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RESEARCH ARTICLE

Production and development of vaccines for *Ornithobacterium rhinotracheale* infection in turkeys

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Özet

Erganiş O, Hadimli HH, Kav K, Sayın Z, Aras Z. Hindilerde *Ornithobacterium rhinotracheale* için aşıların geliştirilmesi ve üretilmesi. ***Eurasian J Vet Sci, 2010, 26, 2, 101-107***

Amaç: Bu çalışmanın amacı bivalan inaktif *Ornithobacterium rhinotracheale* aşıları hazırlamak, kan serumlarında antijenlere karşı antikorların titrelerini ölçmek ve hindilerde *O. rhinotracheale* aşılarının etkinliklerini belirlemektir.

Gereç ve Yöntem: Bivalan inaktif *O. rhinotracheale* aşıları; alüminyum hidroksit, mineral yağlı, alüminyum hidroksit + ginseng ve mineral yağ + ginseng adjuvantları kullanılarak *O. rhinotracheale* serotip A ve B'den hazırlandı. Sterilite ve zararsızlık testlerinden sonra, hindilerde (5. ve 8. haftalarda 0.25ml ve 0.5 ml dozlarla iki kez aşılama ile) aşıların laboratuvar etkinlikleri (çelınç/koruma ve serolojik potens) yapıldı.

Bulgular: Çelınç sonuçlarına göre hindilerde bütün aşıların %100 etkili olduğu bulundu. Aşılı ve aşısız grupların serumlarında titrelerin serolojik ölçümleri için ve saha şartlarında *O. rhinotracheale* enfeksiyonunun teşhisinde lam aglütinasyon, mikro serum aglütinasyon ve ELISA testleri kullanıldı. Adjuvant olarak mineral yağ ve ginseng içeren aşı diğerlerine göre belirgin olarak daha yüksek humoral immune cevap oluşturdu. Aynı zamanda ve mineral yağ + ginseng aşısı özel bir hindi işletmesinde saha denemesinde çok etkili olduğu belirlendi.

Öneri: Kanatlılarda ornitobakteriozisin önlenmesi için *O. rhinotracheale* aşıları kullanılabilir.

Abstract

Erganis O, Hadimli HH, Kav K, Sayin Z, Aras Z. Production and development of vaccines for *Ornithobacterium rhinotracheale* infection in turkeys. ***Eurasian J Vet Sci, 2010, 26, 2, 101-107***

Aim: The purpose of this study was to prepare bivalent inactive *Ornithobacterium rhinotracheale* bacterin vaccines to measure the levels of antibodies against antigens in blood sera and to determine the efficacies of different *O. rhinotracheale* vaccines on turkeys.

Materials and Methods: The bivalent inactivated *O. rhinotracheale* bacterin vaccines were prepared from *O. rhinotracheale* serotype A and B strains using aliminium hydroxide, mineral oil, aluminium hydroxide + ginseng and mineral oil + ginseng. After the sterility and the safety tests, laboratory efficiencies of vaccines (challange/protection and serological potency) were done on the turkeys (twice vaccinated with doses 0.25 ml and 0.5 ml at 5 and 8 weeks, respectively).

Results: According to the challenge results, all the vaccines were found effective at 100%. Slide agglutination, micro serum agglutination and ELISA tests were used for the diagnosis. The vaccine containing mineral oil and ginseng as adjuvant induced significantly greater humoral immune response than others. Also, vaccine containing mineral oil and ginseng as adjuvant was determined to be more effective in the field trials in a company privately producing turkeys.

Conclusion: *O. rhinotracheale* vaccines could be used for prevention of ornithobacteriosis in turkeys.

