DEVELOPMENT AND EXPERIMENTAL ASSAY OF INACTIVATED *SALMONELLA* AND *E. COLI* VACCINE FOR PIGS

Modestas Ružauskas
Lithuanian Veterinary Institute, Department of Bacteriology and Parasitology, Instituto g. 2, Lt-4230 Kaišiadorys
Lietuvos veterinarijos institutas, Bakteriologijos-parazitologijos skyrius, Instituto g 2, LT–4230 Kaišiadorys,
Tel. +370-346-60693 el. pašto adr.: modruzauskas@hotmail.com

Abstract. Bacteriological investigations reveal that often pig infectious diseases are caused by a mixed pathogenic flora. In most cases it is *Salmonella* and also *E. coli.* Monovalent vaccines against pig bacterial diseases are effective, however they cause stress in animals. A bivalent vaccine was developed in Lithuanian Veterinary Institute. Vaccine was produced from local bacterial strains (*Salmonella enterica* subsp. *enterica* ser. Choleraesuis and *E. coli*), which were isolated from Lithuanian pig farms. Emulsigen (MVP laboratories, Inc.) was used as adjuvant for a better immune response. Experimental vaccine was tested on laboratory animals. The laboratory trial of S*almonella* and *E. coli* vaccine using rabbits as experimental animals revealed that the potency to *S. enterica* subsp. *enterica* ser. Choleraesuis was 100 % when the vaccine contained $2 \cdot 10^9$ bacterial cells/ml of *Salmonella* and the same number of *E. coli*. Specific antibody titres in blood sera of vaccinated rabbits were high both for *Salmonella* and *E. coli*. The vaccine had no side effects and was safe.

Keywords: vaccine, *Salmonella*, *E. coli*, pigs.

INAKTYVUOTOS SALMONELIŲ IR E. COLI VAKCINOS KIAULĖMS GAMYBA IR JOS EKSPERIMENTINIAI TYRIMAI

Santrauka. Bakteriologiniai tyrimai rodo, kad dažnai kiaulių infekcines ligas sukelia mišri patogeninė mikroflora. Dažniausiai tai būna *Salmonella* ir *E. coli.* Monovalentinės vakcinos nuo bakterinių kiaulių ligų yra efektyvios, tačiau jos sukelia didesnį stresą gyvūnams. Lietuvos veterinarijos institute buvo atlikti dvivalentės vakcinos tyrimai. Vakcina buvo pagaminta iš vietinių salmonelių (*S. enterica* subsp. *enterica* ser. Choleraesuis) ir *E. coli* (K88, K99 ir 987P) padermių. Geresniam imuniniam atsakui pasiekti panaudotas adjuvantas „Emulsigen" (MVP laboratories, Inc.). Eksperimentinė vakcina išbandyta eksperimentiškai, panaudojant laboratorinius gyvūnus (triušius). Tyrimais nustatyta, kad eksperimento metu vakcinuotų ir po to užkrėstų salmonelėmis išgyvenusių triušių skaičius buvo šimtaprocentinis, kai salmonelių ir *E. coli* koncentracija vakcinoje buvo po 2 mlrd. bakterijų mililitre, švirkščiant triušiams po 1 ml vakcinos. Ši vakcinos dozė užtikrino ir aukštus specifinių antikūnų titrus tiek prieš salmoneles tiek ir prieš *E. coli*. Vakcina buvo saugi ir nesukėlė šalutinio poveikio.

Raktažodžiai: vakcina, salmonelės, *E. coli*, kiaulės.

