GENOTYPING AND DETERMINATION OF RADIATION SENSITIVITY PATTERN OF MULTIDRUG-RESISTANT BACTERIA ISOLATED FROM HUMAN AMNIOTIC MEMBRANE

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ABSTRACT

Background: The transplantation of human amniotic membrane (HAM) is a significant accomplishment in the fields of cosmetic surgery, ocular surgery, epidermis, abdominal and vaginal reconstruction, and cosmetic surgery, as it has the potential to save thousands of lives annually. Nevertheless, the risk of infectious disease transmission using amniotic membrane allografts is a significant concern, as microorganisms can be introduced into the grafts during tissue procurement. **Objectives:** This study aimed at the genotypic characterization of the multidrug-resistant (MDR) membrane-associated bacteria and determining the isolates' radiation sensitivity pattern. **Methods:** A total of 163 bacteria retrieved from 10 amnion samples (5 from vaginal and 5 from cesarean) were subjected to biochemical and genotypic characterization. Antimicrobial and radiation sensitivity patterns of the isolates from different genotypes were determined after exposure to ^{60}Co Gamma irradiation. **Findings:** Cultured bacteria were distinguished into diverse genera belonging to *Enterobacter* spp., *Pseudomonas* spp., *Stenotrophomonas* spp., *Bacillus* spp., and *Staphylococcus* spp. as detected by ARDRA (Amplified Ribosomal RNA Restriction Analysis) followed by 16S rRNA gene sequencing. Isolates showed highest resistance against Cefixime (100%) followed by Ampicillin (77.5%), Ceftriaxone (72.5%), Amoxicillin (70%), Cefuroxime (67.5%), Colistin (65%), Vancomycin (52.5%), Streptomycin 40%), Ciprofloxacin (32.5%), Gentamicin (27.5%), Azithromycin (25%) and Imipenem (7.5%). The D_{10} value for Gram-positive bacteria was higher than that of Gram-negative ones. The survival fraction indicated that the decimal reduction rate of the bacterial level decreased as the radiation dose was increased. Two strains of Gram-positive bacteria, *Staphylococcus* spp. (NS_2 8 and NS_2 21) and one strain of *Bacillus* spp. (CS_8 2) were found to survive at 7 KGy Gamma irradiation. These findings suggested that an 8 KGy irradiation dose was enough to eradicate the bacterial load of the samples. **Conclusions:** This investigation reports the genotypes and radiation sensitivity pattern of AM-associated MDR bacteria, which might be necessary for determining a suitable radiation dose to eliminate the bacteria without hampering the sterility assurance level (SAL).

KEYWORDS: Amniotic membrane, MDR, ARDRA, 16S rRNA, D_{10} value.

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Introduction

Tissue transplantation, one of medicine's greatest achievements, enhances the quality of life for numerous individuals and saves thousands of lives every year. Amniotic membrane (AM) grafting is the most prevalent among the various types of tissue transplantations. The innermost fetal membrane, human AM, lines the amniotic cavity and safeguards the fetus during pregnancy. It is situated close to the amniotic fluid and comprises a single layer of cuboidal epithelial cells that supply the fetus with nutrients. Human amniotic membrane (HAM) is a biological treatment instrument that is widely recognized and is currently being employed in cosmetic surgery, ocular surgery, skin, abdominal, and vaginal reconstruction.

The amniotic membrane is the sole tissue naturally designed explicitly as a universal transplant, assuring the baby's protection and verifying its acceptance by the mother's immune system. Amniotic membranes possess a bacteriostatic effect, are incredibly adherent to the incision, and serve as a barrier to the external environment when used as a dressing material.

The continued demand for AM results from the myriad reasons for its use over the years. The biological properties of this wound dressing are one of the primary factors that render it suitable for regenerative treatments, including serving as a

wound covering, providing indirect protection against infection, and providing distinct relief from discomfort. In addition to preserving the structural and anatomical configuration of regenerated tissues, the clinical application of amniotic membrane contributes to the improvement of healing by reducing postoperative scarring and subsequent loss of function, as well as by providing a plentiful source of stem cells (Rinastiti *et al.*, 2006).

Currently, patient guarantees against communicable infections are necessary for human tissue transplants. This infection may result from introducing undesirable bacterial populations into various stages of membrane allograft preparation. Infection is the most severe complication patients may encounter during surgery. This is why international standards should be followed in the application of manufacturing processes to ensure the safety of the transplants from infections while simultaneously ensuring that they are of high quality.

The microbial burden can be reduced but not eliminated using an aseptic technique in tissue banks. The appropriate sterilization procedure would facilitate the acquisition of sterile allografts and the restriction of infectious diseases. Currently, the most prevalent method for tissue sterilization is gamma radiation (Glyn, 1994; Yusof, 2007). Gamma radiation is the preferred form of exposure due to its numerous advantages. The dose necessary for sterilization is contingent upon the radiation resistance of the contaminants and the initial level of microbial contamination. Although a radiation dose of 25 KGy is the generally accepted and recommended dose by the International Atomic Energy Commission (IAEA) for sterilization, it has been desired to maintain the therapeutic potentialities intact by using a lower dose without compromising the SAL (sterility assurance level) of 10^{-6} (Mukherjee, 1994) as well as ensuring the safety of tissue transplantation. The current study aimed to investigate the prevalence of bacterial contaminants in amniotic membrane samples and the genotyping of the isolated bacteria. Besides these, bacterial species' antimicrobial and radiation sensitivity patterns were determined. This information may be advantageous in predicting an appropriate radiation dose to protect the amnion graft from any unintended bacterial contamination.

Methodology

Collection of Amnions

A total of 10 human amniotic membrane samples were collected from Gonoshasthaya Samaj Vittik Medical College in Savar and the Maternal and Child Health Training Institute in Azimpur, Dhaka. The samples consisted of five vaginal and five cesarean sections. Experienced medical personnel have taken the placenta from the donor under strict aseptic conditions from both the vaginal and cesarean delivery sections. Previously, the donors were serologically screened for potentially communicable diseases, such as syphilis, hepatitis B and C viruses, and the human immunodeficiency virus (HIV). Before the arrival of newborns, hospitals maintained a supply of standard sterile saline (0.9% NaCl) in numerous plastic containers. The donor ID, hospital registration number, and placenta sample categories (vaginal and cesarean) were all clearly indicated on each container. Amniotic membrane samples (glossy, translucent, and thinner) were collected into the containers shortly after the delivery and temporarily stored in a freezer below -20°C. The containers containing membranes were promptly transported to the tissue banking laboratory by placing them in a cool box.

Sample Preparation

All of the experiments conducted in this investigation were performed in the laboratories of the Institute of Tissue Banking and Biomaterial Research, Atomic Energy Research Establishment, located at Savar. Initially, the amnion samples were removed from the freezer and maintained at a standard temperature to facilitate thawing. Subsequently, the amniotic membrane was surgically separated from the chorion. To proceed with this phase of processing, blunt dissection was implemented. This stage was conducted using instruments that had been previously sterilized and in the most optimal aseptic condition.

