Human skin bacterial flora differ with altitudes in different ethnic groups of Nepal

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ABSTRACT

Background: Largest organ of human body, the skin, is colonized by millions of microorganisms, most of which are not only harmless but also beneficial to the host. Human skin microbes depend upon geographical variations, ethnicity and various host factors. Despite several studies on human skin microbiota in various parts of the globe, it has not been studied in Nepalese population. **Aims and Objective:** To identify skin bacterial normal flora in different ethnic groups residing in different altitude of Nepal. **Materials and Methods:** We cultured skin swabs of 166 randomly selected volunteers belonging to 10 major ethnic groups from 3 distinct geographical altitudes of Nepal, viz. Bharatpur (415 m from sea level), Kathmandu (1,400 m from sea level) and Lukla (2,860 m from sea level). The isolated organisms were characterized and tested for their susceptibility against different antibiotics. **Results:** Altogether 231 bacterial isolates were characterized from 166 skin samples. Among them, 140 isolates (60.60 %) were Gram positive and 91 isolates (39.40 %) were Gram negative bacteria. *Staphylococcus aureus* (35.49%) was the most dominant skin bacterial flora followed by *Escherichia coli* (22.51%) and *Streptococcus* spp. (17.75%). Medium altitude Kathmandu exhibited the highest growth (120 isolates) followed by low land Bharatpur (66 isolates) and high land Lukla (51 isolates) which is statistically significant (p value = 0.0124). The antibiotic susceptibility testing against 14 antibiotics exhibited the Gram positive isolates were the most sensitive to Imipenem (94.93 %) whereas the least sensitive to Cephalexin (31.36 %) and the Gram negative isolates were the most sensitive to Amikacin (100%) whereas the least sensitive to Amoxycillin (28.57 %). **Conclusion:** Our data indicates that the skin bacterial normal flora; which is directly exposed to external environment; has significant relationship with altitudes where individuals live. The result desires further study for the adaptability of normal flora found in different altitudes. Some bacterial commensals were found resistant even against new generations of antibiotics as well, and hence can cause life-threatening infections if they happen to cross the skin physical barrier.

Key words: Normal flora; Ethnicity; Altitudes

INTRODUCTION

Skin is a diverse ecosystem extending about 1.8 m² surface area in adult human. In general, skin is cool, acidic and desiccated. Skin primarily contributes as physical barrier, protecting sensitive organs from foreign organisms, toxic elements and physical injury while retaining nutrients.[1] Skin supports the growth of commensal bacteria, which, in turn, protects skin from pathogenic bacteria.[2-4]

Human skin microbiota also produces modulators of immune system, such as short-chain fatty acids and

metabolites of tyrosine or tryptophan; up regulates regulatory T lymphocytes,[5] and produces bacteriocin to out compete skin pathogens.[6]

Human skin bacterial communities appear to be diverse. The diversity is obscure owing to the complexity of the physiology of the skin.[7] Skin exhibits distinct and diverse habitats for normal flora depending upon skin thickness, invaginations, folds and density of hair follicles and glands.[1,8] Skin microbial diversity is also influenced by the environment, as well as host sex, species, behavioral factors and genetics.[9-11]

Physiological and anatomical differences between male and female cutaneous environments such as sweat, sebum and hormone production, partially account for the microbial differences seen among the genders.[9]

Intrapersonal variation in microbial community membership and structure between symmetric skin sites is lesser than the interpersonal variation.[12]

Skin is the interface between the human and environment. The skin of the human living in high altitude is exposed to various extreme conditions such as hypobaric pressure, hypoxia, high ultraviolet radiation; and cold and dry environment.[13] These conditions exert a substantial selective pressure on skin microbiome and hence the skin microbiomes differ with altitude in terms of diversity and number.[14]

This study aims to isolate and identify the skin bacterial flora from 10 different ethnic groups residing in 3 distinct altitudes of Nepal; ranging from low land *Terai* region to high mountains. It will help to trace the microbial diversity in them.

