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Efficacy of inactivated *Parapoxvirus ovis* paraimmune activator as a prophylaxis against mastitis and therapy for subclinical mastitis in dairy cattle

Tuğçe Demirlek[1]*, Ahmet Sabuncu[2], Sinem Özlem Enginler[2], Baran Çelik[3], Ömür Koçak[4]

[1] The Institute of Medical Sciences, Istanbul University, Istanbul, Turkey; [2] Department of Obstetrics and Gynaecology, Faculty of Veterinary Medicine, Istanbul University-Cerrahpaşa, Istanbul, Turkey; [3] Department of Microbiology, Faculty of Veterinary Medicine, Istanbul University-Cerrahpaşa, Istanbul, Turkey; [4] Department of Animal Breeding and Husbandry, Faculty of Veterinary Medicine, Istanbul University-Cerrahpaşa, Istanbul, Turkey.

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Abstract

Mastitis is the most important disease in dairy cattle industry because of its high economic losses both in herd management, milk and milk products. The aim of this study was to determine the efficacy of inactivated *Parapoxvirus ovis* (IPPVO) Para immune activator as a prophylaxis against mastitis and as the therapy for subclinical mastitis in dairy cattle. The prophylactic effects of IPPVO were investigated in California mastitis test (CMT). Healthy Holstein cows were divided into A1 (n = 30) and A2 (n = 30) subgroups. In addition, 90 subclinical mastitis Holstein cows were divided into subgroups of B1, B2, and B3 to investigate the efficacy of IPPVO treatment. A significant difference in CMT levels was observed ($p < 0.01$) 30 day after treatment in A groups. The difference in somatic cell count (SCC) levels between the A groups 15, 30 day after treatment was significant ($p < 0.01$). The results of the CMT among the B groups showed no statistically significant difference ($p > 0.05$). The results of the SCC tests showed no statistically significant difference ($p > 0.05$) among the B groups on days 0, 9 and 15 after treatment. Coagulase-negative *Staphylococcus* (n = 53) and *Escherichia coli* (n = 30) were the most prevalent bacteria isolated in this study. In conclusion, IPPVO, although had no additional effect when used in combination with antibiotics could possibly be used instead of antibiotics and to protect cattle from subclinical mastitis, however, it is not known how long this prophylaxis effect could last.

Introduction

Mastitis is the most important disease in dairy cattle industry because of its high economic losses both in herd management, milk and milk products.[1-3] Several microorganisms, among other factors, are involved in the onset of mastitis.[4,5] An inactivated *Parapoxvirus ovis* D1701 strain (IPPVO) is a microbial-derived nonspecific immune-modulating's virus that stimulates natural immunity, especially that against infectious diseases.[6] The use of IPPVO in calves has been found to elevate malondi-aldehyde in blood parameters related to oxidative stress leading to lipid peroxidation, a decrease in blood glutathione levels, and a decrease in antioxidant levels.[7-9] The IPPVO has an effect on the release of proinflammatory and anti-inflammatory cytokines from human immune cells of T-lymphocytes and natural killer cells. Accordingly, it has a regulatory impact on inflammation and immune-logical responses.[10] In addition, it is believed that the immunostimulatory effects of IPPVO also stimulates the immune system of mammary tissue.[11]

The IPPVO has been proved to be an immune-stimulator,[12] but has not been studied as a prophylaxis for mastitis or in the treatment of subclinical mastitis. The aim of this study was to investigate that IPPVO could positively affect a prognosis when used in combination with antibiotics in the treatment of subclinical mastitis and provide protection of dairy cattle from the formation of mastitis when used prophylactically.