Introduction. Bacteriological investigations reveal that often pig diseases are cause by a mixed pathogenic flora (Dobilas et al., 1999, 2000). *Salmonella* and *E. coli* can be mentioned as the most common agents of digestive tract infections (Kennan et al., 1995, Pastoret et al., 1997). The genus *Salmonella* consists of two species: *S. enterica* and *S. bongori*. Species names were arbitrarily given to serovars for convenient reasons in medical practice (Popoff, 2000). Although primarily intestinal bacteria, *Salmonella* and *Escherichia coli* are widespread in the environment and commonly found in farm effluents, sewage and in any material subject to faecal contamination. Salmonellosis and colibacillosis has been recognized in all countries, but appears to be most prevalent in areas of intensive animal husbandry, especially of poultry or pigs and dairy cattle reared in confinement. Salmonellosis and colibacillosis can affect all species of domestic animals; young animals and pregnant and lactating animals are the most susceptible. Some serovars only affect certain hosts, e.g. *S. choleraesuis* or *E. coli* O149:K91 K88ac in pigs, although most serovars may cause disease in a wide range of animal species. Enteric disease is the commonest clinical manifestation but a wide range of clinical signs, which include acute septicaemia, abortion, arthritis, and respiratory disease, may be seen. Many animals, however, may also be infected but show no clinical illness. Such animals may be important in relation to the spread of infection between herds and as causes of human food poisoning. By this reason active immunization of various animal species is desirable. As salmonellosis and colibacillosis are very common diseases between animals and human, an important attention has been paid both to *Salmonella* and *E. coli* (Kennan et al., 1995, Dobilas et al., 1999, 2000, Ružauskas et al., 2000, Hughes et al., 2002). Bacteriological investigations in Lithuanian pig herds showed that the most spread serotype among *Salmonella* is *Salmonella enterica* subsp. *enterica* ser. Choleraesuis. *E. coli* containing pili antigens K88 (F4), K99 (F5) and 987 P (F6) are the most common among *Escherichia* spp. (Ružauskas et al., 2002).

Many inactivated vaccines are used against salmonellosis and colibacillosis and some live vaccines are available commercially. While inactivated vaccines have been used successfully in a number of circumstances, the efficacy of some may be law and

therefore local bacterial strains and some oil or alhydrogel adjuvants have been used. Field efficacy data are often lacking, although laboratory testing may provide a useful indication. Innocuity tests are performed in laboratory animals and, in the case of inactivated vaccines, sterility tests using bacteriological enrichment media are carried out. For initial experiments of inactivated bacterial vaccines laboratory animals are widely used (Pastoret et al., 1997). Monovalent vaccines against pig salmonellosis, and colibacillosis were tested at the Lithuanian Veterinary Institute. The clinical and epizootological investigations proved the efficacy of these vaccines in specific prophylaxis of infectious diseases of pigs (Barzelis et al., 1997, Dobilas et al., 1998, 2000).

Vaccination of pigs and sows with monovalent vaccines against individual diseases separately induces greater stress, whereas veterinarians have difficulties in determining vaccination schemes. Besides, monovalent vaccines are relatively more expensive than the bivalent or polyvalent ones (Ružauskas, Virgailis, 1999).

The aim of the present work was to develop bivalent *Salmonella* and *E. coli* vaccine using swine origin isolates, to determine optimal concentration of bacterial cells in the vaccine and to carry out some initial experimental investigations using laboratory animals.

Materials and methods. The development of bivalent vaccine against pig salmonellosis and colibacillosis was based on the collected data and new experiments. For vaccine production local bacterial strains, which were isolated from swine herds have been used. All isolated bacterial strains were identified by historical records and characterized by phenotypic markers. Typical strains of stable phenotypic characteristics were selected. These strains are stored in the collection of microorganisms of the Lithuanian Veterinary Institute. Their growth, antigenic, biochemical and toxigenic properties as well as lethal doses for laboratory animals were established (Dobilas et al., 1999).

For vaccine production one strain of *Salmonella enterica subsp. enterica ser. Choleraesuis* and one strain of *E. coli* that contains pili antigens K88, K99, and 987 P were used. Each master seed were tested to ensure its identity and safety. Safety of the vaccine was determined in the course of experiment by observing vaccinated rabbits.

Selected strains were incubated separately into fermenter. Buffered peptone water (Liofilchem, Italy) enriched with 5 % of glucose was used as growing media. Sterile air and Na OH were supplied for a better growing and to guarantee proper concentration of pH. Bacteria were incubated for 12 hours. After that they were concentrated by centrifugation. Formaldehyde was added as an inactivator. Thiomersal (Sigma) was used as a preservative. Commercial adjuvant "Emulsigen" (MVP Laboratories Inc., USA), which is optimal for these bacteria, was used as immunostimulator (Osek et al., 1994, Ružauskas, 1996, Dobilas et al., 2000).

An optimal *Salmonella* and *E. coli* concentration in the vaccine was determined. For this purpose bivalent, inactivateded *Salmonella* and *E. coli* vaccines with adjuvant "Emulsigen" were produced with the following ratios of *Salmonella* and *E. coli* (Table 1).

