UNIVERSITI PUTRA MALAYSIA

# DEVELOPMENT OF AN RT NESTED PCR-ELISA DIAGNOSTIC TEST FOR THE DETECTION OF NEWCASTLE DISEASE VIRUS

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# DEVELOPMENT OF AN RT NESTED PCR-ELISA DIAGNOSTIC TEST FOR THE DETECTION OF NEWCASTLE DISEASE VIRUS

By

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**This thesis is specially dedicated to**

**my beloved family and**

**friends .....**

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| | |
|---|---|
| ABTS | 2,2'- azinodi-ethylbenzothiazolinesulphonate acid |
| BCP | 1-bromo-3-chloropropane |
| bp | base pairs |
| $^{o}C$ | degree Celcius |
| cDNA | complimentary DNA |
| DEPC | diethyl pyrocarbonate |
| $dH_2O$ | distilled water |
| DIG | digoxigenin |
| DNA | deoxyribonucleic acid |
| dNTP | deoxynucleoside triphosphate |
| EDTA | ethylenediamine tetraacetic acid |
| $EID_{50}$ | egg infective dose 50% |
| ELISA | enzyme linked immunosorbent assay |
| F | fusion |
| fg | fentogram |
| FIP | inner primer |
| FOP | outer primer |
| x *g* | x gravity |
| h | hour |

| | |
|---|---|
| HA | haemagglutination |
| HN | haemagglutinin-neuraminidase |
| HI | haemagglutination -inhibition |
| kb | kilobase pairs in duplex nucleic acid, kilobases in single-stranded nucleic acid |
| KCl | potassium chloride |
| $KH_2PO_4$ | di-potassium hydrogen phosphate anhydrous |
| L | large |
| M | molar |
| $MgCl_2$ | magnesium chloride |
| MTP | microtiter plate |
| µl | microliter |
| min | minute |
| mM | milimolar |
| $Na_2HPO_4$ | di-natrium hydrogen phosphate |
| ND | Newcastle disease |
| NDV | Newcastle disease virus |
| ng | nanogram |
| NP | nucleoprotein |
| NTE | natrium choride-Tris-EDTA buffer |
| P | phosphoprotein |
| PBS | phosphate-buffered saline |

| | |
|---|---|
| PCR | polymerase chain reaction |
| PD | primer-dimer |
| pfu | plaque forming unit |
| pg | picogram |
| RNA | ribonucleic acid |
| RNase | ribonuclease |
| RT-PCR | reverse transcriptase-polymerase chain reaction |
| s | second |
| SD | standard deviation |
| TBS | Tris-buffered saline |
| $T_m$ | melting temperature |
| U | unit |
| UPM | Universiti Putra Malaysia |
| UV | ultraviolet |
| V | volt |
| VRI | Veterinary Research Institute |
| VVNDV | viscerotropic velogenic Newcastle disease virus |
| v/v | volume per volume |
| w/v | weight per volume |

Abstract of thesis submitted to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science.

**DEVELOPMENT OF AN RT NESTED PCR-ELISA DIAGNOSTIC TEST FOR THE DETECTION OF NEWCASTLE DISEASE VIRUS**

By

**KHO CHIEW LING**

**June 1999**

Chairperson: **Associate Professor Khatijah Mohd. Yusoff, Ph.D.**

Faculty: **Science and Environmental Studies**

Newcastle disease virus causes an economically important poultry disease known as Newcastle disease in Malaysia. The velogenic strain of this virus causes 100% mortality in infected chicken. Therefore, a rapid and sensitive diagnostic method is necessary for the detection of the virus. In this study a sensitive one tube reverse transcription nested PCR was developed by using two nested pairs of primer which were designed from the consensus fusion gene sequences. The outer primer sequences are 5'-TACACCTCATCCCAGACAGGGTC-3' (FOP1) and 5'-AGGCAGGGGAAGTGATTTGTGGC-3' (FOP2). The inner primer pair with the sequence of 5'-TACTTTGCTCACCCCCCTT-3' (FIP1) and 5'-CATCTTCCCAACTGCCACT-3' (FIP2) were labeled with biotin and digoxigenin at their 5' ends respectively. The PCR condition for the outer primers is 90°C/30 s, 67°C/30 s and 72°C/30 s for 20 cycles in which a 532 bp PCR product was generated. While for the inner primers, the PCR condition used is 90°C/30 s, 55°C/30 s and