Isolation and Preservation of Bacteria

MacConkey agar (MAC), Eosin methylene blue agar (EMBA), Manitol salt agar base (MSAB), and Nutrient Agar (NA) were the four solid bacterial media that were prepared. Plates of each medium were inoculated with 0.1 ml of each liquid under strict aseptic conditions of laminar airflow. The samples were distributed using a sterile glass rod spreader, and the plates were incubated at 37°C for 24 hours. Bacterial colonies were observed on various media to identify and differentiate between distinct types of bacterial flora. Colonies were kept for cultural, morphological, and biochemical identification. The isolates were streaked aseptically on Nutrient agar and incubated overnight at 37°C. After incubation, one colony was pulled out aseptically with a sterile loop and suspended in nutrient broth (NB). The 20% glycerol stock culture was prepared in a 1 ml sterile Eppendorf tube. Duplicate vials of each strain were stored at -80°C.

Morphological and Biochemical Characterization

The cultural characterization of each colony was conducted by examining its size, pigmentation, shape, margin, elevation, and opacity. The Gram staining technique was employed to ascertain the morphological characteristics of bacteria, which are classified according to their Gram character (Gram-positive or Gram-negative).

Conventional biochemical tests, such as Oxidase, Catalase, IMVIC (Indole, Methyl-red, Voges Proskauer, and Citrate assays), and Triple Sugar Iron tests, were performed to identify the isolates. The bacterial isolates were subsequently identified using Bergey's Manual of Determinative Bacteriology.

Antimicrobial Susceptibility Test

The antimicrobial susceptibility testing used the Kirby Bauer method (Barry, 1985), a widely recognized disc diffusion technique (Baur *et al.*, 1966), following the procedures outlined in the NCCLS Manual of Antimicrobial Susceptibility Testing (2018). The antibiotic discs were purchased from Oxoid, UK, and utilized following the manufacturer's prescribed protocols. The following discs, each with specific microgram quantities, were used for the antibiotic sensitivity assay: Gentamicin (CN-10), Amikacin (AK-30), Streptomycin (S-10), Imipenem (IMP-10), Cefixime (CFM-30), Cefuroxime (CXM-30), Vancomycin (VA-30), Azithromycin (AZM-15), Colistin (CT-10), Ampicillin (AMP-10), Ciprofloxacin (CIP-5), Doxycycline (DO-30), Amoxicillin (AML-10), Ceftriaxone (CRO-30). Based on biochemical test results, presumptively identified isolates were checked to detect antimicrobial resistance.

Radiation Sensitivity Pattern of the Isolates

Following isolation and identification, forty-one bacterial isolates from the amnion were evaluated for comparative radiation resistance across radiation doses ranging from 1 to 8 KGy. Bacterial colonies were enumerated at two intervals: before radiation and subsequent to radiation. In the untreated population, cells were incubated for 2 to 3 hours at 37°C; subsequent plating was conducted with suitable dilutions, followed by an additional incubation for 24 hours at 37°C, during which viable cells were assessed. Plating was conducted on the irradiated population following exposure to various radiation levels. D_{10} Value and Radiation Sterilization Dose (RSD) were also determined.

DNA Extraction of the Isolates

DNA of 163 isolates was extracted from the bacteria grown overnight. The bacterial DNA was extracted using a QIAamp DNA micro kit (QIAGEN, USA), according to Haque *et al*., 2024. The concentration of the extracted genomic DNA was measured as ng/µL using Nanodrop (Thermo Scientific, USA). The ratio between the readings at 260 nm and 280 nm (A260\280) indicates the purity of the extracted DNA. A ratio of approximately 1.8 suggests that the DNA is not contaminated with RNA or protein.

Molecular Fingerprinting of the Isolates by Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Two universal primers, 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′), were used to amplify the target gene fragments of the 16S rRNA. Agarose gel electrophoresis (1.2% wt/ vol) was used to verify the presence of PCR products. ARDRA is a rapid technique that entails the digestion of the amplified 16S rRNA gene with a suitable restriction enzyme. Agarose gel electrophoresis separates the size of the resulting restriction fragments, resulting in the formation of characteristic restriction fragment length polymorphs (RFLP) (Kullen *et al.*, 1997). The *Alu*1 restriction enzyme (Promega, USA) was used to complete the digestion of the 16S rRNA gene product of the isolates. The resulting digestion products were observed under UV light at 302 nm. Following agarose gel electrophoresis with a 1.5% agarose (w/v) gel that was run for 90 minutes at 75V and stained with ethidium bromide (0.5 µg/ml). The restriction patterns were analyzed to cluster the isolates by sorting identical bands. A 100 bp and a 1 kb DNA ladder (Promega, USA) were employed to analyze various restriction fragments.

16S rRNA Gene Sequencing and Phylogenetic Analysis

Seven (7) isolates were selected randomly from different ARDRA groups for bacterial ribosomal (16S rRNA) gene sequencing. PCR products of the isolates were purified, and DNA sequencing was conducted at First Base Laboratories (Malaysia) utilizing the Applied Biosystems' premier capacity genetic analyzer (ABI PRISMR 377 DNA Sequencer) platforms in conjunction with the BigDyeR Terminator v3.1 cycle sequencing kit chemistry (Hoque et al., 2022). Employing Molecular Evolutionary Genetics Analysis (MEGA) version 7.0 for extensive datasets (Kumar et al., 2016), the 16S rRNA gene sequences, derived from all individual bacterial isolates, were aligned with one another and with pertinent reference sequences taken from the NCBI Database. A maximum-likelihood tree was constructed and shown with default parameters using MEGA 7.0 software.

Results

Cultural Characterization

One hundred sixty-three colonies were isolated from the respective agar plates used in this study. Representative bacterial colonies were subcultured on nutrient agar media and observed for their characteristics.

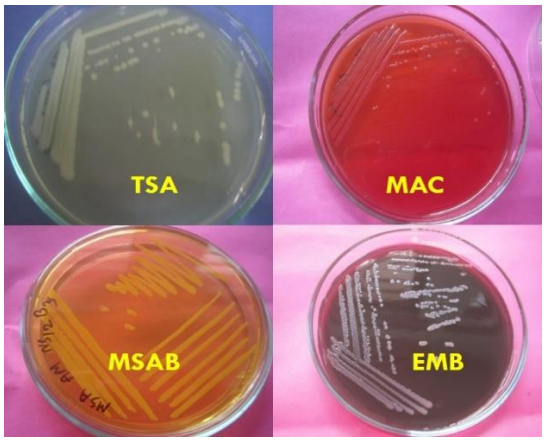

Figure 1. Bacterial colonies on different representative Media.