Materials and Methods

Animals. Holstein cows (n = 150) from a dairy farm aged 3-6 years and in their first through sixth month of

***Correspondence:**

Tuğçe Demirlek. DVM
The Institute of Medical Sciences, Istanbul University, Istanbul, Turkey
E-mail: tugcedemirlek@hotmail.com

lactation were used in this study. The cows were milked twice a day and kept individually in 10.00 m^2 stalls. The animals were received a complete diet prepared according to their nutritional requirements by Department of Animal Breeding, Faculty of Veterinary Medicine, Istanbul University-Cerrahpaşa. An approval by Istanbul University Animal Research and Ethics Committee was obtained on November 24, 2015 (verdict number: 2015/99).

Selection of the animals and experimental design. The milk samples with clots, blood clots, blood, or pus were evaluated as clinical mastitis and were excluded from the study. The study was conducted on the milk samples of healthy cows and cows with subclinical mastitis. Sixty cows with all mammary lobes testing negative and suspicious for California mastitis were accepted as "healthy" and were confirmed by microbiological analysis. One mammary lobe from each animal was selected and included in the study. Ninety cows with only one mammary lobe testing positive for subclinical mastitis were accepted for this study. Thus, 60 CMT (-) (Group A) and 90 CMT (+) (Group B) cows were used. Group A (prophylaxis group), the CMT (-) cows, was divided into the following two subgroups: A1, experimental group (n = 30) and A2, control group (n = 30). A volume of 2.00 mL Zylexis® (Zoetis, Istanbul, Turkey), an immunostimulant containing IPPVO, was administered intramuscularly (IM) to A1 cows on days 0, 2, and 9. The same amount of normal saline was administered to A2 cows on the same days. Milk samples were collected from both groups on days 0 and 9, 15, and 30 after treatment. Group B, was divided into the following three subgroups: B1 (n = 30), B2 (n = 30), and B3 (n = 30). B1 was given 2.00 mL IPPVO IM on days 0, 2, and 4; B2 was given 2 mL IPPVO IM on days 0, 2, and 4 with an intramammary antibiotic added to this treatment on days 0 and 1. According to antibiogram results, combination of 200 mg ampicillin sodium and 200 mg dicloxacillin sodium (Vilsan, Istanbul, Turkey; BID) was administered to seven cows, and 100 mg cefoperazone dihydrate (Zoetis; SID) was given to 23 cows. Only the intramammary antibiotic was administered to B3 on days 0 and 1. According to the results of the antibiogram in group B3, 200 mg ampicillin sodium and 200 mg dicloxacillin sodium (Vilsan; BID) was given to five cows, and 100 mg cefoperazone dihydrate (Zoetis; SID) was given to 25 cows. Milk samples were collected on days 0, 9 and 15 after treatment from all three groups.

Milk sample evaluation. A strip cup test, CMT, SCC, electrical conductivity (EC) test and microbiological examinations were conducted on the collected milk samples. Antibiotic susceptibility testing of each isolate was also used to determine antibiotic susceptibilities for the study. The first few streams of foremilk were discarded, the paddle was held in place to fit the teat, and a few milliliters of milk were taken. The control samples were those with no abnormal milk odor or color.

