Studies on Newcastle Disease Virus : Thermostablity and Molecular Diagnostics

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Athesis submitted to the university of Khartoum in fulfillment for the Degree of Doctor of Philosophy in veterinary medicine

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October2005

Dedication

To my family...with love.

Acknowledgement

I wish to express my sincere gratitude to my supervisor Prof. Imadeldin Elamin Aradaib for his constant enthusiasm, close supervision and support. Thanks and gratitude also extends to my co-supervisor Prof. Abdelrahim Mohamed ElHussein for his personal interest, generous guidance and advice. It has been a privilege to work under their supervision. Thanks are extended to Dr. Sobhi Ahmed Mohamed Kheir due to his kind help and useful suggestions. Prof. Mohamed Mohamed Salih Elgabalabi, the director general of the Central Veterinary Research Laboratory is greatly thanked for his kind help. I am deeply grateful to my intimate and sincere friend Dr. Diaeldin Ahmed Salih who supplied me with valuable literature from South Africa. The help offered by my colleagues in the Central Veterinary Research Laboratory is greatly appreciated. Namely, Dr. Magdi Badawi and Dr. Omer Othman. My colleagues in The Molecular Biology Laboratory are certainly acknowledged for their nice cooperation and support. Thanks also extended to all staff members of the Viral Vaccine Production Compound for their continuous help. Special thanks go to Mrs. Altoma for her patience on printing and preparation of the thesis.

I owe a great deal thanks and I am deadly indebted to all members of my family for unlimited patience and hard time afforded to me to accomplish this work. I am asking God to pay them health and wellbeing.

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Abstract

In the first series of experiments the Komarov strain of Newcastle disease (ND) virus was subjected to selection for heat resistance. Four cycles of heat selection at 56^{o}C were carried out. Residual virus obtained after the last cycle was lyophilized. The stability of haemagglutinin and infectivity at room and at refrigeration temperatures was assessed. Effect of some stabilizers (lactalbumen hydrolysate, lactose, sucrose and skimmed milk powder) on stability of virus haemagglutinin activity at both temperatures was also studied. On heat selection the haemagglutination titre of surviving virus raised from 7log2 after the first cycle to10log2 after the fourth cycle. Results showed that haemagglutinin activity of non-stabilizer added product maintained a titre of ≥8log2 up to 20 days under refrigeration and for 7 days at room temperature (4 days and ≤1 day for parent virus respectively) while activity in lactalbumen hydrolysate, lactose and sucrose-added products were 9, 9 and 7 weeks and 6, 7 and 8 at both temperatures, respectively. For infectivity ≥8log2 titre was maintained up to 50 days under refrigeration and for 12 days at room temperature (2-5 days and ≤2 days for parent virus respectively). The haemagglutination test could not be accurately performed on the skimmed milk-added product. The results showed that Komarov strain of ND virus could be successfully selected for heat resistance and its stability may be enhanced by addition of suitable stabilizers.

In the second series of experiments, a reverse transcriptase (RT) polymerase chain reaction (RT-PCR) was developed to detect field isolates of Newcastle disease virus

(NDV) grown in vero cell culture or embryonated chicken eggs (ECE). Five Sudanese isolates of NDV designated (OB, KU, GR, A12, and A105) and five vaccine strains including Komarov, B1, LaSota, Clone30 and Clone79 were used in this study. A pair of primers (nd1 and nd2), targeting a fragment in the F gene of NDV, was designed for PCR amplification. The RT-PCR assay resulted in amplification of a 356 bp PCR product from RNAs of Sudanese and vaccine strains of NDV. However, nucleic acid extracts of infectious bursal disease (IBD) virus, non-infected Vero cells or ECE failed to produce the specific 356 bp PCR product. The described RT-PCR assay was a simple procedure that involved a single amplification step. In addition, the developed RT-PCR assay provides a rapid, sensitive, and specific method for detection of an outbreak of the disease in susceptible birds.

Finally, a nested RT-PCR assay for detection of Newcastle disease virus in clinical samples was evaluated. Clinical samples include blood, tracheal swabs and cloacal swabs, liver, spleen, heart, lung, kidney, bursa and brain. Tissue homogenates were made from all samples in sterile phosphate buffered saline containing antibiotics. After centrifugation of homogenates the supernatant fluids were used for virus isolation in embryonated chicken eggs and for viral RNA extraction as well. Two steps of nested RT-PCR were performed. In the first step a pair of primers (nd1 and nd2) flanking a 356 bp long were used to amplify specific region in the F gene of the virus. In the second step, another (nested) pair of primers (nd3 and nd4) was employed to produce 216 bp amplification products for confirmation the authenticity of the first primers to the Newcastle disease virus. For diagnosis the nested RT-PCR assay was proved to be accurate, sensitive, rapid and less expensive when compared with virus isolation.

ملخص الأطروحة

في التجربة الأولى تمت دراسة إمكانية الحصول على فيروس مقاوم للحرارة من العترة اللقاحية كوماروف من فيروس مرض شبه طاعون الطيور (النيوكاسل) عن طريق الانتخاب لمقاومة الحرارة. تم إجراء أربع دورات انتخاب في درجة حرارة 56مº لمدة ساعة. كانت عيارية الفيروس المقاوم للحرارة بعد الدورة الأولى 2^{7} وارتفعت بعد الدورة الرابعة إلى2^{10} .لم يتم الحصول على فيروس حي بعد تعرض العترة اللقاحية لدرجة حرارة 56مº لأكثر من ساعة.

بعد تجفيد المحصول الناتج من دورة الانتخاب الرابعة أجريت بعض الاختبارات لمعرفة ثبات العيارية لبروتين تلازن الدم والمقدرة على عدوى أجنة الدجاج للفيروس المنتخب على مدى فترات حفظ مختلفة في درجتي حرارة الغرفة والتبريد الاعتيادي. كذلك تمت دراسة اثر إضافة مواد مثبتة على زيادة مقاومة العترة المنتخبة للحرارة. بالنسبة للفيروس المنتخب (بدون إضافة مواد مثبتة) كانت عيارية بروتين التلازن ثابتة عند 2^{8} لمدة 20 يوم تحت درجة التبريد (2-8مº) ولمدة 7 أيام في درجة حرارة الغرفة (28-32مº) و بمقارنتها مع العترة ألام كانت 4 أيام واقل من يوم عند درجتي الحرارة المذكورتين بالتتابع. أما في حالة المواد المثبتة المضافة، فقد كانت فترة الثبات عند نفس العيارية 9 أسابيع عند استعمال اللاكتالبيومين هايدرولايسيت و 9 أسابيع عند استعمال اللاكتوز و 7 أسابيع عند استعمال السكروز في درجة حرارة التبريد الاعتيادي، و 6 و7 و8 أسابيع في درجة حرارة الغرفة لنفس المواد المذكورة بالتتابع. في حالة إضافة بدرة اللبن خالي الدسم لم نتمكن من إجراء اختبارات التلازن بصورة جيدة و بالتالي لم تقارن نتائجها مع المواد الأخرى. أما عند دراسة ثبات المقدرة على عدوى أجنة الدجاج للفيروس المنتخب فقد استمرت عيارية الفيروس عند 2^{8} لمدة 50 يوم تحت درجة التبريد و لمدة 12 يوم عند درجة حرارة الغرفة (2-5 أيام و اقل من يومين للعترة اللقاحية ألام بالتتابع).

أثبتت نتائج التجربة الأولى أنه يمكن انتخاب فيروس مقاوم للحرارة من العترة اللقاحية متوسطة الضراوة (كوماروف) لفيروس مرض النيوكاسل. كما أنه أمكن تعزيز مقدرة هذه العترة لمقاومة الحرارة بإضافة مواد مثبتة.

من ناحية أخرى تم إجراء دراسة لتقييم تفاعل البلمرة المتسلسل العكسي للكشف عن فيروسات مرض النيوكاسل الحقلية و العترات اللقاحية كوسيلة تشخيص سهلة، سريعة و أكثر حساسية من طرق التشخيص الأخرى. أجري الكشف على الفيروس بهذه الطريقة بعد زراعته في أجنة الدجاج و الخط الخلوي(فيرو)، و من ثم استخلاص الحمض النووي للفيروسات لإجراء تفاعل البلمرة. استخدم في هذه التجربة زوج من البوادئ مستهدفا جزء من جين بروتين الالتحام بطول 356 زوج قاعدي. كل الفيروسات الحقلية المسماة؛ الأبيض، كوكو، قرنفلي،12أ و 105أ و كذلك العترات اللقاحية؛ كوماروف، لاسوتا، كلون30، كلون 79 و ب1 أمكن الكشف عنها بهذه الطريقة. أظهر هذا الزوج من البوادئ خصوصية لهذه الفيروسات حيث لم يتم الكشف عن فيروسات أخرى مماثلة مثل فيروس مرض التهاب البورصة المعدي، كما لم يستهدف أي جزء من الأحماض المستخلصة من أنسجة أجنة الدجاج أو الخط الخلوي.

