UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF IN SITU PCR FOR VIRUS DETECTION, STRAIN DIFFERENTIATION AND PATHOGENESIS OF NEWCASTLE DISEASE AND INFECTIOUS BURSAL DISEASE IN CHICKENS

HUSSEIN ABDALLAH ELAWAD MAHMOUD

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By

HUSSEIN ABDALLAH ELAWAD MAHMOUD

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

January 2017

DEDICATION

To our friend

Amanullah Akhtar,

who travelled to the other world leaving a sunken wound.

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 IN SITU PCR FOR VIRUS DETECTION, STRAIN DIFFERENTIATION AND PATHOGENESIS OF NEWCASTLE DISEASE AND INFECTIOUS BURSAL DISEASE IN CHICKENS

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January 2017

Chairman : Professor Mohd Hair-Bejo, PhD
Faculty : Veterinary Medicine

In molecular methods, there is great probability of virus-specific detection and identification. This is due to genus and family specific genome sequences. *In situ* PCR appeared into being to comprise PCR power of amplification and *in situ* hybridization ability to localize target sequence. This new method paves the way to detect minute quantities of nucleic acids in undamaged cells.

The objectives of this study were to develop *in situ* PCR in light microscopy for detection, strains differentiation, and tissue tropism determination of Newcastle disease virus (NDV) and infectious bursal disease virus (IBDV). The method was used to determine pathogenesis of velogenic NDV (vNDV) and very virulent IBDV (vvIBDV) infections.

In the first experiment specific pathogen free (SPF) chickens were infected with vNDV by intranasal administration of 0.1 mL of 10^5 EID50 /0.1 mL of isolate AF2240. Tissue samples were collected and fixed in 10% buffered formalin for histological examination, immunoperoxidase staining (IPS) and *in situ* PCR. *In situ* PCR was successfully developed with probe specific for vNDV. *In situ* PCR (score of 2.17 ± 0.06) was significantly (p<0.05) more sensitive than IPS (score of 1.51 ± 0.40). Histological changes were consistent with the presence of virus.

In the second experiment, SPF chickens were inoculated orally with of 0.1 mL of $10^{7.5}$ EID50/mL of vvIBDV (UPM0081 isolate). Tissue samples were collected and fixed in 10% buffered formalin for histological examination, IPS, and *in situ* PCR. *In situ* PCR was developed with probe specific for vvIBDV. *In situ* PCR (score of 2.85 ± 0.29) was significantly (p<0.05) more sensitive than IPS (score of 1.80 ± 0.09). Histological changes were consistent with the presence of virus.

In the third experiment, 24 SPF chickens were infected with vNDV (0.1 mL) by intranasal administration of 10^5 EID_{50}/0.1mL of vNDV AF2240 isolate. Fifteen SPF chickens were kept as control. Chickens were sacrificed at various intervals and tissue samples were collected for histological examination, IPS, and *in situ* PCR. *In situ* PCR revealed that at hr 2 post inoculation (pi), virus tended to enter trachea and respiratory tract leading to primary viraemia and invading other organs. At hr 4 pi, virus had entered liver and spleen. However, brain and heart were involved only at hr 6 pi. Secondary viraemia probably started as early as hr 12 pi since vNDVwas positive in all collected tissues. The study showed the descending order of tissues according to positive signal scoring was: trachea, caecal tonsil, liver, bursa of Fabricius, intestine, proventriculus, lung, spleen, thymus, kidney, heart, and brain. IPS findings were almost similar to *in situ* PCR but less sensitive. Histological changes were consistent with the presence of virus.

