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Original Research Article

A report evaluating the effects of hot-spring water on growth of *Staphylococcus aureus*

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A B S T R A C T

Effects of hot spring water from Bakreswar and Taptapani were studied in vitro on growth related properties of two reference strains of *Staphylococcus aureus* with seasonal variations. Colony counts were found over 87 times lower in summer and winter; and 20 times lower in rainy season in case of water from Bakreswar than control. In case of Taptapani they were found to be 216 and 62, respectively. Human plasma coagulation efficiency with alkaline hot spring water had confirmed the coagulase activity for both the strains. The attachment property was found 31 times lower in experimental conditions than in control in summer and winter, but it became 24 times in rainy season. Immature biofilms were formed in plasma with/without hot spring water, but colony counts from those biofilms were significantly less ($P<0.001$). So it may be inferred that hot spring water exhibited bacteriostatic / bactericidal effects depending on the concentration and inhibitory effects on plasma coagulation and attachment of *S. aureus*. As microorganisms got sterilized, this result might totally represent the importance of some ions of hot-spring water for bactericidal activity in a higher pH condition, not an organic factor.

Keywords

Hot-spring water, *Staphylococcus aureus*, biofilms

Introduction

Staphylococcu aureus are gram positive cocci, catalase and coagulase positive and oxidase negative. Due to its structure and ability to produce a number of enzymes and toxins, it is uniquely suited to be a very common pathogenic strain for human beings. That pathogenicity is characterized by beta type haemolysis on blood agar, golden yellow pigment formation, production of coagulase, phosphatase and deoxyribonuclease, mannitol fermentation, gelatin liquefaction and tellurite reduction. It forms a wall of fibrin clot around the lesion and thick creamy pus is formed in staphylococcal infection site, among which cutaneous and deep infections, skin exfoliative diseases, etc., are common. For long time bathing in hot spring water was believed to be a remedy for skin infections. Staphylococcal colonization is an important co-relate of severity of atopic dermatitis – a common dermatosis.

The purpose of the present study is to evaluate and compare the *in vitro* effects of hot spring water, collected from two sources (situated at a long distance from each other) on *S. aureus* in terms of growth rate, coagulation of plasma, immature biofilm formation and the attachment property. So, the study can appraise the usefulness of hot spring water in the management of some infective dermatoses in a scientific manner, confirming the activity of a biocide, especially with antibacterial properties.

Materials and Methods

Hot spring water collection

Bakreswar

23^0 53' 0" North, 87^0 22' 0" East, Birbhum, West Bengal, India, Asia.

Taptapani :

19^0 30' 0" North, 84^0 24' 0" East, Ganjam, Orissa, India, Asia.

Hot spring water was collected directly from the source in sterilized glass bottles, then transported and stored in dark condition at room temperature.

Chemical analysis of sample water

Method- As described in APHA (1998).

Filtration of hot spring water

The stored water was filtered through nitrocellulose (Millipore) membrane filters having pore size of 0.25µ. These were routinely used to make the sample water bacteria-free before use in media, because the pore size is smaller than that of the smallest bacteria.

Bacterial strains

Two reference strains of *S. aureus* were used (NCTC 6571 and NCTC 8532). They both are of coagulase positive type, confirmed by slide agglutination test and tube agglutination test. Each strain was used to examine both samples of the hot spring water separately.

Bacterial suspension for inoculation

S. aureus strains were allowed to grow in tryptic soy broth at 37^oC for 24 hr without shaking. Following incubation, the bacterial cells were harvested by centrifugation at 6000 rpm for 10 min at 4^oC, then re-suspended in sterile saline solution and centrifuged as per above description. The washed bacteria were re-suspended in 1 ml of sterile normal saline in polypropylene micro centrifuge tubes. This was further diluted and compared with McFarland's standard to bring the standard inoculum to 2.5×10^3 cfu.

