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Original Article

Isolation and characterization of bacteria from human amniotic membrane and determination of radiation sensitivity of isolates.

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ABSTRACT: The possibility of infectious diseases transmission through tissue allograft is very crucial in good tissue banking practice. The present study was therefore aimed to elucidate the antibiotic and radiation sensitivity pattern of amniotic membrane (AM) associated bacteria for choosing a suitable radiation dose to reduce the bioburden level effectively. Based on biochemical characteristics, thirty bacterial isolates were presumptively identified as *Klebsiella* spp., *Staphylococcus* spp., *E. coli*, *Bacillus* spp., *Moraxella* spp. and *Citrobacter* spp. They showed high resistance to Penicillin (100%), Ampicillin (90%), Vancomycin (87%) and Streptomycin (80%). Most of the isolates were sensitive to Ciprofloxacin, Imipenem and Polymixin B. Two strain of gram-positive bacteria *Staphylococcus* spp. (AMI01 and AMI11) and one strain of *Bacillus* spp. (ASI08) were found to survive at 7 kGy gamma irradiation. The D_{10} value range of gram-positive isolates was 0.89 to 0.94 whereas for gram-negative bacteria the range was 0.63 kGy to 0.90 kGy. Bacterial load was reduced in decimal reduction rate with the increment of radiation dose and 8.0 kGy gamma irradiation dose was found enough to eradicate the bioburden associated with the amnion samples.

Keywords: Amniotic membrane, Bioburden, Gamma irradiation, D_{10} value, Sterilization, PCR

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INTRODUCTION

Human tissue transplantation is no more an imaginary project of medical research in this 20th century. It has been established itself as a significant part of treatment which is able to save thousands of lives by healing wounds, scars, injuries and more on. Among the other types of tissues such as bones, heart, kidney, amniotic membrane transplantation is playing a crucial part especially in burn healing which is becoming more and more acute in our country.

Human amniotic membrane (HAM) is a unique material, which is comprised of the innermost layer of the placenta and lines the amniotic cavity. Its cuboidal epithelial cell layer contiguous to amniotic fluid delivers all the essential nutrients to the fetus. This membrane has potential bacteriostatic properties, lack of immunogenicity and anti-inflammatory action. These unique properties of the amniotic membrane have made it a novel; tissue engineering biomaterial

that has been used from centuries back to present days in tissue regeneration by transplantation. The inclusion of modern technologies in medical science has made it even more promising in the field of clinical treatments like burn healing, ocular reconstruction, periodontal operation, anti-aging, wound healing. Amniotic membrane is a rich source of stem cells which plays a vital role not only in maintaining the structural and anatomical configuration of regenerated tissue but also in fast healing of the wound[1]. So, human amniotic membrane transplantation has shaped a new hope not only to the patients but also to the doctors and scientists as to start a new era of advancement in life-saving.

Now, transplants of human tissue require patient guarantees against communicable infections. This infection may occur due to the incorporation of the unwanted bacterial population into different steps of membrane allograft preparation. The most serious complication found in patients of any kind of surgery is an infection. For this reason, manufacturing processes should be applied in accordance with international standards offering safety from infections, yet maintaining a high quality of the transplants.

Maintenance of proper aseptic technique at different stages of tissue grafts processing can only reduce the level of bioburden but could not totally eliminate the microbial load. Because of several beneficial factors, exposure to gamma radiation is preferred for tissue grafts sterilization[2,3]. A radiation dose of 25 kGy was recommended by International Atomic Energy Agency IAEA[4] for tissue grafts to limit the infectious diseases and to obtain contamination free allografts. But to keep the potential properties and the integrity of the tissue grafts intact it has been analyzed to apply lower dose without conceding the SAL[5] (sterility assurance level) of 10^{-6}. Therefore, it is necessary to know the level of microbial contamination at the initial stage and the radiation resistance pattern of the contaminants to determine the suitable dose required for complete elimination of microorganism without destroying the properties of the amniotic membrane. The present study was designed to investigate common bacterial contaminant associated with vaginal and cesarean amnion samples. Additionally, we have also observed the antimicrobial and radiation sensitivity pattern of bacterial species found in both types of amnion samples which might be helpful to predict a suitable radiation dose to prevent any unwanted bacterial contamination of amnion graft.

METHODOLOGY

Collection of samples

A total of twelve amnion samples (six from a cesarean section and another six were collected from vaginal delivery section) were collected from two hospitals (Maternal and Child Health Training Institute, Azimpur and Gonoshasthaya Samaj Vittik Medical College, Savar) of Dhaka city (Figure 1).

Figure 1. Sample collection locations (A. Maternal and Child Health Training Institute, Azimpur and B. Gonoshasthaya Samaj Vittik Medical College, Savar)

Placenta from both the vaginal and cesarean delivery sections has been retrieved under strict aseptic conditions from the donor by experienced medical personnel. Previously the donors were screened serologically for potentially communicable diseases including human immunodeficiency virus, hepatitis B and C viruses and syphilis.