In the fourth experiment, 24 SPF chickens were infected with vvIBDV (0.1 mL) by oral administration of $10^{7.5}$ EID50/0.1 mL (UPM0081 isolate). Fifteen SPF chickens were kept as a control group. Chickens were sacrificed at various intervals and tissue samples were collected for histological examination, IPS, and *in situ* PCR. IBDV was detected in intestine, junction of proventriculus and gizzard and caecal tonsil at hr 2 pi using *in situ* PCR leading to primary viraemia and invasion of liver, kidney, and bursa of Fabricius. At hrs 4 and 6 pi, virus reached spleen and thymus, respectively. It was detected in muscle on day 1 pi. Hence, secondary viraemia might occur during the period 12 to 24 hrs pi. Virus tissue tropism was summarized briefly in bursa of Fabricius, thymus, caecal tonsil, liver, junction of proventriciulus and gizzard, spleen, kidney, intestine, and muscle. IPS findings indicated sequence of detection of virus, in different tissues, was similar to *in situ* PCR findings. Histological changes were consistent with findings of *in situ* PCR and IPS.

In fifth experiment, 15 SPF chickens were divided into 3 groups. One group was inoculated with vNDV (0.1 mL) by intranasal administration of 10^5 EID_{50}/0.1 mL of vNDV AF2240 isolate, another group was inoculated with lentogenicNDV strain (lNDV) (V4 isolate) and a third group was kept as control. Tissue samples were collected for detection of virus by *in situ* PCR with probe specific for vNDV, positive results were obtained only when

tissues were infected with vNDV. Similarly, probe specific for INDV strain showed positive results only for tissues infected with INDV. It demonstrated that specific probes have ability for differentiation of NDV strains.

In the sixth experiment, 15 SPF chickens were divided into 3 groups. One group was inoculated with vvIBDV strain by oral and intraocular administration of 1 mL ($10^{4.83}$ EID50/0.1 mL) of UPM0081 isolate, another group was inoculated with caIBDV strain (V877 isolate) and a third group was kept as control. Tissue samples were collected for detection of virus by *in situ* PCR with probe specific for vvIBDV, positive results were obtained only when tissues were infected with vvIBDV. Similarly, probe specific for caIBDV strain showed positive results only for tissues infected with caIBDV. It demonstrated that specific probes have ability for differentiation of IBDV strains.

It was concluded that the study has successfully developed *in situ* PCR for detection and differentiation of NDV and IBDV strains. *In situ* PCR was more sensitive than IPS in detection of the virus. Tissue tropisms of the virus and disease pathogenesis were established.

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

PEMBANGUNAN PCR IN SITU UNTUK PENGESANAN VIRUS, PEMBEZAAN STRAIN DAN PATHOGENESIS PENYAKIT NEWCASTLE DAN PENYAKIT BURSA BERJANGKIT DALAM AYAM

Oleh

HUSSEIN ABDALLAH ELAWAD MAHMOUD

Januari 2017

Pengerusi : Profesor Mohd Hair-Bejo, PhD
Fakulti : Perubatan Veterinar

Dalam kaedah molikul, terdapat kebarangkalian besar pengesanan dan pengenalan khusus virus. Ini disebabkan oleh genus dan keluarga urutan genom khusus. PCR *in situ* wujud untuk membentuk kuasa PCR penguatan dan kemampuan penghibridan *in situ* untuk mengesan penyentempatan urutan sasaran. Kaedah baharu ini membuka jalan untuk mengesan kuantiti kecil asid nukleik dalam sel yang tidak rosak.

Objektif kajian ini adalah untuk membangunkan PCR *in situ* dalam mikroskopi cahaya untuk pengesanan, pembezaan strain, dan penentuan tropisme tisu virus penyakit Newcastle (NDV) dan virus penyakit bursa berjangkit (IBDV). Kaedah ini digunakan untuk mementukan patogenesis jangkitan velogenik NDV (vNDV) dan sangat getir IBDV (vvIBDV).

