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DEVELOPMENT OF SYBR GREEN I REAL-TIME PCR METHOD FOR DETECTION AND DIFFERENTIATION OF NEWCASTLE DISEASE VIRUS PATHOTYPES

TAN SHEAU WEI

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DOCTOR OF PHILOSOPHY
UNIVERSITI PUTRA MALAYSIA

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By

TAN SHEAU WEI

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

AUGUST 2008

This dissertation specially dedicated to,

My late father, Tan Ke Eng,
who always lives in my heart.

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

DEVELOPMENT OF SYBR GREEN I REAL-TIME PCR METHOD FOR DETECTION AND DIFFERENTIATION OF NEWCASTLE DISEASE VIRUS PATHOTYPES

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TAN SHEAU WEI

AUGUST 2008

Chairman: Professor Aini Ideris, PhD

Faculty: Veterinary Medicine

Newcastle disease (ND) which is caused by Newcastle disease virus (NDV) is a highly contagious viral disease of domestic poultry, cage, aviary and wild birds. ND outbreaks have led to substantial losses in the poultry industry. NDV can be classified into three major pathotypes: velogenic, mesogenic and lentogenic. Velogenic strains are highly virulent and may lead to 100% mortality in infected chicken whilst mesogenic and lentogenic strains cause mild clinical or inapparent infections, respectively. Early detection and differentiation of NDV pathotypes are very important during monitoring of suspected ND cases or during disease outbreaks. In this study, SYBR Green I real- time polymerase chain reaction (PCR) was developed for detection and differentiation of NDV pathotypes. Velogenic-specific primers (NDVIF2 & NPV2N) and lentogenic-specific primers (NDVIF2 & NPL2N) were designed to detect specific sequence of velogenic strains and lentogenic/vaccine strains, respectively.

After establishing the optimum condition of the real-time PCR, the assay was performed on 22 previously characterized NDV strains. All the velogenic strains were only detected by using velogenic-specific primers (NDVIF2 & NPV2N) with threshold cycle (Ct) ranged from 12.92 to 22.76 and melting temperature between 85.6°C to 86.4°C. Similarly, all the lentogenic/vaccine strains were only successfully detected when lentogenic-specific primers (NDVIF2 & NPL2N) were used. All the lentogenic/vaccine strains amplified with the lentogenic-specific primer had a Ct value ranged from 11.93 to 18.73 and Tm between 87.2°C to 87.6°C. No amplification was found when the NDV velogenic-specific primers and lentogenic-specific primers were used to amplify avian influenza virus (AIV), infectious bronchitis virus (IBV) and infectious bursal disease virus (IBDV). This revealed that both velogenic- and lentogenic-specific primers were pathotype specific and no unrelated viral RNA can be amplified. The newly developed assay had a dynamic detection limit which spans over a 5 $\log_{10}$ concentration range. The velogenic and lentogenic amplifications showed high PCR efficiency of 98.8% and 103%, respectively. Mean coefficient variation (CV) of reproducibility tests for velogenic amplification and lentogenic amplification was around 1% and 2%, respectively. The SYBR Green I real-time PCR was 10-fold more sensitive when compared to the conventional detection method using agarose gel electrophoresis. Turnaround time for the developed assay was approximately 2.5 hours including reverse transcription, PCR amplification and melting curve analysis.

Clinical samples from the experimental infected chickens as well as the suspected field cases were collected and then tested on the developed assay. In the experimental infection with lentogenic NDV F strain, virus could be detected 3 days post infection

UPM

(p.i.), followed by day 4, 5 and 10 p.i. For the SPF chickens infected with high doses of velogenic NDV strain AF2240 (10^5 to 10^3 ELD_{50}/0.1 ml), the virus can be detected as early as day 2 p.i., followed by day 3 and 4 p.i. All the infected chickens were dead on day 4 p.i. For the chickens group infected with low doses (10^2 to $10^{0.5}$ ELD_{50}/0.1 ml), the virus can be detected starting on day 4 p.i., followed by day 5, 7, 10, 11 and 12 p.i. All the infected chickens were dead on day 12 p.i. The assay was able to detect the viruses as early as day 2 before the observation of clinical signs. This is an important achievement as early detection can prevent further spread of the disease. A total of 41 suspected NDV field cases were tested with the developed assay, 33 cases were NDV negative and 8 cases were positive for velogenic NDV. The results were correlated well with the virus isolation method and F cleavage site sequence analysis. All these 8 isolates possess two pairs of dibasic amino acids at the position 112 to 116 of the F cleavage site, and a phenylalanine residue at the position 117. This F cleavage site analysis revealed that all of the 8 NDV isolates belonged to velogenic group.

