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Microbiological Quality Assessment at Different Stages of Frozen Amniotic Allograft Processing for Safe Tissue Banking Activities

Md Arifuzzaman*, Naznin Akhtar and SM Asaduzzaman

Institute of Tissue Banking and Biomaterial Research, Atomic Energy Research Establishment (AERE), Savar, Dhaka-1349, Bangladesh

Abstract

Human Amniotic Membrane (HAM) is widely used as biological dressing material in reconstructive skin surgery, abdominal and vaginal reconstruction, plastic and cosmetic surgery and in ophthalmologic surgery. The objective of this study was to assess microbial quality of HAM during different stages of frozen HAM allograft processing and determination of antimicrobial susceptibility of isolated microorganisms. For this purpose, twelve amniotic sacs were collected from normal vaginal delivery of seronegative mother from Azimpur maternity, Dhaka, Bangladesh.

Initial bioburden was determined by using Nutrient Agar (NA), McConkey Agar, Eosin Methylene Blue (EMB) Agar, Potato Dextrose Agar (PDA). Total Viable Bacterial Count (TVBC) was calculated and Initial bacterial load was ranged from 39 to 5.25 × 10^3. No fungus was found. A total 28 bacterial isolates were selected. These bacterial isolates were identified on the basis of cultural (e.g. colony size, shape, opacity), morphological (e.g. gram reaction, cell shape and arrangement) and biochemical characterization (e.g. catalase, oxidase, carbohydrate fermentation, Methyl Red (MR) test and Voges Proskauer (Vp) test). Of them, eight bacterial isolates were identified as *Staphylococcus aureus*, two were *Staphylococcus epidermidis*, nine were *Escherichia coli*, three were *Salmonella typhimurium*, one was *Enterobacter aerogenes*, one was *Pseudomonas aeruginosa*, four were *Acinetobacter baumanii*. Then, antimicrobial susceptibility pattern of isolated microorganisms were determined against ten antibiotics which includes Amphicillin, Streptomycin, Gentamycin, Neomycin, Imipenem, Vancomycin, Cloxacillin, Polymixin-B, Penicillin-G and Ciprofoxacin. It was found that, all bacterial isolates were sensitive to streptomycin and Penicillin-G. Thus, Streptomycin-Penicillin-G (Strep-P) cocktail was formulated and was used for the preparation of frozen AM. Then, bioburden was again determined by spread plate technique using the same media. Bacterial load in the processed HAM were ranged from 33 to 3.94 2. After then, HAM was preserved by using Dulbeco's Modified Eagles Media (DMEM) and glycerol (1:1 ratio) and was stored at -80°C. Microbial quality of the preserved samples were checked at 07, 14, 21 & 30 days and no bioburden was found. Thus, it can be said that the antibiotic cocktail was suitable to remove the culturable microorganisms associated with HAM.

Keywords: Human amniotic membrane; Frozen amniotic membrane; Antibiotic cocktail; Bioburden; Sterility

Introduction

Amniotic Membrane (AM) is the thin, tough, transparent and innermost lining of the fetal membrane which functions as a boundary to save the fetus from different types of infections and traumas [1]. Structurally, HAM possesses a stromal matrix which is avascular, a basement membrane and an epithelial monolayer [2]. Generally, living mothers who deliver a live baby through the elective cesarean section and vaginal section, are donors of HAM [3]. HAM has several exclusive properties e.g. promotion of epithelialization, anti-inflammatory, anti-fibrotic, anti-adhesive & antimicrobial activities [4]. However, its epithelium also secretes several types of growth factors including interleukin 6 (IL-6) and interleukin 8 (IL-8). Besides, amniotic basement membrane and stroma consist of collagen type I, III, V, VII, laminin, and fibronectin [5]. AM transplantation is regarded as an ideal dressing or wound healing related therapy among the physicians. Previously, different types of fistula such as vaginal or entero cutaneous fistula was successfully treated by AM transplantation [6,7]. However, unique antibacterial properties of AM have made it an ideal therapeutics in case of wound treatment [8]. AM Transplantation (AMT) is very much beneficial for the treatment of different types of ophthalmic disorders such as persistent epithelial defects, conjunctival defects etc [9]. However, there are several types of processed AM including heat dried AM, freeze-dried AM, preservation of AM in cold glycerol and cryopreserved or frozen AM [10]. Among them, cryopreserved AM is extensively used for the treatment of different ophthalmic disorders due to the presence of maximum biological properties compared to other methods of processing and preservation [11]. Low temperature preservation in the deep freezer plays an important role to maintain the viability of AM for a long time [12]. Biochemical studies of cryopreserved AM confirmed the presence of Epidermal Growth Factor (EGF), Transforming Growth Factor alpha (TGF α), Keratinocyte Growth Factor (KGF), Hepatocyte Growth Factor, basic Fibroblast Growth Factor (bFGF), Transforming Growth Factor β1 and β2 [13]. Moreover, due to preservation at very low temperature all epithelial cells of AM are destroyed which leads to the loss of immunogenicity of AM [14]. In cryo condition AM manifests its capacity to promote migration and adhesion of corneal epithelium due to the presence of laminin 5 [15]. However, some safety prerequisites should be ensured to make cryopreserved AM as an ideal allograft for the treatment of different ophthalmic disorders [13] Microbial contamination of AM may occur due to the lack of aseptic techniques in tissue collection, procurement, and processing [16]. Sometimes microbial flora originated from a donor, operation theater or hospital personnel are responsible for microbial contamination of AM grafts. Bacterial infections inhibit the process of wound healing and tissue regeneration, antimicrobial properties are a valuable characteristic of AM. The post-surgical environment is susceptible to bacterial or fungal colonization

