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Original Research Article

A Study on IFN-γ and IL-10 Gene Expression Changes in *Gallus gallus domesticus* Embryo Infected with *E. coli* and its Possible Alteration by Bakreshwar Hot Spring Water

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Abstract

In search of effective treatment against *E. coli* there are some conventional medicinal treatments available but it becomes difficult to fight against MDR strains as they are resistant to most of the antibiotics. It was found that Bakreshwar hot spring water has some therapeutic properties against various infections, mostly against skin infections. Bakreshwar hot spring having a pH range of 8.1-8.5 and temperature which varies from 35°-71°C, contains several chemical compounds. Our aim of this work was to study whether Bakreshwar hot spring water can cure infections caused by *E. coli* in *Gallus gallus* embryo in relation to their gross appearances and IFN- γ and IL-10 gene expression changes. The sample water was collected around March, 2023. To study the therapeutic activity of Bakreshwar hot spring water, different experimental sets were set up with 14days embryonated eggs. Freshly prepared 0.5Mcfarland E. coli culture was inoculated into the eggs and in curative set, 100µl hot spring water was injected to check any alterations. Allantoic fluid was collected and mRNA was extracted the very next day. cDNA was synthesized from the extracted mRNA. Along with master mixture (primers, nuclease free water, Syber green) the cDNA was run at RT-PCR and C_T value was obtained. The mean gene expression shows that IL-10 and IFN- γ both gene expression was decreased in curative set after treatment with water as compared to control sets. These finding suggests that Bakreshwar hotspring water treatment can be a potential method to control *E. coli* caused infections.

Keywords: Bakreshwar hot spring, *E. coli* infection, Uropathogenic *E. coli*, UTI, Interleukin-10, Interferon- γ.

INTRODUCTION

A "Hot spring" is a natural lentic system that is produced when geothermally heated groundwater emerges from the earth's crust. Among other organisms, thermophiles substantially mostly choose hot springs as their niche. Many of the organisms are still unknown and also unculturable which are found in this water. There are many hot springs around the world such as Yellowstone national park hot spring (USA), Monterry hot spring (Peru), El Dorado (Arizona), Kapishya hot spring (Zambia)etc. As well as there are many hot springs located in India such as Manikaran, Panamik, Kheer ganga hot spring, Tattapani, Bakreshwar and so on. Bakreshwar is a popular tourist destination which is located (Lat. 23º 52' 48" N; Long. 87º 22' 40" E) in Dubrajpur, Suri Sadar subdivision of Birbhum district, West Bengal, India (Chaudhuri,2017). In Bakreshwar, there are many hot springs located there, Agni Kund is one of them.

This hot spring water has a temperature of 35-66.5°C and a pH of nearly 9. This water is found mainly alkaline, which contains low to moderate level of Na(30-100ppm), K(<4.8ppm), Bicarbonate (HCO_3) and SO_4(<10 ppm) as compared to chloride (Cl). It also contains moderate TDS (Total dissolved solid) and Silica (60-82 ppm), Ca, Mg, Fluorine (9-12 ppm) etc (Mukhopadhyay *et al*., 2012).

Nosocomial infections are responsible for 0.7–10% of deaths as compared to 0.1–4.4% of all the deaths occurring in hospitals. Interestingly, 10–30% of patients in the Indian population are susceptible to such fatal infections. *E. coli* is the most common pathogen causing diarrhoea, neonatal septicaemia, UTI, bacteraemia and urosepsis. It is responsible for 80% of

community-acquired UTIs and 30% of nosocomial infections (Nagarjuna *et al*., 2015). This statistic shows that how much disaster can be caused by *E. coli*.

In a report, it was shown that in 2010, India was the world's largest consumer of antibiotics for human health with 12.9 x 10^9 units of antibiotic consumption (~10.7 units per person) (Laxminarayan *et al*., 2016). *Escherichia coli* is a member of the *Enterobacteriaceae* family, the enteric bacteria, which are facultative anaerobic Gram-negative bacteria, commonly found in the intestinal tract of warm-blooded animals including humans (Silva, 2011). *E. coli*, a near-ubiquitous colonizer of the gastrointestinal tract in children and adults has often been used in studies of the incidence of antibiotic resistance in commensal bacteria (Oluyege *et al*., 2015).