في تجربة ثالثة تمت محاولة الكشف عن فيروس النيوكاسل مباشرة من العينات المأخوذة من أنسجة الطيور مستحدثة العدوى باستخدام تقنية تفاعل البلمرة المتسلسل الداخلي. تمت عدوى الطيور بفيروس ضاري من فيروسات النيوكاسل و بعد ظهور الأعراض تم اخذ عينات من الدم، القصبة الهوائية، فتحة المجمع، الكبد، الطحال، الرئة، الكلية، المخ و عضلة القلب. تم استخلاص الحمض النووي مباشرة من هذه الأنسجة و من ثم الكشف عن الفيروس عن طريق استهداف جزء من جين بروتين الالتحام بزوج من البوادئ (كما في التجربة السابقة). اختبرت خصوصية وحساسية هذا الزوج بزوج آخر من البوادئ صمم ليستهدف جزء داخلي بطول 216 زوج قاعدة. بهذه الطريقة تم الكشف عن الفيروس في كل الأنسجة. بمقارنة هذه النتائج مع نتائج طريقة العزل التقليدية أمكن إثبات أن هذه التقنية اكثر دقة وحساسية وأسرع من الطرق التقليدية لتشخيص المرض معمليا.

مجمل نتائج هذه الدراسة أوجدت بعض الحلول لمشكلة صعوبة التشخيص المعملي لمرض النيوكاسل من ناحية، وكذلك أضاءت الطريق لاستحداث لقاح مقاوم للحرارة وسهل التداول من ناحية أخرى. كل ذلك في سبيل تقليل الأضرار الاقتصادية الناتجة عن انتشار المرض في الحقل و إحكام السيطرة عليه بمكافحته في قطيع الدواجن المحلية.

CHAPTER I

GENERAL

LITERATURE REVIEW

1.1. Introduction:

Diseases in general are the most retarding factors for intensive poultry industry. Outbreaks of viral origin are certainly very deleterious ones. The high mortality rates within birds during early stages of life, the decreased productivity in late stages and impairing flock immunity that render birds susceptible to other pathogens are the major economical drawbacks due to these infections.

Since its emergence in Sudan in 1951, Newcastle disease (ND) became a constant threat for poultry industry and hindered expansion in this field. ND is very common among rural and commercial chicken flocks (Fadol 1991) and pigeons (Ballal *et al.*, 1996).

Routine diagnosis of ND commonly practiced is based upon clinical signs or, sometimes, lesions which may be inadequate. Laboratory tests mainly involve primary isolation in embryonated chicken eggs and subsequent identification by haemagglutination inhibition (HI) test. Seldom virus pathotyping by conventional tests are carried out merely at the Central Veterinary Research Laboratory (CVRL). Due to the wide range of clinical pictures of the disease, such diagnostic procedures are no longer convincing because they could not differentiate between field viruses and live vaccine strains (of different virulence) commonly used in the field. In addition, these techniques are known to be slow, laborious and require *in vivo* manipulation. Moreover, specific-pathogen free source of embryonating chicken eggs and live chicks are needed to carry out pathogenicity tests to confirm diagnosis. Thus, the identification of the virus and estimation of its pathogenicity may not be of value in epidemiological assessment of an outbreak concerning the source of the virus and its spread.

The universal use of live vaccines of variable virulence in commercial poultry is extremely complicating the assessment of geographical distribution of epizootic and enzootic ND viruses. This problem is further aggravated by the lack of a unique and precise definition of the disease which could be acceptable to all countries (Alexander 1991a).

The development of molecular biological techniques afforded actual and potential alternative techniques for diagnosis of ND and accordingly the usage of live animals in pathogenicity tests became increasingly unacceptable (Aldous and Alexander, 2001). These techniques will clearly discuss the three points of ND diagnosis (detection, characterization and epidemiology) and their advantages appear to be overwhelming in ND diagnosis despite the fact that exclusive variations among ND viruses still pose some technical problems.

Since early sixties - of the last century - a strategy of ND control was featured out but hitherto no practical or systematic control programmes have been commenced. Immunization against ND in Sudan was started in 1952 by the use of an imported wet vaccine (Ali, B.H., 1978). A mesogenic strain of ND virus (Komarov) was introduced from Lebanon in early sixties and enrolled in the manufacture of a lyophilized live vaccine at the CVRL. This strain conferred a good protective level of immunity to vaccinates. Yet, some technical limitations linked to its manufacture and handling in the field are still present. It is usually dispensed in large doses and it needs a cold chain delivery. Hence, its usage is restricted to the large scale production and the big farms. Moreover, the disease profile is complicated by the fact that different live and killed vaccines of different virulence are still imported by the private sector and delivered for

sale without official or veterinary supervision. These vaccines are optionally used with variable vaccination protocols and irrespective to the nature of viruses in a locality.

This research work aimed to study the response of the ordinary mesogenic vaccine strain (Komarov) for selection for heat resistance and the effect of addition of stabilizers in accordance to maintain its infectivity during handling without cold preservation. In another aspect, some molecular diagnostic tools that are rapid and sensitive for detection of ND virus were evaluated and compared with the conventional diagnostic procedures.

1.2. Classification of Newcastle disease virus:

1.2.1. The order; Mononegavirale:

At the eight International Congress of Virology held in Berlin in 1990, the International Committee on Virus Taxonomy (ITCV) established a new order (Mononegavirale) composed of four families (Filoviridae, Paramyxoviridae, Rabdoviridae and Bornaviridae) which are monoparatite, negative stranded ribonucleic acid (RNA) viruses (Pringle, 1991). The paramyxovirus study group of ICTV justified the grouping of the first three families under this order, but later Bornaviridae was included despite some differences (de La Torrie 1994, Schneemann *et al*., 1995 and Pringle 1997). The mechanism of gene expression control is the major character that differentiates Bornaviridae from other families.

Genomes of member viruses of the order Mononegavirale posses a conserved gene order. The re-engineered genomes of monopartite negative stranded RNA viruses by

reverse genetic techniques are returned during prolonged propagation in vitro (Conzelmann, 1996 and Schnell *et al*., 1996).

1.2.2. The Avian Paramyxoviruses:

Avian paramyxoviruses are typed species belonged to the genus Rubulavirus (Aviulavirus), which is greatly similar to the other two genera in the subfamily Paramyxovirinae (Paramyxovirus and Morbilivirus) of the family Paramyxoviridae (Pringle 1997). Recent work involving study of the whole Newcastle disease (ND) virus genome sequence has suggested that avian paramyxoviruses are sufficiently different from other rubulaviruses to warrant placing them in a separate genus (de Leeuw and Peeters 1999). ND virus lacks a gene encoding for small hydrophobic protein occasionally present in members of rubulaviruses (Lamb and Kolakofsky 1996).

Nine serotypes were identified and named avian paramyxovirus type 1 to type 9 (APMV-1 to 9). Newcastle disease was known to be caused by APMV type 1. In 1956 the APMV type 2 was isolated from chicken with acute laryngeotrachietis in Yucaipa, USA (Bankowski *et al*., 1960 and Dinter *et al*., 1964). The APMV type 3 was recovered from turkeys in Ontario, Canada in 1964 (Tumova *et al*., 1979) and from parakeets in The Netherlands in 1975.

It is important to distinguish between APMV-1 and APMV-3 as some cross relationship is present. Alexander *et al*., (1983) and Box *et al*., (1988) reported strong correlation between APMV-1 and APMV-3 titres in species systematically vaccinated against ND (Layers and breeders) and they hypothesized that the APMV-3 antibodies detected are, in fact, vaccine-induced antibodies to APMV-1 detected nonspecifically.

Also, Maldonado *et al*., (1994) reported that most of antibodies against APMV-3 detected in a survey in Southern Spain corresponded to vaccine induced APMV-1 antibodies.

During 1970s most of the other avian paramyxoviruses were isolated from different avian species as a result of a survey or surveillance (Alexander, 1991a). Avian paramyxovirus type 4 and type 6 were detected from ducks in Hong Kong, type 5 from budgerigar in Japan and type 7 from doves in Tennesse, USA.

Generally the member viruses of paramyxoviridae are responsible for the most devastating diseases of both animal and man and they cause, relatively, higher morbidity and mortality than any other group of viruses (Murphy, 1999).

1.3. Morphology of Newcastle disease virus:

Newcastle disease virus is spherical or polymorphic in shape and approximately 120-300 nanometer (nm) in diameter (Waterson and Cruickshank, 1963). The virion is composed of an outer lipoprotein coat (envelope) contained an inner nucleocapsid (core). The envelope is derived from the host cell plasma membrane and covered with large peplomers (Spikes) 8-200 nm in length (Klenk and Choppin, 1970, Murphy, 1999). The electron microscopy profile showed a herring-bone shaped helical symmetry of the nudeocapsid 600-800 nm length and 18 nm in diameter (Alexander, 1991b).

Disruption of purified virus preparations with sodium dodecyl-sulphate (SDS) and subsequent electrophoresis in polyacrylamide gel (PAGE) reveals at least seven polypeptides one of them is a host-derived protein (actin) (Alexander and Collins, 1981).

The total ND virus particle weigh about $500x10^6$ Daltons (Da) and its density in sucrose is 1.18-1.20 g/cm^3 (Alexander, 1991b), while the genome alone weigh about $7.5x10^6$ Da (Paul *et al*., 1979).

