International Journal of Pharmaceutical Science Invention
ISSN (Online): 2319-6718, ISSN (Print): 2319- 670X
www.ijpsi.org Volume 12 Issue 4 ǁ Jul. –Aug. 2023 ǁ PP. 23-29

A Study on IFN -γ and IL-10 gene expression changes in *Gallus gallus domesticus* embryo infected with *Candida albicans* and its possible alteration by Bakreshwar Hot Spring Water

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ABSTRACT

Background : *Hot spring's water possesses numerous therapeutic benefits such as smoothening of the dry and rough skin and also helps to treat dry scalp, arthritis, acidity, digestive issues etc. The Bakreshwar hot springs located in Birbhum district of West Bengal is also popular for its medicinal values.*

Aim : *To study about the antimicrobial effect of Bakreshwar hot spring water against immune-pathogenecity caused by Candida albicans in the context of IFN -γ and lL-10 gene expression changes in Gallus gallus domesticus embryo.*

Materials & methods : *The 14th day fertilised chick eggs were cleaned and candling was done. Then the eggs were marked and kept in an incubator for overnight. On the next day, eggs were inoculated with fungal suspension and then challenged with Bakreshwar water. Then harvesting was done to collect the allantoic fluid. Then RNA extraction and cDNA synthesis followed by RT PCR was performed.*

Result : *After infection of chick embryo Gallus gallus domesticus with Candida albicans, IL-10 and IFN-Y gene expression were moderately increased in comparison to controls but when it was challenged with Bakreshwar water, IL-10 and IFN –γ gene expressions became normal.*

Conclusion :*From our study, we can conclude that there is an antimicrobial property present in the water of Bakreshwar hot spring and due to this, it can be used against Candida infections.*

Keywords: ***B****akreshwar hot spring, Candida albicans, Gallus gallus domesticus, antimicrobial property, IFN –γ, IL-10.*

Date of Submission: 12-07-2023 Date of acceptance: 29-07-2023

I. INTRODUCTION

A hot spring is produced when geothermally heated groundwater emerges from the earth's crust. Since ancient times, hot springs have been used for various medicinal purposes. In India, people bathe in hot springs to treat skin diseases and stomach problems and rheumatic disorders. Hot spring baths for three and more days have significant therapeutic effects among patients with musculoskeletal disorders, including rheumatoid arthritis. Physicians who are currently working in the area of diagnosis and treatment of patients in government and public facilities of the southern region of India to consider hot spring bath treatment for those patients with complaints of musculoskeletal pain, nonspecific arthritis, and rheumatoid arthritis (Achamyeleshet al., 2021).
There are many hot springs located in India from those some famous hot springs are listed below : (Asha et. al., 2020)

LOCATION(STATE)	NAME OF THE HOT SPRING
Maharashtra	Ganeshpuri, Vajreshwari
Himachal Pradesh	Manikaran, Khirganga, Tapri
Karnataka	Bendruteertha, Irde, Bandaru
Madhya Pradesh	Chavalpani, Anhoni, Dhunipani
Chhattisgarh	Tatapani
Bihar	Suryakund, Gaya
Sikkim	Yumthang, Phurchachu, Borang
West Bengal	Bakreshwar, Tantloi, Kendughata

Uttarakhand	Gaurikund, Taptkund, Suryakund
Arunachal Pradesh	Hot spring of dirang area
Orissa	Taptapani, Atri, Deulajhari
Jharkhand	Tattahotspring,Jarom, Brahmakund
Tamil Nadu	Mannargudi
Kerala	Varkala

Figure.1 Location of Bakreshwer hot spring

Bakreshwar is a popular pilgrimage destination near Tarapith, located (Lat. 23º 52' 48" N; Long. 87º 22' 40" E) in the Birbhum district, West Bengal, India **(**Figure.1). Bakreshwar is known for its ten hot springs (Agnikund,SoubhagyaKund,Suryakund, Brahmakund, etc.) which has varying temperatures (35°C - 71°C). The thermal water at Bakreshwar is alkaline with low to moderate sodium, HCO3 and SO4 as compared to Chloride. Bakreshwar water shows profuse gaseous activity with 0.31 to 1.33% Helium content. It is observed that in the hot springs areas also show high fluoride content. It is because of this they are well known for their activity in healing skin infections. Hot springs at Bakreshwar show pH of 9, 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 (Total dissolved solid) and silica ranging from 60 to 82 ppm. Fluorine content is high 9 to 12 ppm and needs caution before supply for direct uses (Mukhopadhyay et. al., 2012).