Dalam eksperimen pertama, ayam bebas patogen khusus (SPF) telah dijangkiti vNDV melalui intranasal (0.1 mL) 10^5 EID50/0.1 mL daripada strain AF2240. Tisu sampel telah dikumpulkan dan diawet dalam 10% formalin berpenimbal untuk pemeriksaan histologi, pewarnaan immunoperoxidase (IPS) dan PCR *in situ*. PCR *in situ* telah berjaya dibangunkan dengan probe yang direka khusus untuk vNDV. PCR *in situ* (2.17 ± 0.06) adalah ketara ($p<0.05$) lebih sensitif daripada IPS (skor 1.51 ± 0.40). Perubahan histologi adalah konsisten dengan kehadiran virus.

Dalam eksperimen kedua, ayam SPF telah disuntik secara oral dengan 0.1 mL, $10^{7.5}$ EID50/mL vvIBDV asingan UPM0081. Sampel tisu telah dikumpulkan dan diawet dalam 10% formalin berpenimbal untuk pemeriksaan histologi, IPS, dan PCR *in situ*. PCR *in situ* telah dibangunkan dengan prob yang direka khusus untuk vvIBDV. PCR *in situ* (skor 2.85 ± 0.29) adalah signifikan ($p<0.05$) lebih sensitif daripada IPS (skor 1.80 ± 0.09). Perubahan histologi adalah konsisten dengan kehadiran virus.

Dalam eksperimen ketiga, 24 ekor ayam SPF telah dijangkiti vNDV (0.1 mL) melalui intranasal (10^5 EID_{50}/ 0.1mL) daripada asingan vNDV AF2240. Lima belas ekor ayam SPF telah diasingkan sebagai kelompok kawalan. Ayam dikorbankan dalam pelbagai jarak dan sampel tisu dikumpulkan untuk pemeriksaan histologi, IPS dan PCR *in situ.* PCR *in situ* menampakkan bahawa pada jam 2 selepas inokulasi (pi), virus cenderung untuk memasuki trakea dan saluran pernafasan menuju ke viraemia primer dan menyerang organ lain. Pada jam 4 pi, virus memasuki hati dan limpa. Bagaimanapun, otak dan jantung terlibat hanya pada jam 6 pi. Viraemia sekunder mungkin bermula seawal jam 12 pi sejak vNDV positif dalam semua tisu yang dikumpulkan. Kajian ini menunjukkan bahawa tertib menurun tisu menurut penskoran signal positif ialah: trakea, tonsil sekum, bursa Fabricius, usus, proventrikulus, peparu, limpa, timus, ginjal, jantung, dan otak. Penemuan IPS hampir serupa dengan PCR *in situ* tetapi kurang sensitif. Perubahan histologi adalah konsisten dengan kehadiran virus.

Dalam eksperimen keempat, 24 ekor ayam SPF dijangkiti vvIBDV (0.1 mL) melalui oral $10^{7.5}$ EID50/0.1 mL (asingan UPM0081). Lima belas ekor ayam SPF telah disimpan sebagai kelompok kawalan. Ayam dikorbankan pada pelbagai jarak dan sampel tisu dikumpulkan untuk pemeriksaan histologi, IPS dan PCR *in situ*. IBDV dikesan di dalam usus, persimpangan proventrikulus dan hempedal dan tonsil sekum pada jam 2 pi menggunakan PCR *in situ* menuju ke viraemia dan menyerang hati, ginjal dan bursa Fabricius. Pada jam 4 dan 6 pi, virus masing-masing mencapai limpa dan timus. Virus dikesan dalam otot pada hari 1 pi. Oleh itu, viraemia sekunder mungkin berlaku dalam tempoh 12 hingga 24 jam pi. Tropisme tisu virus telah diberi ringkasan singkat dalam bursa Fabricius, timus, tonsil sekum, hati, persimpangan proventrikiulus dan hempedal, limpa, usus dan otot. Dapatan IPS menunjukkan urutan pengesanan virus, dalam tisu yang berbeza, adalah sama dengan dapatan PCR *in situ*. Perubahan histologi adalah konsisten dengan penemuan PCR *in situ* dan IPS.