Newcastle disease virus is sensitive to lipid solvents as phospholipids represent 20-25% of envelope components while carbohydrates are about 6% and-like other paramyxoviruses- it is labile to heat, desiccation and disinfectants (Murphy, 1999). Infectivity of ND virus can be readily inactivated by ethyl ether, formalin and ultraviolet light (Paul *et al*., 1979). Other studies showed that ND is stable on storage for several days at 4^oC, for weeks at -20^oC and for years at -60^oC, but is less rapidly inactivated at 56^oC than other viruses of its family (Picken, 1964 and Lomniczi, 1975).

Haemagglutination and elution mediated by glycoprotein spikes are important biological properties of ND virus. Red blood cells of chicken, guinea pig and human type O are most susceptible to agglutination activity but elution, or even haemolysis, rapidly occur at room temperature or at 37^oC (Granoff and Henle, 1954).

Newcastle disease virus can be readily grown in the allantoic sac of embryonated chicken egg (Liu and Bang, 1953) and in many cell cultures of chicken and mammalian origins (Chanock, 1955, Brandt, 1961 and Wheelock and Tamm, 1961).

1.4. The genome of ND virus:

The complete genome of ND virus is composed of 15186 nucleotides (Phillips *et al*., 1998). It is a single linear molecule of negative sense RNA. There are six genes separated by conserved non-coding sequences that contain termination polyadenylation and initiation signals (Murphy, 1999). Proteins coded by these genes are nucleocapsid

protein (NP), Phosphoprotein (P), Matrix protein (M), fusion protein (F), Haemagglutinin-neuraminidase protein (HN) and large polymerase protein (L) (Millar and Emmerson 1988). Additional products designated V and W proteins may be produced by an RNA-editing event that occurs during transcription of P protein (Steward et al., 1993). The gene order is as follows;

1- Leader sequence of 55 bases long.
2- Nucleoprotein gene, 1746 bases, codes for nucleoprotein (53-56kDa), which protects the genome RNA.
3- Phosphoprotein gene, 1451 bases, codes for phosphorylated protein (53-56kDa) which is essential for RNA transcription.
4- Matrix gene, 1241 bases, produces matrix protein (38-40kDa) responsible for virus stability.
5- Fusion gene, 1792 bases, produces fusion protein (55-67kDa) that involved in cell fusion, virus penetration, cell to cell spread and induction of protective immunity.
6- Haemagglutinin-neuraminidase gene, 2002 bases, codes for two proteins (72-75kDa).These are: a) Haemagglutinin protein responsible for attachment to cells, haemagglutination and productive immunity. b) Neuraminidase protein responsible for virion release and destruction of inhibitors.
7- Large gene, 6703-6761 bases long, codes for a large protein (200kDa), which acts as RNA directed RNA polymerase.

1.5. Virus replication and infectivity:

The virion attachment to cell sialoglycoprotein or glycolipid receptor is mediated by HN protein that shares in the fusion process although its interaction with F protein is still controversial (Cobaleda *et al*., 2002). Studies revealed that a virus-type specific HN-F mechanism is necessary for efficient fusion process (Horvath *et al*., 1992, Hu *et al*., 1992) while another study showed that the fusion activity in a reconstituted viral envelope is exclusively F dependant (Cobaleda *et al*., 2002). The HN protein composed of three characteristic domains; an external large hydrophilic glycoprotein and two small (transmembrane hydrophobic and internal hydrophilic) domains (Sakaguchi *et al*., 1989, Morrison and Portner 1991). The external domain contains (beside the stalk region) the functional sites for cell attachment and neuraminidase activity (Takimoto *et al.*, 2000).

The fusion protein (F) is produced as a precursor protein F0 which should be cleaved by tissue or host cell proteases during transportation through the Golgi membranes into two disulphide-linked F1 and F2 forms or subunits (Morrison and Portner 1991). This process exposes the hydrophobic domain at the amino terminus of F1 subunit which is essential for the biological activity of the mature protein (Scheid *et al.,* 1978). Four domains of F1 polypeptide have to be involved into fusion process; the N-terminal peptide and three heptads repeat (HR) regions of the ectodomain (HR1, HR2 and HR3) (Champers *et al.,* 1990, Buckland and Wild 1989, Sergel, *et al.,* 2000). The HR3 has been proposed to be linked with the HN protein during the fusion activities (Sergel *et al.,* 2000) but in a later study a mutant HR region escaped the need for this mechanism. Toyoda, *et al.,* (1988) and Yusoff *et al.,* (1989) mapped the five major epitopes (A1-A5) on the fusion protein of NDV that are involved in fusion inhibition and neutralization. They found that the stretch of amino acids from residues 157-171 and individual residues

72, 78, 79 and 343 were critical for both structure and function of these epitopes. These findings had been confirmed and the three dimensional structure of the F protein was determined by Chen, *et al.,* (2001). The F glycoproteins of paramyxoviruses undergo extensive post-translation modifications in addition to the proteolytic cleavage of F0 into the active farms. These include cleavage of the signal sequence (Blumberg *et al*., 1985), glycosylation (Mountcastle, *et al*., 1971), fatty acid acylation (Schmidt, 1982 and Chatis and Morrison, 1982) and possibly rearrangement of intermolecular disulphide bonds (McGinnes, *et al*., 1985). Modifications to F2 include blockage of the N-terminus (Scheid *et al*., 1978) and trimming of the C-terminus by carboxypeptidase (Kohama, *et al.,* 1981). Determination of the amino acid sequence of ND virus F protein may suggest the locations of some of these processing events (Champers *et al*., 1990). In general, the fusion peptide is highly conserved among paramyxoviruses and is considered to be directly involved in mediating membrane fusion (Lamb 1993).

After penetration the whole nucleocapsid remains intact and containing its RNA-dependant RNA-polymerase (L protein) then six to ten discrete unprocessed m RNA transcribed by sequential interrupted synthesis from a single promoter to code for protein synthesis (Murphy, 1999). A positive-sense full RNA sequence is also synthesized to act as template for genome replication which is entirely cytoplasmic. Peeters, *et al*., (2000) mentioned that early in infection, transcription results in the synthesis of a short non-translated leader RNA and six mono-cistronic subgenomic mRNAs which are translated to produce viral proteins. The viral mRNAs are generated by a sequential and discontinuous RNA synthesis mechanism involving conserved cis-acting transcription start and stop signals. Later in infection when viral proteins accumulate, RNA synthesis

switches from transcription to translation or replication which is mediated by limited concentration of NP. This protein associate with viral RNA polymerase or with nascent positive sense RNA (Leader RNA) and prevent polyadenylation and reinitiation (Murphy 1999).

Newly synthesized NP molecules are recruited by the polymerase complex and sequentially added to the growing RNA chain. As a result of binding of NP, the polymerase complex ignores the transcription start and stop signals, resulting in the synthesis of full-length antigenomic RNA which, in turn, serves as template for the synthesis of full-length genomic RNA (Lamb and Kolakofsky 1996).

Following maturation (nucleocapsid formation), the HN and F glycoproteins arranged in patches on the cell membrane. Matrix (M) protein and other non-glycosylated proteins associated with altered cell membrane followed by alignment of nucleocapsid beneath the M protein then budding and release of virions occur (Peeples, 1988).

1.6. Newcastle disease:

1.6.1. Definition:

Newcastle disease is an infection of poultry caused by any strain of avian paramyxovirus type 1 (APMV-1) with an intracerebral pathogenicity index (ICPI) in one day old chicks greater than 0.7 (Commission of European Communities 1992). The same definition is approved by The Office de International Epizooties (OIE), but another option for strain virulence determination is delivered; multiple basic amino acids sequence of at least three arginine or lysine between residues 113-116 at the C-terminus

of F2 and phenylalanine at residue 117 which is the N-terminus of F1 protein should be present (OIE Manual, 2000).

1.6.2. Epizootology:

Domestic avian species including chickens, ducks, geese, turkeys and pigeons beside parrots, ostrich and doves are mostly affected by ND. The less susceptible species are penguins, owls, falcons, eagles, storks, sparrows and song birds while water fowl, koels, shags, gulls, coots and cranes are least susceptible (Kaleta and Baldauf 1988).

Emergence of ND and its first panzootic started at mid 1920s from South-east Asia, Java (Kranveld, 1926) and England (Doyle, 1927) and took over 30 years to cover most countries of Europe, Middle East and North America (Alexander 1991a). In 1930 similar signs to ND were reported in chicken in North America and termed pneumoencephalitis which was confirmed later by serological tests to be ND (Beach 1944).

The second panzootic emerged at early 1960s and covered most countries by 1973. This shorter period of spread is referred to the great expansion in poultry industry in many countries associated with the international trade of psittacine birds that involved in a rapid airborne shipment of the virus (Francis, 1973 and Walker *et al*., 1973). The disease was reported in Japan, Korea and Taiwan in 1962, in Near East and East Mediterranean in 1967 and in Latin America and Europe during early 1970s. These outbreaks involved vaccinated and non-vaccinated flocks as well and many questionable observations concerning the host range and reservoir of infection and control measures

were raised since then. It had been mentioned that some procedures adopted to control the disease in one area at a time may fail to be efficient in another place and that the vaccines and vaccination schedules may not be adequate for mass protection (Chu and Rizk 1972).

The large numbers of pigeons and doves reared in many countries had been ignored for long time as a potential source of ND till the flare up of the third panzootic started in late 1970s in the Middle East (Kaleta *et al*., 1985). Affected pigeon flocks showed only nervous signs. The spread to chicken was reported in many countries during early 1980s and contaminated feed by infected pigeons was accused to be the source of infection (Alexander et al., 1985).