Candida spp is a common commensal fungus which is commonly found mostly on the skin and mucosal surfaces of the gastrointestinal and urogenital tract. They have been considered to be an important pathogen due to their resistance ability at present towards broad spectrum of antifungals. In 1992 and 1993 and from 1998 to 2000, it was observed in the first surveillance period that, *C. albicans* accounted for 52% of the isolates while *C. glabrata* accounted for only 12%. In the second round of the surveillance period, *C. albicans* accounted for 45% but *C. glabrata* had a shift up, to 24% (Punithavathy et. al.,2012). ICUs are the epicentre of invasive Candida infection due to an increasing population of immunocompromised patient. A survey of the epidemiology of sepsis conducted in USA indicated that the incidence of fungal sepsis increased 3 fold between 1979 and 2000 (Kaur et. al.,2014). National Nosocomial Infection Survey (NNIS) observed that *Candida* spp. were the 4th most common cause of nosocomial blood stream infection during the 1990s. However, in more recent studies, it has been considered that *Candida* spp. are the 3rd most frequent nosocomial blood stream isolates (Atriwa et. al., 2021).*C. albicans* was the predominant species; it was isolated in 63% of the episodes of candidemia (Olafuret. al., 2003).Among all the nosocomial infection fungal infections are account for 8% and *Candida* is responsible agent in80% of the cases. Nosocomial candidemia is still associated with an extremely high crude mortality rate over 60%, while the attributable mortality rate may be as high as 49% (Lark et. al., 2000, Gudlaugsson et. al.,2003)

Previous research on the Bakreshwar hot spring water have reported that water of Bakreshwar hot spring exhibited bacteriostatic/ bactericidal effects depending on the concentration and inhibitory effects on

plasma coagulation and attachment of *Staphylococcus aureus*(Manna et. al., 2014).According to that information we wanted to study the change in the gene expression of IL-10 and IFN -γ in the *Gallus gallus domesticus* embryo, infected with *Candida albicans* and then challenging the infection with water of Bakreshwar hot spring.

II. MATERIALS & METHODS

Procedure :

1. Collection of water from Bakreshwar hot spring : Water of Bakreshwar hot spring (Agnikund temp.-68°C, pH-11) was collected within a sterile plastic container and the lid of the container was sealed avoid any contamination at room temperature.

2. Details of the fungus used for the study : *Candida albicans* ATCC (American Type Culture Collection) 10231 was collected for the study from Peerless Hospitex Hospital and Research Centre Limited, Kolkata, India. The fungal strain was sub-cultured in the biological safety cabinet and kept overnight at 37°C in incubator.

3. **Collection of eggs of *Gallus gallusdomesticus***: 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.

4. **Cleaning of eggs:** eggs were cleaned thoroughly with distilled water using cotton.

5. **Candling of eggs : U**sing a torch, air sac of the eggs were marked. It can also be done to differentiate between live (blood vessels are visible) and dead (black spots are visible) eggs.

6. **Marking of control, preventive and curative sets :**

Control set : 6 eggs were used as control, 3 were marked as 'without water' and other 3 were marked as 'with water'.

Infection set : 3 eggs were marked as Candida ATCC (10231) 1, 2 and 3

Curative set : 3 eggs were marked as Candida ATCC (10231) 1 with water, Candida ATCC (10231)2 with water and Candida ATCC (10231)3 with water.

7. **Incubation of eggs :** eggs were incubated overnight at 37°C and to keep the humidity 60-80%, a tray filled with distilled water were placed below the eggs in the incubator.

8. Inoculation of eggs : Before inoculation Air sacs of the eggs were cleansed by using 70% ethyl alcohol followed by providone iodine. Then a puncture at the center of the air sacs was formed using a sterile needle.

Control sets marked as **'without water'** were kept as it was.

Control sets marked as **'with water'** were inoculated with 100µl of Bakreshwar water using a sterile 1ml syringe.

Infection sets were inoculated with 10 µl 0.5McFerland standard fungal (*Candida albicans* ATCC 10231) suspension.

