

Original Research Article

A Study on IFN- γ and IL-10 Gene Expression Changes in *Gallus Gallus Domesticus* Embryo Infected with *Klebsiella Pneumoniae* and its Possible Alteration by Bakreshwar Hot Spring Water

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Abstract: **Background:** Hot spring's water is enriched with minerals which has many therapeutic values. Bakreshwar is well known for its ten hot springs of varying temperatures (35°C-71°C). Bathing repeatedly in hot springs can help us to fight against skin infection, rheumatic disorders, joint pain etc. **Objectives:** The present study aimed to explore the changes in the gene expression of IL-10 and IFN- γ in the *Gallus gallus domesticus* embryo, infected with multi-drug-resistant *Klebsiella pneumoniae* and by using the Bakreshwar hot spring water to make any curative changes in the target gene expression or not. **Methodology:** 14 days old embryonated eggs of *Gallus gallus domesticus* was inoculated with freshly prepared 0.5 Mc Farland standard suspension of *Klebsiella pneumoniae* and curative set was treated with Bakreshwar Hot Spring water, collected from Agnikund (65°C). Then the embryonated eggs were harvested following the Institutional Ethical Committee guidelines and the allantoic fluid was collected. On the next day, RNA extraction was performed and cDNA synthesis followed by RT PCR was performed to study the cytokine gene expression. **Results:** After infection of chick embryo *Gallus gallus domesticus* with multi drug resistant *Klebsiella pneumoniae*, both IL-10 and IFN- γ gene expression was increased, but in curative sets that were challenged with Bakreshwar water, IL-10 gene expression was decreased and IFN- γ was increased. **Conclusion:** Hot spring water of Bakreshwar has antimicrobial effect on multi drug resistant *Klebsiella pneumoniae* and it can normalize the cytokine balance with prolonged protection with raised IFN- γ .

Keywords: *Klebsiella pneumoniae*, *Gallus gallus domesticus*, Bakreshwar Hot Spring water, cytokines, allantoic fluid.

INTRODUCTION

Hot springs are defined as a naturally occurring spring produced by heated groundwater from the earth's crust [1]. It is also known as Geothermal spring. Hot springs are the natural habitat for the thermophiles.

Nowadays, water therapy is used in many countries that have a variety of mineral springs considerably different in their hydrogeological origin, temperature, and chemical composition to treat different kind of disease like skin infections, stomach pain, arthritis, joint pain etc.

Bakreshwar is a popular tourist destination which is located (Lat. 23° 52' 48" N; Long. 87° 22' 40" E) in Dubrajpur, Suri Sadar subdivision of Birbhum district, West Bengal, India [2].

Bakreshwar is renowned for its ten hot springs (Table 1) which has various temperatures (35°C - 71°C).

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Table 1: Ten Bakreshwar Hot springs (Kund) are listed in this Table [3]

AREA	TEMPERATURE
Agnikund	65-66.5°C
Soubhagyakund	45- 47.5°C
Kharkund	58- 66°C
Amrita kund	---
Suryakund	55-57°C,
Brahmakund	43-45°C
Swet gangn	35°C
Paphara gangn	---
Bhairabkund	55-59°C.
Baitarini ganga	---

Some Characteristics Feature of Thermal Water at Bakreshwar Hot Springs:

The thermal water of all the Hot springs is alkaline with a pH between 7.4 and 9.2; Bakreshwar water shows profuse gaseous activity (Table 2); chloride content in hot springs in Bakreshwar ranges from 30 ppm to 100 ppm, SO₄ content is low <10 ppm; Thermal water from Bakreshwar contains high Na ranging from 30-100 ppm, low K < 4.8 ppm and low Ca and Mg, moderate TDS and silica ranging from 60 to 82 ppm; Fluorine content is high 9 to 12 ppm and needs caution before supply for direct uses [3].

Table 2: Compositions of hot spring gases from Bakreshwar: [4]

GAS	BAKRESHWAR (VOL%)
Nitrogen	92.20
Helium	1.37
Argon	2.10
Oxygen	0.90
Methane	3.43

Klebsiella pneumoniae is a virulent bacterium belonged to Enterobacteriaceae family. Morphologically, it can be described as a gram-negative, non-motile, rod-shaped, encapsulated bacteria [5].