In north-east India, prevalence of antibiotic resistant *E. coli* is very high. In a survey in rural areas of Sikkim, the report says that adults carried more antibiotic resistant commensal than the young ones. Resistance rate to at least one microbial agent is (75.7% to 54.7%) and resistance rate to multidrug (30.4% to 14%) between adult vs young ones (Singh *et al*., 2018). ATCC strains are sensitive to all drugs whereas MDR strain means that is resistant to more than two drugs. In case of *E. coli* MDR strain, it is found that these strains are resistant to almost all drugs except some last line resorts like Polymyxin B, Colistin, Chloramphenicol etc. In another study, it was reported that commensal *E. coli* isolates from new borne children less than one month of age without any significant exposure to the antibiotics were highly resistant to single antibiotic doses like ampicillin (100%) and co-trimoxazole (96%) (Tule *et al*., 2017).

It was evaluated the high antibiotic (Ampicillin, cefoxitin, nalidixic acid, polymyxin-B etc.) resistance nature in commensal *E. coli* isolates from human, animals, and water by disk diffusion method and reported that commensal *E. coli* from all sources displayed resistance to all the antibiotics tested except polymyxin-B. It was also reported a higher incidence of antibiotic resistance in human isolates as compared to that from environment or animals (Purohit *et al*., 2017). So, to find a remedy against *E. coli* MDR strains is very necessary in recent times.

As there is no previous fruitful research about Bakreshwar water used for treatment against diseases caused by *E. coli.* So, the aim of this project is to study the IFN-γ and IL-10 gene expression changes in *Gallus gallus domesticus* embryo infected with *E. coli* and its possible alteration by Bakreshwar hot spring water. In egg model experiment, in case of bacterial infection, after 6-7 hours the sample was putrified so that it was difficult to study about all genes as they have longer incubation period than the time of incubation we provided. So, our study is about these two (IFN-γ and IL-10) gene alterations.

Study Procedure

Different experimental sets were designed where each set contains three eggs.

Control Sets: These three sets mainly used as control sets where each of those sets contained three embryonated chick eggs. These sets are -1stControl set in which 14days embryonated eggs were used without any inoculum, 2ndBacterial control set where the 0.5 opacity McFarland. *E. coli* culture was injected in these fertilized eggs, 3rd Therapeutic water control sets where only Bakreshwar water sample was inoculated into the eggs. The previously mentioned control sets were in used for the subject experiment from which the data is taken accordingly. Curative Set: In this set, the three eggs are injected with *E. coli* suspension firstly and after 1hour 100µl Bakreshwar water was again injected by a sterile syringe of 1ml volume in to the eggs to check whether this water could make any curative changes in the target genes expression or not.

Collection of water sample: Water sample was collected from Bakreshwar hot spring (Agni Kund), Birbhum, West Bengal around March, 2023 in a 100 mL sterile sample collecting container.

Bacterial strain: Used for this work is *Escherichia coli* (MDR) that was collected from a stool sample atPeerless Hospitex Hospital and Research Centre Limited, Kolkata, India. The antibiogram table (Table 1) of the strain is given below:

Table 1: Antibiogram of isolated *Escherichia coli* (MDR) strain

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Amoxicilin/ Clavulanic Acid	>=32	R	Meropenem	8	R
Piperacillin/ Tazobactam	>=128	R	Amikacin	>=64	R
Ceforoxime	>=64	R	Gentamicin	>=16	R
Cefuroxime Axetil	>=64	R	Ciprofloxacin	>=4	R
-Cefixime		R	+Levofloxacin		R
Cefiriaxone	>=64	R	Tigecycline	<=0.5	S
Cefoperazone/ Sulbactam	>=64	R	Fosfomycin	<=16	S
Cefepime	>=32	R	Colistin	<=0.5	I
-Doripenem		R	+Polymyxin B		I
Ertapenem	>=8	R	Trimethoprim/	<=20	S

