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A Study on IFN-γ and IL-10 Gene Expression Changes in *Gallus gallus* Embryo Infected with *Staphylococcus aureus* and Its Possible Alteration by Bakreshwar Hot Spring Water

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Abstract | **Original Research Article**

***Introduction*:** Hot springs have been used for medicinal purpose since ancient times. Bathing in the hot springs is effective in treating skin infections, arthritis etc. Bakreshwar, located in Birbhum district of West Bengal is known for its ten hot springs of varying temperature (35°C-71°C). These hot springs have relatively high sulphur content and are believed to heal skin infections. ***Aim*:** To study whether Bakreshwar hot spring water can effectively act on *Gallus gallus* embryo infected with *Staphylococcus aureus.* ***Methodology*:** Fourteen days old embryonated eggs were inoculated with freshly prepared suspension of *Staphylococcus aureus*. The hot spring water, collected from Agnikund(65°C), was used to check whether it can cure the bacterial infection effectively. Following harvesting morbid anatomy study was done followed by allantoic fluid collection, RNA was extracted and cDNA was synthesized. This cDNA was then used to study the cytokine gene expression changes. ***Result*:** By comparing cytokine gene expression in control and curative sets, it was observed that there were improvements in general appearances of the embryos and IL-10 and IFN-γ expressions were enhanced in bacterial control set whereas in the curative set the gene expressions were much less. ***Conclusion*:** It is thus inferred that hot spring water has curing effect in *Staphylococcus aureus* infection and is also capable of normalizing the cytokine balance in such infection.

Keywords: Bakreshwar Hot Spring Water, Cytokines, *Staphylococcus Aureus, Gallus Gallus* Embryo.

INTRODUCTION

The search for non-conventional source of energy lead to the discovery of over 350 hot springs in India. Tattapani Hot Spring in Chhattisgarh, Manikaran in Himachal Pradesh etc. are some of the important geothermal vents in India. Besides these, Bakreshwar located in Birbhum district of West Bengal is one of the most promising geothermal areas in Eastern India [1]. Hot springs are formed due to volcanic or tectonic activities characterized by high temperature that is a natural habitat for abundant microbial communities mostly thermophiles [2. 3].

Bakreshwar, (Fig.1) located in the Birbhum district (Lat.23°52'N; Long.87°25'E), is a popular pilgrimage place known for its different hot springs characterized by similar chemical composition and varying temperature that ranges between 35°C – 71°C [4]. The water shows a moderately alkaline pH 8.8 and the chloride content varies between 30ppm to 100ppm [4]. These hot springs have relatively high sulphur content, and is believed to have therapeutic effects against skin infections, arthritis etc. Previous studies reported the presence of green pigment producing bacteria from the hot springs of Bakreshwar [4]. Cyanobacterial species were also reported in the hot springs [5].

Staphylococcus aureus is a Gram-positive bacterium that remain an important pathogen responsible for a wide array of serious infections. *S.aureus* has also been associated with severe skin infections such as atopic dermatitis (eczema) [6]. It is a leading cause of nosocomial infections.