Morphological Characterization

Microscopic observation of the bacterial isolates revealed Gram-positive and Gram-negative bacterial contaminants in the amnion membrane by Gram staining. These bacteria were further categorized into the following types according to their morphological appearance, such as Gram-positive cocci, Gram-positive bacilli, and Gram-negative bacilli (Figure 2).

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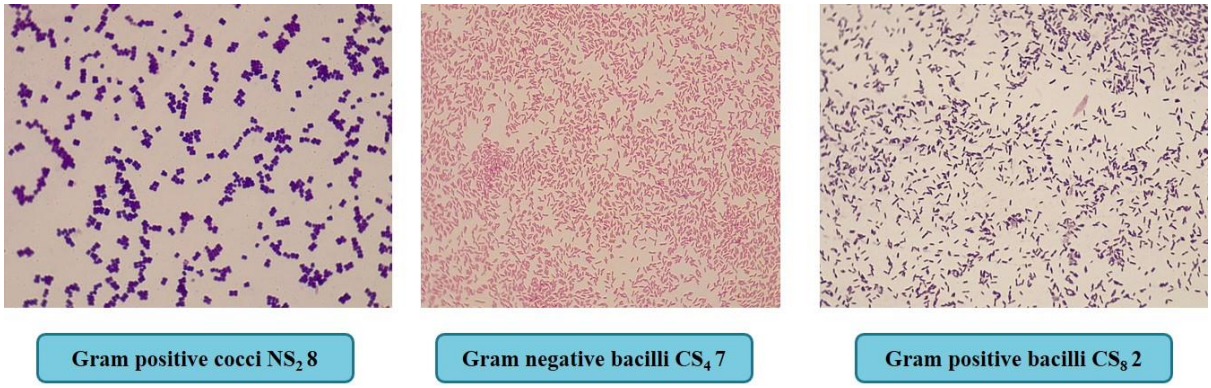

Figure 2. Microscopic 100x view of isolated bacteria after Gram staining using a Leica DM500 microscope (Germany).

Among the 69 Gram-positive bacteria, 37 of them were found in the vaginal amnion, and 32 were found in cesarean amnion. In the case of 94 Gram-negative bacteria, 53 of them were found in the vaginal amnion, and 41 were found in the cesarean amnion.

Biochemical Characterization

Nine biochemical tests were carried out to determine the identity of bacterial contaminants retrieved from amniotic membrane samples. After completing different biochemical tests, the following presumptive bacterial population was determined. (Bergey's manual of determinative bacteriology, 2012). A total of 163 bacterial isolates were studied for the biochemical tests. Among 163 bacterial isolates, *Staphylococcus* spp. was the most prevalent contaminant, 34% of total isolates (Table 1).

Table 1. Predominance of contaminating bacteria on the amniotic membrane based on biochemical tests.

Group	Presumptive identification	Percentage (%)
1	*Staphylococcus* spp.	34
2	*Enterobacter* spp.	24
3	*Pseudomonas* spp.	20
4	*Klebsiella* spp.	11
5	*Bacillus* spp.	8
6	*Stenotrophomonas* spp.	3

The prevalence of Bacterial flora can be depicted as:

***Staphylococcus* spp.> *Enterobacter* spp.> *Pseudomonas* spp.> *Klebsiella* spp.> spp.> > *Bacillus* spp. > *Stenotrophomonas* spp.**

Antibiotic Sensitivity Pattern Analysis

In this study, bacterial isolates were also tested for their antibiotic sensitivity. Fourteen antibiotics from 8 different groups were used to test 40 bacterial isolates derived from the amniotic membrane (Figure 3). The result was interpretative, following the CLSI 2022 guidelines. All the isolates showed multidrug resistance (MDR). Isolates showed highest resistance against Cefixime (CFM) (100%) followed by Ampicillin (AMP) (77.5%), Ceftriaxone (CRO) (72.5%), Amoxicillin (AMC) (70%), Cefuroxime (CXM) (67.5%), Colistin (CT) (65%), Vancomycine (VA) (52.5%), Streptomycin (S) (40%), Ciprofloxacin (CIP) (32.5%), Gentamicin (CN) (27.5%), Azithromycin (AZM) (25%), Doxycycline (DO) (10%), Amikacin (AK) (10%) and Imipenem (IMP) (7.5%) (Figure 4). Different groups of bacteria showed differences in their antibiotic sensitivity pattern. Seven isolates of *Staphylococcus* spp. (NS_1 11, NS_2 21, NS_3 1, CS_5 3, NS_6 3, NS_7 1, CS_9 4) showed resistance against Colistin, Azithromycin, Ampicillin, and Ciprofloxacin, while three isolates (NS_3 1, CS_5 3, CS_{10} 1) showed sensitivity against Gentamicin, Amikacin, Streptomycin, and Imipenem. Five isolates of *Stenotrophomonas* spp. (NS_3 13, CS_5 8, NS_7 8, CS_8 15, CS_{10} 5) showed resistance against Cefixime, Cefuroxime, Vancomycin, Amoxicillin, and Ceftriaxone, while three isolates (NS_1 4, NS_2 12, CS_8 15) showed sensitivity against Gentamicin, Amikacin, Imipenem, and Azithromycin. Three isolates of *Pseudomonas* spp. (NS_1 2, NS_7 6, CS_{10} 18) were resistant against Gentamicin, Streptomycin, Cefixime, Cefuroxime, Vancomycin, Amoxicillin, and Ceftriaxone, while four isolates (NS_2 3, NS_1 2, NS_7 6, CS_{10} 18) were sensitive to Amikacin, Imipenem, Azithromycin, and Doxycycline. *Klebsiella* spp. NS_2 9, CS_5 10, NS_6 8, and CS_{10} 3 were sensitive to Gentamicin, Amikacin, Streptomycin, Imipenem, Ampicillin, Ciprofloxacin, and Doxycycline. Most of the *Bacillus* spp. (NS_1 20, NS_1 16, CS_4 15) were resistant to Gentamicin, Amikacin, Streptomycin, Cefixime, Cefuroxime, Vancomycin, Colistin, Ampicillin, Ciprofloxacin, and Ceftriaxone, but a few are (NS_3 4, CS_8 18, CS_8 2) were sensitive to Gentamicin, Amikacin, Imipenem, Doxycycline and Ciprofloxacin, Streptomycin, Imipenem, Cefuroxime, and Vancomycin.