Curative sets were inoculated with 10 µl 0.5McFerland standard fungal (*Candidaalbicans* ATCC 10231) suspension and after 1 hour incubation again inoculated with 100µl of Bakreshwar water using a sterile 1ml syringe.

9. Incubation period : eggs were incubated in the incubator for 5 hours.

10. Collection of allantoic fluid : The embryonated eggs were ethically and aseptically harvested on the 17th day with sterile scissors and forceps and the allantoic fluid was collected by ethically dissecting the Chorio-Allantoic membrane with sterile 5 mL syringes and transfer to Falcon tube. The fluids were stored at -80°C for further study. This whole work was done in biosafety cabinet (Class ll A2 Systronics,India).

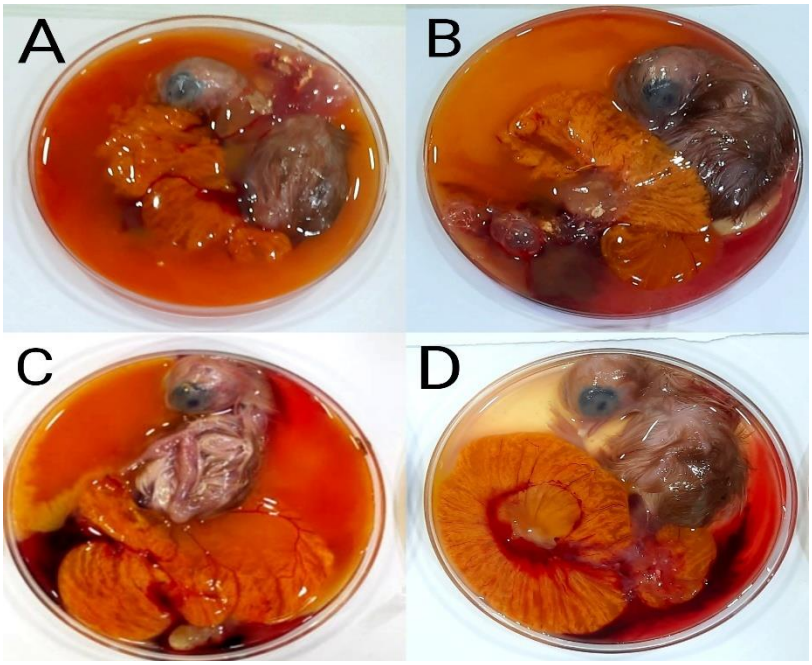
11. RNA extraction from allantoic fluid : The total Ribonucleic Acid (RNA) was extracted using RNA isoplus and the whole extraction was carried following the protocol of the manufacturer (Takara, USA).

12. RNA quantification : 10 µl of transferred RNA was quantified using Ultraviolet- vis spectrophotometer (Carrywin UV-Vis 60, Agilent, Singapore)using the absorbance ratio at 260 nm by 280 nm.

13. 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.

14. 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 Polymerase Chain Reaction (PCR) (BioRad, CFX-96 instrument, USA) against the house keeping gene β-actin(DebaSMita et.al.,2022) The gene expression quantification was based on the formula 2^-(ΔCt1-ΔCt2) where Ct denotes Cycle threshold.

III. RESULT



Changes in the morbid anatomy of *Gallus gallus domesticus* embryo :

Figure 1. Gross appearance (representative) of embryo in different experimental sets. (A) Control (without water); healthy well developed embryo and no haemolysis was observed (B) Control (with water) ; healthy embryo and slight haemolysis was observed ; (C) Infection sets (*Candida albicans* ATCC 10231) ; severe haemolysis was observed along with necrosis and putrefaction. (D) Curative set (*Candida albicans* ATCC 10231) inoculation followed by challenge with Bakreshwar water) ; no haemolysis was observed and the embryo was well developed.

Changes in the cytokine gene expression : IL-10 and IFN-γ gene expression have been represented in the bar chart diagrams. The figure 2 prepared from the RT-PCR values that indicates after infection with *Candida albicans* ATCC 10231, IL-10 and IFN-γ gene expression were increased 70.27 and 259.80 times respectively in the *Gallus gallus domesticus* embryo. Whereas, the figure 3 indicate that in the curative sets where Bakreshwar water was used against the infection with *Candidaalbicans*, there was a major decrease shown in both IL-10 and IFN-γ gene expression with a value of 1.08 and 90.35 times respectively.