MATERIALS AND METHODS

Study subject
One hundred and sixty-six healthy volunteers, 80 males and 86 females, of 10 ethnic groups, age 18-25 as inclusion criteria, with no history of dermatologic disorders (mild acne or other common features other than forehead acceptable) or other chronic medical disorders and with no current skin infections and antibiotic therapy were selected for our study. The ethnic groups under study are among major ethnic groups of Nepal; Brahmin (n=32), Chhetri (n=30), Newar (n=21), Tamang (n=17), Chaudhary (n=14), Gurung (n=12), Magar (n=10), Dalit (n=10), Kirat (n=11) and Sherpa (n=9). The volunteers were asked to fill up form to interrogate their behavioral and physiological information. Written consented was taken from the volunteers for sample collection and data publication prior to sample collection. Ethical approval was received from Nepal Health Research Council (Regd no. 311/2018).

Geographical regions
The sample sites were Bharatpur, Kathmandu and Lukla of Nepal as indicated in the map 1.

Kathmandu, the capital city of Nepal, is the medium altitude site located 1400 m (4600 feet) above sea level. The GPS coordinate is 27.7172° N and 85.7172° E.

Bharatpur is a low altitude city in Chitwan district of Nepal, only 208 m (682 feet) above sea level. It is situated in the mid-southern part of Nepal, 87 km southwards from Capital city, Kathmandu. The GPS coordinate for Bharatpur is 27.6847° N and 84.4173° E.

Lukla is a high altitude village in Solukhumbu district, the gateway to the highest peak of the world, Mount Everest. It
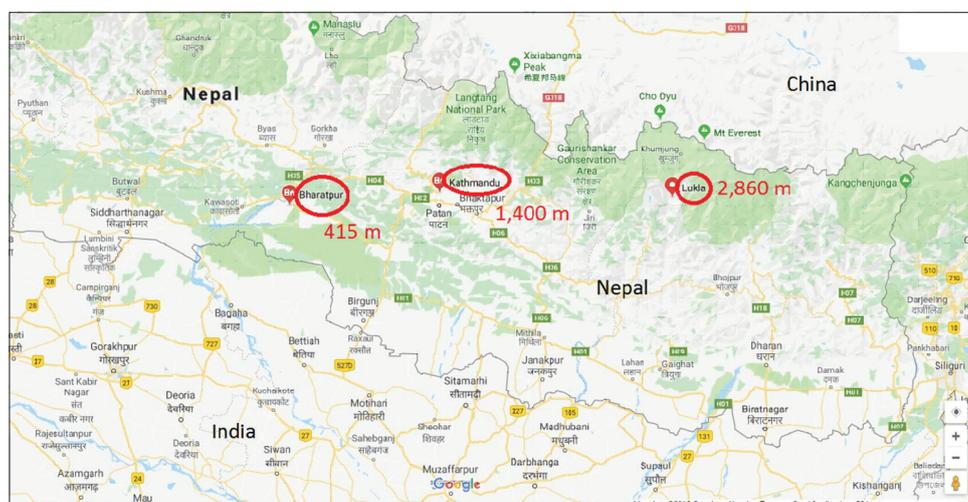
Map 1: Map showing sampling sites as indicated with Red circle

Table 1: Diversity of Skin Microbiome on different altitudes

S. No.	Organism	Bharatpur (*n*=60)		Kathmandu (*n*=55)		Lukla (*n*=51)		Total (*n*=166)	
		n	%	*n*	%	*n*	%	*n*	%
1	*Staphylococcus aureus*	16	26.66	33	60	33	64.70	82	35.49
2	*Escherichia coli*	30	50	22	40	0	0	52	22.51
3	*Streptococcus* spp	7	11.66	27	49.09	7	13.72	41	17.75
4	*Klebsiella oxytoca*	2	3.33	10	18.18	0	0	12	5.19
5	*Micrococcus luteus*	0	0	8	14.545	1	1.96	9	3.89
6	*Neisseria* spp	0	0	3	5.45	2	3.92	5	2.16
7	*Acinetobacter baumannii*	5	8.33	0	0	0	0	5	2.16
8	*Bacillus subtilis*	0	0	5	9.09	0	0	5	2.16
9	*Citrobacter freundii*	1	1.66	4	7.27	0	0	5	2.16
10	*Enterobacter aerogenes*	0	0	5	9.09	0	0	5	2.16
11	*Proteus mirabilis*	4	6.66	1	1.81	0	0	5	2.16
12	*Staphylococcus epidermidis*	1	1.66	0	0	2	3.92	3	1.20
13	*Pseudomonas aeruginosa*	0	0	2	3.63	0	0	2	1.20
	Total isolates	66		120		45		231	

is 2860 m (9380 feet) from sea level. The GPS coordinates for Lukla is 27.6857° N and 86.7278° E. It is 138 km East from Kathmandu.