Regarding distribution of ND, the situation cannot be static because some virulent viruses may be masked by vaccination. Spradbrow (1988) emphasized such difficulties involved in monitoring the disease and he concluded that the disease spread worldwide but only the countries of Oceania are relatively free from infection.

By the advance of molecular techniques, phylogenetic studies carried on different isolates from many places around the world illustrated the general relatedness between them. Toyoda *et al.*, (1989) studied the complete sequence of F and HN genes of ND virus. Ballagi *et al.*, (1996) carried a restriction site analysis of the 75% region from F gene amplified by polymerase chain reaction (PCR) for more than 200 ND virus isolates and grouped them as follows:

Group I: Lentogenic water fowl and chickens isolates

Group II: Include all North American virulent and avirulent isolates appeared prior to 1960.

Group III: Some isolates from the Far East.

GroupIV: The early European strains (Herts 33 and Italian) and their descendants.

Group V: Viruses reported in imported psittacine and epizootics of chickens at early 1970s.

Group VI: These are isolates from Middle East in late 1960s and later isolates from Asia and Europe.

The viruses of the first four groups are responsible for the first panzootic in late 1920s. While the last two, V and VI, are categorized to be the cause of the second panzootic. Viruses which encountered in the third panzootic are placed in a distinct subgroup in group IV.

Such groups are actually genotypes. Novel VIIa, VIIb and VIII genotypes and subtypes were reported in Far East, Europe and South Africa in late 1980s and later, another six genotypes were characterized (VIf, VIg, VIIc, VIId, VIIe and IX) by Liu et al (2003).

Virus transmission takes place by aerosol and ingestion of contaminated feed and water (Alexander 1991a). Inhalation transmission of pneumotropic viruses occur due to aerosols set by infected birds (Beard and Hanson, 1984), but bird to bird transmission of avirulent enteric and pigeon variant viruses occur usually without respiratory signs (Alexander et al., 1984).

The significance of vertical transmission of ND virus is still controversial. Infected embryos from naturally infected layers with virulent virus were reported (Lancaster and Alexander, 1975 and Beard and Hanson 1984). Coman (1963) and French *et al.*, (1967) reported infected chicks hatched from eggs infected with lentogenic

vaccinal viruses. This usually complicates the assessment of vertical or transovarian transmission. However, LaSota strain was isolated from the reproductive organs after vaccination (Raszewska, 1964).

The modes of introduction or spread of the virus include the movement of live birds (pet, game, racing and commercial birds), movement of other animals and people, equipments, poultry products, airborne, contaminated poultry feed and vaccines (Alexander, 1991a).

Permenant carrier state of ND is common in turkeys (Lancaster, 1963) but long term carrier with infrequent shedding of the virus may be possible in chickens if continuous supply of susceptible birds occur in a population (Heuschele and Easterday, 1970, Hanson 1974).

1.6.3. Pathogenesis:

During the first few days (3-4 days following exposure) a viremia of low magnitude could be detected in some exposed chicken (Heuschele and Easterday 1970).

The susceptibility of precursor fusion protein (F0) of the virus to be cleaved by different tissue proteases reflects the severity of ND symptoms, lesions and the rate of spread in tissues. Trypsin-like enzymes are essential for F0 cleavage of less virulent viruses; therefore they primarily multiplicate in respiratory and intestinal tracts (Roger and Oakeley, 2000). The cleavage motif with pairs of amino acids is commonly used as signal for processing a variety of polypeptide precursors in a wide range of cell types (Docherty and Steiner, 1982), whereas use of the single basic amino acid cleavage motif, seen in less virulent strains, is comparatively rare and apparently restricted to a few cell types (Schwartz, 1987). Hence, differences in virulence of different strains of ND viruses

can be ascribed to variable distribution of the proteases in host cells required for proteolytic activation of their F0 protein (Glickman, *et al*., 1988, Morrison, 1988, Nagai, *et al*., 1989 and Toyoda, *et al.,* 1987).

Collins, *et al.,* (1993) found that virulent viruses usually have a motif 112 R/K-R-Q-K/R-R-F 117 in chicken isolates or 112 G-R-Q-K-R-F 117 in pigeon isolates while in low virulent viruses the sequence is 112 G/E-K/R-Q-G/E-R-L 117. The wide variation in pathogenicity of some pigeon PMV-1 isolates for chickens is not related to such difference in amino acid motif at F1/F2 cleavage site nor due to production of HN0 and this means that the double pair of basic amino acids in the F1/F2 cleavage site is not necessary for the full expression of virulence (Collins, *et al*., 1994). Cleavage of F0 protein usually takes place between residue 116 and 117. Other studies showed a positive correlation between virulence or pathogenicity and a high content of basic amino acid residue at the cleavage site of F protein similar to other paramyxoviruses (Glickman *et al*., 1988). Schaper *et al*., (1988) stated that the property of neurovirulence of some isolates may not depend exclusively on the sequence of amino acids at F0 cleavage site because a minor (2 amino acid changes) difference between two uneven isolates (Texas GB and Beaudette C) was found, but a molecular property of HN protein could be an important factor in neurovirulence as they reported eleven amino acid changes between the two isolates. Despite that Kawamura, *et al*., (1987) reported no apparent correlation between antigenicity of HN protein and virulence.

Avirulent strains of ND viruses produce a haemagglutinin-neuraminidase precursor protein (HN0) with a 45 residue extension at its C-terminus relative to the shortest HN protein, 571 amino acid in length, of virulent strain (Scanlon *et al*., 1999).

Activation of the HN0 involves proteolytic removal of the C-terminal extension (Alexander, 1990). The HN proteins of virulent and low-virulent forms of ND viruses have the potential to be translated with C-terminal extension up to six amino acids (Gotoh, *et al*., 1988, Jorgensen, *et al*., 1987, McGinnes, *et al*., 1987 and Sakaguchi *et al*., 1989).

1.6.4. Shift in virulence:

A group of viruses responsible for the outbreaks diagnosed in two flocks of laying chicken in Ireland (Alexander *et al*., 1992) and outbreaks in Australia between 1998 and 2000 (Gould *et al.,* 2000) were suggested to be arisen from avirulent viruses endemic in these areas, as they were found to be very closely related genetically and antigenically. Moreover, new outbreaks in vaccinated poultry had been referred to the emergence of new genotypes or antigenic variants (Li *et al*., 2002, Liang *et al*., 2002, Panshin *et al*., 2002 and Panshin *et al*., 2001). Li *et al*., 2002 reported that a mainland China strain (CHA 7/96) is an antigenic variant responsible for recent outbreaks of ND in vaccinated flocks. In a cross protection test these authors reported only 40% protection against clinical signs conferred by LaSota strain when vaccinates challenged by CHA 7/96. They concluded that the antigenic differences between strains representing new genotypes or sub genotypes and the vaccine strain might influence the level of virus replication and shedding in vaccinated birds that could be an important contributing factor to vaccine break in the field. However, more work is needed to be undertaken to understand the role played by the emergence of new genotypes or subtypes in frequently occurring vaccination failure.

There is an instance of possible dynamic shift in ND virus populations although how such shifts occur has not been proven (Young, 2002). The genetic materials of all organisms, including viruses, are subjected to alteration by mutation. RNA genome, such as that of ND virus, seen more susceptible to such alterations because the genome itself is replicated (Spradbrow, 1992). During replication progeny viruses with variant genomes may be produced which will remain unnoticed in virus population until selection pressure, such as change in the environment (intensification of poultry industry) or host characteristics (breed or altered disease or immune status) favors a shift in the predominant virus type (Westbury 2001).

1.6.5. Clinical forms and pathotypes:

A broad range of clinical signs, varying from asymptomatic to systemic infections causing 15 to 100% mortality can be seen in ND outbreaks (Brandly, 1964). Experimentally, Beard and Hanson (1984) divided ND infections into five groups based on clinical signs usually observed. Viscerotropic velogenic infections characterized by severe hemorrhagic lesions in intestinal tract, neurotropic velogenic infections of high mortality following respiratory and nervous signs, mesogenic infections with respiratory and nervous signs (but usually with low mortality), lentogenic respiratory infections without apparent signs and asymptomatic enteric infections. The capacity of the virus to cause such a wide variation in severity of signs has been attributed to many factors including the host, age of birds, health status of the flock, environmental conditions and other concurrent infections (McFerran and McCracken, 1988). Consequently, the ideal clinical forms are rarely observed in the field. During the panzootic caused by viscerotropic velogenic pathotype from 1970 to 1973, marked severe respiratory signs

were reported in Great Britain and Northern Ireland (Allen, *et al.,* 1978, McFerran and McCraken 1988), while in other countries these symptoms were absent.

The viscerotropic velogenic pathotype (Doyle's form) may cause edema around the eyes and death. Green diarrhoea is frequently seen in birds not dying early in infection and prior to death, muscular tremors, torticollis, paralysis of legs and wings and opisthotonous become apparent and mortality frequently reaches 100% in fully susceptible flocks (Alexander,1992)

The Beach's form of ND (neurotropic velogenic pathotype) had been reported in North America (Beach 1942). It is characterized by sudden onset of severe respiratory signs followed by neurological symptoms a day or two later. Egg production falls drastically and diarrhoea usually absent. Morbidity may reach 100% but mortality is usually around 50% in adult bird and 90% in young chickens.