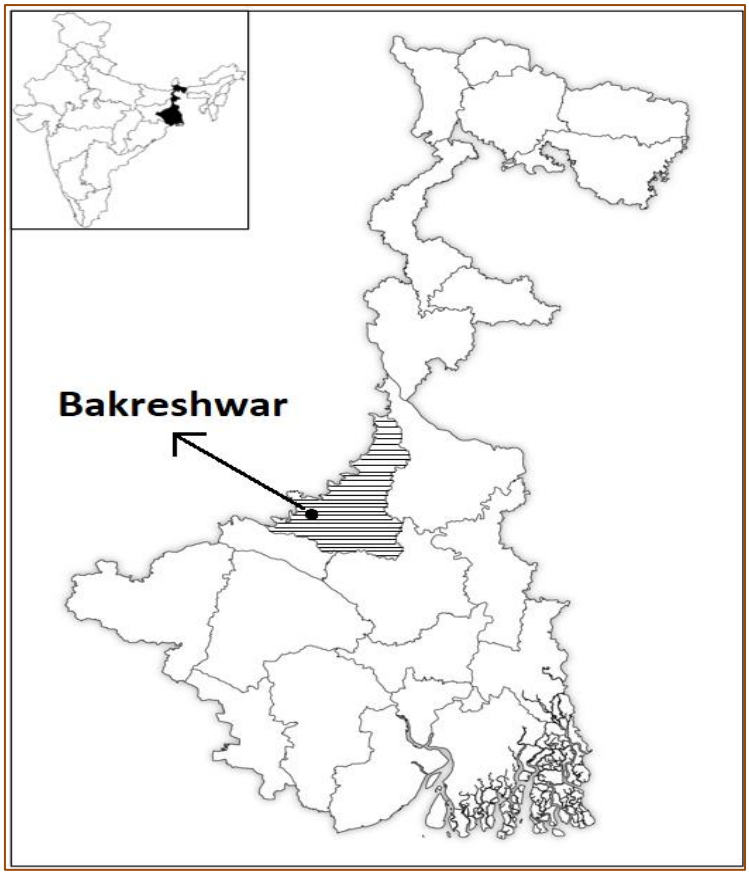

Fig.1: Location of hot spring of Bakreshwar in West Bengal

Staphylococcal infection is associated with significant morbidity and mortality rate [6]. An assessment from 2012 in USA has shown that *S. aureus* bacteria has an incidence rate that ranges between 20-50 cases per 10,00,000 population per year and around 10% to 30% of these patients die out of Staphylococcal infection [7]. According to a recent survey from 2017, the annual number of deaths due to *S. aureus* in USA was reported to be 20,000 [8]. Strains of *S. aureus* that are resistant to β-lactam antibiotics for example methicillin-resistant *S. aureus* (MRSA) are most important clinically [9]. While hospital-acquired MRSA infections are declining in certain countries like USA, China, Europe many other countries, yet there are some poorly developed countries for example some African countries, where these kinds of infections are on the rise [8]. In Scandinavian countries, the annual incidence of MRSA infection is approximately 26/1,00,000 population [10].

Previous report has shown the inhibitory effect of hot spring water on *S. aureus.* Kubota *et al.,* (1997) reported that skin infection of atopic dermatitis patient improved in 76% cases through bathing in hot spring water. They also revealed this organism on skin surface disappeared in 20 cases out of 42. These findings showed strong bactericidal activity of hot spring against *S. aureus.*[11]

The purpose of our present study is to evaluate and compare the in-vitro therapeutic effects of Bakreshwar Hot spring water collected from Agnikund on embryonated chick egg after challenging with *Staphylococcus aureus* (ATCC 29213) and also study the cytokine gene expression. Thus, this study can assess the importance of Bakreshwar water in the management of common pathogenic strains.

MATERIALS AND METHODS

Collection of water sample:

Water sample was collected from the hot spring of Bakreshwar, West Bengal during June 2023 in sterile plastic container. The temperature of water was around 65°C.

Bacterial strain:

Staphylococcus aureus ATCC 29213 was collected for research work from Peerless Hospitex Hospital and Research Centre Limited, Kolkata, India.

Procurement of eggs:

Fourteenth day old embryonated chick eggs (*Gallus gallus domesticus*) were procured from The State Poultry Farm, Tollygunge, Kolkata in a thermocol insulating box to maintain 38°C temperature. The eggs were properly cleaned with distilled water. After cleaning, the eggs were candled to determine the live and dead status and the air sacs were marked. Finally, the eggs were incubated at 38°C maintaining 60-80% humidity [12].