Sample collection

Subjects were instructed not to wash face with anything, including water alone, for a 4 hour interval prior to sampling, but otherwise were allowed using not wet materials, for example handkerchief. Informed consents were obtained from all participants. All procedure done in aseptic way. Sterile cotton swab, soaked in sterile 0.9% sodium chloride with 0.1% Tween-20 solution, was vigorously rubbed in 2 cm X 2 cm area of the middle forehead for around 1 minute.[15]

Skin swab samples were collected from human samples of Kathmandu (n=55), Bharatpur (n=60) and Lukla (n=51) and processed in Kantipur General Hospital Laboratory, Kathmandu; Balkumari College Microbiology Laboratory, Chitwan and Pasang Lhamu Memorial Hospital Laboratory, Lukla respectively.

Sample processing

Skin swabs were inoculated in Nutrient, MacConkey, Blood and Chocolate Agar culture plates and incubated at 37° C for 24 hours. Colonies were observed, noted and sub cultured for obtaining pure culture. The isolates were identified by using microbiological tools and techniques as described in the Bergey's manual of systematic bacteriology which involves morphological appearance of the colonies, staining reaction and biochemical properties. Each of the organisms was isolated in pure form before performing biochemical and other tests. The biochemical tests used for the identification include Catalase test, Oxidase test, Sulfide Indole and Motility (SIM) test, Methyl red test, Voges Proskauer test, Citrate utilization test, Oxidation Fermentation test, Triple Sugar Iron (TSI) test and Nitrate reduction test.[16-17]

Antibiotic susceptibility testing

The antimicrobial susceptibility testing of the isolates towards various antimicrobial disks was done by modified Kirby-Bauer disk diffusion method using Mueller Hinton agar (MHA). Pure culture colony was grown into Mueller Hinton broth tube at 37° C for 2-4 hours until the turbidity of the suspension was matched with the turbidity standard of McFarland tube number 0.5. The bacterial suspension was inoculated into MHA using carpet culture technique and antibiotic discs were place over the carpet culture in aseptic way. The antibiotic discs used against Gram positive organisms were Amoxycillin, Cephalexin, Cefotoxin, Cefixime, Cefotaxime, Ceftriaxone, Co-trimoxazole, Ertythromycin, Gentamycin, Ciprofloxacin, Ofloxacin, Norfloxacin, Cloxacillin and Imipenem; and for Gram negative organisms were Amoxycillin, Cefixime, Cefotaxime, Ceftriaxone, Ceftazadime, Co-trimoxazole, Nitrofurantoin, Chloramphenicol, Amikacin, Gentamycin, Ciprofloxacin, Norfloxacin, Ofloxacin and Imipenem. The plates were left covered for few minutes after incorporating antibiotic discs which was then incubated at 37° C for 24 hrs. The plates were observed for zone of inhibition around antibiotic discs and measured in millimeter to compare with standardized zone interpretative chart provided by the company.

Data analysis

Data was entered and analysed using the Statistical Package for Social Sciences (SPSS), v23.0 (IBM SPSS Statistics, Armonk, NY). Descriptive statistics were applied to find frequencies, percentages and means. Qualitative variables, such as type of bacteria and antibiotics were expressed as frequencies and percentages. Chi-square tests were applied to compare the percentages of two categorical variables. Level of significance was set at $p < 0.05$.

