Scientific Works. Series C. Veterinary Medicine. Vol. LXIX (2), 2023
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

PRODUCTION AND EFFICACY EVALUATION OF A *PASTEURELLA* AUTOVACCINE FOR SHEEP

George MOGOȘ[1], Anca BULGARU[1], Elena NEGRU[1], Costin STOICA[2], Mihai DANEȘ[3], Doina DANEȘ[1]

[1]Faculty of Veterinary Medicine/University of Agronomic Sciences and Veterinary Medicine, Splaiul Independentei 105, District 5, Bucharest, Romania
[2]Romvac Company, Centurii Road 7, Voluntari, Ilfov, Romania
[3]Faculty of Veterinary Medicine/Spiru Haret University, 256 Basarabia Avenue, District 2, Bucharest, Romania

Corresponding author email: mogoshge@yahoo.com

Abstract

The aim of the current study was to develop an auto-vaccine based on two Pasteurella strains (P. multocida and P. spp.), previously isolated from an outbreak of respiratory infection in sheep. Preliminary tests were performed to assess the pathogenicity of the isolates on Balb/C mice. For the vaccine production, the Pasteurella strains were cultured on brain-heart infusion (BHI) medium supplemented with horse serum, were inactivated with formaldehyde and adjuvanted with aluminium hydroxide. In vitro and in vivo tests were performed to determine the sterility, safety and efficacy of the finished product. The results showed that the autovaccine had minimal side effects on both laboratory animals, and the target species; also, the biological product was successful in protecting vaccinated mice against the two pathogenic strains in a challenge test.

Key words*: evolution Pasteurella multocida, Pasteurella spp., pneumonia, sheep, vaccine.*

INTRODUCTION

One of the most prevalent bacterial illnesses in small ruminants, pasteurellosis is an important respiratory infection and has a wide geographic distribution, occurring in temperate, subtropical, and tropical regions. The first case of pneumonic pasteurellosis in sheep was documented in 1931, but it wasn't until the 1960s that serotyping and biotyping were important in defining the disease's epidemiology (Aitken, 2007). In sheep, *Mannheimia haemolytica* strains are usually responsible for pneumonic pasteurellosis in all age groups, while *Pasteurella trehalosi* strains induce systemic infection in lambs between the ages of 6 and 10 months. The species *Pasteurella multocida*, considered in rare cases to be the causative agent of respiratory infections in sheep and goats, includes a group of microorganisms characterized by antigenic diversity and multi-host predilection (Weiser et al., 2003). In the upper respiratory tract microbiome of numerous animal species, *P. multocida* is frequently present as a commensal. Environmental factors, stress, viral or mycoplasma infections favour bacterial invasion of lung tissue and the emergence of pneumonia. *P. multocida* can infect and cause illness in people and a variety of animal species (Boumart et al., 2021). As the cause of pneumonia in sheep and goats, the bacterial species has been subjected in a number of studies (Watson and Davies, 2002; Özyildiz et al., 2013; Valadan et al., 2014; Hailu et al., 2017; Boumart et al., 2021). According to Odugbo et al. (2006) and Sadeghian et al. (2011), clinical signs include anorexia, dyspnea with head and neck tightness and open-mouthed breathing, nasal discharge, coughing, listlessness, and death. Diarrhoea has occasionally been reported (Valadan et al., 2014). The best strategy of control, given the sporadic character of the illness, is targeted immunoprophylaxis by routine vaccination (Aitken, 2007). Due to *Pasteurella*'s great antigenic diversity and weak immunogenicity, vaccination against pasteurellosis is challenging (Mânzat, 2001). The purpose of the current study was to produce a vaccine based on two *Pasteurella* strains, previously isolated from an outbreak of respiratory infections in

sheep. The isolates, identified as *P. multocida* and *P.* spp. using Api 20 E and Api 20 NE biochemical tests, were found responsible for the reoccurring respiratory syndrome affecting a flock of sheep, where antimicrobial therapy was unsuccessful long-term (Mogoș et al., 2022). The researchers aimed to determine if the bacterial isolates were suitable for the production of an auto-vaccine, as well as if the finished product was safe to administer to animals and had good immunogenic qualities.

MATERIALS AND METHODS

To determine the pathogenicity of the two *Pasteurella* isolates, 60 Balb/C mice were split into three batches: Batch 1 - control, Batch 2 - *P.* spp. test and Batch 3 - *P. multocida* test. Batches 2 and 3 were subdivided into 4 groups (a, b, c, and d, respectively) of 5 animals each, each group to be inoculated with a different concentration of bacteria (Table 1).

