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

By Nabangshu Shekhar Das, Janmajoy Dey, Dr. Md. Mahbubur Rahman,
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Abstract - Present studies were conducted to estimate the bioburden level of human amniotic membranes; and to detect inhibitory effects of antibiotic and radiation on the bacterial flora associated with amniotic membrane. Bacterial counts per gram of the samples (25) were found 5.42×10^2 to 8.87×10^6 CFU/gm. Fifteen different colonies were isolated from the samples and identified up to the genus level based on several morphological, physiological and biochemical characteristics where the isolates were identified as *Bacillus* sp. (5), *Staphylococcus* sp. (3), *Micrococcus* sp. (2), *Pseudomonas* sp. (2), *Achromobacter* sp. (1), *Alcaligenes* sp. (1) and *Citrobacter* sp. (1). In vitro Inhibitory activities of three different antibiotics were screened against the isolates where all isolates were found sensitive to Gentamicin. Except *Alcaligenes* sp. and *Achromobacter* sp., all isolates were found resistant to Ampicillin. Similarly all isolates were also found resistant to Cloxacillin except *Micrococcus* sp. and *Pseudomonas* sp. Isolates were treated in an incremental series of γ -radiation doses from 2.5-25 kGy where all Gram positive bacteria were killed at 15 kGy.

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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Abstract - Present studies were conducted to estimate the bioburden level of human amniotic membranes; and to detect inhibitory effects of antibiotic and radiation on the bacterial flora associated with amniotic membrane. Bacterial counts per gram of the samples (25) were found 5.42×10^2 to 8.87×10^6 CFU/gm. Fifteen different colonies were isolated from the samples and identified up to the genus level based on several morphological, physiological and biochemical characteristics where the isolates were identified as *Bacillus* sp. (5), *Staphylococcus* sp. (3), *Micrococcus* sp. (2), *Pseudomonas* sp. (2), *Achromobacter* sp. (1), *Alcaligenes* sp. (1) and *Citrobacter* sp. (1). In vitro inhibitory activities of three different antibiotics were screened against the isolates where all isolates were found sensitive to Gentamicin. Except *Alcaligenes* sp. and *Achromobacter* sp., all isolates were found resistant to Ampicillin. Similarly all isolates were also found resistant to Cloxacillin except *Micrococcus* sp. and *Pseudomonas* sp. Isolates were treated in an incremental series of γ -radiation doses from 2.5-25 kGy where all Gram positive bacteria were killed at 15 kGy. Gram negative bacteria showed more sensitivity than the positives and were killed at 5 kGy. Based on these studies it could be concluded that antibiotic decontamination should not be regarded as the way of obtaining sterility due to resistance of bacteria against antibiotics. Gamma-radiation assures complete sterility.

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

1. 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) Antibigram of the Bacterial Isolates

Antibiogram profile of the selected isolates was examined for three commercial antibiotics viz., Ampicillin (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 A₂, A₃ and A₆ formed small colonies, another three isolates A₄, A₇ and A₉ made large colonies while the remaining isolates were moderate in size (Table 2). Isolate A₈ was rhizoid and two other isolates A₄ and A₇, 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. A₁, A₅, A₇, A₁₃ and A₁₄), 20% were *Staphylococcus* sp. (isolates no. A₃, A₆ and A₁₀), 13.33% were *Pseudomonas* sp. (isolate no. A₄ and A₉) and *Micrococcus* sp. (isolates no. A₂ and A₁₂). The remaining isolates were identified to be *Achromobacter* sp. (isolate no. A₁₁), *Alcaligenes* sp. (isolate no. A₁₅) and *Citrobacter* sp. (isolate no. A₈).

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	Sporulation	Catalase reaction	Oxidase reaction	O-F test	
A1	+	R	+	+	+	-	F	<i>Bacillus</i> sp.
A2	+	C	-	-	+	+	NR	<i>Micrococcus</i> sp.
A3	+	C	-	-	+	-	F	<i>Staphylococcus</i> sp.
A4	-	SR	+	-	+	+	NR	<i>Pseudomonas</i> sp.
A5	+	R	+	+	+	-	F	<i>Bacillus</i> sp.
A6	+	C	-	-	+	-	F	<i>Staphylococcus</i> sp.
A7	+	R	+	+	+	-	F	<i>Bacillus</i> sp.
A8	-	R	+	-	+	-	F	<i>Citrobacter</i> sp.
A9	-	R	+	-	+	+	O	<i>Pseudomonas</i> sp.
A10	+	C	-	-	+	-	F	<i>Staphylococcus</i> sp.
A11	-	R	+	-	+	+	O	<i>Achromobacter</i> sp.
A12	+	C	-	-	+	+	NR	<i>Micrococcus</i> sp.
A13	+	R	+	+	+	+	NR	<i>Bacillus</i> sp.
A14	+	R	+	+	+	+	NR	<i>Bacillus</i> sp.
A15	-	Fi	-	-	+	-	NR	<i>Alcaligenes</i> 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. A₁₅) and *Achromobacter* sp. (isolate no. A₁₁), all other organisms were found to be resistant against to Ampicillin (Table

4). Two isolates *Micrococcus* sp. (isolate no. A₂) and *Pseudomonas* sp. (isolate no. A₄) 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 (5µg/disc)	Gentamicin (10µg/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. A₉), 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 x10 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 x10 ²	NG	NG	NG	NG	NG
A6	S P	7.8x10 ²	NG	NG	NG	NG	NG
	P P	3.2 x10 ²	NG	NG	NG	NG	NG
A7	S P	TMGC	TMGC	1.4x10 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	5.5x10 ²	NG	NG	NG	NG	NG
	P P	1.56 x10 ²	NG	NG	NG	NG	NG
A10	S P	TMGC	6.65 x10 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 x10 ²	NG	NG	NG	NG	NG
	P P	2.94 x10 ²	2.40 x10 ²	NG	NG	NG	NG
A14	S P	TMGC	TMGC	2.25 x10 ²	NG	NG	NG
	P P	TMGC	TMGC	2.86 x10 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 *Achromobacter* 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-Goclawski 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. A₁₅) and *Achromobacter* sp. (isolate no. A₁₁). Similarly, all of the isolates were resistant to Cloxacillin except for two isolates *Micrococcus* sp. (isolate no. A₂) and *Pseudomonas* sp. (isolate no. A₄). 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, except one of the two isolates of *Pseudomonas* sp. (isolate no: A₉), 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, *Achromobacter* 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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Trade of Threatened Himalayan Medicinal and Aromatic Plants-Socioeconomy, Management and Conservation Issues in Garhwal Himalaya, India

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Abstract - Collection and trade of medicinal and aromatic plant species (MAPs) is age old practice throughout the world to increase household income. Over harvesting decreased their populations and a number of species became threatened in natural habitat. Survey was conducted on trade of such threatened MAPs from wild in Garhwal Himalaya (Uttarakhand), India. Information's were collected on MAPs trade, channels involved and profit at each level of transaction. Even after ban on commercial exploitation of some MAPs, trade continued through illegal ways. Harvesting starts from July and continue till November. Further, harvesting prior to seed dispersal decreased chance of recruitment of new individual, due to which abundance of these species is decreasing with alarming rate.

Keywords : CITES, illegal trade, threatened MAPs, trade channels, rotational harvest, sustainable utilization.

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Trade of Threatened Himalayan Medicinal and Aromatic Plants-Socioeconomy, Management and Conservation Issues in Garhwal Himalaya, India

R. S. Chauhan^α, B. P. Nautiyal^σ & M. C. Nautiyal^α

Abstract - Collection and trade of medicinal and aromatic plant species (MAPs) is age old practice throughout the world to increase household income. Over harvesting decreased their populations and a number of species became threatened in natural habitat. Survey was conducted on trade of such threatened MAPs from wild in Garhwal Himalaya (Uttarakhand), India. Information's were collected on MAPs trade, channels involved and profit at each level of transaction. Even after ban on commercial exploitation of some MAPs, trade continued through illegal ways. Harvesting starts from July and continue till November. Further, harvesting prior to seed dispersal decreased chance of recruitment of new individual, due to which abundance of these species is decreasing with alarming rate.

Total eighteen species are in active trade from study areas, out of which five are common for all sites. Number of MAPs traded from each site directly influenced by abundance of species in natural habitat. 18% of annual cash income generated from MAPs related activities in these areas. Market demand for *Aconitum heterophyllum*, *Nardostachys jatamansi* and *Picrorhiza kurrooa* is much higher than supply. Percentage of profit varied at each level from site to site and species. Traders earned maximum percentage of profits whereas collectors also received good profit, due to which they were engaged in illegal trade. Rotational harvesting may be applied for sustainable utilization. Agrotechnology has been developed for few MAPs, but incentive should be given to farmers to initiate cultivation of such species for *ex situ* conservation.

Keywords : CITES, illegal trade, threatened MAPs, trade channels, rotational harvest, sustainable utilization.

1. INTRODUCTION

People settled in the high altitude areas of Himalayan regions have limited options to earn money for their daily needs, due to uneconomic nature of cereals production i.e. low returns from agricultural production. Only few varieties of Millets, Buckwheat, Pulses, Amaranths, Barley, etc. are cultivated crops in small quantities in these regions due

to small and fragmented land holdings. Animal husbandry is another important income generating source for them. However, integrated agriculture system, animal husbandry, etc. is unable to satisfy the basic minimum subsistence needs of the peoples and poverty is common in these areas. Despite the abundance of natural resources, most of its peoples are marginalized and still live on subsistence level (Singh 2006). In the past four decades, human population in Indian Himalaya has increased up to 2.7 fold (Nandy and Rao 2001), whereas cereal crop production in mountain agriculture become uneconomic and decreasing regularly (Chauhan 2010). Due to the low agricultural production, lack of industrial development, poverty and unemployment, peoples migrate to exploit biodiversity to improve their socioeconomy. MAPs collection provides extra source of income, which compensate low agricultural production. It has been estimated that many village communities derive as much as 10-50% of their household income from the sale of the forest products (Olsen and Helles 1997; Kuniyal et al 2005; Williams et al 2005; Adhikari et al 2007; Christensen and Heilmann-Clausen 2009). Annual cash income in itself proved to be positively correlated with income from wild products (Olsen and Larsen 2003; Pandit and Kumar 2010).

Demand of herbal medicine is increasing throughout the world. Annual turnover of herbal medicine in India is estimated 1,77,000 MT for which 960 plant species are in active trade (Ved and Goraya 2008). Such increased demand creates a pressure on natural population, due to which many species declined in their number and abundance and entered into various degrees of threat (Uniyal et al 2006). On account of high degree of threat CITES has notified 11 Indian MAPs in its schedule for care including *Nardostachys jatamansi*, *Picrorhiza kurrooa*, etc. To conserve such threatened species, Government of India imposed ban on collection of such MAPs from natural habitats but the ban imposed for conservation purpose; promoted illegal trade. There are many reports in daily newspapers for seizing of banned MAPs, extracted illegally from wild sources, whereas large part of illegal trade escapes.