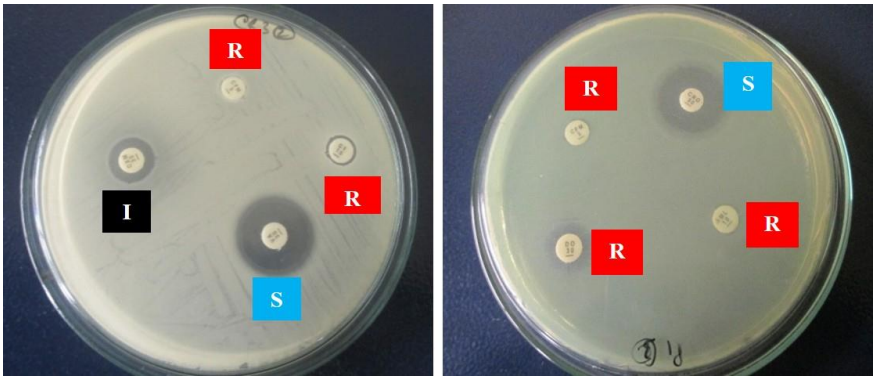

Figure 3. Antibiotic sensitivity pattern of bacterial isolates on Mueller-Hinton agar.

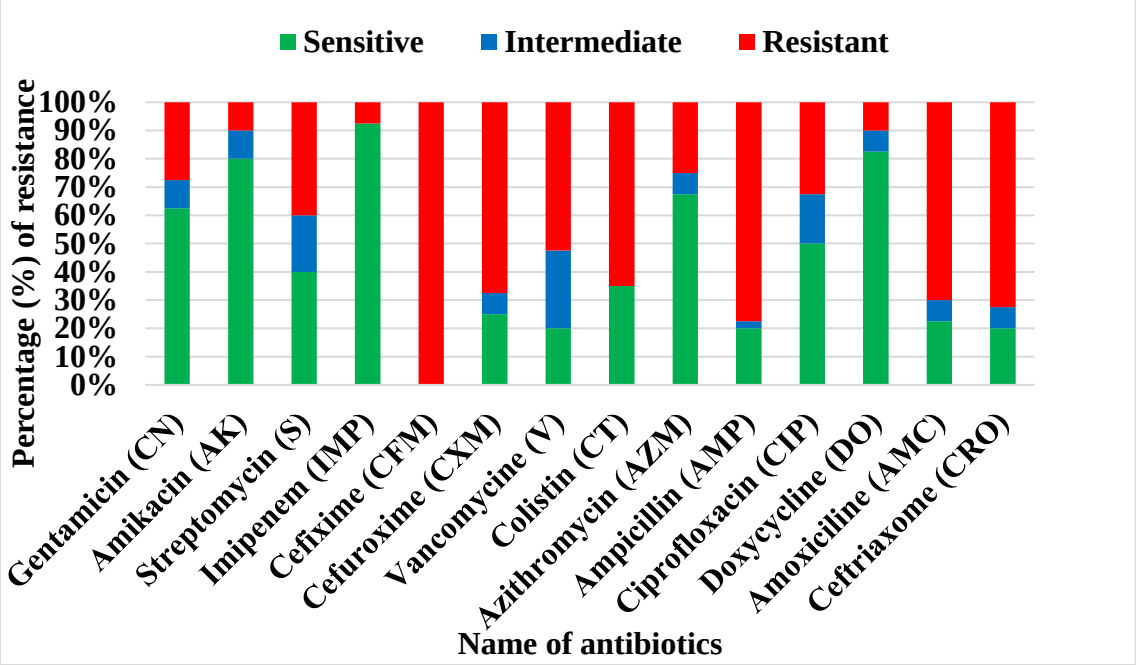

Figure 4. Diagrammatic representation of the antibiotic resistance pattern of isolated bacteria.

Determination of Radiation Sensitivity Pattern of Isolates

Determination of Untreated Population (N_0) and Irradiated Population (N) of Bacterial Microflora Isolated from Amnion before and after Gamma Irradiation

To determine the D-value of a bacterial isolate from the amnion sample, bacterial colonies were counted at two stages: before radiation (N_0) and after radiation (N). As radiation is the process that decreases the bacterial population, the results depict the decline of the bacterial load after gamma radiation.

Tables 2 and 3 represent the bacterial load of samples in two stages, before and after radiation, by the spread plate method. Results showed a chronological decline in bacterial population with the increase in radiation dose.

Table 2. Bacterial population (N_0) before radiation.

ID	Presumptive Identification	No. of Bacteria (CFU/ml)
NS_2 8	*Staphylococcus* spp.	$8.5x10^8$
CS_{10} 17	*Pseudomonas* spp.	$1.2x10^8$
NS_7 8	*Enterobacter* spp.	$9.5x10^8$
CS_9 1	*Klebsiella* spp.	$6.7x10^8$
NS_2 21	*Staphylococcus* spp.	$9.8x10^8$
NS_1 13	*Stenotrophomonas* spp.	$6.7x10^8$
CS_8 2	*Bacillus* spp.	$7.8x10^8$
NS_1 20	*Bacillus* spp.	$8.8x10^8$

NS_2 12	*Enterobacter* spp.	$9.2x10^8$
CS_5 3	*Staphylococcus* spp.	$7.4x10^8$
NS_1 5	*Pseudomonas* spp.	$2.3x10^8$

Table 3. Quantification of bacterial population after irradiation.

Isolate Isolates ID	Number of bacterial population after irradiation (CFU/ml) Radiation Dose (KGy) 1	2	3	4	5	6	7	8
NS_2 8	$1.8x10^8$	$4.6x10^7$	$8.2x10^5$	$4.8x10^5$	$7.4x10^4$	$3.9x10^3$	$1.3\ x10^2$	0
NS_2 21	$2.1X10^8$	$5.1x10^7$	$8.6x10^5$	$5.2x10^5$	$7.6x10^4$	$4.3x10^3$	$1.4x10^2$	0
CS_5 3	$1.5x10^8$	$4.0x10^7$	$7.9x10^5$	$4.6x10^5$	$6.9x10^3$	$4.8x10^2$	0	0
CS_8 2	$1.8x10^8$	$3.9x10^7$	$8.1x10^5$	$5.0x10^5$	$7.3x10^3$	$4.6x10^3$	$1.8x10^2$	0
NS_1 13	$1.3x10^8$	$1.5x10^7$	$4.2x10^5$	$7.8x10^3$	$6.0x10^2$	0	0	0
CS_9 1	$1.2x10^8$	$3.4x10^7$	$5.9x10^5$	$1.3x10^4$	$4.0x10^3$	$1.2x10^2$	0	0
NS_1 20	$1.8X10^8$	$5.6x10^6$	$8.2x10^4$	$3.8x10^3$	$2.1x10^2$	0	0	0
NS_2 12	$1.8X10^8$	$4.0x10^6$	$3.8x10^5$	$2.9x10^4$	$5.1x10^3$	0	0	0
NS_7 8	$1.6x10^8$	$4.6x10^7$	$7.4x10^5$	$5.6x10^4$	$3.9x10^3$	$9.0x10^2$	0	0
CS_{10} 17	$1.9X10^7$	$6.1x10^5$	$8.1x10^4$	$2.3x10^2$	0	0	0	0
NS_1 5	$4.1x10^7$	$3.9x10^6$	$7.1x10^4$	$1.02x10^4$	$5.8x10^3$	0	0	0

Determination of logarithmic Survival fraction and Decimal Reduction value (D_{10})

Gamma radiation is one of the most frequently employed methods for destroying microbial organisms. In general, the decimal reduction dose or D_{10} value (Thronley 1963) describes radiation's lethal effect on microorganisms. The survival rate of bacterial isolates was determined to assess the progressive decrease in their rate as the gamma irradiation dose increased.

