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Gene Profiling of Toll Like Receptor in Indigenous Chicken and Japanese Quail

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ABSTRACT

Toll like receptors (TLRS) are trans membrane proteins that detect the invading pathogens by binding conserved microbial derived molecules that induce signaling cascades to & pro inflammatory gene expression TLRs utilize leucine rich-motifs (LHRS) for ligand binding. The samples liver and spleen were collected from the bird's indigenous chicken and Japanese quail from the moor market, Chennai. In the present study, DNA was isolated from the samples and DNA bands were separated in 0.8% gel. DNA was quantified, calculated and the DNA quantity was above 200ng. PCR was done for both TLR3 and TLR5 using outer primer as templates. Then the using outer primer, internal primers ip1, ip2, ip3 and ip4 were designed and used for both TLR3 and TLR5. And with the same, bulk was run for the internal primers to elute the amplified and purified DNA. The amplified DNA was extracted from 2% gel and then DNA was purified. It was sequenced using BLAST. Toll – like receptor (TLR) family are vital to immune function through the sensing of pathogenic agents and the initiation of immune response. And they are capable of sensing a wide range of microbes and quickly produce anti-microbial chemokine's and cytokines. The TLR biology on various species modulates that system favorably affect a disease condition. So the TLR opens an exciting era of drug development. Avian TLR biology was different from mammals. There are opportunities to develop species –specific adjuvants and pathogen control strategies will be an impact on animal and human health. Gene profiling of toll-like receptors in indigenous chicken and Japanese quail was carried out in tamilnadu vetinery university, Chennai.

1. Introduction

Immunity broadly involves resistance shown and production offered by host organism against the infectious disease. The immune system consists of a complex network of cells and molecules, and their interaction. The organism is capable of distinguishing the self from non-self, and eliminates non-self. It is broadly divided into two types` innate (non-specific) immunity and adaptive or acquiring (specific) immunity. Innate immunity is not specific and toll-like receptor perform a vital role as sentinels of the innate immune system in the host organism through the recognition of pathogen associated molecular patterns [PAMPs]. Totally 143 non-redundant TCR sequences were there and it represent 26 TLR genes from 30 different species. There were 10 TLRS in chickens and the TLR3, 4, 5 and 7 are directly orthologs of the single TLR2 of mammals (Nicholas Temperly et al., 2008).

2. Methodology

2.1 Sample Collection:

Sample were collected from the indigenous chicken and Japanese quail from the moor market. The parts collected were spleen, lungs, liver, kidney, heart, intestine and stored in-70◦c

2.2 Requirements:-

- **Rpmi Medium:-**
  Rpmi medium (bio chrome USA) used for preparation of media for isolation of tissues.
- **DEPC Water:** (di ethyl pyro carbonate)
  Di ethyl pyro carbonate 0.1% is taken and then added to 100ml triple distilled water and then autoclave to remove DEPC.
- **TAE:** (Tris acetate EDTA buffer)
- **Stock 50X:**

Tris base	-	242g
Glacial acetic acid	-	57.1ml
0.5m EDTA (pH8)	-	100ml
Triple distilled water	-	made up to 1lit

- **Working Solution 1X:** Stock solution of TAE buffer 50×20ml triple distilled water.
- **TBE Buffer**: (Tris borate EDTA)
- **Stock 10X**
  - Tris base - 108g
  - Boric acid - 55g
  - Triple distilled water - 1litre
- **Working solution 1X:**
  - Stock solution TBE buffer 10X-100ML+900ml triple distilled water.
- **Ethidium Bromide (Sigma Solution):**
  Prepare stock solution of 5mg/ml ethidium bromide using TBE working solution and the prepare a working solution in concentration range of 0.5-1mg/ml using TBE working solution.
- **Gel Loading Buffer:**
  - Bromophenol blue - 0.25gm
  - Xylene cyanol - 0.2g

- Sucrose - 40g
- 1X TBE buffer to 100ml

- **Gel Casting: 0.8%Gel:**
  - Agarose - 0.2g
  - TAE buffer - 25ml

- And the TAE was added to agarose and kept in micro oven for 30seconds.
- Then 2µl of ethidium bromide was added and then the gel was casted.