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The trade of medicinal plants had attracted the attention of scientist as well as development planners on their potential to improve rural livelihoods throughout the world. A number of studies are available regarding medicinal plant trade from different parts of the world including India (Edwards 1993; Olsen 1998; Olsen and Larsen 2003; Williams et al 2005; Kala et al 2006; Uniyal et al 2006; Pandit and Kumar 2010; Heubach et al 2011), whereas pattern of trade especially in threatened high altitude species is rarely available. Olsen and Bhattarai (2005) reported that local trader can have a negative margin in one year and positive in another. Larsen and Olsen (2007) found that empirical data does not paint a clear picture of unfair harvester exploitation and advocated a need for more stringent price data collection because many studies assumed exploitative nature of middlemen but none of the reference provide suggestion as to what a fair margin is?. Holley and Cherla (1998) emphasized the need to investigate the socioeconomic profiles of the economic agents in a review of medicinal plant sector in India. Trade data and household survey is important in order to effective conservation (Olsen 2005). In view of above, present paper provide information's on role of MAPs in household income to improve socioeconomy of hill peoples, pattern of trade especially in threatened high altitude species along with policy management for conservation of such species.

II. MATERIAL AND METHODS

a) Study Sites

The study area falls (between 29°26'-31°28' N and 77°49'-80°6' E) in Uttarakhand, India (Figure 1). Alpine and sub alpine sites covering surrounding areas of Har-Ki-Doon (HKD) and Dayara (DR) in Uttarkashi district, Panwali Kantha (PK) in Tehri district, Madhyamaheshwer (MD) in Rudraprayag district and Joshimath (JM) in Chamoli district are known as potential zone of wild MAPs in western Himalaya. Population in these areas is distributed in villages from lowland (1000 m asl) to high altitude regions (2500 m asl). Peoples of high altitude regions migrate to MAPs producing areas with their cattle's in the temporarily settlements during May to October and exploits MAPs to increase their cash income. MAPs collected in villages of such areas are traded throughout the markets. Therefore, these areas were selected and surveyed for present studies.

b) Methods

Each of the selected sites was visited frequently during 2008 to 2010 and data were collected. Information's were collected on MAPs trade from all sites and voucher specimen were maintained, authenticated by Dr. P. Prasad of Botanical Survey of India, Dehradun and deposited in same herbarium (BSD 201-238). Based on the trade volume and number of

collectors engaged, species that were in active trade in all zones was selected for detail investigation *i.e.* profit at each level of transaction. These species are 1) *Aconitum heterophyllum* Wall. ex Royle, 2) *A. balfourii* (Bruhl) Muk (synonym *A. atrox*), 3) *Nardostachys jatamansi* DC., 4) *Picrorhiza kurrooa* Benth ex Royle, and 5) *Rheum* spp. (*Rheum emodi* and *R. moorcroftianum*). At least two collectors of 50% families from two villages of each study site were interviewed for estimation of annual household income as cash. Data regarding involvement of households, contribution of MAPs related activities in annual income, season and method of collection of MAPs, channels involved in trade, methods of sale, etc. was collected by open ended questionnaire and standardized interviews. Respondents were assured that the data will be used only for research purpose and their identity will not be disclosed. Data on involvement of households engaged and percentage share in annual income through MAPs related activities were collected from these villages, pooled and average values was calculated. Channels involved in trade was identified and treated as proposed by Olsen and Bhattarai (2005). Ten to fifteen collectors, 3-4 middlemen and traders were interviewed from each of the selected zones for data collection and at least 50% of them interviewed in every study year. 'Species area curve method' was applied to collect data in a sigmoid form as suggested by Williams et al (2005).

Income of collectors was estimated on the basis of net income with the sale of selected species minus input cost (time consumed for collection of particular species @Rs. 120 per day as common labor charges in these sites). Profit percentage of all channels was calculated based on the difference in sale price received minus purchase price. Net profit at different level of traders was estimated by deducting expenses during journey *i.e.* loading, transport, expenses on journey and other charges. Selling price was analyzed on the sites of collection and their trade route, as personal observation at least once in every year. Existing market of this region (Wholesale market of Dehradun, Haridwar, Saharanpur and retail market of Badrinath and Rishikesh) was surveyed, because collected MAPs are traded in these markets. Variation in prize of MAPs throughout the study period was pooled and average values was considered for further analysis. To calculate final data on profit percentage of different channels, data of net income at species level at each channels from each sites were pooled separately and average value were calculated as percentage profit.

III. RESULTS

Collection and trade of MAPs is age-old practice and believed to be 5000 year old. However, number of species traded fluctuated regularly. Before 1994, collection of MAPs in these areas was made by

licensed contractor with payment of royalty to forest department and sale through district level cooperative societies. Later on some of the plants categorized as threatened and their collection was banned from natural habitats. Even after ban, peoples settled in high altitude regions migrate to MAPs growing areas, exploit a number of species and sale them to market in illegal way. Almost 30-40% families (varies with altitude of villages and their option to other source of income) are directly linked to MAPs related activities *i.e.* collection, drying, grading or selling, etc. They generated 18% of total annual cash income through MAPs related activities (Figure 2). There was a stark difference in abundance (availability as well as density) of MAPs from site to site. These differences directly influenced cost of collection and number of species traded along with trade pattern from each site. Collection of MAPs made frequently throughout the summer season although major proportion were extracted from August to November. During the months of August to November, peoples made team to collect MAPs, moved to sites and stayed there for few weeks in natural caves or in tents. Collections were made in a destructive way (as underground part is economic in these perennial species) by uprooting whole plants even before the maturation of seeds. Currently eighteen MAPs species are in trade from study areas. List of important MAPs with their trade name, distribution range, economically useful part with sites of collection is presented in Table 1.

The traders of MAPs employed local agent (local traders), who made contact with collectors. Collectors dig out MAPs from nature, supplied them to local traders (sometime directly to traders if local trader is not present as in DR and PK sites, Fig. 3). Local traders supplied MAPs to traders. Different channel involved in the trade of these species are presented in Figure 3. Local traders were often moneylenders or local merchants of household requirements. The collectors were mostly local inhabitants but sometimes persons hired by the local traders which extracted plants destructively. Collectors dug out material from nature, dried it partially or completely and carried the produce to local trader's house. The produce further moved to other channels accordingly. Sometime MAPs may also bypass through one or more channels as indicated in Figure 3. Volume, market rate and value of collected species, annual demand and deficit are presented in Table 2. Study reveals that volume of collected MAPs varied from site to site depending on availability of species. Total worth of 5 selected species is Rs. 1,17,21,400 annually from this region. Among these species, sale of *P. kurrooa* provided highest income of Rs. 34,98,750. *A. balfourii* was second important income generating species with value of Rs. 33,60,000. Collection of *A. heterophyllum* was much lower in quantity as compared to other species but had high

market rate and trade reached up to Rs.13,10,400. Collection of *Rheum* spp. showed lowest income among selected species. Observations revealed that there is a gap between demand and supply of selected species. This gap was much higher in *A. heterophyllum*, *N. jatamansi* and *P. kurrooa*.

Table 3, show details of profit percentage of channels involved in trade, at each site. Profit analysis of collectors indicates that they got lowest profit of 2.24% in MD with the sale of *A. heterophyllum* and maximum (59.52%) in PK with the sale of *A. balfourii*. Local traders are important channel in trade of MAPs, which purchase material from collectors. They earned lowest profit of 6.66% in JM with the sale of *N. jatamansi* and highest (29.41%) in MD with the sale of *Rheum* spp. Traders obtained maximum profit of 92.80% in PK with the sale of *A. balfourii* and minimum profit 2.70% in HKD with the sale of *Rheum* spp. *A. balfourii* was the species with maximum profit for traders in most of the sites while *Rheum* spp. was the source of minimum profit in four sites. The mode of transportation on roads depends on the volume of the produce and distance to be covered *e.g.* Trucks, Bus or Jeep. The traders then send produce to wholesaler in Dehradun, Haridwar, Saharanpur, Ram Nagar or Delhi market. In case of market, wholesale dealer earned maximum profit of 36.84% with the sale of *Rheum* spp. and profit of 10.0-18.66% with the sale of other MAPs. In retail market, maximum profit was 53.84% for the sale of *Rheum* spp. and minimum profit of 5.66% with the sale of *A. heterophyllum*. Other three species provided almost similar (13.63%-17.97%) profit to retail shopkeepers.

IV. DISCUSSION

Collection and trade of MAPs is as old as human civilization and an integral part of socioeconomy in rural areas. However, pattern of trade changed from time to time *i.e.* in the form of exchange of food material with the peoples of low altitude areas in ancient time, exchange as money in later stage, collection via licensed contractor and now in illegal way. Poor economy with good profit compelled peoples in illegal harvesting of threatened MAPs. It is also estimated that a total 2200MT of MAPs covering 100 species are traded from Uttarakhand (Ved and Goraya 2008). In general, the income generated through MAPs related activities (only 18%) is a small part, but due to lack of other sources of income it makes important contribution. MAPs collection provides cash business and sometime advance monetary benefits to local economy. Mostly traders paid incentives to collectors as advance money for collection of MAPs at the start of season. Edwards (1993) also reported almost similar pattern for MAPs collection in Nepal. MAPs also act as 'saving' to be cashed in contingencies and thus constitute the larger part of household income in time of need (Olsen 1998).

Large part of population was found linked to MAPs work in Nepal (Larsen and Olsen 2007). Engagement of only 30-40% families in MAPs related activities is lower as compared to other parts of the world, which may be due to changing nature of policy and decrease in abundance of MAPs. Collection of MAPs continued throughout the summer season with destructive harvesting practice is also reported earlier (Rai et al 2000).

Variation in the numbers and volume of MAPs collected from site to site may be due to availability (abundance and density of species) in nature, profit earning and preference in terms of physical labor of particular species. Our observation reveals that few sites are rich whereas others are poor in availability of MAPs. Collection of *A. balfourii* and *P. kurrooa* is easier as compared to *A. heterophyllum*, *N. jatamansi* and *Rheum* spp. A difference in the relative abundance and diversity of plants sold by street traders and shop traders is also reported in Johannesburg, South Africa (Williams et al 2005). The volume of collected MAPs and deficit reveals that the study areas are under heavy harvesting pressure. Due to manifold increase in market demand, collection period increased from few months to all growing seasons (July-November) against the principle of active constituents and quality. However, collectors have the opinion that collection is difficult during October-November as compared to August- September due to cold and freezing of soil. Only underground parts are useful in these species and plants were uprooted through destructive harvesting system. Almost 66% of total species sourced from wild reported to be harvested destructively (Ved and Goraya 2008). Further, harvesting prior to seed dispersal decreased chance of recruitment of new individual, due to which abundance of these species is decreasing with alarming rate. Occurrence of trade channels and unequal distribution of profit among market channels is also reported for other species and regions (Holley and Cherla 1998; Olsen 1998; Olsen and Bhattarai 2005; Kuniyal et al 2005).

Lack of storage facilities and technology is major factor, which accelerate sale of medicinal plants even when price may not be better. Collectors are sometime compelled to sell produce with minimum profit due to uncertainty in MAPs trade. Availability (abundance) of species at collection sites also fluctuated profit percentage, as abundance is directly related to cost of collection. Shiva (1998) has the opinion that wholesalers have a virtual monopoly on the Jari-buti trade and control the price, paid to the small traders. Sharma (2004) also reported that market price, trade and channels of *Cordyceps* sp. collection are not transparent in Indian subcontinent. The overall study indicates that traders earned maximum percentage of profits. Contrary to exploitation of collectors in MAPs trade, here collectors also got good profit with the sale of MAPs, due to which they take risk of illegal collection.