In the year of 1882, *Klebsiella pneumoniae* was first described by Carl Friedlander as an encapsulated bacillus after isolating the bacterium from the lungs of those who had died from pneumonia. Originally named Friedlander's bacillus, it was not until 1886 when the bacterium garnered the name *Klebsiella*. *Klebsiella pneumoniae* is referred as a major bacterial pathogen responsible for hospital outbreaks worldwide. Approximately 11.8% of all hospital-acquired pneumonia in the world, is caused by *Klebsiella pneumoniae*, the organism accounts for 3% to 8% of all nosocomial bacterial infections [6, 7]. *Klebsiella pneumoniae* is an important causative agent of nosocomial infection, urinary tract infections (UTI) and septicemia, which can be life-threatening [8].

The polysaccharide capsule, which covers the entire bacterial surface, generally considered as a most important virulence factor in *Klebsiella pneumoniae*. Recent studies have shown that expression of capsule impedes the ability of *Klebsiella pneumoniae* to adhere to and invade epithelial cells in vitro [8]. Virulence of the bacterium provides a wide array of factors that can lead to infection and antibiotic resistance. The polysaccharide capsule of the organism is allowed the bacteria to evade opsono-phagocytosis and serum killing by the host organism. To date, 77 different capsular types have been studied, and those *Klebsiella* species without a capsule tend to be less virulent [9]. Polysaccharide capsules have been shown to enhance bacterial survival in the gut by multiple mechanisms [10].

The aim of our present study is to evaluate and compare the in-vitro therapeutic effects of Bakreshwar Hot spring water collected from Agnikund (65°C) on embryonated chick egg after challenging with multi drug resistant *Klebsiella pneumoniae* and also study the cytokine gene expression of IL-10 and IFN- γ .

MATERIAL AND METHODS

Study Procedure:

- 1. Collection of water sample:** Water sample was collected from Bakreshwar (Lat. 23° 52' 48" N; Long. 87° 22' 40" E) hot spring (Agnikund-65°C to 66°C) in Birbhum, West Bengal during month of March, 2023 in a 50ml sterile plastic container.
- 2. Collection of eggs of *Gallus gallus domesticus*:** The 14th day fertilised chick eggs were purchased from The State poultry farm, Tollygaunge, Kolkata. The eggs were carried in a thermocol insulating box to maintain the temperature at 38°C.

3. **Bacterial strain used:** Multi drug resistant *Klebsiella pneumoniae* (MDR-KP) was collected from the Microbiology department of Peerless Hospitex Hospital and Research Centre Limited.

The antibiogram (Table 3) of the isolated *Klebsiella pneumoniae* (MDR-KP) is mentioned below:

Table 3: Antibiogram of isolated *Klebsiella pneumoniae*

Susceptibility information	Analysis time: 9.45 hours				Status: final
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Amoxicillin/ Clavulanic acid	>=32	R	Meropenem	>=16	R
Piperacillin/ Tazobactam	>=128	R	Amikacin	>=32	R
Cefuroxime	>=64	R	Gentamicin	>=16	R
Cefuroxime axetil	>=64	R	Ciprofloxacin	>=4	R
+Cefixime		R	+Levofloxacin		R
Ceftriaxone	>=64	R	Tigecycline	4	R
Cefoperazone/ Sulbactam	>=64	R	Fosfomycin	128	R
Cefepime	>=32	R	Colistin	>=0.5	R
+Doripenem		R	+Polymixin B		R
Ertapenem	>=8	R	Trimethoprim/ Sulfamethoxazol	>=320	R
Imipenem	>=16	R			R
AES Findings					
Confidence	Consistent				

Different experimental sets were prepared where each set contains with three chick eggs:

- **Control Set:** control set that contained only the 14th day old embryonated eggs of *Gallus gallus domesticus*.
 - **Therapeutic control water set:** Therapeutic water set where embryonated eggs were inoculated with Bakreshwar water.
 - Third set of eggs were inoculated with freshly prepared multi drug resistant *Klebsiella pneumoniae* (MDR-KP) bacterium suspension.
 - **Curative set:** At first, these sets of eggs were inoculated with multi -drug -resistant *Klebsiella pneumoniae* (MDR-KP) suspension and after one hour of inoculation, challenged with Bakreshwar water to check whether this Hot spring water can make any curative changes in the target gene expression or not.
4. **Cleaning & Candling of eggs:** Firstly, eggs were cleaned thoroughly with distilled water using cotton. Then, Candling was performed to differentiate between live and dead eggs with the help of a torch.
5. **Inoculation of the eggs:** Before inoculation, the marked air sac areas of eggs were thoroughly cleaned and disinfected using 70% ethyl alcohol followed by iodine. A hole was puncture at the canter of the air sacs was formed using a sterile needle.
- 1 Control set marked as 'without water 'were kept as it was.
 - 2 Control set marked as 'with water 'inoculated with 100 microliters of Bakreshwar water using a sterile 1ml syringe through the punctured hole into the egg.
 - 3 3rd set of eggs were inoculated with 10 µl 0.5McFerland standard multi-drug resistant *Klebsiella pneumoniae* (MDR-KP) suspension.
 - 4 Curative sets were inoculated with 10 µl 0.5McFerland standard bacterial (multi-drug-resistant *Klebsiella pneumoniae*) suspension and after 1 hour incubation again inoculated with 100µl of Bakreshwar water using a sterile 1ml syringe through the punctured hole into the egg.
6. **Incubation periods:** Eggs were incubated in the incubator at 37°C for five hours. According the protocol of Ethical Committee, prior to harvesting, the eggs were kept at 4°C for 1 hour for dissection. After 6 hours, the eggs were brought out from the fridge.
7. **Harvesting of the eggs and Allantoic Fluid Collection:** Following inoculation, the embryonated eggs were harvested in the 17th day with sterile scissors and scalpel and the allantoic fluid was collected from each egg by dissecting the Chorio-Allantoic membrane and kept the fluid in falcon tube with sterile 5ml syringes. This fluid was stored at -80°C for further analysis.
8. **RNA Extraction:** Total RNA was extracted using RNA isoplus and the whole extraction was carried out following the manufacturer's protocol (Takara, USA). Quantification of the total RNA yield was done using Ultraviolet-Vis spectrophotometer (Agilent, Singapore) by measuring the absorbance ratio at 260nm by 280nm.
9. **cDNA synthesis:** This total RNA was then converted to cDNA using cDNA reverse transcriptase synthesis kit (Biorad, USA) in conventional PCR (T100, Biorad, USA conventional PCR).

10. RT-PCR Study: After synthesizing cDNA, to examine the gene expression of the cytokines IL-10 and IFN- γ using Real Time Polymerase Chain Reaction (Biorad, CFX-96, instrument, USA) [12] against the house keeping gene β -actin. The gene expression quantification was based on the formula $2^{-(\Delta Ct1-\Delta Ct2)}$ where Ct denotes Cycle threshold.

RESULTS

The cytokine gene expression was studied in different experiment sets

After infection of chick embryo *Gallus gallus domesticus* with *Klebsiella pneumoniae* (MDR) IFN- γ gene expression was mildly increased to 7.86 times. After challenge with Bakreshwar water, IFN- γ gene expression was remarkably increased up to 1756.55 times. IL-10 gene expression was also increased to 14.53 times after *Klebsiella* infection but this was decreased to 3.67 times after challenge with Bakreshwar water Fig. 1 and 2).

The expressions of cytokines have been represented in Fig 1 and Fig.2.

