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Arsenic Poisoning Alters the Composition of Skin Microbial Flora of Human

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ABSTRACT

Arsenic toxicity causes immunosuppression and skin lesions in human beings. These two factors govern the composition of normal skin microbial flora. Therefore, the aim of the investigation was to determine, if there is any alteration of skin bacterial composition in arsenicosis patients compared to control healthy population. The microbial load and composition in skin swab samples, collected from three anatomical sites of 15 arsenicosis patients and 10 healthy control populations were analyzed by using culture techniques in nutrient agar and selective media. Finally, the organisms were identified by biochemical test and/or PCR methods. The results revealed that the total microbial count was much higher in arsenicosis patients over control populations. Composition of the bacterial isolates revealed that the Gram-negative bacterial load increased significantly in arsenicosis patients compared to control populations. In contrast, a decrease of Gram-positive bacterial load in arsenicosis patients was observed. Various genera of the Gram-negative bacteria- *Proteus* sp., *Enterobacter* sp., *Acinetobacter* sp., *Escherichia coli*, *Pseudomonas* sp., *Moraxella* sp., *Klebsiella* sp. and *Neisseria* sp. were isolated from arsenicosis and control individuals. The statistical calculation of the rate of occurrence of individual Gram-negative organism associated with the arsenicosis patients over control populations revealed that *E. coli* and *Pseudomonas* sp. ($|Z| = 2.16$) were the most predominant candidates. Other candidates like *Acinetobacter* sp., *Moraxella* sp., *Klebsiella* sp. and *Neisseria* sp. were found in arsenicosis patients but were absent in controls. The results clearly support our hypothesis and conclude that arsenic poisoning alters composition of skin microbial flora.

Key words: Arsenicosis, skin microbiota, Gram-negative bacteria, Gram-positive bacteria, bacterial composition

INTRODUCTION

Inorganic Arsenic (As) is a natural element of earth's crust, species of which are found throughout the environment. More than 100 million people worldwide have been estimated to be chronically exposed to arsenic through drinking water containing high levels of arsenic and food-chain arsenic (Chowdhury *et al.*, 2000; Alaerts *et al.*, 2001; Islam *et al.*, 2004a, b; Chowdhury *et al.*, 2006). Exploitation of groundwater from wells has resulted in mobilizing the arsenic and led to mass poisoning in the arsenic affected region which is defined by the generic term arsenicosis (Rahman *et al.*, 2001) and the process may take between 5 and 15 years to reveal clinical manifestations of arsenicosis (British Geological Survey, 1998; Mazumder *et al.*, 1998b).

ATSDR (1997) has ranked arsenic as the highest in the priority on a list of top 20 hazardous substances. Almost every physiological system in the human is adversely affected by prolonged exposure to high doses of arsenic (Gorby, 1994; Morton and Dunnette, 1994). According to epidemiologic studies and clinical observations, arsenic is associated with increased risk for certain types of human cancers including epidermoid carcinomas of skin, lung cancers and possibly liver cancers (Chen *et al.*, 1992; Yu *et al.*, 2006; Chen *et al.*, 2010).

There is strong evidence from epidemiological studies of an association between chronic exposure to inorganic arsenic and hyperpigmentation, hyperkeratosis and neoplasia in the skin as well as other diseases (Vega *et al.*, 2001; Dastgiri *et al.*, 2010). Hyperpigmentation and keratoses caused by arsenic are quite distinctive. Hyperpigmentation is marked by raindrop-shaped discolored spot, diffuse dark brown spots, or diffuse darkening of the skin on the limbs and trunk (Mazumder *et al.*, 1998b). Patchy hyperpigmentation, a pathologic hallmark of chronic exposure, may be found anywhere on the body but occurs particularly on the eyelids, temples, axilla, neck, nipples and groin (ATSDR, 1990). Simple keratosis usually appears as bilateral thickening of the palms and soles while in nodular keratosis, small protrusions appear on the palms and soles, with or without nodules on the dorsum of the hands, feet, or the legs (Mazumder *et al.*, 1998a).

The trivalent inorganic form of arsenic, arsenite [As(III)] and the pentavalent form, Arsenate [As(V)], are both contaminants of groundwater and foods but arsenite is the more toxic of the two (Joshi *et al.*, 2003; Mahata *et al.*, 2004; Spallholz *et al.*, 2004). Several studies have also demonstrated that arsenic is immunotoxic (ATSDR, 1999). Arsenic toxicity has effects on lymphocytes (Ostrosky-Wegman *et al.*, 1991) and interferes with the antigen-presenting function of splenic macrophages (Sikorski *et al.*, 1991). It is also able to alter the response of IgM and IgG antibody-forming cells to sheep erythrocytes and the proliferative response of lymphocytes to phytohemagglutinin (Savabieasfahani *et al.*, 1998; Gohar and Mohammadi, 2010).