A number of plastic containers containing normal sterile saline (0.9% NaCl) were kept ready at hospitals before the delivery of new borne. Each container was labeled with placenta sample types (vaginal and cesarean), donor ID and hospital registration number (Figure 2).

Figure 2. Amniotic samples flooded with saline in a sterile plastic container

Amnion samples (glossy, translucent, and thinner) were then collected into those containers shortly after the delivery and preserved temporarily in a freezer below −20°C.

Transport of samples to the laboratory

The sterile containers with membranes were placed in a cool box and transported to the tissue banking laboratory as quickly as possible. All the experiments were carried out in the laboratories of the Institute of Tissue Banking and Biomaterial Research (ITBBR) of Atomic Energy Research Establishment, Savar, Bangladesh.

Sample procurement

At the beginning of the processing, amnion samples were kept out from the freezer at normal temperature to allow the samples to thaw. Following that amniotic membrane was surgically separated from chorion. Blunt dissection was used to follow this step of processing. The strict aseptic condition was maintained at every stage. The weight of AM and chorion of each sample was taken to help the calculation of bio-burden of different processing steps. Finally, samples (AM and chorion) were taken into small beakers containing distilled water and shaken into an orbital shaker approximately for 25-30 minutes for the blood to come out. Then we were left with two different types of solution that could represent the bio-burden of each sample during sample collection and processing. After that those solutions for each sample were serially diluted to 10^{-5} for carrying out the following tests (Figure 3).

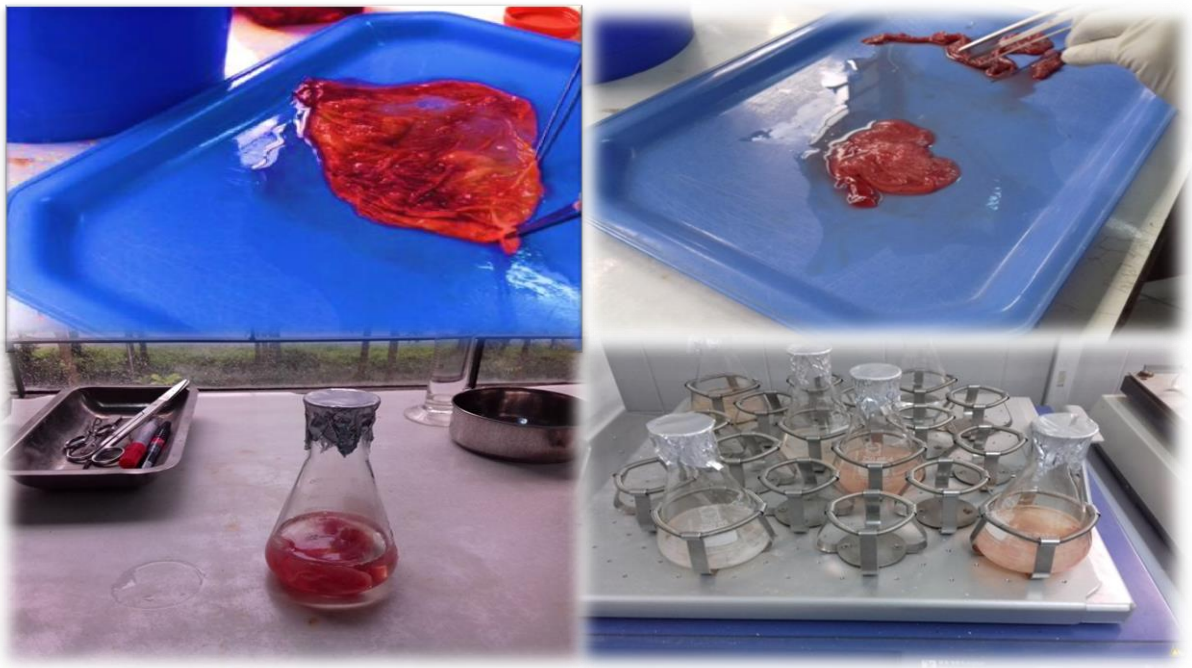

Figure 3. Different stages of sample processing

Media preparation

To observe the bio-burden of the samples total five types of solid bacterial media were prepared named Tryptose soya agar (TSA), MacConkey agar (MA), Eosynemethyline blue agar (EMBA), Salmonella-Shigella agar (SSA) and Mannitol salt agar base (MSAB).

Bioburden count

One hundred microliter (100 µl) of each liquid was inoculated on plates of each media under strict sterile condition using the spread plate technique. Then plates were incubated at 37^0C for 24-48 hours.

Isolation of bacteria

Different media used previously were again prepared to support the growth of a pure single colony. Different colonies were grown on respective media by the help of streaking plate method. Colonies were preserved in slant tubes at a cold temperature for further cultural morphological and biochemical identification.

Cultural and morphological characterization

Individual single colonies were considered for the following characterization- size, pigmentation, shape, margin, elevation, opacity. Morphological characteristics were determined by Gram staining technique and microscopic examination.[6]

Biochemical characterization

For the sake of identification, associated bacterial flora underwent a series of biochemical tests. A total of 30 isolates were randomly selected from vaginal or cesarean placenta samples. Following that those isolates were identified according to Bergey's manual of determinative bacteriology[7].