Higher profit obtained by the collectors and traders in DR and PK, as compared to other sites could become possible due to lack of one channel (local traders) in trade chain. The study supports earlier studies (Sharma and Tewari 1996; Gupta 1997) that highest share of profits to collectors can be ensured with less number of middlemen. Retail market showed less profit percentage in most of the species, which may be due to bypass transaction of produce that decreased number of channels.

Collection of medicinal plants raises a number of complex environmental and social issues that must be addressed locally on a case-by case basis (WHO 2003). Income from MAPs is integral part in the socioeconomy of the peoples settled in such areas. Apart from healthcare, medicinal plants are mainly alternative income generating source of underprivileged community (Lacuna-Richman 2002), therefore, strengthening of this sector may benefit and improve the living standard of poor peoples (Kala et al 2006). In case, the root is not harvested at maturity stage, it starts decaying from the core (Rundel and Witter 1994). Therefore, ecologically non-destructive systems of collection may be employed and the ban imposed on collection may be replaced with rotational harvesting system to promote sustainable utilization of biodiversity. Due to socioeconomic importance of biodiversity, Conservation of Biological Diversity (CBD) also agreed on a framework that biodiversity should be utilized for human welfare in a sustainable manner (Kate and Laird 2000). Subedi and Koontz (1999) also supported rotational harvesting for MAPs in Nepal with minimum 20% plant undisturbed. Ghimiri et al (2005) indicates that sustainable commercial harvest of *P. kurrooa* may be feasible while *N. grandiflora* appears very sensitive to harvest. Larsen and Olsen (2007) concluded that the present level of information renders impossible conclusion on whether the resource is degraded or not, status of MAPs was essentially based on participant's perceptions of change in various species parameters such as population size and not equivalents to scientific evidences for the status of any medicinal plants species. In such case careful management is required for sustainable harvesting of high altitude perennial medicinal plants (Ghimiri et al 2008). Further, recently developed cultivation technology of few high altitude MAPs (Nautiyal et al 2001, 2003, 2005; Chauhan and Nautiyal 2005; Chauhan et al 2008) will also be helpful in conservation of these species if technology transferred to interested growers properly. However, farmers need incentive for cultivation of these species.

V. CONCLUSIONS

Illegal MAPs trade continues in Himalayan regions even after imposing ban, to restrict it. Thousands of plants of these species are uprooted

every year through destructive harvesting system. Good profit at each channels encourage peoples to involve in illegal trade. MAPs trade showed their importance in socioeconomy of these regions. Thus without providing optional sources of income generation for local population, complete ban on MAPs collection seems impossible. On the other hand conservation of natural resources is also important issue to restrict biodiversity loss. Furthermore, collection practices should also ensure the long term survival of wild populations and their associated habitats. Management plans for collection should provide a framework for setting sustainable harvest levels and describe appropriate collection practices. In such condition, rotational harvesting after few years interval (only after seed dispersal) may be useful. Cultivation of such species should be promoted, as it is viable option to meet the increasing demand, improvement in socioeconomy and conservation of these species. If MAPs cultivation starts once, illegal collection will automatically discouraged. To meet the challenges in MAPs sector, scientific studies should be conducted for- 1) the impact of illegal collection on natural resource, 2) resource assessment survey for evaluation of the regulation potential, and 3) What is the limit of sustainable harvesting? It should be determined at species level.

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Table 1 : General information on MAPs in trade in Western Himalaya

S.N.	Plant species	Trade name	Useful part	Distribution range (m asl)	Sites of collection*
1.	<i>Aconitum balfourii</i>	Mitha Bish	Tubers	2500-4000	1,2,3,4,5
2.	<i>Aconitum heterophyllum</i>	Atis	Tubers	3000-4500	1,2,3,4,5
3.	<i>Allium sp.</i>	Farn	Arial shoots	2800-4200	2,5
4.	<i>Angelica glauca</i>	Choru	Underground	2500-3800	1,2,3,4
5.	<i>Arnebia benthami</i>	Lal Jari	Underground	3000-4000	1,4
6.	<i>Dactylorhiza hatagirea</i>	Salampanja	Tuber	2800-4000	1,2,3
7.	<i>Fritillaria roylei</i>	Kakoli	Tuber	2800-4000	1,3
8.	<i>Habaenaria spp.</i>	Riddhi-Bridddhi	Tuber	2200-3000	2,3,5
9.	<i>Malaxis muscifera</i>	Jeevak	Tuber	2500-3700	2,5
10.	<i>Morina longifolia</i>	Dhoop	Whole plant	2800-3800	2,5
11.	<i>Nardostachys jatamansi</i>	Jatamansi	Underground	3300-5000	1,2,3,4,5
12.	<i>Picrorhiza kurrooa</i>	Kutki	Rhizomes	2800-4500	1,2,3,4,5
13.	<i>Podophyllum hexandrum</i>	Bakarchimka	Underground	2800-3800	1,2
14.	<i>Polygonatum sp.</i>	Mahamaida	Underground	2200-4000	1,2,5
15.	<i>Polygonum sp.</i>	Kanthla	Tuber	3000-4200	3,4,5
16.	<i>Rheum spp.</i>	Archa, Dolu	Underground	2800-4200	1,2,3,4,5
17.	<i>Swertia spp.</i>	Chirata	Whole plant	2000-3500	2,5
18.	<i>Thalictrum spp.</i>	Mamira	Underground	2800-3700	1,5

* Site of collection - 1-HKD, 2- DR, 3- PK, 4- MD, 5-JM

Table 2 : Quantity (qt.), value (Rs.), demand and deficit of selected MAPs in Western Himalaya

Sites	Quantity of selected species (qt.)				
	<i>A. heterophyllum</i>	<i>A. balfourii</i>	<i>N. jatamansi</i>	<i>P. kurrooa</i>	<i>Rheum spp.</i>
HKD	1.0	33	18	5.5	18
DR	0.45	58	4.25	10	6
PK	1.50	25	18	48	18
MD	1.35	32	39	60	13
JM	0.38	12	35	32	32
Total	4.68	160.0	114.25	155.5	87.0
Market rate (Rs.)/kg	2800	210.0	250.0	225.0	80
Value (Rs.)	13,10,400	33,60,000	28,56,250	34,98,750	6,96,000
Demand	24.0	189.0	317.0	470.0	91.0
Deficit	19.32	29.0	202.75	314.5	4.0

Table 3 : Profit percentage of different channels involved in trade of MAPs in Western Himalaya

Sites	Categories	<i>A. heterophyllum</i>	<i>A. balfourii</i>	<i>N. jatamansi</i>	<i>P. kurrooa</i>	<i>Rheum spp.</i>
DR	Collector	20.8	54.54	33.89	36.84	12
	Local trader	-	-	-	-	-
	Trader	64.22	88.91	18.06	24.46	4.39
HKD	Collector	8.87	45	25	27.77	11.11
	Local trader	24.07	29.31	10.71	17.39	25
	Trader	42.26	72.41	19.76	19.72	2.70
MD	Collector	2.24	34.88	15.0	31.11	13.33
	Local trader	13.46	23.72	15.94	10.16	29.41
	Trader	53.70	77.72	17.37	24.46	13.77
PK	Collector	11.08	59.52	39.13	43.67	25
	Local trader	-	-	-	-	-
	Trader	78.59	92.80	17.09	29.60	6.14
JM	Collector	12	42.22	33.33	37.5	20
	Local trader	15.71	15.62	6.66	36.0	25
	Trader	47.05	74.41	16.27	26.42	78.57
Drug Shop	Wholesaler	10.41	18.66	10.0	12.5	36.84
	Retailer	5.66	17.97	13.63	13.63	53.84

- Local traders not found in sites

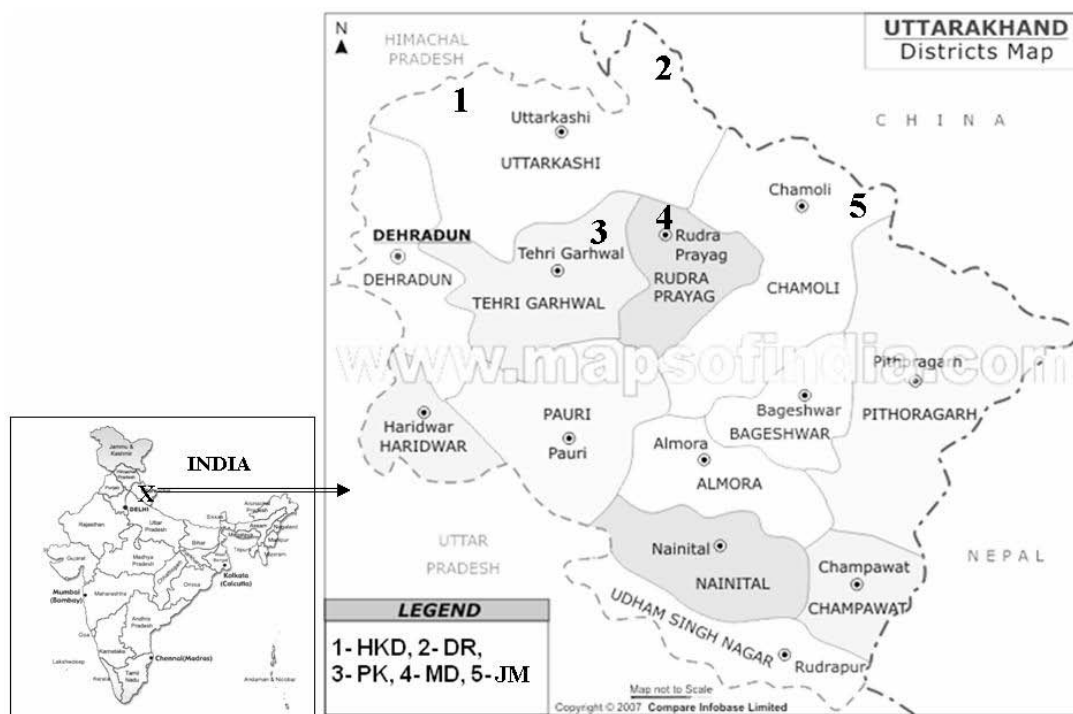


Figure 1 : Location map showing study sites in Uttarakhand (India)

