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Keywords : *amniotic membranes, bioburden, antibiogram, gamma-radiation, radiation doses.*

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Bioburden of Human Amniotic Membranes and Inhibition of the Associated Bacteria using Antibiotics and Gamma-Radiation

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Keywords : amniotic membranes, bioburden, antibiogram, gamma-radiation, radiation doses.

I. Introduction

Human amniotic membranes or foetal membranes are used as biological skin substitutes or dressings (Bennett et al 1980; Singh et al 2004). Amniotic membranes are obtained from screened human placenta after delivery. The histological structure of amniotic membrane is similar to that of the skin and its application is not associated with immunological problems (Matsui et al 1989). Amniotic membrane is semi-permeable whose attachment properties onto the surface of a tissue are excellent. The membrane transplant can be attached onto many wound surfaces without stitching. The translucence of the transplant enables direct follow-up of the healing process through the membrane transplant (Goebel and Schubert 1990).The anti-inflammatory proteins secreted by the amniotic membrane form an effective barrier against external microbial attacks. Amniotic membrane transplant also promotes the formation of epithelium and reduces the development of scar tissue and blood vessels. Application of amniotic membrane may also prevent or reduce the level of bacterial contamination in the wound bed. They may also prevent fibrosis during ocular surface construction. The application of amnion membrane as a biological dressing speeds the re-epithelialization and prevents invasive bacterial infection (Andonovska et al 2008).

Microbial quality of amniotic membrane is one of the most important considerations for its clinical application. As it comes into contact with the open wounds, it needs to be perfectly sterile to avoid contamination as well as transmission of any disease. In order to minimize the risk of disease transmission, careful donor-screening, proper tissue processing and sterilization of tissue allograft are necessary (Dziedzic-Goclawska and Stachowicz 1997). Sterilizations of tissue allograft are generally done by using chemicals, heat, UV, and ionizing radiation. Antibiotic decontamination and γ-radiation are used for achieving high sterility assurance level. Although, antibiotic decontamination is considered as a suitable method for treating tissues but, it is only effective against bacteria, and its effectiveness is dependent on the constituent of antibiotics. It is therefore important to assess the degree of preprocessing bacterial contamination, and to procure the tissue under conditions that minimize contamination. Gamma radiation is another common method for sterilization of tissue (Yusof 1994). Conventionally, a radiation dose of 25 kGy is the generally accepted dose for sterilization, but to keep intact the biomechanical and other properties of amniotic membrane, it has been proposed to use a

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lower dose without compromising a high sterility assurance level (SAL) of 10^6. However, initial bioburden level and radiation resistances of the contaminants determine the dose required for sterilization. The behaviors of the microbial population on exposure to antibiotics and ionizing radiation are the matter of greatest relevance in antibiotics and radiation sterilization practice. Thus, it is important to assess the specific antibiotic resistance and the general radiation resistance of bacteria present in amniotic membrane. This paper, therefore, aims to estimate the bioburden level of amniotic membranes; identification of the isolated bacteria as well as to depict antibiotic-sensitivity and radiation-sensitivity of the bacterial flora associated with amniotic membrane.

II. Materials and Methods

a) Sample Collection and Preparation

Human Amniotic sacs were collected from operation theatres of three different hospitals: Ganashastho Hospital, Savar, Azimpur Maternity Hospital and Nitor Hospital, Dhaka. The membranes were obtained only from clinically acceptable donors (mothers) after their delivery and kept in plastic containers with sterile physiological saline (0.9% NaCl), and preserved temporarily in a freezer below -20°C. The containers were placed in a cool box and transported immediately to the Tissue Banking and Biomaterial Research Unit of Bangladesh Atomic Energy Research Establishment, Savar, Dhaka. Amnion sample was separated (glossy, translucent and thinner membrane) from the chorion (opaque and thicker) under aseptic condition. The membranes were washed separately in sterile physiological saline several times using orbital shaker to remove blood.