Skin is the first line body defense of human and intact healthy skin is impermeable to many pathogenic as well as opportunistic microorganisms (Tortora *et al.*, 1998). The most superficial layer of the epidermis, the stratum corneum, is composed of flattened dead cells (corneocytes or squames) attached to each other to form a tough horny layer analogous to a wall of bricks (corneocytes) and mortar (lipids) and serves as the primary protective barrier (Larson, 1999). However, certain microorganisms still can grow, harbor and create the micro-environment on skin causing the formation of normal skin microbial flora. This flora is mostly Gram-positive in nature and behaves synergistically with human skin and antagonistically with Gram-negative pathogens (Brooks *et al.*, 2001). The bacterial flora of skin consists primarily of staphylococci, micrococci, corynebacteria and propionibacteria (Davis, 1996; Cogen *et al.*, 2008; Willey *et al.*, 2008). *Staphylococcus aureus* is widespread in nature and commonly found on human skin, skin glands and mucous membranes (Davis, 1996; Nagase *et al.*, 2002). The distribution of these skin bacteria in normal healthy individuals has been detailed previously (Willey *et al.*, 2008). However, traumatic injuries produce breaks in the skin that may lead to life-threatening infectious diseases. Persons with burns, surgical incisions, or other serious wounds are highly susceptible to infections. Wounds expose underlying tissues to dangerous pathogens. In addition, the reduced blood supply to the traumatized tissue often impairs the ability of protective immune and phagocytic cells to reach the affected area (McKane, 1996).

We hypothesized that various skin lesions and consequent disruption and immunopathogenicity due to chronic ingestion of inorganic arsenic will change the composition of the human skin normal flora. The present study was therefore undertaken to investigate the composition of normal flora

of skin in patients with arsenicosis and was compared with that of the healthy individuals and thereby attempt to find out any correlation of skin normal flora with arsenic poisoning.

MATERIALS AND METHODS

Subjects and clinical samples: To assess the effect of arsenic on the skin surface microbial population and composition, skin swab samples from 15 arsenicosis patients as subjects and 10 healthy individuals as control were randomly collected over a period of six months (May-October, 2006). Among these participating subjects, 12 subjects were male and 3 were female. The Subjects in this study comprised of 15 (N) individuals who were arsenicosis patients with characteristic histopathological changes in skin and had been exposed to arsenic contaminated water for at least 6-8 years (designated as Type A). Melanosis and keratosis characterized by reddish rash and pigmentation on skin and often in sever cases, skin rupture were apparent in these subjects and they had been picked from Skin and Dermatology out door unit of Bangabandhu Sheikh Mujib Medical University (BSMMU) Hospital (n = 7) as well as affected locality (Nowanagar and Nazirpur village, Sonmandi Union, Sonargon Upazila, Narayangonj, (n = 8) under the supervision of a clinical practitioner of Skin and Dermatology Unit of BSMMU. A control group comprising of healthy individuals (designated as Type B) was also included in this study. Sampling sites were carefully selected and samples were solely collected from sites where characteristic lesions were evident of arsenicosis. These sites included three anatomical sites: fore arm, finger web and upper chest.

Patients' history: All patients in this study were supplied with a questionnaire. Personal information with arsenic related medical history was obtained and documented from all participants of the study which included name, sex, age and occupation, treatment taken, safety measures adopted and participants' knowledge on arsenic transmission, prevention and medication.

Sampling procedure: Skin swab samples from both subjects and control group were initially taken from different skin surface sites by moistening the sterile swab into the nutrient broth containing 10% Tween 80 and skin micro flora were collected by rubbing the moist cotton swab for at least 8 times over the surfaces. Sterile screw capped test tubes containing nutrient broth with 10% Tween 80 were used to immerse and carry these swabs to the laboratory.

Quantitative and qualitative analyses: Following incubation for 4 hours in a shaker incubator (at 37°C, 120 rpm), quantitative bacterial analysis was done by 10-fold serial dilution using spread plate method on Trypticase soy agar containing 0.1% Tween 80. Cultural, morphological and biochemical characteristics of the bacterial culture were observed similarly using different selective, differential and biochemical media.