Colony numbers of *S. aureus*

The inoculums were spread as lawn culture in nutrient agar (NA) plates using the serial tenfold dilution method. Colony counts were made after aerobic incubation for 24 hrs at 37^oC.

Observation of plasma coagulation by *S. aureus*

Two *S. aureus* strains were allowed to grow in tryptic soy broth (TSB) with hot spring water according to the guidelines of micro-dilution method, in tissue culture plates. Growth was checked after aerobic incubation for 24 hrs at 37^oC.

Effect of hot spring water on the growth rate of *S. aureus*

Cell suspensions of two *S. aureus* strains were used to inoculate TSB either alone

(control) or supplemented with hot spring water (in controlled pH) in tissue culture plates. After incubation for 24 hrs at 37^{o}C, the colony numbers of the organism were counted.

Effect of hot spring water on immature biofilms

One milliliter aliquots of cell suspensions of *S. aureus* strains containing 2.5 × 10^3 cfu were inoculated into 2.5 ml of human plasma on 1.77 Cm^2 cover slips on tissue culture plates. After incubation for 24 hrs at 37^{o}C, membranous structures (immature biofilms) were formed on the cover slips. Then gentle wash with 1 ml sterile saline solution for five times was done. Immature biofilms were then placed in 2.5 ml of human plasma either alone (control) or supplemented with hot spring water. After incubation for 24 hrs at 37^{o}C, the *S. aureus* cells attached to each cover slip were washed gently with 1 ml sterile saline solution ones and sonicated at 60% power for 45 sec at 4^{o}C. The colonies of the organism stripped from the cover slips were counted as in 2.5. Plasma coagulation was observed simultaneously.

Inhibitory effect of hot spring water on attachment of *S. aureus*:

One milliliter of cell suspensions of *S. aureus* strains containing 2.5 × 10^3 cfu were inoculated into 2.5 ml of human plasma either alone (control) or supplemented with hot spring water on 1.77 cm^2 cover slips placed on tissue culture plates. After incubation for 24 hrs at 37^{o}C, the numbers of *S. aureus* cells attached to each cover slip were determined.

Sets of experiments

All the experiments from 2.5 to 2.9 were done in triplicate sets and the average value of the three was taken as the result.

Statistical test

Software Statistica.

Results and Discussion

Physical nature of the samples collected from hot springs of Taptapani and Bakreswar was shown in Table 1.

Colony No. of *S. aureus*

The control sample of 1 ml contained 2.5 × 10^3 cfu.

Effect of hot spring water on growth rate of *S. aureus*

Growth rate of *S. aureus* in control and hot spring water was measured in terms of colony count and found lower than control. Changes in different seasons were recorded and presented in Table 2 A, B, C. Results after adjustment of pH was given in Table 3.

***S. aureus* induced plasma coagulation**

The *S. aureus* strains showed detectable plasma coagulation in plasma with alkaline hot spring water after 24 hrs of incubation. The result was confirmed repeatedly in all sets of experimentation and no seasonal variation was found.

Effect of hot spring water on immature biofilm formation by *S. aureus*

These parameters were measured in terms of how many cells were in that film and were expressed by colony count and plasma coagulation.

The colony count of *S. aureus* in immature

biofilm was 50% lower than that of the control in media containing hot spring water from Bakreswar and 33 times lower in media containing that of Taptapani. No significant variation was found between summer and winter seasons (Table 4 A, B, C).

Effect of hot spring water on attachment by *S. aureus*

Hot spring water had shown inhibitory effect on attachment of *S. aureus* on glass surface. After 24 hrs of incubation, colony counts of attached *S. aureus* cells were found to be 77 and 210 times lower than that of control in media with water from Bakreswar and Taptapani, respectively. No significant seasonal variation was found.