In the attempt to improve the efficacy of the developed assay, internal amplification control (IAC) was incorporated into the developed real-time PCR assay for detection of PCR inhibitors. The potential of simultaneous detection of IAC and NDV target was investigated. The simultaneous detection was achieved based on the melting curve analysis. The co-amplified products exhibited two distinguished melting peaks at 86.36±0.13°C and 91.42±0.21°C which corresponded to NDV NP gene product and IAC KanR gene product, respectively.

In conclusion, this study successfully developed a SYBR Green I real-time PCR for NDV pathotypes detection and differentiation. The virus can be detected directly from clinical samples without the need of virus propagation in chicken embryonated eggs. Owing to these advantages, the developed assay will contribute significantly in the control and prevention of the spread of the disease. ND-infected birds can be rapidly isolated from the healthy bird in the case of field outbreaks, if the causal agent is detected at the early stage of the outbreak. Consequently, spread of the disease and economical losses can be prevented.

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

PEMBANGUNAN PCR MASA NYATA SYBR GREEN I UNTUK PENGESANAN DAN PEMBEZAAN PATOTIP VIRUS PENYAKIT NEWCASTLE

Oleh

TAN SHEAU WEI

OGOS 2008

Pengerusi : Profesor Aini Ideris, PhD

Fakulti : Perubatan Veterinar

Penyakit Newcastle (ND) yang disebabkan oleh virus penyakit Newcastle (NDV) merupakan penyakit yang mudah menjangkiti ternakan ayam, burung-burung peliharaan dan burung-burung liar. Penyakit Newcastle telah menyebabkan kerugian yang serius dalam industri ternakan ayam. NDV boleh dibahagikan kepada 3 patotip utama: velogenik, mesogenik dan lentogenik. Strain velogenik adalah sangat virulen dan boleh menyebabkan 100% kematian pada ayam yang dijangkiti manakala strain mesogenik dan lentogenik masing-masing hanya menyebabkan jangkitan yang biasa atau jangkitan yang tidak ketara. Pengesanan dan pembezaan patotip NDV pada peringkat awal adalah sangat penting dalam mengawal wabak ND. Dalam kajian ini, PCR masa nyata SYBR Green I telah dibangunkan untuk mengesan dan membezakan patotip NDV. Primer velogenik (NDVIF2 & NPV2N) dan primer lentogenik (NDVIF2 & NPL2N) telah direka untuk pengesanan jujukan nukleotida yang spesifik pada strain velogenik dan strain lentogenik/vaksin masing-masing.

Selepas menetapkan keadaan optimum bagi PCR masa nyata, 22 strain NDV di mana patotipnya telah ditentukan dahulu digunakan dalam kajian ini. Primer velogenik (NDVIF2 & NPV2N) hanya dapat megesan strain velogenik dengan had kitaran (Ct) berjulat antara 12.92 ke 22.60 dan suhu peleburan (Tm) di antara 85.6°C dan 86.4°C. Manakala, primer lentogenik (NDVIF2 & NPl2N) hanya mengesan strain lentogenik/vaksin dengan Ct berjulat antara 11.93 ke 18.73 dan Tm di antara 87.2°C dan 87.6°C. Kedua-dua velogenik dan lentogenik primer tidak mengesan virus-virus lain seperti virus selsema burung (AIV), virus penyakit berjangkit bursa (IBDV) dan virus penyakit berjangkit bronkitis (IBV). Ini menunjukkan primer-primer tersebut adalah sangat spesifik dan tidak menghasilkan amplifikasi apabila RNA daripada virus lain digunakan. Keadah yang dibangunkan dalam kajian ini mempunyai had pengesanan meliputi julat kepekatan sebanyak 5 $\log_{10}$. Amplifikasi velogenik dan lentogenik masing-masing menunjukkan kecekapan PCR 98.8% dan 103%. Purata koefisien varian (C.V.) bagi ujian reproduksibiliti dalam amplifikasi velogenik dan lentogenik masing-masing adalah 1% dan 2%. PCR masa nyata SYBR Green I yang dibangunkan ini adalah 10-kali lebih sensitif daripada kaedah pengesanan tradisional iaitu gel agarose elektroforesis. Kaedah ini boleh disiapkan dalam masa kira-kira 2.5 jam termasuk transkripsi berbalik, amplifikasi PCR dan analisis lengkungan peleburan.