The CMT was conducted on any mammary lobes without visible changes to detect those without mastitis or with subclinical mastitis. To conduct CMT, the first milk was discarded and 2.00 mL subsequent milk was drained into the test chamber with 1.00 - 2.00 mL CMT solution (INOBIO, Siauliai, Lithuania). The samples were stirred and any observed changes were evaluated. The mammary lobes with CMT (–) were recorded as non-mastitic (healthy) and those with CMT (+) were recorded as having subclinical mastitis. Only one mammary lobe from this group was included in the study and followed up. The somatic cells were counted (10^3 cell mL^{-1}) using the Somacount 150 counting device (Bentley Instruments Inc., Chaska, USA), which uses flow cytometry. The EC value of the milk was measured using the LactoStar milk analyzer (Funke-Gerber, Berlin, Germany). Each milk sample was streaked onto Columbia Agar with 5.00% sheep blood (Thermo Fisher Scientific, Wesel, Germany), MacConkey Agar (Thermo Fisher Scientific), Dermatophyte test medium (HiMedia, Einhausen, Germany), and Saboroud dextrose agar (HiMedia) for microbiological examination and incubated for 24 - 72 hr under aerobic and micro-aerobic conditions at 37.00 ˚C.[13,14] Isolates were preliminarily identified based on hemolysis characteristics, the catalase test, the cytochrome-oxidase test, and Gram staining. Species were identified using either standard bacteriological methods[13,14] or gram-positive combo (PMIC/ID-100) and gram-negative combo panels (NMIC/ID-123) using the BD Phoenix™ automated biochemical identification system (BD Diagnostic Systems, Sparks, USA). The antibiotic susceptibilities of each isolate were determined using the disc diffusion test[15] and BD Phoenix™ (BD Diagnostic Systems). Ampicillin, ampicillin/sulbactam, amoxicillin, amoxicillin/clavulanic acid, cefoperazone, ceftiofur, enrofloxacin, erythromycin, gentamycin, streptomycin, and tetracycline discs (HiMedia) were used to determine antibiotic susceptibilities.

Statistical analyses. The Chi-squared test was used to compare the CMT results. The Student's *t*-test was used for the A groups that were CMT (–) and the one-way analysis of variance test was used to compare the B groups that were CMT (+). Duncan's multiple range test was used to control intergroup significance. SPSS Software (version 13.0; SPSS Inc., Chicago, USA) was used to analyze the data.

Results

The results of this study showed that the difference in CMT between groups A1 and A2 on days 0,9 and 15 day after treatment was not statistically significant ($p > 0.05$), however, on day 30 after treatment, the difference in CMT (0) and CMT (suspicious) between A1 and A2 groups was statistically significant ($p < 0.01$), (Table 1). Any microbiological agent was not isolated on days 0 and 30 in group A.

In this study, SCC (10^3 cells mL^{-1}) results of A groups on day 0 were found as 67.47 ± 11.20 in group A1 and 65.67 ± 10.18 in group A2. On day 9, 62.73 ± 9.34 in group A1 and 153.70 ± 9.34 in group A2, on day 15, 60.17 ± 10.88 in group A1 and 248.93 ± 81.77 in group A2, on day 30, 64.47 ± 18.59 in group A1 and 315.67 ± 104.54 in group A2. According to SCC data, a statistically significant difference ($p > 0.05$) was not observed at days 0 and 9 but was observed ($p < 0.01$) on days 15 and 30 after treatment between A1 and A2.

Electrical conductivity results (msec per cm) of A groups on day 0 were found as 2.84 ± 0.07 in group A1 and 2.66 ± 0.06 in group A2, on day 9, 2.84 ± 0.07 in group A1 and 2.65 ± 0.06 in group A2. On day 15, 2.80 ± 0.07 in group A1 and 2.67 ± 0.06 and on day 30, 2.79 ± 0.07 in group A1 and 2.67 ± 0.06 in group A2. The result of the EC tests showed a significant difference between A1 and A2 on day 0 ($p < 0.05$). The difference between the groups on the other days according to EC results was not statistically significant ($p > 0.05$).

The results of the CMT tests among the B groups showed no statistically significant difference ($p > 0.05$), (Table 2). Somatic cell count (10^3 cell mL^{-1}) in the milk samples from the three B groups on days 0, 9 and 15 after treatment tended to decrease, and the difference among the groups was not statistically significant ($p > 0.05$), (Table 3). There was a statistically significant difference in EC results between group B3 and the other B groups on days 0,9, and 15 after treatment ($p < 0.01$), (Table 4).

All CMT (+) samples (n = 90, all B groups) were positive for microbiological growth on day 0. In 58 samples, a single bacterium was present, and in 32 samples, two different microorganism species were cultured. The isolated bacteria species and their distribution among the B groups are shown in Table 5. *Escherichia coli* (n = 30) and *Staphylococcus* spp. (n = 28) were determined to be the most isolated bacteria species, followed by *Streptococcus agalactiae* (n = 9) and *Klebsiella pneumoniae* (n = 7).