***Corresponding author:** Md Arifuzzaman, Institute of Tissue Banking and Biomaterial Research, Atomic Energy Research Establishment, Savar, Dhaka-1349, Bangladesh, Tel: +880-1827806384; Fax: +887790034; E-mail: arifbio366@gmail.com

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or contamination, and hence the risk for developing infections and secondary scarring and organ dysfunction is high. To diminish the possibility of microbial contamination, it is mandatory to take several precautionary steps including careful donor screening, proper tissue processing and tissue allograft sterilization. In cryopreservation method, AMs are treated with antibiotics at different concentrations for their decontamination which is very much effective against different types of fungal and bacterial contaminations. So, it is very important to process cryopreserved AM and to assess its microbiological quality during its different processing stages to ensure an effective and sterile tissue allograft for ophthalmic and massive burns and surgical wound associated skin dysfunctions treatment. Thus, the objective of this study was to determine the microbial quality of AM during its different stages associated with cryopreservation.

Materials and Methods

Sample collection

Human amniotic sacs were collected from Azimpur Maternity Hospital, Dhaka, Bangladesh after normal vaginal delivery of healthy seronegative (free from HBV, HSV, HIV, HPV & HCV virus) mother. Shortly after collection amniotic sacs were transferred to the Institute of Tissue Banking & Biomaterial Research (ITBBR), Atomic Energy Research Establishment (AERE), Savar, Dhaka under sterile condition.

Processing of cryopreserved AM

In ITBBR, at first, AM was separated from chorions by using sterile forceps. After being a separation of AM from the chorion, they were then cut into small sizes (~2x2 cm) by using sterile scalpel blade and repeatedly washed by using sterile Phosphate Buffer Saline (PBS). Then, initial bioburden was determined by spread plate technique using Nutrient agar, McConkey agar, EMB agar, and Potato Dextrose Agar (PDA). After then, AM samples were washed again by using PBS and bioburden was also determined again by same technique using same media. Bacterial isolates were selected during the initial and secondary wash and analyzed them by gram staining. Finally, they were washed with PBS containing antibiotic cocktail (penicillin 50 µg/mL, streptomycin 50 µg/mL, gentamicin 25 µg/mL, neomycin 100 µg/mL and amphotericin B 2.5 µg/mL). After then, final bioburden was determined by the same way. Before preservation, AM was fixed with sterile nitrocellulose paper and was then put into falcon tubes containing Dulbecco's Modified Eagles Media (DMEM) and glycerol (1:1 ratio) and were then stored at -80°C. All procedures were done under laminar airflow cabinet. Microbial quality checking of every samples were performed periodically between 07 days, 14 days, 21 days and 30 days.

Cultural characterization

Cultural characteristics of bacterial isolates were studied by inoculating the colonies on Nutrient Agar, McConkey Agar, Eosin Methylene Blue Agar, Salmonella-Shigella Agar, Mannitol Salt Agar Base media plates and incubated at 35°C for 24 to 48 hours. After incubation, colonies on media plates were observed for size, pigmentation, shape, edge, elevation, opacity etc.

