

IN VITRO EVALUATION OF ANTIBACTERIAL ACTIVITY OF BOVINE AMNIOTIC MEMBRANE HOMOGENATE

By

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ABSTRACT

Misuse of antibiotics has been increasing drastically since the 90s of the past century, especially after the beginning of the COVID -19 pandemic. This led to the emergence of antimicrobial resistance (AMR) which increased the demand of finding natural alternatives for antibiotics that could be safely used. One of the candidates that has shown antimicrobial properties is the amniotic membrane (AM). Amniotic membrane homogenate (AMH) is one of the forms of amniotic membrane extracts (AME) that can be easily prepared and applied. In this work, we tested the antibacterial activity of bovine AMH (BAMH) against standard strains of some pathogens that have great clinical importance to humans and animals using the agar well diffusion method.

Keywords:

Amniotic membrane, Amniotic membrane homogenate, Antimicrobial activity.

INTRODUCTION

Antimicrobial resistance (AMR) has been a threat to world health and development for the past 20 years. One of the top 10 worldwide public health hazards to humanity, according to the World Health Organization (WHO), is AMR. The overuse and abuse of antimicrobials in veterinary and human medicine have added to the expanding global spectacle of AMR since the invention of the first antibiotics, which were hailed as life-saving miracle medications (Ferri *et al.*, 2017).

The bovine placenta's innermost layer, also known as the chorio-amnion, is composed of chorion and a thin membrane made of collagen called the amnion (Wells *et al.*, 2022). AM is a

metabolically active tissue with anti-inflammatory, anti-microbial, anti-fibrotic, and epithelialization-promoting properties that make it particularly well-suited for many clinical uses (Niknejad *et al.*, 2008).

AM cells secrete antimicrobial peptides (AMPs) that have a great role in the defense and protection of fetus (Frew and Stock 2011; Ramuta *et al.*, 2021). Numerous AMPs, such as Human-Beta Defensins, elafin, secretory leucocyte protease inhibitors and histone H2B have been found in fresh amniotic membranes and may protect wounds by preventing bacterial development and regulating immune response (Mao *et al.*, 2017).

A homogenate is a combination of cells and extracellular matrix that has undergone mechanical disruption (Ramuta *et al.*, 2021). Different strategies, such as freeze-drying or cryopreserving the AM, have been designed to extend its shelf life. AME is a different strategy that could be simply sterilized by filtration to be preserved (Dadkhah Tehrani *et al.*, 2021).

To maintain their survival, bacteria have developed sophisticated drug resistance mechanisms gained through numerous biochemical pathways (Munita and Arias 2016). Principal nosocomial pathogens, according to epidemiological evidence, include *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* (WHO 2011). Beside *Salmonella* spp., they are substantial sources of infection and diseases in a wide range of animal hosts, having a large negative influence on both agriculture and public health (Bélanger *et al.*, 2011; Drózdź *et al.*, 2021; Haag *et al.*, 2019; Osman *et al.*, 2012).

Accordingly, this study aimed to investigate the potential antibacterial effect of bovine amniotic membrane homogenate on bacterial pathogens using agar gel diffusion test.

MATERIAL AND METHOD

Studied bacteria:

The amniotic membrane tissue samples were first suspended as explained below, the antimicrobial effects of suspensions were tested on five standard bacterial strains including four pathogens: *E. coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 9027), *Staphylococcus aureus* (ATCC 6538), *Salmonella* Typhimurium (ATCC 17028), in addition to *Micrococcus*

luteus (ATCC 10240). The standard bacterial strains were obtained from Egyptian Drug Authority (EDA) laboratories.

Table (1): The bacterial strains used in this study.

Bacterial strain	Genotype and/or Phenotype Features	Gram's reaction	Reference/Source.
<i>Micrococcus luteus</i>	Reference strain	Positive	ATCC 10240
<i>Staphylococcus aureus</i>	Reference strain	Positive	ATCC 6538
<i>Salmonella Typhimurium</i>	Reference strain	Negative	ATCC 17028
<i>Escherichia coli</i>	Reference strain	Negative	ATCC 8739
<i>Pseudomonas aeruginosa</i>	Reference strain	Negative	ATCC 9027

1. Preparation of bacterial suspensions:

Standardized bacterial suspensions corresponding to the turbidity of McFarland tube number 0.5, ($\sim 1.5 \times 10^8$ CFU/ml) were prepared in saline. Bacterial suspensions were spread-plated onto Mueller-Hinton agar plates. Testing the antibacterial effects were applied by delivering 100 μ l of each amniotic membrane homogenate into a corresponding well in the seeded agar.