Table 1. **Concentration of *Salmonella* and *E. coli* in the vaccines**

Number of the vaccine group	Concentration of bacteria in vaccine (bacterial cells/ml)	
	Salmonella	*E. coli*
1	$1x10^9$	$1x10^9$
2	$2x10^9$	$2x10^9$
3	$4x10^9$	$4x10^9$

Tests for vaccine sterility were done according to Lithuanian standards in force.

For vaccine potency tests rabbits aged 3 – 4 months were used. There were 3 experimental groups, four rabbits in each. The rabbits were vaccinated with prepared bivalent vaccine of different concentrations (Table 1). The first group of rabbits were vaccinated with the 1 ml of vaccine, which contained 10^9 bacterial cells (b.c.) of *Salmonella* and the same number of *E. coli*. The second group were vaccinated by the same dose, but concentration of *Salmonella* and *E. coli* was $2x10^9$ b.c./ml of each strain. The third group of rabbits were vaccinated with $4x10^9$ b.c./ml of each strain.

The vaccination was repeated fourteen days after the first vaccination using the same vaccines and doses. Side effects were observed in the course of vaccination. On the 21-th day after the second vaccination the rabbits were infected using lethal doses (LD_{100}) of pathogenic strain of *Salmonella enterica* subsp. *enterica* ser. Choleraesuis. Three nonvaccinated rabbits were infected with the same pathogenic *Salmonella* strain as a control group. Infected animals were observed each day. Potency of the vaccine counted by the number (percent) of survivors in all rabbit groups. Bacteriological tests from faeces of survivors were carried out with the aim to find carriers of salmonella. They were repeated every week.

According to our data, *E. coli* strains are not highly virulent to rabbits. By this reason efficacy of the *E. coli* component was determined according to some indices of humoral (specific antibody titres) and cell immunity (number of leucocytes), which were induced by rabbit's vaccination during experiment.

In the course of experiment the blood of experimental animals was collected and some immunological, chemical and also morphological indices of blood were determined.

The titres of specific antibodies to *E. coli* and *Salmonella* were determined using the classical agglutination test. The number of leucocytes and erythrocytes was determined by counting in Goriajev's chamber. The content of haemoglobin was determined by the colorimetric method (Gabrijolavičius, 1991).

Statistical analysis was carried out by computer programme Sigma Plot (Jandel Scientific, version 1.02a).

Research results. From all strains of isolated *Salmonella enterica* subsp. *enterica* ser. Choleraesuis most of them have had stabile morphological, biochemical, antigenic and pathogenic properties. One

strain, which had typical and stabile phenotypic characteristics, was selected for vaccine preparing. Isolated *E. coli* strains had not such stabile properties as *Salmonella* had. Most of them dissociated into R growing form and lose their ability to agglutinate with pili diagnostic antisera. For preparing of the vaccine one strain of *E. coli,* which had stabile phenotypic characteristics and have had good agglutinating properties with K88, K99 and 987P diagnostic antiserum was selected.

Before vaccination no specific antibodies against the pathogenic *Salmonella* and *E. coli* were detected in rabbit blood sera (the initial dilution of sera - 1:12,5).

On the 21-th day after the second vaccination the titres of specific antibodies were determined. They are shown in table 2.

It can be seen (Table 2) that specific antibody titres against *Salmonella* as well as against *E. coli* were rather high, but the highest means were reached when the vaccine concentration was $4 \cdot 10^9$ b.c./ml ($2 \cdot 10^9$ bacterial cells of *Salmonella* and the same number of *E. coli*).

The content of haemoglobin before and after the vaccination in rabbit blood changed unevenly without any statistical reliability. This implies that vaccination had no greater influence on the content of haemoglobin in blood.

The number of erythrocytes before vaccination and after it in the rabbit blood remained almost unchanged. This means that vaccination produced no effect on the number of erythrocytes in the blood. The number of leucocytes increased in all rabbit groups (Table 3).

The increase of the number of leucocytes reveals that the organism is prepared to resist the infection. No adverse reactions after vaccine application were observed, except temporary slight increase of body temperature, that is normal reaction after vaccination. No allergic reactions occurred. The vaccine was sterile and pure.

Part of the rabbits, infected with pathogenic *Salmonella* strain after vaccination, died, the rest of them survived depending on the content of administered antigen. This is shown in Table 4.