The mesogenic infections (Beaudette forms of ND) usually characterized by respiratory signs and drop in egg production and quality. Nervous signs are not common. Mortality is low except in very young susceptible birds and usually influenced by exacerbating conditions.

Adult birds do not show any symptoms in case of lentogenic infections (Hitchner form), but severe respiratory signs may be seen in young birds with mortality up to 30%. Vaccination or infection of broilers relatively late in their life can produce colisepticemia or airsacculitis (Alexander, 1992).

General (non-specific) clinical signs were reported in pigeons (Vindevogel and Duchatel, 1988) and chickens (Alexander *et al*., 1985) during the panzootic of 1980s with different viruses. In both species the predominant clinical features were diarrhea and

nervous signs but respiratory symptoms were unnoticed in uncomplicated cases. In turkeys, which are susceptible as chickens, signs are usually less severe (Box *et al*., 1970). Ducks and geese showed relative resistance, although they were infected, even to the virulent strains for chicken. However, an outbreak of severe disease in ducks had been reported by Higgins (1971). Outbreaks in different species of game birds caused by virulent ND viruses, which appeared similar to that in chicken, were reported (Lancaster 1966 and Beer 1976).

Alexander *et al.,* (1992) reported unusual symptoms during an outbreak in the Republic of Ireland in 1990. Slight drop in feed consumption and few sick birds were the initial symptoms followed by severe depression, green diarrhoea and death after two or three days of disease onset. Egg production dropped by 50% and eggs showed loss of shell color. The disease spread slowly between cages with low daily mortality rate (≤ 1%).

1.6.6. Lesions:

Gross lesions of ND depend on strain virulence or pathotype and the other factors influencing clinical features of the disease (Alexander, 1992). There are no pathognomonic lesions and even they may be absent completely. The prominent lesions, if present, are hemorrhagic and necrotic enteritis in jejunum, ileum and posterior half of duodenum and/or in the proventriculus and caeca. Cross-pathological changes are not always present in the respiratory tract, but when observed consist predominantly of hemorrhagic lesions and marked congestion of the trachea (Alexander and Allan 1974). Airsacculitis observed even in infections with mild strains and thick air sacs containing catarrhal or caeseous exudates is not uncommon (Beard and Hanson 1984). Chicken and

turkey layers infected with velogenic strains usually reveal egg peritonitis (yolk in the abdominal cavity) and follicles are often flaccid and degenerative beside hemorrhages and discoloration of the reproductive organs (Alexander, 1992).

Hemorrhages on the surfaces of liver and spleen without any other gross lesions were reported in unusual outbreaks (Alexander *et al*., 1992). On microscopy the only finding is viral encephalitis.

1.6.7. Diagnosis of ND:

Diagnostic evidences as a prerequisite to impose successful control measures could never be based upon clinical observations, lesions or mere detection of the virus. Also, detection of specific antibodies to ND virus has limited diagnostic value since it gives little information about strain virulence or vaccination status (Alexander 1992). Unless isolation and/or detection and characterization of the virus by conventional or advanced techniques are discussed with the concurrent environmental factors the proper diagnosis of ND could never be reached.

1.6.7.1. Isolation and characterization:

For propagation of ND virus from field samples the allantoic cavity of embryonated chicken egg and different types of cell and organ cultures are commonly used (Paul and Hope, 1979). Different types of cytopathic effects were usually observed. Syncytia and complete destruction of cell monolayer is common. However, some ND viruses did not produce cytopathic effect (Durand and Eisenstork, 1962). If less virulent virus were suspected to be present trypsin should be added to maintenance medium to promote growth and multiplication of the virus.

Conventional methods of virus pathotyping are based on the ability of virus to induce infection for chick embryo or live chicks. They include the mean death time (MDT) of egg embryos, intracerebral pathogenicity index (ICPI) in one day old chicks and intravenous pathogenicity index (IVPI) of 6 weeks old chicks. The average values and means according to the virulence of viruses for each test were discussed by Alexander and Allan (1974).

1.6.7.2. Serology:

Single radial immunodiffusion test was described by Chu *et al.,* (1982) and single radial haemolysis test was performed by Hari Babu (1986). The agar gel precipitation test was tried by Gelb and Cianci (1987). They prepared a precipitating antigen for ND virus by concentrating the allantoic fluid harvest of the virus by hydrochloric acid or ultracentrifugation followed by extraction with suitable detergents. The antigen was found to be stable for 6 months at -20^{o}C or +4^{o}C and withstood 20 freezing and thawing cycles.

Serum neutralization, as the most accurate test, for diagnosis of ND was applied by Beard (1980) in chick embryos and plaque neutralization was established by Beard and Hanson (1984).

Seromonitoring to assess vaccination response or to detect seroconversion as a result of infection is routinely done by haemagglutination inhibition (HI) test. This test is performed in a microtitre V bottomed plates and recommended by OIE to fulfill the standards of international trade (OIE manual, 2000). Beta procedure incorporating a known virus strain and titre was usually carried out to measure serum antibody levels to

ND virus. Sometimes filter paper-dried blood samples could be used successfully in microtitre HI test (Park, *et al.,* 1987).

1.6.7.3. Detection of viral antigens by monoclonal antibodies:

Monoclonal antibodies (Mabs) directed against highly conserved epitopes had been prepared by many workers for confirmation the diagnosis of ND (Russel and Alexander, 1983, Ishida, *et al.,* 1985, Meulemans, *et al.,* 1987 and Lama, *et al*., 1988). Specific Mabs used for rapid detection and differentiation between vaccine and other field viruses had been prepared (Srinivasapa *et al*., 1986, Erdei, *et al*., 1987). Alexander, *et al.,* (1985a) used a Mab which inhibit the majority of NDV isolates and strains but not the variant virus responsible for the current pigeon panzootic while Collins, *et al*., (1989) in contrast produced a Mab against the pigeon variant which gave high HI titres to isolates of that variant but not any other APMV-1 virus. Scanlon, *et al.,* (1999) stated that the antisera targeting pathotype specific characteristics of the C-termini of F2 polypeptides of ND virus have the potential to form the basis of rapid and robust pathotyping assays of virus isolates. They added that antisera to various portions of the HN0 extension accurately predicted the length of the C-terminal extension but did not enable pathotyping. However, the HN/HN0-specific antisera would certainly compliment anti-F2 for detection of ND virus and may have a role in epidemiological studies based on Mabs classification rather than for pathotyping applications. The advantages of Mabs assays could be summarized as follows:

1) Rapid diagnosis when single highly specific Mab for individual ND virus strain or isolate is used.

2) Can be incorporated in a simple test, such as HI, which is performed easily and inexpensively.
3) Immediate identification of the virus could be achieved in initial diagnostic screening if multiple Mabs are available.
4) An alternative for inhumane conventional pathotyping methods with consequent savings in cost and time.
5) Unlike other biological and biochemical parameters, Mabs can distinguish isolates and place them into meaningful groups in terms of biological and epizootological properties (Abenes, *et al.,* 1983; Hoshi *et al*., 1983, Nishikawa *et al*., 1983, Russell and Alexander 1983, Ishida et al., 1985, Srinivasappa, *et al*., 1986, Meulemans, *et al.,* 1987 and Lana *et al*. 1988).

However, the ultimate benefit by using available Mabs to detect the antigenic variations among NDVs is their capability to identify any new viruses that may arise- with sufficient differences to infect and cause disease in birds immunized with current vaccines- early enough to allow alternative control measures to be taken (Alexander 1990).

1.6.7.4. Molecular biological techniques:

Recently, a wide range of molecular techniques were validated for the diagnosis of ND by many workers. The polymerase chain reaction technique (PCR) was applied in order to detect and identify ND viruses for the first time by Jestin and Jestin (1991) targeting a specific region in the F gene coding for the cleavage activation site of fusion protein precursor (F0). Further studies based on reverse-transcription RT-PCR have been developed with subsequent analysis of the product by restriction enzyme endonuclease

(Ballagi-Pordany *et al*., 1996; and Kant *et al*., 1997), hybridization with probe (Jarecki Black *et al.,* 1992 and Aldous *et al*., 2001) and/or sequencing (Toyoda *et al*., 1989; Collins *et al.,* 1993 and King and Seal 1997).

RT-PCR has been approved as a reliable diagnostic and pathotyping tool specially when accompanied by restriction enzyme analysis. Nanthakumar *et al.*, (2000) carried out restriction enzyme digestion in RT-PCR amplification products with *Bgl I* and *Hha I* to type field and vaccine strains into velogenic, mesogenic and lentogenic groups without any exceptions. However, some isolates showed unexpected passiveness for some endonucleases' action (Jestin and Jestin 1991).

Kho *et al*., (2000) performed RT-nested PCR enzyme linked immunosorbent assay (RT-nPCR-ELISA) technique for rapid and sensitive detection of ND viruses. They used a colorimetric detection system of the product instead of electrophoresis techniques and thus escaped the more hazardous aspects of post-PCR analysis. The technique has been developed to work directly on tissue samples. The authors calculated their method of PCR product detection to be 10-fold more sensitive than the electrophoresis method. This nested PCR assay was claimed to be 100-fold more sensitive than standard PCR or even 1000-fold as described by Aradaib *et al.,*(1998).