72°C/15 s for a total of 30 cycles for the amplification of a labeled 280 bp PCR product. The primer pairs used are highly specific enabling the identification of all the three different pathotypes of NDV. No cross-reactions with other avian infectious agents such as infectious bronchitis virus, infectious bursal disease virus, influenza virus, and fowl pox virus were observed. The detection limit of this one tube nested PCR technique was 3 pfu/ml of NDV by agarose gel electrophoresis detection method and was about 100 fold more sensitive compared to that of a non-nested RT-PCR. To facilitate the detection of the PCR product, the amplified PCR product was then subjected to a colorimetric detection method using ELISA where the labeled PCR product was captured in a streptavidin coated microtiter plate and was detected by using anti-digoxigenin-peroxidase enzyme conjugate and 2,2'- azinodi-ethylbenzothiazolinesulfonic acid (ABTS) as the substrate. Comparisons between the detection methods of agarose gel electrophoresis and ELISA showed that the latter was 10-fold more sensitive than the former. The efficacy of the nested PCR-ELISA was also compared with the conventional NDV detection method (HA test) and non-nested RT-PCR by testing against a total of 35 tissue specimens collected from ND-symptomatic chickens. With the cutoff value of 0.154 having been calculated from 15 known negative samples, 21 of 35 (60%) samples were tested to be NDV positive by nested PCR-ELISA. One of these positive samples, however, was negative by nested PCR and gel detection method. Only 8 of 35 (22.9%) samples were tested positive by non-nested RT- PCR and 2 of 35 (5.7%) samples were positive by the conventional HA test. Due to the high sensitivity of nested PCR-ELISA for the detection of NDV

from tissue specimens, a PCR-ELISA based diagnostic test may be a useful screening test especially in dealing with large number of samples.

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**PENGEMBANGAN UJIAN DIAGNOSTIK TINDAKBALAS RANTAIAN POLIMERASE PENYARANGAN ELISA (NESTED PCR-ELISA) UNTUK MENGESAN VIRUS PENYAKIT NEWCASTLE**

Oleh

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Pengerusi: **Profesor Madya Khatijah Mohd. Yusoff, Ph.D.**

Fakulti: **Sains dan Pengajian Alam Sekitar**

Virus penyakit Newcastle menyebabkan penyakit Newcastle atau sampar ayam yang amat penting dari segi ekonomi di Malaysia. Strain velogenik virus ini boleh mengakibatkan 100% kematian pada ayam yang dijangkiti. Oleh itu, satu teknik diagnostik yang cepat lagi sensitif adalah amat diperlukan untuk mengesan virus ini. Dalam kajian ini, satu teknik yang dikenali sebagai transkripsi berbalik tindakbalas rantaian polimerase penyarangan (RT nested PCR) yang sensitif telah direka. Teknik ini menggunakan dua pasang primer (primer luaran dan primer dalaman) yang direka berasaskan jujukan nukleotida pada gen pertaupan. Jujukan nukleotida pasangan primer luaran adalah 5'-TACACCTCATCCCAGACAGGGTC - 3' (FOP1) dan 5'-AGGCAGGGGAAGTGATTTGTGGC - 3' (FOP2). Jujukan nukleotida pasangan primer dalaman adalah 5'- TACTTTGCTCACCCCCCTT - 3' (FIP1) dan 5'-CATCTTCCCAACTGCCACT - 3' (FIP2) yang masing-masing telah dilabelkan dengan biotin dan digoxigenin pada setiap hujung 5'. Keadaan tindakbalas rantaian