Two isolates of *Staphylococcus* spp. (NS_2 8, CS_5 3 and NS_2 21) and two isolates of *Bacillus* spp. (CS_8 2 and NS_1 20) isolated from amnion were tested for their resistance to gamma radiation. Two *Staphylococcus* spp. (NS_2 8and NS_2 21) and *Bacillus* spp. (CS_8 2) showed high resistance against gamma radiation and could grow after exposure to 7 KGy (Table 4).

Table 4. Logarithmic survival fraction of Gram-positive and negative isolates.

Bacterial Type	Isolate ID	Logarithmic survival fraction-log S							
		Radiation dose (Gy)							
		1	2	3	4	5	6	7	8
Gram-Positive	*Staphylococcus* spp. NS_2 8	-0.67	-1.26	-3.01	-3.24	-4.06	-5.33	-6.81	0
	Staphylococcus spp. CS_5 3	-0.67	-1.28	-3.06	-3.28	-4.11	-5.36	-6.84	0
	Staphylococcus spp. NS_2 21	-0.72	-1.26	-3.00	-3.20	-4.22	-5.03	0	0
	Bacillus spp. CS_8 2	-0.64	-1.30	-2.98	-3.19	-5.03	-5.23	-6.63	0
	Bacillus spp. NS_1 20	-0.68	-2.28	-3.36	-4.48	-5.23	-6.80	0	0
Gram-negative	*Klebsiella* spp. CS_9 1	-0.74	-1.29	-3.06	-4.71	-5.22	-6.75	0	0
	Stenotrophomonas spp. (NS_1 13)	-0.71	-1.65	-3.20	-4.93	-6.05	0	0	0
	Enterobacter spp. NS_2 12	-0.70	-2.22	-4.04	-5.38	-6.64	0	0	0
	Enterobacter spp. NS_7 8	-0.77	-1.31	-3.11	-4.22	-5.38	-7.02	0	0
	Pseudomonas spp. CS_{10} 17	-0.80	-2.29	-3.17	-5.71	0	0	0	0
	Pseudomonas spp. NS_1 5	-0.74	-1.77	-3.51	-4.35	-4.59	0	0	0

Two isolates of *Enterobacter* spp. (NS_2 12and NS_7 8), One isolate of *Klebsiella* spp. (CS_9 1), *Pseudomonas* spp. (CS_{10} 17 and NS_1 5), *Stenotrophomonas* spp. (NS_1 13), and *Pseudomonas* spp. (NS_1 5) isolated from amnion were also tested for their resistance to gamma radiation. Gram-negative bacteria showed lower resistance to gamma radiation than Gram-positive bacteria, except *Stenotrophomonas* spp. (NS_1 13) which showed a tolerance of 7 KGy (Table 4).

Determination of D_{10} values

The D_{10} value is a critical concept in the field of radiation microbiology and is used to measure the radiation resistance of a microorganism, specifically indicating the dose of radiation required to reduce the viable population of a microorganism by 90% (or by a factor of 10). In other words, the D_{10} value represents the radiation dose at which only 10% of the original microbial population remains viable. The D_{10} value for Gram-positive isolates ranged from (1.45-1.57) KGy. The highest value was found for *Bacillus* spp. (CS_8 2) 1.57 KGy. On the other hand, the D_{10} value for Gram-negative isolates ranged from (1.25-1.41) KGy. The highest value was found for *Enterobacter* spp. (NS_2 12) 1.41 KGy (Table 5).

Table 5. D_{10} values for representative Gram-positive and Gram-negative bacterial isolates.

Type of Bacteria	Isolate ID	Sub-lethal dose	D_{10} value
Gram-Positive	*Staphylococcus* spp. NS_2 8	7	1.48
	Staphylococcus spp. CS_5 3	7	1.49
	Staphylococcus spp. NS_2 21	6	1.44
	Bacillus spp. CS_8 2	7	1.57
	Bacillus spp. NS_1 20	6	1.45

Gram-negative	*Klebsiella* spp. CS_9 1	6	1.34
	Stenotrophomonas spp. (NS_1 13)	5	1.40
	Enterobacter spp. NS_2 12	5	1.41
	Enterobacter spp. NS_7 8	6	1.29
	Pseudomonas spp. CS_{10} 17	4	1.25
	Pseudomonas spp. NS_1 5	5	1.33

Logarithmic Reduction of Bioburden

A graphical representation has been prepared to present the effect of radiation on the isolated bacteria. The horizontal axis (X-axis) presents different radiation doses used in this research, and the vertical axis (Y-axis) represents the logarithmic growth rate after the corresponding radiation dose.

