

Table

Colony count of *E. coli* in stool of healthy volunteers, arsenic exposed controls and arsenicosis patients both before and after probiotics supplementation

Group	n	Colony count of <i>E. coli</i> in stool ($\times 10^{11}$ CFU/g of stool)		p value ^a
		Before	After	
Healthy volunteers	9	11.1 \pm 1.4	12.1 \pm 0.8	0.027
Arsenic exposed controls	15	7.3 \pm 2.0	10.5 \pm 1.9	0.000
Arsenicosis patients	14	3.2 \pm 1.5	6.7 \pm 2.5	0.000

Data are presented as mean (\pm SD); Healthy volunteers vs. arsenic exposed controls (before supplementation): p= 0.000; Healthy volunteers vs. patients of (before supplementation): p= 0.000; ^acompared the data of before supplementation with 12 weeks after supplementation

Table

Arsenic level in stool of healthy volunteers, arsenic exposed controls and arsenicosis before and after probiotics supplementation

Group	n	Stool arsenic level (μ g/g)		p value ^a
		Before	After	
Healthy volunteers	9	0.9 \pm 0.4	1.1 \pm 0.5	0.176
Arsenic exposed controls	15	5.1 \pm 1.2	5.7 \pm 1.3	0.106
Arsenicosis patients	14	4.4 \pm 1.3	5.3 \pm 0.8	0.019

Data are presented as mean (\pm SD). Patients vs. arsenic exposed controls (before supplementation): p= 0.404; Healthy volunteers vs. patients (before supplementation): p= 0.000; ^acompared the data of before supplementation with 12 weeks after supplementation

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The colony count of *Escherichia coli* in the stool of palmar arsenical keratosis following probiotics supplementation

The colony count of *Escherichia coli* in the stool of palmar arsenical keratosis following probiotics supplementation

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Abstract

The role of probiotics supplementation for 12 weeks on the colony count of *Escherichia coli* in the stool of moderate palmar arsenical keratosis (14), arsenic exposed controls (15) and healthy volunteers (9) were studied. The mean colony counts of *E. coli* in the stool of arsenic exposed controls and patients were reduced to 7.3×10^{11} and 3.2×10^{11} CFU/g respectively in comparison to healthy volunteers (11.1×10^{11} CFU/g). After probiotics supplementation, the *E. coli* count was significantly increased in patients ($p=0.000$) and arsenic exposed controls ($p=0.000$) in comparison to healthy volunteers ($p=0.027$). Patients excreted less arsenic through stool and supplementation with probiotics showed increased excretion of arsenic only in patients. The level of arsenic in nail of patients was more than the arsenic exposed controls and healthy controls. Probiotics supplementation did not show any significant change. This study suggests that arsenicosis reduced the stool colony count of *E. coli*, less excretion of arsenic and probiotics supplementation reversed the processes.

Introduction

Bangladesh has already experienced the largest global catastrophe due to the presence of excessive amounts of arsenic in drinking water and food (Misbahuddin et al., 2011). The situation is deteriorating as the new cases of arsenic poisoning are still being reported in different parts of the country.

There is no satisfactory treatment regimen for arsenicosis. Some experts had recommended the use of spirulina (Misbahuddin et al., 2006), corn (Chowdhury et al., 2009), spinach (Umar, 2007), vitamin E (Ahmad et al., 1998), selenium (Nasir et al., 2002), *N. sativa* oil (Basher et al., 2014), garlic (Misbahuddin et al., 2013), zinc (Kamaluddin and Misbahuddin, 2006), α -lipoic acid (Tabassum, 2006), or other anti-oxidants enriched

with vitamins like A (Hall, 1946; Ahmad et al., 1998), C (Ahmad et al., 1998; Saha, 2006), and E (Ahmad et al., 1998), along with discontinuation of arsenic contaminated water in the treatment of arsenicosis. These require prolong duration of treatment and develop recurrence after discontinuation of drug.

We selected gut flora for the therapeutic purposes as bacteria living within the intestinal lumen are known to play an important role in host homeostasis.