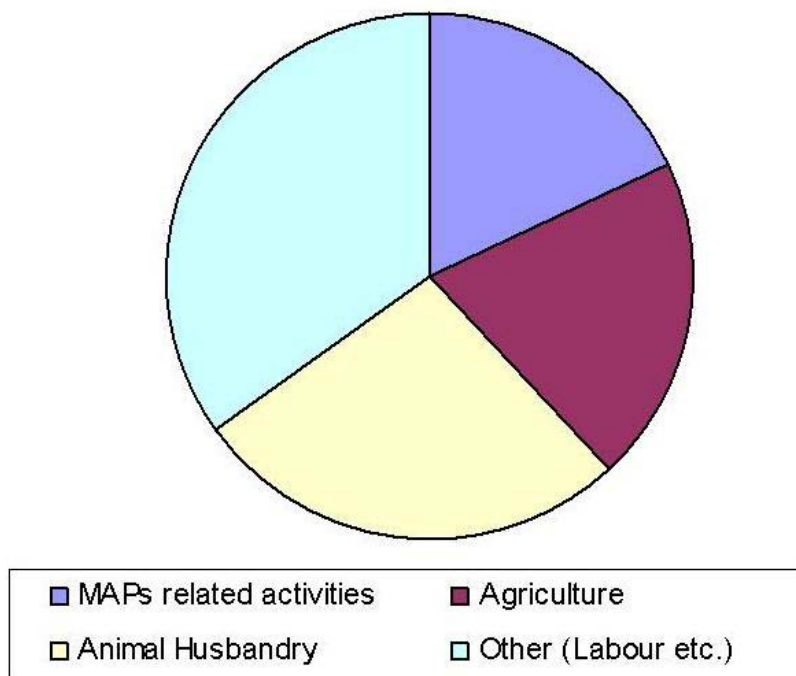


Figure 2 : Percentage share of MAPs in household's income in study areas

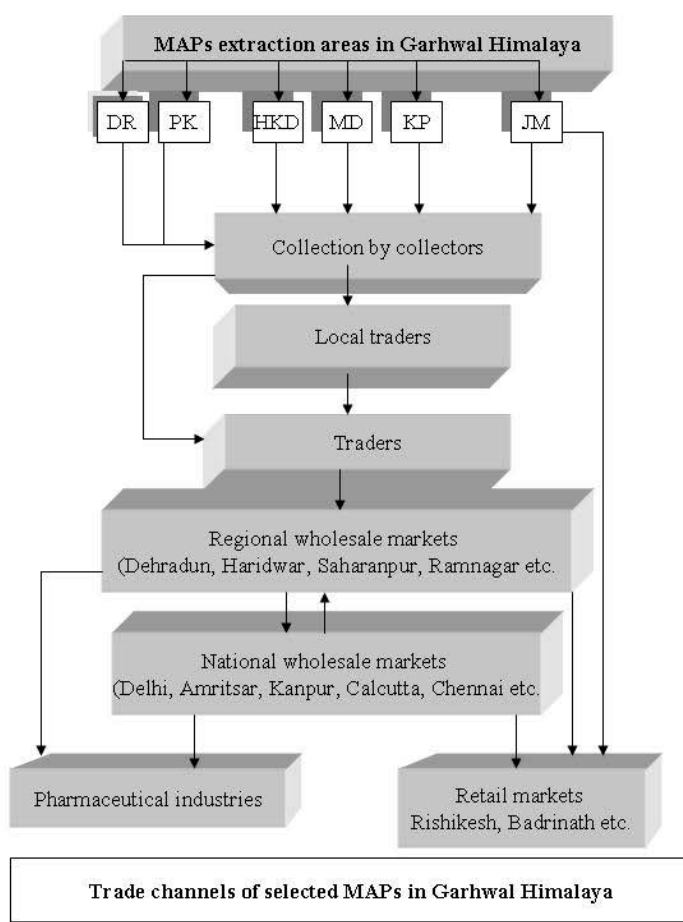


Figure 3 : Trade channels of selected MAPs in study areas



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Efficacy of Chlorhexidine in Reduction of Microbial Contamination in Commercially Available Alginate Materials – In-Vitro Study

By Sunitha Kollu, Veena Hedge & Kalyana Chakravarthy Pentapati

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Abstract - Objective: To evaluate the prevalence of contamination, minimum inhibitory concentration (MIC) of chlorhexidine against the isolated microbes and also to assess the surface activity of chlorhexidine in three commercially available alginate impression materials.

Materials and Methods: Twelve sealed packets of three different routinely used alginate impression materials were used. Three 0.1 gram samples were aseptically obtained from each packet. These were inoculated in glucose broth, thioglycolate broth and sabouraud's dextrose agar tubes and were incubated for seven days. Surface activity of chlorhexidine in alginate mix was assessed using the Agar well technique.

Keywords : *alginate, bacteria, chlorhexidine, fungi, Irreversible hydrocolloid.*

GJMR-C Classification : *NLMC Code: QW 25.5.M6*



EFFICACY OF CHLORHEXIDINE IN REDUCTION OF MICROBIAL CONTAMINATION IN COMMERCIALY AVAILABLE ALGINATE MATERIALS IN-VITRO STUDY

Strictly as per the compliance and regulations of:



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Results: A total of 24 out of 36 samples inoculated on various media showed growth, with an average contamination frequency of 67%. Bacillus species was highest in all three brands, followed by aerobic spore bearing bacilli and coagulase negative staphylococcus. Pseudomonas species was detected in two brands. All dilutions at 20μg/ml or above were clear of turbidity at 0.02% concentration, 10μg/ml or above were clear of turbidity at 0.05% concentration and all dilutions were clear of turbidity at 0.1% concentration.

Conclusion: The level of contamination with microorganisms in commercially available alginate materials is high. 0.1% Chlorhexidine solution mixed in alginate can be effective alternative for disinfection impression material.

Keywords : alginate, bacteria, chlorhexidine, fungi, Irreversible hydrocolloid.

1. INTRODUCTION

Dental practitioners, patients, and laboratory personnel are subject to notable risks with respect to infectious diseases, which can be spread by saliva or blood from contaminated impression material, particularly irreversible hydrocolloid impression material like alginates (Wang et al, 2007). A growing concern regarding the control of cross-infection in dentistry can be seen in literature. Laird and Davenport have stated that, it is often impossible to sterilize dental materials contaminated during manipulation in the mouth. Dental impressions are one such kind where the

sterilization is not possible and can therefore act as a means of transmitting infectious agents from patients to those who handle them subsequently. In order to prevent the transmission of these infectious agents, effective infection control procedures should be exercised by all dental health care personnel (Ghani F, Hobkirk JA and Wilson M, 1990).

Saliva and blood can be a source of high concentrations of potentially infective pathogens that can cause Common cold, Herpes, Hepatitis B virus (HBV), Pneumonia, Tuberculosis, and are the suspected mode of transmission of Acquired Immune Deficiency Syndrome (AIDS). The dental clinics have responsibility for infection control and if not practiced, a cycle of cross-contamination may occur, thereby exposing dental health care personnel and patients to infection.

American Dental Association (ADA, 1985) proposed guidelines to limit cross-contamination during dental clinical procedures like impression disinfection. But unfortunately enough information regarding infection control and sterility of materials received by the dentist from the manufacturer, is not readily available. Further study in this area is warranted because of the increasing number of subjects who were immunocompromised due to disease process, chemotherapy or elderly individuals and are, thus very prone to normal or opportunistic infections.

The increasing emphasis on infection control has created interest in the possible hazard of the clinical use of commercial available alginate impression material. The need of prevention of transmission of microorganisms from the patient's oral fluids to the dental health care personnel via impression materials has been studied previously (Wang et al, 2007; Ghani F, Hobkirk JA and Wilson M, 1990). Previous studies have demonstrated microbial contamination in commercially available alginate impression material (Rice CD, Dykstra MA and Feil PH, 1992; Casemiro LA et al, 2007; Jafari AA et al, 2012).

Several methods of disinfection for alginate impression materials were proposed. Spray and immersion methods are the two most widely used techniques in clinical practice. However, these conventional strategies present several disadvantages like loss of surface detail and dimensional inaccuracy of the impression (Wood PR, 1992). Due to the difficulties

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with the disinfection of alginate impression materials, self-disinfecting alginate impression materials were developed. Studies have shown that this technique demonstrated better dimensional stability than spray and immersion techniques, and saved disinfection time (Touyz LZ and Rosen M, 1991; Rosen M and Touyz LZ, 1991; Poulos JG and Anton off LR, 1997).

Therefore, the need of hour was to develop a self-disinfecting irreversible hydrocolloid impression material with broad spectrum antimicrobial agent in order to provide a blanket cover against the spread of infection while recording the impression and handling. Hence, we aimed to evaluate the prevalence of contamination, minimum inhibitory concentration (MIC) of chlorhexidine against the isolated microbes and also to assess the surface activity of chlorhexidine in three commercially available alginate impression materials.

II. MATERIALS AND METHODS

Twelve sealed packets of three different routinely used commercially available alginate impression materials were purchased from the local vendor. The materials tested were Tropicalgin(Zhermack), Microflex (Septodont) and Zelgan (Dentsply) henceforth, referred as Brand A, B and C.

The packaging was examined to ensure that it had not been tampered. Each packet was shaken well before opening it. The packets were opened, under a laminar flow chamber and three 0.1 gram samples were aseptically obtained from each packet. First sample of 0.1 gram was inoculated in 20ml of glucose broth; the second sample was inoculated into 20ml of thioglycolate broth, and vortexed for 10 seconds to disperse the irreversible hydrocolloid. The third sample was inoculated aseptically into 20ml sabouraud's dextrose agar tubes which were incubated at 22°C and 37°C for seven days, for the isolation of fungus. Ten sabouraud's dextrose agar tubes were inoculated with *Candida albicans* as positive control. Ten sham inoculated sabouraud's dextrose agar tubes served as negative control. The isolated fungus was identified by standard procedures (Moore GS and Jaciow DM, 1979). Ten sham inoculated glucose broth tubes and equal number of sham inoculated thioglycolate broth tubes served as negative controls. Ten glucose broth tubes and thioglycolate broth tubes were each challenged with *Acinobacter* and *Bacteroidsfragilis* respectively to serve as positive controls. All media were incubated at 37°C for 18 to 24 hours and later subcultures were done on blood agar. The isolated bacterial growth was identified by using standard microbiological techniques (Bailey and Scott, 1994; Mackie and McCartney, 1989).

In this study Pour plate technique (Bailey and Scott, 1994) was used for colony counting. Five

allocates of 10mg each were made under aseptic conditions from each container, and each of these allocates were spread evenly on the base of an agar plate using a sterile spatula. The inoculums was covered completely with agar medium and allowed to set. The plates were incubated for 24 hours and colony counts were done following standard technique (Miles AA, Misra SS and Irwin JO; 1938).

The minimum inhibitory concentration (MIC) of chlorhexidine against *Bacillus* species, Aerobic spore bearing bacilli and Coagulase negative staphylococcus which were isolated from the alginate powder, was determined by using Broth dilution method (Bailey and Scott, 1994). A constant volume (1ml) of different concentrations of the chlorhexidine was incorporated into 5ml of glucose broth in test tubes to get the final concentrations of (80µg/ml, 40µg/ml, 20µg/ml, 10µg/ml, 5µg/ml and 1µg/ml while 0µg/ml served as a control) to which 1ml of the isolated standardized bacterial suspension was added. The efficacy of chlorhexidine was also tested against the standard strains (*Escherichia coli*, ATCC No: 25922; *Staphylococcus aureus*, ATCC No: 25923; *Pseudomonas aeruginosa*, ATCC No: 27853) (Robert Cruickshank, 1992). Inhibition of bacterial growth was determined by noting the turbidity in broth solution after overnight incubation.