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Anahtar kelimeler:*Ornithobacterium rhinotracheale*, hindi, vaccine, ginseng

Keywords: *Ornithobacterium rhinotracheale*, turkey, vaccine, ginseng

► Introduction

The disease of respiratory tract is one of the most important problems in poultry industry (DeRosa et al 1996, Chin and Droual 1997, Hadimli et al 2003). Bacterial agent in these infections are isolated as a primary and/or secondary etiological agent. *Ornithobacterium rhinotracheale* (ORT) is an infectious agent that has been ascribed an aetiologic role in the respiratory disease complex in poultry (Hinz et al 1994, Van Beek et al 1994, Hafez 1996, Travers et 1996, Van Empel et al 1996, Sprenger et al 2000, Erganiş et al 2002a, Szalay et al 2002). ORT, a pleomorphic gram-negative, rod-shaped bacterium, is generally isolated from the respiratory tract of most of affected birds (Van Beek et al 1994, Van Veen et al 2004). The major economic losses due to ORT infection results from the rejection of carcasses for consumption, growth retardation, and mortality (Van Empel 1998). The infection of ORT could form several clinical signs such as tracheitis, airsacculitis, pericarditis, sinusitis, and exudative pneumonia (Van Empel and Hafez 1999).

Various pathogens (Turkey rhinotracheitis virus, Newcastle Disease virus, *Escherichia coli, Bordetella avium,* etc.) have been identified as causing respiratory disease, acting either as a primary or secondary role (Van Empel and Hafez 1999). ORT can be a primary or secondary etiological agent depending on strain virulence, adverse environmental factors (poor management, inadequate ventilation, high stocking density, poor litter condition, poor hygiene, high level of ammoniac), immune state of the flock, and presence of other infectious agents (Van Beek et al 1994, Travers et al 1996). The primary role of ORT in respiratory disease is questionable.

ORT was identified by Vandamma et al (1994) after phenoypic and genotypic characterizations including protein profiles, and DNA-DNA or DNA-rRNA hibridizations (Vandamme et al 1994). Up to now, 18 different serotypes, designed A-O, have been reported (Van Empel and Hafez 1999). In chickens and turkeys, more than 95% of the isolates are of serotype A (Van Empel and Bosch 1998). Vaccines against the infection was also produced for chickens (Van Empel and Bosch 1998) and turkeys (Sprenger et al 2000).

Because of the infections of ORT can horizontally and vertically be transmitted into flocks and animals in a short period of time, the animals are needed to be vaccinated (Van Empel and Bosch 1998, Van Veen et al 2004). Vaccines has been freguently prepared from serotype A for chickens and most common strains can be used for turkeys. However, inactive bacterins with mineral oil or aluminium hydroxide has been usually used prepared from serotype A (van Empel 1998).

The purpose of this study was to prepare bivalent inactive ORT bacterin vaccines by local strains, to measure the levels of antibodies against antigens in blood sera and to determine the efficacies of different ORT vaccines on turkeys.

► Materials and methods

Animals

Turkeys with no clinical respiratory abnormalities were included in the study. Turkeys were divided into two groups for trials of challenge and serological monitoring. Then, challenge groups (n=50) were again divided 5 groups, each of them consists of 10 turkeys. Also, serological monitoring groups (n=50) were divided into 5 groups; each of them consists of 10 turkeys.

Vaccines and vaccination

ORT serotype A and serotype B were separately grown into Brain Heart Infusion Broth (Oxoid), supplemented with bovine serum 5%. Bacterial concentrations were adjusted to $1.2x10^9$ cells/ml. Formalin (0.3-5% v/v) was added to inactivate bacteria (Anonim 1996, Van Veen et al 2004). Cultures of ORT serotype A and B strains was mixed with equal volume, mixed antigens were absorbed with aluminium hydroxide (4%) or mineral oil and then were added to ginseng extract (4 mg/ml) to all mixtures (Hadimli et al 2005a, Hadimli et al 2005b).

Bivalent inactivated ORT bacterin vaccines were prepared from ORT serotype A and B strains using aliminium hydroxide ($Al[OH]_3$), mineral oil (MO), $Al[OH]_3$+ ginseng (G) and MO + ginseng (G).

Table 1. The program of vaccination and challenge for turkeys.

