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Vadivoo VS
Assistant Professor, Veterinary University Training and Research Centre, Tamil Nadu Veterinary and Animal Sciences University, Tirupur, Tamil Nadu, India

Dhinakar Raj G
Director of Animal Health Studies, Tamil Nadu Veterinary and Animal Sciences University, Madhavaram milk Colony, Chennai, Tamil Nadu, India

Balasubramanian S
Director of Clinics, Tamil Nadu Veterinary and Animal Sciences University, Madhavaram milk Colony, Chennai, Tamil Nadu, India

Suresh Subramanion B
Professor and Head, Livestock Business Management, Madras Veterinary College, Chennai, Tamil Nadu, India

Kumanan K
Professor and Head, Department of Bioinformatics & ARIS Cell, Madras Veterinary College, Chennai, Tamil Nadu, India

Correspondence
Vadivoo VS
Assistant Professor, Veterinary University Training and Research Centre, Tamil Nadu Veterinary and Animal Sciences University, Tirupur, Tamil Nadu, India

Elucidation of possible antibacterial effects of statins against primary pathogens of mastitis in cows

Vadivoo VS, Dhinakar Raj G, Balasubramanian S, Suresh Subramanion B and Kumanan K

Abstract
Statins act as selective and competitive inhibitors of HMG-CoA reductase thus inhibiting the synthesis of cholesterol in the liver. In addition to this activity, the other pleiotropic effects of statins are anti-inflammatory, immunomodulatory, antioxidant, antimicrobial activity and anticoagulant activities. The aim of our study was to assess the effect of statins on the bovine mastitis causing pathogens. Simvastatin one of the classes of statins was used in this study to elucidate its antimicrobial property against *Staphylococcus aureus* isolated from bovine mastitis milk. Simvastatin at the minimum concentration of 31.25 µg/ml produced a maximum percent reduction in the growth of the *S. aureus* based on the cfu/ml. The maximum inhibitory effect of the Simvastatin was noticed at 4 hours. Simvastatin produced inhibition of all the four *S. aureus* isolates from mastitis milk. Based on this pilot *in vitro* study, Simvastatin at concentration of 31.25 µg /ml had an antibacterial effect on the *Staphylococcal* isolates from mastitis milk.

Keywords: Simvastatin, antimicrobial effect, *Staphylococcus aureus*, bovine mastitis.

Introduction
Statins (HMG-CoA reductase inhibitors) are today some of the most prescribed drugs to humans in the world due to their beneficial effects on cardiovascular disease. (Blahaa and Martin, 2013) [1]. Statins bind to HMG-CoA reductase and inhibit the rate limiting step of the mevalonate pathway, thus diminishing cholesterol production (Liao, 2005) [6]. Initially research on statins originated with the intention of developing new antibiotics as the inhibition of HMG-CoA reductase, results in the death of microorganisms (Endo, 2010)[3]. The discovery of statins and their potent cholesterol-lowering abilities soon led to their clinical use in preventing cardiovascular diseases instead (Endo, 2010) [3]. In recent years however, interest returned to the inherent antimicrobial effects of statins in humans (Jerwood and Cohen, 2008) [4]. The antibacterial properties of atorvastatin (ATV), fluvastatin (FLV), lovastatin (LVS), pitavastatin (PTV), pravastatin (PRV), rosuvastatin (RSV), and simvastatin (SMV) are the currently registered drugs for lowering cholesterol in humans, thus likely to affect the statin-bacteria-human-environment continuum (Ko *et al.*, 2017) [5].
Mastitis is considered to be the most economically important disease affecting productive cows in the dairy industry. It affects 50% of the herd population and it has been estimated that the mastitis alone can cause approximately 70% of all avoidable losses incurred during milk production (Sumathi *et al*., 2008) [10]. One of the causes for mastitis is bacterial infection caused by organisms such as *Staphylococcus* sp., *Escherichia coli, Streptococcus* sp., *Pseudomonas* sp., etc. Most of these bacteria have developed resistance due to the indiscriminate use of antibiotics leading to chronic mastitis which leads to the extensive destruction of the udder.
Since the resistance of mastitis pathogens to antimicrobial agents is a well-documented challenge in dairy cows, the present study is proposed to elucidate the possible beneficial antibacterial effects of Statins, the HMG-CoA reductase inhibitors, against primary pathogens of mastitis.