Surface activity of chlorhexidine in alginate mix was assessed using the Agar well technique (Bailey and Scott, 1994). The powder of alginate impression material was spatulated in standard proportions with distilled water containing varying concentrations of chlorhexidine (0%, 0.01%, 0.02%, 0.05%, and 0.1%) for 30 seconds and poured into petridish such that the prepared blocks had a flat and smooth surface. The mixing was done according to the powder/liquid ratio (10g/23ml) recommended by the manufacturer.

The Specimens were divided into five groups in each test: specimens mixed with (0.01%, 0.02%, 0.05%, and 0.1%) of chlorhexidine solution served as test groups and specimens mixed with distilled water (0%) of chlorhexidine served as control group. Group allocations were consistent for all tests. Then impression disks, 6mm in diameter by 2mm thickness, were prepared. After that, wells of the same size as the impression disk were cut into nutrient agar plates previously inoculated with the appropriate microorganisms under sterile conditions. On each agar plate, five wells were cut and specimen was selected from each of four test groups (0.01%, 0.02%, 0.05%, and 0.1%) and put into four of the agar wells respectively. The control group (0%) was placed in the fifth well of each plate. Three independent assays were performed for each microorganism (n=3). Finally, all plates were incubated in the appropriate aerobic environment for 24 to 48 hours at 37°C. After incubation, clear zones or inhibitory areas were observed in the culture plates around the specimens and measured to

evaluate the antibacterial effect. Standard strains of the following microorganisms were used: *Staphylococcus aureus* (ATCC No.25923), *Pseudomonas aeruginosa* (ATCC NO.27853), *Escherichia coli* (ATCC No.25922) and *Candida albicans*.

III. RESULTS

A total of 24 out of 36 samples inoculated on various media showed growth, with an average contamination frequency of 67%. The three brands showed wide variation in contamination frequency, contamination average of Brand A (Tropicalgin), Brand B (Microflex) and Brand C (Zelgan) were 83, 67 and 50% respectively (Table 1).

There was an average of 0.733 colonies in each 10mg sample placed on nutrient agar plates for the three brands, yielding an average contamination of approximately 73.3 colony forming units (CFUs) per gram of materials. Brand C showed the lowest concentration of organisms at 40 CFUs per gram, followed by Brand B, 80 CFUs per gram and Brand A being the highest (100) CFUs per gram.

Among the various aerobic bacteria isolated, *Bacillus* species contamination was observed with maximum frequency in all three brands of alginate powder, followed by aerobic spore bearing bacilli and coagulase negative staphylococcus. *Pseudomonas* species was detected in two brands which is a known pathogen to cause nosocomial infections. However, there were no anaerobic bacteria isolated from any of the three brands of alginate impression material. Among the fungi isolated *Aspergillus fumigatus* and *Aspergillus Niger* were observed to be the commonest contaminants followed by *Mucor* and *Rhizopus* (Table 2).

In our study, the lowest dilution of chlorhexidine which inhibited the bacterial growth of three test strains of bacteria (*Bacillus* species, aerobic spore bearing bacilli and coagulase negative *Staphylococcus*) isolated from the irreversible hydrocolloid impression material and against the standard strains was observed to be 20µg/ml at 0.02% concentration, 10µg/ml at 0.05% concentration.

All dilutions at 20µg/ml or above were clear of turbidity at 0.02% concentration, 10µg/ml or above were clear of turbidity at 0.05% concentration and all dilutions were clear of turbidity at 0.1% concentration, indicating inhibition of bacterial growth on overnight incubation and were confirmed by subcultures on blood agar.

The dilutions of (0µg/ml, 1µg/ml, 5µg/ml and 10µg/ml) at 0.02% concentration and dilutions of (0µg/ml, 1µg/ml, 5µg/ml) at 0.05% concentration showed turbidity of broth solution, indicating bacterial growth after overnight incubation at 37°C, which was confirmed later by subcultures on blood agar plates.

Well-defined zones of inhibited growth became apparent after this incubation period and allowed for consistent measuring of inhibitory fields. The results demonstrated that zones of growth inhibition around all the test specimens were observed on all plates. No zones of inhibited growth were observed around the control wells on all agar plates. ANOVA test revealed that the inhibition zones tested became significantly larger ($p < 0.001$) for each microorganism when the concentrations of chlorhexidine solution were raised from 0.01% to 0.1% (Table 3).

IV. DISCUSSION

Our study showed 67% of contamination from three different brands of commercially available alginate impression material. This was comparable to the findings of Rice et al, who reported a 60% contamination from four commercial brands of irreversible hydrocolloid (Rice CD, Dykstra MA and Feil PH, 1992). However, Jafari et al reported overall 75% bacterial contamination with wide range of bacteria and fungi which are known to cause opportunistic infections (Jafari et al, 2012). Similarly, in our study the isolated organisms were avirulent members of environmental and normal skin flora, which might have a pathogenic potential in immunocompromised patients. The organisms isolated in our study were aerobic spore bearing bacilli, *Bacillus* species, coagulase negative *Staphylococcus*, *Pseudomonas* species, *Aspergillus* species, *Mucor* and *Rhizopus*. However, no anaerobic bacteria were isolated in our study which was contrary to the findings of Rice and co-workers, who reported 6% contamination with anaerobic bacteria (Rice CD, Dykstra MA and Feil PH, 1992; Rice CD et al, 1991).

The level of contamination was demonstrated by the number of colony forming units (CFUs) from the positive samples. The average quantity of irreversible hydrocolloid powder used in routine clinical use is approximately 15gms or about 150 times larger than the tested material in this study. This would yield a much higher concentration of organisms per clinical sample of material. The CFU's (40-100 CFUs/gm) in our study were in corroboration with the study by Rice and co-workers et al who found that the concentration of organisms varied from 12-82 CFUs/gm of contaminated sample (Rice CD et al, 1991).

It was observed that 0.1% of chlorhexidine was sufficient to inhibit the growth of most of the isolated organisms from the alginate powder and the three standard tested bacteria. The MIC determined in this study was against a limited bacterial species so it needs further evaluation against a wider spectrum of organisms. Lower concentration of the disinfectant would be more acceptable to the patient and was shown to be effective in previous study than the routinely used concentrations of 0.2% (Tanaka et al., 1994).

Flanagan et al, tested the antibacterial effects with chlorhexidine or quarternary ammonium compounds (Flanagan et al, 1998). The results revealed that the quarternary ammonium containing alginates were completely effective against all five test microorganisms. The alginate containing chlorhexidine killed all the gram-negative bacilli and majority (95-99%) of the gram-positive cocci and yeast. Previous studies have also reported that irreversible hydrocolloids with chlorhexidine and quaternary ammonium were effective in reducing surface growth (Rice, Dykstra and Feil, 1992; Cserna A et al, 1994; Tobias, Browne and Wilson, 1989). Based on the findings from our study, we conclude that materials like alginate can be contaminated with bacteria and fungi during various phases of manufacturing and packing. So it is necessary to maintain aseptic conditions at the manufacturing level. Techniques like gamma ray irradiation should be employed to sterilize the alginate material. Interim audits of the various batches of alginates should be done by the manufacturer to ensure aseptic conditions in the packets. Guidelines should be made for clinicians and dental auxiliary personnel who handle the powder for mixing. Wherever possible, individual packets of alginate powder for each impression shall be supplied by the manufacturer to prevent cross contamination in dental clinics. Further studies are needed to evaluate the possibility of incorporating chlorhexidine into alginate powder so that only water can be used as previously. Studies are also needed to evaluate patient acceptability with incorporated disinfectants. 0.1% Chlorhexidine solution for mixing alginate can be a suitable alternative for disinfection of impression material.

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Table 1 : Frequency of contamination in three types of alginate impression material

Brand	Positive in Culture
	(n=12)
A	10(83%)
B	8(67%)
C	6(50%)

Table 2 : Growth in Thioglycolate broth & Glucose broth in three brands of alginate impression material

Type of Bacteria	Average (%)	Brand A	Brand B	Brand C
Coagulase negative Staphylococcus	19.44	4	2	1
Aerobic spore bearing Bacilli	30.56	6	3	2
Bacillus species	36.11	6	4	3
Pseudomonas species	5.56	1	1	0
Escherichia coli	8.33	2	1	0
Aspergillus fumigatus	25	4	3	2
Aspergillus	13.89	3	2	0
Mucor	11.11	2	1	1
Rhizopus	5.56	1	1	0

Table 3 : Mean diameter of inhibition zone (mm) and SD for each bacterial species and group

Species	0.01%	0.02%	0.05%	0.1%	p-value
Staphylococcus aureus	12±0.9	14.5±0.98	18.5±1.1	21.4±1.2	<0.001
Pseudomonas aeruginosa	10.7±1.2	14.3±0.78	16.3±0.84	19.4±1.2	<0.001
Escherichia coli	11.4±1.4	13.5±1.1	16.0±1.43	18.1±1.51	<0.001
Candida albicans	12.9±1.1	19.3±1.2	27.0±2.3	30.1±1.32	<0.001



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An Investigation of the Factors that Influence Maternal Encouragement of Healthy Diets for Preadolescent Daughters

By Dr. Lauren M. Hamel & Prof. Sandi W. Smith

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Abstract - Childhood overweight has reached epidemic levels in the United States. Twenty percent of U.S. children are overweight or obese. This problem is likely to carry into adulthood with increased risk of certain cancers and cardiovascular problems. It has also been demonstrated that diets of preadolescent girls are strongly influenced by their mothers in terms of communication, modeling, and regulation. Using the Theory of Planned Behavior as a framework, the factors that influence mothers to communicate encouragement of a healthy diet for their preadolescent daughters were studied in an effort to reduce female child overweight and subsequent negative consequences. A websurvey (N = 104) with mothers with at least one preadolescent daughter was conducted. Attitude toward encouraging their daughters to eat healthily, the most likely subjective norm, and perceived behavioral control all significantly influenced intention to communicate encouragement to daughters to eat healthily. Implications of these findings are discussed.

Keywords : mother-daughter communication, encouragement, diet, childhood obesity, theory of planned behavior.

GJMR-B Classification : NLMC Code: WB 400



AN INVESTIGATION OF THE FACTORS THAT INFLUENCE MATERNAL ENCOURAGEMENT OF HEALTHY DIETS FOR PREADOLESCENT DAUGHTERS

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An Investigation of the Factors that Influence Maternal Encouragement of Healthy Diets for Preadolescent Daughters

Dr. Lauren M. Hamel^a & Prof. Sandi W. Smith^σ

Abstract - Childhood overweight has reached epidemic levels in the United States. Twenty percent of U.S. children are overweight or obese. This problem is likely to carry into adulthood with increased risk of certain cancers and cardiovascular problems. It has also been demonstrated that diets of preadolescent girls are strongly influenced by their mothers in terms of communication, modeling, and regulation. Using the Theory of Planned Behavior as a framework, the factors that influence mothers to communicate encouragement of a healthy diet for their preadolescent daughters were studied in an effort to reduce female child overweight and subsequent negative consequences. A web-survey (N = 104) with mothers with at least one preadolescent daughter was conducted. Attitude toward encouraging their daughters to eat healthily, the most likely subjective norm, and perceived behavioral control all significantly influenced intention to communicate encouragement to daughters to eat healthily. Implications of these findings are discussed.

