ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Biological Properties and Experimental Substantiation the Regimes of Industrial Cultivation *Enterococcus spp.*

A. V. Skorikov[1], N. N. Zabashta[1], N. A. Yurina[1], O. Y. Chernyh[2], A. F. Dmitriev[3]

[1]Krasnodar Research Centre for Animal Husbandry and Veterinary Medicine
350055, Russia, Krasnodar, Znamensky Village, 4 Pervomaiskaya Street
[2]Kuban State Agrarian University named after I. T. Trubilin
350044, Russia, Krasnodar, 13 Kalinina Street
[3]Stavropol State Agrarian University
355017, Russia, Stavropol, 12 Zootechnicheskiy Lane

Abstract

This article presents the results of study the main biological properties of enterococcal strains isolated as a result of laboratory tests from piglets and young pigs with a clinic of infectious diseases. A comparative analysis of industrial enterococcus strains was also carried out at the enterprise of the biological industry of the Russian Federation in the manufacture of vaccines for the prevention of enterococcal infection of pigs. A scheme of cultivation and methods of inactivation enterococcal groups have been developed: *Streptococcus faecalis 13, 345, 356, Sokolovo, Konstantinovsky*. Regulatory and technical documentation was developed to use it in the development and production of inactivated vaccines.

Keywords: enterococcus, biological properties, strains, development, regimes, cultivation, pigs.

INTRODUCTION

For the first time, bacteria representing gram-positive cocci isolated from the intestine, had been described by the French microbiologist Thiercelin in 1899. In 1930, scientists attributed enterococcus to streptococcus group D [1], in 1984, during study the DNA of enterococcus and streptococcus, enterococcus were assigned to a separate genus Enteroccus, which includes 34 species [2]. Several serological groups of bacteria referred to the genus Enteroccus are representatives of the normal microbiocenosis of the intestine of animals and humans. These bacteria are involved in metabolic processes in the intestine, synthesize vitamins, participate in the excretion of antimicrobial peptides, hydrolyze lactose and eliminate pathogenic microorganisms. Therefore, bacteria of the genus Enteroccus are used for the probiotic drugsproduction to preventthe infectious diseases of animals and humans [1, 3-6]. At the same time, some researchers include individual serological groups and enterococcus strains to the most dangerous microorganisms that are able to cause infectious diseases of animals and humans [7-12], the leading of gastrointestinal diseases [7, 8, 13, 14]. Enterococcus are pathogens of infectious and inflammatory diseases of animals and humans, mainly due to the production of virulence factors by the microbial cell: hemolysin, bacteriocin, plasmid-coding protein, which participates in the adhesion and aggregation of enterococcus, cytolysin, enzymes that cause the aggression of this bacterium - protease, hyaluronidase and gelatinase - an extracellular zinc of endopeptidase similar to that of elastase which is produced by Pseudomonas aeruginosa [1, 4, 10, 15-19].

In this regard the study of biological properties and the development of new biological products against the infections caused by microorganisms of the genus Enteroccus with the aim of their prevention in pig production is actual [20].

MATERIALS AND METHODS

Diagnostic material was selected at agricultural enterprises at the Krasnodar Territory. The material had been obtained from pigs which were clinically ill or died from infectious and inflammatory diseases. The studies were conducted in the veterinary laboratories of the Federal State Scientific Institution "Krasnodar Scientific Center of Animal Husbandry and Veterinary Medicine", in the laboratory of the biological and technological control department of the Federal State Unitary Enterprise "Armavir Biofactory". The studies were conducted in accordance with the methodological recommendations for laboratory diagnosis of streptococcosis in animals (1990). A selective differential medium was used to isolate and incubate the enterococcus. It was grown in a medium: gall esculin agar, agar with potassium telluride, 5% blood agar, meat peptone agar, half-liquid Hottinger agar. The strains of microorganisms were isolated on the basis of morphological, cultural and biochemical properties. Biochemical properties were studied by the test systems "STREPTOtest" and "EN-COCCUStest" from Pliva-Lachema, Czech Republic. A comparative analysis of morphological, cultural and biochemical properties was conducted in the development of regimens for culturing enterococcus. Epizootic and production strains of microorganisms and individual strains for industrial cultivation were isolated. Matrix flasks, glass cylinders with a capacity of 20 liters, industrial reactors, semi-liquid agar and Hottinger broth were used in the process. Sterility of cultivation enterococcus was studied in accordance to GOST 28085-89 (GOST-Russian National Standard). The strains of *Streptococcus faecalis 13, 345, 356 Sokolovo, Konstantinovsky*, were used for the cultivation of enterococcus.