Morphological characterization: Morphological characteristics were determined by Gram staining technique and microscopic examination.

Biochemical characterization: Several biochemical tests were performed to identify bacterial isolates which includes: catalase test, oxidase test, Citrate test, indole test, Methyl Red (MR) test, Voges Proskauer (VP) test, motility test, Kligler Iron (KI) agar test and carbohydrate fermentation test.

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 (Barry and Thornsberry). It is a modification of Baur's method [17]. Commercially available discs and Mueller-Hinton agar (Oxoid Limited, England) were used for the antimicrobial assay. Total ten types of antibiotic discs were used in this study (Table 1).

Sterile antimicrobial disks were dispensed onto the surface of the inoculated agar plate. Each disk was pressed down individually to ensure complete contact with the agar surface. The disk placed in the agar surface was not closer than 24 mm from center to center. The plates were inverted and placed in an incubator set to 35°C within 15 min after the disks were applied. After 16-18 h of incubation, each plate was examined for the zone of inhibition, uniformly circular with a confluent lawn of growth. The diameters of the zones of complete inhibition (judged by the unaided eye) were measured, including the diameter of the disk. Zones are measured to the nearest whole millimeter. Faint growth of tiny colonies, which can be detected only with a magnifying lens at the edge of the zone of inhibited growth, was ignored. The sizes of zones of inhibition were interpreted by referring to zone diameter interpretive standards from NCCLS 2000 (Table 2) organisms are reported as susceptible, intermediate or resistant to the agents that have been tested.

Antibiotic cocktail formation

On the basis of the result of antibiotic susceptibility pattern of

Antimicrobial Groups	Antimicrobial agents	Disc conc. (µg)	Zone interpretation (diameter in mm)		
			S	M	R
Aminoglycosides	Gentamycin (CN)	10	≥ 15	13-14	≤ 12
	Streptomycin (S)	10	≥ 15	12-14	≤ 11
Carbapenems (carboxypenems)	Imipenem (IMP)	10	≥ 16	14-15	≤ 13
Glycopeptidase	Vancomycin (VA)	30	≥ 15	12-14	≤ 11
Quinolones	Ciprofloxacin (CIP)	5	≥ 21	16-20	≤ 15
Penicillins	Ampicillin (AM)	10	≥ 17	14-16	≤ 13
	Penicillin-G (P)	10	≥ 29	-	≤ 28
Polypeptides	Polymixin B (PB)	300	≥ 12	9-11	≤ 8
Quinolones (Fluoroquinolones)	Ciprofloxacin (CIP)	5	≥ 21	16-20	≤ 15
	Neomycin (N)	-	≥ 26	23-25	≤ 22

Note: S: Susceptible, M: Medium, R: Resistant

Table 1: Lists of antibiotic discs used in the experiment.

Sample No.	Initial bioburden count (c.f.u/g)					
	NA	Mac	EMB	PDA	SSA	MSAB
1	1.386×10^3	1.37×10^3	1.26×10^3	Nil	Nil	Nil
2	1.107×10^3	3.3×10^3	2.26×10^3	Nil	Nil	Nil
3	8.56×10^2	3.18×10^2	3×10^2	Nil	Nil	Nil
4	4.74×10^3	3.20×10^3	3.26×10^3	Nil	1.98×10^2	Nil
5	3.4×10^3	5.25×10^3	2.63×10^3	Nil	Nil	Nil
6	1.7×10^3	2.3×10^3	1.5×10^3	Nil	7.5×10^1	3.9×10^1
7	2.34×10^3	3.01×10^3	2.9×10^3	Nil	5.9×10^1	Nil
8	4.06×10^3	3.92×10^3	3.06×10^3	Nil	Nil	NIL
9	5.11×10^3	4.07×10^3	3.18×10^3	Nil	Nil	2.9×10^1
10	2.43×10^3	4.34×10^3	2.90×10^3	Nil	Nil	6.7×10^1
11	1.56×10^3	2.67×10^3	2.89×10^3	Nil	Nil	Nil
12	2.78×10^3	2.99×10^3	3.01×10^3	Nil	Nil	Nil

Note: NA: Nutrient Agar, Mac: McConkey Agar, EMB: Eosine Methylene Blue, PDA: Potato Dextrose Agar, SSA: Salmonella-Shigella Agar, MSAB: Mannitol Salt Base Agar

Table 2: Initial bioburden count.

