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RESEARCH ARTICLE

The production and development of vaccines for *Ornithobacterium rhinotracheale* infection in commercial broilers

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Özet

Erganiş O, Hadimli HH, Kav K, Sayın Z. Piliçlerde *Ornithobacterium rhinotrachealae*'ye karşı aşı üretimi ve geliştirilmesi. **Eurasian J Vet Sci, 2011, 27, 2, 99-105**

Amaç: Çalışmanın amacı bivalan inaktif *Ornithobacterium rhinotracheale (O. rhinotracheale)* aşıları hazırlamak, kan serumlarında antijenlere karşı antikorların titrelerini ölçmek ve etçi piliçlerde *O. rhinotracheale* aşılarının etkinliklerini belirlemektir.

Gereç ve Yöntem: Bivalan inaktif *O. rhinotracheale* aşıları; alüminyum hidroksit ($Al[OH]_3$), mineral yağlı (MO), alüminyum hidroksit + ginseng ($Al[OH]_3$ + G) ve mineral yağ + ginseng (MO + G) adjuvantları kullanılarak *O. rhinotracheale* serotip A ve B'den hazırlandı. Sterilite ve zararsızlık testlerinden sonra, etçi piliçlerde (ilk gün 0.1 mL doz ile bir kez aşılama ile) aşıların laboratuvar etkinlikleri (çelınç/koruma ve serolojik potens) kontrol edildi.

Bulgular: Çelınç sonuçlarına gore; etçi piliçlerde bütün aşıların %100 etkili olduğu bulundu. Adjuvant olarak mineral yağ ve ginseng (MO + G) içeren aşı diğerlerine göre belirgin olarak daha yüksek humoral immune cevap oluşturdu.

Öneri: Bu çalışmada, birinci günde etçi piliçlerin aşılanması *O. rhinotracheale* enfeksiyonlarına karşı etkili şekilde koruyabildiği gösterilmiştir.

Abstract

Erganis O, Hadimli HH, Kav K, Sayin Z. The production and development of vaccines for *Ornithobacterium rhinotracheale* infection in commercial broilers. **Eurasian J Vet Sci, 2011, 27, 2, 99-105**

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

Materials and Methods: The bivalent inactivated *O. rhinotracheale* bacterin vaccines were prepared from *O. rhinotracheale* serotype A and B strains using aluminium hydroxide ($Al[OH]_3$), mineral oil (MO), aluminium hydroxide + ginseng ($Al[OH]_3$ + G) and mineral oil + ginseng (MO + G). After the sterility and the safety tests, laboratory efficiencies of vaccines (challange/protection and serological potency) were controlled on the broilers (vaccinated at first day with a dose of 0.1 mL).

Results: According to the challenge results, all the vaccines were found to be effective as 100% for broilers. The vaccine containing mineral oil and ginseng (MO + G) as adjuvant induced significantly greater humoral immune response to the antigens than others.

Conclusion: The study showed that vaccination of broilers chicken at the first day age may effectively protect against *O. rhinotracheale* infections.

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Keywords: *Ornithobacterium rhinotracheale,* broiler chicken, vaccine, ginseng

► Introduction

Ornithobacterium rhinotracheale (O. rhinotracheale) is a bacterial pathogen of poultry, linked to the respiratory disease complex of broilers and the economic losses associated with that disease complex (Van Beek et al 1994, Vandamme et al 1994, Hafez 1996, Chin and Droual 1997, Van Veen et al 2000). *O. rhinotracheale* is a pleomorphic gram-negative, rod-shaped bacterium. Up to now, 18 different serotypes, designed from A to O, have been reported (Van Empel and Hafez 1999, Erganiş 1988, Erganiş ve Hadimli 2000). In chickens and turkeys, more than 95% of the isolates are of serotype A (Van Empel et al 1997, Van Empel 1998, Erganiş ve Hadimli 2000).

The bacteria has been isolated from chickens, turkeys, chukar, quail, ducks, geese, ostriches, guinea fowl, pheasants, rooks, pigeons, and possibly from many other species of birds. *O. rhinotracheale* infections are probably seen worldwide (Odor et al 1997, Hafez et al 2000, Canal et al 2005, Allymehr 2006). In Turkey, this bacteria have isolated from broilers, broilers breeders and layers (Ak and Turan 2002, Erganiş et al 2002a, Turan and Ak 2002, Ürşen and Çarlı 2004, Türkyılmaz 2005) and detected the DNA by polymerase chain reaction from field cases (Özbey et al 2005). Also, the serologic presence of *O. rhinotracheale* infection has been determined (Ürşen and Çarlı 2004, Türkyılmaz 2005).