Age (Week)	Vaccination and challenged	Time of sampling
5.	1. vaccine (0.25 ml)	1.
8.	2. vaccine (0.5 ml)	2.
11.	Challenged	3.
12.	Challenged	///////////
14.	//////////////	4.
17.	//////////////	5.
20.	//////////////	6.
23	//////////////	7.

For experimental trials, the turkeys by ORT vaccines were subcutaneously vaccinated twice with dose 0.25 ml and 0.5 ml at back neck at 5 and 8 weeks, respectively. Controls were similarly vaccinated with sterile saline (Table 1).

For field trials, the MO+G vaccine was administered to determine field efficacies in a a company privately producing turkeys (n:1100). The turkeys were subcutaneously vaccinated with dose of 0.5 ml at back neck at 6 weeks. After 3 weeks, half of vaccinated group (n:550 turkeys) were secondly vaccinated with same

dose vaccine. The time of vaccination was chosen as at 6 and 9 weeks, because extra labor was not brought for participating farmers to the project and most suitable timing concerns due to flocks has to do under field conditions.

The sterility and safety tests

The *O. rhinotracheale* vaccines in steps were performed microbiological analysis (aerobic, microaerofilic, anaerobic, mycoplasma and micotic microorganisms) for sterility. Also, adverse reactions after the vaccination in vaccinated animals were recorded by the observation of animal behaviour and local reactions (Anonim 2004).

Challenge

The isolates of live ORT (serotypes A and B) for challenge trials were chosen different strains from selected vaccines isolates. Turkey poults were challenged by spraying to mouth, nose and eyes, with 1.2x109 cfu of ORT after 21 days from second vaccination (at 11 weeks), and observed during 20 days (Table 1). Then, all turkey were euthenasized and internal organ samples of animals were cultured for reisolation of ORT.

Sampling

Blood samples were regularly taken from turkeys at before and after vaccination. Serological monitoring was made at intervals 3 weeks until 23 weeks in turkeys (Table 1).

Serological Monitoring

Serological efficacies of 4 different ORT vaccines in turkeys were determined by 3 serological (slide agglutination, micro serum agglutination and ELISA) tests.

Slide agglutination test

To prepare antigen for the slide agglutination test, ORT strains (serotypes A and B) were separately grown into Brain-Heart Infusion at 37^0C for 48 h in 10% CO_2. The microorganisms were harvested by centrifugation 2500 g for 50 min and were washed with phosphate buffer solution (PBS; pH:7.2) three times. The concentration of each isolates were adjusted to $2x10^9$ cfu/ml and inactivated with 0.3% formalin. After staining with Rose Bengal dye, both monovalent and bivalent slide agglutination antigens were prepared and 5 or 10 ml of antigens were bottled to vials with prospectus (Back et al 1998, Erganis and Hadimli 2000, Erganis et al 2002b).

For the slide agglutination test, 25 µl of antigen and 25 µl of serum were mixed on a glass slide. After rotating slide, presence or absence of agglutination within 1 to 2 min was recorded (Erganis and Hadimli 2000, Erganis et al 2002b).

Micro (mSAT) serum agglutination test

To prepare antigen for the serum agglutination test, ORT strains (serotypes A and B) were separately grown into Brain-Heart Infusion at 37^0C for 48 h in 10% CO_2. The microorganisms were harvested by centrifugation 2500 g for 50 min and were washed with phosphate buffer solution (PBS; pH:7.2) three times. The concentration of each isolates were adjusted to $2x10^7$ cfu/ml and inactivated with 0.3% formalin. Also, the protein value and optic density at 630 nm of ORT antigen were determined as 4 mg/ml and 1.0, respectively. Then, antigens were stained with safranine 0.005% (C.I: 50240 The British Drug Houses ltd. BG). Both monovalent and bivalent serum agglutination antigens were prepared and 50 or 100 ml of antigens were bottled to bottles with prospectus (Back et al 1998, Erganis and Hadimli 2000, Erganis et al 2002b).

For the serum agglutination test, the two-fold dilutions of serum samples were made with PBS in microplate and serum agglutination test antigen were added to wells. The microplate was incubated at 37^0C overnight before evaluation (Erganis and Hadimli 2000, Erganis et al 2002b).

