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Original Research Article

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

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Abstract: Bakreswar hot spring is a naturally occurring hot spring located at Birbhum district in West Bengal, India. The surface temperatures of the hot springs varies between 35°C to 66°C. The water of hot spring is alkaline with pH 9 and has curative properties. *Pseudomonas aeruginosa* is an important pathogen causing hospital acquired infections and in this study we used multi-drug resistance (MDR) isolate. The motive of the study is to compare the IL-10 and IFN-γ gene expression changes in *Gallus gallus domesticus* embryo infected with *Pseudomonas aeruginosa* by using the Bakreswar hot spring water.The curative properties of the hot spring water collected from Agnikund (65°C) was studied on 14thdays old chick eggs infected with *Pseudomonas aeruginosa*. After harvesting and allantoic fluid collection, RNA extraction was done and cDNA synthesis and RT-PCR were performed. After infection of the chick embryo with MDR *Pseudomonas aeruginosa*, both IL-10 and IFN-Y gene expressions were increased, but in the curative sets those were challenged with Bakreswar hot spring water IL-10 and IFN- γ both gene expressions were decreased. From this study we may conclude that hot spring water of Bakreswar has antimicrobial activity against *Pseudomonas aeruginosa* due to which it decreses the cytokine gene expressions.

Keywords: *Pseudomonas Aeruginosa*, Bakreswar Hot Spring, Allantoic Fluid, *Gallus Gallus Domesticus*, Cytokine Gene Expression.

INTRODUCTION

Hot spring, hypothermal spring or geothermal spring is a spring produced by the emergence of geothermally heated ground water on to the surface of the earth. The ground water is heated either by shallow bodies of magma or by earth's crust (Rao *et al.*, 2021; Mukherjee *et al.*, 2012).

India has some natural occurring hot springs.

STATE	NAME OF HOT SPRINGS
West Bengal	Bakreswar
Himachal Pradesh	Manikaran, Garamkund, Tapri
Sikkim	Yumthang, Borang , Taramchu
Uttarakhand	Gaurikund, Taptkund, Suryakund
Madhyapradesh	Chavalpani, Anhoni, Dhunipani
Maharashtra	Ganeshpuri, Akloli, Vajreshwari
Bihar	Gaya, Suryakund
Karnataka	Bendruteertha, Bandaru
Andhrapradesh	Ushnagudam
Kerala	Varkala
Tamilnadu	Mannargudi
Orissa	Atri, Deulajhari

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In Eastern India hot spring is located at Bakreshwar in Birbhum district West Bengal. It shows temperature 35°C – 66°C on the surface. The thermal water of Bakreswar is alkaline with low to moderate Na, HCO3 and SO4 compared to chloride. Bakreswar water shows unlimited gaseous activity with 0.31-1.33% He. Water shows pH 9, chloride content 30 ppm – 100 ppm, SO4 content is low less than 10 ppm, also contains high Na ranging from 30-100 ppm, low k less than 4.8 ppm and low Ca and Mg, moderate total dissolved solid and Silica ranging from 60-82 ppm. Fluorine content is also high 9-12 ppm and Li content is high.

In this experiment we collected water from Agnikund, which is one of the hot spring of Bakreswar (Mukhopadhyay *et al.*, 2012).

Agnikund	65°- 66.5°C
Kharkund	58°- 66°C
Suryakund	55°-57°C and others

The *Pseudomonas* genus comprises more than 120 species that are rod shaped, gram negative, aerobic and motile with single polar flagella that are favoured humid environment including water and soil. These gram negative bacteria have two membranes such as a peptidoglycan thin layer surrounded by an outer membrane containing lipopolysaccharide (LPS). The latter is made up of three units: a hydrophilic polysaccharide, O-antigen, lipid A which is responsible of these pathogens. *Pseudomonas aeruginosa* strains mostly associated with human infections are y-proteobacterium (Ghssein *et al.*, 2022). It is usually a multi-drug resistance (MDR) opportunistic pathogens that causes nosocomial acute or chronic infection in those individuals who are immunocompromised with chronic obstructive pulmonary disease (COPD), Cystic fibrosis, cancer, burns, sepsis and ventilator associated pneumonia (VAP) (Qin S *et al.*, 2022; Moradali *et al.*, 2022).*Pseudomonas* is also an environmentally resilient microorganisms that can grow in nutrient – deficient conditions and live in a temperature range 4° - 42°C. The difficult adaptability of *Pseudomonas* helps them to survive on dry, abiotic surfaces in hospitals. Hospital acquired *Pseudomonas aeruginosa* causes dermatitis, bacteraemia and infection of respiratory and urinary tracts (Liao *et al.*, 2022). The complex structure of *Pseudomonas aeruginosa* biofilm contributes an additional factor to the pathogenicity of this microorganisms (Tuon *et al.*, 2022). They able to form biofilms which produce extracellular matrix and difficult to eradicate with antibiotic treatment (Ciofu *et al.*, 2019; Rasamiravaka *et al.*, 2015). The main purpose of the study is to compare the IL-10 and IFN-Ɣ gene expression changes in *Gallus gallas*embryo infected with *Pseudomonas aeruginosa*using the Bakreswar hot spring water to make any curative changes in the target gene or not.