Probiotics are viable bacteria given orally to improve health. As a result, they maintain the balance of gut flora. Studies show that symptoms of arsenicosis are rare in age group of less than 7 years (Chowdhury et al., 2000; Rahman et al., 2001). So, there may be a relation between lactobacilli and arsenicosis. So, if probiotics are



given to arsenicosis, there will be multiplication of good bacteria. Increased bacterial multiplication is associated with increased bacterial activity. So, in this study, the probiotics (containing *Lactobacilli bugaicus* and *Bifidobacterium*) was chosen as they are commercially available, cheap and easily tolerated by patients. There is now strong evidence for their use in treating and preventing some human diseases (Boyle et al., 2006). But no studies regarding colony count of *E. coli* or arsenic concentration in stool were carried out yet. Whether chronic exposure to arsenic causes any change to *E. coli* count or stool arsenic level in arsenicosis is unknown.

So, the aim of the study is to find out whether probiotics play any role in patients with chronic arsenical keratosis by estimating the colony count of *E. coli* in stool and by measuring arsenic concentration in stool and nail both before and after treatment.

Materials and Methods

Place and duration of the study

The study was conducted in Kamalla Union, Muradnagar Upazilla of Comilla District, about 110 km from Dhaka. According to a survey report conducted by UNICEF on November 2004, Muradnagar is an arsenic affected Upazilla where a large number of people have been consuming high concentration of arsenic through drinking water. The total area of Muradnagar Upazilla is 339 sq miles with total population of 5,77,971. There are total 440 arsenicosis and 30,199 tube wells, of which 93.5% hand pumped tube wells are contaminated with arsenic (>50 µg/L). Laboratory works were done at the Department of Pharmacology and Department of Microbiology & Immunology, Bangabandhu Sheikh Mujib Medical University. The duration of collecting the samples was from October 2011 to June 2012.

Participants

Total number of patients in the Upazilla Health Complex was 440 of which 105 were suffering from palmar keratosis. In Kamalla Union, there were 52 patients.

Inclusion criteria

Inclusion criteria includes a) both male and female participants, b) age range 28-62 years, c) arsenic exposure was based on the duration of intake of drinking water not from the exposure of ingestion of food materials. Subjects who fulfilled the following criteria was recruited as arsenicosis patients: a) who drank arsenic contaminated water (>50 µg/L) for more than 6 months and b) showing physical signs of moderate palmar keratosis. Subject who fulfilled the following criteria was recruited as arsenic exposed controls: a) who was the relative or family member of the patient, b) showing no physical signs of palmar keratosis, c) shared same tube well water for drinking and cooking purpose with the patient for more than 6 months, d) had same socio-economic background as patient. Subject who fulfilled the following criteria was recruited as normal non-exposed control: a) who drank arsenic safe water (<50 µg/L), b) lived in the same Upazilla, c) had no cutaneous manifestation and d) subjects who voluntarily agreed to participate.

Exclusion criteria

Exclusion criteria includes: a) patients having age <28 and >62 years, b) acutely ill patients with other major health problems, such as tuberculosis, asthma, hypertension. etc., c) patients getting treatment of arsenicosis, d) patients already enlisted as participants in other ongoing clinical trials, e) patients had other dermatological and infectious conditions of skin, such as eczema, psoriasis, contact dermatitis, etc., f) those who did not voluntarily agreed to participate and fulfill the above mentioned criteria.

Finally there were 15 patients, 15 arsenic exposed

Table I

Demographic data and selected characteristics of participants

Characteristics	Healthy volunteers	Arsenic exposed controls	Patients
Number (n)	9	15	14
Age (years)	44.9 ± 9.5	42.0 ± 11.6	46.4 ± 12.0
<i>Gender:</i>			
Male	5	9	8
Female	4	6	6
Duration of arsenic exposure (years)	-	11.6 ± 2.5	13.0 ± 3.3
Duration of symptoms (years)	-	-	6.6 ± 2.6
Amount of arsenic in water (µg/L)	11.7 ± 4.4	243.5 ± 52.0 ^a	243.5 ± 52.0 ^a

Data are presented as mean (± SD); ^apatients and exposed controls had same source of drinking water

controls and 10 healthy volunteers. Drop out cases were: one patient and one health volunteer. Finally 38 participants were involved.