b) Determination of Bioburden in Amniotic Samples and Isolation of Bacteria

Bacterial colonies were isolated from a total of 25 amniotic samples following standard spread plate and pour plate methods. Approximately 1.8 gm to 6.43 gm of tissues were cut at different parts of the samples and taken in 100 ml sterile conical flask containing 20ml sterile physiological saline. The flask (containing the sample) was shaken by orbital shaker for at least 15 minutes. Then 10 ml of that solution (from the mixture) was taken into a blank sterile test tube with the help of glass pipette. Then the sample solution was serially diluted at a particular level in an aseptic condition using Biohazard class II laminar air flow hood and the solutions were thoroughly mixed by the help of vortex. 200 μl (0.2 ml) of original and serially diluted sample was poured onto nutrient agar plate by micropipette and spread onto the nutrient agar plate with the spreader and 0.5 ml of the original and serially diluted sample solution was plated by pour plate method. The plates were incubated at 37°C for 24- 72 hours in inverted position. After incubation, number of the viable bacterial colonies was counted and total number of bacteria per milliliter or per gram of sample was calculated using the standard procedures after Isenberg (1992).

c) Characterization of the Selected Bacterial Isolates

Morphology of the bacterial colonies grown in different plates was carefully observed and several colonies were selected on the basis of their size, pigmentation, form, margin, elevation, and opacity. These colonies were identified up to genus level based on different physio-biochemical tests *viz.*, Gram's test, motility test, oxidase test, catalase test, oxidative-fermentative (O-F) test etc. All of the tests were performed at 37°C and the results were observed after 24 h. Based on the characteristics, the bacterial isolates were initially identified up to genus level following the Manual for the Identification Medical Bacteria (Barrow and Feltham, 1993).

d) Antibiogram of the Bacterial Isolates

Antibiogram profile of the selected isolates was examined for three commercial antibiotics *viz.*, Ampicilin (10 µg/disc), Cloxacillin (5 µg/ disc) and Gentamicin (10 µg/disc). For this purpose, pure colony of the isolates was inoculated in Mueller Hinton broth and incubated in a shaking incubator for 2-3 hours for a mild growth. Hundred microliter of individual bacterial culture was poured on a freshly prepared Mueller Hinton agar plate and spread aseptically by using swab stick. Then, the plate was kept 5-10 minutes to dry the suspension. Then the antibiotic discs were placed aseptically on those plates. The plates were incubated overnight at 37°C. After incubation, diameter of zones of inhibition (mm) for each antibiotic was measured. Interpretation of inhibition zones was done according to Harley (2005). The Gram negative and Gram positive bacterial isolates were considered resistant to Ampicillin when the diameter of inhibition zones were equal to or less than 16 and 28 mm, respectively. Both Gram negative and Gram positive bacterial isolates were considered resistant to Cloxacillin and Gentamicin when the diameter of inhibition zones were equal to or less than 14 and 12 mm, respectively. When the diameter of inhibition zone for any antibiotic was more than the above mentioned ranges the bacterial isolate was considered sensitive to the respective antibiotic.

e) Gamma (γ)-Radiation Sensitivity of the Bacterial Isolates

A loop full pure bacterial colony was inoculated into nutrient broth and incubated in a shaker incubator for 2-3 hours at 37°C for a mild growth (the initiation step of log phase). Then 10ml of the broth culture was taken in sterile test tubes. Each test tube was tagged with isolate number, radiation dose and was sealed in previously irradiated poly bag. Then test tubes were sent to γ radiation unit for radiation where the samples were

irradiated in the series of 2.5,5,10,15,20 and 25 kGy (kilo Gray). After radiation each sample was inoculated on freshly prepared nutrient agar plate by spreading in order to determine the effect of radiation. The plates were incubated overnight at 37°C and observed for growth of any micro-organisms.

III. Results

a) Determination of Bioburden in Amniotic Membranes

In the present study, all of the human amniotic membrane samples were found to be more or less contaminated with bacteria (Table 1). In case of spread plate method, the highest bacterial load was found in sample number AM115 (8.87×10^6 CFU/gm) and the lowest in AM111 (6.10×10^2 CFU/gm). In case of pour plate method, the highest and the lowest figures were observed in sample number AM110 (1.34×10^6 CFU/gm) and AM111 (5.42×10^2 CFU/gm), respectively. During spread and pour plate culture method, some plates contained too much bacterial colony to measure their bioburden.