Table 2. **Specific antibody titres against *Salmonella choleraesuis* and *E. coli* in rabbit blood sera in the course of experiment**

Group of rabbits	Bivalent vaccine concentration (x10^9 bacterial cells/ml)	Specific antibody titres	
		Salmonella	*E. coli*
1	2	1000±400	500±200
2	4	2800±800	1000±400
3	8	2800±800	800

Table 3. **The number of leucocytes in rabbit blood sera in the course of experiment**

Group of rabbits	Bivalent vaccine concentration (x10^9 bacterial cells/ml)	Number of leucocytes before vaccination, 10^9/ L	Number of leucocytes after vaccination, 10^9 /L
1	2	6,75±1,9	7,5±3,8
2	4	6,85±1,9	7,9±1,2
3	8	7,05±2,5	8,1±1,0

Table 4. **Rate of rabbit survival after their infection with pathogenic *Salmonella* strain**

Group of rabbits	Bivalent vaccine concentration (x10^9 bacterial cells/ml)	Infection result		Potency, %
		Survived	Died	
1	2	1	3	25
2	4	4	0	100
3	8	4	0	100
Control	Not vaccinated	0	3	-

As is seen from Table 4 the 100 % potency was observed in rabbits that were vaccinated with vaccine containing not less than $4 \cdot 10^9$b.c./ml. Smaller doses were little effective. The results between 1-th group and 2-th - 3-rd groups are statistically significant ($P<0,05$). All rabbits of the control group died. According to means of specific antibody titres, the number of leucocytes and rabbit survival after infection the optimal ratio of *Salmonella* and *E. coli* in vaccine may be outlined as 1:1, the number of each strain of bacteria being $2 \cdot 10^9$ b.c./ml. Bacteriological investigations showed that after rabbits vaccination, immunity was sterile. No *Salmonella* were found in the faeces of infected animals after all period of investigation (1 month after infection).

Discussion and conclusion. Bacteria family *Enterobacteriaceae* are the most spread pathogenic enteric bacteria, which causes different infections in pigs (Dobilas et al., 1999, 2000). *Salmonella* and *E. coli* were the most frequently isolated pathogenic bacteria from swine origin in Lithuanian pig herds. For example, in 1996-2000 in National Veterinary Laboratory and Lithuanian Veterinary Institute were isolated 122 *Salmonella* strains and 176 pathogenic strains of *E. coli*. The most spread serotype of *Salmonella* is *S. enterica* subsp. *enterica* ser. Choleraesuis. The most common among *E. coli* – serotypes that contain pili antigens K88, K99, and 987P.

Laboratory trial of vaccine against salmonellosis and *E. coli* infections using rabbits as experimental animals revealed that the potency of the vaccine was 100 % to *Salmonella enterica* subsp. *enterica* ser. Choleraesuis infection when the vaccine concentration contained $4 \cdot 10^9$b.c./ml. The highest specific antibody titres to *E. coli* were obtained at the same concentration of the vaccine. So the optimal ratio of *Salmonella* and *E. coli* in the vaccine is 1:1 ($2 \cdot 10^9$ b.c./ml of each strain). The literary data about necessary concentration of bacteria in vaccines are multifarious. It mostly depends on species of bacteria, strains and adjuvants (Osek et al., Pastoret et al., 1997). Different bacterial strains have not equal immunogenicity, but an adjuvant could prolonge the presence of antigen in a body and allows to use smaller antigen concentrations for the development of immunity (Pastoret et al., 1997).

The developed *Salmonella* and *E. coli* vaccine had no adverse effects to rabbits. There were no statistical significance between all groups of rabbits according to common blood indices – the content of haemoglobin and the number of erythrocytes. A considerable increase of specific antibody titres in blood sera implies the development of humoral immunity, whereas the increased number of leucocytes shows cell immunity (Robertsson et al., 1982, McSorley et al., 2002). Statistical significance of these indices was between the first group of rabbits (vaccinated with minimal concentration of bacteria) and the second-third groups ($P<0,05$).

A direct correlation between specific antibody titres in blood sera, number of leucocytes and concentration of the bacteria in the vaccine was determined. Therefore, basing on the results of our investigations we may conclude that bivalent *Salmonella* and *E. coli* vaccine was safe and effective under experimental conditions for laboratory animals.