Preservation of the isolates

Each isolate was streaked aseptically on Nutrient agar plate and was incubated overnight at 37^0C. Following incubation, cells were scrapped out aseptically with a sterile loop and suspended thoroughly in Luria broth (LB) containing 20% glycerol in small screw-capped vials. For each strain, duplicate vials were prepared and maintained at -80^0C.

Antimicrobial susceptibility test

The antimicrobial susceptibility of the test isolates was determined in vitro by using the standardized agar-disc-diffusion method known as the Kirbey Bauer[8]. It is a modification of Baur's method[9]. Commercially available discs and Mueller-Hinton agar (Oxoid Limited, England) were used for the antimicrobial assay. Total of seven antibiotic discs were used in this study.

DNA extraction of the isolates

Chromosomal DNA of thirty isolates was extracted from cell suspensions of bacteria grown overnight. Boiled DNA of the isolates was prepared as described previously[10]. Briefly, isolates were grown in TSB or nutrient broth at 37^0C for overnight. One (1.0) ml culture was taken in a centrifuge tube and centrifuged for 5 min at 10000 rpm (2-3 times). The supernatant was removed and washed with DH_2O. Two hundred microliter (200 µl) PCR grade water was added and dissolved by gentle shaking and boiled at 100^oC for 10 minutes. After boiling, the tubes were placed in ice for 10 minutes and then centrifuged at 10000 rpm for 10 minutes. Finally, supernatant was taken in fresh centrifuge tubes and stored at -20^o C. Extracted DNA was used as templates in PCR amplification[10].

Polymerase Chain Reaction (PCR)

PCR using universal primers for bacterial 16S rRNA gene was done for molecular characterization of isolates. For the test, universal primer sets (27F and 1492R) were used. After mixing, the reaction mixture with the template DNA, the PCR tube containing reaction mixture was capped and centrifuged briefly to spin down the contents. The PCR tubes were then placed in a thermal cycler (Gene Atlas, Germany). After completion of PCR, the tubes were stored at -20^0C until further analysis.

Agarose gel electrophoresis

The PCR products were visualized under, Red protein simple gel documentation system (USA) after 1% w/v agarose gel electrophoresis (30 minutes, 100V) and staining with ethidium bromide (0.5 µg/mL).

Radiation sensitivity pattern of the isolates

After isolation and presumptive identification, bacterial isolates of amnion were screened for comparative radiation resistance from 1.0 to 8.0 kGy of radiation doses. Bacterial count was taken before and after radiation. For the unirradiated population, cells were incubated for 2 to 3 hours at 37^0C; plating was performed with appropriate dilutions and again incubated for 24 hours at 37^0C, and viable cells were estimated. For the irradiated population, plating was done after the exposure of different radiation doses. Assay for D_{10} Value and Determination of Radiation Sterilization Dose (RSD).

RESULTS

Enumeration and isolation of bacteria

The total bioburden in cesarean amnion and vaginal samples was recorded as 2.5 - 4.83 log CFU/ml and 3.0-4.62 log CFU/ml, respectively. Among them, thirty bacterial isolates were gram stained for microscopic observation and the result revealed that most predominant bacteria were found as gram-negative cocci (40%), followed by gram-negative bacilli (30%), gram-positive cocci (23%) and gram-positive bacilli (7%).

Biochemical Characterization of bacteria

Based on biochemical characteristics (Catalase, Oxidase, Citrate, MR, VP, KIA, Motility, Indole, Mannitol test), a total of six groups of bacteria were identified. Among the isolates, *Klebsiella* spp. (30%) was found to be the most prevalent bacteria. The prevalence of bacterial flora can be depicted as follows: *Klebsiella* spp. >*E. coli* >*Moraxella* spp.

>*Staphylococcus* spp. >*Bacillus* spp. >*Citrobacter* spp. **(**Figure 4**).**

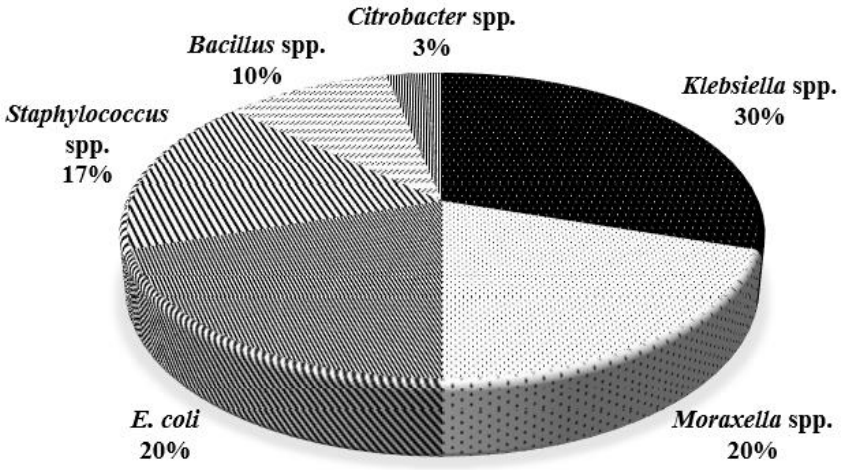

Figure 4. Prevalence of bacteria associated with amnion samples

Antibiogram Profiling

Antibiotic sensitivity pattern of the isolated bacteria from amnion samples was investigated. Total 8 antibiotics of different groups were used to test those 30 bacterial isolates shown in Table 1.