Four different experimental sets were prepared where each of the sets consists of three eggs. The different experimental sets include- a) 1st control set that includes only the 14th day old embryonated eggs of *Gallus gallus domesticus.* b) 2nd therapeutic water set includes of embryonated eggs inoculated with Bakreshwar water. c) 3rd set of eggs were inoculated with freshly prepared bacterial suspension. d) 4th set of eggs comprises of the curative sets where the eggs were first inoculated with bacterial suspension and then challenged with Bakreshwar water one hour after inoculation.

Inoculation of the eggs

Preceding inoculation, the eggs were thoroughly cleaned and disinfected using 70% ethyl alcohol followed by iodine and then re-wiped with 70% ethyl alcohol. The inoculation site in the air sac region was punctured using a sterile needle. Freshly grown bacterial suspension was prepared maintaining 0.5 McFarland standard. 10microlitre of bacterial suspension was inoculated in the 3rd and 4th set of eggs.

The fourth set was the curative set, so after one hour of inoculation they were treated with 100 microliters of Bakreshwar water. Finally, all the sets were incubated for a period of 5hours at 37^{O}C.

Harvesting of the eggs and Allantoic Fluid Collection

According the protocol of Ethical Committee, prior to harvesting, the eggs were kept at 4°C for 1 hour for dissection. Following inoculation, harvesting was carried out using sterile scissors and scalpel. After study of the external appearance of the embryo, the allantoic fluid was collected with a 5ml sterile syringe in sterile Falcon tubes. This fluid was stored at -80°C for further analysis.

RNA Extraction and cDNA synthesis

Total RNA extraction from the allantoic fluid was carried out following the manufacturer's protocol (Takara, USA) using RNA isoplus. Quantification of the total RNA yield was done using Ultraviolet-vis spectrophotometer (Agilent, Singapore) by measuring the absorbance ratio at 260nm by 280nm. This total RNA was then converted to cDNA using cDNA reverse transcriptase synthesis kit (Biorad, USA) in conventional PCR (T100, Biorad, USA conventional PCR) [12,13].

The cDNA thus synthesized were used to examine the gene expression of the cytokines IL-10 and IFN-γ using Real Time Polymerase Chain Reaction (Biorad, CFX-96, instrument, USA) against the housekeeping gene β-actin (Chatterjee *et.al*.,2022). The gene expression quantification was based on the formula 2^-(ΔCt_1-ΔCt_2) where Ct denotes Cycle threshold.

ΔCt_1 = Target gene – Housekeeping gene

ΔCt_2 = Test control – Housekeeping control

RESULT

The cytokine gene expression was studied in different experimental sets. The most important finding in this experiment was elevated expression of IL-10 and IFN-γ in the bacterial control sets that were inoculated with bacterial suspension. The IL-10 increased to 1423.14 and the IFN-γ increased to 39.649 times as compared to the control set. On the other hand, in the curative sets that were treated with Bakreshwar water, it was found that IL-10 and IFN-γ cytokine gene expression markedly decreased to 1.0006 and 3.462 respectively. This proves that the Bakreshwar water have therapeutic activity due to which the cytokine gene expression has decreased to almost normal level. The expressions of cytokine genes are represented in bar diagrams below:

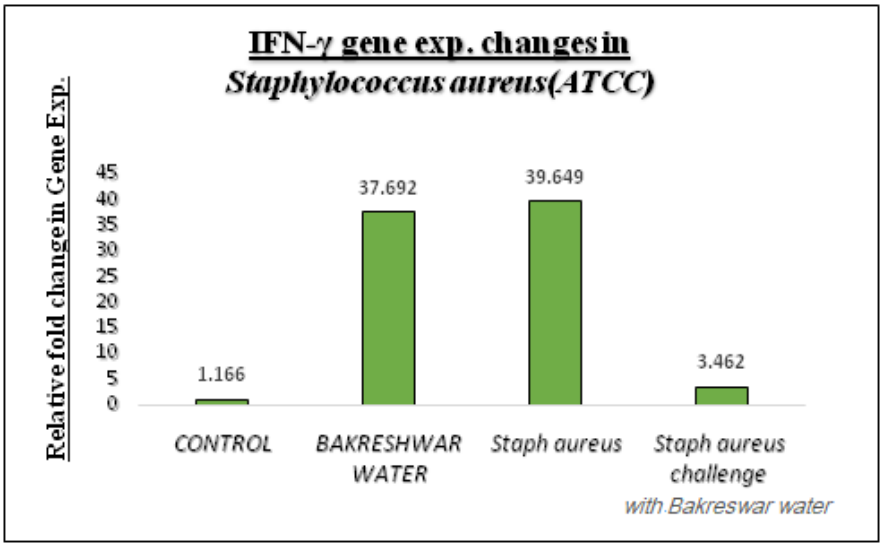

Fig. 2: Showing IFNγ- expression changes in different experimental set

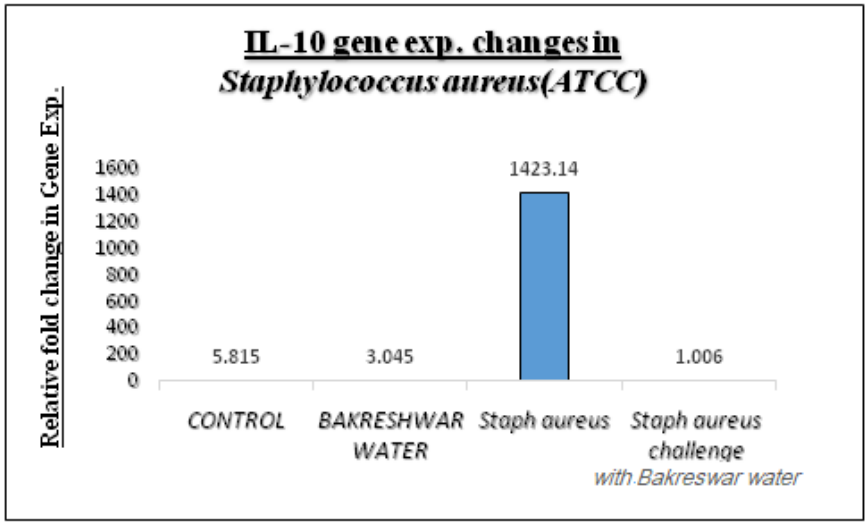

Fig. 3: Showing Il-10 expression changes in different experimental sets

The gross anatomy of the embryo after harvesting of the egg was observed in different experimental sets. In the bacterial control sets, necrosis and putrefaction was observed in the embryo after harvesting. Haemolysis was observed in the control set. Whereas in the curative sets, haemolysis was comparatively less and no putrefaction was observed. The curative sets showed healthy growth of the embryo.