Age of the study populations

The mean (\pm SD) age of patients was 46.4 ± 12.0 years, whereas it was 42.0 ± 11.6 years in arsenic exposed controls and 44.9 ± 9.5 years in healthy volunteers. That is, the mean age of three groups is more or less similar (Table I).

Duration of arsenic exposure and symptoms

The mean duration of arsenic exposure in patients and arsenic exposed control subjects were almost similar (13.0 ± 3.3 and 11.6 ± 2.5 years). The mean duration of symptoms of palmar keratosis in patients was 6.6 ± 2.6 years. All patients ($n = 14$) showed the manifestation of keratosis in both palms.

Levels of arsenic in drinking tube well water and nail sample

The mean amount of arsenic in the hand pumped tube well water of healthy volunteers, arsenic exposed controls and patients were 11.7 ± 4.4 ; 243.5 ± 52.0 and 243.5 ± 52.0 $\mu\text{g/L}$ respectively. Patients and arsenic exposed controls shared drinking water from same source.

Ethical considerations

A protocol containing the aims and objectives, research questions and procedures were submitted and approved by the Institutional Review Board of Bangabandhu Sheikh Mujib Medical University. All the participants were informed about the nature and purposes of the study and what outcome they could expect being a participant. To make sure that the subjects completely understood, a written consent was obtained from each person included in this study.

Study procedure

Detailed history, clinical examinations (mainly based on moderate palmar arsenical keratosis) were done and recorded on data collection sheet and result sheet. Laboratory investigation for arsenic concentration in water was done after preserving the each sample in a labeled plastic container. Probiotics capsules were supplied to each patient/day for 12 weeks without any interruption. Nail and stool samples were collected twice from each of the subject before and after taking probiotics capsules.

Periodic monitoring of patients

Each patient visited the Temporary Arsenic Clinic (a patient's house) at regular (2 weeks) and the improvement and adverse effects of the treatment regimen were noted down. In the mean time, communications were maintained with the participants by cell phone.

Collection of specimens

In this study, water, nail and stool specimens were collected from all participants and then transported to the Department of Pharmacology and stored in a refrigerator until analysis.

Collections of water specimens

About 50 mL of drinking water was obtained from each participant's tube well and stored in plastic containers with 1 drop HNO_3 and marked with ID numbers. Then the samples were transported to the laboratory and stored in a refrigerator until analysis.

Collection of nail specimens

The participants at first washed and dried their hands, nail samples were collected from all the fingers and were stored in a sterile dry container and they were then transported to the laboratory, stored in a refrigerator until time for analysis.

Collection of stool specimens

Stool was collected in sterile, properly labeled container as soon as it was passed by the participants and kept in refrigerator at -20°C until analysis. The specimen was brought to the laboratory with maintaining the temperature by ice box.

Distribution of drugs

Each patient received a plastic bag containing 15 capsules (drugs for 2 weeks). They were advised to swallow one capsule daily, with sufficient amount of drinking water (about 150 mL). Immediately after taking the drug, putting tick marks in the appropriate space printed on a separate sheet.

Parameters studied

The following parameters were studied: a) estimation of total amount of arsenic in drinking water and nail; b) estimation of total amount of arsenic in stool; c) estimation of colony count of *E. coli* in stool both before and after probiotics supplementation.

Methods of studying biochemical parameters

The total amount of arsenic in drinking water, nail and stool was estimated by SDDC method (AOAC, 1975). The estimation of colony count of *E. coli* in stool was done by the method described elsewhere (Buchanan et al., 1986; Choudhry et al., 2009).

Dilution and culture of stool

One gram of fecal matter was taken in sterile labeled test tube containing 1 mL of normal saline for dilution. Then the test tube containing fecal matter was thoroughly mixed by vortex and then centrifuged at $700 \times g$ for 10 min. Supernatant decanted into sterile labeled test tubes containing 1 mL of normal saline.