MATERIALS AND METHODS

Procedure

Collection of water sample:

Bakreswar hot spring water was collected from Agnikund(65°- 66°C)in March,2023 in a sterile plastic container.a

Collection of eggs:

The 14th day fertilized eggs of *Gallus gallas domestics*were purchased from State poultry farm, Tollygunge, Kolkata. The eggs were carried in a thermocol insulating box to maintain the temperature 38°C.

Bacterial strain:

Multi-drug resistant *Pseudomonas aeruginosa* was collected from the Microbiology department of Peerless Hospital Research Centre Limited.

The antibiogram of the isolated *Pseudomonas aeruginosa* is given below:

Source: URINE

Antimicrobial	**MIC**	**Interpretation**	**Antimicrobial**	**MIC**	**Interpretation**
Piperacillin/ Tazobactam	>=128	R	Gentamicin	>=16	R
Ceftazidime	>=32	R	Ciprofloxacin	>=4	R
Cefoperazone/ Sulbactam	>=64	R	Levofloxacin	>=8	R
Cefepim	>=16	R	Minocycline		R
Aztreonam	>=64	R	Tigecycline	>=8	R
+Doripenem		R	Fosfomycin	>=256	R
Imipenem	>=16	R	Colistin	>=4	R
Meropenem	>=16	R	+Polymixin B		R
Amikacin	>=64	R	Trimethoprim/ Sulfamethoxazol		R

AES Findings	
Confidence	Consistent

Different Types of Sets Were Prepared Such As –

1. Control set: 14th day old embryonated three eggs of *Gallus gallus domesticus*.

2. Therapeutic water set: Therapeutic water set where embryonated eggs were inoculated with Bakreswar water.
3. Third set of eggs were inoculated with freshly prepared multi drug resistant (MDR) *Pseudomonas aeruginosa* bacterium suspension.
4. Curative sets : At first the sets of eggs were inoculated with multi drug resistant (MDR) *Pseudomonas aeruginosa* suspension and after 1hour of inoculation challenged with Bakreswar water to check whether this hot spring water can make any curative changes in the target gene expression or not.

Cleaning of the eggs:

Collected 14th day fertilized eggs were kept in thermocol insulating box and firstly they were cleaned by distilled water and cottons.

Candling of eggs:

Air sac of the eggs were marked and the eggs were examined to differentiate between live and dead eggs. If blood vessels are visible it is live and if black spots are visible it means dead egg.

Incubation of eggs:

The eggs were incubated over night at 37°C and to maintain the humidity a tray filled with distilled water were placed under the eggs in the incubator.

Inoculation of eggs:

Before inoculation Air sacs of the eggs were cleaned by using 70% ethanol and providone iodine. Then a hole was made at the centre of the air sacs with a sterile needle.

Control sets were kept as such.

Water control sets were inoculated with 100 µl of Bakreswar water using a sterile 1ml syringe.

Therapeutic sets were inoculated with 10 µl 0.5 McFarland standard bacterial (*Pseudomonas aeruginosa* -MDR) suspension.

Curative sets were inoculated with 10 µl 0.5 McFarland standard bacterial (*Pseudomonas aeruginosa* – MDR) suspension and after 1 hour incubation again inoculated with 100 µl of Bakreswar water using a sterile 1ml syringe. Then the eggs were incubated in the incubator for 4 hours.

Collection of allantoic fluid:

In a biosafety cabinet (Class II A2 Systronics, India) the embryonated eggs were ethically harvested with the sterile scissors and forceps and the allantoic fluid was collected by dissecting the corio-allantoic membrane (CAM) with sterile 5 ml syringe and transfer to a falcon tube. The fluids were stored at -80°C for further experiment.

RNA extraction:

The total RNA was extracted using RNA isoplus and the whole extraction was executed following the protocol of the manufacturer (Takara,USA).