An antibiotic susceptibility test was conducted on 120 of the 122 isolated bacteria (test was not performed on the remaining two *Malassezia* spp. because they were fungi species). The sensitivity ratios of the bacterial species to the tested antibiotics are shown in Table 6 (supplement 1).

Table 3. Somatic cell count (10^3 cells mL^{-1}) of milk sample results for groups B1, B2 and B3 (n = 30). Data are shown as mean ± SD.

Day	Group B1	Group B2	Group B3	*p-value*
0	1169 ± 182	1306 ± 137	939 ± 111	> 0.05
9	787 ± 140	704 ± 138	924 ± 181	> 0.05
15	947 ± 167	513 ± 137	738 ± 143	> 0.05

Table 4. Electrical conductivity (msec per cm) results for groups B1, B2 and B3 (n = 30). Data are shown as mean ± SD.

Day	Group B1	Group B2	Group B3	*p-value*
0	3.95 ± 0.09 [b]	4.05 ± 0.11 [b]	4.45 ± 0.10 [a]	< 0.01
9	3.84 ± 0.09 [b]	3.97 ± 0.11 [b]	4.39 ± 0.11 [a]	< 0.001
15	3.86 ± 0.09 [b]	3.94 ± 0.10 [b]	4.35 ± 0.11 [a]	< 0.01

[ab] Mean values within the same row with different superscript small letters are significant.

Discussion

Mastitis is an economically devastating disease in dairy cattle and their breeding programs.[16,17] Several infectious microorganisms in addition to environmental factors play a role in the etiology of the disease,[17], therefore, prevention and treatment are important, especially because vaccination for this disease is ineffective.[5,17-21] The commercial preparation containing the inactive Parapoxvirus ovis D1701 strain is nonspecific immunostimulatory and stimulates natural immunity to infectious diseases. The inactivated Parapoxvirus ovis paraimmunity activator stimulates proliferation of lymphocytes and increases the release of interferons and interleukins from lymphocytes. It has been reported that IPPVO is effective in cell binding of microorganisms causing infectious diseases, activation of cells in the immune system, communication between cells, identification of cells and lowering of antioxidant levels.[8,10]

Table 1. California mastitis test (CMT) results for groups A1 and A2.

Day	CMT scores										*p-value*
	Group A1 (experiment group)					Group A2 (control group)					
	0	Suspicious	+1	+2	+3	0	Suspicious	+1	+2	+3	
0	18	12	0	0	0	20	10	0	0	0	> 0.05
9	21	9	0	0	0	18	8	2	2	0	> 0.05
15	22	8	0	0	0	14	11	2	3	0	> 0.05
30	23[a]	4[b]	3	0	0	13[c]	12[d]	2	3	0	< 0.01

[abcd] Mean values within the same row with different superscripts are significant at $p < 0.05$.

Table 2. California mastitis test results for groups B1, B2 and B3.

Day	Group B1					Group B2					Group B3					*p-value*
	0	Suspicious	+1	+2	+3	0	Suspicious	+1	+2	+3	0	Suspicious	+1	+2	+3	
0	0	0	19	10	1	0	0	10	19	1	0	0	16	14	0	> 0.05
9	2	11	7	10	0	2	12	7	8	1	1	11	9	6	3	> 0.05
15	7	6	4	11	2	6	15	3	4	2	4	12	2	12	0	> 0.05

Table 5. Number of the isolated bacteria in groups B1, B2 and B3.