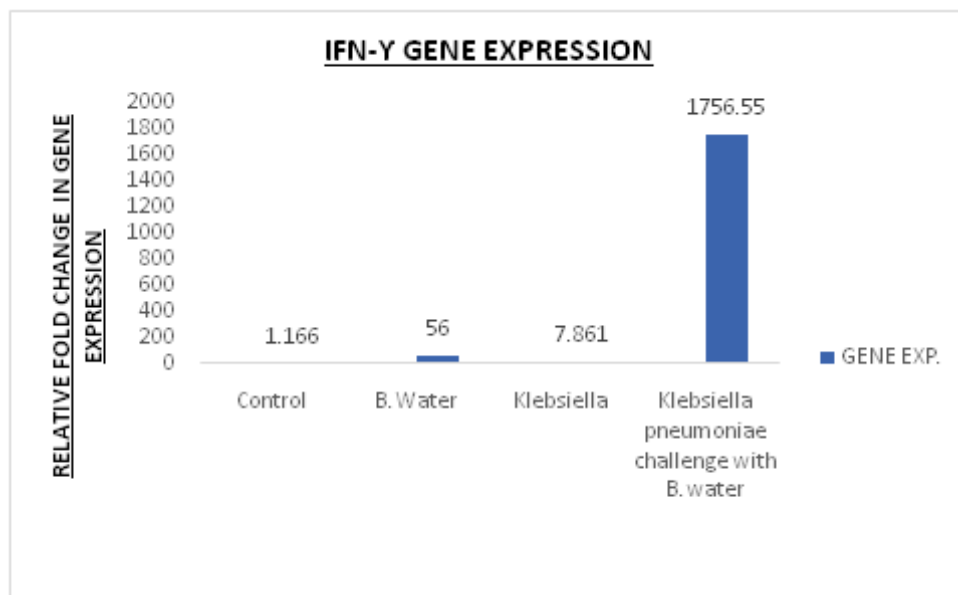


Fig. 1: IFN- γ changes in different experimental sets

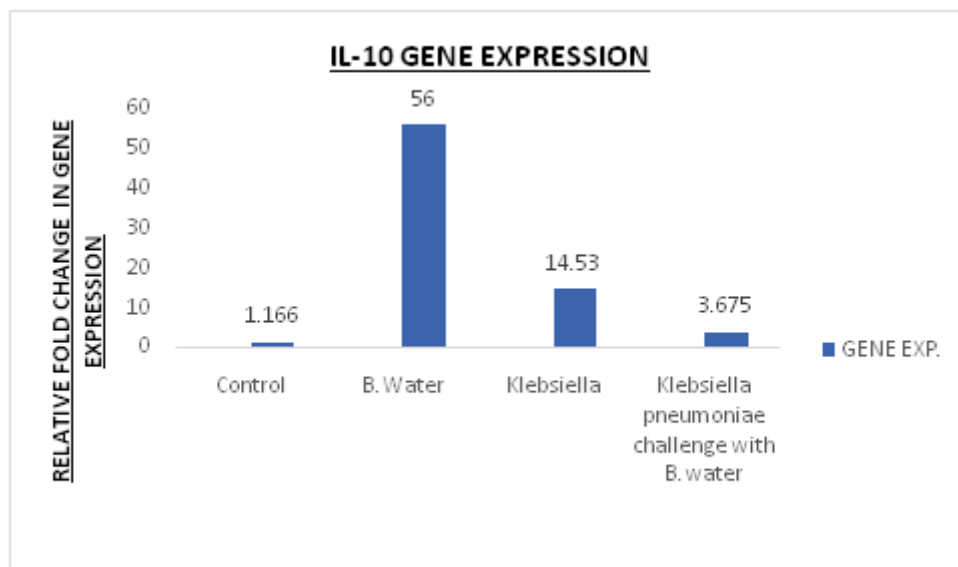


Fig. 2: IL-10 gene expression changes in different experimental sets

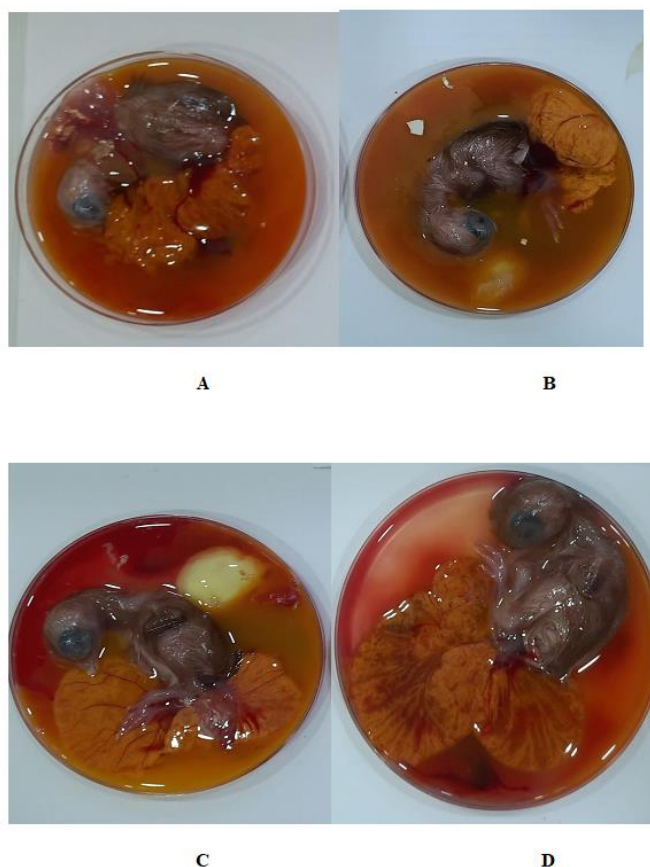


Fig. 3: Gross appearance of the embryo in different experimental sets A) Control B) Control with water C) Bacterial control set D) Curative set

The gross anatomy of the embryo in different experimental sets was determined. In the control set that was infected with only bacteria, severe haemolysis and necrosis were observed. But in the curative set very slight haemolysis was observed and the embryo was well developed.

DISCUSSION

From the previous research work, it was reported that polysaccharide capsules are very important component of bacteria that has been found to play an important role in regulating immune response [10].

Polysaccharide capsules can alter immune responses to bacteria, even minor differences between capsules can completely alter the immune responses as each is recognised as immunologically distinct.

Bacteroids fragilis, a gram-negative bacterium which has polysaccharide capsules that are phase variant and are able to switch between different capsules.

Capsular polysaccharides of *Bacteroids fragilis* that are composed of glycans are firmly attached to the cell surface and having polysaccharide antigen [10]. Effects of bacterial polysaccharide capsule on the immune system has been observed in case of *Bacteroids fragilis*. It enhances antigen presentation by upregulating MHC II, CD80, and CD86; phagocytosed by APCs and displayed on MHC II to activate CD4 + T cells in a TLR2-dependent manner; corrects CD4 + T cell deficiencies and TH1/TH2 imbalance in germ-free mice by upregulating the production of IFN- γ + TH1 T cells through CD11c + DCs and the IL-12/STAT4 pathway; represses TH17 responses; induces IL-10, producing FoxP3 + Tregs in a TLR2-dependent manner; protects against TNBS-induced colitis and *Helicobacter hepaticus*- induced colitis. Thus, Antigen presentation and T cell activation plays an important role in immune system [13-16].