Isolated colony ID	Size	Form	Elevation	Margin	Surface	Color	Gram staining	Shape
FAM 01	1.5 mm	Circular	Raised	Entire	Shiny & smooth	Light Yellow	Gram positive	round
FAM 02	0.9 mm	Circular	Raised	Entire	Shiny & smooth	White	Gram positive	cocci
FAM 03	1.1 mm	Circular	Umbonate	Wavy	Shiny & smooth	Yellow	Gram positive	diplococci
FAM 04	1.2 mm	Circular	Raised	Entire	Shiny & smooth	Pink	Gram negative	rod
FAM 05	0.8 mm	Circular	Raised	Entire	Shiny & smooth	Reddish brown	Gram negative	rod
FAM 06	1 mm	Circular	Raised	Entire	Shiny & smooth	Dark blue	Gram negative	rod
FAM 07	0.6 mm	Circular	Raised	Entire	Shiny & smooth	Light brown	Gram negative	rod
FAM 08	1.2 mm	Circular	Raised	Entire	Shiny & smooth	White	Gram positive	cocci
FAM 09	0.5 mm	Circular	Raised	Entire	Shiny & smooth	White	Gram positive	cocci
FAM 10	0.7 mm	Circular	Raised	Entire	Shiny & smooth	Green metallic sheen	Gram negative	rod
FAM 11	1 mm	Circular	Raised	Entire	Shiny & smooth	Blue	Gram negative	rod
FAM 12	1.1 mm	Circular	Raised	Entire	Shiny & smooth	White	Gram negative	rod
FAM 13	0.5 mm	Circular	Raised	Entire	Shiny & smooth	Pink	Gram negative	rod
FAM 14	0.6 mm	Circular	Raised	Entire	Shiny & smooth	White	Gram negative	rod
FAM 15	0.7 mm	Circular	Raised	Entire	Shiny & smooth	Pink	Gram positive	cocci
FAM 16	0.8 mm	Circular	Raised	Entire	Shiny & smooth	Blue	Gram negative	rod
FAM 17	0.56 mm	Circular	Raised	Entire	Shiny & smooth	Blue	Gram negative	rod
FAM 18	0.67 mm	Circular	Raised	Entire	Shiny & smooth	White	Gram negative	rod
FAM 19	0.72 mm	Circular	Raised	Entire	Shiny & smooth	White	Gram negative	rod
FAM 20	1.1 mm	Circular	Raised	Entire	Shiny & smooth	Whitish	Gram negative	rod
FAM 21	0.9 mm	Circular	Raised	Entire	Shiny & smooth	White	Gram negative	rod
FAM 22	0.45 mm	Circular	Raised	wavy	Shiny & smooth	Yellow	Gram positive	cocci
FAM 23	0.7 mm	Circular	Raised	Entire	Shiny & smooth	Green metallic sheen	Gram positive	cocci
FAM 24	0.52 mm	Circular	Raised	Entire	Shiny & smooth	Green metallic sheen	Gram negative	rod
FAM 25	0.48 mm	Circular	Raised	Entire	Shiny & smooth	Blue	Gram negative	rod
FAM 26	0.9 mm	Circular	Raised	Entire	Shiny & smooth	Green metallic sheen	Gram negative	rod
FAM 27	1 mm	Circular	Raised	Entire	Shiny & smooth	Bright yellow	Gram positive	cocci
FAM 28	1 mm	Circular	Raised	Entire	Shiny & smooth	Bright yellow	Gram positive	cocci

Table 3: Cultural and morphological characterization.

isolated microorganisms, an antibiotic cocktail will be formulated for the processing of frozen AM.

Processing of frozen AM and microbiological quality analysis

AM samples were washed several times with sterile PBS solution. Then, samples were cut into small pieces by using sterile scalpel blade. After then, small pieces of AM samples were finally washed with sterile PBS containing antibiotic cocktail. Final bioburden was determined by similar procedure of initial bioburden determination and by using similar media. Then, Total Viable Bacterial Count (TVBC) was determined.

Preservation of HAM and periodic microbial quality check

For preservation, small pieces of AM samples were put into plastic falcon tubes containing with Dulbecco's Modified Eagles Media (DMEM) and glycerol (1:1 ratio) and was stored at -80°C. Periodic microbial quality checking of every sample was done between 07, 14, 21 & 30 days interval.