Table 2 : Bacterial isolates in different ethnic groups

Ethnic groups	No. of samples	S. aureus	E. coli	Streptococcus spp	K. oxytoca	A. baumanii	C. freundii	P. mirabilis	S. epidermidis	Micrococcus spp	E. aerogenes	Neisseria spp	B. subtilis	Pseudomonas spp	Total
Arya	32	18	11	9	5	1	1	1	1	1	1	2	1	1	54
Chhetri	30	14	12	9	3	2	1	1	1	1	1	2	1	0	49
Newar	21	11	5	6	2	0	1	1	0	2	1	1	2	1	33
Tamang	17	9	7	6	1	0	1	1	0	1	1	0	0	0	26
Chaudhary	14	4	7	4	0	1	0	1	0	0	0	0	1	0	18
Gurung	12	6	3	2	0	0	0	0	0	1	1	0	0	0	13
Magar	10	3	4	1	0	0	0	0	0	1	0	0	0	0	9
Dalit	10	4	3	2	1	1	0	0	0	1	0	0	0	0	12
Sherpa	9	6	0	1	0	0	0	0	0	0	0	0	0	0	7
Kirat	11	7	0	1	0	0	0	0	1	0	0	0	0	0	10
	166	82	52	41	12	5	5	5	3	9	5	5	5	2	231

RESULTS

Microbial diversity on different altitudes

The most predominant skin aerobic bacterial flora was found to be *Staphylococcus aureus* isolated from 49.9% samples, followed by *Escherichia coli* and *Streptococcus* spp isolated from 31.32% and 24.69% samples respectively as shown in Table 1.

Skin swab samples from Kathmandu and Lukla exhibited *Staphylococcus aureus* as the most predominant skin flora from 60% and 64.70% samples respectively whereas that from Bharatpur exhibited *E. coli* as the most predominant skin flora growing in 50% samples.

Kathmandu samples (n=55) grew 120 isolates followed by 66 isolates observed from Bharatpur samples (n=60). The least number of isolates were observed from Lukla samples (n=51) that grew only 45 isolates. This relationship of total bacterial isolates from different altitudes of Nepal was found to be statistically significant (p value = 0.0124).

Regarding the diversity of bacterial normal flora, skin samples from Kathmandu (n=55) exhibited 10 bacterial genus, the highest among three altitudes. Bharatpur (n=60) and Lukla (n=51) skin samples grew 7 and 4 genus of skin bacterial flora respectively. The relationship is, however, statically insignificant (p value = 0.8868).

Microbial diversity on different ethnic groups

Out of 10 ethnic groups, 8 ethnic groups exhibited *S. aureus* as the most predominant bacterial flora and 2 ethnic groups exhibited *E. coli* as the most predominant one as shown in Table 2. The relationship between the numbers of isolates with the ethnic groups, however, is found to be non-significant with p-value 0.9991.

Gender wise distribution of skin microbiome

The bacterial flora isolated in skin of male was found slightly higher in ratio than that of female. 80 male samples exhibited 115 isolates and 86 female samples exhibited 116 isolates. The data observed, however, was statistically insignificant (p value = 0.9913). *S. aureus* was the most predominant isolates in both of the cases as shown in Table 3.

The number of isolates on the basis of altitude, however, was significant for both male and female samples separately with p-values 0.1632 and 0.2401 respectively. Kathmandu samples exhibited the highest number of isolates followed by Bharatpur and Lukla samples in both genders.

Coorelation of human skin microbiota with selected habits

The diversity of skin microbiome was found to be slightly higher in Non-alcoholic people with respect to Alcoholic.

Table 3: Skin Normal flora distribution on sex basis

S. No.	Skin Microbiome	Male (*n* = 80)	Female (*n* = 86)	Total
1	*S. aureus*	38	44	82
2	*Streptococcus* spp	20	21	41
3	*Micrococcus luteus*	4	5	9
4	*Bacillus* spp	4	1	5
5	*S. epidermidis*	1	2	3
6	*E. coli*	30	22	52
7	*K. oxytoca*	6	6	12
8	*Proteus mirabilis*	2	3	5
9	*Acinetobacter baumannii*	0	5	5
10	*Enterobacter aerogenes*	3	2	5
11	*Citrobacter freundii*	3	2	5
12	*Neisseria* spp	3	2	5
13	*Pseudomonas aeruginosa*	1	1	2
	Total	115	116	231