The most important finding from our present experiment is elevated expression of IL-10 and IFN- γ in the control sets that were inoculated with bacterial suspension.

After infection of *Gallus gallus domesticus* embryo with *Klebsiella pneumoniae* (MDR), IFN- γ gene expression was mildly increased to 7.86 times. After challenge with Bakreshwar water, it was remarkably increased up to 1756.55

times. IL-10 gene expression was also increased to 14.53 times after *Klebsiella* infection but this was decreased to 3.67 times after challenge with Bakreshwar water.

The remarkably increased IFN- γ may indicate body immune activity may be due to presence of capsules of bacteria which is a known virulence factor and which may increase IFN- γ .

There are evidences that polysaccharide antigen (PSA) of capsule can increase IFN- γ level significantly and the *Klebsiella* capsular antigen is also made up of polysaccharide.

Decrease of IL-10 gene expression after challenge with Bakreshwar water is due to decrease pathogenicity in the embryo by the water.

CONCLUSION

IL-10 and IFN- γ have anti-inflammatory and pro-inflammatory properties that plays an important role against different infections. From this study, we can conclude that the hot spring water of Bakreshwar has some therapeutic values due to remarkably increased IFN- γ gene expression and decreased IL-10 gene expression in curative set, It may indicate body immune activity due to presence of capsule of the bacteria, which is a known virulence factor.

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Author Contributions

Author PS performed the experimental work, collected the data and written the first draft of the manuscript. Author DC and BS guided technically PS throughout the research study. Author KP arranged all the resources for the research investigation and offered the administrative support. Author SD designed the entire research investigation, interpreted the findings, and checked the final version of the manuscript. All authors have checked the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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