The treatment of infection *O. rhinotracheale* is really difficult and can not be achieved effectively through antibiotic use. It is notified that the sensitivity of *O. rhinotracheale* strains to antibiotics is very variable and depends on the source of the strain (Devriese and Herdt 2001, Dodouyt et al 1995, Sariano et al 2003). Vaccination may be a promising control measure against *O. rhinotracheale* infections. Vaccination with autogenous inactivated oil adjuvanted vaccines was successful in reducing outbreaks of *O. rhinotracheale* (Cauwerts et al, 2002, Bischop et al 2004, Schuijffel et al 2005 Schuijffel et al 2006, Erganis et al 2010).

The purpose of this study was to prepare bivalent inactive *O. rhinotracheale* bacterin vaccines by local strains, to measure the levels of antibodies against antigens (*O. rhinotracheale* serotypes A and B) in blood sera and to determine the efficacies of *O. rhinotracheale* vaccines on broiler chickens.

► Materials and Methods

- *Animals*

Broiler chicks, with no clinical respiratory abnormalities, were included in the study. Study protochol was approved by Ethic Commettee of Selcuk University Veterinary Faculty. Broilers were divided into two groups for trials of challenge and serological monitoring. Then, challenge groups (n=50) were again divided into 5 groups, of which each having 10 broilers. Also, serological monitoring groups (n=75) were divided into 5 groups, of which each having 15 broilers.

- *Vaccines and vaccination*

O. rhinotracheale serotype A and serotype B were separately grown into Brain Heart Infusion Broth (Oxoid), supplemented with bovine serum 5%. Bacterial concentrations were adjusted to 1.2 x 10^9 cells/mL. Formalin (0.3-5% v/v) was added to inactivate bacteria (Storm and van Empel 1996, Erganiş et al 2010). Each bacteria culture were mixed with equal volume, mixed antigens were absorbed with aluminium hydroxide (4%) (Vetal, Adıyaman, Turkey) or mineral oil (Montanide ISA 50, Seppic, France) and then ginseng extract (4 mg/mL) (Solgar, Leonia, NJ, USA) were added to mixtures (Hadimli et al 2005a, Hadimli et al 2005b, Erganiş et al 2010). For experimental trials, the broiler chicks were vaccinated by *O. rhinotracheale* vaccines at first day with a dose of 0.1 mL. Controls were similarly vaccinated with sterile saline.

- *The sterility and safety tests*

The microbiological analysis (aerobic, microaerofilic, anaerobic, mycoplasma and micotic microorganisms) for *O. rhinotracheale* vaccines in steps were performed for sterility. Also, adverse reactions after vaccination in vaccinated animals were recorded by observation of animal behaviour and local reactions (Anonymus 2004, European Pharmacopaeia 2005).

- *Challenge*

The isolates of *O. rhinotracheale* (serotypes A and B) for challenge trials were chosen different strains from selected vaccines isolates. Broiler chicks were challenged at 15 days by spraying to mouth, nose and eyes, with 1.2x10^9 cfu of *O. rhinotracheale,* and were observed for 20 days.

- *Sampling*

Blood samples were regularly taken from broilers before and after vaccination. Serological monitoring was made at intervals 15 days until 10 weeks in broilers.

- *Serological monitoring*

Serological efficacies of 4 different *O. rhinotracheale* vaccines in broilers were determined by 3 serological tests (slide agglutination, serum agglutination and ELISA).

- *Slide agglutination test*

To prepare antigen for the slide agglutination test, the *O. rhinotracheale* strains (serotypes A and B) were separately grown into Brain-Heart Infusion at 37 ^{0}C 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 (Erganiş and Hadimli 2000, Erganiş et al 2002b).

- *Micro serum agglutination test (mSAT)*

To prepare antigen for the serum agglutination test, the *O. rhinotracheale* strains (serotypes A and B) were separately grown into Brain-Heart Infusion at 37 ^{0}C 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 densicity at 630 nm of *O. rhinotracheale* 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, Erganiş and Hadimli 2000, Erganiş et al 2002b).

For the micro 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 ^{0}C overnight before evaluation (Erganiş et al 2002b).