Table 4: Resistivity of Gram positive Organisms against various antibiotics

S. No.	Antibiotics	Total isolates tested	Resistant isolates	Percentage of resistance isolates
1	Amoxycillin	140	42	30.00
2	Cephalexin	140	43	30.71
3	Cefoxitin	140	29	20.71
4	Cefixime	140	14	10.00
5	Cefotaxime	140	17	12.14
6	Ceftriaxone	140	14	10.00
7	Co-trimoxazole	140	11	7.85
8	Erythromycin	140	21	15.00
9	Gentamycin	140	14	10.00
10	Ciprofloxacin	140	22	15.71
11	Ofloxacin	140	26	18.57
12	Norfloxacin	140	23	16.42
13	Cloxacillin	140	18	12.86
14	Imipenem	140	**7**	5.00

Samples from 79 non-alcoholic volunteers showed 117 isolates whereas 87 Alcoholic volunteers' samples showed 114 isolates. However, the difference was statistically insignificant (p value = 0.9480). *S. aureus* is the most predominant isolates in both alcoholic and non-alcoholic volunteers.

The diversity of Skin microbiome was found some higher in Smokers with respect to Non-Smokers, though statistically the data was insignificant (p value = 0.9858). *S. aureus* is the most predominant isolates in both Smokers and non-smokers.

ANTIBIOTIC SUSCEPTIBILITY PATTERN OF THE ISOLATES

Antibiotic susceptibility pattern of Gram positive isolates
Out of 14 common antibiotics tested against Gram positive isolates, Imipenem was the most sensitive antibiotic with sensitivity of 95.00 % (133 out of 140 isolates), followed by Cefixime and Ceftriaxone and Gentamycin with equal sensitivity of 90.00 % (126 out of 140 isolates). Likewise, the least sensitive antibiotic includes Cephalexin with resistivity of 30.71 % (43 out of 140) isolates, followed by Amoxycillin which exhibited resistivity of 30 % 42 out of 140 isolates. Detail information has been exhibited in Table 4.

Antibiotic susceptibility pattern of *S. aureus*
Out of 14 common antibiotics used against the most abundant skin bacterial normal flora, *S. aureus*, Imipenem and Co-trimoxazole were the most susceptible antibiotic with susceptibility of 91.46% (75 out of 82 isolates), followed by Ceftriaxone and Gentamycin with equal susceptibility of 86.58 % (71 out of 82 isolates). Likewise, the least susceptible antibiotic includes Amoxycillin with resistivity of 31.71 % (26 out of 82 isolates), followed by Cefotoxin which exhibited resistivity of 29.27 % (24 out of 82 isolates).

Antibiotic susceptibility pattern of Gram negative isolates
Out of 14 common antibiotics used against Gram negative isolates, Amikacin was the most susceptible antibiotic with susceptibility of 100 % (91 out of 91 isolates), followed by Nitrofuratoin and and Imipenem with equal susceptibility of 98.90 % (90 out of 91 isolates). Likewise, the least susceptible antibiotic includes Amoxycillin with resistivity of 28.57 % (26 out of 91 isolates), followed by Cefixime, Co-trimoxazole and Norfloxacin which exhibited resistivity of 18.68 % (17 out of 91 isolates).

Antibiotic susceptibility pattern of *E. coli*
Out of 14 common antibiotics used against the most abundant Gram negative isolates, *E. coli*, Amikacin was the most susceptible antibiotic with susceptibility of 100 % (52 out of 52 isolates), followed by Nitrofuratoin, Chloramphenicol and and Imipenem with equal susceptibility of 98.07 % (51 out of 52 isolates). Likewise, the least susceptible antibiotic includes Amoxycillin with resistivity of 32.69 % (17 out of 52 isolates), followed by Co-trimoxazole which exhibited resistivity of 23.07 % (12 out of 52 isolates).

DISCUSSION

Skin bacterial isolates have been found to be higher in Kathmandu with respect to Bharatpur and Lukla, which we conclude is due to altitude related factors. Skin bacteria significantly grew more comfortably in mild altitude rather than extreme ones. Kathmandu lies in the altitude of 1400 m (4600 feet), whereas Bharatpur and Lukla which lie at altitude of 208 meter (682 feet) and 2860 m (9380 feet) respectively above sea level. The barometric pressure, temperature, along with other several factors affects the growth of skin microbiome that grows in margin between the natural environment and human body.

