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Evaluation of Biofilm Formation in Different Capsular Genotypes of *Pasteurella multocida* and the Effects of *Matricaria camomilla* L. on it

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Abstract: In the present study, biofilm formation in different capsular genotypes of *Pasteurella multocida* and the effects of *Matricaria camomilla* (L.) on it, have been studied. At first, capsular genotypes were confirmed by multiplex PCR and then bioflm formation was evaluated by Fonseca method. In order to investigation of antibacterial effects of the 20% aqueous essence of *Matricaria camomilla* (L.) was used in disk diffusion method. The effects of the essence on biofilm formation were measured in the concentrations of 0.2, 0.35 and 0.5 mg of the essence. The results showed biofilm formation was evident in the entire of capsular genotypes. However, there was not significant different among different capsular genotypes ($p>0.05$). Although the essence had no antibacterial effects on the bacteria, there was significant difference in comparison to the positive control of capsular genotype B, in concentration of 0.5 biofilm formation ($p<0.05$).

Key words: Capsular genotype · Biofilm formation · Ovine · *Pasteurella*

INTRODUCTION

Pasteurella multocida is a flora of digestive system of domestic animals and can cause a wide variety of diseases in different hosts primarily and secondarily [1]. Different factors have been regarded in relation to *Pasteurella multocida* [2]. Some of these factors help the bacteria with attaching to different surfaces like external cell membrane proteins, fimbria [2] and the bacterial capsule [3].

Normally capsule is a hydrated polysaccharide and binds to lipid A molecules and cell membrane phospholipid through covalent bounds. Having many hydroxyl groups the contributory monosaccharides in capsular polysaccharides, enable them to produce various structures by makink variety in nature and composition of monosaccharides. Also the bounds and their branches have made them quite variable. This variety even becomes wider regarding their bounds with organic and non-organic compounds [3]. Based on this capsular variety, *Pasteurella multocida* has five genootypes namely A, B, D, E, F [2].

The capsule is characterized by antiphagocytosis, dryness resistence, interaction with complement system and attachming ability to host cells [3]. There is little information on the attaching ability of these bacteria and its localization significance in host. Regarding the significance of the capsule as a thick external polysaccharide (EPS) in making a tight attachment to surfaces and biofilm formation, this study is to evaluate the biofilm formation in different capsulated genotypes of *Pasteurella multocida* [4]. On the other hand, regarding the growing resistance of these bacteria against antibiotics [5] *Matricaria camomilla* was chosen to be studied as a biofilm formotion inhibitor.

MATERIALS AND METHODS

Capsule typing in Pasteurella Multocida: In this experiment, two of each capsulated type of bacteria namely A, B, D and one non-capsulated type were prepared. To get ensured about the bacteria genotypes their molecular type was confirmed by multiplex PCR [6].

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Based on the suggested protocol by Townsend *et al.* [7], PCR process dose not require DNA extraction, so in this method 0.4µl bacteria containing BHI culture was used as template.

The primers used in this study were mixtures of 6 pairs of primers. To make this, equal quantities of each primer were mixed in Eppendorf tubes and used as Mix primer in PCR process. Each 50µl of PCR reaction included 0.4 µl of glycerinated stock of isolates as template, dNTPs (10mM), Buffer (10X), $MgCl_2$ (50 mM), 1µl Mixprimer, Taq DNA polymerase (5U/µl) and Distillated Water to increase the final quantity to 50µl. Primary denaturation temperature was 95°C for 5 minutes, 35 complete cycle including denaturation temperature of 95°C for 30 seconds, annealing temperature of 54°C for 30 seconds, extension temperature of 72°C for 30 seconds. At the end, a final extension temperature of 72°C for 5 minutes was designed and performed using a thermocycler gradiant (TechNet).

PCR product was electroforised electrophorised for 60 minutes using agarose gel in concentration of 2% The acquired gel was stained by ethidium bromide and images were made using UVTech [7].

Essence Preparation: Aqueous essence of *Matricaria Camomilla* in concentration of 20% was obtained from Giyah Essence Co.

Evaluation of Antibacterial Effects Using Disk Diffusion Method: Evaluation of antibacterial effects of seven *Pasteurella multocida* isolates determined by disk diffusion method. After prepring of Muller-Hinton agar media the surface of all media subcultured by swabs from 0.5 Macfarland standard bacterial suspension. Then 10µl of 20% stock of essence was transferred on sterile blank disks. In the next stage, disks were placed on media surface and incubated 24 hours at 37°C. The diameter of inhibition zone was measured.

Measurement of Biofilm Formation: To evaluate the biofilm formation the suggested method of Fonseca *et al.* [8] was used. To do this, 24 hour old cultured bacteria were used to prepare bacteria suspension in concentration of OD_{620} = 0.01 by spectrophotometry and 190µl of Muller-Hinton broth filled in 96 well plate and 10µl of bacteria suspension (OD_{620} = 0.01) was added. The plate then was incubated at 37°C for 24 hours. After 24 hours the plate was taken, its contents were removed and washed well three times with distilled water. Then 200µl of safranin of 0.025% was added and washed with distilled water after two minutes. Then 200µl of ethanol-stone (50/50 v/v) was added to each well and the OD was determined by ELISA reader at 492nm.