Table 1. Antibiotic resistance pattern of both gram-positive and negative isolates

Group Name	Isolate ID	Antibiotics sensitivity profile*		
		Resistance	Intermediate	Sensitive
Gram positive	01, 11, 14	AMP, P, VA	S	IPM, CN, CIP, PB
	02	VA, P	AMP	IPM, CN, CIP, S, PB
	03	-	-	AMP, IPM, CIP, S, CN, VA, P, PB
	08	P, PB	-	AMP,IPM,CIP,S, CN, VA
	09	AMP, P, VA, S	PB	IPM, CN, CIP
	21	AMP, P	-	IPM, CN, CIP, S, VA, PB
Gram negative	04, 06, 07, 23	AMP, P, VA,	S	IPM, CN, CIP, PB
	05	IPM, AMP, P, VA	S	CN, CIP, PB
	10, 13, 15, 18, 27	AMP, P, VA, S	-	IPM, CN, CIP, PB
	12	CN, IPM, CIP, PB	-	AMP, P, VA, S
	16, 26	IPM, AMP, P, VA	S	CN, CIP, PB
	17, 19, 22, 25, 29, 30	AMP, IPM, P, S, VA	-	CIP, CN, PB
	20	AMP, IPM, CIP, S, CN, VA, P, PB	-	-
	24	AMP, IPM, P, VA	-	CN, CIP, S, PB
	28	IPM, AMP, P, S, VA	-	PB, CN, CIP

The antibiogram test results were interpreted according to the CLSI 2007 guidelines[11]. Antibiotic sensitivity pattern of the isolates may be different depending on the type of bacteria. All the isolates of both Gram positive and Gram-negative bacteria showed 100% resistance against Penicillin (P). According to the resistance profile, Vancomycin (VA) was the second most resistant (95.45%) and Ampicillin, (AMP) was positioned right after that (90.90%). Gram-positive bacterial isolates were found to be resistant against Penicillin-G (P) (87.5%), Ampicillin (AMP) (62.5%), Vancomycin (VA) (62.5%) and Streptomycin (S) (12.5%). Some were found to be moderately resistant to Streptomycin (S) and Polymixin (PB). Most of the Gram-positive isolates were sensitive against imipenem (IPM), Ciprofloxacin (CIP), Polymixin (P) and Gentamycin (CN).

Gram-negative bacterial isolates of the present study were found more dangerous than Gram-positive ones. Isolate *Moraxella* spp. (AMI20) was resistant against all antibiotics tested in the study. Thirteen isolates among 22-gram negative bacteria appeared resistant against imipenem (IPM) (59.09%). This is a serious threat to antibiotic cocktail treatment if used for tissue grafts decontamination as imipenem (IPM) is used to kill a wide range of both Gram-positive and Gram-negative bacteria. All Gram-negative isolates were moderately resistant to Streptomycin (S). Most of the isolates were sensitive against Gentamycin (CN) and Ciprofloxacin (CIP) (90.90%) (Figure 5).