Jarecki Black, *et al*., (1992) designed a universal radiolabel oligonucleotide probe for the detection of ND virus but they mentioned that a limitation of this technique is its inability to differentiate between vaccinated and naturally infected birds. Later, Jarecki Black and King (1993) produced another probe that distinguished virulent viruses from avirulent ones. The probe was designed to complement the cleavage activation site of F gene of strain Texas GB. This probe detected 36 similar virulent isolates but not any low

virulent virus. No cross reactivity was seen between the probe and other common avian viruses.

Oberdörfer and Werner (1998) also implemented a technique for detection and differentiation of ND viruses using probes. The aim was to develop a fast screening method for large number of samples. The probes designed were pathotype specific which were labeled with digoxgenin (DIG) and the labeled products were detected using anti-DIG alkaline phosphatase conjugate in an immunoassay. The authors recognized the potential problems associated with false negatives due to genome variability preventing primers binding, but they discussed the possibility of designing new primers to hybridize with new subtypes as they arise.

Aldous *et al.*, (2001) used fluorogenic probes (Taq Man^{M}) which gave good correlation with sequence data and the ICPI. Moreover, this assay is fast and did not require post-PCR handling of the product (Heid *et al.*, 1996, Petrik *et al.*, 1997)

Sequencing and sequence analysis for specific parts of viral genome, specially the region that codes for the cleavage site in F gene, were applied for different field isolates and vaccine strains of ND viruses.

Direct sequencing of ND virus cDNA for virus detection was reported by Stauber *et al*., (1995). The authors used RT-PCR and sequencing to screen vaccine preparations for the presence of ND virus and concluded that this system could be used to screen poultry vaccines and remove the requirement for current methods of indirect *in vivo* assays. Seal *et al*., (1995) used degenerate oligonucleotide primers for the amplifications of sections of the matrix and fusion genes, including the cleavage site encoding region.

Following phylogenetic analysis of the aligned sequences, it was possible to group the viruses for epidemiological studies and predict the pathotype of each virus reliably.

Martin *et al*., (1996) used RT-PCR and sequencing to differentiate between nine ND field virus isolates from USA. Unlike the results obtained in the same study by traditional characterization they were able to identify minor genetic heterogenicity in these lentogenic isolates. This may be of considerable significance as the recent problems in Australia have suggested that virulent viruses emerged from pools of endemic viruses of low virulence (Kirkland, 2000, Westbury 2001). Some viruses of low virulence may therefore represent a much greater risk than others, as they require fewer point mutations for this to occur (Alexander, 2001).

Stram *et al*., (1998) used divergence/similarity percentage of F gene sequence for pathotyping different ND viruses. They grouped two Israeli isolates as velogenic which had not been classified by conventional MDT and ICPI due to inconsistent results (Nanthakumar *et al*., 2000).

Ali and Reynolds (2000) developed a multiplex PCR assay for simultaneous detection of ND viruses and avian pneumovirus (APV) causing turkey rhinotrachietis (TRT), since infections with these two viruses are common in USA. They employed 309 bp from cDNA on the F gene of NDV and 631 bp on M gene of APV. This allows the detection of more than one virus at a time which lowers the cost and time.

1.6.8. Control of Newcastle disease:

After the second panzootic of ND and its bad consequences on developing poultry trade and industry control measures had been arisen. At first, some countries imposed strict regulations for introduction of birds and later produced vaccines and developed

vaccination regimes (Alexander, 1992). However, proper control measures for ND should be based fundamentally upon an adequate diagnosis. This means that just isolation or detection of virus or serological evidence of infection are insufficient and characterization of virus virulence at any level is essential for planning control procedures as they differ between virulent and avirulent viruses (Aldous and Alexander, 2001).

The appropriate combination of vaccination and prevention procedures is the most satisfactory approach for ND control (Chu and Rizk, 1972). Such procedures include good husbandry, hygiene and biosecurity (Young *et al.,* 2002).

1.6.9. Vaccination against ND:

1.6.9.1. Protocol of vaccination:

The best protocol to break infection cycle in endemic areas is to vaccinate chicken (Asplin 1953) and turkeys (Koncicki *et al.*, 1987) at early ages. Three vaccinations should be carried at 5-10, 15-22 and 28-36 days of age for broilers according to the level of maternal immunity. The third vaccination is better to be with mesogenic strain. Alexander (1992) mentioned that lentogenic live vaccines, such as V4 or HB1, are recommended for use as primary vaccines while mesogenic strains such as Mukteswar and Komarov are suitable for use in birds already primed.

In laying flocks revaccination should be done with inactivated or live mesogenic vaccines if immunity declined or uneven. Revaccination also must be carried out immediately for infected flocks (Mahasin *et al.,* 1980).

When planning for vaccination programmes is attempted, consideration should be given to the type of vaccine, immune and disease status of birds, level of protection

required in relation to suspected challenge with field viruses (Nagai *et al*., 1989). However, two programmes were proposed by the OIE according to disease circumstances (OIE manual 2000). One, if the disease is mild and sporadic, live Hitchner B1 administered intraocular or by aerosol at 1 day of age, live HB1 or LaSota at 18-21 day of age in drinking water, LaSota in drinking water at 10 weeks of age and inactive oil-emulsion vaccine at point of lay. When the disease is severe and widespread, the same protocol up to 21 days of age is adopted, revaccination at 35-42 days of age with live LaSota in drinking water or as aerosol and at 10 weeks of age an inactivated vaccine or mesogenic live vaccine is administered and repeated at the time of lay. Generally, better secondary response could be obtained when a mesogenic vaccine is used. Komarov strain, which is the mildest among them showed the best protective level of immunity and safety for grower and layer hens (Komarov and Goldsmith 1946).

Vaccination failure for ND had been reported by Rathore *et al.,* (1987). They referred the mortalities seen after three days of vaccination with less attenuated vaccines to the immunosuppression caused by intercurrent IBD infections with or without aflatoxicosis, defective vaccination procedures and leakage to the cold chain transportation of vaccines. Vaccinated birds exposed to virulent ND virus may also become infected and excrete virulent virus although they still remain healthy. Such birds may, therefore, be considered a source of infection for unvaccinated birds, so it is better to vaccinate all batches of birds in a farm at one time (Young *et al.*, 2002).

1.6.9.2. Live attenuated vaccines:

Strains from lentogenic and mesogenic viruses have been subjected to selection and cloning to fulfill different criteria in their production and application (OIE manual 2000).

Lentogenic live vaccines must contain a high concentration of virus per dose ($10^{7.2}$ EID_{50}) to overcome maternal or residual immunity which is 10 to 100 times higher than generally accepted. This could be achieved by using antibody free eggs for vaccine production, adding skimmed milk to protect virus during freeze-drying and improve vacuum sealing of vials and ampoules (Chu and Rizk 1972).

All mesogenic vaccine viruses have two pairs of basic amino acids at the C-terminus of F2 protein and ICPI values of around 1.4. This means that infection of birds with such viruses would fall within the definition of clinical ND according to the OIE standards but as these vaccines are primarily used in endemic countries, this may not be necessarily to preclude their use. In emergency situations where severe outbreaks occur the losses or other disadvantages involved in primary use of more virulent strains may be acceptable (Alexander 1992).

However, the use of live vaccines may be restricted by legislations in developed countries (OIE manual 2000). The European commission decision No. 93/152/EEC restricted the use of live vaccines in member states of European Union by 1995 to those for which the master seed has been tested and shown to have an ICPI of < 0.4 if no fewer than 10^7 mean egg infections dose (EID_{50}) are recommended per dose or < 0.5 if no fewer than 10^8 EID_{50} are administered to each bird.

1.6.9.3. Inactive vaccines:

Both virulent and avirulent strains are used as seed virus to produce killed vaccines. Although from the aspect of safety, the use of avirulent strains appears more suitable (OIE manual 2000). As much larger amount of antigen is required for immunization (no virus multiplication occurs) the selection of a high yield strain is essential for the production of a potent vaccine. The Ulster 2C is very suitable for this purpose (Gough, *et al.,* 1977).

In the manufacture of inactive vaccines the harvested allantoic fluid is treated with either formaldehyde (typical final concentration is 1:1000) or beta propiolactone (typical final concentration is 1:2000-1:4000). The time required should be sufficient to insure complete inactivation. Efficiency of inactivation process could be tested in embryonated chicken eggs; aliquots of 0.2 ml from each batch should be passaged three times in eggs and residual live virus detected (Allan *et al.,* 1978). Most of the inactivated vaccines are not concentrated and are usually emulsified with mineral or vegetable oils (Cross, 1988). The oil phase usually consist of nine volumes of highly refined mineral oil such as Marcol 52, Drakeol 6VR and Bayol F plus one volume of emulsifying agent such as Arlacel A, Montanide 80 and Montanide 888 (Palya and Rweyemamu, 1992). The aqueous phase is the inactivated virus to which a non-ionic emulsifier, such as Tween 80, is added. The oil aqueous phase ratios are usually 1:1 to 1:4. However, the final product should be easy in use (stable and not highly viscous).