Statistical analysis: The mean bacterial population is calculated from duplicate cultures taken from right and left sides of the body from each site in each subject. Demographic variables such as gender and age were assessed. Age was categorized by decades into three groups, with the youngest one being under twenty one years and the oldest group over 50 years. Age group under 10 years is excluded from this study. To assess the significance of occurrence of isolates between the two groups of subjects by test hypothesis, following equation of hypothesis is followed:

$$Z = \frac{P_A - P_B}{\sqrt{P(1-P)(1/n_1 + 1/n_2)}}$$

Where:

- Z = Rate of significance
- n = Total number in a group (n₁ = in arsenicosis patient group, n₂ = in healthy control group)
- P_A = Occurrence per subject in arsenicosis group, P_A = a/total number
- P_B = Occurrence per subject in arsenicosis group, P_B = b/total number
- P = (a+b) / (1/n₁ + 1/n₂)

If |Z| >1.96, the result is assumed to be significant.

RESULTS

As arsenic toxicity impairs immune system and damage skin texture, it had been hypothesized that both of these factors governs skin microbial flora to change. Various types of skin lesions were found in these subjects due to drinking of arsenic polluted water for a prolonged period. Of the total 15 subjects, red rashes were found in 8 patients, pigmentation problem in 11 and skin rupture in 7. Detailed observations revealed presence of two or more of these symptoms in the patients at a time.

Total microbial load comparatively higher in arsenicosis patients: The pattern of microbial load is similar in both arsenicosis patients and control population, where the order of microbial load was: Fore arm > Finger web > Upper chest. The total microbial count was much higher in arsenicosis patients over control populations. In arsenicosis patients, the most heavily loaded microbial site was fore arm containing 7.05 log₁₀ cfu cm⁻², whereas microbial load of the upper chest and finger web were 3.55 and 5.2 log₁₀ cfu cm⁻², respectively. When compared to arsenicosis patients, microbial count was found significantly lower in the skin of control populations. The fold increase of microbial load in arsenic patients over control populations maintains the order: Finger web > Fore arm > Upper chest = 2.6 > 2.39 > 1.78 (Table 1).

Table 1: Total bacterial count

Anatomical sites	Type A (Arsenicosis patients)		Type B (Healthy subjects)		Ratio of type A: type B (A/B) (log ₁₀ cfu cm ⁻²)
	Range (log ₁₀ cfu cm ⁻²)*	Mean (log ₁₀ cfu cm ⁻²)	Range (log ₁₀ cfu cm ⁻²)	Mean (log ₁₀ cfu cm ⁻²)	
Fore arm	4.3-9.8	7.05	2.3-3.6	2.95	2.39
Upper chest	2.8-4.3	3.55	0.8-3.2	2.00	1.78
Finger web	3.5-6.9	5.20	1.5-2.5	2.00	2.60

*Total bacterial count has been assayed in terms of log₁₀ i.e., total bacterial count = log of (colony per plate × dilution factor/square centimeter); cfu: colony forming unit

Table 2: Occurrence of Gram-negative and Gram-positive isolates (n = 159)

Subjects	Gram (-) ve species		Gram (+) ve species			Total
	Gram(-) ve rods	Other than rods	<i>Staphylococcal</i> spp.	<i>Streptococcal</i> spp.	Other Gram (+) ve spp.	
Type A (n = 127)	50	7	27	16	27	70
Type B (n = 32)	10	0	8	5	9	22
Total (n = 159)	60	7	35	21	36	92

Gram-negative bacteria predominate over Gram-positive in arsenicosis patients: To verify whether there was any change of microbial composition of Gram-positive or negative microbes on the skin, we isolated a total of 159 of which 127 were isolated from 15 arsenicosis patients and that of 32 colonies from 10 control populations. Total 67 out of total 159 isolates were Gram-negative species and 92 were Gram-positive (Table 2). The fold increase of Gram-negative bacterial load on the arsenicosis patients was found 1.75 times higher over the increase of Gram-positive bacteria (Table 2).

Escherichia coli and Pseudomonas sp. predominate in arsenicosis patients: As the load of Gram-negative bacteria was found higher in arsenicosis patients over control, the frequently occurring Gram-negative microbes present in arsenicosis patients were identified. Various species and genera of the Gram-negative isolates were identified based on their culture characteristics and biochemical properties. The identified floras were *Proteus* sp., *Enterobacter* sp., *Acinetobacter* sp., *E. coli*, *Pseudomonas* sp., *Moraxella* sp., *Klebsiella* sp. and *Neisseria* sp. (Table 3).