Table 1. Batch organization and dose administered to mice for the pathogenicity test

Batch	Group	Dose
1 - Control	1a	PBS - 1 ml
	1b	PBS - 1 ml
	1c	PBS - 1 ml
	1d	PBS - 1 ml
2 - *Pasteurella* spp.	2a	2,000 CFU*/ml - 1 ml
	2b	200 CFU/ml - 1 ml
	2c	20 CFU/ml – 1 ml
	2d	2 CFU/ml - 1 ml
3 – Pasteurella multocida	3a	2,000 CFU/ml - 1 ml
	3b	200 CFU/ml - 1 ml
	3c	20 CFU/ml - 1 ml
	3d	2 CFU/ml - 1 ml

Legend: CFU/ml - colony forming units per millilitre

The inoculums were prepared by culturing the two bacterial strains in BHI medium for 24 hours at 37°C. The concentration of the cultures was determined using the serial dilutions method. To prepare the inoculums for the pathogenicity test, dilutions were made from each culture in PBS, in order to obtain suspension of 2000 colony forming units per mililiter (CFU/ml), 200 CFU/ml, 20 CFU/ml and 2 CFU/ml. The mice within the test groups were administered 1 ml of the respective dilution intraperitoneally, and the mice belonging to the control group were injected by the same route with 1 ml of sterile PBS each (Table 1). The clinical status of infected animals and their behaviour were monitored for 3 days post-inoculation. The time of appearance of the first clinical signs of disease, their evolution and the outcome were noted. Animals that survived the control infection, as well as animals included in the control group, were euthanized on day 8 post-inoculation.

Post-mortem examinations were performed and samples were collected from the liver, kidney and lungs of each mouse. The samples were cultured on blood agar plates. To determine the bacterial load in the different experimental groups, tissues harvested from lungs, liver and kidney were aseptically homogenized in normal saline to obtain a 10% suspension. Serial dilutions were made and cultured on BHI agar to determine the bacterial titer (CFU / g of tissue).

For the preparation of the *Pasteurella* autovaccine, the bacterial isolates were cultured on BHI medium supplemented with 10% horse serum. The cultures were incubared for 24 hours at 37°C, and then centrifuged 4000 rpm for 20 minutes at a temperature of 4°C. The supernatant was removed and the bacterial mass was washed 3 times in sterile PBS. The bacterial titer was determined for each bacterial suspension and formalin was added to a concentration of 0.2% for inactivation, followed by incubation at 37°C for 72 hours. The inactivated cultured were homogenized and 20% v/v aluminium hydroxide was added as an adjuvant. The pH was adjusted to 7.3 using sodium hydroxide solution.

The safety for laboratory animals was assessed on 25 Guinea pigs, divided into 5 batches of 5 animals/batch. Batches I - IV were administered subcutaneously a different dose of vaccine each, as follows: batch I - 0.5 ml, batch II - 1 ml, batch III - 1.5 ml, and batch IV - 2 ml. The animals in batch 5 represented the control group and were administered 1 ml of PBS/animal. All the animals included in the experiment were vaccinated twice, the booster being administered 3 weeks after the first

vaccination. The animals were monitored daily throughout the experiment and an additional 14 days after the booster, to assess any adverse reactions following vaccination, such as an increase in body temperature, loss of appetite, depression, and other local or systemic reactions attributed to the vaccination.

Safety tests were also performed on the target species. For this experiment, 11 healthy sheep were selected of different ages and physiological status. The test subjects were 2 adult males, two pregnant females, two lactating females and two lambs. The animals were administered two doses of vaccine (2 ml/dose), 21 days apart, subcutaneously. The control group consisted of three sheep (1 male, 1 female and 1 lamb), which were inoculated with sterile PBS following the same protocol. The animals' health status was monitored daily for 35 days following the first vaccination.