Table 1 : Bacterial load in human amniotic membrane samples

Sample ID	Sample Weight	Total bacterial count (CFU/gm of sample)	
		Spread Plate	Pour Plate
AM101	3.94	2.08×10^5	5.48×10^4
AM102	2.32	7.75×10^4	2.24×10^5
AM103	5.59	1.79×10^4	3.14×10^5
AM104	1.9	1.36×10^6	1.89×10^4
AM105	4.31	2.25×10^5	1.20×10^4
AM106	3.95	4.53×10^6	4.15×10^5
AM107	5.03	1.59×10^4	1.19×10^5
AM108	3.0	6.67×10^4	5.07×10^4
AM109	6.04	5.20×10^5	-
AM110	5.23	3.26×10^5	1.34×10^6
AM111	2.95	6.10×10^2	5.42×10^2
AM112	2.32	1.5×10^3	1.62×10^3
AM113	6.43	1.29×10^3	7.52×10^3
AM114	3.17	8.20×10^2	1.03×10^2
AM115	2.03	8.87×10^6	-
AM116	3.4	8.82×10^5	-
AM117	1.84	1.65×10^4	4.91×10^5
AM118	4.08	6.17×10^3	4.02×10^5
AM119	3.88	1.17×10^3	9.34×10^2
AM120	2.00	3.03×10^3	-
AM121	3.50	2.63×10^3	1.58×10^3
AM122	4.25	1.99×10^3	1.06×10^3
AM123	1.76	3.9×10^3	3.1×10^3
AM124	5.00	4.68×10^2	3.55×10^2
AM125	2.95	1.17×10^3	9.34×10^2

b) Characterization of Bacterial Isolates

From 25 different batches of amniotic samples 15 bacterial colonies were selected according to their colonial morphology. In this experiment, three isolates A_2, A_3 and A_6 formed small colonies, another three isolates A_4, A_7 and A_9 made large colonies while the remaining isolates were moderate in size (Table 2). Isolate A_8 was rhizoid and two other isolates A_4 and A_7, were irregular and all other were circular. Except four (one of which was brownish in color and three of which were cream) isolates, all isolates were whitish in pigmentation.

Several physico-biochemical tests were performed to identify the selected bacterial isolates up to genus level. In the present study, 66.67% of the bacterial isolates were Gram positive and 33.33% were Gram negative (Table 3). All the Gram negative bacteria were rod shaped. All of the isolates were catalase positive, 53.33% were oxidase positive and 60.00% were motile. Forty percent of the isolates gave no reaction on O-F test while 46.67% of the isolates were fermentative and 13.33% were oxidative. Spore forming organisms were counted to be 33.33%.

Based on the physico-biochemical characteristics 33.33% isolates were resembled to *Bacillus sp.* (isolate no. A_1, A_5, A_7, A_{13} and A_{14}), 20% were *Staphylococcus* sp. (isolates no. A_3, A_6 and A_{10}), 13.33% were *Pseudomonas* sp. (isolate no. A_4 and A_9) and *Micrococcus* sp. (isolates no. A_2 and A_{12}).The remaining isolates were identified to be *Achromobactor* sp. (isolate no. A_{11}), Alcaligenes sp. (isolate no. A_{15}) and *Citrobacter* sp. (isolate no. A_8).

Table 2 : Colonial morphology of selected bacterial isolates

Isolate number	Colony Morphology					
	Size	Form	Margin	Pigmentation	Elevation	Opacity
A_1	Medium	Circular	Entire	Whitish	Thin/Flat	Opaque
A_2	Small	Circular	Entire	Brownish	Raised	Opaque
A_3	Small	Circular	Undulate	Whitish	Convex	Opaque
A_4	Large	Circular	Undulate	Whitish	Centrally Raised	Opaque
A_5	Medium	Circular	Entire	Whitish	Thin/Flat	Opaque
A_6	Small	Circular	Undulate	Whitish	Convex	Opaque
A_7	Large	Irregular	Undulate	Cream	Flat	Opaque
A_8	Medium	Rhizoidal	Undulate	Cream	Flat	Opaque
A_9	Large	Irregular	Undulate	Cream	Thick	Opaque
A_{10}	Moderate	Circular	Entire	White	Flat	Opaque
A_{11}	Moderate	Circular	Entire	Cream	Slightly thick	Opaque
A_{12}	Moderate	Circular	Entire	Whitish	Slightly thin	Opaque
A_{13}	Moderate	Circular	Entire	Whitish	Slightly thick	Opaque
A_{14}	Moderate	Irregular	Entire	Whitish	Flat	Opaque
A_{15}	Moderate	Circular	Entire	Cream	Centrally raised	Opaque