- *ELISA*

The presence of IgG antibodies against *O. rhinotracheale* antigens in broilers were measured by using a modified ELISA, which were prepared in our laboratory. The *O. rhinotracheale* strains (serotypes A and B) were separately grown into Brain-Heart Infusion at 37 ^{0}C for 72 h in 10% CO2. 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 suspansion of each isolates were inactivated with 0.5% formalin. Then, the protein values of *O. rhinotracheale* 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 µL/well of *O. rhinotracheale* antigens; agitation killed bacteria, suspended in carbonate-bicarbonate buffer (pH:9.6) at 4 mg/mL. Immunoplates were incubated at 37 ^{0}C for 1 h and overnight at 4 ^{0}C. After washing 5 times with phosphate buffer solution-tween 20 (PBS-T; 50mM 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.

Blood sera samples of broiler 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-chicken (whole molecule, SouthernBiotech, Cat. No: 6110-05, Birmingham, USA) at 1: 8000 was added to each well and incubated at 37 ^{0}C for 1 h. After washing, 100 µL 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. Positive and negative serum standards were added to each plate (Hafez et al 1999).

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

Age (Week)	Vaccination and challenged	Sampling
1 days	once vaccine (0.1 mL)	1.
2.	challenge	2.
3.	challenge	//////
4.	//////	3.
6.	//////	4.
8.	//////	5.
10	//////	6.

- *Statistical analysis*

Analysis of variance (ANOVA) was used to determine the significance within the groups ($p<0.05$).

► Results

In challenge trials, while no mortality and morbidity were observed in vaccinated broilers, the ratios of mortality in 20% and morbidity in 50% were determined in controls, respectively (Table 2). While no re-isolation of *O. rhinotracheale* was made from respiratory organs (lung and trache) of vaccinated broilers, *O. rhinotracheale* isolates were recovered from 50% in non-vaccinated broilers (Table 3).

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
Control	5/10	2/10

Table 3. The isolation of bacteria from lungs and/or trachea of the challenged broilers

Groups	ORT	Others
$Al(OH)_3$	0/10	9/10
MO	0/10	0/10
$Al(OH)_3$ + G	0/10	3/10
MO + G	0/10	2/10
Control	5/10	36/10

In experimental trials, the titers of antibodies of all *O. rhinotracheale* vaccine to serotypes A and B of *O. rhinotracheale* antigens were significantly higher than in controls. In addition, the levels of antibodies compromised for ORT vac MO + G *O. rhinotracheale* vaccine were found significantly greater by mSAT and ELISA than other vaccinal antibody levels, when humoral responses of all vaccine were compared.

► Discussion

The infection of *O. rhinotracheale* could be form several clinical signs such as tracheitis, airsacculitis, pericarditis, sinusitis, and exudative pneumonia (Travers et al 1996, Van Empel et al 1996). The major economic losses due to *O. rhinotracheale* infection result from the rejection of carcasses for consumption, growth retardation, and mortality (Travers et al 1996).

Various studies on the control of *O. rhinotracheale* infection in poultry through vaccination have been described (Storm and van Empel 1996, Van Empel and Bosch 1998, Cauwerts et al 2002, Lopes et al 2002, Bissschop et al 2004, Schuijfeel 2005, Schuijfeel et al 2005, Erganiş et al 2010). Vaccination of chickens with inactivated vaccines was effective against experimental *O. rhinotracheale* challenge (Van Empel and Van den Bosch 1998). *O. rhinotracheale* vaccines are efficacious against an infection with distinct serotypes, but their ability to induce broad cross-protection is variable (Schuijfeel et al 2005). However, development of more efficacious vaccines is complicated by the total lack of knowledge of the pathogenesis of the

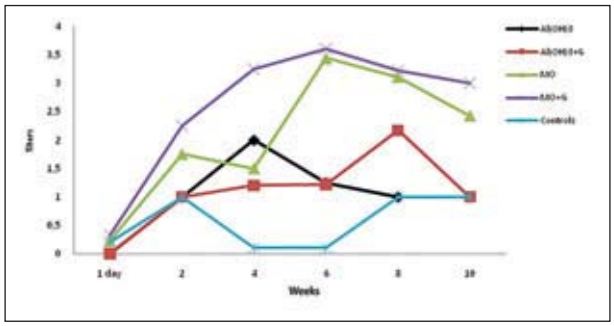

Figure 2. The titers of antibodies to O. rhinotracheale (serotypes A and B) antigens by ELISA in broilers.

infection and the limited knowledge of the host immune response to *O. rhinotracheale* infection (Murthy et al 2007).