The efficacy test for the auto-vaccine was performed using four batches (A, B, C and D) of 10 Balb/C mice each. Batches A and B represented the control animals, and were administered two doses of sterile PBS, 0.5 ml/animal, s.c., 21 days apart. Batches C and D contained the test subjects, which were vaccinated with two doses 0.5 ml auto-vaccine/animal, at 21 days interval. Thirty-five days after the first inoculation, mice belonging to group A and group C were administered intraperitoneally a suspension of *P. multocida* with a concentration of 200 CFU/ml at a dose of 1 ml/animal. Mice belonging to groups B and D were administered intraperitoneally a suspension of *P.* spp. with a concentration of 200 CFU/ml at a dose of 1 ml/animal. Animals were clinically monitored for 7 days after the control infection, noting clinical signs and time of death. At the end of the 7 days of monitoring, all animals in agony, which during the course of the experiment showed clinical signs specific to *Pasteurella* infection, were considered dead and were euthanized. At the end of the experiment, all remaining live animals were euthanized. All of the mice included in the study were subjected to necropsy. Biological samples were collected for bacteriological examination during the necropsy. In the case of mice from the control groups, bacteriological examination was performed to demonstrate the presence of the pathogen. In vaccinated mice, bacteriological examination was carried out to confirm or exclude the presence of the pathogen in the tissues of the animals.

RESULTS AND DISCUSSIONS

The pathogenicity test results for the two strains of *Pasteurella* showed that both isolates caused the death of over 50% of the inoculated mice at a dose of 20 CFU/ml.

In group 2a, 100% mortality was recorded within 24 hours of inoculation; for group 2b, 100% mortality was recorded within 30 hours; for group 2c, 60% mortality was recorded within 36 hours, with surviving mice showing severe clinical signs of respiratory failure. Mice in group 2d survived throughout the experiment without clinical signs of disease (Table 2).

Following the experiment, it was determined that the bacterial isolate of *P.* spp. is pathogenic for mice at a dose of 20 CFU/ml.

In group 3a, all individuals died within 16 hours of inoculation, achieving 100% mortality; all individuals in group 3b died within 22 hours of inoculation, achieving 100% mortality; 4 of the 5 mice belonging to group 3c died within 28 hours, with a mortality rate of 80%, and the fifth mouse survived to the end of the experiment, showing severe respiratory distress; all individuals belonging to group 3d survived, 2 of them showing signs of apathy (Table 3).

Table 2. Pathogenicity test results for *Pasteurella* spp.

Infectious dose (CFU/ml)	Dead subjects	Mortality rate
2000 CFU/mL	5/5	100%
200 CFU/mL	5/5	100%
20 CFU/mL	3/5	60%
2 CFU/mL	0/5	0%

Legend: CFU/ml - colony forming units per millilitre

Table 3. Pathogenicity test results for *Pasteurella multocida*

Infectious dose (CFU/ml)	Dead subjects	Mortality rate
2000 CFU/mL	5/5	100%
200 CFU/mL	5/5	100%
20 CFU/mL	4/5	80%
2 CFU/Ml	0/5	0%

Legend: CFU/ml - colony forming units per millilitre

The experiment demonstrated that the isolated *P. multocida* strain is pathogenic for mice at a dose of 20 CFU/ml. Post mortem examinations revealed predominantly vascular lesions, including congestion, haemorrhages and oedema in most internal organs in the mice that died from the infection. The lung was the main organ affected, with pulmonary oedema and lobar congestion found in most cases. The liver was congested, with haemorrhagic areas and in some cases areas of focal necrosis. The kidneys contained haemorrhages and areas of necrosis. Most of the cadavers showed lesions characteristic of sepsis, such as absence of blood coagulation and splenomegaly. In mice infected with *P. multocida*, the lesions found at necropsy were more severe compared to lesions caused by *P.* spp. infection.

No pasteurellas were isolated from the tissues and organs of the control subjects.

For the subjects in batch 3, infected with *P. multocida*, for group 3a the mean bacterial titre obtained from liver, kidney and lung tissues was 2 x 10^9 CFU/g. For samples collected from cadavers belonging to group 3b, the mean bacterial titre was 7.16 x 10^8 CFU/g, with the highest concntration of bacteria in the kidneys. The mean bacterial titre for for the tissue samples of group 3c was 8.37 x 10^5 CFU/g. For group 3d, from individuals infected with the 2 CFU/ml dose and euthanised after 72 hours, no pasteurellas were recovered.

For the subjects belonging to batch 2, infected with *P.* spp., the mean bacterial titer obtained from tissue samples of mice in group 2a was 4.46 x 10^8 CFU/g. For samples collected from cadavers belonging to group 2b, the mean bacterial titre was 2.67 x 10^7 CFU/g. The mean bacterial titre for the tissue samples belonging to group 2c was 7.1 x 10^4 CFU/g. No pasteurellas were recoverd from the organs of the mice belonging to group 2d, infected with the 2 CFU/ml dose and euthanised after 72 hours.