Table II

Colony count of *E. coli* in stool of healthy volunteers, arsenic exposed controls and arsenicosis patients both before and after probiotics supplementation

Group	n	Colony count of <i>E. coli</i> in stool ($\times 10^{11}$ CFU/g of stool)		p value ^a
		Before	After	
Healthy volunteers	9	11.1 \pm 1.4	12.1 \pm 0.8	0.027
Arsenic exposed controls	15	7.3 \pm 2.0	10.5 \pm 1.9	0.000
Arsenicosis patients	14	3.2 \pm 1.5	6.7 \pm 2.5	0.000

Data are presented as mean (\pm SD); Healthy volunteers vs. arsenic exposed controls (before supplementation): p= 0.000; Healthy volunteers vs. patients of (before supplementation): p= 0.000; ^acompared the data of before supplementation with 12 weeks after supplementation

Table III

Arsenic level in stool of healthy volunteers, arsenic exposed controls and arsenicosis before and after probiotics supplementation

Group	n	Stool arsenic level (μ g/g)		p value ^a
		Before	After	
Healthy volunteers	9	0.9 \pm 0.4	1.1 \pm 0.5	0.176
Arsenic exposed controls	15	5.1 \pm 1.2	5.7 \pm 1.3	0.106
Arsenicosis patients	14	4.4 \pm 1.3	5.3 \pm 0.8	0.019

Data are presented as mean (\pm SD). Patients vs. arsenic exposed controls (before supplementation): p= 0.404; Healthy volunteers vs. patients (before supplementation): p= 0.000. ^acompared the data of before supplementation with 12 weeks after supplementation

Table IV

Arsenic level in nail of healthy volunteers, arsenic exposed controls and arsenicosis patients both before and after probiotics supplementation

Group	n	Arsenic level in nail (μ g/g)		p value ^a
		Before	After	
Healthy volunteers	9	0.9 \pm 0.4	0.8 \pm 0.5	0.254
Arsenic exposed controls	15	4.1 \pm 1.1	3.8 \pm 1.0	0.234
Arsenicosis patients	14	4.8 \pm 0.9	4.5 \pm 1.0	0.224

Data are presented as mean (\pm SD); Patients vs. arsenic exposed controls (before supplementation): p= 0.189; Healthy volunteers vs. patients (before supplementation): p= 0.000; ^acompared the data of before supplementation with 12 weeks after supplementation

From here 1 mL of fluid was transferred serially to the test tubes containing 1 mL of normal saline. From the 7th test- tube 10 μ L of sample μ L was taken by sterile wire loop and inoculated in MacConkeys agar media and incubated at 37°C for 24 hours.

Then colonies of bacteria were counted as follows:

If there 350 colony is grown, the colony count is
 $= 350 \times 10^7/\text{mL} = 350 \times 10^9/\mu\text{L}$ or $3.5 \times 10^{11}/\mu\text{L}$

Statistical analysis

Statistical analyses of the results were performed using SPSS software version 17 for windows. Paired 't' test was done for comparisons among the same groups. One-way analysis of variance (ANOVA) followed by Bonferroni test was done for comparison among the different groups. P value <0.05 was considered as significant.

Results

The colony count of *E. coli* in stool of healthy volunteers was $11.1 \times 10^{11} \pm 1.4 \times 10^{11}$ CFU/g of stool (Table II). It was significantly (p=0.000) decreased in arsenic exposed controls ($7.3 \times 10^{11} \pm 2.0 \times 10^{11}$ CFU/g of stool) and patients ($3.2 \times 10^{11} \pm 1.5 \times 10^{11}$ CFU/g of stool). After probiotics supplementation for 12 weeks, colony counts of *E. coli* in stool of all the three groups were significantly increased.

The mean arsenic level in stool of patients was 4.4 ± 1.3 μ g/g, which was less than arsenic exposed controls (5.1 ± 1.2 μ g/g). The amount was very little in healthy volunteers (0.9 ± 0.4 μ g/g) in comparison with other two groups. That means, patients can excrete less arsenic through stool. Supplementation with probiotics showed significant increase in excretion of arsenic in the stool of patients (Table III).