ELISA

The presence of IgG antibodies against ORT antigens in broilers and turkeys were measured by using a modified ELISA, which were prepared in our laboratory. ORT strains (serotypes A and B) were separately grown into Brain-Heart Infusion at 37^0C for 72 h in 10% CO_2. The microorganisms were harvested by centrifugation 3000 g for 30 min and were washed with phosphate buffer solution (PBS; pH 7,2) three times. The suspension of each isolates was inactivated with 0.5% formalin. Then, the protein values of ORT antigens were determined by DC protein assay kit (Bio-Rad Lab, Cat No. 500-0116, USA) as 4 mg/ml (Lowry et al 1951).

In brief, 96-well immunoplates (Nunc C bottom Immunplate 96 well, 446612) were coated with 100 l/well of ORT antigens; agitation killed bacteria, suspended in carbonate-bicarbonate buffer (pH: 9.6) at 4 mg/ml. Immunoplates were incubated at 37^0C for 1 h and overnight at 4^0C. After washing 5 times with phosphate buffer solution-Tween 20 (PBS-T; 50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH: 8), 100 µl of 3% bovine serum albumin (BSA) were added to the wells and incubated for 45 min at room temperature. Plates were again washed three times for 5 min with PBS-T.

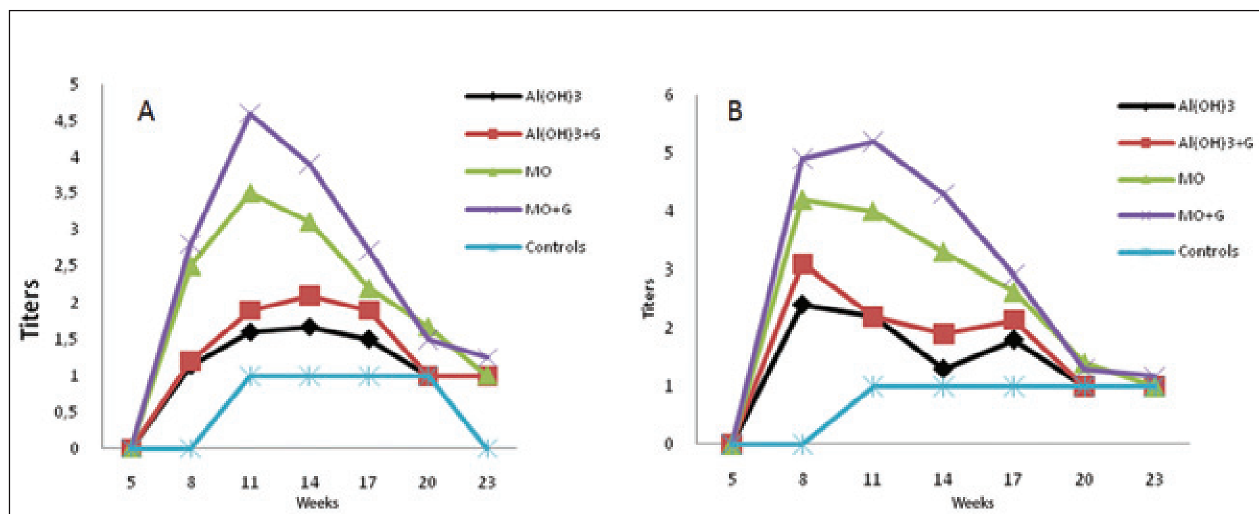

Figure 1. The titers of antibodies to O. rhinotracheale (serotypes A and B) antigens by mSAT in experimental groups.