1.7. Immunity:

The transmission of maternally derived antibodies (MDAbs) in birds takes place from immunized hen to their offspring through the vitelline membrane circulation (Heller

et al., 1977). These MDAbs normally last for few weeks after hatching and protect young birds against infectious organisms or it modifies infection with virulent viruses.

The level of MDAbs in chicks is correlated to the level of antibodies in their laying flocks (Chu *et al.,* 1973, Danchev and Arnaudov, 1974). The peak titre in chicks is normally detected at the second or third day after hatch by HI test (Mahasin 1990). Allan et al., (1978) reported that MDAbs HI titre of 2^4 to 2^6 (mean log of $2^{5.2}$) provides 100% protection for chicks against challenge.

Vaccination of birds with some strains may protect them from challenge with others. Alexander, *et al.,* (1992) demonstrated that vaccination with Hitchner B1 strain defends birds against challenge with strain 34/90 that differs from vaccine strain in most of F and HN antigenic epitopes. However, the antibodies directed against either of the functional surface glycoproteins (HN and F polypeptides) may be cabable of neutralizimg ND viruses (Russel, 1988). Monoclonal antibodies specific for F polypeptides epitopes were found to induce stronger neutralization than that for HN polypeptides in *in vitro* and *in vivo* tests.

The level of serum antibodies depends mostly on the strain type (Alexander, 1991). In infected birds, serum antibodies could be detectable after 6-10 days of infection or vaccination and the peak response at about 3-4 weeks. Decline in antibody titre also varies with type of the strain and titre achieved but generally is much slower than the response. HI antibodies may persist for a year in birds recovered from mesogenic virus infection. Reinfection or immunization at the beginning of antibodies declining is essential for better secondary response (Allan et al., 1978).

The early protection against challenge after active immunization is cell-mediated which can be detectable two or three days after infection but strong secondary response dose seem to occur (Timms and Alexander 1977, Ghumman and Bankowski 1976). This may explain the early protection of vaccinated birds before detectable antibodies seen (Allan and Gough 1976). Thus the importance of cell-mediated immunity in protection conferred by vaccine is still not clear (Alexander 1991).

1.8. Thermostable vaccines:

The thermostability of a substance is defined as its ability to remain active at a particular level after treatment by defined temperature for specific period of time (Osol 1972). For viruses the thermostability is measured by their ability to infect cell culture or living animal after being exposed to a constant temperature (e.g.56^{o}C) for different time intervals. However, a virus is considered thermostable if the decrease of infectivity titre is less than 1 log 10 following exposure for one hour at 56^{o}C (Rovozzo and Burke 1973, Burleson *et al.*, 1992). Therefore, thermostability of a vaccine is expressed by the length of time it will retain an infectivity titre sufficient to induce protective response at a particular time.

It is known that exposure to heat will alter both the protein and the nucleic acid of viruses. The contributions of these targets seem to be different according to temperatures. The poliomyelitis virus exhibits little inactivation at temperature below 44^{o}C and the viral RNA is the target, but at 44^{o}C both viral proteins and nucleic acid are affected (Spradbrow 1992b). Moreover, heat energy is required to inactivate viral enzymes and denature DNA viruses.

Spradbrow, (1992b) mentioned that after a series of studies on Aujeszky's disease virus and rhinopneumonitis virus (Equine herpes virus type 4) it could be suggested that heat resistant virions are not true mutants but they are a fraction from the original population that their habit was masked unless selection for heat resistance occurred.

Original studies on heat resistance of NDV were made by Goldman and Hanson (1955). The heat labile Najarian (human) strain was the starting material. By stepwise reduction of labile population they successfully produced variants of heat stable haemagglutinin that survived exposure at 56^{o}C for 300 minutes and their infectivity enhanced from 60 to 240 minutes. Although they considered these variants as mutants, but later, Hanson (1988) pointed out that it might not be a fact. Moreover, King, (2001) found that thermostability of NDV could be raised by subsequent heat treatment cycles. He reported an increase in haemagglutinin thermostability from ≤10 minutes to ≥120 minutes at 56^{o}C in two to four cycles of treatment, while infectivity of some isolates were raised from 60 minutes in the first cycle to > 120 minutes by the third cycle.

For epidemiological studies, the thermostability of the NDV haemagglutinin and infectivity could be a successful parameter in the absence of other markers (King, 2001). By nucleotide sequence analysis the vaccine viruses could be phylogenetically categorized and differentiated from other viruses possessing haemagglutinin thermostability (Seal *et al.*, 1996 and Werner et al., 1999).

Some strains of NDVs, such as V4 and Ulster strains, showed relative heat resistance (Spalatin and Hanson 1976). The haemagglutinin of these strains was stable for 120 minutes at 56^{o}C while of other lentogenic strains (B1, LaSota and F strains) survived only for 5 minutes. However, when chickens infected with V4 or Ulster virus a distinct

subpopulation could be detected from different organs; viruses yielded from tracheal swabs were eluted rapidly from chicken red blood cells and had a labile haemagglutinin(5 minutes survival at 56^{o}C), but viruses from cloacal swabs were eluted slowly and had heat stable haemagglutinin character of parental strain. The standard vaccine strains B1, LaSota and F did not display this heterogenicity (Spradbrow 1992a).

1.9. Vaccination against the disease in the Sudan:

First attempts to use vaccines against ND in Sudan were done in 1952 just after the first report of the disease. A total of 4700 doses imported from South Africa were used in some provinces (Ali, B H 1978). Local production of ND vaccine started in 1958 when Muketswar strain was used as seed virus to produce wet live-attenuated egg-adapted vaccine. In early sixties the Komarov strain was introduced from Lebanon and involved in the production of a live lyophilized vaccine at the Central Veterinary Research Laboratory (CVRL). This vaccine was recommended to be administered by nasal route (Karrar and Mustafa 1964). Elamin *et al.*, (1993) found that this strain will induce a high level of antibodies and conferring a protective level against challenge if applied by intranasal or intramuscular routes but not by oral route. The lentogenic F strain was used for early vaccination (mainly for broilers) in endemic areas since mesogenic strains could not be used safely before 3-4 weeks of life (Mahasin *et al.,* 1980). However, for layers a poster dose by the mesogenic Komarov strain should be done 4 weeks later (Khalafalla *et al.,* 1994). Although Beaudette *et al.*, (1949) pointed that chicken under 8 weeks of age should not be vaccinated with mesogenic strains, Haroun and Hajer (1989) reported no adverse reaction when K strain administered to one

day old chicks. Nevertheless, except if there is a low level of MDAbs (<5.2 log2) the primary vaccination with lentogenic strains is preferred to be delayed up to the third week of life (Zakia *et al.*, 1983).

1.10. The impact of rural poultry in the control of ND:

Despite that many vaccines against ND are commercially available but their delivery in rural sub-Saharan Africa has often proved economically unsustainable and technically inappropriate because the maintenance of the cold chain needed could not be achieved effectively (Spradbrow 1994). So, continuous presence of ND virus among extensive and rural flocks poses a real threat to the control procedures of the disease (Roger and Oakeley 2000).

The epidemiological features of ND within the back yard or rural poultry remain unclear and complicated by the role of wild birds (Martin 1992, Awan *et al*., 1994). Spradbrow (1994) found that ND virus could be maintained in a relatively small population of chicken. The turnover of birds within a flock would result in about 30% potentially unprotected birds over 16 weeks (Oakeley 1998). Therefore, vaccination of rural flocks should be considered every 4 months or it may follow a seasonal or hatching pattern (Wilson *et al.*, 1987, Oakeley 1998).

As mentioned before, the aims of this research work are: a) to study the response of the candidate Komarov strain (an ordinary mesogenic vaccine virus) for selection for heat resistance and the effect of addition of stabilizers on maintaining its infectivity during handling without cold preservation. b) to evaluate some molecular diagnostic tools in detection of ND virus and compare them with the conventional diagnostic procedures.

CHAPTER II

GENERAL

MATERIALS AND METHODS

2.1. Newcastle disease viruses:

Four lentogenic and one mesogenic vaccine strains were obtained from the CVRL as lyophilized products. Other five field isolates of the virus were obtained from the same source. All viruses were propagated in ECEs and Vero cell line. Table (2-1) showed information about these viruses.

2.2. Virus propagation in embryonated chicken eggs:

The embryonated chicken eggs (ECE) were supplied by the Viral Vaccine Production Department of the CVRL. ECE were 9-10 days of incubation and used routinely for vaccine production. All vaccine strains and field viruses were propagated in the allantoic cavity and harvested at the third day of inoculation.

2.3. Virus propagation in Vero cell line:

This cell line was obtained from the above source and used for production and titration of some other live vaccines. Vero cells were sub cultured in 25 ml Falcon tissue culture flasks. Glasgow minimal essential medium with additives were used for growth and maintenance of cells. All viruses were adapted to this cell line by 2-3 successive passages. One freeze-thaw cycle was carried out on infected cells when obvious cytopathic effect observed followed by centrifugation. Cell-free supernatant was dispensed in 1.5 ml microcentrifuge tubes and kept at -20^{o}C till used.

Table (2-1):

The Sudanese field isolates and vaccine strains used in the experiments.