The nail arsenic level of arsenicosis patients was $4.8 \pm 0.9 \mu\text{g/g}$, which was more than the arsenic exposed controls ($4.1 \pm 1.1 \mu\text{g/g}$) and healthy controls ($0.9 \pm 0.4 \mu\text{g/g}$). When the result was compared with different groups, the comparison between healthy volunteers and patients and healthy volunteers and arsenic exposed controls were highly significant. After 12 weeks supplementation of probiotics, there was no significant change (Table IV).

In this study, there was no significant side effect from the use of probiotics.

Discussion

The present study showed that arsenic exposure decreased the colony count of *E. coli* in stool. It was severely reduced in the stool of patients. In addition, excretion of arsenic in the stool of arsenic exposed control and patient was increased. However, patients showed less excretion than arsenic exposed controls. After 12 weeks supplementation with probiotics, both *E. coli* count and stool arsenic level were significantly increased in patients.

This was the first study regarding the count of *E. coli* in the stool of arsenicosis. There were only two studies showing the effect of arsenic on gut flora. Choudhry et al. (2009) conducted an *in vivo* study in rat where as Upreti et al. (2006) conducted an *in vitro* study on rat stool. Both studies showed inhibitory effect of arsenic on gut flora. There was a similarity with those two studies with the present study regarding the inhibitory effect of arsenic on *E. coli*.

When a person consumes arsenic through drinking water and food the concentration of arsenic in gut increases. Though, most of the bacteria in our gut are anaerobic, but in this study no attempt was made to isolate or identify the anaerobic bacteria due to lack of laboratory facilities, only *E. coli* was considered which is a facultative aerobe. Growth and multiplication of bacteria is an important parameter to study the response to toxic insults as it reflects directly to the viability of the bacterial population. The study conducted by Upreti et al. (2006) also revealed a concentration-dependent inhibition on *in vitro* exposure of gut flora to arsenic. Here this study showed that in chronic exposure to arsenic, the colony count of *E. coli* was decreased in arsenic exposed controls and severely decreased in arsenicosis patients in relation to healthy volunteers. As the study was carried out for a short period of time, the duration of inhibitory effect of arsenic could not be evaluated.

Studies carried out suggest that bacteria play a role in arsenic detoxification (Rowland and Davies, 1981). Microbes have been shown to reduce or detoxify a wide

range of toxic metals such as, chromium, mercury, cobalt, lead and arsenic by detoxification and elimination from the body (Upreti et al., 2004). Before consumption of probiotics stool arsenic level was less which might be due to inhibition of gut bacterial count by chronic arsenic exposure resulting in decreased bacterial detoxification of arsenic (Choudhry et al., 2009).

Though a few studies have been carried out to see the interactions between gut flora and arsenic (Upreti et al., 2006), still then researchers findings showed that hepatic methylation of inorganic arsenic produce MMA^{III} and DMA^{III} which are considered to be more toxic (Stylbo et al., 2002; Vahter, 2002; Gamble et al., 2005). On the other hand, gut flora produce MMA^V and DMA^V (Bently and Chasteen, 2002) which are relatively non-toxic and finally TMA^{III} in gaseous form (Qin et al., 2006) whose volatilization lowers arsenic concentration in the body. Moreover, some bacteria possess the ability to enzymatically oxidize arsenite to less toxic arsenate (Upreti et al., 2006).

Manipulation of the human intestinal flora offers potentially to improve health issue through a variety of mechanisms (Salminen et al., 1998). Probiotic bacteria are known to be promoters of the host body's defense mechanism by stabilizing the local microflora, triggering a humoral immune response and constructing a barrier against immunological disorder. Here in this study, it could be assumed that there was multiplication of probiotic bacteria in the digestive tract making an increase of beneficial bacteria in the gut and thus increased stool arsenic excretion.

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Conflict of Interest

Authors declare no conflict of interest

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