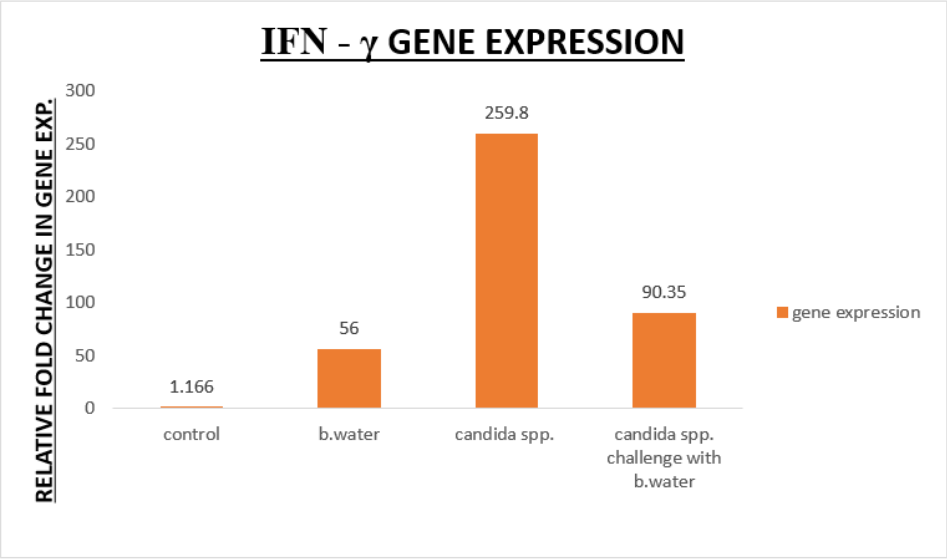


Figure.2: IFN-γ changes in different experimental sets

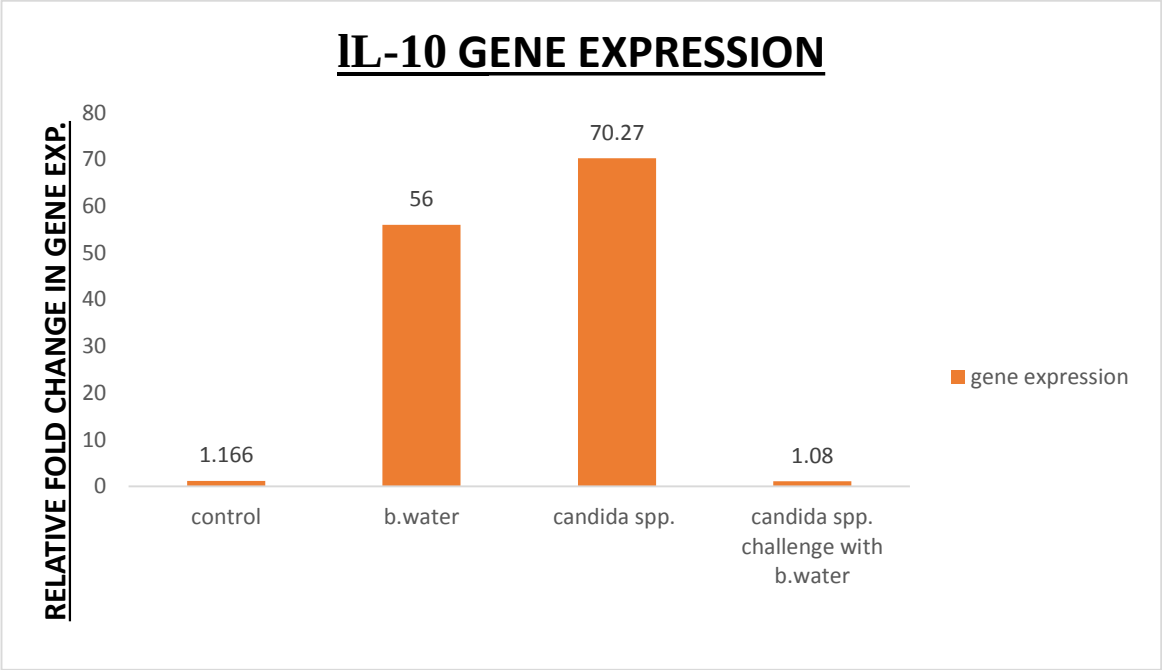


Figure.3 : IL-10 changes in different experimental sets

IV. DISCUSSION

So far, the *Gallus gallus domesticus* embryo's cellular immune response against any strain of *Candida* spp. is hardly investigated. Therefore, though the aim our study was to investigate about the antimicrobial effect of Bakreshwar water, it also covered the response of IL-10 and IFN- γ gene expression in the Gallus gallus domesticus embryo through infecting with *Candida albicans* ATCC 10231.