Dalam eksperimen kelima, 15 ekor ayam SPF telah dibahagikan kepada 3 kelompok. Satu kelompok telah disuntik dengan vNDV (0.1 mL) melalui intranasal 10^5 EID_{50}/0.1mL daripada asingan vNDV AF2240, kelompok lain telah disuntik dengan strain lentogenik NDV (lNDV) (asingan V4) dan kelompok ketiga disimpan sebagai kelompok kawalan. Sampel tisu yang

dikumpulkan untuk pengesanan virus oleh PCR *in situ* dengan prob khusus bagi vNDV, memperolehi keputusan yang positif hanya untuk tisu yang dijangkiti vNDV. Begitu juga, bagi prob lNDV memberi keputusan yang positif hanya untuk tisu yang dijangkiti lNDV. Ini menunjukkan bahawa prob khusus mempunyai kemampuan untuk perbezaan strain NDV.

Dalam eksperimen keenam, 15 ekor ayam SPF telah dibahagikan kepada 3 kelompok. Satu kelompok telah disuntik dengan strain vvIBDV melalui oral dan intraokular (1 mL) $10^{4.83}$ EID_{50}/ 0.1mL) daripada asingan UPM0081, kelompok lain telah disuntik dengan strain caIBDV (asingan V877). dan kelompok ketiga disimpan sebagai kelompok kawalan. Sampel tisu yang dikumpulkan untuk pengesanan virus oleh PCR *in situ* dengan prob khusus bagi vvIBDV, memperolehi keputusan yang positif hanya untuk tisu yang dijangkiti vvIBDV. Begitu juga, bagi prob caIBDV memberi keputusan yang positif hanya untuk tisu yang dijangkiti caIBDV. Ini menunjukkan bahawa prob khusus mempunyai kemampuan untuk perbezaan strain IBDV.

Kesimpulannya, kajian ini telah berjaya membangunkan PCR *in situ* untuk pengesanan dan pembezaan strain NDV dan IBDV. PCR *in situ* adalah lebih sensitif daripada IPS dalam pengesanan virus. Tisu tropism virus dan patogenesis penyakit telah tertubuh.

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I certify that a Thesis Examination Committee has met on 4 January 2017 to conduct the final examination of Hussein Abdallah Elawad Mahmoud on his thesis entitled "Development of in Situ PCR for Virus Detection, Strain Differentiation and Pathogenesis of Newcastle Disease and Infectious Bursal Disease in Chickens" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilments of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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APMV–1	Avian paramyxovirus serotype 1
BALT	Bronchial associated lymphoid tissues
bp	Base pair
CD	Cluster of differentiation CD4+ and CD8+ T lymphocytes
caIBD	Classical infectious bursal disease strain
cDNA	Complementary deoxy ribonucleic acid
DAB	Diaminobenzidine
DIG	Digoxigenin
DNA	Deoxy ribonucleic acid
EID50	Egg infective dose by 50%
F gene	Fusion gene
g	Gram
GALT	Gut (Gastrointestinal tract) associated lymphoid tissue
G-C	Guanine-cytocine
HE	Haematoxylin and eosin
HIV	Human immunodeficiency virus
IBD	Infectious bursal disease
ICPI	Intracerebral pathogenicity index
IgM+	Immunogobuline M
IPS	Immunoperoxidase staining
lNDV	Lentogenic Newcastle disease virus strain
MALT	Mucosa-associated lymphoid tissues
mL	Millilitre
mRNA	Messenger ribonucleic acid
NBT/BCIP	Nitroblue tetrazolium 5 - bromo 4 - chloro 3 - indolyl phosphate
ND	Newcastle disease