The cultivation process for the two bacterial isolates resulted in a final concentration of 3 x 10^9 CFU/ml *P. multocida* and 2 x 10^9 CFU/ml *P.* spp. for the bacterial suspensions. After confirming the sterility of the bulk product, the vaccine was dispensed into brown glass vials with rubber stoppers and aluminium caps (Figure 1).

Figure 1. Pasteurella vaccine - finished product

During the safety trials on laboratory animals, throughout the monitoring period, no change in the general condition of the vaccinated Guinea pigs was observed both after the first dose and after the booster. The subjects showed no modification in appetite and defecation and urination were within physiological limits. In 30% of the vaccinated animals, belonging to batches 3 and 4, a slight increase in body temperature was observed within 24-48 hours after the first vaccination, which subsided after 48 hours without any intervention.

The safety trial on the target species revealed no change in the general condition of the vaccinated individuals after the first dose or after the booster. In vaccinated animals, a mild inflammatory reaction was observed at the site of administration within the first 24-72 hours after vaccination, which subsided after 72 hours without treatment. Body temperature remained within physiological limits throughout the clinical monitoring period. No local or systemic reactions were observed in all individuals belonging to the control groups during the 35 days of observation. The results of the *in vivo* tests performed to assess the safety of the *Pasteurella* auto-vaccine have confirmed that the product can be safely administered to both laboratory animals and target species, regardless of the individuals' age and physiological status.

The challenge test performed to determine the efficacy of the auto-vaccine resulted in all mice belonging to batches A and B (control batches) dying within 25 hours of infection. All individuals belonging to batches C and D survived throughout the monitoring period without showing clinical signs of disease.

Necropsy and bacteriological examination of biological samples collected from the dead mice following the control infection established the cause of death as *Pasteurella* septicaemia. Biological samples collected from vaccinated mice subjected to control infection and euthanized were found to be free from *Pasteurella* during bacteriological examination.

The results obtained during the challenge test have demonstrated the fact that vaccination with a high concentration of bacterins has successfully immunized the test subjects, and was able to prevent death, clinical signs of disease and tissue colonization by the two pathogens. Further research will determine the degree of seroconversion that can be obtained by vaccinating the target species with the auto-vaccine.

In the efforts to control pasteurellic pneumonia in sheep, vaccination is the best practical alternative to antibiotic therapy, used both to control and decrease the incidence of the disease and to minimize the use of antimicrobials in economically valuable animals (Verma and Jaiswa, 1998; Kehrenberg et al., 2001). Attempts to produce an effective vaccine against *P. multocida* have not always been successful, with researchers concluding that immunization against sheep pasteurellosis requires either the identification of a bacterial strain that elicits a broad-spectrum immune response or the discovery of an attenuated strain suitable for vaccine production, while establishing optimal cultural conditions to allow expression of a common immunizing antigen. With such strains not having yet been identified, the best alternative remains preparing auto-vaccines from pathogenic strains isolated from the field (Cameron and Bester, 1983). The studies of Berhe et al., (2017) also support this idea, having demonstrated that most animals in a geographical area were infected with at least 4 serotypes of *P. multocida*, which is why they recommend immunising animals with a product containing all the strains or serotypes responsible for the infection. The use of auto-vaccine against pasteurellosis is also supported by a study which shows that following the use of a commercial *Pasteurella* vaccine in a disease outbreak, when tested by indirect haemagglutination using strains from the outbreak, an immune response was obtained in only 16.7% of the vaccinated herd (Qasim et al., 2022).

CONCLUSIONS

The *P. multocida* and *P.* spp. bacterial isolates were pathogenic for mice at the minimum dilution of 20 CFU/ml, and were declared suitable for vaccine production.

For immunoprophylactic purposes, a *Pasteurella* auto-vaccine was prepared from the two bacterial strains isolated from the outbreak.

The auto-vaccine was formulated to a final concentration of 3 x 10^9 CFU/ml *P. multocida* and 2 x 10^9 CFU/ml *P.* spp., inactivated with formalin and adjuvanted with aluminium hydroxide.

To establish the safety of the product administration in animals, tests were performed on both laboratory animals and the target species. The tests showed that the vaccine did not cause systemic adverse reactions and that local reaction at the inoculation site were transient and of low intensity.

To determine the efficacy of the product, a challenge test was performed on mice, whereby the vaccinated individuals remained clinically healthy and necropsy investigation showed no changes specific to the exposure of mice to *Pasteurella*. Necropsy and bacteriological investigation of mice in the control group established the cause of death as septicaemia caused by *P. multocida* and *P.* spp. infection, respectively.