Serum samples of turkeys were diluted as 1/10, 1/20 up to 1/40960 and 100 µl from each dilution were added to the wells and the plates were incubated at 37^{0}C for 1 h. After washing, 100 µl of rabbit anti-turkey IgG horseradish peroxidase conjugate (whole molecule, Sigma, Cat. No: A-9792,USA) at 1:8000 was added to each well and incubated at 37^{0}C for 1 h. After washing, 100 µL of substrate solution (TMB A and B; Kirkegaard and Perry, Gaithersburg, MD) was added as substrate and plates were reincubated for 10 min at room temperature. Finally, 50 µL of 2M H_2SO_4 as a stop solution were added to all wells and plates were immediately read in a microplate autoreader (Anthos Labtec Instruments, A 5022, Salzburg) at 450 nm. The positive and negative serum standards were added to each plate (Hafez et al 1999).

Statistical analysis

Analysis of variance (ANOVA) and Duncan test was used to determine the significance within the groups. $p<0.05$ was accepted as statistically significance.

Table 2. The results of morbidity and mortality in challenge of vaccinated and non-vaccinated animals.

Groups	Morbidity	Mortality
$Al(OH)_3$	0/10	0/10
MO	0/10	0/10
$Al(OH)_3$ +G	0/10	0/10
MO + G	0/10	0/10
Controls	2/10	1/10

Table 3. The re-isolation of bacteria from lungs and/or trachea of the challenged turkeys

Groups	ORT	Others*
$Al(OH)_3$	0/10	9/10
MO	0/10	0/10
$Al(OH)_3$ +G	0/10	8/10
MO + G	0/10	2/10
Controls	2/10	6/10

*different microorganisms

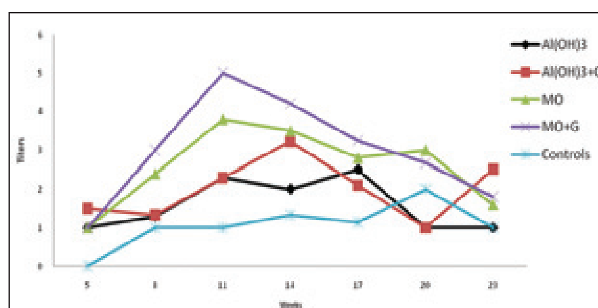

Figure 2. The titers of antibodies to O. rhinotracheale (serotypes A and B) antigens by ELISA in experimental groups.

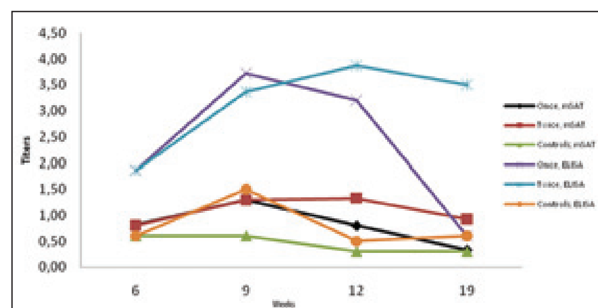

Figure 3. The titers of antibodies by mSAT and ELISA in vaccinated once or twice turkeys in the field trial.

► Results

In challenge trials, no mortality and morbidity were observed in vaccinated turkeys. In controls, the ratios of mortality and morbidity were in 10% and 20%, respectively (Table 2). While no re-isolation of ORT was made from respiratory organs (lung and trachea) vaccinated of turkeys, ORT isolates were recovered from 20% in non vaccinated broilers (Table 3). In experimental trials, the levels of antibodies to ORT in blood sera of vaccinated turkeys were significantly determined higher by both mSAT and ELISA than non-vaccinated animals ($p<0.05$) (Figure 1 and 2). In field trial, before vaccination, blood serum samples in separated groups of vaccine and controls were approximately determined as positive 50% by mSAT

Table 4. Data of different flocks suffered ORT infection in field turkeys of same age (6 weeks), which administered vaccination and/or antibiotic treatment for 5 days.

Flocks No	Number	Vaccine	Number of dead	Mortality %	Gender
1	2670	(-)	256	9.59	female
2	1792	(-)	105	5.86	female
3	1588	(-)	101	6.36	female
4	3070	(-)	132	4.23	male
5*,***	966	(-)	32	3.70	male
6**,***	1104	(+)	27	2.70	male
7	932	(-)	57	6.11	male
8	530	(-)	27	5.09	male
9	12200	(-)	593	4.86	mix

*same flocks, **after transport, enrofloxacin as antibiotic administered for 5 days to vaccinated animals, ***after transport, the deaths at 6 and 11 days started in vaccinated and non vaccinated animals, respectively.