NDV	Newcastle disease virus
nt	Nucleitide
OIE	*Office Internationale des Epizooties*
PB	Phosphate buffer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pi	Post-inoculation
RNA	Ribonucleic acid
RT-PCR	Reverse transcription- polymerase chain reaction
SDS	Sodium dodecyl sulphate
SE	Sandard error
SPF	Specific pathogen free
SSC	Saline sodium citrate
vNDV	Velogenic Newcastle disease virus strain
vvIBD	Very virulent infectious bursal disease strain
WB	Western Blot
µl	Microlitre
µm	Micrometre

CHAPTER 1

INTRODUCTION

Rapid change was observed in production of animal products, processing, consumption and marketing and rapid growth was observed in livestock production over last four decades (Narrod *et al*., 2007). At present, consumption in developing countries is roughly one-third the meat and one-quarter of the milk products *per capita* compared to consumption in the richer North. By 2020, developing countries contribution to whole world meat consumption will be 63% instead of 52% right now. By 2020, consumption of meat and consumption of milk in developing countries will increase 107 and 177 million metric tons (mmt), respectively, more than it was in 1996/1998 (Delgado, 2003). However, poultry has been leading growth in livestock production in both developed and developing countries. There was an increase in poultry meat production in East and South East Asia and in Latin America particularly in China and Brazil (Narrod *et al*., 2007). It was anticipated that an increase of 3.6 to 3.5% *per annum* would ensue in poultry meat production and consumption in developing countries between 2005 and 2030. Rise is attributed to introduction of modern methods of intensive production, genetic improvements, improvement in preventive applications, disease control and biosecurity measures, elevation in income and human population, and urbanization (Narrod *et al*., 2007).

On the other hand, chickens, in all corners of the world, are permanently exposed to diseases of various kinds. Diseases sometimes have regional impact or even an impact on world trade. Namely, some diseases cause catastrophic consequences such as highly pathogenic avian influenza (HPAI strain H5N1), which is expected to threaten poultry industry throughout current century. Other examples of threats comprise Newcastle disease (ND), which is endemic all over the world, and infectious bursal disease (IBD). In general, great losses are associated with flock mortality, disruption in supply to markets, declines in consumption and seriously affected profitability. All these factors stimulate investment and scientific research in diagnostic resources to result in available sophisticated diagnostic procedures (Simon and Durham, 2010).

Diagnostic virology, on its long way of progress, encountered numerous landmarks; starting with tissue culture, cell culture, histopathology, electron microscopy and serology to end up with molecular diagnosis. Molecular methods are characterized by ability to detect viable viruses and even dead viruses which are impossible to cultivate. In molecular methods, there is great probability of virus-specific detection and identification. This is due to genus and family specific genome sequences (Storch, 2000). Polymerase

chain reaction (PCR), with resounding fame for almost two decades, is still not satisfactory to morphologists whose interests are associated with conserved tissues (Morel and Raccurt, 2003). Direct hybridization assays without amplification suffers from lack of sensitivity (Storch, 2000). Then *in situ* PCR appeared into being to comprise PCR power of amplification and *in situ* hybridization ability to localize a target sequence. This new method paved way to detect minute quantities of nucleic acids in undamaged cells (Morel and Raccurt, 2003). But, *in situ* PCR is still in stage of development. It requires more refining (Takeuchi *et al*., 2006). *In situ* PCR technique has recently been developed to allow performance of a reaction directly on tissue. An exquisitely sensitive detection of viral nucleic acid is carried out in tissues, whereas, this technique is done in a few number of laboratories at present (Storch, 2000). It is hoped that this technique will allow detection of extremely low levels of nucleic acids, subsequently investigation of early pathogenesis (Zhang *el al.,* 2002). Exquisiteness of *in situ* PCR was verified by Zhang *et al*. (2002) when they managed to detect IBDV at 6 hours, 12 hours, 16 hours, 28 hours post-infection in the bursa of Fabricius, caecal tonsil, thymus and spleen, liver, kidney and thigh muscle. In comparison to *in situ* hybridization which was applied for detection of IBDV in tissues collected 40 hour pi (Zhang *el al.,* 2002). In other study conducted by Brown *et al*. (1999), *in situ* hybridization was applied for detection of 3 different isolates of viscerotropic velogenic ND virus (vvNDV) on day 2 pi, zero out of 3 isolates were detected on brain and heart, 1 out of 3 isolates was detected in airosac and lung and 3 out 3 isolates were detected in eyelid, spleen and caecal tonsil.