Table 3 : Morphological, physiological and biochemical characteristics of the selected bacterial isolates

Isolate number	Morphological, physiological and biochemical characteristics of the isolates							Presumptive identification
	Gram's staining	Cell shape	Motility	Sporul-ation	Catalase reaction	Oxidase reaction	O-F test	
A1	+	R	+	+	+	-	F	*Bacillus* sp.
A2	+	C	-	-	+	+	NR	*Micrococcus* sp.
A3	+	C	-	-	+	-	F	*Staphylococcus* sp.
A4	-	SR	+	-	+	+	NR	*Pseudomonas* sp.
A5	+	R	+	+	+	-	F	*Bacillus* sp.
A6	+	C	-	-	+	-	F	*Staphylococcus* sp.
A7	+	R	+	+	+	-	F	*Bacillus* sp.
A8	-	R	+	-	+	-	F	*Citrobacter* sp.
A9	-	R	+	-	+	+	O	*Pseudomonas* sp.
A10	+	C	-	-	+	-	F	*Staphylococcus* sp.
A11	-	R	+	-	+	+	O	*Achromobacter* sp.
A12	+	C	-	-	+	+	NR	*Micrococcus* sp.
A13	+	R	+	+	+	+	NR	*Bacillus* sp.
A14	+	R	+	+	+	+	NR	*Bacillus* sp.
A15	-	Fi	-	-	+	-	NR	*Alcaligenes* sp.

+ = Positive; - = Negative; R = Rod; SR = Short Rod; C = Coccid; Fi = Filamentous; O = Oxidative; F = Fermentative; NR = No Reaction

c) Antibiogram Profile of Bacterial Isolates

Except *Alcaligenes* sp. (isolate no. A_{15}) and *Achromobacter* sp. (isolate no. A_{11}) all other organisms were found to be resistant against to Ampicillin (Table 4). Two isolates *Micrococcus* sp. (isolate no.A_2) and *Pseudomonas* sp. (isolate no. A_4) was found sensitive for Cloxacillin. However, all isolates were found to be sensitive to Gentamicin.

Table 4 : Antibiogram profile of the bacterial isolates

Isolate number	Zone of inhibition in millimeter (mm) against antibiotics		
	Ampicillin (10μg/disc)	Cloxacillin(5μ/disc)	Gentamicin(10μ/disc)
A_1	16(Rap)	22(S)	23(S)
A_2	0(Rap)	0(Rc)	18(S)
A_3	20(Rap)	26(S)	28(S)
A_4	7(Ran)	7(Rc)	18(S)

A_5	18(Rap)	25(S)	20(S)
A_6	14(Rap)	28(S)	24(S)
A_7	26(Rap)	22(S)	22(S)
A_8	5(Ran)	29(S)	25(S)
A_9	20(Ran)	24(S)	22(S)
A_{10}	13(Rap)	22(S)	20(S)
A_{11}	27 (S)	48(S)	48(S)
A_{12}	20(Rap)	24(S)	23(S)
A_{13}	24(Rap)	38(S)	40(S)
A_{14}	25(Rap)	42(S)	41(S)
A_{15}	26 (S)	32(S)	33(S)

μg: microgram.
S: Sensitive to antibiotics
Ran: Ampicillin resistant Gram negative bacteria (zone less than or equal to 16 mm)
Rap: Ampicillin resistant Gram positive bacteria (zone less than or equal to 28 mm)
Rc: Cloxacillin resistant bacteria (zone less than or equal to 14 mm)
Rg: Gentamicin resistant bacteria (zone less than or equal to 12 mm)

d) Sensitivity of the Isolates to γ- Radiation

Relatively higher doses of γ-radiation were required for inactivation of Gram positive bacteria; especially growth of *Bacillus* sp. was observed even after 10kGy radiation treatment, but at 15 kGy all the isolates were killed. In case of Gram negative bacteria, expect one of the two isolates of *Pseudomonas* sp. (isolate no. A_9), which showed growth after first dose of γ radiation; the isolate was killed at 5kGy radiation treatment. All other isolates were killed by 2.5kGy.

