. M. Peiris, H. Chen & Y. Guan, 2007. Evolution and molecular epidemiology of H9N2 influenza A viruses from quail in southern China, 2000 to 2005. *Journal of Virology*, **81**, 2635–2645.

Paper received 29.09.2013; accepted for publication 09.12.2013

Correspondence:

Dr. Mohammad Abbaszadeh Hasiri
Assistant Professor of Small Animal Internal Medicine
Department of Clinical Studies,
School of Veterinary Medicine,
Shiraz University,
P.O. Box 71345-1731, Shiraz, Iran
Tel: +98 711 2288660, +98 9177070881,
Fax: +98 711 2286950
e-mail : abbaszadeh@shirazu.ac.ir,
vetmail@gmail.com