2. Bovine amniotic membrane homogenate (BAMH) preparation:

The method performed by **Capistrano da Silva et al. (2021)** was followed with few modifications and without the addition of any antimicrobial substances. A placenta was retrieved aseptically before it contacted the ground during vaginal birth from a healthy multiparous cow that gave birth to a normal, full-term calf. Within a few hours of calving, AM was collected in a sterile bag and transported to the laboratory while cold with a minimal delay. Upon arriving to the laboratory, the sample was immediately frozen until further processing. To be processed, Bovine AM (BAM) was let to thaw at room temperature, washed several times in sterile normal saline (NaCl 0.9 %) and distilled water in a laminar airflow cabinet. The membrane was aseptically minced into approximately equal cuts as shown in Fig. (1). AM sections were separated into 50 ml sterile tubes and immersed in sterile normal saline as shown

in Fig. (2). Tubes were frozen at -80°C and thawed at room temperature repeatedly for mechanical disruption of cells (Moravvej *et al.*, 2021; Ngangan and McDevitt 2009). The constituent of each tube was homogenized 3 min using a tissue homogenizer (Capistrano da Silva *et al.*, 2021; Ramuta *et al.*, 2020). The homogenites Fig. (3) were stored in a deep-frozen state till filtration and sterilization by syringe filters ($0.22\text{ }\mu\text{m}$) to be evaluated for antimicrobial activity (Dadkhah Tehrani *et al.*, 2021).



Fig. (1): Sections of BAM.

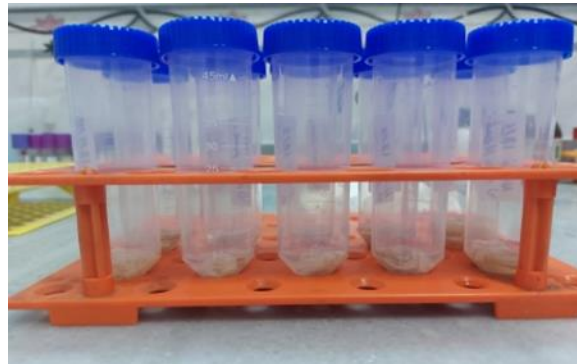


Fig. (2): AM sections immersed in sterile saline.

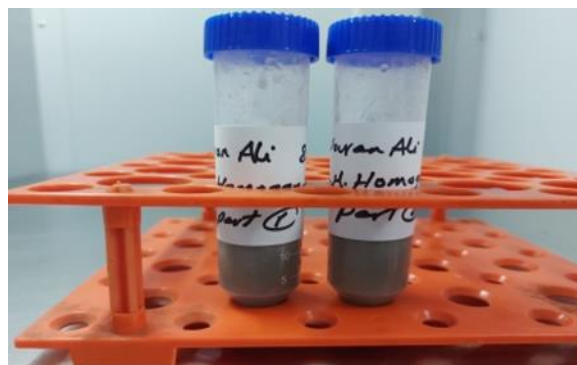


Fig. (3): Result of homogenization.

3. Sterility testing:

The bovine amniotic membrane homogenate (BAMH) filtrate was tested for its sterility according to the United States Pharmacopeia (USP) method. Briefly, 1 ml of the filtrate was inoculated onto a fluid thioglycolate tube and incubated at 37° C for 14 days considering +ve and -ve controls (**Pharmacopeia, 2014**).

4. Evaluation of the antimicrobial activity of BAMH:

This study adapted the traditional disk diffusion assay to assess the diffusion ability of antimicrobial components present in the amniotic membranes instead of antibiotic susceptibility. The effect of BAMH on the growth of *S. aureus*, *E. coli*, *P. aeruginosa* and *Salmonella* spp. **was tested as described by Šket *et al.* (2019)**. A swab of an overnight bacterial culture of each strain was spread onto a Mueller-Hinton agar plate. After dryness at room temperature, 100 µl of undiluted BAMH was delivered into a corresponding 6 mm well made in the agar plate. The test plates were incubated at 37 °C for an overnight to be examined the next day for inhibition zones. The test was repeated three times.

RESULTS

1. Sterility test:

Sterility of the preparations was indicated by absence of microbial growth in the inoculated thioglycolate broth tubes.