There were a few reports from Japan, USA, Germany and Canada available in the world literature. But those all were with acidic water. Hence such comparative study using alkaline hot spring water had got no support from which our results may be tallied. A correlation between severity of atopic dermatitis / eczematous lesions and the density of the *Staphylococcus aureus* colonization has been demonstrated by Williams el al. (1990) and Akiyama *et al.* (1996). In 1997, Kubota *et al.*, reported that the skin symptoms of atopic dermatitis patients improved in 76% of cases through acidic hot spring water bathing and acidic hot spring water acted against *S. aureus* because this organism on the skin surface disappeared in 20 cases and decreased in 10 cases out of 42 cases after the balenotherapy. Kawabata *et al.* (1985) discussed a lot about the enzymatic properties of Staphylo-thrombin, which is an active molecular complex formed between staphylo-coagulase and human prothrombin. Their findings and inferences were very much helpful to build up the molecular basis of the enzymatic action of this study. According to them, the hot spring water showed strong bactericidal activity against *S. aureus in vitro*. In order to clarify the mode of action further, they showed that the bactericidal activity of hot spring water was expressed by manganese and iodide ions in acidic condition (pH 2.0–3.0). Silver (0.32%) and sucrose (70%) were shown to be effective in eliminating *S. aureus* in a biofilm by Akiyama *et al.* (1998b). They also put light on the effects of acetic acid and role of fibrinogen and fibrin on biofilm formation by *S. aureus* (Akiyama *et al.*, 1997b; 1999b). Calcium oxide and magnesium oxide had inhibited plasma coagulation by *S. aureus*, zinc oxide could tell upon its attachment property, as well as the role of povidone iodine on density of *S. aureus* and lesion severity in cases of atopic dermatitis – all were discussed by the same team from time to time (Akiyama *et al.*, 1997a, 1998a, 1999a). Inoue *et al.* (1999) gave an idea about the use of manganese and iodide ions for the treatment of acute atopic dermatitis caused by *S. aureus*.

The results of the present study may suggest that the type and amount of non-living components (as the sample water was rendered bacteria-free by autoclaving) were very important for the bactericidal activity on those very two bacterial strains. In summer and winter they showed better efficacy which may be attributed to dilution and decrease in pH of hot spring water by rain water. All the parameters tested had shown encouraging results confirming that among those two sources, alkaline hot spring water from Taptapani was more effective or had naturally got more powerful antibacterial activity. On the other hand, a decent scientific elucidation towards a future *in vivo* study about the therapeutic approach to prevent the colonization of *S. aureus* on human epidermal layer with the help of

clinical experts in a hospital was acquired. Actual findings lead to the query that is combination of Na^+ and SO_4^- effective for healing or restricting the growth. Therefore, the next question is whether Na^+ might build a favorable environment for the action of healing or itself acted as a healing agent, i.e., more Na^+ more positive result. This requires further investigation. It is common belief that SO_4^- is effective and act as healing agent. But in our study, effectiveness of the water of Bakreswar as healer was found less, though it contained more SO_4^-. This raises a question whether SO_4^- should be administered strictly in optimum dose for maximum effectiveness. Hence, finding out the right dose for administration of SO_4^- as a local application should be taken up for further investigation and is very much likely to open up new avenues in field of dermatology regarding effective use of SO_4^- .

Table.1 Physical nature of the samples from Taptapani (T) and Bakreswar (B)

Season	Summer		Rainy		Winter	
Source	T	B	T	B	T	B
Temperature at ^{0}C (When collected)	68–72	80–82	60–62	72–74	44–48	60–62
pH	8.6	7.9	7.9	7.2	8.2	7.6
Taste	Metallic					
Smell	Sulphureous					

Chemical analysis of sample water

In mg/L	Na^+	K^+	SO_4^-	Cl^-	PO_4^-
Taptapani	133.9	1.8	25	7.8	<0.01
Bakreswar	79.9	2.6	63	9.7	<0.01

Table.2 Colony count of *S. aureus* cells after 24 hrs of incubation in different seasons