Isolated microorganisms	B1	B2	B3	Total
Corynebacterium spp.	1	-	1	2
Escherichia coli	13	3	1	17
Escherichia coli-Staphylococcus warneri	-	-	1	1
Escherichia coli-Klebsiella pneumoniae	-	1	-	1
Escherichia coli- Staphylococcus auricularis	-	-	1	1
Escherichia coli - Staphylococcus hominis	-	1	1	2
Escherichia coli-Staphylococcus spp.	1	1	-	2
Klebsiella pneumoniae	-	1	-	1
Klebsiella pneumoniae- Escherichia coli	-	1	-	1
Klebsiella pneumoniae- Staphylococcus sciuri	-	2	-	2
Klebsiella pneumoniae-Staphylococcus spp.	2	-	-	2
Malassezia spp. - Escherichia coli	-	-	1	1
Malassezia spp. - Proteus spp.	-	1	1	2
Proteus spp. - Escherichia coli	-	-	1	1
Staphylococcus auricularis- Streptococcus pyogenes	-	1	1	2
Staphylococcus capitis	-	1	-	1
Staphylococcus haemolyticus	-	-	1	1
Staphylococcus haemolyticus-Staphylococcus spp.	-	1	1	2
Staphylococcus hyicus	-	1	-	1
Staphylococcus hyicus- Staphylococcus caseolyticus	-	1	-	1
Staphylococcus schleiferi	-	-	1	1
Staphylococcus sciuri	-	-	1	1
Staphylococcus sciuri- Staphylococcus xylosus	-	1	-	1
Staphylococcus spp.	3	3	10	16
Staphylococcus spp. – Streptococcus agalactiae	2	1	-	3
Staphylococcus warneri- Staphylococcus spp.	-	2	1	3
Staphylococcus xylosus	-	2	4	6
Streptococcus dysgalactiae	-	2	-	2
Streptococcus dysgalactiae- Staphylococcus auricularis	-	-	1	1
Streptococcus spp.	-	-	1	1
Streptococcus uberis	3	2	-	5
Streptococcus agalactiae	2	1	-	3
Streptococcus agalactiae- Escherichia coli	3	-	-	3

In addition, IPPVO has been used in various animals for immunomodulation. Cows, cats, dogs, horses, mice and catfish have positive effects on the immune system.[6] In a study investigating the immunostimulatory effects of IPPVO, it was reported that the agent stimulates interferon release, its effect may last for three weeks and may stimulate the mammary gland immune system.[11] In cows, mastitis is the first reaction to the entry of pathogens from the nipple, neutrophil migration from the blood to the site of infection occurs.[22,23] The most common cells in healthy mammary glands are macrophages and they are the observer of the mastitis agents. Macrophages and epithelial cells secrete chemical stimulants and carry out the migration of neutrophils into the field. Tumor necrosis factor, production and release of interferons and interleukins together with the inflammatory reaction begins.[24] The resulting inflammatory reaction increases the vascular permeability and causes the migration of neutrophils to the mammary gland.[25] In this study, we believe that IPPVO stimulates the udder defense system by causing interferon increase.

In the present study, a statistically significant difference was observed on day 30 ($p < 0.01$) when there was no significant difference on day 0, 9 and 15 according to the CMT results in A groups ($p > 0.05$). On day 9 and 15 the difference between the CMT results in both groups was statistically insignificant suggested that either the infection was not encountered or the cows were protected even if the infection was encountered. The statistically significant difference between A1 and A2 on day 30 after treatment was attributed to the fact that IPPVO had a prophylactic effect in A1.

The increase in SCC in A2 group 15 and 30 days after treatment might be an indicator of the onset of mastitis, however, the negative result of bacteriological isolation on these days thought us to be any other reason instead of bacteriological growth. Accordingly, the statistical significance of the difference between the two groups was interpreted to reflect the prophylactic effect of IPPVO in group A1. SCC results paralleled those of CMT, which confirmed both 30-day findings.

Aydın and İşcan[16] and Philpot and Nickerson[26] have reported that CMT and SCC results can overlap in mastitis cases. In this study, a correlation between CMT and SCC results showed that the data could be safely interpreted in the diagnosis and follow-up of mastitis cases. In the light of our study results using the microbiological examination methods would be more valuable for diagnosis and follow up of the disease.