RESULTS AND DISCUSSION

Control and production of enterococcus strains, selected for cultivation, were stored in lyophilized form. It was stored on a half-liquid Hottinger agar in the laboratory of the Biological and Technological Control Department of the Armavir Biofactory. For matrix brood, enterococcus strains was disseminated into meat-peptone agar in Petri dishes, then it was incubated in a thermostat for 18-24 hours at a temperature of (37.0 ± 0.5) ° C. Microscopy of microorganisms smears was performed at a magnification of 16-56 times, then it was selected from 3 to 5 colonies of the typical S-shaped form. It was passed to meat peptone agar, meat peptone broth and semi-liquid agar, and it was incubated 16-18 hours at (37 , 0 ± 0.5) ° C. Growing cultures of enterococcus in semi-liquid agar were packaged into ampoules or Pasteur pipettes, followed by sealing and storage at a temperature from 2 ° C to 8 ° C.

Enterococcus strains used for cultivation, according to their morphological properties, were Gram-positive cocci, arranged in pairs of smears or in the chainsform of various lengths. On Hottinger agar and casein-yeast agar, enterococcus formed S-shaped colonies that were smooth dewty with straight edges; on semi-liquid agar growth was observed in the form of

white strands that permeate the nutrient medium; in the Hottinger broth, enterococcus caused intense turbidity of the medium. Enzymatic properties of enterococcus strains were noted by fermentation with the formation of: glucose, lactose, sucrose, maltose, mannitol and sorbitol; it did not ferment raffinose, arabinose, xylose and dulcine. Virulent properties of the strains were characterized by the death of white mice weighing 13-14 g after intraperitoneal injection of a 15-20 hour enterococci culture at a dose of 3 billion / cm3 for 10 days.

To prepare a growsmedium for culturing enterococcus we used a Hottinger broth with the addition of 0.5% peptone, 0.54% sodium chloride, 0.5% chemically pure dibasic sodium phosphate and 10% water, pH of the medium to the values of 7.6-7, 8 was achieved by a 10% solution of caustic sodium. The medium was allowed to stand for 15 minutes, then it was filtered through filter plates and fed to sterile reactors. There, the medium was subjected to a temperature treatment at a temperature of 118-112 ° C. In a nutrient medium, the content of amine nitrogen was 220-250 mg % within 50 minutes.

To obtain the culture of production enterococcus strains in vials, enterococcus strains from ampoules or sealed pipettes were prepared for preparation of matrix dilution into tubes with 4-5 cc of nutrient medium. The crops were cultivated at (37 ± 05) ° C for 6-8 hours. Simultaneously with inoculation into test tubes, cultures were sowed on Petri dishes with Hottinger agar. This was done to control the purity of growth. The crops were cultivated at (37 ± 0.5) ° C for 16-18 hours. At the end of the cultivation, the morphology of colonies and the purity of culture growth were visually assessed.

Cultured enterococcus cultures were transferred to vials from 100-150 cm3 of nutrient medium. Simultaneously, the culture was inoculated into test tubes with nutrient media of meat peptone agar, meat peptone broth, meat peptone liver broth and cultivated for 6-8 hours at (37 ± 1)°C. Before sowing the cultures in vials, the purity of the growth was controlled by microscopy of smears stained by Gram.

To obtain the brood of production Enterococcus strains in bottles, from vials were inoculated into 20 liter bottles containing 10-12 liters of nutrient medium at the rate of 3-7% to its volume. The crops were cultured for 16-18 hours at (37.0 ± 1.0) ° C. To control growth purity simultaneously with culture in a bottle, cultures were inoculated into test tubes with nutrient media: peptone agar meat, peptone broth, meat, peptone liver broth, on Petri dishes with Hottinger agar. Crops was cultivaited for 16-18 hours at 37.0 ± 1.0 ° C. At the end of the cultivation, the morphology of colonies and the purity of culture growth were visually assessed.