polimerase (PCR) untuk pasangan primer luaran adalah 90°C/30 s, 67°C/30 s dan 72°C/30 s sebanyak 20 pusingan dimana tindakbalas ini akan menghasilkan produk PCR yang bersaiz 532 pasangan bes Manakala keadaan PCR untuk pasangan primer dalaman pula adalah 90°C/30 s, 55°C/30 s and 72°C/15 s sebanyak 30 pusingan untuk mengamplifikasikan produk PCR berlabel yang bersaiz 280 pasangan bes Primer yang digunakan adalah amat spesifik di mana ia berupaya mengenal pasti ketiga-tiga patotaip NDV yang diuji Tiada tindakbalas silang diperhatikan apabila teknik ini diuji terhadap virus-virus "infectious bronchitis", "infectious bursal disease", "avian influenza" dan "fowl pox" Had pengesanan teknik ini berserta dengan penggunaan elektroforesis gel agarose ialah 3 pfu/ml NDV Had pengesanan ini adalah lebih kurang 100x lebih sensitif berbanding dengan teknik "non-nested RT-PCR" Demi kemudahan mengesan produk PCR, kaedah pengesanan secara kalorimetri (ELISA) telah digunakan, di mana ia melibatkan pemerangkapan produk PCR yang berlabel di dalam piring mikrotiter yang terlebih dahulu diselaputi dengan streptavidin Produk PCR yang terperangkap itu kemudian dikesan dengan menggunakan enzim berkonjugat peroksidase anti-digoxigenin dan ABTS sebagai substrat Perbandingan kaedah pengesanan produk PCR di antara elektroforesis gel agarose dan ELISA menunjukkan bahawa kaedah ELISA adalah 10x lebih sensitif berbanding dengan yang terdahulu Keberkesanan teknik "nested PCR-ELISA" ini juga dibandingkan dengan kaedah mengesan NDV secara konvensional iaitu ujian HA dan "non-nested PCR" dengan menguji 35 sampel tisu yang dikutip daripada ayam yang menunjukkan simptom ND Dengan nilai "cutoff" sebanyak 0 154 yang dikira daripada 15 sampel

negatif yang dikenali, 21 daripada 35 (60%) sampel tisu didapati positif terhadap NDV melalui teknik "nested PCR-ELISA". Walau bagaimanapun satu daripada 21 sampel yang positif ini didapati negatif apabila dikesan secara "nested PCR" berserta elektroforesis gel agarose. Hanya 8 daripada 35 sampel (22.9%) adalah positif terhadap NDV melalui teknik "non-nested RT-PCR", manakala 2 daripada 35 sampel (5.7%) adalah positif dengan ujian HA. Memandangkan sensitiviti tinggi yang ditunjukkan oleh teknik "nested PCR-ELISA" terhadap pengesanan NDV dalam sampel tisu, teknik ini berpotensi sebagai ujian penyaringan terutamanya berhubungan dengan jumlah sampel yang banyak.

# CHAPTER 1

## INTRODUCTION

Since first isolated in 1926, Newcastle disease virus (NDV) remains a hazard to the poultry industry of many countries. In Malaysia, Newcastle disease (ND) is still endemic throughout the country despite the introduction of various vaccination strategies in commercial poultry farming. It is believed that the backyard type operation where a vaccination programme is seldom extended to and the migration of wild birds are the main culprits of the disease recurrence.

The poultry industry forms a major component of the livestock industry in Malaysia. According to data released from Department of Veterinary Services, Malaysia, in 1996 (Livestock / Livestock Products Statistics, 1996), the ex-farm value of poultry production in Malaysia is estimated to be RM 2.7 billion. The poultry industry contributed more than 60% of the total value of livestock in 1996. Poultry meat and eggs are consumed chiefly as the main source of protein for Malaysians. Currently, Malaysia is self sufficient in poultry eggs and meat production. The total local consumption of poultry meat has increased from 242.88 metric tonne in 1987 to 486,59 metric tonne in 1996 and per capita consumption also increased sharply from 17.86 kg in 1987 to 29.23 kg in 1996.

Due to the expanding population, the demand for poultry products is expected to expand substantially from year to year. In view of this, the problem of ND which may cause a great loss to the poultry owners must be taken seriously.

Correct and easy diagnosis of ND surely would pave way for a more effective control of the disease. Unfortunately, rapid detection of NDV at the onset of the disease has always been hampered by lack of a sensitive and fast detection method. Clinical signs of ND vary according to the age of the bird and the pathotypes of NDV involved. Symptoms always involve both the respiratory and nervous systems in addition to the effects in egg production and eggshell quality. These typical symptoms, however, cannot be assumed as definite proofs that the responsible agent is NDV. In fact there are other avian diseases which also produce some similar symptoms to ND; such as avian encephalomyletis, infectious bronchitis, infectious coryza and laryngotracheitis (North, 1978).