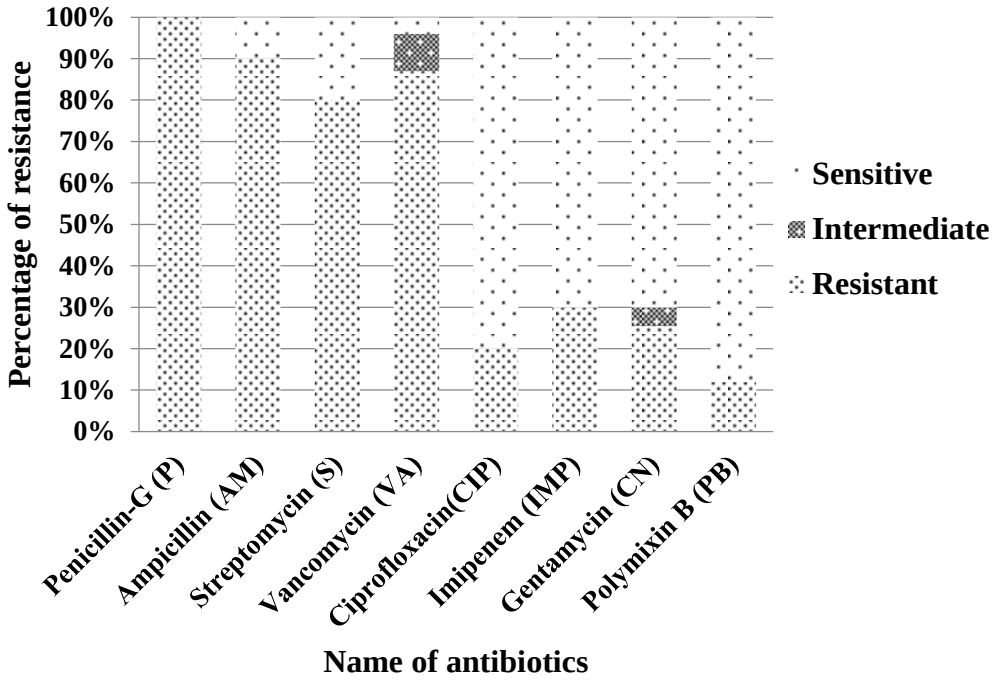

Figure 5. Antibiotic resistance pattern of representative isolates

Radiation sensitivity pattern of the isolates

Eleven isolates were randomly selected from 30 isolates for the radiation sensitivity test. They were exposed to 1-9 kGy gamma radiation using a Co-60 gamma source. Before gamma sterilization, the highest count was observed in *Bacillus* spp. (ASI08) as $7.1x10^8$ cfu/ml and the lowest count in *Moraxella* spp. (AMI18) as $2.2x10^6$ cfu/ml (Table 2).

Table 2. Determination of bacterial population (N_0) before radiation using spread plate method

Isolates ID	Presumptive identification	CFU/ml of bacterial suspension
AMI01	*Staphylococcus* spp.	$5.6x10^7$
AMI07	*Klebsiella* spp.	$6.2x10^7$
ASI08	*Bacillus* spp.	$7.1x10^8$
AMI11	*Staphylococcus* spp.	$2.5x10^7$
AMI12	*Klebsiella* spp.	$5.3x10^7$
AMI16	*E. coli*	$4.3x10^6$
AMI17	*E. coli*	$5.0x10^6$
AMI18	*Moraxella* spp.	$2.2x10^6$
AMI24	*Klebsiella* spp.	$1.1x10^7$
AMI26	*Moraxella* spp.	$2.5x10^6$
AMI30	*Citrobacter* spp.	$3.5x10^6$

All the isolates were able to survive radiation dose up to 5 kGy except Gram-negative *Moraxella* spp. (AMI18). Five Gram-negative isolates (AMI12, AMI16, AMI17, AMI24, AMI30) were found to tolerate up to 6 kGy. On the other hand, only three isolates (AMI01, ASI08, AMI11) survived 7 kGy all being Gram-positive. So, it appears that Gram-positive isolates were more resistant to gamma irradiation than the Gram-negative ones (Table 3).

Table 3. Survival rate of the isolates after radiation exposure

Isolate ID	No. of the colony/ml of bacterial suspension after irradiation Radiation Dose (kGy)							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
AMI01	$1.2x10^7$	$1.04x10^7$	$9.2x10^5$	$7.2x10^5$	$9.6x10^4$	$6.2x10^3$	25	-
AMI07	$5.8x10^7$	$3.5x10^6$	$6.1x10^5$	$4.2x10^4$	$5.1x10^3$	-	-	
ASI08	$2.5x10^7$	$2.2x10^7$	$2.5x10^6$	$2.1x10^6$	$1.9x10^5$	$1.5x10^4$	$1.01x10^2$	-
AMI11	$2.4x10^7$	$2.1x10^7$	$2.3x10^6$	$1.1x10^6$	$1.9x10^5$	$1.3x10^4$	$1.1x10^2$	-
AMI12	$2.1x10^7$	$2.3x10^6$	$5.1x10^5$	$5.2x10^4$	$6.0x10^3$	$2.2x10^2$	-	-
AMI16	$2.4x10^6$	$2.3x10^5$	$4.2x10^4$	$3.1x10^3$	$5.2x10^2$	$1.2x10^2$	-	-
AMI17	$4.5x10^6$	$2.1x10^6$	$2.3x10^4$	$5.1x10^3$	$5.2x10^2$	$4.0x10^2$	-	-
AMI18	$1.2x10^6$	$1.89x10^5$	$1.3x10^4$	$1.1x10^2$	-	-	-	-
AMI24	$1.0x10^7$	$3.1x10^5$	$5.2x10^4$	$1.2x10^3$	$2.5X10^2$	3.5X10	-	-
AMI26	$2.2x10^6$	$2.3x10^5$	$5.1x10^4$	$5.2x10^4$	$6.0x10^3$	-	-	-
AMI30	$3.5x10^6$	$6.1x10^5$	$4.2x10^4$	$5.1x10^3$	$3.3x10^2$	9.1x10	-	-

Determination of D_{10} value and radiation sterilization dose (RSD) of the isolates

Gamma radiation is one of the most commonly used methods employed for the destruction of microbial cells. The killing effect of radiation in microorganisms is generally expressed by the decimal reduction dose or D_{10} value[12]. The survival rate of bacterial isolates was calculated to observe their gradual decrease with the increment of gamma irradiation dose.