Sampel klinikal daripada eksperimen jangkitan ayam dan beberapa kes daripada ladang yang disyaki telah dikumpulkan dan diuji menggunakan kaedah yang dibangunkan ini. Dalam eksperimen jangkitan dengan strain lentogenik F, virus boleh dikesan pada hari ke-3 selepas jangkitan (p.i.), diikuti dengan hari ke-4, ke-5 dan ke-10 p.i. Bagi kumpulan ayam SPF yang dijangkiti dengan dos tinggi strain velogenik

AF2240 (10^5 to 10^3 ELD_{50}/0.1 ml), virus boleh dikesan pada hari ke-2 p.i., diikuti dengan hari ke-3 dan hari ke-4 p.i. Semua ayam dalam kumpulan ini mati selepas hari ke-4 p.i. Bagi kumpulan ayam SPF yang dijangkiti dengan dos rendah strain velogenik AF2240 (10^2 to $10^{0.5}$ ELD_{50}/0.1 ml), virus boleh dikesan bermula pada hari ke-4 p.i., diikuti dengan hari ke-5, hari ke-7, hari ke-10, hari ke-11 dan hari ke-11 p.i. Semua ayam dalam kumpulan ini mati selepas hari ke-12 p.i. Kaedah PCR masa nyata ini dapat mengesan virus seawal hari ke-2 p.i. sebelum tanda-tanda klinikal ditunjukkan. Ini merupakan suatu pencapaian penting kerana pengesanan pada peringkat awal dapat mencegah penyakit Newcastle merebak. Sejumlah 41 kes NDV dari ladang yang disyaki telah diuji menggunakan kaedah yang dibangunkan, 33 kes adalah NDV negatif dan 8 kes positif bagi NDV velogenik. Keputusan tersebut adalah sejajar dengan keputusan-keputusan daripada kaedah pencilan virus dan analisis jujukan nukleotida di bahagian pencelahan protein F. Kesemua 8 isolat NDV yang positif dalam PCR nyata masa memiliki dua pasang asid amino basik di kedudukan 112 ke 116 dan residu Phe di kedudukan 117 pada bahagian pencelahan protein F. Jujukan asid amino pada bahagian pencelahan protein F ini menunjukkan kesemua 8 isolat NDV adalah velogenik.

Kawalan amplifikasi dalaman (IAC) telah dimasukkan dalam kaedah PCR masa nyata yang dibangunkan untuk mengesan perencat PCR. Potensi pengesanan IAC dan target NDV secara serentak telah diuji. Pengesanan secara rentak ini dicapai melalui analisis lengkungan peleburan. Produk bagi amplifikasi serentak ini mempamerkan 2 tahap peleburan yang berbeza pada 86.36±0.13°C dan 91.42±0.21°C, masing-masing mewakili produk gen NP bagi NDV dan gen KanR bagi IAC.

Kesimpulannya, kajian ini telah berjaya membangunkan satu kaedah PCR masa nyata SYBR Green I untuk pengesanan dan pembezaan patotip NDV. Virus dapat dikesan secara langsung daripada sample klinikal dan pertumbuhan virus dalam telur tidak diperlukan. Kaedah yang dibangunkan ini akan memberi sumbangan dalam mengawal dan mencegah kemerebakan penyakit Newcastle. Ayam yang dijangkiti penyakit Newcastle dapat dipisahkan daripada ayam-ayam yang sihat dengan segera, sekiranya agen jangkitan dapat dikesan pada peringkat awal berlakunya wabak penyakit. Dengan demikian, kemerebakan penyakit and kerugian ekonomi dapat di cegah.

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I certify that an Examination Committee has met on 29 August 2008 to conduct the final examination of Tan Sheau Wei on her Doctor of Philosophy thesis entitled "Development of SYBR Green I real-time PCR method for Detection And Differentiation of Newcastle Disease Virus Pathotypes" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (High Degree) Regulation 1981. The Committee recommends that the candidate be awarded the Doctor of Philosophy.

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DECLARATION

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

TAN SHEAU WEI

Date:

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