It is reported that natural protection against *O. rhinotracheale* infection is largely based on the development of a humoral immune response. Therefore, chicken humoral immunity to *O. rhinotracheale* is a key component in protection against infection (Schuijffel et al 2005). When *O. rhinotracheale* vaccines without any adjuvant administered to animals, the serological and protective responses could be poor (Murthy et al 2007). In this study, the antibodies titres in all vaccinated animals were found to be higher, but the bacterin in MO and MO+G induced high and long-lasting responses. Our findings are very well correlated with the reports of Murthy et al (2007).

Schuijfeel et al (2006) found that all genes encoding the eight antigens were highly conserved among different *O. rhinotracheale* serotypes, but the different antigens were not expressed by all serotypes. They were analyzed the immunogenicity of these eight recombinant proteins by subunit vaccination. Vaccination of chickens with single-antigen vaccines demonstrated that the Or77 antigen was protective against serotypes that expressed Or77 in vitro, suggesting that the protein has strong potential as a vaccine antigen (Schuijfeel et al 2006).

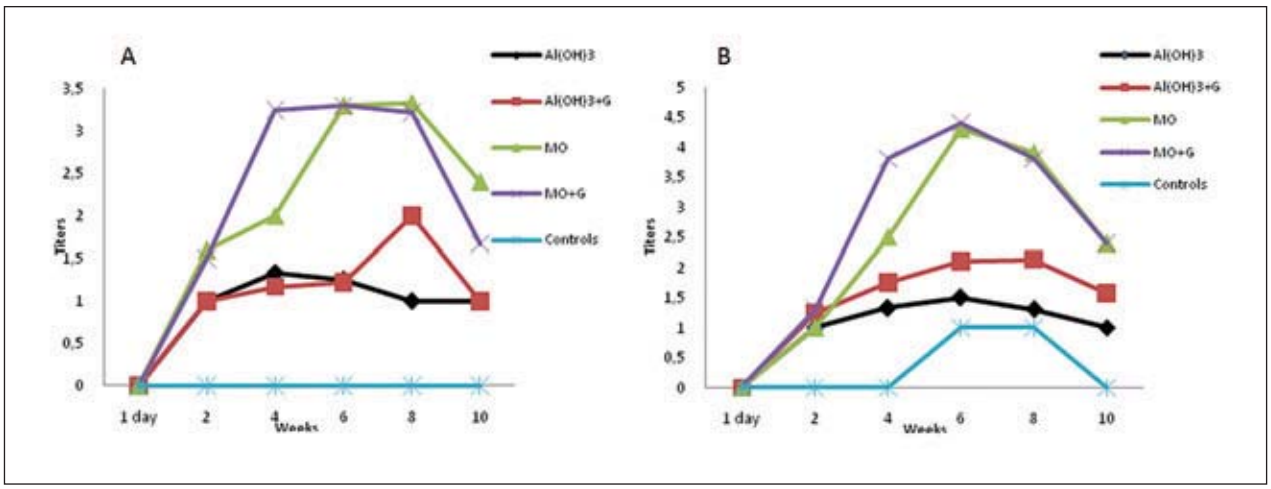

Figure 1. The titers of antibodies to O. rhinotracheale (serotypes A and B) antigens by mSAT.

The some of broiler producers in Turkey is frequently facing threat due to emerging respiratory diseases that result in severe economic losses. They tried to use several antibiotics against *O. rhinotracheale* infection, but sometimes, they could fail or meet ineffectivity. Hence, this study is aimed at the effect of different inactivated *O. rhinotracheale* vaccines on immune response and the determination of ratios of mortality and morbidity after challenge with *O. rhinotracheale* in broilers.

Storm and Van Empel (1996) pointed out that swelling of head in 5 of 9 animals, the small yellowish spots of the air sac in 7 animals and at the enjection site were seen in control group. Also, they noted that except swelling of head in the 2 animals, air sac and other organs were clean in vaccinated broilers. In the present study, no adverse reactions after the vaccination were recorded by the observation of animal behaviour. It is known that some adjuvants in inactivated vaccines may cause inflamatory responses and local reactions but these reactions would be transient (Bisschop et al 2004).

Travers et al (1996) reported that while no mortality were observed, but growth retardation, joint lesions and lung infection were encountered in patogenicity experimental of 3 *O. rhinotracheale* isolates. Van Empel et al (1996) notified that growth retardation, joint lesions and lung infections were observed in aerosol challenge traials, but similar lesions occurred more severe in the presence of viral infection.