Table 5 : Growth of bacterial isolates after Gamma-radiation

Isolate Number	Plating	Growth of bacterial isolates (CFU/ml) after different doses of γ-radiation (kGy)					
		2.5	5	10	15	20	25
A1	S P	TMGC	2.00×10^2	40	NG	NG	NG
	P P	TMGC	TMGC	NG	NG	NG	NG
A2	S P	NG	NG	NG	NG	NG	NG
	P P	40	NG	NG	NG	NG	NG
A3	S P	96	NG	NG	NG	NG	NG
	P P	24	NG	NG	NG	NG	NG
A4	S P	NG	NG	NG	NG	NG	NG
	P P	NG	NG	NG	NG	NG	NG
A5	S P	TMGC	NG	NG	NG	NG	NG
	P P	1.1×10^2	NG	NG	NG	NG	NG
A6	S P	7.8×10^2	NG	NG	NG	NG	NG
	P P	3.2×10^2	NG	NG	NG	NG	NG
A7	S P	TMGC	TMGC	1.4×10^2	NG	NG	NG
	P P	TMGC	TMGC	NG	NG	NG	NG
A8	S P	NG	NG	NG	NG	NG	NG
	P P	NG	NG	NG	NG	NG	NG
A9	S P	5.5×10^2	NG	NG	NG	NG	NG
	P P	1.56×10^2	NG	NG	NG	NG	NG
A10	S P	TMGC	6.65×10^2	NG	NG	NG	NG
	P P	TMGC	TMGC	NG	NG	NG	NG
A11	S P	NG	NG	NG	NG	NG	NG
	P P	NG	NG	NG	NG	NG	NG
A12	S P	NG	NG	NG	NG	NG	NG
	P P	NG	NG	NG	NG	NG	NG
A13	S P	6.95×10^2	NG	NG	NG	NG	NG
	P P	2.94×10^2	2.40×10^2	NG	NG	NG	NG
A14	S P	TMGC	TMGC	2.25×10^2	NG	NG	NG
	P P	TMGC	TMGC	2.86×10^2	NG	NG	NG
A15	S P	NG	NG	NG	NG	NG	NG
	P P	NG	NG	NG	NG	NG	NG

SP : Spread Plate; PP: Pour Plate; TMGC: Too much growth to count; NG: No Growth;

IV. Discussion

Bacterial infection is a severe obstacle of amniotic allograft application. To minimize the risk of this complication, it is important to ensure all grafts are safe from bacterial contamination prior to transplantation. In the present study, 25 amniotic membranes were examined where all of the samples were found more or less contaminated with bacteria. Average numbers of bacteria per gram of sample were found in the ranges of 5.42×10^2 to 8.87×10^6 CFU. These results indicated that amniotic tissues were not sterile. It has been reported that pregnant women without infection and intact amniotic membranes may sometimes (1.4–29%) have microorganisms in their amniotic fluid (Dunlow and Du 1990). Further, tissue may get contaminated from various sources during procurement, processing, handling and packaging. Microbial contaminants may thus arise from the donor tissue itself, the environment and the personnel (Yusof 1999). In this study, microbial loads in the amniotic membranes were found to differ from sample to sample. Begum and Islam (1999) also reported that the bioburden of amniotic membranes may vary from specimen to specimen and from country to country.