RNA quantification:

10 µl of RNA was quantified using Ultra violet – vis spectrophotometer (CarrywinUV-Vis 60, Agilent, Singapore) using the absorbance ratio at 260 nm and 280 nm.

cDNA synthesis

Using cDNA reverse transcriptase synthesis kit (Biorad, USA) in conventional PCR (T100, Biorad, USA conventional PCR), the total RNA was then converted to cDNA.

RT-PCR

Using cyber green reagent the cDNA were utilised to perform the semi quantitative gene expression analysis of the following cytokine parameters namely, IL-10 and IFN-γ using the Real – time PCR (Biorad, CFX-96 instrument, USA) against the house keeping gene beta- actin (Chatterjee *et.al.*,2022). The gene expression quantification was based on the formula 2^-(ΔCt1 - ΔCt2) where Ct is threshold.

RESULTS

Changes in cytokine gene expression:

After infection with *Pseudomonas aeruginosa* (MDR) IL-10 gene expression was increased 32.105 times but after challenge with Bakreswar water it became 0.297. Similarly after *Pseudomonas aeruginosa* (MDR) infection IFN-γ gene expression was increased 855.09 times but after challenge with Bakreswar water it was significantly decreased to 20.189 times (Fig.1 and 2).

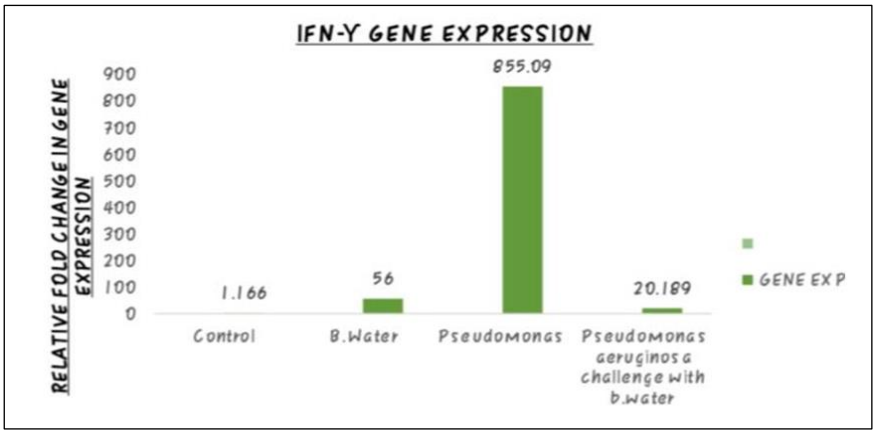


Figure 1: IFN-Y changes in different experimental sets

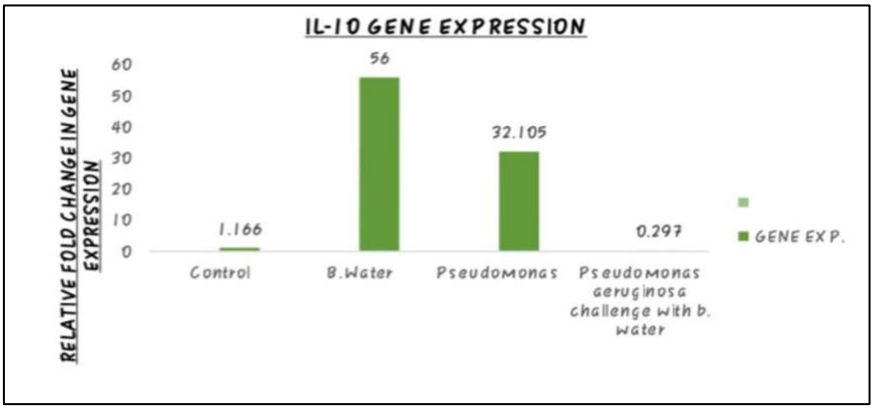
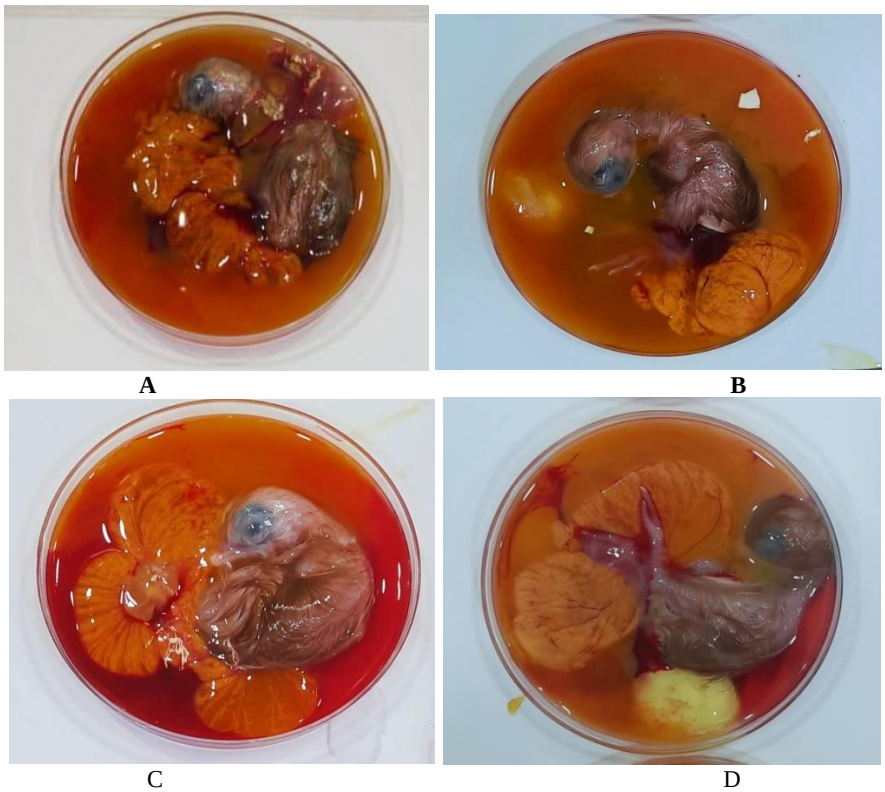