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I. INTRODUCTION

The prevalence of childhood obesity is dramatically increasing in the United States (Jolliffe, 2004; Ogden, Carroll, Curtin, Lamb, & Flegal, 2008; Ogden & Carroll, 2010). In 2010 the Centers for Disease Control and Prevention (CDC) reported that overall prevalence of obesity in children between the ages of 6 and 11 had increased from 6.5 percent in 1980 to 19.6 percent in 2008. The immediate and long-term health effects of being overweight are numerous. Among the most well known serious effects are the increased risk of certain types of cancers, such as colon and breast cancers, an increased risk for type-two diabetes, cardiovascular disease as well as high blood pressure and high cholesterol levels (CDC, 2007; Dietz, 1998). A main factor that influences childhood weight problems is the diet of the child (CDC, 2007). A child who consumes high fat, high calorie foods is more likely than a child

who does not consume as much fat or high caloric foods to incur a large number of negative health conditions.

The biological and physical consequences of childhood overweight and obesity are typically more severe in girls than in boys (Dietz, 1998), and although children as young as seven have been shown to diet, have concerns over weight, and exhibit body dissatisfaction, this is more common in girls than boys (Collins, 1991; Rolland, Farnill, & Griffiths, 1996; Thompson, Corwin, & Sargent, 1997). In addition to physical health maladies, overweight children tend to also suffer psychosocially. More specifically, overweight children, again especially female children, tend to experience discrimination more so than normal size children (Reilly, et al., 2003) especially in adolescence (Strauss 2000). This discrimination can result in decreased self esteem among overweight children which can then lead to other problems including decreased academic achievement (Rosenberg, Schooler, & Schoenbach, 1989) and social isolation (CDC, 2007). These effects can also progress into adulthood (CDC, 2007; Reilly et al., 2003).

Mothers play a crucial role in the eating behavior of young children of both genders (Cutting, Fisher, Grimm-Thomas, & Birch, 1999; Fisher & Birch, 1999), including through direct instruction about what is good to eat and what is not good to eat (Hays, Power, & Olvera, 2001). It has been demonstrated that girls as young as five have ideas about dieting that are strongly influenced by their mothers (Abramovitz & Birch, 2000). The most successful point of intervention for weight problems is during childhood when the problem is still developing or before the problem develops (Guo et al., 2000). Due to the findings that female children are more likely to experience immediate and prolonged psychosocial and physical effects of being overweight, the current research effort focuses on this population rather than grouping girls and boys together. Similarly, because mothers have been shown to have a great deal of influence on eating perceptions and behaviors of girls, the current effort focuses on mothers rather than grouping both parents together.

What follows is a summary of literature regarding maternal influence on the eating habits of preadolescent daughters, a description and rationale for

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the theoretical framework utilized, the predications based on the theory and previous research, a description of the web survey that was conducted, the results of the analysis, and a discussion of the findings.

a) Maternal Influence on Food Consumption

The eating habits of young girls are strongly influenced by their mothers in a variety of ways. This includes the mothers' own eating habits, food restriction and regulation (Birch & Fisher, 2000; Cutting et al., 2007; Hill et al., 1990), and the type of communication that occurs between mothers and daughters. Communication research has established that when communication between mother and daughter is more positive and open, daughters are less likely to suffer from disordered eating (Vidovic, Juresa, Begovac, Mahnik & Tocil, 2005). Conversely, it is also been found that negative maternal communication is related to disordered eating attitudes and behavior in daughters (Kicher & Crowter, 2001; Prescott & Le Poire, 2002). The current study was undertaken in an effort to gain greater understanding of the predictors of positive, maternal communication with daughters regarding diet.

The findings reported here can be utilized in future efforts to craft messages aimed at mothers with preadolescent daughters to inform and persuade them that they hold great influence over their daughters' diets. Moreover, it can be communicated to mothers that they should use that influence by communicating their desires for their daughters to make healthy choices for food. Finally, that communication should be in a constructive and encouraging manner rather than in a destructive and restrictive manner.

b) Theory of Planned Behavior

As the previous section demonstrates mothers have a great deal of influence on the eating habits of their preadolescent daughters. In an effort to determine what influences a mother to encourage a healthy diet for her preadolescent daughter, the Theory of Planned Behavior (TPB) (Ajzen, 1985; 1991) was utilized as the guiding framework for this research. Succinctly put, the TPB predicts that behavioral intentions and subsequent behavior, are determined by attitude toward that behavior, normative perceptions about what important others think about performing the behavior, and perceived barriers to performing that behavior. This theory is particularly well suited for the current issue because it has been shown to be a reliable predictor of intentions and behavior over time for a variety of behaviors (Albarracin, Johnson, Fishbein, & Muellerleile, 2001; Armitage & Conner, 1999; Hausenblas, Carron, & Mack, 1997). In particular, it has found to be useful in predicting communication behavior within the family context – which is the focus of the current study (Park & Smith, 2007). Moreover, recent research has indicated that the TPB is well suited for predicting maternal intention to discuss safe sex issues with their daughters

(Askelson, et al., 2011). Given the theory's history of consistently predicting behavioral intent, and specifically maternal intent to communicate, it was utilized in this case as well.

Attitude is the extent to which a person is favorable or unfavorable regarding engagement in a particular behavior. Favor or disfavor is determined by taking into account the anticipated outcomes (behavioral beliefs) of the behavior and whether those are positive or negative (outcome evaluations). Beliefs are created through associations with certain attributes of attitude toward the behavior. According to the TPB, an individual will favor behaviors with positive and desired outcomes and disfavor behaviors with negative or undesirable outcomes. In addition, favor and disfavor of a behavior is also determined by the likelihood of the expected outcomes occurring. Attitude has been found to significantly influence eating behaviors in a variety of populations (Backman, Haddad, Lee, Johnston, & Hodgkin, 2002; Gracey et al., 1996; Patrick & Nicklas, 2005; Schifter & Ajzen, 1985). A previous focus group study in this area indicated that mothers think encouraging their daughters to eat healthily is important and worthwhile but that it can be a difficult and unpleasant process (Hamel, 2009; Hamel & Smith, 2009).

Subjective norm is the function of one's normative beliefs. Normative beliefs are the perceptions an individual holds of whether important referent others in his or her life believe that he or she should engage in the behavior of interest (Ajzen, 1985; 1991; Park & Smith, 2007). In addition to identifying these important people, the level at which an individual feels motivated to comply with these referents must be understood. This is the degree to which the individual is likely to follow what he or she perceives the important referents would want them to do and/or do themselves. This perception of what behaviors others expect them to perform results in a perceived social pressure to act in accordance with those others. Subjective norm has been found to significantly influence eating intentions and behaviors (Backman et al., 2002; Schifter & Ajzen, 1985). Previous research indicated that this population viewed their mothers, mothers-in-law, pediatricians, and mothers of their daughters' schoolmates as their most influential subjective norm referents. Their husbands, their own fathers, siblings, fathers-in-laws, close friends, and dentists were mentioned somewhat less often (Hamel, 2009; Hamel & Smith, 2009).

The traditional conceptualization of the source of the subjective norm component of the TPB is someone who is seen as trustworthy or experienced regarding the behavior of interest. However, previous research revealed a different source of normative influence at work in this situation. Although certain sources were seen as trustworthy and preferred by the participants (such as their own mothers and

pediatricians), other reported sources were described as being more common or more likely referents (such as mothers in law and mothers of daughters' schoolmates), but not necessarily the most valued. Focus group discussions in previous research focused on some of these 'more likely referents' and found that despite not being a preferred source of subjective norms by the participants, they may nonetheless possess normative influence (Hamel, 2009; Hamel & Smith, 2009). The current research is designed to further investigate the relative influence of both types of referents found in previous studies.

Perceived behavioral control (PBC) is determined by what the individual perceives to be a barrier(s) to performing the behavior in question. In addition, his or her control beliefs will determine how likely the barrier(s) will be to deter the individual from performing the behavior. The individual's control belief strength or the extent to which an individual believes something will act as a barrier to a behavior; and his or her control belief power or the extent to which an individual believes a barrier will make it more difficult to enact a behavior, will determine the individual's overall PBC. These perceived barrier beliefs can be the result of individual past experiences and are often influenced by second-hand information from others. PBC has been found to significantly influence eating intentions and behaviors (Backman et al., 2002; Gracey et al., 1996; Patrick & Nicklas, 2005; Schifter & Ajzen, 1985). The barriers reported in previous research were mothers' own schedules, their daughters' schedules, the cost of healthy foods, the time needed to prepare healthy foods, and their daughters' dislike of healthy foods or preference for unhealthy foods. The barriers discussed were not direct barriers to encouragement; however they were barriers to providing a nutritional environment that allowed this type of encouragement (Hamel, 2009; Hamel & Smith, 2009).

Finally, the behavioral intention component of the model has to do with how likely the individual is to perform the behavior in question. It is predicted that an individual's attitude, subjective norm, and PBC should result in one's intention to engage in a particular behavior. In this context the behavior of concern is encouraging one's daughter to eat a healthy diet.

To gain further understanding of this population and as a measure to determine the generalizability of the qualitative data obtained from the focus group study (Hamel, 2009; Hamel & Smith, 2009), a Web survey was conducted. Based on the previous study findings and previous research on the TPB (Albarracín, et al., 2001; Armitage & Conner, 1999; Askelson, et al., 2011; Hamel, 2009; Hamel & Smith, 2009; Babrow, Black, & Tiffany, 1990; Courneya, 1995; Hausenblas, Carron, & Mack, 1997; Park & Smith, 2007) the following hypotheses were posed:

H1: Mothers' attitude toward healthy eating will be a significant positive predictor of their behavioral intention to encourage a healthy diet for their preadolescent daughter(s).

H2: Mothers' subjective norm regarding healthy eating will be a significant positive predictor of their behavioral intention to encourage a healthy diet for their preadolescent daughter(s).

H3: Mothers' perceived behavioral control regarding healthy eating will be a significant positive predictor of their behavioral intention to encourage a healthy diet for their preadolescent daughter(s).

II. METHOD

a) *Participants*

A sample of 104 mothers with at least one daughter between the ages of 6 and 11 participated in the study. The mean age of participants was 38.8 years and the standard deviation was 5.7. The sample was comprised of 84.6% Caucasian, 7.7% Latin, 4.8% African, 1.0% Asian, and 1.9% other. In terms of education 39.4% completed graduate school, 13.5% had some graduate school, 26.9% completed college or other post high school training, 15.4% had some college or post high school training, 3.8% completed high school, and 1.0% had some high school. This sample was recruited through a snowball sampling method through a health and risk communication research center and also through personal contacts of the authors. Specifically, an invitation email was sent to potential participants that included a summary of the study and a link to the web survey. There was also a message at the end of the email requesting women forward the invite on to others they knew that fit the inclusion criteria.

b) *Measures*

All scales were comprised of seven-item Likert response scales. Items were adapted from Ajzen (2007) and created to validate the responses of the focus groups regarding attitude, subjective norm, and PBC of encouraging one's daughter to eat healthily.