There was no significant difference ($p > 0.05$) between A groups according to EC results. It was understood that there was a parallelism between all these data and that they were compatible with diagnostic methods. When SCC and CMT scores and EC results were evaluated together in group A, a discrepancy was identified among the EC results. EC values are explained as an increase in conductivity resulting from an increase in Na^+ and Cl^- ratios in milk.[16,17,19,27,28]

According to the study conducted by Küplülü *et al.,*[29] the CMT results and EC results were parallel, while Timurkan has shown that CMT and EC results were incompatible and has stated that it was insufficient to use only EC results to diagnose subclinical mastitis.[30] In our study, the mismatch between the EC values and CMT and SCC results was similar to that in the Timurkan's study.[30]

When the CMT results between groups B were tested, there was no statistically significant difference ($p > 0.05$) between the groups suggesting that IPPVO might be just as effective whether combined with antibiotics.

There was no statistically significant difference among the groups on days 0, 9 and 15 on evaluation according to SCC results ($p > 0.05$). The statistical difference between the groups B2 and B3 when using IPPVO according to SCC and CMT scores showed that the use of IPPVO was not effective when used in combination with antibiotics in sub-clinical mastitis treatment. However, finding no difference between B2 and B3 groups and B1 group suggested that IPPVO could be used alone instead of antibiotics and had a treating feature as much as antibiotics.

When EC results were compared, there was a statistically significant difference ($p < 0.001$) between groups B3 and other B groups on day 9. On the 15th day there was a statistically significant difference ($p < 0.01$) between the B3 group and the other B groups. When SCC and CMT scores and EC results were evaluated together from all B groups, inconsistencies were found among the results, which was attributed to the unreliability of the interpretation of mastitis using EC values. It has been reported that IPPVO applications reduce the symptoms of inflammation in various animals and allow the recovery of sick animals within a shorter time period.[6] It was interpreted that inflammation was reduced after IPPVO treatment in groups B1 and B2, but that inflammation was not reduced in B3, the group without IPPVO treatment, however, it has also been suggested that using the EC values alone for evaluating mastitis is insufficient.[19,31]

In worldwide studies, *S. aureus*, coagulase-negative *staphylococcus* (CoNS), *E. coli, Enterobacter* spp., *Streptococci, K. pneumoniae*, and *Bacillus* spp. have been reported to be the most frequently isolated bacteria.[5,32-34]

In this study, coagulase-negative *Staphylococcus* (n = 53) and *E. coli* (n = 30) were determined as the most isolated bacteria species. It has been reported that coagulase-negative *Staphylococcus* spp. have been isolated as the primary pathogen in subclinical mastitis cases in several countries.[35] In this study, an antibiotic sensitivity test revealed that the isolated bacteria were most sensitive to enrofloxacin, cefoperazone, and ceftiofur and most resistant to amoxicillin, ampicillin, and tetracycline,[36-40] but the sensitivity of bacteria to antibiotics varies greatly among different countries. In general, the bacteria isolated in mastitis and their antibiotic susceptibilities have been evaluated, and reported that the number of resistant isolates in developed countries are less than that in middle and underdeveloped countries.[41,42]

In conclusion, in the treatment of mastitis, IPPVO, although had no additional effect when used in combination with antibiotics; it could possibly be used instead of antibiotics. The results suggested IPPVO could be used to protect cattle from subclinical mastitis, however, it is not known how long this prophylaxis effect can last. Therefore, more long-term studies are necessary to confirm its efficacy. When the costs of immune activators, the cost of treatment, and yield loss from mastitis were compared, it was believed that the animal had to be monitored over several lactation periods to avoid long-term economic losses. In the future, the idea that immunomodulator drugs, which are thought to be included in mastitis-control programs, are open to development in dairy cattle industry.

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Conflict of interest

The authors hereby declare there is no conflict of interest which affects the outcome of this paper.

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