Two isolates of *Staphylococcus* spp. (AMI01, AMI11) and one isolate of *Bacillus* spp. (ASI08) characterized from amnion were tested among eight-gram positive isolates for logarithmic survival fraction. *Staphylococcus* spp. (AMI11) showed high resistance against gamma radiation and could grow even after being exposed to 7 kGy (Table 4).

Table 4. Logarithmic survival fraction of representative isolates from amnion

Type of bacteria	Isolate ID	Logarithmic survival fraction-log S Radiation dose (kGy)							
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Gram positive	**AMI01**	-0.67	-0.72	-1.79	-1.88	-2.60	-4	-6.31	-
	ASI08	-1.45	-1.50	-2.45	-2.53	-3.57	-4.68	-6.82	-
	AMI11	-.01	-.07	-1.03	-1.36	-2.12	-3.28	-5.36	-
Gram negative	**AMI07**	-0.03	-1.24	-2.00	-3.17	-4.07	-	-	-
	AMI12	-0.41	-1.39	-2.01	-3.0	-3.95	-5.39	-	-
	AMI16	-0.25	-1.30	-2.04	-3.15	-3.92	-4.60	-	-
	AMI17	-0.04	-0.37	-2.33	-2.99	-3.9	-4.1	-	-
	AMI18	-0.26	-1.06	-2.23	-4.3	-	-	-	-
	AMI24	-0.04	-1.56	-2.32	-3.96	-4.64	-5.5	-	-
	AMI26	-.05	-1.03	-1.69	-2.62	-	-	-	-
	AMI30	-0.11	-0.89	-2.04	-3.0	-4.13	-5.60	-	-

The sub-lethal dose was 7 kGy for all the gram-positive representative isolates. The D_{10} value range for them was between (0.80-0.94). *Staphylococcus* spp. (AMI11) showed the highest D_{10} as 0.94 and the lowest value was 0.80 *Bacillus* spp. (ASI08). *Staphylococcus* spp. (AMI01 and AMI11) were more resistant against radiation than *Bacillus* spp. (ASI08) in this study (Table 5).

Table 5. D_{10} values of bacterial isolates and RSDs required for 10^{-6} sterility assurance level (SAL)

Type of bacteria	Isolate ID	Sublethal dose	D_{10} value (kGy)	RSD (kGy), Bioburden	
				100	1000
Gram-positive	AMI01	7	0.90	7.2	8.1
	ASI08	7	0.80	6.4	7.2
	AMI11	7	0.94	7.5	8.5
Gram-negative	AMI7	5	0.64	5.12	5.76
	AMI12	6	0.78	6.24	7.02
	AMI16	6	0.90	7.2	8.01
	AMI 17	6	0.89	7.12	8.01
	AMI18	4	0.63	5.04	5.67
	AMI 24	6	0.85	6.8	7.65
	AMI26	5	0.94	7.52	8.46
	AMI 30	6	0.92	7.36	8.28

In the case of Gram-negative isolates, three of *Klebsiella* spp. (AMI07, AMI12, and AMI24), two of *E. coli* (AMI16, AMI17) and *Moraxella* spp. (AMI18, AMI 24) and one *Citrobacter* spp. (AMI30) were investigated for logarithmic survival fraction. *Klebsiella* spp. (AMI12 and AMI 24), *E. coli* (AMI16 and AMI17) and *Citrobacter* spp. (AMI30) showed relatively high resistance than other Gram-negative ones. These five isolates survived up to 6 kGy doses of gamma irradiation (Table 4). The sub-lethal dose for the representative Gram-negative representative isolates ranged from 4-6 kGy. The D_{10} value range for them was between 0.64-0.94. *Moraxella* spp. (AMI30) showed the highest D_{10} as 0.94 and the lowest value was also found for *Moraxella* spp. (AMI18) as 0.64 (Table 5).

The calculation of radiation sterilization dose (RSD) is necessary to achieve sterility assurance level (SAL) of 10^{-6} for bioburden level of 100 and 1000 (Table 5). The range of calculated RSD for the isolates was (5.67-8.5). The highest RSD value was found in gram-positive isolate *Staphylococcus* spp. (AMI11). The RSD value 8.5 kGy was required for 1000 bioburden to eliminate the bacterial species AMI11 to achieve the SAL. The lowest value of calculated RSD was 5.67 for gram-negative *Moraxella* spp. (AMI18) (Table 5). The growth rate of all bacterial isolates growth was reduced in logarithmic rate with the increase of radiation dose and finally declined at radiation dose 8.0 kGy. It has been clearly shown that there is an inverse proportional relationship of bioburden count with the radiation dose (Figure 6).