A. Summer (Strain A- NCTC 6571, Strain B- NCTC 8532)

Water used	pH	Strain of *S. aureus*	Cfu (no. × 10^2/ml)		
			Set-1	Set-2	Set-3
Control	7.0	A	25	25	25
		B	25	25	25
Bakreswar	7.9	A	0.24	0.26	0.22
		B	0.27	0.27	0.25
Taptapani	8.6	A	0.14	0.15	0.13
		B	0.09	0.08	0.19

P<0.001

B. Winter (Strain A- NCTC 6571, Strain B- NCTC 8532)

Water used	pH	Strain of *S. aureus*	Cfu (no. × 10^2/ml)		
			Set-1	Set-2	Set-3
Control	7.0	A	25	25	25
		B	25	25	25
Bakreswar	7.9	A	0.3	0.31	0.29
		B	0.4	0.38	0.38
Taptapani	8.6	A	0.17	0.19	0.16
		B	0.1	0.11	0.09

C. Rainy (Strain A- NCTC 6571, Strain B- NCTC 8532)

Water used	pH	Strain of *S. aureus*	Cfu (no. × 10^2/ml)		
			Set-1	Set-2	Set-3
Control	7.0	A	25	25	25
		B	25	25	25
Bakreswar	7.9	A	1.2	1.3	1.44
		B	0.9	1.1	1.3
Taptapani	8.6	A	0.4	0.33	0.5
		B	0.4	0.45	0.4

Table.3 Colony count of *S. aureus* cells after 24 hrs of incubation after adjustment of pH (Strain A- NCTC 6571, Strain B- NCTC 8532)

Water used	pH	Strain of *S. aureus*	Cfu (no. × 10^2/ml)		
			Set-1	Set-2	Set-3
Control	7.0	A	25	25	25
		B	25	25	25
Bakreswar	7.0	A	16	16.6	16.1
		B	17.2	17	17.4
Taptapani	7.0	A	14.4	14.1	14.5
		B	14.8	14.6	15.1

No significant seasonal variation was detected.

Table.4 Effect of hot spring water on immature biofilm formation by *S. aureus* cells (in terms of colony count and plasma coagulation)

A. Summer (Strain A- NCTC 6571, Strain B- NCTC 8532)

Time	Media	pH	Cfu (no. × 10^2/ml)		Plasma Coagulase
			St.-A	St.-B	
Pre-incubation	Plasma	7.4	25	25	--Ve
Post incubation	Plasma + distilled water (Control)	7.0	22	22	+Ve
	Plasma + Bakreswar water	7.6	0.7	0.7	+Ve
	Plasma + Taptapani water	8.2	0.54	0.59	+Ve

B. Winter (Strain A- NCTC 6571, Strain B- NCTC 8532)

Time	Media	pH	Cfu (no. × 10^2/ml)		Plasma Coagulase
			St.-A	St.-B	
Pre-incubation	Plasma	7.4	25	25	--Ve
Post incubation	Plasma + distilled water (Control)	7.0	22	22	+Ve
	Plasma + Bakreswar water	7.6	0.76	0.72	+Ve
	Plasma + Taptapani water	8.2	0.51	0.58	+Ve

C. Rainy Season (Strain A- NCTC 6571, Strain B- NCTC 8532)

Time	Media	pH	Cfu (no. × 10^2/ml)		Plasma Coagulase
			St.-A	St.-B	
Pre-incubation	Plasma	7.4	25	25	--Ve
Post incubation	Plasma + distilled water (Control)	7.0	22	22	+Ve
	Plasma + Bakreswar water	7.6	1	1.1	+Ve
	Plasma + Taptapani water	8.2	0.8	0.82	+Ve

Table.5 Number of *S. aureus* cells attached on cover slips after incubation of 24 hrs (Strain A- NCTC 6571, Strain B- NCTC 8532)

Media	pH	Cfu (no. × 10^2/ml)	
		St.-A	St.-B
Plasma only (Control)	7.4	22	22
Plasma + Bakreswar water	7.6	0.3	0.27
Plasma + Taptapani water	8.2	0.1	0.11

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