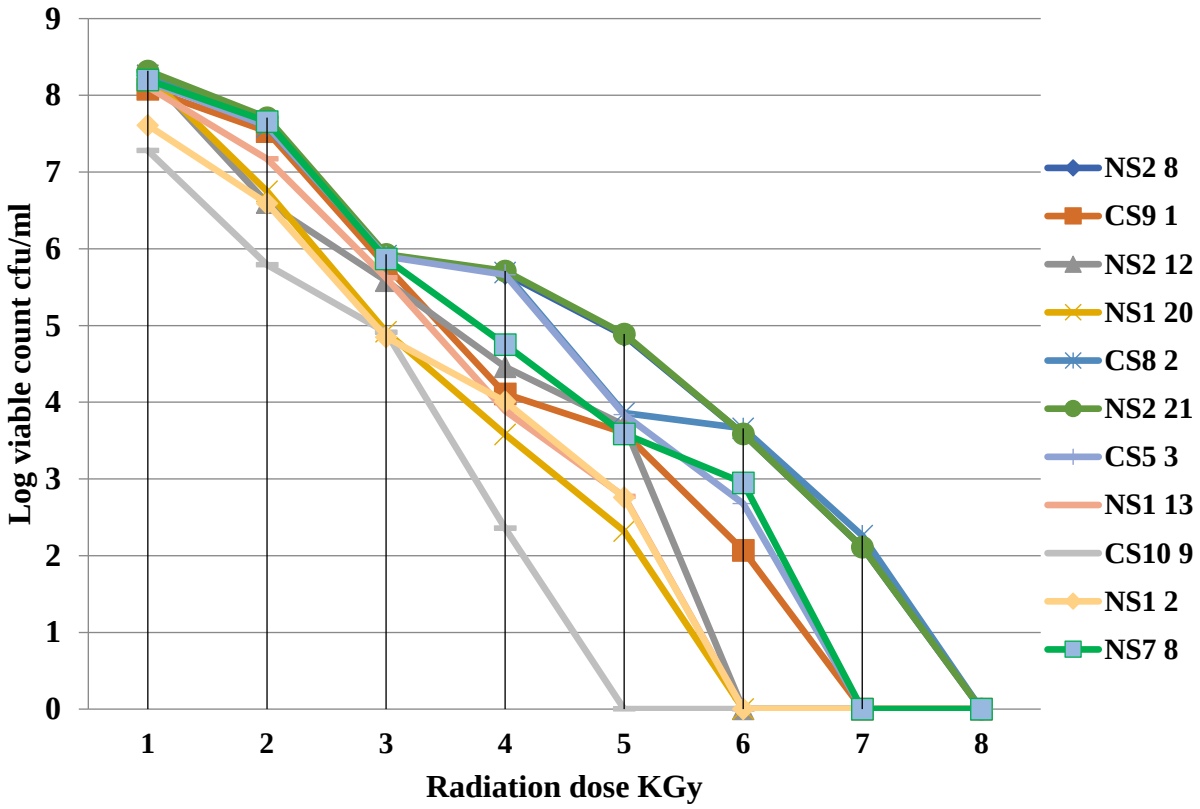

Figure 5. Logarithmic reduction of bioburden counts with the increase of radiation dose.

The graph above shows the inversely proportional relationship of bioburden counts with the radiation dose. All bacterial isolates' growth rate was reduced at a logarithmic rate with the increase of radiation dose and finally declined at a radiation dose of 8 KGy (Figure 5). It has been unequivocally demonstrated that the radiation dose is inversely proportional to the bioburden count.

Molecular Characterization of the Isolates

PCR amplification of the 16S rRNA Gene of the Isolates

Templates DNA from each of the 82 isolates were subjected to amplification for their 16S rRNA gene using universal primers 27F and 1492R. Approximately 1400-1465 bp PCR product was successfully amplified from each isolate (Figure 6).

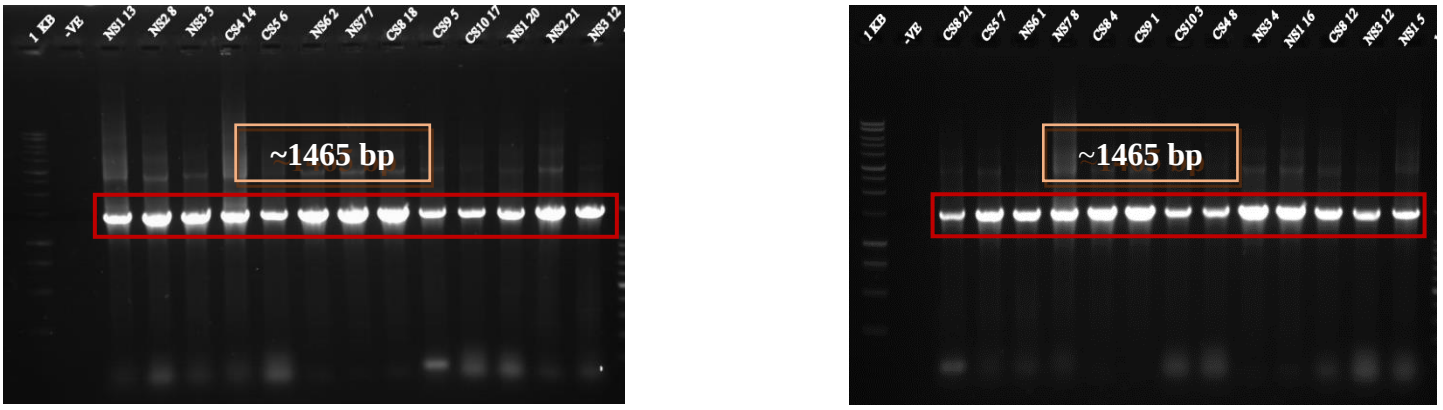

Figure 6. The PCR-specific amplicon of the 16S rRNA gene was electrophoresed on a 1% agarose gel. An isolate code was indicated on each lane. A 1 kb DNA ladder (Promega, USA) was used as the marker.

ARDRA of the Isolates

Restriction digestion with the *Alu* 1 enzyme of the approximately 1400-1465 bp amplified fragment of the 16S rRNA gene showed different restriction patterns for all 163 isolates. According to the restriction pattern, both the vaginal amnion and cesarean amnion isolates were distinguished into seven groups (Figure 7).

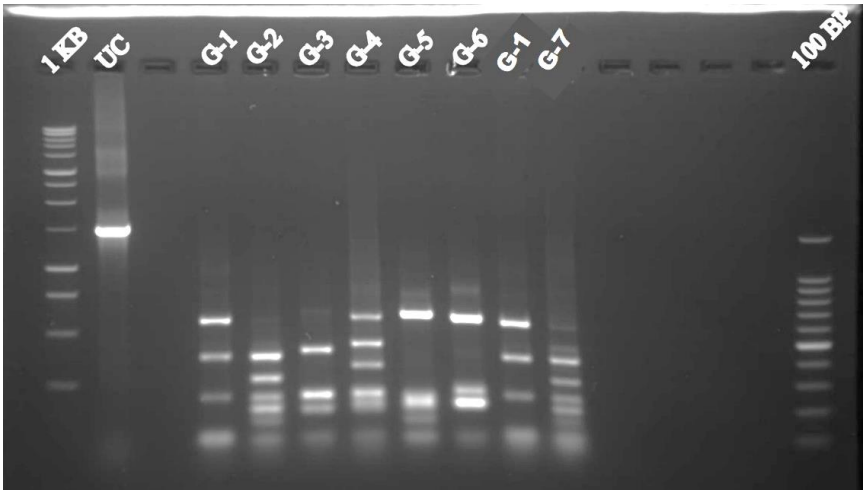

Figure 7. Numerous fragments (approximately 1400-1465 bp) of isolates were derived from the 16S rRNA gene PCR product through Alu 1 enzyme digestion. This section illustrates representative categories. A control was set up by incubating uncut experimental DNA under identical conditions. An isolated code was designated by each lane. The markers employed was 1 kb and 100 bp.