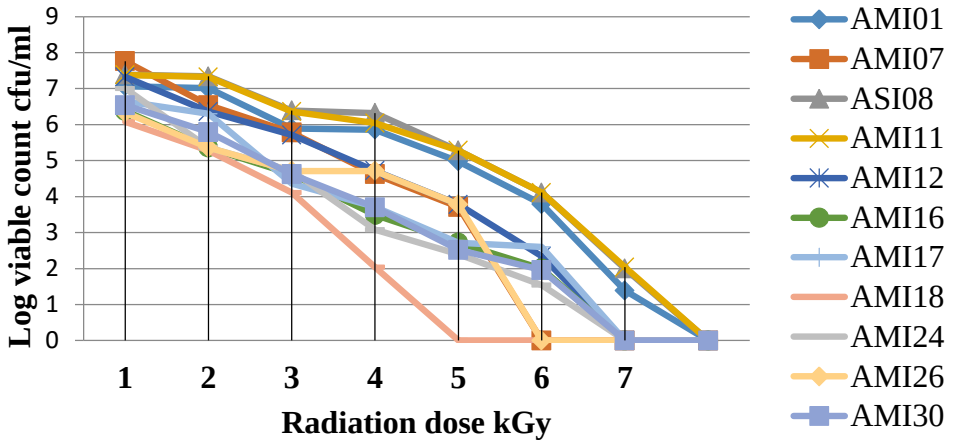

Figure 6. Logarithmic reduction of bioburden counts with the increment of radiation dose

16S rRNA PCR Analysis

After 1% agarose gel electrophoresis, all the bacterial isolates were positive for amplification of a 1500bp fragment of 16S rRNA specific genes in PCR using the universal primer 27F and 1492R (Figure 7).

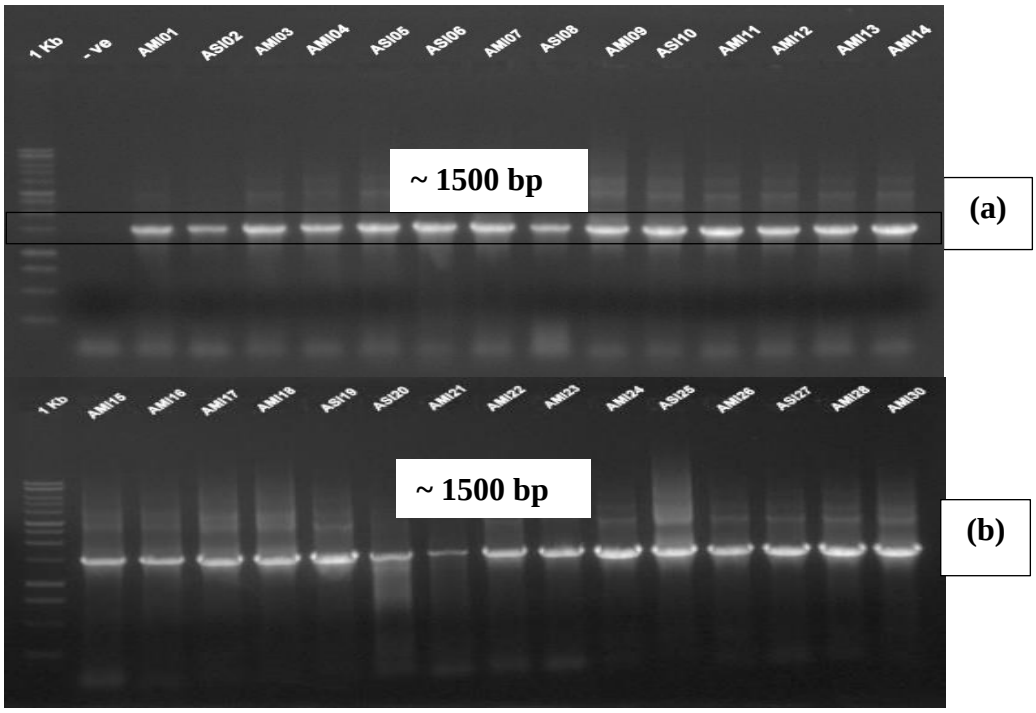

Figure 7 (a and b). PCR amplification of 16S rRNA gene of bacteria isolated from both vaginal and caesarian amnion samples

For molecular characterization of these amplified fragments of the isolates will be sequenced and the phylogenetic analysis will be done further using bioinformatics tools.

DISCUSSION

Tissue bank has been playing an important part to provide human connective tissues for clinical use with the guaranteed quality from the moment of retrieval up to the treatment as allograft over years. In spite of all the strict maintenance to ensure the aseptic condition of storage and processing for tissue allograft; bacterial contamination still exists as a major threat. This may result in prolonged hospitalization; organ failure or even death can occur. In most cases, infection occurs after a graft implant. There is always a chance that microorganisms could invade the grafts if any single step of the chain is broken such as-tissue procurement, processing, handling, storage before supply or during surgery in spite of proper donor screening. The major aim of this study was to investigate the level of bioburden on amniotic membrane and the antibiotic and radiation sensitivity pattern of membrane-associated isolates for determining a suitable radiation dose to reduce the bioburden level effectively without compromising the SAL.