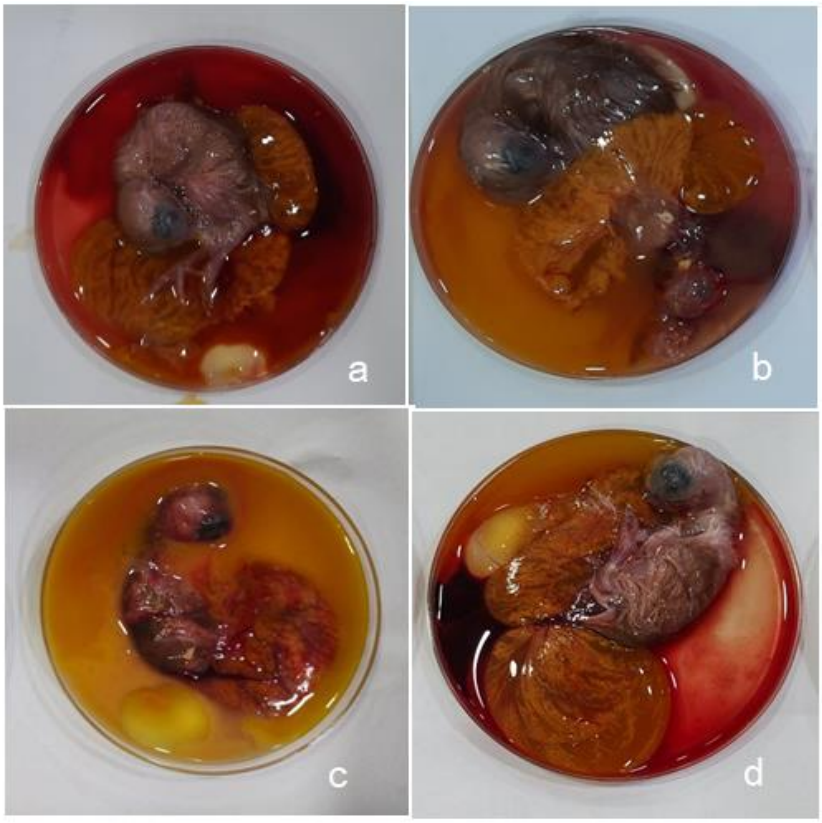

Fig. 4: Morbid anatomy of the chick embryo in different sets, a) Control: healthy and well developed embryo b) Control with Bakreswar water: No putrefaction observed c) Bacterial control set: Embryo necrosed and putrified d) Curative set: Healthy and moderately developed embryo.

DISCUSSION

Many pro-inflammatory and anti-inflammatory cytokines such as IL-7 , IL-10, IFN-γ etc. are expressed during *Staphylococcus aureus* infection that decreases inflammation in the damaged tissues. But in this study, we are mainly targeting IL-10 and IFN-γ as these two cytokines are expressed during 4hours of infection while others take longer time to express. IL-10 is a potent anti-

inflammatory mediator that plays an important role in host immune response. IL-10 is specifically produced by CD4+ T cells. IL-10 mainly lower the inflammation and prevent tissue damage. Previously it has been reported that *S. aureus* profoundly activates $FOXP3^{+}$ CD4+ T cell that was able to produce IL-10 and IFN-γ during infection [14]. IFN-γ also acts as defense mechanism against any kind of bacterial infection [15]. But this IFN-γ that is increased during staphylococcal infection may have adverse effect [16].

S. aureus produces four hemolysins – α, β, γ, δ among which β-hemolysin encoded by Hlb gene is one of the major hemolysins that stimulate the production of IFN-γ by $CD56^{bright}$ NK cells [17]. Sasaki *et al.*, in 2000 observed that mice that was deficient in IFN-γ during *S. aureus* infection have higher survival rates than the wild type ones [18]. Though in most of the cases this Hlb gene is not expressed due to integration of bacteriophage in the structural Hlb gene, yet in some cases, precise excision of bacteriophage lead to the re-induction of Hlb gene that is the main cause of pathogenesis [17].

In the present study, we have observed that when the chick embryo was infected with *S. aureus*, Il-IL-10 and IFN-γ gene expression increased 1423.14 folds and 39.64 folds as compared to control set. In the curative set, when the chick embryo was treated with hot spring water after infection, IL-10 and IFN-γ decreased significantly. Bakreshwar water control is used to observe any preventive and curative changes of IL-10 and IFN-γ gene expression after challenge with *Staphylococcus aureus* ATCC 29213. IL-10 gene expression decreases to 1.006 while IFN-γ gene expression to 3.462. This decrease of IL-10 and IFN-γ after challenging with Bakreswar water is due to decreased pathogenicity in the embryo.

CONCLUSION

From this study, we can thus conclude that hot spring water collected from Bakreshwar can effectively act on *Staphylococcus aureus* infections and maintain the cytokine balance.

Acknowledgement

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Authors Contribution

SD carried out the experiment, collected all the data and wrote the manuscript. DC and BS did experimental analysis. KP was involved in correcting the manuscript. Satadal Das conceived and designed the whole study, and also helped in data interpretation.

Conflict of Interest

The authors declare that the research was conducted in absence of any conflict of interest.

Source of Fund: There is no source of fund.

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