16S rRNA gene phylogeny

Representative isolates from each genotype were selected for comprehensive 16S rRNA gene sequence analysis. Each isolate's 16S rRNA gene PCR product was sequenced using the 27F and 1492R primers. Partial sequences obtained with forward and reverse primers were merged to generate full-length sequences (1400-1450 bp) and compared to the GenBank database of the National Center for Biotechnology Information (NCBI) using the basic local alignment search tool (BLAST). The sequences of the isolates and other close relative reference sequences retrieved from the database were aligned using ClustalW and exported to MEGA7 for sequence analysis. The aligned sequences showed an excellent correlation to the conserved regions. A phylogenetic tree was constructed in MEGA7 software using the Neighbor-Joining algorithm and 1000 bootstrap replicates to deduce a close phylogenetic relationship. Based on 16S rRNA gene sequencing, the cultured bacteria were classified into a variety of genera, including *Enterobacter* spp., *Pseudomonas* spp., *Stenotrophomonas* spp., *Bacillus* spp., and *Staphylococcus* spp. (Figure 8).

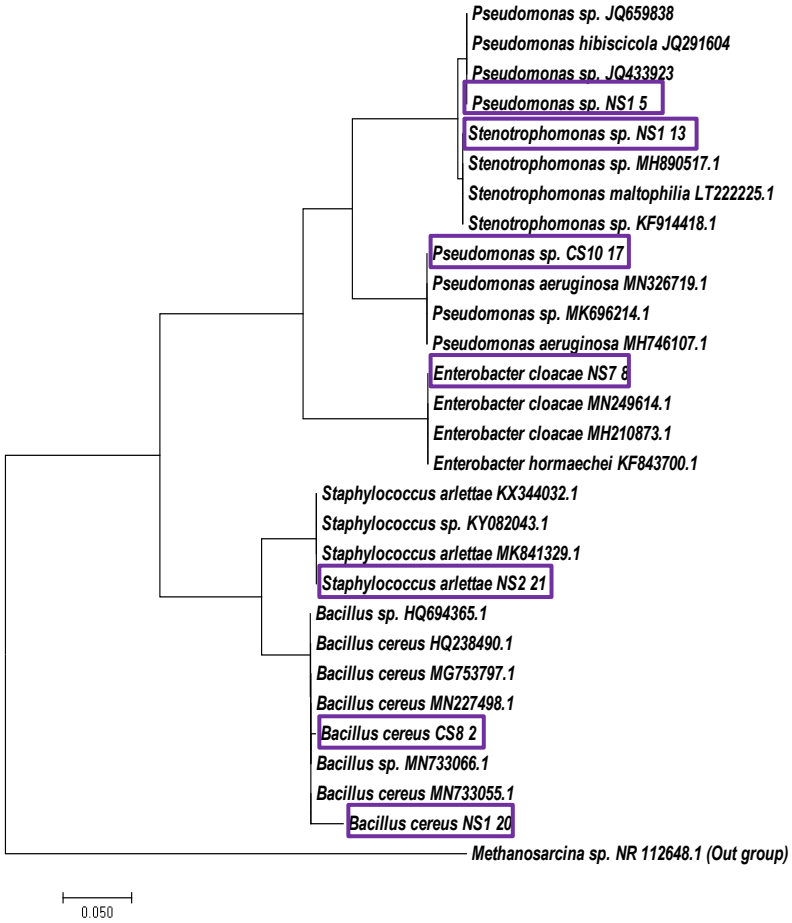

Figure 8. Phylogenetic tree of 16S rRNA gene sequences from isolates of human amniotic membrane and closely related reference isolates sourced from the database, accompanied by accession numbers. The computer generated the tree utilizing the Neighbor-Joining technique in MEGA 7, with the Methanosarcina sp. sequence as an out-group. Bootstrap values (n = 1000 repeats) are displayed at branch nodes, and the scale bar represents the number of changes per nucleotide location. The violet hue signifies the isolation. Isolated bacteria will be further characterized for radiation resistance and antimicrobial resistance gene analysis.

Discussion

For many years, transplanting a broader variety of human tissues has been done to improve and save lives. Because of their unique qualities and characteristics, the amniotic membrane's function in transplantation is well known (Gajiwala and Sharma, 2003). Furthermore, because the amnion is made of a fragile material, it is light and does not trigger immunological reactions. The amnion makes Early patient mobilization possible so the body can absorb quickly. It may also adapt effectively to the body's shape (Singh *et al.*, 2003).

Impermeability to microorganisms yet permeability to the transfer of oxygen and water vapor are crucial physical characteristics for an amnion to be suitable for clinical application (Singh *et al.*, 2003). Amniotic membrane grafting is one of the most common types of tissue transplantation. Even though tissue banks have thorough documentation of all storage and processing protocols, it is impossible to completely rule out the possibility of infection from bacterial contamination. Long-term hospitalization may end in organ failure or possibly death. After a graft implant, infection usually follows. Microbes may enter the grafts from various sources during tissue collection, processing, handling, storage, or surgery, even with donor screening. Microorganisms may be present in the amniotic fluid of pregnant women who are experiencing preterm labor and have an intact amniotic membrane (Dunlow *et al.*, 1990).

Mycoplasma hominis, *Fusobacterium* spp., and *Ureaplasma urealyticum* were the most frequently isolated microorganisms from the amniotic cavity in women with preterm labor and intact membranes (Romero *et al.*, 1989). In addition to *Staphylococcus aureus*, *Streptococcus agalactiae*, *Peptostreptococcus* spp., *Gardnerella vaginalis*, *Streptococcus viridans*, and *Bacteroides* spp., the amniotic fluid contained other microorganisms. Bacterial agents were responsible for approximately one-half of the infections associated with human tissue transplants, with 90% of these infections caused by aerobic organisms. (Wang *et al.*, 2007). The graft contamination can be influenced by environmental exposure, underlying diseases, and the host defense mechanism in a ratio of 2 to 5% (Russu *et al.*, 2007).

All of the amniotic membrane samples were obtained under aseptic conditions from seronegative donors (HIV, HBV, and VDRL) in our study. Despite adhering to appropriate tissue banking protocols, donor tissues were not sterile. Most of the

samples were contaminated with *Staphylococcus* spp. This result is consistent with the findings of other researchers who have researched amniotic membranes (Singh *et al.*, 2007; Begum *et al.*, 1999), as they have previously summarized the staphylococcal contamination in amnion grafts. Aghayan *et al*. 2013 also identified Staphylococci as the most prevalent amniotic membrane organism. Hasan M Zahid *et al*. (2012) have also reported the predominance of higher numbers of *Streptococcus* spp. on amnion.

In this study, ten amnion samples were collected (five vaginal and five cesarean). Bacterial contamination was found in all the samples. After morphological and biochemical

characterization, eight groups of bacteria were presumptively identified as *Staphylococcus* spp., *Bacillus* spp., *Pseudomonas* spp., *Klebsiella* spp., *Stenotrophomonas* spp. Among 163 bacterial isolates, we found *Staphylococcus* spp. The most prevalent contaminant was 34% of the total isolates in this study. Still, the prevalence of Gram-negative bacteria was higher than Gram-positive bacteria. This research's interest is similar to the articles from the Tissue Bank of Iran (Hamid *et al.*, 2011). Their finding suggests that the prevalence of Gram-positive bacteria was higher in the amniotic membrane. At this point, our findings showed a difference as we found that the number of Gram-negative bacteria was higher than that of Gram-positive bacteria. This finding is similar to the findings of Tasnim *et al*., 2019.