In this study, twelve amnion samples were analyzed (six were from the vaginal section and another six were from cesarean section). Both gram positive and gram-negative bacteria were isolated and identified from the human amniotic membrane[13,14]. In this particular point, the findings of the present study showed similarity as both gram negative and positive bacterial species were retrieved and gram-negative bacteria was the most prevalent than the gram-positive contaminant. *Klebsiella* spp. showed the highest number according to the biochemical test result. *E. coli* was also found that could indicate the possibility of hospital infection. A recent study showed 27% foetal mortality due to *E. coli* infection in pregnant women.[15] *Ureaplasma urealyticum*, *Fusobacterium* spp., and *Mycoplasma hominis* have been reported as the most common microbial species isolated from the amniotic cavity of women having preterm labor and intact membranes[16,17] isolated 90% aerobic organisms from the amniotic fluid such as *Streptococcus agalactiae*, *S. viridans*, *Peptostreptococcus*, *Staphylococcus aureus*, *Gardnerella vaginalis and Bacteroides* spp. which are responsible for approximately half of the infections occurred during human tissue transplantation. There are also reports on the most predominant bacterial contaminant *Streptococcus* spp. and *Staphylococcus* spp. on amnion grafts[13,18, 19,20]. *Staphylococcus* spp., *Bacillus* spp. and *Citrobacter* spp. were found as a major contaminant of amnion[21] which has close proximity to our findings.

About 2-3% tissue grafts contamination has been occurred due to an environmental factor, donor infected with diseases and the impaired host defense mechanism[22]. The major source of the contamination may come from patient's skin, nasal flora, airborne particles from operation theatre personnel[23,24]. In this study, all the amniotic membrane samples were collected from seronegative (HIV, HBV, and VDRL) donors and were procured under strict aseptic condition. Despite maintaining good tissue banking practices, the amnion tissues were found to be highly contaminated with bacteria. The possible sources of contamination might be unhygienic handling of clinical samples, inappropriate disinfection system of surgical utensils, cross-contamination between samples etc.

Proper use of disinfectants, sterilization procedure, and antibiotic treatment might be considered in order to reduce contamination level at different stages of tissue procurement[25]. Antibiotic treatment has been used as a traditional way to decontaminate and for storage of membrane allograft. In this study total, eight antibiotics were tested against 30 bacterial isolates. All were found multidrug-resistant (MDR) with an alarming level of resistance. They were 100 % resistant to Penicillin-G (P). Isolates were also showed a high level of resistance against Ampicillin (A), Streptomycin (S) and Vancomycin (V). Lower resistance against antibiotic was observed in gram-positive bacteria than that of gram-negative ones. Most of the isolates were sensitive against imipenem (I), Ciprofloxacin (C) and Polymixin (PB). Multidrug-resistant *Staphylococcus* spp. was evident in other researches such as Aghayan et al. in 2012. Previous studies have also demonstrated about multidrug resistance of *E. coli* and *Klebsiella* spp. from different ranges of samples [26,27,28].

Due to various positive factors, gamma irradiation is one of the best methods for the sterilization of tissue allografts. Usually, the appropriate dose for the sterilization of the tissue grafts is 25 kGy. But few tissue banks favor lower radiation dose without compromising SAL 10^{-6} to keep potential properties of tissues intact[29]. The mechanical properties and osteo-inductive capacity of grafts may be destroyed if radiation dose is increased greater than 25 kGy. If the radiation dose is decreased to 9.2 kGy, sterility (10^{-6} SAL) of tissue allograft could be achieved because it causes less hamper to the biomechanical properties of amnion grafts[30]. 17.6 kGy gamma radiation had also been applied for amnion allografts. Nabangshu et al. (2013) demonstrated that a much higher radiation dose to kill bacterial isolates as 15 kGy. In our findings, 8.0 kGy is suitable to eliminate both gram positive and negative bacteria.

CONCLUSION

The presence of multidrug-resistant and radiation resistant strains on amnion samples could be a potential threat to tissue transplantation in Bangladesh. There is an urgent need for the epidemiological monitoring to prevent the spread of these MDR and radiation resistant pathogens in the hospital environment. Further investigation might play a vital role in determining a suitable radiation dose as well as in obtaining sterile allograft for safe tissue transplantation.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest

The authors declare no conflict of interest.

Human and Animal Rights and Informed Consent

We used human amniotic membrane following the approval of the ethical committee of the Institute of Tissue Banking and Biomaterial Research (ITBBR) under Bangladesh Atomic Energy Commission. The ITBBR performs its research and developmental activities with human tissue samples (amniotic membrane and human bone) under the authority of Bangladesh Atomic Energy Commission with the strong cooperation of International Atomic Energy Commission (IAEA). Besides, two government bills had been passed entitled "Human Organ/Tissue Donation and Transplantation Act (5/1999)" and "Safe Blood Transfusion Act (12/2002)". ITBBR strongly follows the guideline of IAEA regarding Tissue Banking set up, American Association of Tissue Bank (AATB) and the European Association of Tissue Bank (EATB). Furthermore. The human samples are collected after taking permission and filling up the patient's consent form.

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