2% Gel:

- Agarose - 0.5g
- TAE Buffer - 25ml

- **Proteinase K Solution:**
  Dissolve 15g 0f proteinase K and dissolve in 1ml of distilled water. Initially pre incubate at 37◦c for 2hours and store at-20◦c
- **Absolute Ethanol:**
  Absolute ethanol of 96% were taken,

- **Methods:**

1. DNA Extraction using Genomic DNA Extraction Kit for Tissues

1.1 Tissue Dissociation:

- Take 10mg of the tissue (spleen, liver) and chop it into small piece and put it in the micro centrifuge tube.
- Grind and taken tissue sample with the help of modern and pestle along with liquid nitrogen (LN2)
- Along with it add 200µl of GT buffer while grinding.

1.2 Lysis:

- Add 20µl of proteinase k and vortex immediately
- Incubate at 60◦c for 30 minutes for the cell lysis. (During incubation invert the tube every 10min)
- Add 200µl of GB buffer and mix by vortex for 5v seconds.
- Incubate at 70◦c for 20min until the sample lysate is clear. (During incubation invert the tube every 5min)
- Preheat DEPC H2O (25µl/sample) at 70◦c
- If the sample is insoluble then centrifuge at maximum rpm

1.3 DNA Binding:

- Add 200µl ethanol (96%) to make sample lysate clear and suddenly mix by vortexing. If precipitate acquires break it by pipetting.
- Place a GD column in collection tube which is already preheated
- Centrifuge at 13,000rpm for 2min
- Discard the flow through and place a new collection tube

2. Wash

- Add 400µl of W1 buffer to GD column and centrifuge at full speed 13,000rpm for 30 seconds.
- Discard the flow through GD column
- Add 600µl of wash buffer in the GD column and centrifuge at 13,000rpm for 3min to dry the column matrix

3. DNA Elution:

- Transfer dried GD column into a 1.5ml micro centrifuge tube.
- Then add 25µl of preheated elution buffer. Allow it to stand for 5min until elution buffer is absorbed by matrix.
- Centrifuge at full speed 13,000rpm for 30seconds to elute the purified DNA.

4. RNA Degradation:

- (It is don before DNA binding)
- After 70◦c incubation, add 4C of Rnase A to sample lysate and mix by vortexing.
- Incubate at room temperature. (If RNA free genomic DNA is preferred add this step)

5. DNA Quantification:

DNA was quantified using **cubit Invitrogen flurometer DNA**

- Buffer - 198µl
- Fluoresce dye - 1µl
- (Quibit Invitrogen)
- Sample - 1µl
- Add 198µl of quantifying buffer, 1µl of fluorescent-dye 1µl of sample.
- Mix it by vortexing
- Transfer the sample to the quantifying tube.
- Measure the concentration of 1µl of sample DNA in cubic flurometer

6. DNA Elution From the Agarose Gel:

(DNA elution was done using the kit **Tarus scientific genie)**

- The amplified DNA was cut from the agarose gel.
- To it add 3 volume of extraction buffer and incubated it for 20min at 50◦c, simultaneously preheat the elution column.
- The preheat elution column transfer the content.
- Centrifuge it to 10,000rpm for 3min.
- Again add another 300µl extraction buffer and centrifuge at 10,000rpm for 3min.
- Discard the follow through the column.
- Add 400µl of wash buffer and centrifuge at 10,000rpm for 3min.
- Discard the flow through the column and dry the column using centrifuge.
- Elute it in 25µl DEPC H2O after leave the column with DEPC H20 for 3min in the column.
- Then centrifuge at 10,000rpm for 1min and the amplified DNA is obtained

7. TLR For Indigenous Chicken and Japanese Quail:

Outer Primer (Op) Full Length

- PCR master mix 12.5µl was taken and 7.5µl of DEPC H2O was added in a 0.5ml PCR tubes.

- To it add 1µl of TLR3 full length forward primer (FP) and full length reverse primer was added
- Then the DNA extracted the template 3µl was added to the mixture.
- It was vortex so that the mixture gets mixed thoroughly.(note: There should not be any air bubbles)

The PCR was run in the following condition for the outer primer.

