Determination of effects of amniotic membrane homogenate on the growth of the uropathogenic *Escherichia coli*, *S. aureus* and *S. marcescens* strains

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ABSTRACT

The antimicrobial effect of the amniotic membrane (AM), the innermost part of placenta, has been explored to a limited extent so far. To quantify and better understand the mechanisms behind antibacterial effect of AM, we followed the growth of three common pathogens, uropathogenic *Escherichia coli* (UPEC), *Staphylococcus aureus* and *Serratia marcescens* in liquid and on solid culture medium with and without the addition of the AM homogenate. We monitored the effect of AM homogenate on bacterial growth using plate count method (liquid culture medium) and agar diffusion method (solid culture medium). Our results clearly demonstrated that AM homogenate had bacteriostatic effect on studied UPEC and *S. aureus*. Interestingly, when used in lower concentrations, the AM homogenate had bactericidal effect on *S. aureus* and bacteriostatic effect on UPEC. In contrast, *S. marcescens* was completely resistant to the growth-inhibitory substances of AM homogenate. In fact, its growth was slightly accelerated in liquid culture medium in the presence of AM homogenate. Obtained results illustrated that AM homogenate could be used for treatments and prevention of UPEC and *S. aureus* infections. Moreover, the established liquid culture medium assay can be used as a time- and cost-effective method for personalized evaluation of drug effect on the growth of chosen bacterial strains with parallel testing of resistance or susceptibility to multiple drugs.

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1 Bacterial strains

Escherichia coli DL94 is a wild type uropathogenic clinical strain (UPEC DL94), isolated from urine of a patient with a urinary tract infection. UPEC DL94 was obtained from the strain collection of the Molecular Genetics Research Group, Department of Biology, Biotechnical faculty, University of Ljubljana, Slovenia. *Staphylococcus aureus* EXB V54 and *Serratia*

marcescens EXB V15 are wild type strains retrieved from the Microbial Culture Collection Ex, Department of Biology, Biotechnical Faculty, Slovenia.

2 Growth conditions

Bacterial strains were kept on Mueller-Hinton agar (Formedium, Nutrient Agar, Hunstanton, Norfolk, United Kingdom; Biolife, Mueller Hinton Broth, Milan, Italy) at 4 °C and were transferred to fresh agar plates monthly. Liquid cultures in Mueller-Hinton broth (Biolife, Mueller Hinton Broth, Milan, Italy) were grown overnight at 37 °C with aeration (180 rpm). Before use, overnight cultures were diluted 500-fold and grown at 37 °C on Mueller-Hinton agar (Formedium, Nutrient Agar, Hunstanton, Norfolk, United Kingdom; Biolife, Mueller Hinton Broth, Milan, Italy) or in Mueller-Hinton broth without shaking.

3 Amniotic membrane homogenate preparation

The study on human AM was approved by the National Medical Ethics Committee of Republic of Slovenia and obtained with written informed consent at the time of elective caesarean sections from healthy volunteers. All volunteers were serologically negative for HIV, syphilis, and hepatitis B and C. Immediately after Caesarean section the human amniotic membrane (AM) was manually separated from chorion, washed with sterile phosphate buffered saline (PBS) and cut into approximately 3 cm × 3 cm sized pieces. After measuring the volume of AM pieces and addition of sterile PBS (ratio 1:3) the AM pieces in PBS were homogenized with a homogenizer (600W) for 3-4 minutes. The obtained homogenate was stored at –80 °C (patent pending, Ramuta and Kreft, 2019). In this work, we refer to so-prepared AM homogenate as undiluted or H1. Before the use, we diluted H1 with PBS 1:1 (marked H2) and 1:3 (marked H3). The preparation of AM homogenate was carried out aseptically. All cryopreserved samples used in the experiments went only through one freeze-thaw cycle.

4 Enumeration of bacteria with plate count method

The plate count method was used to estimate the concentration of viable bacteria in a sample. First, 100 µl of sample were transferred into 900 µl of sterile saline solution (0.9 % NaCl) and after mixing, further diluted until the concentration of viable bacteria was less than 10 bacteria per 1 ml. Samples were diluted between 10-fold and 10,000,000-fold, depending on the strain and incubation time of the sample. Then, 100 µl of several dilutions were plated onto Mueller-Hinton agar and incubated overnight at 37 °C. The following day, we counted the number of bacterial colonies on Mueller-Hinton agar and calculated the colony-forming unit (CFU) per 1 ml of sample.

5 Effect of AM homogenate on the growth of bacteria in liquid medium

The overnight bacterial culture (UPEC, *S. aureus*, or *S. marcescens*) was diluted 500-fold into fresh Mueller-Hinton broth and transferred to a 96-well microplate so that each well contained 100 µl of the diluted culture. Then to each well containing the diluted culture, 100 µl of either saline solution, PBS, H1, H2 or H3 was added. The microplate was incubated without shaking at 37 °C for seven hours and at the chosen time points the bacterial concentration was estimated by the plate count method. Results were obtained from at least 3 independent experiments, using 3 biological repeats of AM homogenate and 3 technical repeats for each tested bacterial strain.

Schematic protocol for determination of growth-inhibitory effect of AM homogenate on UPEC, *S. aureus* and *S. marcescens* in liquid culture medium. Abbreviations: CFU, colony forming unit; H1, undiluted AM homogenate; H2, 2-fold diluted AM homogenate; H3, 4-fold diluted AM homogenate.

6 Effect of AM homogenate on the growth of bacteria with agar diffusion method

The overnight bacterial culture was spread on the Petri dish with Mueller-Hinton agar. After 10-20 minutes, 5 µl and 10 µl of undiluted AM homogenate (H1) were applied at different spots (each volume three times). The Petri dishes were incubated at 37 °C overnight and next day the zones of inhibited or reduced growth were measured. Each strain was tested in at least three biological repeats.

7 Statistical analysis

The statistical program GraphPad Prism 7 (GraphPad, Software) was used to calculate arithmetic mean, standard deviation and standard error of values for bacterial growth curves and to evaluate the effect of AM homogenate on the growth of bacterial strains. With one-way ANOVA it was determined whether the CFU/ml of cultures with added substance (PBS, H1, H2 or H3) were statistically different (p-value smaller than 0.05) from those with added saline solution at all time points. Logarithmic values were used to lower the variance difference between samples.