Results

Initial bioburden count

Initial microbiological analysis was performed on 12 AM samples

and Total Viable Bacterial Count (TVBC) was determined (Table 2). No fungus was determined during the bioburden analysis

Cultural and morphological characterization

Twenty eight bacterial isolates were selected from twelve different samples. And they were designated as FAM 01 to FAM 28 respectively and most of the isolates were between (0.5-1.5) mm, circular with shiny and smooth surfaces (Table 3) on the other hand, among the twenty eight isolates we found ten isolates are gram positive cocci shape and rest eighteen isolates are gram negative rod shape (Table 4).

On the basis of this result, 01, 02, 08, 09, 15, 19, 23, 28 isolates are resembled as *Staphylococcus aureus*. 03, 22 isolates are resembled as *Staphylococcus epidermidis*. 04, 05, 10, 11, 14, 20, 24, 25, 27 isolates are resembled as *Escherichia coli*. 16, 13, 26 isolates are resembled as *Salmonella typhimurium*. 07 isolate is resembled as *Enterobacter aerogenes*. 21 isolate is resembled as *Pseudomonas aeruginosa*. 06, 12, 17, 18 isolates are resembled as *Acinetobacter baumanii*.

Antibiotic susceptibility analysis of the bacterial isolates

In this study, we isolated seven different genuses of bacteria. These are: *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus* spp. (*Staphylococcus aureus*, *Staphylococcus epidermidis*), *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, *Acinetobacter baumanii*. Then, Antimicrobial susceptibility patterns of these bacterial isolates were analysed by using ten antibiotic disks. Then the following result was observed, *Staphylococcus aureus* is resistant against three antibiotics (Neomycin, Cloxacillin, Amphicillin) and is sensitive to seven antibiotics (Imipenem, Gentamycin, Polymixin-B, Vancomycin, Penicillin-G, Ciprofloxacin and Streptomycin). *Staphylococcus epidermidis* is resistant against five antibiotics (Imipenem, Neomycin, Cloxacillin, Amphicillin, Polymixin-B) and is sensitive to five antibiotics (Gentamycin, Penicillin-G, Vancomycin, Ciprofloxacin and Streptomycin). *Salmonella typhimurium* is resistant against five antibiotics (Neomycin, Cloxacillin, Amphicillin, Gentamycin, Vancomycin) and is sensitive to five antibiotics (Polymixin-B, Penicillin-G, Imipenem, Ciprofloxacin and Streptomycin). *E. coli* is resistant against all antibiotics except Streptomycin and Penicillin-G. *Acinetobacter baumanii* is resistant against all antibiotics except Polymixin-B, Streptomycin and Penicillin-G. *Enterobacter aerogenes* is sensitive to Streptomycin, Penicillin-G and Imipenem and resistance against rest of the antibiotics. *P. aeruginosa* is sensitive to Ciprofloxacin, Penicillin-G Streptomycin and is resistant against rest of seven antibiotics (Table 5).

Follicular kinetics

The animals treated with ethyl acetate at low dose level caused a statistically less significant ($P<0.05$) reduction in the number of small antral and graafian follicles with concomitant significant increase in the number of atretic follicles of the same stage. At high dose caused a highly significant decrease ($P<0.001$) in the number of healthy small preantral, large preantral, small antral, graafian follicles and active and fresh corpora lutea with a concomitant significant increase in the number of atretic follicles of the same stage. The result also showed a significant reduction in the total number of follicles in the ethyl acetate extract treated ovary (Table 6).