Evaluation of Essence Effect on Biofilm Formation: 190µl of each prepared cultures were filled in wells of 96 well plates. Then non-essenced culture (control positive), cultures of 0.2µg/ml, 0.35µg/ml, 0.5µg/ml concentration and non-essenced, non-bacterial suspension containing (control negative), were poured in the first, second, third, fourth and fifth w ell respectively. In the next stage, 10µl of fresh bacterial suspension with concentration of OD_{620} = 0.01 was added to first to forth well and instead of bacterial suspension, distilled water was added to fifth well. The plate incubated at 37°Cand then removed from incubator after 24 hours and the procedure was continued under sterile hood. Contents of wells were removed and washed with distilled water. At this stage bacteria could have formed the biofilm except in fifth well which did not contain bacteria suspension. Then 200µl of 0.025% safranin was added and let to remain for 2 minutes in each well. In this stage, biofilms are stained. Safranin was removed after 2 minutes and wells washed three times using distilled water. 200µl of ethanol-stone (50/50) was added to each well for 15 minutes. At this time stains of safranin which staines the biofilm were released and could be read by ELISA reader at 492nm of wave length. Thus, the more biofilm was formed, the more stained it was and even got more stained adding ethanol-stone and resulted in much more absorption in ELISA reader.

Statistical Calculation: Each experiment was repeated 12 times for biofilm and 3 times for *Matricaria Camomilla* effects and statistically analyzed using ANOVA method and Duncan's multiple range tests. The acquired difference was compared by SPSS 16 software. The amounts of $P<0.05$ were taken into account.

RESULTS

Bacteria Capsule Typing by Molecular Method: Bands with 460bp of length at stained gel with ethidium bromides showed that isolates were *Pasteurella multocida*. Bands of DNA appearing in PCR product gel in 1044bp, 760bp, 657bp shows A, B, D capsulated types, respectively.

Table 1: Comparison of mean OD of frequency in different types of *Pasteurella multocida* compared to negative control in *Matricaria camomilla* essence free culture in 492nm wave length

No.	Bacteria genotype	Mean OD of frequency in different types
1	*Pasteurella multocida* type A	0.068
2	*Pasteurella multocida* type B	0.066
3	*Pasteurella multocida* type D	0.062
4	*Pasteurella multocida* type U	0.065
5	control	0.053

Table 2: OD values in samples in different concentrations of *Matricaria camomilla* essenceand positive control in 492nm wave length

		Mean for essence samples in concentration			
No.	Bacteria genotype	0.2 µg/ml	0.35µg/ml	0.5 µg/ml	Mean for essence samples (positive control)
1	*Pasteurella multocida* type A	0.099	0.124	0.092	0.092
2	*Pasteurella multocida* type B	0.113	0.122	0.087	0.095
3	*Pasteurella multocida* type D	0.01	0.120	0.093	0.082
4	*Pasteurella multocida* type U	0.098	0.093	0.080	0.081

Results of this study by multiplex study confirmed the types of capsule of isolates.

Results of evaluating antibacterial effects of *Matricaria camomilla* essence on different capsular type of *Pasteurella multocida* in disk diffusion method did not show any inhibition zone for these concentrations.

Results of evaluating antibacterial effects of *Matricaria camomilla* essence on the bacteria by disk diffusion method showed no haleye adame roshd in the mentioned concentrations.

Results of measuring biofilm formation showed in positive control, incubator absorbance was much more than in negative. The difference was significant statistically ($P<0.05$) (Table 1).

Results of Evaluating Biofilm Formation Rate: In this method biofilm formation by the bacteria in culture containing 3 concentrations of essence (0.2m µg/l, 0.35 µg/ml, 0.5ml) was measured. Absorption wave were compared to positive control and the rates of biofilm formation for each group was estimated. Results showed that *Matricaria mamomilla* in concentration of 20% does not have a significant effect on biofilm formation in types A and D *Pasteurella multocida* ($P<0.05$) but, whereas its effect on type B is significant ($P<0.05$).

DISCUSSION

Some researches concern the important role of biofilm formation in pathogenesis of diseases by *Pasteurella multocida* [9, 10]. Also many researches show that protein profile of external membrane proteins in *Pasteurella multocida* bacteriae which have formed biofilm is different with free cells [9, 11]. Although it seems these proteins has a significant role in biofilm formation in bacteria, the researches show that the tight attachment of bacteria to surfaces requires attaching to polymers of external membrane polysaccharides. This enables these polymers to attach to surfaces without ligands as a nonspecific attachment [4].

The capsule compounds is not the same in all genotypes of *Pasteurella multocida* [3, 7]. Results from this study showed that the variety in the compounds of the capsule in these genotypes does not affect their attaching ability to plystyrene surfaces.

Today, it is demonstrated that *German Matricaria camomilla* not only has effects on gram positive bacteriae but also avoids gram negative bacteriae to gather [12, 13] and form biofilm [14]. This means that *Matricaria camomilla* cannot be considered as an affectless plant on gram negative bacteria. Previous studies shows that this plant avoids biofilm formation in *Pseudomonas aeruginosa, E.coli and Helicobacter pylori.* In this study, antibiofilm effect of *Matricaria camomilla* is significant only on *Pasteurella multocida* type B in concentration of 0.5µg/ml of *Matricaria camomilla* 20%. This is possibly due to low concentration of effective compounds in *Matricaria camomilla.* Higher concentrations may affect as an antibiofilm agent.

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