Figure 2: IL-10 changes in different experimental sets

Changes in morbid anatomy of Gallus gallus domesticus:



A **B**

C D

Figure 3: Gross appearance of the embryo in different experimental sets

A) Control B) Control with Bakreswar 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

Interleukin -10 (IL-10) is a potent anti-inflammatory cytokine which acts by inhibiting synthesis of pro-inflammetorycytokines of monocytes, macrophages, T-cells, neutrophils, mast cells, dendritic cells, as well as human alveolar macrophages. IL-10 also down regulates the antigen presenting capacity of monocytes by down regulating MHC class-II molecules and co-stimulatory ligands for T-cells(Casaultaet.al.). IL-10 also balances cytokine gene expression by proinflamatory cytokine.

E. Frederick Wheelock was first to describe Interferon-γ (IFN-γ) as a phytohemagglutinin induced virus inhibitor produced by white blood cells after they have been stimulated (Jorgovanovic et.al.2020). IFN-γ is a pleiotropic molecule with associated anti-proliferative, pro-appoptotic and anti-tumor mechanisms (Castrol *et al.,* 2018). It has also a role in recognising and eliminating pathogens. Additionally cancer cells are destroyed by IFN-γ activity via induction of an anti-proliferative state (Gunjan *et al.*, 2018).

In our study, we have explored cytokine gene expression after challenging *Gallus gallus domesticus* embryo with Bakreswar water infected with *Pseudomonas aeruginosa* (MDR).

As, IL-10 and IFN-γ both are expressed within 4 hours. We targeted only these cytokine gene expression in this study because other cytokine gene expression cannot be studied within 4-5 hours time. This restriction of time period is due to experiment with pathogenic bacteria *Pseudomonas aeruginosa* (MDR) which multiply rapidly in embryonated egg and cause necrosis and purification. We also used normal control and the Bakreswar water (Agnikund-68°C) control. Bakreswar water control is used to observe preventive and curative changes of IL-10 and IFN-γ gene expression after bacterial challenge. After infection IL-10 gene expression was increased in comparison to the controls. After infection with *Pseudomonas aeruginosa (*MDR) IL-10 gene expression was increased 32.105 times but after challenge with Bakreswar water it became 0.

After infection with *Pseudomonas aeruginosa* (MDR) IFN-γ gene expression was increased 855.09 times but after challenge with Bakreswar water it was decreased to 20.19 times.

In general, IL-10 and IFN-γ they have protective roles against different infection. After giving Bakreswar water inflammatory changes ameliorated in *Gallus gallus* embryo.

CONCLUSION

We have studied in this experiment that Bakreswar hot spring water has ability to reduce infection of *Pseudomonas aeruginosa (*MDR) in cytokine gene expression in *Gallus gallus* embryo. So we can conclude from our study that Bakreswar hot spring water has therapeutic and anti- microbial activity against many pathogenic microorganisms.

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AUTHOR CONTRIBUTIONS

Author SN performed experimental work, collected the data and written the first draft of the manuscript. Author DC and BS guided technically SN 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 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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