Isolated Colony ID	Catalase test	Oxidase test	Citrate test	Motility test	Indole test	Urease test	KIA test	MR test	VP test	Glu test	Lac test	Fru test	Man test
FAM 01	+	-	-	-	-	+	PS & YB	-	+	+	+	-	+
FAM 02	+	-	-	-	-	+	PS & YB	-	+	+	+	-	+
FAM 03	+	-	-	-	+	+	PS & YB	-	+	+	+	+	+
FAM 04	-	-	-	+	+	-	YS & YB	+	-	+	-	+	+
FAM 05	-	-	-	+	+	-	YS & YB	+	-	+		+	+
FAM 06	+	-	+	+	-	-	YS & YB	+	-	-			
FAM 07	+	-	+	+	-	-	PS & YB	-	+	+	+	+	+
FAM 08	+	-	-	-	-	+	PS & YB	-	+	+	+	-	+
FAM 09	+	-	-	-	-	+	PS & YB	+	-	+	-	+	+
FAM 10	-	-	-	+	+	-	YS & YB	+	-	+	-	+	+
FAM 11	-	-	-	+	+	-	YS & YB	+	-	+	-	+	+
FAM 12	+	-	+	+	-	-	YS & YB	+	-	-	+	+	+
FAM 13	+	-	+	+	-	-	PS & PB	+	-	-	+	+	+
FAM 14	-	-	-	+	+	-	YS & YB	+	-	+	-	+	+
FAM 15	+	-	-	-	-	+	PS & YB	+	+	+	+	-	+
FAM 16	+	-	+	+	-	-	PS & PB	-	-	+	-	+	+
FAM 17	+	-	+	+	-	-	YS & YB	+	-	-	+	+	+
FAM 18	+	-	+	+	-	-	YS & YB	+	-	-	+	+	+
FAM 19	+	-	-	-	-	+	PS & YB	-	+	+	+	-	+
FAM 20	-	-	-	+	+	-	YS & YB	+	-	+	-	+	+
FAM 21	+	+	+	+	-	-	YS & YB	-	-	+	+	+	+
FAM 22	+	-	-	-	-	+	PS & YB	-	+	+	+	-	+
FAM 23	+	-	-	-	-	+	PS & YB	-	+	+	+	-	+
FAM 24	+	-	-	-	-	+	YS & YB	-	+	+	+	-	+
FAM 25	-	-	-	+	+	-	YS & YB	+	-	+	-	+	+
FAM 26	+	-	+	+	-	-	YS & YB	+	-	-	+	+	+
FAM 27	-	-	-	+	+	-	YS & YB	+	-	+	-	+	+
FAM 28	+	-	-	-	-	+	PS & YB	-	+	+	+	-	+

Note: MR: Methylene red, VP: Voges prouskeur, Glu: Glucose, Fru: Fructose, Lac: Lactose, Man: Mannitol, YS: Yellow slant, YB: Yellow butt, PS: Pink slant, PB: Pink butt

Table 4: Biochemical characterization.

Identified bacterial strain	Sensitive	Resistance
Staphylococcus aureus	IMP, CN, PB, VA, P, CIP, S	N, OB, AM
Staphylococcus epidermidis	CN, P, VA, CIP, S	IMP, N, OB, AM, PB
Escherichia coli	S, P	IMP, N, OB, AM, PB, CIP, VA, CN
Salmonella typhimurium	PB, P, IMP, CIP, S	N, OB, AM, CN, VA
Enterobacter aerogenes	S, P, IMP	N, PB, OB, AM, CIP, VA, CN
Pseudomonas aeruginosa	CIP, P, S	N, PB, OB, AM, VA, CN, IMP
Acinetobacter baumanii.	PB, S, P	IMP, N, OB, AM, CIP, VA, CN

Note: IMP: Imipenem, PB: Polymixin-B, CIP: Ciprofloxacin, AM: Amphicillin, N: Neomycin, VA: Vancomycin, CN: Gentamycin, OB: Cloxacillin, S: Streptomycin, P: Penicillin-G.

Table 5: Antibiotic susceptibility analysis of the isolates.

Sample No.	Bioburden count during processing of frozen AM (c.f.u/g)					
	NA	Mac	EMB	PDA	SSA	MSAB
1	1.57×10^{2}	1.99×10^{2}	2.30×10^{2}	Nil	Nil	Nil
2	2.8×10^{2}	1.87×10^{2}	1.97×10^{2}	Nil	Nil	Nil
3	5.66×10^{1}	3.45×10^{1}	3.48×10^{1}	Nil	Nil	Nil
4	3.94×10^{2}	2.96×10^{2}	1.98×10^{2}	Nil	0.7×10^{1}	Nil
5	2.7×10^{2}	1.29×10^{2}	2.21×10^{2}	Nil	Nil	Nil
6	2.9×10^{2}	2.01×10^{2}	1.79×10^{2}	Nil	0.5×10^{1}	0.33×10^{1}
7	2.08×10^{2}	1.78×10^{2}	2.49×10^{2}	Nil	0.4×10^{1}	Nil
8	2.56×10^{2}	1.89×10^{2}	1.46×10^{2}	Nil	Nil	Nil
9	3.68×10^{2}	2.67×10^{2}	3.15×10^{2}	Nil	Nil	0.15×10^{1}
10	2.01×10^{2}	1.95×10^{2}	1.96×10^{2}	Nil	Nil	0.29×10^{1}
11	2.43×10^{2}	1.59×10^{2}	1.29×10^{2}	Nil	Nil	Nil
12	1.92×10^{2}	1.45×10^{2}	1.79×10^{2}	Nil	Nil	Nil

Note: NA: Nutrient Agar, Mac: McConkey Agar, EMB: Eosine Methylene Blue, PDA: Potato Dextrose Agar, SSA: Salmonella-Shigella Agar, MSAB: Mannitol Salt Base Agar

Table 6: Bioburden count during processing of frozen AM.