Seeding and cultivation of the matrix culture in the reactor was carried out in the following sequence: tested for purity, streptococcal matrix cultures were inoculated into the reactor at a rate of 8-10% to the volume of the nutrient medium, with the addition of 5% glucose. Each enterococcus strains was cultured in a separate reactor. The volume of the nutrient medium did not exceed 50-70% of the volume of the reactor. The nutrient medium before the inoculation was heated to a temperature (38 ± 0.5) ° C, the pH corresponded (7.4 ± 0.2). Cultivation of the cultures was carried out at 37 ° C, for 18-20 hours with constant stirring. Stirring started from the second hour of cultivation and continuous aeration at the rate of enrichment of the nutrient medium with air in a ratio of 1: 2. From the third hour of cultivation, the concentration of microbial bodies was determined. Cultivation was stopped when the concentration of microbial cells of the bacterial mass was at least 10 billion / cm3. At the end of the cultivation, the morphology of colonies and the purity of growth of agar cultures were visually assessed. The culture grown in the reactor was free from extraneous microflora, in Gram-stained smears only gram-positive diplococcus and streptococcus were present.

The grown enterococcal cultures were inactivated with formalin. Formalin was diluted with physiological saline to 0.25-0.3% concentration. For this purpose, technical formalin was used in accordance with GOST 1625-75 (GOST-Russian National Standard) with formaldehyde content of at least 36.0%. Inactivation of the cultures was carried out for 5-7 days at a temperature of (37 ± 0.5)°C. In the process of inactivation, the cultures were mixed for 5 minutes 1-2 times a day. After the inactivation, the culture was tested for inactivation by seeding on nutrient media (meat peptone broth, meat peptone agar, meat peptone liver broth and Saburo slant agar) by sampling from each reactor. In the absence of growth on nutrient media, inactivation of cultures was considered complete. Upon completion of the inactivation, enterococcal cultures were pumped to a collection reactor. At the same time, sterility and constant mixing were observed. The storage period of bacterial mass after inactivation should not exceed 10 days.

Immunogenic activity of enterococcus was tested on white mice weighing 16-18 g. The inactivated biomass was administered to mice at doses of 0.1; 0.2 and 0.3 cm3 in the left groin area intraperitoneally. Injections were administered three times with an interval between it of 5 days. Immunization was subjected to 15 white mice, 4 mice were used as a control. Ten days after the last injection, 12 vaccinated mice were selected and challenged simultaneously with the control group of mice intraperitoneally at a dose of 3 billion / cm3 with a culture of the control strain of *Streptococcus faecalis No. 356*. Before infection, the enterococcal culture was cultured in test tubes on broth for 24-36 hours. The inactivated biomass was considered active if from the 12 immunized white mice, after the control infection, no more than 3 animals died under the condition of death of all control animals. If more than 3 animals perished from among the vaccinated, a second test was carried out using the same procedure. The observation period for the experimental animals was 10 days after the infection.

CONCLUSION

As a result of this work, the technology of industrial cultivation of enterococcus for the production of inactivated vaccines was developed. The results obtained in the course of the experiment were used in the development of normative and technical documentation for the manufacture, control and application of an inactivated vaccine against enterococcal infection of piglets.

REFERENCES

1. Murray, B.E., The life and times of Entorococcus, *Clin.Microbiol.* 1990, *3*, 46-55.
2. Jordens, J.Z., Faecal carriage and nosocomial spread of vancomycin-resistant Enterococcus faecium, *J. Antimicrob. Chemother.* 1994, *34*, 515-28.
3. Ermolenko, E.I., Bakteriotsiny enterokokkov: problem i perspektivy [Bacteriocins of enterococcus: problems and perspectives], *Vestnik Sankt-Peterburgskogo Universiteta* [Bulletin of St. Petersburg University] 2009, *11(3)*, 184-201.
4. Poshvina, D.V., Sycheva, M.V., (2014) Vidovaya kharakteristika I proteoliticheskaya aktivnost' enterokokkov [Species characteristic and proteolytic activity of enterococcus], *Vestnik veterinarii* [Bulletin of veterinary medicine] 2014, *2(69)*, 40-43.
5. Popaganni, M., Ribosomallysynthezed peptides with antimicrobial properties: biosynthesis, structure, function, and applications, *Biotechnol. Adv.* 2003, *21(6)*, 465-499.
6. Jaouani, I., Ablassi, M.S., Ribeiro, S.C., (2015) Safety and technological properties of bacteriocinogenic enterococci isolates from Tunisia, *J. Appl. Microbiol.* 2015, *119(4)*, 1089-1100.
7. Bondarenko, V.M., Suvorov, A.N., *Simbioticheskiye enterokokki I problem enterokokkovoy opportunisticheskoy infektsii* [Symbiotic

