UNIVERSITI PUTRA MALAYSIA

CANDIDATE GENES AND GENE EXPRESSION ANALYSIS FOR RESPONSE TO Salmonella enteritidis CHALLENGE IN YOUNG MALAYSIAN INDIGENOUS CHICKENS

REZA TOHIDI

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By

REZA TOHIDI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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DEDICATION

TO

I dedicate this dissertation to my dear parents and wife who have sacrificed their good life for my progress and for their encouragement and support during this process.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

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Chairman : Associate Professor Dr. Ismail Bin Idris, PhD

Faculty : Agriculture

Salmonella enteritidis (SE) is a common cause of food-borne disease in humans and loss of growth in poultry. The main source of infection for humans is contaminated food especially poultry products. Protocol to control salmonellosis is similar that of other infectious diseases. However, resistance of the bacteria to antibiotics and non-effectiveness of vaccination have been reported. The effective method for inhibition of salmonellosis is increasing of genetic resistance of poultry to *Salmonella* through genetic selection programs that may be performed based on phenotypic or genotypic data. There are two major problems in the traditional selection for disease resistance. First, the heritability for resistance to salmonellosis is low and second, collection of data is generally not feasible, hazardous and impractical. Marker-assisted selection (MAS) has been introduced to improve these kinds of traits. For this goal, the

choice of a suitable molecular marker or a candidate gene that being polymorphic for that is necessary. On the other hand, indigenous chickens have priority in selection programs for improving genetic resistance to disease, as these genetic types have been evolutionary adapted to the local environment. Based on these hypotheses, the objective of this study was to evaluate the ability to resist SE infection in Malaysian village and red jungle fowl chickens using candidate gene analysis. Three studies were designed for this goal. In the first study, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to investigate the levels of diversity in 16 candidate genes responsible for immunological reactions in chicken. These genes were inducible nitric oxide synthase (*iNOS*), natural resistance-associated macrophage protein 1 (*NRAMP1*), immunoglobulin light chain (*IgL*), transforming growth factor β family (*TGFβ2*, *TGFβ3*, *TGFβ4*), interleukin 2 (*IL2*), inhibitor of apoptosis protein 1 (*IAP1*), toll like receptor (*TLR4*), myeloid differentiation protein 2 (*MD2*), interferon γ (*IFNγ*), caspase 1 (*CASP1*), lipopolysaccharide induced tumor necrosis factor (TNF)-α factor (*LITAF*), tumor necrosis factor related apoptosis inducing ligand (*TRAIL*), cluster of differentiation 28 (*CD28*) and prosaposin (*PSAP*). Fourteen candidate genes were polymorphic and in Hardy-Weinberg equilibrium (HWE). The results of the current study showed that *CD28* and *PSAP* were monomorphic in both breeds, and *LITAF* was monomorphic in the red jungle fowl. The minimum and maximum of the observed heterozygosity belonged to *IL2* and *TGFβ4* in red jungle fowl, respectively. Allele 'T' of *LITAF* was fixed in red jungle fowl and allele 'C' of *iNOS*, 'T' of *CASP1*, 'G' of *IL2* and 'A' of *MD2* were present at low frequencies in red jungle fowl. Based on the Shannon information index (I),

nine candidate genes were highly polymorphic in both breeds. However, *iNOS* and *IL2* were highly polymorphic in only village chickens.

In the second study, the association of different genotypes of 14 polymorphic candidate genes acquired from the first study with SE counts in the cecum, spleen and liver of the chickens was investigated. For this study, one day-old chicks were inoculated with 1×10^7 cfu/ml SE phage type 13a. The samples were collected seven days after inoculation. The bacterial counts in the cecum are more than in the spleen and liver ($P < 0.01$). Overall, the most significant associations were found for cecum and spleen SE counts. The polymorphisms in *iNOS*, *NRAMP1*, *TGFβ2*, *TGFβ3*, *IL2*, *IFNγ*, *TLR4* and *TRAIL* were associated with SE load. For liver, six genes (*iNOS*, *TGFβ2*, *TGFβ3*, *TGFβ4*, *TLR4* and *TRAIL*) had significant associations with SE counts.

In the third study, the levels of expressions of five genes including *NRAMP1*, *TLR4*, *IFNγ*, *IgY* and *IL8* in cecum, spleen and liver 48 hours after inoculation with 1×10^7 SE in both breeds were analyzed. Real time reverse transcription PCR was used to quantify the fold change in mRNA expression. The results showed that all the genes were expressed highly 48 h after inoculation in the cecum of both breeds. However, *IgY* was significantly expressed in spleen. Moreover, the fold change in expression of *IL8* was significantly higher in the liver of village chickens and red jungle fowl chicks ($P < 0.05$).