A total of 57 Gram-negative isolates were recovered from arsenicosis patients' skin, of them 20 (35.08%) were associated with red rash skin, 20 (35.08%) with pigmented skin and 17 (29.84%) with ruptured skin. The significant proportion of occurrence of the individual organism associated with the arsenicosis patients over control populations was calculated using statistical method, the test of null hypothesis (if $|Z| > 1.96$, the results seem to bear the significant difference between two groups). The results were presented in Table 3 indicating that *E. coli* ($|Z| = 2.16$) and *Pseudomonas* sp. ($|Z| = 2.16$) were the most predominant Gram-negative bacteria in the arsenicosis patients compared to control populations.

Microbial species found on the skin surface of the arsenicosis patients and the control:

The normal skin represents a distinct ecosystem with characteristic micro flora. The resident micro flora of human skin comprises several bacterial genera, including staphylococci, micrococci, corynebacteria and propionibacteria (Davis, 1996; Cogen *et al.*, 2008; Willey *et al.*, 2008). Results in the previous section clearly indicate an alteration of the composition of commensal microflora. Therefore, we examined the composition of different Gram-positive and negative bacteria present on arsenicosis patients' skin. The results were presented in Table 4. Among all the Gram-positive

Table 3: Frequency of occurrence of Gram-negative isolates in subjects

	No. of patients	<i>Proteus</i> sp.	<i>Enterobacter</i> sp.	<i>Acinetobacter</i> sp.	<i>E. coli</i>	<i>Pseudomonas</i> sp.	<i>Moraxella</i> sp.	<i>Klebsiella</i> sp.	<i>Neisseria</i> sp.	Total isolates
Type A (patients) rash	8	5	3	3	2	2	3	1	1	20 (35.08%)
Pigment	11	4	3	4	2	3	2	2	-	20 (35.08%)
Skin rupture	4	2	4	2	4	3	1	1	-	17 (29.84%)
Total (n1)	15	11	10	9	8	8	6	4	1	
Type B	10	4	4	-	1	1	-	-	-	1
Control (n2)										
Z		1.65	1.31		2.16	2.16				

Arsenicosis patients were further sub-divided into three groups depending on their lesions, rash, pigmentation or skin-rupture which can be found in more than one participants. $|Z|$ is the significant rate of proportion between the two groups where $|Z| > 1.96$ indicates that the difference between two groups is significant

Table 4: Presumptive types of bacteria isolated (n = 159)

Organisms	Type A (Arsenicosis patients)	Type B (normal healthy subjects)	Total
	No. of isolates (%) n = 127	No. of isolates (%) n = 32	
Gram-positive members			
<i>Staphylococcus aureus</i> (β -hemolytic)	13* (10.24)	4 (12.5)	17
<i>Streptococcus</i> sp. (β -hemolytic)	10 (7.87)	4 (12.5)	14
<i>Staphylococcus epidermidis</i> (γ -hemolytic)	9 (7.08)	3 (4.38)	12
<i>Micrococcus</i> sp.	8 (6.30)	3 (4.38)	11
<i>Propionibacter</i> sp.	7 (5.51)	2 (6.25)	9
<i>Bacillus</i> sp.	6 (4.72)	2 (6.25)	8
<i>Lactobacillus</i> sp.	6 (4.72)	2 (6.25)	8
<i>Staphylococcus</i> sp. (β -hemolytic)	5 (6.30)	1 (3.13)	6
<i>Staphylococcus</i> sp. (α -hemolytic)	3 (2.36)	1 (3.13)	4
<i>Streptococcus</i> sp. (γ -hemolytic)	3 (2.36)	1 (3.13)	4
<i>Streptococcus</i> sp. (α -hemolytic)	3 (2.36)	-	3
Gram-negative members			
<i>Proteus</i> sp.	11(8.66)	4 (12.5)	15
<i>Enterobacter</i> sp.	10 (7.87)	4 (12.5)	14
<i>Acinetobacter</i> sp.	9 (7.08)	-	9
<i>E. coli</i>	8 (6.30)	1 (3.13)	9
<i>Pseudomonas</i> sp.	8 (6.30)	1 (3.13)	9
<i>Moraxella</i> sp.	6 (4.72)	-	6
<i>Klebsiella</i> sp.	4 (3.15)	-	4
<i>Neisseria</i> sp.	1 (0.79)	-	-
Total	127	32	159