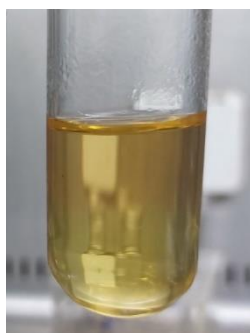


Fig. (4): Sterile thioglycollate medium with BAMH.

2. Evaluation of the antimicrobial activity of BAMH:

No observable clear zones of inhibition were observed by naked eyes around wells containing BAMH in of the Mueller-Hinton agar plates, cultivated with all tested strains that showed normal growth (Table 2) and Fig. (5).

Table (2): Activity of BAMH against tested strains in agar well diffusion test.

Bacterial strain	Zone of inhibition in mm
<i>Staphylococcus aureus</i>	0-
<i>Salmonella spp.</i>	0-
<i>Escherichia coli</i>	-0
<i>Pseudomonas aeruginosa</i>	0-



Fig. (5): Normal bacterial growth of tested strains without clear zones of inhibition.

DISCUSSION

Several studies targeting the antimicrobial activity of AM have been conducted, especially those evaluating its homogenate form since it is one of the easiest and safest forms of AME that could be produced and sterilized by syringe filtration (**Dadkhah Tehrani et al., 2021; Ramuta et al., 2020; Ramuta et al., 2021; Šket et al., 2019**).

In our work, we performed sterility test following syringe filtration with the method of USP to ensure the sterilization process (**Pharmacopeia, 2014**). BAMH was prepared using sterile materials (**Šket et al., 2019; Ramuta et al., 2020; Capistrano da Silva et al., 2021; Ramuta et al., 2021**) without the addition or use of antibiotics or chemicals for decellularization, depending only upon physical method by repetitive freezing-thawing cycles and homogenization process (**Ngangan and McDevitt 2009; Moravvej et al., 2021**).

No antibacterial effect was observed with any of the tested strains employing the agar diffusion method on Mueller-Hinton agar plates.

The positive antimicrobial activity observed in others' work could be attributed to the use of antibiotics for preparation of AM sections or to the differences between tested bacterial strains. For example, **Šket et al. (2019)** tested human AM homogenate against *S. marcescens* in a liquid culture medium and obtained negative results, **Ramuta et al. (2021)** demonstrated that AM homogenate had antibacterial activity against only 7 out of 11 tested multidrug-resistant strains. Variations in bacterial strains, origin of AM and methods of evaluation could all lead to differences in results.

In another experiment, researchers reported antibacterial properties of human amniotic chorionic membrane (hACM) and split-thickness human skin homogenates against *P. aeruginosa* and *E. coli*. However, after incubating the bacterial cultures with these homogenates for 18-24 hours, they did not observe any antibacterial effect in either homogenate (**Robson and Krizek, 1973**).

The current data could be coordinated with a previous study by **Talmi et al. (1991)**, who used a modified disk diffusion assay to investigate the effect of fresh human amniotic chorion membrane (hACM), human amnion membrane (hAM), and synthetic polyurethane membranes on various bacteria. Those bacteria included coagulase-positive *Staphylococcus* species,

Escherichia coli, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Proteus mirabilis*. The study found that all the membranes inhibited bacterial growth directly underneath them, but no clear zone of inhibition was observed around the membranes. This led the researchers to conclude that, the membranes' antimicrobial effect likely stemmed from their ability to adhere to the bacterial surface.

The initial investigations focused on replicating clinical applications by testing fresh (FAM) and cryopreserved (cAM) amniotic membrane patches against various bacterial strains. However, fAM and cAM patches surprisingly showed no observable antimicrobial effect on any tested bacteria. This was evidenced by the absence of both inhibited bacterial growth and detectable antimicrobial zones under the patches (Ramuta *et al.*, 2020).