An optimal concentration of the vaccine was $2 \cdot 10^9$ b.c./ml of *Salmonella* and the same number of *E. coli*.

References

1. Barzelis L., Dobilas J., Virgailis M. Investigations of immunoprophylaxis against salmonellosis in pigs using Salmonella strains isolated in Lithuania. Ecological Effects of Microorganism Action. Materials of international conference. Vilnius, 1997. P. 349-352.
2. Dobilas J., Barzelis L. Kolibakteriozės paplitimas ūkiuose ir iš vietinių kamienų sukurtos *E. coli* vakcinos efektyvumo tyrimai. Veterinarija ir zootechnika. Kaunas, 1998. N. 5. P. 5-11.
3. Dobilas J. A., Barzelis L., Ružauskas M., Virgailis M. Epizootiniai gyvulių ir paukščių užkrečiamųjų ligų tyrimai, išskirtų mikroorganizmų identifikavimas ir jų saugojimas mikroorganizmų kolekcijoje. Botanica Lituanica, Supplementum 3. Vilnius, 1999. P. 21-26.
4. Dobilas J. Epidemiology of swine colibacteriosis and salmonellosis in Lithuania and description of preventive vaccines. Veterinar medicines raksti. Jelgava, 2000. P. 30-35.
5. Dobilas J. Gyvulių kvėpavimo ir virškinimo sistemų bakteriniai susirgimai bei jų specifinė profilaktika. Actual veterinary problems in modern pig industry. Proceedings of international conference. Kaišiadorys, 1999. P. 12-21.
6. Gabrijolavičius V. Gyvulių vidaus ligų klinikinė diagnostika. Vilnius: Mokslas, 1991. P. 253-276.
7. Hughes E. A., Galan J. E. Immune Response to Salmonella: Location, Location, Location. Immunity. 2002. Vol 16. P. 325.
8. Kennan R., Soderlind O., Conway P. Presence of F 107, 213 P and AV 24 fimbriae strains of Escherichia coli isolated from Swedish piglets with diarrhoea. Veterinary microbiology. 1995. Vol. 2. P. 123–129.
9. Manual of standards Diagnostic Tests and Vaccines. OIE, 2000.
10. McSorley S. J., Asch S., Castalonga M., et al. Tracking Salmonella-Specific CD4 T Cells In Vivo Reveals a Local Mucosal Response to a Disseminated Infection. Immunity. 2002. Vol 16. P. 328.
11. Osek J., Truszczynski M., Tarasiuk K., Pejsak Z. Evaluation of different vaccines against porcine colibacillosis. Proceedings of the 13th IPVS Congress. Bangkok, 1994.
12. Pastoret P. P., Blancou J., Vannier P., et al. Veterinary Vaccinology. Elsevier. Amsterdam-Lausanne-New-York-Oxford-Shannon-Tokyo, 1997.
13. Popoff M.Y. Antigenic Formulas of the *Salmonella* serovars. WHO Collaborating Centre for Reference and Research Salmonella. Paris, 2001. P. 3-10.
14. Robertsson J. A., Svenson S. B., Renstrom L. H. et al. Defined salmonella antigens for detection of cellular and humoral immune responses in salmonella infected calves. Research Veterinary Science. 1982. N. 33. P. 7.
15. Ružauskas M., Ambrozevičienė-Butrimaitė Č., Kiudulas V. Patogeninių *Escherichia coli* bakterijų paplitimas Lietuvos kiaulių fermose ir jų atsparumas antimikrobinėms medžiagoms. Žemės ūkio mokslai. Spaudoje.
16. Ružauskas M. Impact of some factors to efficacy of vaccines against swine pasteurellosis. Laboratorinė ir klinikinė imunologija. Tarptautinės mokslinės konferencijos tezės. Vilnius-Birštonas, 1996. P. 68.
17. Ružauskas M., Virgailis M., Kiudulas V. Biochemical characteristics of *Enterobacteriaceae* isolated from pigs in Lithuania. Biologija. Vilnius, 2000. N. 2 (priedas). P. 257-259
18. Ružauskas M., Virgailis M. Production and laboratory trial of salmonella and pasteurella vaccine for pigs. Proceedings in Veterinary Medicine. Jelgava, 1999. P. 295-300.

2002 09 16