Table 5. The comparison of data of weight gain, mortality and FCR in different 2 hens after slaughtered.

Flocks*	Number	Slaughtered Age (day)	Weight gain (g)	Mortality %	FCR	Total feed consumption (kg)
Vaccinated	1000	134	15.381	10.40	2.02	31.500
Non vaccinated	837	134	15.700	12.54	2.06	27.150

*Although the seropositivity was determined before the vaccination, no infection was bserved clinically in vaccinated and non vaccinated turkeys, which grown in same place.

and ELISA. Other words, the vaccinated turkeys had a subclinical infection of ORT. In field trial, the levels of antibodies of turkey vaccinated with only a dose were similar to vaccinated twice of turkey, but it is emphasized that twice vaccination is important to increase humoral responses since especially 19 weeks (Figure 3). Blood serum samples in control group tested to be positive approximately 50% of hens, it is indicate that it remained seropositive during the trial. After transportation, it expressed that infections of ORT (re-infection) increased in many hens, depending on the import-handling stress, and the ratio of mortality were less in vaccinated turkeys (Table 4). When vaccinated turkeys compared with other hens without stress of vaccination, the lower of losses can be connected with effects of specific or non-specific immunstimulation (related to Montanid ISA50 and/or ginseng). The values of weight gain, feed conversion ratio (FCR) and feed consumption were better in vaccinated animals than non vaccinated slaughtered turkeys. Also, the ratio of mortality was lower in vaccinated animals (Table 5).

► Discussion

ORT can cause several respiratoric disesases in poultry such as tracheitis, airsacculitis, pericarditis, sinusitis, and exudative pneumonia (Van Empel and Hafez 1999). The major economic losses due to ORT infection results from the rejection of carcasses for consumption, growth retardation, and mortality. Travers et al (1996) reported that while no mortality was observed, but growth retardation, joint lesions and lung infection were encountered in patogenicity of 3 ORT isolates. Van Empel et al (1996) notified that growth retardation, joint lesions and lung infections were observed in aerosol challenge trials, but similar lesions occurred more severe in the presence of viral infection.

The some of turkey producers in Turkey is frequently facing threat due to emerging respiratory diseases that result in severe economic losses. They tried to use with several antibiotics against ORT infection, but sometimes, they could be failure or ineffectivity

Since the pathogenicity of ORT strains could not be precisely determined, the availability of live vaccine is discussed (Van Empel 1998, Van Empel and Bosch 1998, Lopes et al 2002). Van Empel (1998) suggested that live vaccine experiments in animals did not developed any damage, as well as immunity. Because of cross-protection is among serotypes and relationship could be between protection and antibodies, it could be prepare new recombinat vaccines (Schuijffel et al 2005, Schuijffel et al 2006). However, using the temperature sensivitive mutant strain of ORT was found to be promising as live vaccine (Lopes et al 2002).

Bacterins with mineral oil was proved to protect the aerosol challenge in vaccinated broiler chicks or poults of turkey in experimental trials (Hafez et al 1999, Anonim 1996). Also, the ratio of mortality in field trials was significantly lower in vaccinated turkeys at 3-7 weeks than non vaccinated animals. In addition to this, vaccination at 2-6 week carried out a protection against challenge at 19 weeks for inflamation of air sacs and pneumonia (Van Empel and Bosch 1998). It is important that breedings must be vaccinated for protection of progency derived from broilers or turkeys (Van Empel and Hafez 1999).

It is known to be a relationship between increased with age and development of resistance to ORT in-

fection (Van Empel and Hafez 1999). Therefore, the sooner the vaccination is done in infected animals, earlier is immunization provided due to immune stimulation before transmission of ORT infection.