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
			Sulfamethoxazole		
Imipenem	8	R			
AES Findings					
Confidence: -	Consistent				

Procuring of eggs: Firstly, 14day fertilized eggs (*Gallus gallus domesticus*) were brought from state poultry farm, Tollygaunge in a thermocol insulating box maintaining 38°C.The eggs were washed by distilled water. Candling was performed to differentiate between live and dead eggs. Air sac was marked properly with marker. Then these eggs were kept in incubator maintaining 60-80% humidity overnight at 38^0c.

Preparation of inoculum: From the stock cultures, E. coli was streaked on agar plate and the plate was incubated overnight for 37^0c for 24hours. The inoculum was made up to0.5 McFarland suspension in the 4ml of saline water.

Inoculation: The air sac of the eggs was disinfected with alcohol (70%) followed by povidone Iodine solution and pinpoint hole with a sterile syringe was made on those sites. 10µl of inoculum was injected into the 3rd control set and curative set through the hole. The eggs were kept for incubation at 38°c. After one hour, the water sample was injected only into the curative set of eggs by 1mL of sterile syringe and kept them again for incubation for 5hours. Then the eggs were kept in refrigerator in 4°c for an hour.

Collection of allantoic fluid: After 6 hours, the embryonated eggs were brought out from the refrigerator and harvested with sterile scissors and forceps. Allantoic fluid was collected from each egg by ethically dissecting the chorio-allantoic membrane and put the fluid in 5 ml sterile falcon tube and the fluids were stored at-80^oC overnight for further study.

Molecular biology study: After liquifying the stored allantoic fluid, 200µl from each sample was taken in respective eppendorfs and 1ml RNAisoplus was added into it. The rest of the mRNA extraction process was carried out by the manufacturer's protocol (Takara, USA). The total RNA yield was measured by using UV-visspectrophotometer (Agilent, Singapore) by the absorbance ratio at 260nm by 280nm. Then, the total RNAwas then converted to cDNA using cDNA reverse transcriptasekit (Bio-Rad, USA) in conventionalPCR (T100, Bio-Rad, USA conventional).

Then the master mix (it contains nuclease free water, iTaq Syber green, Primers) was given in each well of microtiter plate according to the genes and the cDNA synthesized were added accordingly. To examine the gene expression of the cytokines IL-10 and IFN-γagainst the house keeping gene β-actin, Real Time-PCR (Bio-Rad, CFX-96, instrument, USA) was run (Marone *et al*., 2001). We got the cycle threshold value (C_t value) of each sample. The quantitative gene expression values were calculated using the formula $2^{(-\Delta C_t)}$ where $\Delta C_t=(\Delta C_t1-\Delta C_t2)$. Here,$C_t1$=Gene of interest; C_t2=Housekeeping gene. So,the main formula we used here was given below-$\Delta C_t=C_t1$(gene of interest)-C_t2(housekeeping gene).Here, the House keeping gene was β-actin and the gene of interest was IFN-γ and IL-10 respectively.

RESULT

Morbid anatomy analysis:

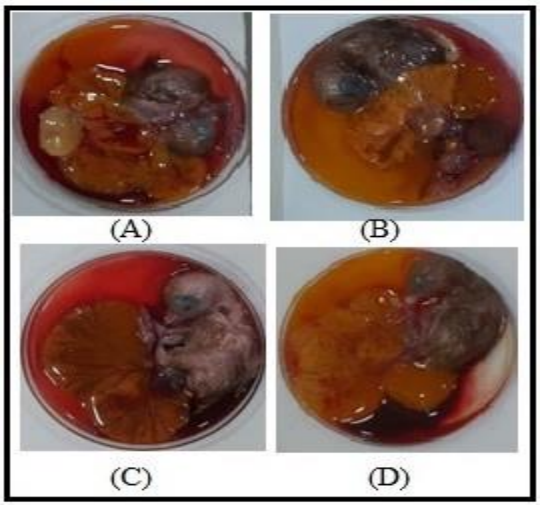

Fig 1: Gross appearance of embryo of different experimental sets

The figure (Fig 1) presents Gross appearance of embryo of four different experimental sets-(A) Control setof 14 days embryonated eggs, no hemolysis was shown; (B) Therapeutic control set where the water sample was injectedonly, hemolysis was shown;(C) Bacterial control set where, the *E. coli* culture was inoculated, hemolysis was shown here; In (D) Curative set with Bakreshwar water, no hemolysis was shown.

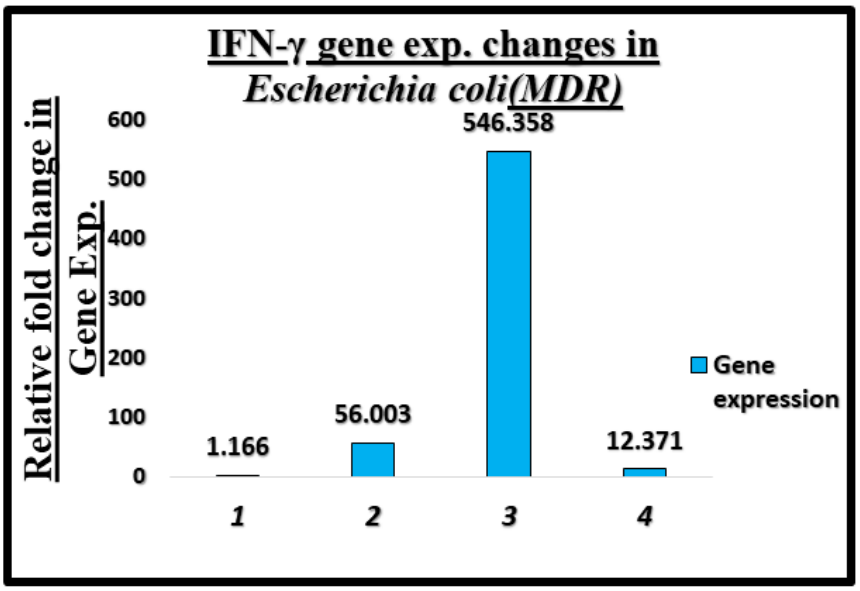

Fig 2: IFN- γ changes in different experimental sets (1) Control, (2) Bakreshwar water control, (3) *E. coli* control set, (4) Curative set

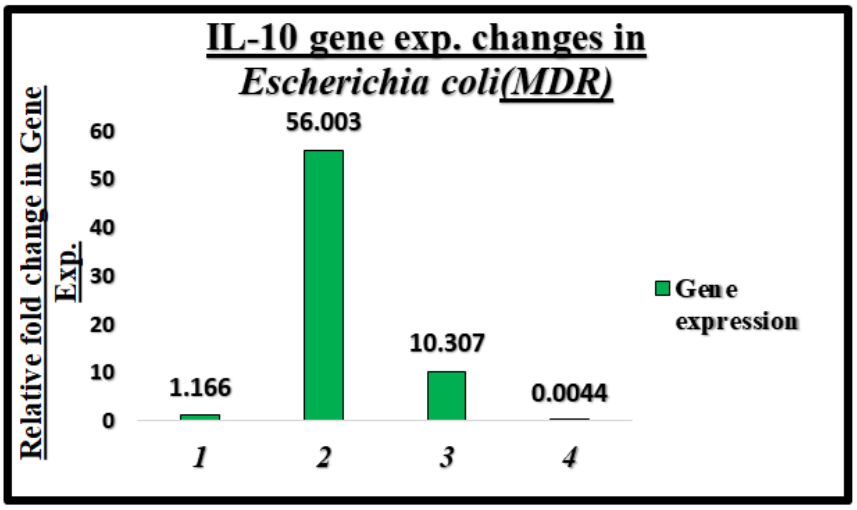

Fig 3: IL-10 changes in different experimental sets (1) Control, (2) Bakreshwar water control, (3) *E. coli* control set, (4) Curative set