Fifteen different bacterial colonies were selected on the basis of their morphological characteristics including size, margin, pigmentation, opacity, elevation etc. These isolates were identified up to genus level based on their physico-biochemical characteristics, where 33.33% isolates were resembled to *Bacillus* sp., 20% were *Staphylococcus* sp. and 13.33% were *Pseudomonas* sp. and *Micrococcus* sp. The remaining isolates were identified as *Achromobactor* sp., *Alcaligenes* sp. and *Citrobacter* sp. Singh *et al*. (2006) isolated 20 bacterial isolates from 70 different batches and characterized the isolates where three Gram-positive cocci were identified as *Staphylococcus*, seven strains of Gram-positive bacilli were identified as *Bacillus,* one Gram-positive bacillus was *Corynebacterium* and one isolate was identified as *Clostridium*, seven strains were oxidase-positive, Gram-negative bacilli; three of these isolates were *Pseudomonas* and four were *Alcaligenes*. Other Gram-negative bacilli and oxidase-negative isolates were identified to be *Citrobacter*, *Proteus* and *Flavimonas* which was almost similar to our present findings.

Risk of infectious disease transmission with tissue allograft is a major concern in Tissue Banking practice. Microorganisms can be introduced into the grafts during tissue collection, processing and storage. Several steps should be undertaken by Tissue Banks, including careful donor-screening, proper tissue processing and sterilization of tissue allografts to minimize the risk of disease transmission (Dziedzic-Goclawska and Stachowicz, 1997). In Tissue Banking, several methods are applied for sterilization of tissue allograft including chemicals, heat, UV, and ionizing radiation. Although heat (autoclaving, boiling etc.) is an effective sterilizing agent, it has not been routinely used for tissue allograft because it impairs the mechanical properties of grafts, destroys the osteoinductive capacity of tissue and reduces tissue graft incorporation. Antibiotic decontamination is considered to be a suitable method for treating tissue such as heart valves, skin, and amnion and antibiotic disinfection is currently the method of choice for amnion. In order to evaluate the effect of antibiotics, the bacterial isolates were examined against Ampicillin, Cloxacillin and Gentamicin where, all organisms were found resistant against Ampicillin except *Alcaligenes* sp. (isolate no. A_{15}) and *Achromobacter* sp. (isolate no. A_{11}). Similarly, all of the isolates were resistant to Cloxacillin except for two isolates *Micrococcus* sp. (isolate no. A_2) and *Pseudomonas* sp. (isolate no. A_4). However, all isolates were found to be sensitive to Gentamicin.

Bacterial isolates were tested for survival in an incremental series of radiation doses from 2.5 to 25 kilo-Gray (2.5, 5, 10, 15, 20 and 25kGy). Relatively higher doses of γ-radiation were required for Gram positive bacteria; especially growth of *Bacillus* sp. was observed even after 10kGy, but at 15 kGy all the isolates were killed. In case of Gram negative bacteria, all isolates were killed by 2.5kGy γ radiation, expect one of the two isolates of *Pseudomonas* sp. (isolate no: A_9), which was killed at 5kGy dose. Whitby (1993) reported that Gram-negative bacteria are usually much more sensitive to gamma-radiations than gram-positive, which supports the present study. Variation in the lipid content of cell wall of gram-positive and gram negative bacteria may be responsible for the variation in the radiation sensitivity. In this study, sterilization of amniotic allograft contaminated with *Bacillus* sp, *Staphylococcus* sp*, Pseudomonas* sp*, Achromobactor* sp, *Alcaligenes* sp, *Citrobacter* sp, and *Micrococcus* sp*.* was successfully achieved with lower radiation level than reported earlier.

A radiation dose of 25 kGy is the generally accepted dose for sterilization, but a lower dose is proposed to keep intact the biomechanical and other properties of allograft. Use of lower RSD (radiation sterilization dose) will lessen the effects on physical properties of amnion (Hilmy et al. 2003). Use of lower RSD (radiation sterilization dose) will lessen the effects on physical properties of amnion (Hilmy et al. 2003). Thus, findings of the present study will provide tools for further investigation.

From the present study, it could be concluded that amniotic tissue collected from delivery patients were contaminated with different bacterial isolates that might cause infection if transplanted in untreated state. Antibiotic should not be considered as the best choice for sterilization of the grafts due to the resistance of bacteria against antibiotics. Sterilization of the amniotic

grafts contaminated with bacteria could be achieved with 15kGy radiation level which was lower than reported earlier. Therefore, this finding will provide tools for future investigation as researchers are interested to lower the gamma-radiation dose.

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