The preliminary trial results described in the current paper demonstrate that the auto-vaccine produced with the field isolates was safe to administer to animals and successful in protecting the vaccinated mice in the challenge test.

REFERENCES

Aitken, I. D. (2007) *Diseases of sheep*, Fourth Edition, Oxford, U. K.: Blackwell Publishing House.

Berhe, K., Weldeselassie, G., Bettridge, J., Christley, R. M., Abdi, R. D. (2017). Small ruminant pasteurellosis in Tigray region, Ethiopia: marked serotype diversity may affect vaccine efficacy. *Epidemiology & Infection, 145*(7): 1326-1338.

Boumart, Z., Bamouh, Z., Semmate, N., Tadlaoui, K. O., El Harrak, M. (2021). Biological Characterisation and Pathogenicity of a *Pasteurella multocida* Isolate from Sheep in Morocco. *Journal of Agricultural Science and Technology A,11*: 53-64.

Cameron, C. M., Bester, F. J. (1983). The inefficacy of polivalent *Pasteurella multocida* vaccines for sheep. *Onderstepoort J. vet. Res., 50*: 101-104.

Hailu, S. M., Kitila, D. B., Gemeda, A. E., Tarekegn, M. (2017). *Pasteurella* organism: Its isolation and identification from pneumonic lungs of goats in Ethiopia. *Journal of Advanced Veterinary and Animal Research, 4*(2): 147–154.

Kehrenberg, C., Schulze-Tanzil, G., Martel, J. L., Chaslus-Dancla, E., Schwarz, S. (2001). Antimicrobial resistance in *Pasteurella* and *Mannheimia*: epidemiology and genetic basis. *Veterinary Research, 32*: 323–339.

Manzat, M. (2001). Boli *Infectioase ale Animalelor – Bacterioze*. Bucharest, RO: Brumar Publishing House.

Mogoş, G., Bulgaru, A., Negru, E., Dinu, H., Daneş, M., Daneş, D. (2022). Isolation and identification of two Pasteurella strains, responsible for an outbreak of pneumonia in sheep. *Scientific Works. Series C. Veterinary Medicine, 68*(2): 78 - 82.

Odugbo, M. O., Odama, L. E., Umoh, J. U., Lamorde, A. G. (2006). *Pasteurella multocida* pneumonic infection in sheep: Prevalence, clinical and pathological studies. *Small Ruminant Research, 66*(1–3): 273-277.

Özyildiz, Z., Tel, O. Y., Yilmaz, R., Özsoy, Ş. Y., Keskin, O. (2013). Pathological and microbiological investigations of pneumonic pasteurellosis in sheep. *Kafkas Univ Vet Fak Derg, 19*(1): 103-108.

Qasim, M. T., Mahdi Mohammed Alakkam, E., Mahdi Mohammed, M., Hachim, S. K., Sabah Jabr, H., Emad Izzat, S., D Al-Dhalemi, M. (2022). Ovine Pasteurellosis Vaccine: Assessment of the Protective Antibody Titer and Recognition of the Prevailing Serotypes. *Archives of Razi Institute, 77*(3): 1207-1210.

Sadeghian, S., Mokhberdezfouli, M. R., Kojouri, G. A., Bazargani, T. T., Tavasoli, A. (2011). *Pasteurella multocida* pneumonic infection in goat: Hematological, biochemical, clinical and pathological studies. *Small Ruminant Research, 100* (2-3): 189-194.

Valadan, M., Jabbari, A. R., Niroumand, M., Tahamtan, Y., Bani Hashemi, S. R. (2014). Isolation and identification of *Pasteurella multocida* from sheep & goat in Iran. *Archives of Razi Institute, 69*(1), 47-55.

Verma, R. S., Jaiswa, T. N. (1998). Haemorrhagic septicaemia vaccines. *Vaccine, 16*: 1184–1192.

Watson, P. J., Davies R. L. (2002). Outbreak of *Pasteurella multocida* septicaemia in neonatal lambs. *Veterinary Record, 151*: 420-422.

Weiser, G. C., Delong, W. J., Paz, J. L., Shafii, B., Price, W. J., Ward, A. C. S. (2003). Characterization of *Pasteurella multocida* associated with pneumonia in Bighorn sheep. *Journal of Wildlife Diseases, 39*(3): 536–544.