REFERENCES

- Bélanger, L., Garenaux, A., Harel, J., Boulianne, M., Nadeau, E., and Dozois, C. M. (2011):** *Escherichia coli* from animal reservoirs as a potential source of human extraintestinal pathogenic *E. coli*. *FEMS Immunology and Medical Microbiology*, 62 (1), 1-10.
- Capistrano da Silva, E., Carossino, M., Smith-Fleming, K. M., Langohr, I. M., and Martins, B. D. C. (2021):** Determining the efficacy of the bovine amniotic membrane homogenate during the healing process in rabbits' ex vivo corneas. *Veterinary Ophthalmology*, 24(4), 380 -390.
- Dadkhah Tehrani, F., Firouzeh, A., Shabani, I., and Shabani, A. (2021):** A review on modifications of amniotic membrane for biomedical applications. *Frontiers in bioengineering and biotechnology*, 8, 606982.
- Drózdź, M., Małaszczuk, M., Paluch, E., and Pawlak, A. (2021):** Zoonotic potential and prevalence of *Salmonella* serovars isolated from pets. *Infection Ecology and Epidemiology*, 11(1), 1975530.
- Ferri, M., Ranucci, E., Romagnoli, P., and Giaccone, V. (2017):** Antimicrobial resistance: A global emerging threat to public health systems. *Critical reviews in food science and nutrition*, 57 (13), 2857-2876.
- Frew, L., and Stock, S. J. (2011).** Antimicrobial peptides and pregnancy. *Reproduction*, 141(6), 725.
- Haag, A. F., Fitzgerald, J. R., and Penadés, J. R. (2019):** *Staphylococcus aureus* in Animals. *Microbiology spectrum*, 7 (3), 10-1128.
- Mao, Y., Hoffman, T., Singh-Varma, A., Duan-Arnold, Y., Moorman, M., Danilkovitch, A., and Kohn, J. (2017):** Antimicrobial peptides secreted from human cryopreserved viable amniotic membrane contribute to its antibacterial activity. *Scientific reports*, 7(1), 13722.

- Moravvej, H., Memariani, H., Memariani, M., Kabir-Salmani, M., Shoaie-Hassani, A., and Abdollahimajd, F. (2021):** Evaluation of fibroblast viability seeded on acellular human amniotic membrane. *BioMed research international*, 2021, 1-6.
- Munita, J. M., and Arias, C. A. (2016):** Mechanisms of antibiotic resistance. *Virulence mechanisms of bacterial pathogens*, 481-511.
- Ngangan, A. V., and McDevitt, T. C. (2009):** Acellularization of embryoid bodies via physical disruption methods. *Biomaterials*, 30 (6), 1143-1149.
- Niknejad, H., Peirovi, H., Jorjani, M., Ahmadiani, A., Ghanavi, J., and Seifalian, A. M. (2008):** Properties of the amniotic membrane for potential use in tissue engineering. *Eur Cells Mater*, 15, 88-99.
- Osman, K. M., Ata, N. S., Hedia, R. H., AbuElnaga, A. S. M., El-Hariri, M., and Aly, M. A. (2012):** Emergence of an Anti-microbial Resistant *Pseudomonas aeruginosa* from Human and Animal Clinical samples: A zoonotic and public Health Hazard. *Global Veterinaria*, 19, 745-751.
- Pharmacopeia, U. S. (2014):** United States Pharmacopeia and National Formulary (USP 37–NF 32). Rockville, MD: US Pharmacopeia.
- Ramuta, T. Ž, Starčič Erjavec, M., and Kreft, M. E. (2020):** Amniotic membrane preparation crucially affects its broad-spectrum activity against uropathogenic bacteria. *Frontiers in microbiology*, 11, 469.
- Ramuta, T. Ž, Šket, T., Starčič Erjavec, M., and Kreft, M. E. (2021):** Antimicrobial activity of human fetal membranes: From biological function to clinical use. *Frontiers in bioengineering and biotechnology*, 9, 691522.
- Robson M. C., Krizek T. J., Koss N., Samburg J. L. (1973):** Amniotic membranes as a temporary wound dressing. *Surg. Gynecol. Obstet.* 136 904 - 906.
- Šket, T., Ramuta, T. Ž, Starčič Erjavec, M., and Kreft, M. E. (2019):** Different effects of amniotic membrane homogenate on the growth of uropathogenic *Escherichia coli*, *Staphylococcus aureus* and *Serratia marcescens*. *Infection and Drug Resistance*, 3365-3375.
- Talmi Y. P., Sigler L., Inge E., Finkelstein Y., Zohar Y. (1991):** Antibacterial properties of human amniotic membranes. *Placenta* 12 285–288. 10.1016/0143-4004(91)90010-d
- Wells, H. C., Sizeland, K. H., Kirby, N., and Haverkamp, R. G. (2022):** Structure and Strength of Bovine and Equine Amniotic Membrane. *Biology*, 11(8), 1096.
- World Health Organization (2011):** Report on the Endemic Burden of Healthcare-Associated Infection Worldwide.