In the present study, virulent two different strains of *O. rhinotracheale* aerosolly administered to vaccinated and nonvaccinated broilers at 15 days. While it was not observed any mortality and morbidity in vaccinated broilers, mortality and morbidity in controls are in the ratio of 20% and 50%, respectively. It is interesting that no isolation of *O. rhinotracheale* and other microorganisms were recorded from respiratory and internal organs in MO vaccinated groups. Also, the re-isolation of *O. rhinotracheale* was not made from respiratory organs in other vaccinated animals. But, the isolation of *O. rhinotracheale* strains and other microorganisms were observed in controls.

It is known to be a relationship between increased with age and development of resistance to *O. rhinotracheale* infection (Van Empel and Hafez 1999). Therefore, the sooner the vaccination is done in infected animals; early immunization will be provided due to immune stimulation before transmission of *O. rhinotracheale* infection. For this reason, the broiler chicks were vaccinated by *O. rhinotracheale* vaccines at first day in this study.

Van Empel and Van den Bosch (1998) informed that inactive vaccines found to be effective against *O. rhinotracheale* in challenged broiler chicks, the results of vaccination influenced by maternal antibodies. To overcome this problem, using bacterin vaccine with the strong adjuvant such as mineral oil may provide better protection in the presence of maternal antibodies. Bisschop et al (2004) emphisazed that a bacterin vaccine that is applied to broiler-breeder hens to pass on protective immunity to their broiler progeny was tested under large-scale commercial conditions in South Africa. They reported that the progeny of vaccinated hens appeared to perform slightly better under commercial conditions than the progeny of unvaccinated hens. Cauwerts et al (2002) reported that 8 breeder flocks were vaccinated with an inactivated *O. rhinotracheale* vaccine and the other 8 breeder flocks remained unvaccinated as controls. They pointed out that after vaccination, maternal antibodies in the progeny of vaccinated breeder flocks were significantly higher, than the offsprings of non-vaccinated flocks. Also, the ratio of mortality was lower and the mean production index was higher in the broilers derived from vaccinated breeders. Schuijfeel et al (2005) has stated that unravelling of the protective immunity acquired during a natural infection may contribute to vaccine development. Passive transfer of *O. rhinotracheale* specific antiserum to the immune-suppressed birds prior to pathogen challenge significantly decreased morbidity. This protective effect was not observed after administration of control sera containing similar concentrations of immunoglobulins. The authours (Cauwerts et al 2002) pointed out that the applied immune depletion and reconstitution approach is an attractive tool to analyse the nature of the protective immune response.

In this study, the determination of the efficiencies of *O. rhinotracheale* vaccines planned on broiler breeders. But, because of the outbreaks of Avian Flu during 2006-2007 in Turkey, the trials of vaccine efficiency could not overcome on broiler bredeers.

Murthy et al (2007) has investigated the effect of vaccination of chickens with 8 different inactivated vaccines (formalin and thiomersal; and with or without adjuvants such as mineral oil, alum and aluminium hydroxide gel) against experimental *O. rhinotracheale* challenge. They reported that vaccination of layer chicken at the 8th week followed by a booster dose at the 12th week of age can effectively protect against *O. rhinotracheale* infections. Also, they reported that the bacterin in mineral oil adjuvant induced the highest serologic response. When compared with control group, vaccines in mineral oil and other adjuvants in vaccinated animals decrease significantly lesions such as air sacculitis and pneumonia (Murthy et al 2007).

Ginseng (Panax ginseng) has been begun 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, staphylococcal mastitis, *O. rhinotracheale*) (Erganiş et al

2010, Hadimli et al 2005a, Hadimli et al 2005b). Although no significant differences were observed between vaccinated groups, titers of specific antibodies of ginseng extract added *O. rhinotracheale* vaccines were measured greater. It is thought that ginseng increase the bactericidal activity of the immune system, according to the re-isolation studies from lung and tracheal samples of animals vaccinated with and without ginseng.

► Conclusion

The study showed that vaccination of commercial broiler chicks by 4 different of *O. rhinotracheale* vaccines with adjuvants (aluminium hydroxide, mineral oil and ginseng) at one day age by once dose can effectively protect against *O. rhinotracheale* infections. Ginseng also positively affected on increasing of bactericidal activity of the inactive bivalent bacterin vaccines with mineral oil or aluminium hydroxide adjuvants.

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