Hafez et al (1999) noted that vaccination at 7 and 10 weeks were found to be more effective than at 1 and 3 weeks in turkeys vaccinated with two different vaccination programs. Because the levels antibodies to Turkey rhinotracheale virus and Newcastle Disease virus were determined the higher in controls, no many questions were answered for determination of problems. Sprenger et al (2000) subcutaneously administered inactived vaccine to turkey at 6 weeks aged and challenged aerosolly with virulent strain at 14 and 21 weeks. When vaccinated animals compared to controls, they reported that pneumonia and airsacculitis formed less and vaccinated animals protected from pathological lesions. Van Veen et al (2004) reported that the turkeys' poults of vaccinated parents showed significantly fewer respiratory tract lesions at postmortem examination at 16 days of age than that of offsprings of nonvaccinated parents. In addition, all vaccinated young turkeys, regardless of the vaccination status of their parents, were showed significantly fewer respiratory tract lesions at 6 week of age.

In the present study, virulent two different strains of ORT aerosolly administered to vaccinated and non vaccinated turkeys at 11 weeks. While mortality and morbidity in controls are in 10% and 20%, respectively, it was not observed any mortality and morbidity in vaccinated turkeys in challenge trials. Also, the re-isolation of ORT was not made from respiratory organs in vaccinated animals. The results of the present study were parallel with that of other researchers.

In the present, the titers of antibody in all vaccinated turkeys increased considerably at 3 week after first vaccination. After second vaccination, the titers of specific antibodies to ORT were determined to be increasing at 11 weeks. In addition, the levels of antibodies of MO+G ORT vaccine were significantly greater than that of other vaccines when humoral responses of all vaccine were compared.

Six and 9 weeks were chosen for vaccination in field trial, because no extra labor and the most appropriate timing can be for participating turkey hens. But, this period can be close to encountered time for infection of ORT with import-handling stress. For this reason, secondary vaccination time among 3-6 weeks could be recommended as to changeable transportation from hens to hens.

Ginseng (Panax ginseng) has been began to use for human health and animal vaccines as an immunostimulator and antistress drug in cancer therapy (Hu et al 2003, Kim et al 2003, Rivera et al 2003a, Rivera et al 2003b). We have also reported sinergic effect of ginseng extract with aluminium hydroxide in inactive bakterin vaccines (*Salmonella typhimurium* and staphylococcal mastitis) (Hadimli et al 2005a, Hadimli et al 2005b). The adjuvant effects of ginseng on veterinary vaccines have not been come accros in poultry. Althoug no significant differences were observed between vaccinated groups, titers of specific antibodies of ginseng extract added ORT vaccines were greaterly measured. It is thought that ginseng increases the bactericidal activity of the immune system, according to the re-isolation studies from internal organs of turkeys vaccinated with and without ginseng.

According to the results of field trial, twice vaccination interval of 3 weeks could be effective for long-lasting term than a single administration. Although vaccination were made to subclinical animals and once dose to half of hens, the ratio of mortality in vaccinated turkeys were less 2.14% than controls. In other words, 23.54 in lots of turkeys (each turkey approximately 15 kg, total 23.54x15=353,1 kg and one kg of turkey meat is nearly 5 Turkish lira) do not dead in a hens of 1100 animals. If all turkey vaccinate with ORT vaccines, so the more money (353,1x5=1765,5 Tl) may be gain. In Turkey, annual turkey meat production is taken into account, economic gains will be understood.

In the present study, no adverse reactions after the vaccination were recorded by the observation of animal behaviour. But, it was stated that subcutanous injection of ORT vaccines into neck of turkeys was deemed impractical for commercial hens. On the other hand, since the part of turkey neck was consumed as food in Turkey, the injection of the vaccine may cause a tissue damage in this region.

► Conclusion

These results show that 4 different of ORT vaccines with different adjuvants (aluminium hydroxide, mineral oil and ginseng) are very effective against highly pathogenic ORT challenge. Ginseng also positively affected on increasing of bactericidal activity of the inactive bivalent bacterin vaccines with mineral oil or aluminium hydroxide adjuvants. ORT vaccines would be used for prevention of ornithobacteriosis in poultry.

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