Materials and Methods
Source of milk samples: 20 milk samples from clinical mastitis cases were collected from Large Animal Ward of Madras Veterinary College, Chennai, Tamil Nadu.

Isolation of Bacteria: The milk samples were inoculated to nutrient broth and incubated at

37^0C overnight and plated on MacConkey's agar and blood agar plates. Individual colony characteristics and haemolytic patterns on blood agar were noted. The staining and cellular morphological features of organisms were ascertained by microscopic examination of Gram stained smears. Further each culture was subjected to various biochemical tests. The bacteria isolated were identified on the basis of their cultural, morphological and biochemical characteristics.

Determination of antibiotic sensitivity: All the bacteria isolated were tested *in vitro* for their sensitivity to 7 different antibiotics, commonly used in veterinary practice. These included Chloramphenicol, Amoxicillin, Enrofloxacin, Gentamicin, Tetracycline, Penicillin and Ciprofloxacin. The disc diffusion method as described by Miles and Amyes (1996) [8] was employed and the interpretation was made as per the zone size interpretation chart provided by the manufacturer of discs (Himedia, India). For the synergistic effect of statin and the antibiotics, zone of inhibition was checked with combination of Penicillin disc and 31.25 µg Statin, Penicillin disc and 6.25 µL of the solvent Dimethyl sulphoxide (DMSO) and Penicillin disc alone.

Determination of minimum inhibitory concentration for statins: Four isolates of *Staphylococcus aureus* were used in the assay. An overnight broth culture was inoculated in Mueller-Hinton broth and allowed to attain 0.5 optical density (OD) at 600nm with a final concentration of 5X 10^6 CFU/ml.5mg of simvastatin (Ranbaxy, India) was dissolved in 1 ml of DMSO (5mg/ml stock concentration). From this stock solution 100 µL (500µg/ml) was added to a sterile 96 well plate. To all other wells 50 µL of Mueller-Hinton broth was added. Two fold serial dilutions of 500µg/ml of simvastatin was made up to a final concentration of 1.9 µg/ml. For the control well 100 µL of pure DMSO was added to the first well which was further two fold serially diluted with 50 µL of Mueller-Hinton broth up to a final concentration of 1.7 µL. 10 µL of bacterial suspension from the stock concentration was added to each well. The plate was incubated for 2, 4, 6, 8 hours at 37^0C and the action of the test drug and that of DMSO in each dilution was noted by plate counting.

Results and Discussion

A total of 6 bacterial isolates were recovered from 20 mastitis milk samples. The predominant bacterial isolates recovered were *Staphylococcus aureus* (25%) and *Escherichia coli* (10%).The bacterial strains were identified and characterized by biochemical tests. The antibiotic sensitivity pattern of the pathogen isolated from bovine mastitis milk to the different antibiotics are given in table 1.

Table 1: Determination of antibiotic sensitivity for *Staphylococcus aureus* isolates (S1-S5) and *E.coli* isolates from Bovine mastitis milk

S. aureus	**Penicillin**	**Tetracycline**	**Enrofloxacin**	**Ciprofloxacin**	**Gentamicin**	**Amoxicillin**	**Chloramphenicol**
Isolate 1	S	S	I	I	R	R	R
Isolate 2	S	I	S	I	R	R	I
Isolate 3	R	R	S	S	I	R	I
Isolate 4	R	I	I	I	S	S	S
Isolate 5	R	R	S	S	I	R	R
E.coli 1	R	R	R	R	R	R	R
E.coli 2	R	R	R	R	R	R	R

R-Resistance, I-Intermediate, S-Sensitive

The optimum time required for maximum inhibitory effect of Simvastatin on the multiplication of *Staphylococcus aureus* culture are shown in table 2. The maximum inhibitory effect of the Simvastatin was noticed at 4 hours.