Interleukin (IL)-10 may play an important role in response to bacterial and fungal infections by modulating excessive inflammation and antimicrobial defences(Moore et.al.,1993).This cytokineis produced by CD4 and CD8 T lymphocytes, monocytes, macrophages, and B lymphocytes (Levitz et.al.,1996). IL-10 plays a major role in the regulation of immunologic and inflammatory responses, including inhibition of T cell and macrophage functions. A previous research suggested that IL-10 has been also shown to suppress nitric oxide production and candidicidal activity of murine macrophages (Cenci, et.al.,1993)and generally is considered a macrophage deactivator (Bogdan, et.al.,1991).

Interferon-gamma (IFN-γ) is a pleotropic cytokine secreted by CD4 Th1, CD8, γδ T, and natural killer (NK) cells. The properties of IFN-γ include regulation of the immune system and the control of infectious disease(Daniel, et.al.,2009).In our study, we have investigated cytokine gene expression after challenging chick embryo (*Gallus gallus domesticus*) with Bakreshwar water infected with *Candida albicans* ATCC 10231. As IL-10 and IFN- γ both the genes are expressed within 4 hours, we have targeted only these cytokine genes expression in this study. Other cytokine gene expressions cannot be studied within 4-5 hours. This restriction of time period is due to experiment with pathogenic strains *Candida albicans* ATCC 10231 which can rapidly multiply in embryonated egg, causing necrosis and putrefaction.

We have also studied using normal control and Bakreshwar water control that gave a result of cytokine gene expression of 1.66 and 56 respectively. Bakreshwar water control is used to observe any preventive and curative changes of IL-10 and IFN- γ gene expression after challenge with *Candida albicans* ATCC 10231 and also observe any improvement of the detrimental action of bioactive compounds secreted by *Candida albicans* ATCC 10231 in morbid anatomy of the embryo.

IL-10 gene expression

After infection with *Candidaalbicans* ATCC 10231 IL-10 gene expression was increased 70.27 times but after challenge with Bakreshwar water, it became normal with a value of 1.1.

IFN- γ gene expression

After infection with *Candida albicans* ATCC 10231 IFN- γ gene expression was increased upto 259.8 times but after challenge with Bakreshwar water, it was significantly decreased to a value of 90.35.

In general, IL-10 and IFN-γ have protective roles against different infections. After giving Bakreshwar water inflammatory changes may be occurred, due to this there was a decrease in IL-10 and IFN-γ gene expressions.

V. CONCLUSION

Previous research work on Bakreshwar water have shown that there is a presence of a green pigment producing bacteria. This aquatic isolate was found to be Gram negative, short rods and catalase positive. The secondary metabolite produced by that bacteria inhibits the growth of other Gram negative bacteria such as *E.coli, Pseudomonas* spp. and *Rhizobium* spp with maximum zone of inhibition of 26.2 mm for *E.coli*(Swati, et.al.,2012).

From our study, we have studied that water from Bakreshwar hot spring has the ability to reduce infections caused by *Candida albicans* ATCC 10231 in the context of cytokine gene expression in *Gallus gallus domesticus* embryo. So, we can conclude that water from Bakreshwar hot spring has a antimicrobial ability against many pathogenic microorganisms.

ACKNOWLEDGMENT

The authors would like to acknowledge Heritage Institute of Technology for providing the infrastructural facility to carry out the research study.

AUTHOR'S CONTRIBUTION

AM carried out the main experiment and collected the data and involved in the manuscript writing. DC and BS carried out the analysis of the experiment. KP involved in the correction of the manuscript. SD carried out in the design of the study, participated in manuscript correction and interpreted the results.

CONFLICT OF INTREST

The authors declare that there is no conflict of interest.

SOURCE OF FUND

There is no funding available for the research study.

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