Enterococcus and Problems of Enterococcal Opportunistic Infection], Medicine, Moscow 2007.
8. Bukharin, O.V., Valsheva, I.V., Kartashova, O.L., Kharakteristika virulentnogo potentsiala klinicheskikh izolyatov enterokokkov [Characteristics of the virulent potential of clinical isolates of enterococcus], *Zhurnal Mikrobiologii Epidemiologii i Immunobiologii* [Journal of Microbiology Epidemiology and Immunobiology] 2013, *3*, 13-18.
9. Garmasheva, I.L., Kovalenko, N.K., Identifikatsiya i taksonomiya enterokokkov [Identification and taxonomy of enterococcus], *Microbiological journal* 2010, *72(5)*, 49-58.
10. Coburn, P.S., Gilmore, M.S., The Enterococcus faecalis cytolysin: a novel toxin active against eukaryotic and prokaryotic cells, *Cell Microbiol.* 2003, *5(10)*, 661-669.
11. Poulsen, L.L., Risgaard, M., Son, N., Enterococcus faecalis clones in poultry and in humans with urinary tract infections, *Emerging Infectious Diseases Vietnam* 2012, *18(7)*, 1096- 1100.
12. Keis, S., Smith, J.M., Cook, G.M., Characterization of a vancomycin resistant Enterococcus faecalis (VREF) isolate from a dog with mastitis: further evidence of a clonal lineage of VREF in New Zealand, *J. Clin. Microbiol.* 2003, *13*, 3331-3333.
13. Poshvina, D.V., Shepitov,a N.E., Sycheva N.V., Vidovaya kharakteristika i factory persistentsii enterokokkov, vydelennykh ot zhivotnykh v norme i pri patologii [Species characteristic and factors of persistence of enterococcus isolated from norm and pathology animals], *Veterinariya* [Veterinary Medicine] 2015, *6*, 26-30.
14. Sidorenko, S.V., Rezul'taty mnogotsentrovogo issledovaniya antibiotiko-chuvstvitel'nosti enterokokkov [Results of a multicentre study of antibiotic sensitivity of enterococcus], *Antibiotiki i khimioterapiya* [Antibiotics and chemotherapy] 1998, *9*, 9-17.
15. Walsheva, I.V., Geneticheskaya kharakteristika virulentnogo potentsiala enterokokkov kishechnoy mikrobioty cheloveka [Genetic characteristics of the virulent potential of enterococcus in human intestinal microbiota], *Zhurnal Mikrobiologii Epidemiologii i Immunobiologii* [Journal of Microbiology Epidemiology and Immunobiology] 2012, *4*, 44-47.
16. O'Driscoll, T., Crank, C.W., Vancomycin-resistant enterococcal infections: epidemiology, clinical manifestations, and optimal management, *Infection and Drug Resistance* 2015, *8*, 217-30.
17. Qin, X., Singh, K.V., Weinstock, G.M., Effects of Enterococcus faecalis for genes on production of gclatinase and a serine protease and virulence, *Infect. Immun.* 2000, *68*, 2579-2586.
18. Kafil, H.S., Mobarez, A.M., Spread of Enterococal Surface Protein in Antibiotic Resistant Enterococcus faecium and Enterococcus faecalis isolates from Urinary Trackt Infections, *Open Microbiol. J.* 2015, *26(9)*, 14-17.
19. Larsen, H.S., Streptococcaceae, in Mahon, C.R., Manuselis, G., *Textbook of diagnostic Microbiology*, W.B. Saunders, Philadelphia 2000, pp. 345-358.
20. Rusaleev, V.S., Gnevashev, V.M., Pruntova, V.M., Bakterial'nyye vaktsiny v svinovodstve [Bacterial vaccines in pig production], *Veterinariya sel'skokhozyaystvennykh zhivotnykh* [Veterinary of farm animals] 2005, *12*, 21-23.