Table 2: Effect of Simvastatin on *Staphylococcus aureus* at various time intervals

Test Compound	**2 hours $\log_{10}$ cfu/ml**	**4 hours $\log_{10}$ cfu/ml**	**6 hours $\log_{10}$ cfu/ml**	**8 hours $\log_{10}$ cfu/ml**
31.25 µg Simvastatin + 6.2 µL of DMSO+ 10 µL of bacterial suspension	2×10^8	7×10^8	1×10^{11}	5×10^{11}
6.2 µL of DMSO+ 10 µL of bacterial suspension	6×10^{10}	7×10^{11}	8×10^{13}	9×10^{13}
Percent reduction in the multiplication of the bacteria	22.94	25.3	16.13	20.83

The results of the optimum concentration of Simvastatin that produced maximum percent reduction on bacterial growth are given in table 3. Simvastatin at the minimum concentration of 31.25 µg/ml produced a maximum percent reduction in the bacterial growth based on the cfu/ml

Table 3: Effect of various concentrations of Simvastatin and control DMSO on *Staphylococcus aureus*

Simvastatin at different concentrations (µg)	**$\log_{10}$ cfu/ml**	**DMSO at different concentrations (µL)**	**$\log_{10}$ cfu/ml**	**Percent reduction**
500	No growth	100	No Growth	-
250	No growth	50	$1x\ 10^1$	-
125	3×10^4	25	4×10^4	2.72
62.5	2×10^6	12.5	3×10^7	15.73
31.25	1×10^8	6.25	$4x\ 10^{11}$	31.04

The results of Simvastatin at concentration of 31.25 µg/ml on different *Staphylococcus* isolates obtained from mastitis milk at 4 hours time interval and the percent reduction are shown in table 4. All the isolates could be inhibited by Simvastatin with percent reduction ranging from 25 – 38 percent.

Table 4: Effect of Simvastatin on different isolates of *Staphylococcus aureus* obtained from mastitis milk at 4 hours time interval and the percent reduction

Staphylococcus aureus isolates	31.25 µg Simvastatin + 6.2 µL of DMSO + 10 µL of bacterial suspension log_{10} cfu/ml	6.2 µL of DMSO + 10 µL of bacterial suspension log_{10} cfu/ml	Per cent reduction
Isolate 1	7×10^{8}	7×10^{11}	25.3
Isolate 2	2×10^{10}	1×10^{14}	26.43
Isolate 3	6×10^{8}	1.8×10^{14}	38.38
Isolate 4	8×10^{8}	3×10^{13}	33.98

The results of the synergistic action of antibiotic Penicillin, Simvastatin and DMSO by disc diffusion method are shown in table 5 and Fig 1. The disc diffusion method with penicillin discs and combination of the antibiotic disc with 31.25 µg/ml Simvastatin and with DMSO did not show any difference in the zone of inhibition. This indicates that the drug is probably active only against mid logarithmic cultures.

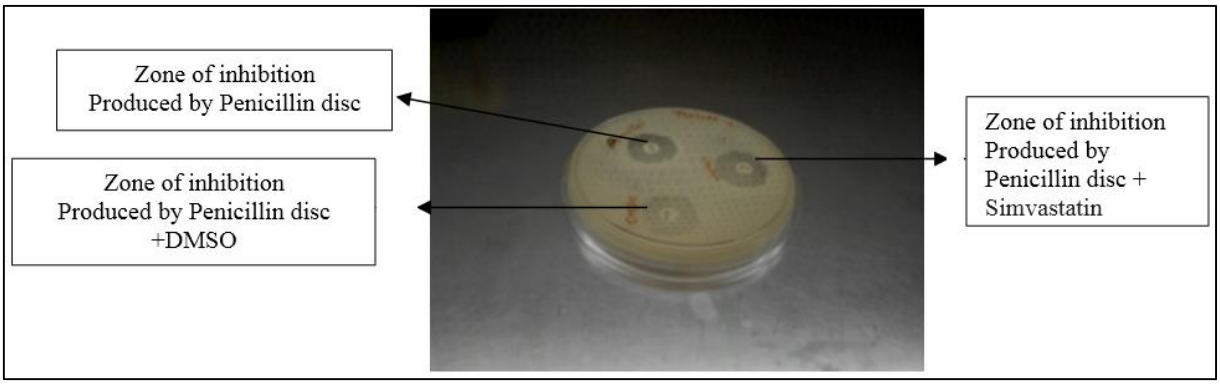

Fig 1: Zone of inhibition to check the synergistic action of antibiotic Penicillin, Simvastatin and DMSO