Attitude. Based on the direction provided by Ajzen (2007), the attitude variable was based on measures of behavioral beliefs multiplied by outcome evaluations. The potential behavioral beliefs were based on previous research (Hamel, 2009; Hamel & Smith, 2009). The scales created to assess behavioral beliefs and outcome evaluations were adapted from Ajzen (2007). Behavioral beliefs items included "encouraging your daughter to eat a healthy diet will: (1) help your daughter have good overall health now; (2) make your daughter less likely to become overweight/obese; (3) help your daughter choose healthy foods to eat instead of unhealthy foods; (4) help prevent her from getting a serious disease, such as cancer, as an adult; (5) place heavy focus on health that will make her too concerned

with her body image". The outcome evaluation scale presented the same five behavioral beliefs set to a 7-point scale from 'good' to 'bad' so participants could evaluate these potential outcomes. These responses were submitted to a confirmatory factor analysis (CFA; Hamilton & Hunter, 1988) to determine if the items were measuring the constructs reliably. After the analysis the fifth item from each scale was removed. The final behavioral belief scale ($M = 6.27$, $SD = .79$, $\alpha = .82$) and the outcome evaluation scale ($M = 6.84$, $SD = .47$, $\alpha = .84$) were each comprised of four items.

Subjective norms. Based on the direction provided by Ajzen (2007), the subjective norm variable was based on measures of normative beliefs multiplied by motivation to comply. Based on findings from the previous research (Hamel, 2009; Hamel & Smith, 2009) subjective norm was measured for two different referents, the person whose opinion the participants' valued most and the person the participant thought would most likely to talk with them about encouraging their daughters to eat healthily.

Two scales were developed because although an individual may serve as one's more important referent, this person may not be the person most likely to comment or give advice on this behavior. Focus group data provided information to develop the list of normative referents (Hamel, 2009; Hamel & Smith, 2009): mother, father, mother-in-law, father-in-law, sister(s), brother(s), daughter's father, husband, pediatrician, dentist, close friends, other mothers, or other. Participants chose from this list for their 1) most valued referent and 2) most likely referent.

Eight items were adapted from Ajzen (2007) assessing the subjective norms (e.g. this person thinks/expects you should encourage your daughter to eat a healthy diet), and motivation to comply with important others (e.g. when it comes to encouraging your daughter to eat a healthy diet, you want to do what this person thinks you should). The responses to each of the subjective norm scales were submitted to CFA. All items were retained for both. The final subjective norm scale for most valued referent ($M = 6.51$, $SD = .74$; $\alpha = .84$) and for the most likely referent ($M = 6.56$, $SD = .58$; $\alpha = .83$) were comprised of three items each. The motivation to comply with the most valued referent ($M = 5.97$, $SD = 1.05$) and motivation to comply with the most likely referent ($M = 5.53$, $SD = 1.30$) were both one-item scales.

Perceived behavioral control. Based on the direction provided by Ajzen (2007), the PBC variable was based on measures of control belief strength multiplied by control belief power. Participants were presented with a list of behavioral barriers (mom's schedule, daughter's schedule, the cost of healthy foods, time, daughters' taste, inability to cook healthy food), pulled from previous research (Hamel, 2009;

Hamel & Smith, 2009). They were then asked to assess their control belief strength (e.g. I expect that the most major barrier I selected will prevent me from encouraging my daughter to eat a healthy diet), control belief power (e.g. my most major barrier will make it more difficult to encourage my daughter to eat a healthy diet) for that barrier. The control belief strength measure ($M = 3.67$, $SD = 1.84$) and control belief power measure ($M = 4.69$, $SD = 1.67$) were both one-item scales.

Behavioral intention. Intention to encourage a healthy diet for one's daughter was measured by a four-item scale (e.g. I plan to encourage my daughter to eat a healthy diet) adapted from Ajzen (2007). The scale was submitted to CFA and it was fully retained ($M = 6.64$, $SD = .57$, $\alpha = .97$). See Table 1 for a summary of the means, standard deviations, and reliabilities of the variables assessed. Also see Table 3 for a correlation matrix of the relationships among the five variables: attitude, the most valued referent, the most likely referent, PBC, and behavioral intent.

III. RESULTS

a) Analysis

Using hierarchical regression analysis, age, education, and ethnicity were entered in the first block as control variables, then the main predictor variables from the TPB (attitude, both subjective norms, and PBC) were entered in the second block with all possible interactions entered into the third block. According to Ajzen (2007) attitude is product of behavioral beliefs and evaluations of those outcomes, subject norm is the product of the normative measures and the motivation to comply, and perceived behavioral control is the product of control belief strength and control belief power, and that formula was followed here. To guard against nonessential multicollinearity the independent variables were mean-centered before they were entered into the equations (Cohen, Cohen, West, & Aiken, 2003). The results are presented in Table 2.

b) Regression Model

The analyses indicated that the overall model was significant, $F(7, 91) = 12.62$, $p < .001$, $R^2 = .49$, adjusted $R^2 = .45$. Block 1 demographics did not contribute significantly to the variance in maternal intent to communicate. When the four main TPB components (attitude, two types of norms, and PBC) were entered into the regression analysis in Block 2, attitude toward the behavior (standardized beta weight: $\beta = .54$, $p < .001$), the subjective norm of the person most likely to provide a message regarding the behavior ($\beta = .18$, $p < .05$) and PBC ($\beta = -.22$, $p < .01$) were statistically significant predictors of maternal intention to communicate with daughters about diet. Block 3 interactions were not significant 'thus' the product terms

did not add to the variance in intent to encourage explained by the independent variables.

These data are consistent with hypothesis one and partially consistent with hypothesis two. Mothers' attitude and their most likely subjective norm are both significant, positive predictors of intent to encourage a healthy diet for their daughters. However, their most valued subjective norm had little influence on intent ($\beta = .09$, ns). Last, the findings are in the opposite direction of hypothesis three such that the more control mothers' perceive the less they intend to talk with their daughters. The adjusted R^2 of the analysis was .45, indicating that 45% of the variance in intent to encourage a healthy diet in one's daughter is explained with the TPB components.

c) *Subjective Norms and Barriers*

Based on the data analysis from previous research (Hamel, 2009; Hamel & Smith, 2009) which indicated that common normative referents might not be the most valued referent, participants in this study were asked to provide the normative referent whose opinion they valued most in regards to them encouraging their daughters to eat healthily, and also the normative referent they thought would be most likely to talk with them about encouraging their daughters to eat healthily. The referent most frequently cited as the person whose opinion the participants value most was their daughter's father ($N = 33$, 32.7%) and the second most common was their daughter's pediatrician ($N = 25$, 24.8%).

The referent most frequently cited as the person most likely to send a message regarding their encouragement of their daughters to eat a healthy diet was their own mother ($N = 21$, 20.6%) and the second most common referent was close friends ($N = 16$, 15.7%).

The barrier most frequently cited was their daughters' taste for healthy foods ($N = 31$, 29.8%) and the second most common barrier was the time needed to prepare healthy foods ($N = 22$, 21.2%).

IV. DISCUSSION

Currently the United States is experiencing a weight problem of epidemic proportion. Decades of evidence indicate that more of U.S. children are heavier than ever before, and that this childhood weight gain is not something that they will grow out of (CDC, 2007; Reilly, 2003). Moreover, not only will an overweight child be more likely to be an overweight adult but that childhood overweight has influence over some major adulthood health concerns such as breast and colon cancers, type two diabetes, and cardiovascular problems (CDC, 2007; Guo, et al., 1994). These consequences are documented for both sexes but girls are more likely to experience the biological and psychological deleterious adulthood problems (Guo, et al., 2000; Strauss, 2000). This problem needs to be

targeted either before it develops or while it is still developing during childhood. Once a girl reaches puberty the negative effects of an overweight childhood are much harder to reverse (Guo et al., 2000). While girls are still in their preadolescent years (between 6 and 11) they are greatly influenced by their mothers in terms of modeling, restriction, regulation (Birch & Fisher, 2000; Cutting et al., 2007; Hill et al., 1990) and communication (Kicher & Crowter, 2001; Prescott & Le Poire, 2002; Vidovic, et al., 2005). The aim of the current research was to determine what influences mothers to communicate their encouragement to eat a healthy diet for their preadolescent daughters. The data from this line of research demonstrates that future persuasive attempts focused on mothers and their influential power regarding their daughters' dietary behaviors could hold promise as an avenue to reduce female childhood overweight/obesity.

a) *Theory of Planned Behavior*

The findings from this study demonstrate the applicability and suitability of the TPB as a framework to approach this problem. Because the hypotheses broke down the three main predictions into their individual components, it is clear to see that this theoretical perspective is ideal for examining a variety of factors that might influence mothers to encourage a healthy diet for their daughters. The findings in this case are in line with what has been found in previous research that has utilized the TPB to assess maternal intention to communicate to their daughters (Askelson et al., 2011).

More specifically, mothers held a strong, positive attitude toward encouraging a healthy diet for their daughters, and attitude was shown to be a positive predictor of intent. These findings are similar to what has been found in related research (Askelson et al., 2011; Backman, et al., 2002; Gracey et al., 1996; Patrick & Nicklas, 2005). In this case, mothers who perceived that encouraging their daughters to eat healthily would lead to better health for their daughters now, help them to choose healthy foods over unhealthy foods, help prevent them from becoming overweight or obese, and help prevent serious conditions such as cancer, were significantly more likely to report they intended to encourage their preadolescent daughters to eat healthily. This finding is important as a foundation for future persuasive attempts. Specifically, this group already believes that encouraging their daughters to eat well is important for a variety of reasons. What is needed now are efforts to influence either the onset or maintenance of that behavior.

This study approached the subjective norm component of the TPB in an innovative way. Based on a previous focus group study (Hamel, 2009; Hamel & Smith, 2009) another norm type in addition to the traditional most valued referent was added. This particular situation involved possible influential referents

who were not necessarily the most valued, but the most likely to offer their opinion on the topic. It was predicted that the perception of what important others do themselves and what the respondent perceives them to expect to do will have an effect on one's behavioral intention. The findings differed based on the type of referent. The most valued referent was not found to predict intent to encourage, contrary to what has been found previously (Backman et al., 2002; Schifter & Ajzen, 1985). However, the referent participants felt they were most likely to get a message from about their daughters' diet did significantly predict intent to encourage. This suggests that the most likely referent can be more influential than the most valued in particular situations. The narrow conceptualization of the subjective norm component of the theory is a common critique of the TPB (Terry & Hogg, 1996). The findings in this case offer a more nuanced picture of the normative influence that could be extended to other behaviors.

Perceived behavioral control negatively predicted intent to encourage. This contrary finding may suggest that this group has a great deal of perceived control over the behavior of encouraging a healthy diet. For instance, the most frequently cited barrier was their daughters' taste for healthy foods and the second most common barrier was the time needed to prepare healthy foods. The greatest barriers found in past research have typically been lack of availability to healthy foods or the higher price of healthy foods (Gracey et al., 1996). Based on self-reported education, this sample was relatively affluent and perhaps did not view lack of availability or high prices as barriers. It could be the case the barriers here are not as severe as barriers as in past research.