Bagasra *et al*. (1992) found that *in situ* PCR has greater potential to determine actual proviral load in peripheral blood at various stages of HIV-1 infection. Furthermore, it has a potential power to evaluate efficacy of various therapeutic interventions. Even some entities in latent or defective forms could be detected. Determination of location of a target sequence in a certain tissue is carried out by using two labeled probes; one probe with a certain colour complementary to infectious agent and another probe with another colour complementary to gene of infected cell (Morel and Raccurt, 2003). There are two types of *in situ* PCR (Figure 1.1); in first type, direct *in situ* PCR; primers themselves, which are used for amplification, are labeled with either biotin or digoxigenin (DIG), or other fluorescein compounds. Here, labeled nucleotide is fused into amplified products during the process of amplification. At the end of reaction, labeled products are visualized with the help of immunohistochemistry (Muro-Cachu, 1997; Takeuchi *et al*., 2006).

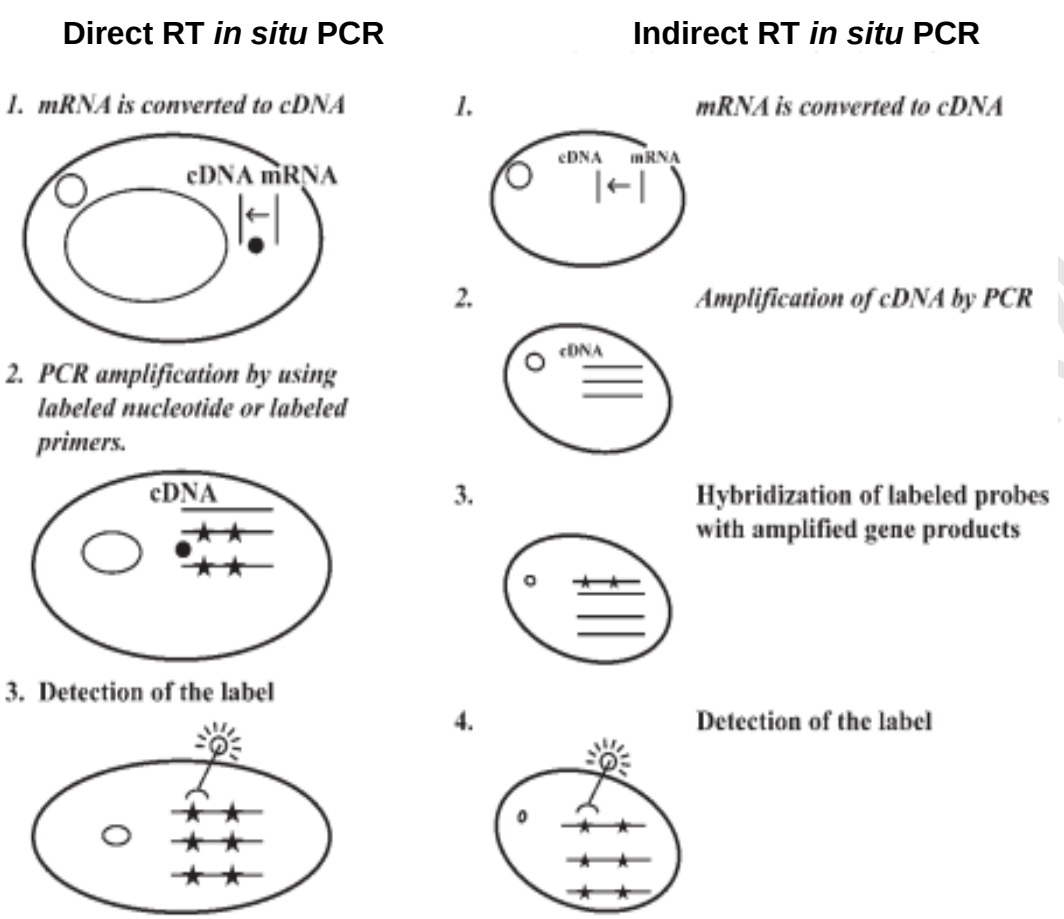

Figure 1.1 : Schematic representation depicting chief stages of direct and indirect RT *in situ* PCR. First, conversion of mRNA to cDNA. Second, amplification;in direct technique; it is labeled amplified product as a result of labeled primer or labeled nucleotide, subsequently, detection by immunohistochemistry; while in indirect technique, conventional amplification is followed by hybridization of labeled probe with PCR product, thus, immunohistochemical detection (Takeuchi *et al*., 2006).

Tereza *et al*. (2008) applied direct *in situ* RT-PCR method and managed to successfully detect, with higher sensitivity than conventional RT-PCR and virus isolation, very virulent IBDV (vvIBDV) from bursa of Fabricius, thymus and liver from IBDV-suspected infection in birds. Primers for *in situ* PCR in such study were labeled with biotin. This technique might clarify uncertain details related to replication and pathogenesis of vvIBDV in chicken.

Second type of *in situ* PCR is indirect; here PCR is carried out with unlabeled primers; then, products of amplification are hybridized with a labeled probe. Then products after hybridization could be observed with immunohistochemistry (Takeuchi *et al*., 2006). In other words, when amplification step has finished, labeled nucleotides (digoxigenin-11-dUTP)