94 – 4min
94 -1min
52 -0.30seconds (annealing)
72 -2min
72 -7min
4◦ -hold

8. Internal Primers IP

- There are four internal primers namely TLR3 IP1, IP2, IP3, and IP4.
- Green dye master mix 12.5µl was taken and to it adds 7.5µl of DEPC H2O.
- Then to it 1µl of TLR3 IP1 FP and 1µl of TLR3 IP1 RP was added.
- 3µl of OP was added to it and vortex thoroughly.

And other PCR condition was given as

94 – 4min
94 – 1min
54 – 0.30min
72 – 1.30min
72 – 7min
4◦ -hold

The same procedure was given for the TLR3 IP2, IP3 and IP4. But different condition was applied.

Sequencing Using BIAST

(Big Dye Terminator vs. 3.1 clean up (Tube method))

- Transfer the reaction product into a 1.5ml tube.
- Make a master mix I 10µl Milli-Q and 2µl of 12mm EDTA per reaction.
- Add 12µl of master mix I to each reaction containing 10µl of reaction. Ensure to the contents are mixed
- Make master mix II of 2µl of 3M NaOH, PH 4.6 and 50µl of ethanol per reaction.
- Add 52µl of master mix II to each reaction.
- Mix the contents well and incubate at room temperature for 15min.
- Decant the supernatant.
- Add 12-15µl of Hi-Di formaldehyde, transfer to sample tubes cover with septa, denature, snap chill and proceed for electrophoresis.

3. Results and Discussion

Gene profiling of toll like receptors in indigenous chicken and Japanese quail was carried out in Tamil Nadu veterinary university, Chennai. The results are produced and discussed in this paper.

DNA Isolation:

The DNA was isolated using liquid nitrogen from liver and spleen as it contains the high amount of DNA. So that DNA concentration should be above 200ng. This work is supported by previous work done in mammalian cells (Sharma, et al., 1993). In his work quantified amount of DNA sample was isolated using the similar procedure.

PCR Technique:

PCR was done for all the internal primers amplification of the DNA using the outer primer as the template. Totally 10 primers were used and out of it 2 were used as template and 5 showed positive result and 3 showed negative result .This was supported by previous work done in chicken (Muhammed Iqubal et al., 2004).

Sequencing Using BLAST

The TLR3 ip1 was sequenced using BLAST, its query length was 822 and for the complete sequence of Gallus the maximum score value and total score was 1291, query coverage is 98%, E value 0 and maximum identity was noted as 95%. For partial sequence the maximum score value and total score value 1288, query coverage is 95%, E value 0 and maximum identity was noted as 96%.

The TLR3 ip2 was sequenced using BLAST, its query length was 779 and for the complete sequence of Gallus gallus maximum score value and total score was 1173, query coverage is 99%,E value 0 and maximum identity was noted as 94% . For partial sequence it has the same value as the complete sequence.

The TLR3 ip3 was sequenced using BLAST, its query length was 787 and for the complete sequence of Gallus gallus maximum score value was 294 and total score was 1007, query coverage is 77%, E value 4e-76 and maximum identity was noted as 100%. For partial sequence it has the same value as the complete sequence.

The TLR5 ip1 was sequenced using BLAST, its query length was 366 and for the complete sequence of Gallus gallus maximum score value and total score was 475, query coverage is 97%, E value 6e-131 and maximum identity was noted as 90%. For partial sequence the maximum score value and total score value 333 query coverage is 68% E value 4e-88 maximum identity was noted as 90%.

The TLR5 ip2 was sequenced using BLAST, its query length was 274 and for the complete sequence of Gallus gallus maximum score value and total score was 150, query coverage is 95%, E value 6e-131 and maximum identity was noted as 76%. (Zheng zhang et al., 2000).

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4. Conclusion

Toll – like receptor (TLR) family are vital to immune function through the sensing of pathogenic agents and the initiation of immune response. And they are capable of sensing a wide range of microbes and quickly produce anti-microbial chemokine's and cytokines. The TLR biology on various species modulates that system favorably affect a disease condition. So the TLR opens an exciting era of drug development. Avian TLR biology was different from mammals. There are opportunities to develop species –specific adjuvants and pathogen control strategies will be an impact on animal and human health.

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