In sum, all of the components of the TPB were significant predictors of mothers' intent to encourage a healthy diet. Clearly personal attitude has a strong impact on whether or not this population will intend to encourage their daughters to eat healthily. This has been found in previous research concerning nutritional behavior. What is surprising is the result of including both the most valued and the most likely normative referents. Although it seems intuitive to assume the most valued person would have the greater effect, in this case it was the most common normative referent who significantly influenced intent. In the case of PBC, the negative relationship between PBC and intent indicates that the more likely a barrier is perceived to act as a barrier and the more severely it is perceived the less likely one will intend to encourage a healthy diet. Statistically speaking, nearly half of the variance of intent was accounted for by the TPB and offers evidence of the suitability of the framework for this research area.

b) Practical Implications

There appear to be several factors which have substantial influence over mothers' intent to encourage

a healthy diet in their daughters. These data are evidence that mothers already have a positive attitude toward encouraging their daughters to eat well and that attitude has great influence over that intent. An area that could be utilized in more practical terms is the use of normative referents, especially in terms of the most likely referents which have more predictive power than most valued referents. In this case the most commonly cited most likely referent was a woman's own mother. This information could be used in educational campaigns especially toward close-knit families informing maternal grandmothers that they have a very important influence over their families.

In addition, the two most valued referents were daughters' fathers and pediatricians. Although they may not have as much predictive power as the most likely referent, they are valued for a reason and campaigns targeting fathers and informing them of their importance in this context as well as reinforcing to pediatricians how their advice is seen as expert and objective and likely to be followed.

The barriers identified in previous research and investigated further in this study were not necessarily barriers to encouragement per se, but more structural in nature. For instance, scheduling and cost barriers for certain foods will not prevent a mother from verbally encouraging her daughter but they do prevent an ideal environment for such encouragement to be likely and effective. Given that the data and findings presented here are meant to inform future persuasive messages, it is important for these future endeavors to take this contextual and structural reality into account.

c) Limitations

The current research is important, but its limitations should be acknowledged. The main drawback is that the dependent variable was the behavioral intention to encourage preadolescent daughters to eat a healthy diet rather than the actual behavior of encouragement. This is a starting point, but the end goal should be able to determine actual encouraging behavior. An improvement upon this research endeavor would be to determine if mothers' attitude, subjective norm, PBC, and intent lead to actually encouraging their daughters to eat healthily.

Additionally, the sample utilized in this case was highly educated and therefore may offer a biased view into mothers' perceptions of encouraging a healthy diet for their preadolescent daughters. Specifically, it could be the case that because these mothers have more training and education than average (and presumably a high socioeconomic status) they may have more nutritional knowledge than average and greater resources to provide a better diet than mothers from lower socioeconomic classes.

V. CONCLUSION

This study offers a solid foundation for future efforts striving to persuade mothers to encourage a healthy diet for their daughters. It was determined that mothers already have a strong, positive attitude toward this behavior and that attitude has a great deal of influence on their intent to encourage. In addition, there is evidence to suggest that normative referents should be utilized as possible sources of persuasive messages. Specifically, the most likely sources to offer their opinions such as mothers and close friends appear to hold the most influence on this population. As a group mothers hold a great deal of influence on their daughters, especially at a young age, and this study is a starting point for future endeavors that can continue to focus on this maternal position as a means to reduce female childhood overweight and obesity.

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Table 1 : Means, Standard Deviations and Reliabilities of the Components of the Theory of Planned Behavior

Variable	Mean	Std. Dev.	Alpha
Subjective Norm 1	6.51	.74	.84
Subjective Norm 2	6.56	.58	.83
Motivation to Comply 1	5.97	1.05	*
Motivation to Comply 2	5.53	1.30	*
Control Belief Strength	3.67	1.84	*
Control Belief Power	4.69	1.67	*
Behavioral Beliefs	6.27	.79	.82
Outcome Evaluation	6.84	.47	.84
Behavioral Intention	6.64	.57	.97

Table 2 : Regression Analysis for Behavioral Intent to Encourage Daughter to Eat a Healthy Diet

Predictors	B	SE	β	T	sr
Ethnicity	.14	.07	.17	2.17	.22
Education	.003	.04	.01	.10	.01
Age	.0	.01	-.01	-.07	-.01
Attitude	0.05	.01	.54	6.8**	.58
MV_ Subjective Norm	.004	.004	.09	1.11	.12
ML_ Subjective Norm	.01	.004	.18	2.14*	.22
Perceived Behavioral Control	-.01	.003	-.22	-2.87*	-.29

$F(7, 91) = 12.62, p < .001, R^2 = .49, \text{adjusted } R^2 = .45$

** $p < .001$

* $p < .05$

MV = most valued referent

ML = most likely referent

Table 3 : Correlation Matrix of Theory of Planned Behavior Components

	Attitude	Most Valued Subjective Norm	Most Likely Subjective Norm	PBC
Attitude				
Most valued Subjective Norm	.19*			
Most Likely Subjective Norm	.29**	.34**		
Perceived Behavioral Control	.02	.06	-.01	
Intent to Encourage	.62**	.26**	.36**	-.19*

* $p < .05$

** $p \leq .01$

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- Very for a short time explain the tentative propose and how it skilled the declared objectives.

Approach:

- Use past tense except for when referring to recognized facts. After all, the manuscript will be submitted after the entire job is done.
- Sort out your thoughts; manufacture one key point with every section. If you make the four points listed above, you will need a least of four paragraphs.



- Present surroundings information only as desirable in order hold up a situation. The reviewer does not desire to read the whole thing you know about a topic.
- Shape the theory/purpose specifically - do not take a broad view.
- As always, give awareness to spelling, simplicity and correctness of sentences and phrases.

Procedures (Methods and Materials):

This part is supposed to be the easiest to carve if you have good skills. A sound written Procedures segment allows a capable scientist to replacement your results. Present precise information about your supplies. The suppliers and clarity of reagents can be helpful bits of information. Present methods in sequential order but linked methodologies can be grouped as a segment. Be concise when relating the protocols. Attempt for the least amount of information that would permit another capable scientist to spare your outcome but be cautious that vital information is integrated. The use of subheadings is suggested and ought to be synchronized with the results section. When a technique is used that has been well described in another object, mention the specific item describing a way but draw the basic principle while stating the situation. The purpose is to text all particular resources and broad procedures, so that another person may use some or all of the methods in one more study or referee the scientific value of your work. It is not to be a step by step report of the whole thing you did, nor is a methods section a set of orders.

Materials:

- Explain materials individually only if the study is so complex that it saves liberty this way.
- Embrace particular materials, and any tools or provisions that are not frequently found in laboratories.
- Do not take in frequently found.
- If use of a definite type of tools.
- Materials may be reported in a part section or else they may be recognized along with your measures.

Methods:

- Report the method (not particulars of each process that engaged the same methodology)
- Describe the method entirely
- To be succinct, present methods under headings dedicated to specific dealings or groups of measures
- Simplify - details how procedures were completed not how they were exclusively performed on a particular day.
- If well known procedures were used, account the procedure by name, possibly with reference, and that's all.

Approach:

- It is embarrassed or not possible to use vigorous voice when documenting methods with no using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result when script up the methods most authors use third person passive voice.
- Use standard style in this and in every other part of the paper - avoid familiar lists, and use full sentences.

What to keep away from

- Resources and methods are not a set of information.
- Skip all descriptive information and surroundings - save it for the argument.
- Leave out information that is immaterial to a third party.

Results:

The principle of a results segment is to present and demonstrate your conclusion. Create this part a entirely objective details of the outcome, and save all understanding for the discussion.

The page length of this segment is set by the sum and types of data to be reported. Carry on to be to the point, by means of statistics and tables, if suitable, to present consequences most efficiently. You must obviously differentiate material that would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matter should not be submitted at all except requested by the instructor.



Content

- Sum up your conclusion in text and demonstrate them, if suitable, with figures and tables.
- In manuscript, explain each of your consequences, point the reader to remarks that are most appropriate.
- Present a background, such as by describing the question that was addressed by creation an exacting study.
- Explain results of control experiments and comprise remarks that are not accessible in a prescribed figure or table, if appropriate.
- Examine your data, then prepare the analyzed (transformed) data in the form of a figure (graph), table, or in manuscript form.

What to stay away from

- Do not discuss or infer your outcome, report surroundings information, or try to explain anything.
- Not at all, take in raw data or intermediate calculations in a research manuscript.
- Do not present the similar data more than once.
- Manuscript should complement any figures or tables, not duplicate the identical information.
- Never confuse figures with tables - there is a difference.

Approach

- As forever, use past tense when you submit to your results, and put the whole thing in a reasonable order.
- Put figures and tables, appropriately numbered, in order at the end of the report
- If you desire, you may place your figures and tables properly within the text of your results part.

Figures and tables

- If you put figures and tables at the end of the details, make certain that they are visibly distinguished from any attach appendix materials, such as raw facts
- Despite of position, each figure must be numbered one after the other and complete with subtitle
- In spite of position, each table must be titled, numbered one after the other and complete with heading
- All figure and table must be adequately complete that it could situate on its own, divide from text

Discussion:

The Discussion is expected the trickiest segment to write and describe. A lot of papers submitted for journal are discarded based on problems with the Discussion. There is no head of state for how long a argument should be. Position your understanding of the outcome visibly to lead the reviewer through your conclusions, and then finish the paper with a summing up of the implication of the study. The purpose here is to offer an understanding of your results and hold up for all of your conclusions, using facts from your research and generally accepted information, if suitable. The implication of result should be visibly described. Infer your data in the conversation in suitable depth. This means that when you clarify an observable fact you must explain mechanisms that may account for the observation. If your results vary from your prospect, make clear why that may have happened. If your results agree, then explain the theory that the proof supported. It is never suitable to just state that the data approved with prospect, and let it drop at that.

- Make a decision if each premise is supported, discarded, or if you cannot make a conclusion with assurance. Do not just dismiss a study or part of a study as "uncertain."
- Research papers are not acknowledged if the work is imperfect. Draw what conclusions you can based upon the results that you have, and take care of the study as a finished work
- You may propose future guidelines, such as how the experiment might be personalized to accomplish a new idea.
- Give details all of your remarks as much as possible, focus on mechanisms.
- Make a decision if the tentative design sufficiently addressed the theory, and whether or not it was correctly restricted.
- Try to present substitute explanations if sensible alternatives be present.
- One research will not counter an overall question, so maintain the large picture in mind, where do you go next? The best studies unlock new avenues of study. What questions remain?
- Recommendations for detailed papers will offer supplementary suggestions.

Approach:

- When you refer to information, differentiate data generated by your own studies from available information
- Submit to work done by specific persons (including you) in past tense.
- Submit to generally acknowledged facts and main beliefs in present tense.



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Methods and Procedures	Clear and to the point with well arranged paragraph, precision and accuracy of facts and figures, well organized subheads	Difficult to comprehend with embarrassed text, too much explanation but completed	Incorrect and unorganized structure with hazy meaning
Result	Well organized, Clear and specific, Correct units with precision, correct data, well structuring of paragraph, no grammar and spelling mistake	Complete and embarrassed text, difficult to comprehend	Irregular format with wrong facts and figures
Discussion	Well organized, meaningful specification, sound conclusion, logical and concise explanation, highly structured paragraph reference cited	Wordy, unclear conclusion, spurious	Conclusion is not cited, unorganized, difficult to comprehend
References	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring



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