*No. of isolates occurring per group, Value in parenthesis are percentages. -: Not present

staphylococcal species, 13 (10.24%) *Staphylococcus aureus* and 9 (7.08%) *Staphylococcus epidermidis* isolates were found, whereas these values were 4 (12.5%) and 3 (4.38%) in control group. β -haemolytic *Staphylococcus* sp. isolate count was 5 (6.30%) in arsenicosis patients but only 1 (3.13) in healthy persons. Alpha, beta and gamma streptococcal species were also observed in both arsenicosis patients and healthy persons. Among them a lower number of β -hemolytic *Streptococcus* sp. was found in arsenicosis patients (7.87%) than healthy persons (12.5%). Beside these staphylococcal and streptococcal species, other Gram-positive isolates were also found from the specimens of both groups. These were *Micrococcus* sp., *Propionibacter* sp., *Bacillus* sp. and *Lactobacillus* sp. Among Gram-negative bacteria, *Proteus* sp. was found to occur in higher number among both the groups (8.66% in arsenicosis patients and 12.50% in control). In contrast, fewer isolates of *Enterobacter* sp. was found (7.87%) in arsenicosis patients than in healthy persons (12.5%) (Table 4).

DISCUSSION

Arsenic is a metalloid and is recognized as one of the top 20 listed toxic substances. Its occurrence in nature through drinking water has made the greatest environmental devastation among all. The scale of this environmental disaster is greater than any seen before; it is beyond the accidents at Bhopal, India, in 1984 and Chernobyl, Ukraine, in 1986 (Smith *et al.*, 2000). Millions of people worldwide are exposed to drinking water containing naturally occurring arsenic. In this study we have made the unique observation that arsenicosis not only affect human by producing malignancies but also affect their normal microbial flora of the skin.

From present study, it is evident that microbial flora of human skin varies quantitatively and qualitatively due to chronic arsenic ingestion through drinking well water. An increased number of bacterial loads in skin sample in arsenicosis patients have been found as compared to the total bacterial count of skin microbial flora of normal healthy subjects. We have also found that the total number of Gram-negative bacteria is rationally increasing over Gram-positive ones in arsenicosis patients. It is interesting to note that some Gram-negative species were present only in arsenicosis patients but never in healthy subjects. Though some Gram-negative isolates e.g., *Proteus* sp., *Acinetobacter* sp., *Escherichia coli* and *Pseudomonas* sp. have been found from skin swab samples, their rate of occurrence have been found significantly higher in arsenicosis patients. The Gram-positive flora usually predominates in normal physical condition in skin though its composition may vary with environment. The Gram-negative bacteria we observed increasing significantly in arsenicosis patients are also present in normal skin (Davis, 1996; Cogen *et al.*, 2008). However, *E. coli* and *Pseudomonas* sp. isolated are the predominant members from the skin of arsenicosis patients. There are reports that some of the species of these two bacteria are As-resistant and can convert more toxic As (III) to As (V) by utilizing As-oxidase enzyme (Chang *et al.*, 2008; Cai *et al.*, 2009; Chauhan *et al.*, 2009; Salam *et al.*, 2009). Present isolated *E. coli* and *Pseudomonas* sp. from arsenicosis patients were found resistant to both As (III) and As (V) at a concentration of more than 20 mM and showed chemotaxis towards As (III) (data not shown) like bacterium *Herminiimonas arsenicoxydans* (Muller *et al.*, 2007). Hashem and Abed (2002) isolated similar bacterial populations as ours' from nails and hair of women who were contaminated with As and Pb. Their results corroborated present findings. Furthermore, poor arsenicosis patients with unhygienic living style are prone to these As-resistance bacteria and thus lead to their colonization. Furthermore, arsenic toxicities have the devastating immunosuppressive effects in human and as the chronic ingestion of arsenic causes various skin related abnormalities including malignancies (cancer), skin lesions and rupturing, it must alter or affect the composition of normal skin flora. All present findings strongly support this hypothesis. The apparent alteration of skin microflora and the predominance of opportunistic and potentially pathogenic Gram-negative species may be responsible for some of the clinical symptoms like gangrene as one of the late stage complications in arsenicosis patients.

In conclusion, the results described here clearly demonstrated that the total bacterial load was higher in arsenicosis patients compared to healthy populations and the normal bacterial flora on skin of arsenicosis patients predominantly shifted from Gram-positive to Gram-negative bacteria. The patho-physiological mechanism of this change needs further elucidation.

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