Table 5: Zone of inhibition for the different *Staphylococcus* isolates (S1-S4) produced by of Penicillin, Simvastatin and DMSO in combinations

	Penicillin (Control) Zone of inhibition mm (A)	Penicillin + 31.25 µg/ml Simvastatin Zone of inhibition mm (B)	Penicillin + 6.25 µL DMSO Zone of inhibition mm (C)	Difference of Zone of inhibition mm (B-C)
Isolate 1	11	13	11	2
Isolate 2	15	17	15	2
Isolate 3	12	13	13	-
Isolate 4	12	14	12	2

Simvastatin at the minimum concentration of 31.25 µg/ml produced a maximum percent reduction in the growth of the *S. aureus* based on the cfu/ml. The maximum inhibitory effect of the Simvastatin was noticed at 4 hours. Simvastatin produced inhibition of all the four *S. aureus* isolates from mastitis milk. Based on the *in vitro* studies, Simvastatin at concentration of 31.25 µg /ml had an antibacterial effect on the *Staphylococcal* isolates from mastitis milk. As far as our literature survey no research has been done on the application of statins as an antimicrobial agent in bovine mastitis. In humans several research has been done on the assessment of the antibacterial effect of statins with different classes; lovastatin, simvastatin, and atorvastatin, alone and in combination with cholesterol on Gram-positive and Gram-negative bacteria isolated from human patients. Lopez *et al.* (2013)[7] concludes that Statin treatment in patients with Staphylococcus aureus bacteremia (SAB) was associated with lower early mortality and persistent bacteremia (PB) and randomized studies are necessary to identify the role of statins in the treatment of patients with SAB. Smit, *et al.* (2017) [9] reported that persons treated with statins experience a decreased risk of community-acquired *Staphylococcus aureus* bacteremia (CA-SAB) as compared with nonusers. Caffrey *et al*., (2017) [2] conducted a national cohort study among patients with *S. aureus* bacteremia, continuation of statin therapy among incident statin users was associated with significant beneficial effects on mortality, including a 54% lower 30-day mortality rate. Simvastatin appears to be the most suitable statin for repurposing as a novel adjuvant antibiotic. However Ko *et al.* (2017) [5], reviews several articles of statins as antimicrobials and concludes that current evidence better supports statins as potential antimicrobial resistance (AMR) breakers, but their role as plausible AMR makers cannot be excluded. Elucidating the mechanism of statins' antibacterial activity is perhaps the most important knowledge gap to address as this will likely clarify statins' role as AMR breakers or makers. The results of Ko *et al.* (2017)[5] states that simvastatin generally exerted the greatest antibacterial activity (lowest MIC) against Gram-positive bacteria when compared to atorvastatin, rosuvastatin, and fluvastatin and atorvastatin generally exhibited similar or slightly better activity against Gram-negative bacteria when compared to simvastatin, but both were more potent than rosuvastatin and fluvastatin.

Conclusion

The major pathogen isolated from bovine mastitis milk was *Staphylococcus aureus* and *E. Coli*. Simvastatin at the minimum concentration of 31.25 µg/ml produced a maximum percent reduction in the growth of the *S. aureus* based on the cfu/ml. The maximum inhibitory effect of the Simvastatin was noticed at 4 hours. Further studies are required to

elucidate the antibacterial effect of the different classes of satins on the different bovine mastitis causing pathogens. A concrete conclusion can be arrived at using statins as an alternate treatment to bovine mastitis by increasing the sample size and also by conducting clinical trials.

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