The primary sources of this contamination are the patient's skin, nasal flora, and airborne particles from the operation theatre personnel (Ha'Eri *et al.*, 1980; Howorth, 1985). To prevent contamination in the allograft, strategies like asepsis, proper use of disinfectants, sterilization procedures, or the pre-operative administration of systemic antibiotics need to be taken (Turgut *et al.*, 2004). This study determined the antibiotic sensitivity patterns of bacteria isolated from human amniotic membranes. Fourteen antibiotics from 8 different groups were used to test 40 bacterial isolates derived from the amniotic membrane. All the isolates showed multidrug resistance (MDR). Isolates showed the highest resistance against Cefixime (100%), followed by Ampicillin (77.5%), Ceftriaxone (72.5%), Amoxicillin (70%), Cefuroxime (67.5%), Colistin (65%), and Vancomycin (52.5%). Isolates were most sensitive to Imipenem (IMP) (92.5%), followed by Amikacin (90%), Doxycycline (90%), Azithromycin (75%), Gentamicin (72.5%), Ciprofloxacin (67.5%), and Streptomycin (60%). This result is similar to the findings of Hamid M. E., where *Staphylococcus* spp. was multidrug-resistant (Hamid *et al.*, 2011). Another study also found multidrug-resistant *Klebsiella* spp. (Heydari *et al.*, 2014). Uncontrolled and extreme consumption of some common antibiotics influences the spread of antibiotic-resistant bacteria (Hillier *et al.*, 2002). Infections caused by resistant bacteria can lead to up to two-fold higher rates of adverse outcomes than similar infections caused by susceptible strains. These adverse outcomes primarily reflect the failure or delay of antibiotic treatment. As disease severity, strain virulence, or host vulnerability increases, these adverse outcomes will be more pronounced. Abuse of antibacterials, like self-prescription, is another significant cause of the spread of antibiotic-resistant properties in bacteria. Multidrug resistance may occur by increased gene expression that codes for multidrug efflux pumps, extruding a wide range of drugs. (Friedman *et al.*, 2016).

Because of several advantages, gamma irradiation has been the most commonly employed method for sterilizing tissue allografts. The International Atomic Energy Agency (IAEA, 1990) specifies a radiation dose of 25 KGy as the reference dose for sterilizing tissue grafts; however, to preserve the biomechanical and other properties of tissues, certain tissue banks opt for a lower radiation dose while maintaining a sterility assurance level (SAL) of 10^{-6}. A Gamma radiation dose over 25 KGy can compromise the mechanical characteristics of grafts and diminish tissue graft integration. A reduced radiation sterilization dosage will mitigate the impact on the physical qualities of amnion transplants. Baker T. F. established that a sterility assurance level (SAL) of 10−6 for tissue allografts could be attained with a minimum dose of 9.2 KGy (Baker et al., 2005), while Singh R. demonstrated that a radiation sterilization dose (RSD) of 17.6 KGy was validated for amnion allografts (Singh et al., 2006).

The present study examined bacterial isolates for survival in 8 radiation doses (1-8) KGy. High radiation treatment makes the allograft more fragile, which is unsuitable for ocular surface treatment. So, a lower radiation dose was tested against the bacterial pathogens retrieved from amniotic samples. We found that radiation dose 1-3 KGy could not reduce the level of contamination that well. Gram-positive isolates were more resistant to gamma irradiation than Gram-negative ones. In Gram-positive representative isolates, the sub-lethal dose was seven KGy for two of the *Staphylococcus* spp. and one of the *Bacillus* spp. Gram, whereas Gram-negative ones ranged from 4-6 KGy, much lower. The result is closely similar to the findings of Atique Binte *et al.*, 2013. In this research, the D_{10} value for Gram-positive isolates ranged from (1.45-1.57) KGy, where the highest value was found for *Bacillus* spp. (CS_8 2) 1.57 KGy, and in the case of Gram-negative isolates, the D_{10} value ranged from (1.25-1.45) KGy, and the highest value was found for *Enterobacter* spp. NS_2 12 (1.41 KGy).

Genomic DNA from the isolates was subjected to PCR to amplify their 16S rRNA gene using universal primers 27F and 1492R. Approximately 1400-1465 bp PCR product was successfully amplified from each isolate.

Restriction digestion with the *Alu*1 enzyme of the approximately 1400-1465 bp amplified fragment of the 16S rRNA gene showed different restriction patterns for the isolates based on the biochemical test results. According to the restriction pattern, both the vaginal amnion and cesarean amnion isolates were distinguished into different groups. Based on 16S rRNA gene sequencing and phylogeny analysis, the cultured bacteria were distinguished into a variety of genera, including *Enterobacter* spp., *Pseudomonas* spp., *Stenotrophomonas* spp., *Bacillus* spp., and *Staphylococcus* spp. These isolates will be further characterized for their radiation and antimicrobial resistance mechanisms.

Conclusion

The application of this novel biological membrane is expanding across multiple fields, including medicine, tissue engineering, regenerative biology, and stem cell research. The clinical utilization of the amniotic membrane maintains regenerated tissues' structural and anatomical integrity. It enhances healing by minimizing postoperative scarring and subsequent functional impairment, thus offering a substantial reservoir of stem cells. Amniotic grafts may cure wounds better than tissue-engineered skin. Opportunistic microbes often compromise

transplantation treatment. Tissue bank aseptic techniques reduce microbial load but cannot eliminate it. The radiation sterilization dose must be chosen carefully to prevent infections and create a sterile, secure allograft. Consequently, the prevalence of multidrug-resistant and radiation-resistant strains in amniotic samples may significantly threaten tissue transplantation in Bangladesh. To facilitate successful tissue transplantation, it is imperative to conduct epidemiological surveillance of the dissemination of these MDR and radiation-resistant pathogens within the hospital environment. Close monitoring and research into these microbes' behavior in clinical environments are essential to mitigate their impact on vulnerable patient populations.

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Conflict of Interest

The authors declare no conflict of interest.

Ethical Statements

The ethical committee of the Institute of Tissue Banking and Biomaterial Research (ITBBR) under the Bangladesh Atomic Energy Commission approved the use of human amniotic membranes. The ITBBR conducts research and development with amniotic membrane and human bone samples under Bangladesh Atomic Energy Commission supervision and with IAEA support. Additionally, the "Human Organ/Tissue Donation and Transplantation Act (5/1999)" and "Safe Blood Transfusion Act (12/2002)" were passed. ITBBR strictly respects IAEA, AATB, and EATB tissue banking guidelines. Furthermore, the patient's consent was taken during the collection of samples.

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