The results of this study indicated that most of the candidate genes were highly polymorphic and only a few of them were monomorphic or lowly polymorphic.

Random mating may be considered as the main reason for this high level of gene diversity. Moreover, some of these candidate genes (*iNOS*, *NRAMP1*, *TGFβ2*, *TGFβ3*, *TGFβ4*, *IL2*, *IFNγ*, *TLR4* and *TRAIL*) were associated with SE burden in young chicks. Thus, they can be appropriate markers in selection programs. On the other hand, the immune genes were expressed after 48 h post-inoculation in the cecum but not in spleen or liver. Therefore, the effects of immune genes should not be investigated for during the early hours after hatching to find suitable candidate genes in young chicks. Malaysian indigenous chickens have been adapted to the tropical environment and based on the result of this study, they can be considered as potential gene pool of alleles responsible for resistance to local diseases.

Abstrak tesis dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

ANALISIS GEN CALON DAN UNGKAPAN GEN BAGI TINDAK BALAS TERHADAP TENTANGAN *Salmonella enteritidis* PADA ANAK AYAM TEMPATAN MALAYSIA

Oleh

REZA TOHIDI

November 2012

Pengerusi : Profesor Madya Dr. Ismail B Idris, Ph.D.

Fakulti : Pertanian

Salmonella enteriditis (SE) merupakan penyebab biasa bagi penyakit bawaan makanan pada manusia dan kehilangan pertumbuhan pada poltri. Sumber utama jangkitan pada manusia adalah makanan yang tercemar terutamanya produk poltri. Protokol pengawalan salmonellosis adalah serupa dengan kaedah pengawalan penyakit berjangkit yang lain. Walaubagaimanapun, kerintangan bakteria terhadap antibiotik dan ketidakberkesanan vaksin telah dilaporkan. Kaedah yang berkesan untuk merencat salmonellosis ialah dengan meningkatkan rintangan genetik poltri terhadap salmonella melalui program pemilihan genetik berdasarkan data-data fenotip dan genotip. Terdapat dua masalah utama dalam pemilihan secara tradisional untuk rintangan terhadap penyakit. Pertama, pewarisan rintangan terhadap *salmonellosis* adalah rendah dan kedua, pengumpulan data secara umumnya adalah sukar untuk dilaksanakan, berbahaya dan tidak praktikal.Untuk meningkatkan ciri-ciri ini pemilihan berbantu penanda telah diperkenalkan. Untuk mencapai matlamat ini

pemilihan penanda molekul yang sesuai atau gen calon yang polimorfik kepada penanda tersebut adalah perlu. Sebaliknya, ayam kampung menjadi keutamaan dalam program pemilihan genetik bagi meningkatkan kerintangan genetik terhadap penyakit oleh kerana jenis genetik ini telah menyesuaikan diri terhadap persekitaran tempatan melalui evolusi. Justeru, berdasarkan hipotesis tersebut, objektif kajian ini adalah untuk menilai keupayaan rintangan ayam kampung dan ayam hutan terhadap SE dengan menggunakan analisis gen calon. Tiga kajian telah direka untuk mencapai objektif ini.

Kajian pertama menggunakan teknik 'Polymerase chain reaction-restriction fragment lenght polymorphism' *(PCR-RFLP)* untuk menilai kepelbagaian dalam 16 gen calon yang memberi kesan kepada reaksi imun ayam. Gen-gen calon tersebut ialah *inducible nitric oxide synthase (iNOS)*, *natural resistance-associated macrophage protein 1* (*NRAMP1)*, *immunoglobulin light chain (IgL)*, *transforming growth factor β family* (*TGFβ2*, *TGFβ3*, *TGFβ4)*, *interleukin 2 (IL2)*, *inhibitor of apoptosis protein 1 (IAP1)*, *toll like receptor (TLR4)*, *myeloid differentiation protein 2 (MD2)*, *interferon γ(IFNγ)*, *caspase 1 (CASP1)*, *lipopolysaccharide induced tumor necrosis factor (TNF)-α factor (LITAF)*, *tumor necrosis factor related apoptosis inducing ligand (TRAIL)*, *cluster of differentiation 28 (CD28)* dan *prosaposin* (*PSAP*). Empat belas gen calon adalah polimorfik dan mengikut keseimbangan Hardy-Weinberg (HWE). CD8 dan PSAP adalah polimorfik di kedua-dua baka. Nilai minimum dan maksimum bagi heterozigositi yang diperhatikan masing-masing dipunyai oleh *IL2* dan *TGFβ4.* Alel 'T' di *LITAF* tidak berubah pada ayam hutan merah, adalah dalam frekuensi yang rendah. Berdasarkan Indeks Informasi Shannon

(I), sembilan gen calon adalah sangat polimorfik di kedua-dua baka. Walaubagaimanapun, *iNOS* dan *IL2* sangat polimormik hanya pada ayam kampong.