are incorporated, thus, consequents are detected by immunohistochemistry. In brief, amplified product is detected by *in situ* hybridization with the help of labeled probes (Figure 1.1). Privilege of *in situ* PCR over *in situ* hybridization is that it allows detection of minimal amount of nucleic acid (Muro-Cachu, 1997). In general, this technique is influenced by target accessibility, probe concentration, hybridization stringency (melting temperature, base composition, specificity of sequence between target and probe, composition of hybridization/washing solution), and kind of label (Muro-Cachu, 1997).

Newcastle disease (ND) and infectious bursal disease (IBD) still lack deep research to comprehend some points in pathogenesis such as why in some NDV isolates, amino acids sequence is similar to sequences in different strains (de Leeuw *et al*., 2003; Brown, 1999). Absence of glycosylation sites in HN protein, how it causes alteration of pathogenicity (Panda *et al*., 2004). How V protein (P gene product) participates in NDV virulence (Huang *et al*., 2003), and how some isolates change behavior of virulence from mild to severe (Kommers *et al*., 2002). Also, what the mechanism of haemorrhage in IBD is (Zeryehun *et al*., 2012). How significant IBDV plays role in apoptosis process (Nieper *et al*., 1999). How long it takes for IBDV to reach target tissues and subsequently how soon other processes of pathogenesis start (Zhang *et al*., 2002). Hybridization technique with specific probes for certain virus strains was applied successfully for differentiation between NDV or IBDV strains by Li and Zhang (2004) and Kataria *et al*. (2000), respectively. It was the hypothesis of present study that *in situ* PCR technique, with the same specific probes, is capable of differentiation of NDV strains and IBDV strains. Moreover, pathogenesis of disease and tissue tropism of the virus could be determined accurately using in *situ* PCR technique.

The objectives of this study were:

1) to develop RT *in situ* PCR in light microscopy for detection of NDV and IBDV.
2) to determine the tissue tropism of velogenic NDV and very virulent IBDV by application of *in situ* PCR.
3) to determine the pathogenesis of velogenic NDV and very virulent IBDV infections by *in situ* PCR.
4) to apply *in situ* PCR for differentiation between NDV strains and IBDV strains.

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