Sample No.	Count after 7 days	Count after 14 days	Count after 21 days	Count after 30 days
1	Nil	Nil	Nil	Nil
2	Nil	Nil	Nil	Nil
3	Nil	Nil	Nil	Nil
4	Nil	Nil	Nil	Nil
5	Nil	Nil	Nil	Nil
6	Nil	Nil	Nil	Nil
7	Nil	Nil	Nil	Nil
8	Nil	Nil	Nil	Nil
9	Nil	Nil	Nil	Nil
10	Nil	Nil	Nil	Nil
11	Nil	Nil	Nil	Nil
12	Nil	Nil	Nil	Nil

Table 7: Bioburden count during microbial quality check of preserved HAM.

Antibiotic cocktail formulation

In antimicrobial susceptibility pattern of isolated bacteria against different antibiotics, it was found that all six isolated bacterial strains were sensitive to Streptomycin and Penicillin-G. Thus, Streptomycin-Penicillin-G (Strep-P) antibiotic cocktail was formulated for the processing of frozen AM.

Bioburden count during processing of frozen AM

Microbial count was significantly reduced due to use of Streptomycin-Penicillin-G (Strep-P) antibiotic cocktail during processing of frozen AM (Table 6).

Bioburden count during microbial quality check of preserved HAM

During preservation at -80°C the value of TVBC was found nil during microbial quality checking between 07, 14, 21 & 30 days interval which ensures the microbial sterility of the preserved HAM sample (Table 7).

Tissue bank deals with the human connective tissues for clinical use with the guaranteed quality from the moment of retrieval up to the use as allograft. Though the storage procedure is well documented in tissue banks, the appearances of infection due the bacterial contamination cannot be excluded. As a result of prolonged hospitalization, organ failure or even death can occur. In most cases, infection occurs after graft implant. Despite thorough donor screening, microorganisms could be introduced into the grafts from various sources during tissue procurement, processing, handling, or storage or at the time of surgery. Even pregnant women with preterm labor and intact amniotic membrane could be able to carry microorganisms in their amniotic fluid [18].

It is mandatory to make tissue grafts free from all types microbial contamination before transplantation which can be assured by

aseptic tissue handling and proper decontamination process. Though tissue bank deals all the procedures of processing and preservation of cryopreserved AM very carefully microbiological contaminations cannot be fully mitigated. Contamination may occur during transportation of tissues from hospitals to tissue bank, during various steps of tissue processing. According to tissue banking standards of American Association of Tissue Banks (AATB), to assure proper sterility of tissue grafts it is mandatory to control microorganisms during and after the processing of the sample. In our study, we found that the rate of contamination is higher during the initial stages of processing which reflects that our tissues were contaminated with different types of microflora during the pre-processing period. On the other hand, a significant reduction of bioburden after treatment with antibiotic cocktail proves the uniqueness of antibiotic treatment as an effective decontamination method during tissue processing. The key sources of these microorganisms are patients skin, different airborne particles of the operation theatre room or normal labor room and different contaminated surgical instruments which were not sterile [19]. Moreover, in our study, we found that morphologically most of our selected bacterial isolates were a gram-negative rod and gram-negative cocci. Some were gram-positive cocci shaped, gram positive and gram negative chain shaped. In a previous study, it was found that gram-positive *Staphylococcus warneri* were most predominant bacteria found in pre-processing samples [20].

Conclusion

In conclusion, it can be said that microbiologically contaminated tissue allografts can cause severe life-threatening health hazards to the recipients. As frozen AM is extensively used for the treatment of different types of ocular diseases, any types of microbial contamination can cause severe infections which may result in permanent loss of vision. So, proper microbial quality check during every step of tissue processing is compulsory. Microbial contamination may also occur due to the lack of carefulness of tissue bank personnel. So, effective training programs with updated knowledge should be raised among tissue bank personnel which will minimize the risk of any type of microbial contamination during every step of tissue processing and preservation.

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