Dalam kajian kedua, hubungkait antara genotip berbeza pada 14 gen calon polimorfik yang di ambil daripada kajian pertama dengan kiraan SE pada sekum, limpa dan hati ayam telah dikaji. Untuk kajian ini anak ayam berumur sehari telah diinokulasi dengan 1 x 10^7 cfu /ml SE phage jenis 13a. Sampel diambil 7 hari selepas inokulasi. Kiraan bakteria di dalam sekum adalah lebih banyak daripada limpa dan hati ($P < 0.01$) Secara keseluruhannya hubungkait yang paling signifikan adalah pada kiraan SE di sekum dan limpa, Polimorfisma pada *iNOS*, *NRAMP1*, *TGFβ2*, *TGFβ3*, *IL2*, *IFNγ*, *TLR4* dan *TRAIL* mempunyai hubungkait dengan kiraan SE. Bagi hati, enam gen (*iNOS*, *TGFβ2*, *TGFβ3*, *TGFβ4*, *TLR4* dan *TRAIL)* telah dikenalpasti mempunyai hubungkait yang signifikan dengan kiraan SE.

Dalam kajian ketiga, aras ekspresi lima gen termasuk *NRAMP1*, *TLR4*, *IFNγ*, *IgY* dan *IL8* dalam sekum, limpa dan hati kedua-dua baka telah dianalisis 48 jam selepas inokulasi dengan 1 × 10^7 SE. Teknik 'Real time Reverse Transcription PCR' telah digunakan untuk mengukur perubahan gandaan dalam ekspresi mRNA. Walaubagaimanapun, hanya *IgY* diekpresikan secara signifikan dalam limpa

Keputusan kajian ini menunjukkan bahawa kebanyakan gen calon adalah sangat polimorfik dan hanya beberapa monomorfik atau kurang polimorfik.

Pengawanan rawak mungkin boleh dianggap sebagai penyebab utama terhadap kepelbagaian gen yang tinggi. Tambahan pula, sebahagian daripada gen-gen calon ini berhubungkait dengan beban SE dalam anak ayam. Maka, gen-gen calon ini boleh menjadi penanda yang sesuai dalam program pemilihan. Sebaliknya, gen imun diekspresi selepas 48 jam pos inokulasi di dalam sekum dan bukannya di limpa atau hati. Oleh yang demikian, kesan gen imun tidak seharusnya dikaji pada jam-jam terawal selepas penetasan dalam mencari gen calon yang sesuai pada anak ayam. Ayam asli Malaysia telah menyesuaikan diri dengan persekitaran tropika dan berdasarkan hasil kajian, baka ini boleh dianggap sebagai kolam gen yang berpotensi untuk alel rintangan terhadap penyakit tempatan.

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I certify that an Examination Committee has met on **date of viva voce** to conduct the final examination of **Reza Tohidi** on his Doctor of Philosophy thesis entitled "Candidate Genes and Gene Expression Analysis for Response to *Salmonella* Enteritidis Challenge in Young Malaysian Indigenous Chicken" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awardwd the degree of Doctor of Philosophy (PhD).

Members of the Examination Committee were as follows:

Halimatun bt Yaakub, PhD
Associated Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Tan Soon Guan, PhD
Professor
Faculty of Biotechnology and Molecular Science
Universiti Putra Malaysia
(Internal Examiner)

Zunita binti Zakaria, PhD
Associated Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

Brian Kirkpatrick, PhD
Professor
Department of Animal Science
University of Wisconsin
The United States of America
(External Examiner)

_____
SEOW HENG FONG, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfillment of the requirements for the degree of Doctor of Philosophy. The members of the Supervisory Committee are as follows:

Ismail B Idris, PhD
Associated Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Jothi Malar Panandam, PhD
Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

Mohd Hair Bejo, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

_____
BUJANG BIN KIM HUAT, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

DECLARATION

I declare that the thesis is my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at Universiti Putra Malaysia or other institutions.

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REZA TOHIDI

Date: 28 November 2012

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