The experiment was done to study about Cytokine gene expression in different experimental sets. After infection of chick eggs (*Gallas gallas domesticus*) with *E. coli*, the IFN- γ gene expression (Fig 2) was increased about 546.35 times. Whereas after challenge with Bakreshwar water, significantly the gene expression was reduced to 12.3 times. On another side, (Fig 3) after infection with *E. coli* the IL-10 gene expression was increased around 10 times. Whereas after treatment with water the gene expression was reduced to 0.0044 times.

DISCUSSION

IL-10 is an anti-inflammatory cytokine molecule which plays a pivotal role against inflammation. In a finding, it was described about a novel modality of IL-10–mediated *E. coli* clearance by deviating the entry of bacteria via CR3 and preventing PGE-2 formation in neonatal meningitis (Mittal *et al*., 2010). That suggests the importance of IL-10 production in early stage of *E. coli* infection.In another work, it was reported that, Urinary tract infection (UTI) by *E. coli* (UPEC) induces IL-10 production as an early

innate immune response to regulate inflammation and promote the control of bladder infection. The major uropathogenic *E.coli* flagellar filament, FliC, as an important bacterial component sensed by the bladder innate immune system responsible for the induction of IL-10 synthesis. The detection of FliC through TLR5(Toll like receptor 5) induces rapid IL-10 production in the bladder, and FliC acts like a potential immune modulator that might have effect in the treatment or prevention of UPEC UTI (Acharya *et al*., 2019). In our work, IL-10 production after bacterial infection increased 10 times than control set. So, it can be reported that an elevated level of IL-10 production may be shown against *E. coli* infection. After Bakreshwar water challenge in curative set, there was a significant decrease in IL-10 gene expression. In curative set, as the infection level decreased that might reduce the IL-10 production too.

The type II interferon or IFN-γ is a proinflammatory cytokine. The level of IFN-γ is increasing in inflamm-ageing process(It is a process mostly developed in older individuals, a condition which results into elevated levels of blood proinflammatory markers those have high susceptibility to chronic morbidity and premature death (Ferrucci, 2018). *E. coli* is a major cause of bacteriaemia and sepsis and those have a high mortality rate. Prevalence of E. coli infections increase with age and become more fatal for elderly patients (Bonten *et al*., 2020). The modulated phagocytic capacity and cytokine release of macrophages is one of the main reasons for the reduced resistance level to infections in elderly aged individuals (Schütze *et al*., 2014). IFN-γ regulates the cytokine release by monocytes and macrophages (Ausler *et al*., 2002) and reduces phagocytosis of different bacterial pathogenic strains including *Streptococcus pneumoniae* (*S. pneumoniae*), *Staphylococcus aureus* (*S. aureus*), and *Escherichia coli* (*E. coli*) (Schütze *et al*., 2021). From these data, we can come to a point that it is an important regulator of the innate immune system and generates resistance in the host against bacterial infections. So, after *E.coli* infection, we get 546 times of elevated level of IFN-γ as response to the infection. Whereas, after Bakreshwar water challenge we got significant decrease in the gene expression because the gene expression is proportional to the infection occurs.

CONCLUSION

IL-10 and IFN-Ɣ are anti-inflammatory and pro-inflammatory cytokines respectively. They have a key role against different bacterial infection. We can conclude from this work that the hot spring water of Bakreshwar has some medicinal values due to significantly decrease IFN-γ gene expression and markedly decrease in IL-10 gene expression in curative set than the bacterial control set. A potential method to fight against *E. coli* infection is enlightened by this work.

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Author's Contribution

The experimental work was performed by Author AC. He collected the data and wrote the first draft of the manuscript. Author DC and BS both guided technically AC throughout the research study. Author KP arranged every resource needed for the research study. Author SD had designed the entire research investigation, interpreted the findings from this work, and checked the final version of the manuscript. All of the authors checked the final copy of the manuscript.

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