The lowest skin microbiome isolates have been observed in Lukla, the highest altitude among three sites of the study. Zeng et al[14] also found the similar result. They found the people at high altitude Tibet plateau (3750–3861 m above sea level) had significantly less diverse skin microbiomes than at low altitude Sichuan basin area (319–1421 m above sea level).

Xing and Hahn[18] observed the similar result that the diversity of Bacterioplankton in four high-altitude Tibetan freshwater lakes was only one-fifth of the Bacterioplankton diversity in seven low-altitudes.

Several factors may influence bacterial diversity at high altitude; for example, the higher UVR intensity at high altitude may reduce bacterial community diversity. Other environmental factors such as temperature and oxygen concentration may also contribute to the altitude associated differences in skin microbiome diversity and structure; however, these factors are all correlated and it is difficult to explain their individual roles in shaping the skin microbiome.[14]

It is important to characterize the skin bacterial flora in mammals living at high altitude to determine whether the skin microbiome has adapted to the extreme environment and whether such adaptations aid in resistance to the development of skin diseases caused by extreme conditions such as UVR.

Eighty-six female samples exhibited 116 isolates whereas 80 male samples exhibited 115 isolates. This shows slightly higher ratio of isolates in male, though the difference is too small to be significant. The slight lower number of isolates in female may be due to application of some skin cosmetics that are antibacterial. Ying and Zeng et al,[19] however, concluded that hormone metabolism, perspiration rate and skin surface pH, can also account for gender wise differences of bacterial flora.

No significant relationship has been observed between the skin bacteria with smoking habit in our study though the isolates were slightly higher in smokers. Less study has been done to co-relate the skin microbiome with smoking habit. However, studies have been done for gut microbiome relating to smoking habits. Lee and Yun et al (2018)[20] observed significant difference of gut microbiome between never and current smokers. They found that current smokers had an increased proportion of the phylum Bacteroidetes with decreased Firmicutes and Proteobacteria compared with never smokers, whereas there were no differences between former and never smokers.

Biederman and Mwinyi et al (2013)[21] observed profound shifts in the gut microbial composition after smoking cessation with an increase of Firmicutes and Actinobacteria and a lower proportion of Bacteroidetes and Proteobacteria on the phylum level. In addition, after smoking cessation, there was an increase in microbial diversity.

Biedermann and Brulisauer et al (2014)[22] found that intestinal microbiota composition in humans is influenced by smoking. They concluded characteristics of observed microbial shifts suggest a potential mechanistic association to alterations in body weight subsequent to smoking cessation.

Eighty-seven alcoholic volunteers exhibited 114 isolates, whereas 79 non-alcoholic volunteers exhibited 117 isolates. This indicates the higher abundance of skin microbiome in non-alcoholic individuals than alcoholic one, though the relationship is not significant. Research has not been focused to relate skin Microbiome with alcohol drinking habits. Andrew and Frederico et al (2014)[23] found significant decrease in oral microbiomes in alcohol drinkers than in alcohol non-drinkers. There has been shift of microbial diversity on alcohol consumption.

Out of 14 common antibiotics used against Gram positive isolates, Imipenem was the most susceptible antibiotic with susceptibility of 95.00 % (133/140), and Cephalexin was the most resistant with resistivity of 30.71 % (43/140). The result was similar to the most predominant Gram positive skin flora, *S. aureus*. Similarly, out of 14 common antibiotics used against Gram negative isolates, Amikacin was the most susceptible antibiotic with susceptibility of 100 % (91/91), and Amoxycillin was the least effective antibiotic with resistivity of 28.57 % (26/91). The result was similar to the most predominant Gram negative skin flora, *E. coli*. Similar finding for the sensitivity and resistivity was reported by Shrestha D. et al.[24] 2016 and Oteo et al.(2001).[25]

CONCLUSION

The result indicates skin bacterial flora varies along the altitude the host human resides. These results lay the groundwork for further studies exploring the possibility of high-altitude adaptation of beneficial skin-associated microbial communities in humans.

Limitations
The research is based on plate culture technique and limited for only aerobic microorganism growing on NA, MA, BA and CA.

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