Although vaccination programs and importation quarantine requirements have provided significant protection against any outbreak, the virus remains a potential threat to the commercial poultry producers as well as the backyard type operation owners. Current diagnosis of NDV infection by conventional virus isolation and serological tests such as hemagglutination inhibition (HI) and serum neutralisation are time consuming, labour intensive and expensive. To overcome these disadvantages, an enzyme-linked immunosorbent assay (ELISA) was developed to detect NDV antibody in the chicken sera (Bidwell *et al.*, 1977; Soula and Moreau,

1981) However, the reported ELISA tests were of limited use, mainly because of non-specific reactions in chicken sera

With the advent in molecular biology based techniques, polymerase chain reaction (PCR) based assay has become a new approach for detecting many veterinary important viruses The first paper to describe the detection of NDV in infected allantoic fluids of embryonated chicken eggs by PCR appeared in the year of 1991 (Jestin and Jestin, 1991), and thereafter there have been several similar publications attesting to the perceived importance of this new diagnostic tool (Hodder *et al*, 1992, Yusoff *et al* , 1993)

The use of reverse transcriptase-PCR (RT-PCR) followed by ethidium bromide staining gel electrophoresis of the amplified product has indeed provided an easy means for detection of various infectious viruses The application of this technique, however, has always been limited on the research bench and is less suitable for routine laboratory where normally, higher degrees of sensitivity and specificity are required for analysing large numbers of clinical samples To fulfil these requirements, nested PCR coupled with a microtiter plate based colorimetric detection system or ELISA has been developed by several research groups to improve both the sensitivity and specificity of their respective diagnostic assays (Wilson *et al*, 1993, Lage *et al*, 1996, Britten *et al*, 1997, Shaio *et al*, 1997)

## Objective of the Study

The development of a rapid, specific, sensitive and relatively inexpensive detection method for the detection of NDV would be advantageous under a variety of circumstances including clinical disease investigation, molecular epidemiological studies for understanding the biology of NDV as well as the enhancement of international trade of poultry. Therefore the objective of the study was to establish a highly sensitive and specific RT nested PCR and colorimetric detection of the PCR product for direct detection of NDV in tissue samples.

This research, therefore, comprised the following steps:

1. establishment of a one tube RT nested PCR;
2. standardisation of the colorimetric detection method or ELISA;
3. sensitivity and specificity evaluation of the RT nested PCR-ELISA; and
4. evaluation of the method efficacy by testing on tissue specimens.

# CHAPTER II

# LITERATURE REVIEW

## General Introduction of Newcastle Disease

Newcastle disease (ND) is a highly contagious and destructive avian disease caused by Paramyxovirus type 1 or better known as the Newcastle disease virus (NDV). This disease has been recognised in most species of free living and also domestic birds throughout the world. Both the exotic and free living nature birds can act as reservoirs for the virus thus making it possible both recrudescence and rapid spread of the ND to domestic birds as well as domestic poultry. Generally, chickens are highly susceptible compared to other avian species. Therefore ND is a serious threat upon the development of poultry industry.

## Occurrence

The outbreaks of ND were first reported for poultry in Batavia (now known as Jakarta), Indonesia in 1926. In the same year Doyle observed a similar disease near

Newcastle-upon-Tyne, England and he named the disease after the locality and demonstrated it to be distinct from any previously known fowl pest (Brandly and Hanson, 1984). Owing to the absence of effective control measures, it spread to many parts of the world within a decade following the first recognition of this disease. In Malaysia, it was believed that the ND made its first apparence in the 1930s'. However, it was initially thought to be a new disease known as 'diptheritic-stomatopharyngitis' (Chong *et al.*, 1960) until it was proven later as ND by Orr and John (1960).

## Importance

ND represents a serious economic challenge to all segments of the poultry industry. In chickens, outbreaks may be so severe with high rates of mortality in the affected flocks. The loses from non-fatal outbreaks, while less spectacular, may be equal to or greater than those where the death loss is high. The greatest loss frequently results from the loss in body weight from the surviving chickens, reduction of the egg production and impaired eggshell and also albumin quality (North, 1978). Besides that, ND also reduces the fertility and hatchability of eggs (Brandly and Hanson, 1984). ND however, is not a serious problem to human. To date, no severe infection has been officially reported in human. Those who had contracted with the disease might show illnesses like headache, flu-like symptoms and mild conjuntivitis (Brandly and Hanson, 1984).