Designation	Type	Virulence	Passage No.	MDT	Year of isolation
OB	Field virus	Velogenic	3	48 h	1974
KU	Field virus	Velogenic	2	36 h	1975
GR	Field virus	Velogenic	2	36 h	1979
A12	Field virus	Unknown	2		2003
A105	Field virus	Unknown	Tissue sample		2003

Komarov	Vaccine	Mesogenic	Standard vaccine strain
LaSota	Vaccine	Lentogenic	Standard vaccine strain
B1	Vaccine	Lentogenic	Standard vaccine strain
Clon30	Vaccine	Lentogenic	Standard vaccine strain
Clon79	vaccine	Lentogenic	Standard vaccine strain

2.4. Experimental chicks:

One day old chicks of Bovans layer breed were obtained from Coral Hatcheries, Khartoum. They were kept under strict hygienic confinement at the CVRL for six weeks till used in experiments. No vaccines were administered.

2.5. Microtitre haemagglutination test:

This test was used to calculate the titres of viruses propagated in embryonated chicken eggs. It was performed to estimate the stability of haemagglutinin of selected heat-resistant vaccine strain (with or without addition of stabilizers) after being stored at two different temperatures for different periods of time. Also, it was used to assess stability of infectivity on storage of the same virus after one passage in ECEs. The test was carried out according to Allan and Gough (1976). Briefly, it was conducted as follows:

a) Phosphate buffered saline (PBS) was dispensed into 96-well polystyrene V-bottomed microtitre plate. Each well received 0.025 ml of PBS.
b) 0.025 ml from each virus suspension (infective allantoic fluid) was placed in the first well of each row. The last row was left without virus.
c) Two fold dilutions of virus suspensions were carried out across the plate.
d) 0.050 ml of 1% chicken red blood cells (RBCs) suspension in PBS was dispensed to each well.
e) Plates were tapped gently to mix the contents of wells and left at room temperature for 40 minutes.

f) When control RBCs in the last row settled, the haemagglutination (HA) titre of each virus was recorded as the highest dilution showing complete HA.

2.6. Viral nucleic acid extraction:

Extraction of viral nucleic acid was carried out by the QIAamp viral RNA extraction kit following the mini spin protocol (QIAGEN GmbH, Hilden, Germany).

a) The lyophilized Carrier RNA was dissolved in 1ml AVL buffer and transferred to the AVL bottle and mixed thoroughly. 560 microlitre (ul) from this preparation were dispensed in each of 1.5 ml microcentrifuge tube and kept at $4^{o}C$.

b) All samples and reagents were equilibrated to room temperature (about$25^{o}C$).

c) 140 ul of virus suspension (purified by centrifugation) was added to the buffer AVL containing Carrier RNA and mixed by pulse vortexing for 15 seconds.

d) The mixture was then left at room temperature for 10 minutes.

e) The tube was briefly centrifuged to remove drops from inside of the lid.

f) 560 ul of pure ethanol was added and mixed by pulse vortexing for 15 seconds followed by brief centrifugation.

g) The QIAamp spin column (in 2ml collection tube) was mounted in a rack and loaded with 630 ul from the mixture then centrifuged at 8000rpm for 1 minute.

h) The column was transferred to another collection tube, the remaining 630 ul of the mixture was loaded and the previous centrifugation step was repeated.

i) 500 ul of buffer AW1 (washing buffer 1) was added to the column and centrifuged at 8000rpm for 1 minute. Another 500 ul of AW2 (washing buffer 2) was added and centrifuged at 14000 rpm for 3 minutes.

j) The spin column was transferred to 1.5 ml microcentrifuge tube and loaded with 60 ul of buffer AVE to elute viral RNAs. After it had been left at room temperature for 1 minute it was centrifuged at 8000 rpm for 1 minute.

k) Finally the microcentrifuge tubes containing RNA extracts were labeled and kept at -20^{o}C till used in RT-PCR.

2.7. Primers:

Two pairs of primers were designed from the published sequence of Miyadera strain by Toyoda *et al.*, (1989). These primers were flanking 356bp and 216bp long regions in the F gene involving the sequence encoding the cleavage activation site of the F0 protein. They were manufactured by Roth Ltd. (Carl Roth GmbH+Co., Karlsruhe, Germany). These primers were used previously for detection and pathotyping some Indian isolates of Newcastle disease virus (Nanthakumar *et al.*, 2000). Table (2-2) shows the primers' designation, sequence and position on F gene.

2.8. Reverse transcriptase polymerase chain reaction (RT-PCR):

Amplification of the target sequence by the first pair of primers (nd1and nd2) was performed by using Titan one-tube RT-PCR system (Roche Diagnostics GmbH, Nonnenwald 2, Penzberg, Germany). Two master mixtures were prepared for each 10 samples as follows:

Table (2-2):

Primers used in RT-PCR and nested PCR for detection of Newcastle disease virus.

Primer	Sequence	Position	Product length
nd1	**$^{5'}$GCA GCT CGA GGG ATT GTG GT$^{3'}$**	158-177	356bp
nd2	**$^{5'}$TCT TTG AGC AGG AGG ATG TTG$^{3'}$**	513-493	
nd3	**$^{5'}$CCC CGT TGG AGG CAT AC$^{3'}$**	282-298	216bp
nd4	**$^{5'}$TGT TGG CAG CAT TTT GAT TG$^{3'}$**	497-478	

Mix 1: 20 ul 10 mM dNTPs.

25 ul 100 mM Dithiothreitol (DDT).

05 ul RNAse inhibitor (5-10 U/reaction).

	130 ul	dd H_2O.
	180 ul	Total volume.
Mix 2:	100 ul	5X RT-PCR reaction buffer.
	40 ul	25 mM Mg cl_2.
	10 ul	Titan enzyme mix (containing AMV transcriptase and blend of Taq and Tgo DNA polymerases).
	100 ul	ddH_2O.
	250 ul	Total volume.

For each sample the reaction mixture (50 ul total volume) composed of 18 ul of mix 1, 25 ul of mix 2, 5 ul from RNA extract and 1 ul from each primer (nd1 and nd2). All reactants were placed in 0.2 or 0.5 ml thin-walled PCR tube, mixed thoroughly by pulse vortexing and briefly centrifuged to collect the sample in the bottom of the tube.

The RT-PCR was performed in Techne PHC-2 thermal cycler (Techne, Princeton, NJ, USA). Initial reverse transcription step was done at 50^{o}C for 30 minutes followed by 40 cycles of amplification. Each cycle include denaturation at 94^{o}C for 1 minute, annealing at 52^{o}C for 1 minute and extension at 68^{o}C for 1minute. Final extension lasts for 10 minutes. The total time of thermal profile took about 200 minutes.

2.9. Nested PCR:

The reaction mixture was composed of 5 ul of amplified product by RT-PCR(the previous step), 1 ul Taq DNA polymerase,1 ul from each primer of the second pair (nd3

and nd4) and 42 ul from the master mixture containing 250 ul 5X PCR buffer, 100 ul Mg cl_2 , 50 ul dNTPs and 1100 ul ddH_2O. the thermal profile for the nested PCR started with initial denaturation at 94^oC for 2 minutes followed by 30 cycles of amplification (94^oC for 1 minute, 55^oC for 30 seconds and 68^oC for 45 seconds) followed by final extension at 68^oC for 10 minutes.

2.10. Agarose gel electrophoresis:

The amplified products of cDNA transcribed from viral RNA molecules by RT-PCR or nested PCR primers were analyzed in agarose gel. The agarose gel preparation, running buffer preparation and electrophoresis were carried out as follows:

a) Preparation of 1% agarose gel:

One gram of agarose powder (molecular biology grade) was suspended in 100 ml of Tris-boric acid-EDTA (TBE) buffer. The suspension was heated in microwave oven for few minutes until the agarose was completely melted. When cooled down 30-40 ml of melted agarose was poured in the gel tray loaded with a comb.

b) Preparation of Tris-boric acid-EDTA:

107.81 gm 0.89 M Tris- (hydroxymethyl)-aminomethane.

55.03 gm 0.89 M Boric acid.

7.44 gm 0.20 M EDTA.$2H_2O$.

Up to 1 litre distilled water.

This mixture (10X TBE) was dissolved on magnetic stirrer then another litre of distilled water was added to prepare 5X buffer concentration. The buffer was then kept at room temperature and used at 1X concentration for gel preparation and electrophoresis.

c) The molecular weight marker:

Per each electrophoresis run two microlitre of one hundred base-pair ladder molecular weight marker (MW marker) was placed in the first lane. Separate distinguishable bands were seen when the gel was stained with ethidium bromide.

d) Electrophoresis:

The agarose gel was submerged in the buffer basin of the electrophoresis apparatus filled with 1X TBE containing 0.001% Ethidium bromide (50 mg/ml). RT-PCR or nested PCR products together with the MW marker were loaded in the gel after being stained with an indicator dye (Bromophenol blue). Constant electric current of 88 mV was then switched on for about 40 minutes.

CHAPTER III

THERMOSTABILITY

Thermostability Trials on a Mesogenic Newcastle Disease Virus

Introduction:

The control of Newcastle disease in rural poultry by using thermostable vaccines in some countries seems to be an interim strategy (Young *et al*., 2002) and the ultimate goal of usage of such affordable vaccines is to increase village chicken production and, in turn, the purchasing power of chicken owners, then more expensive vaccines may come into use. These vaccines were found to be stable for relatively longer periods at specific temperatures and could be supplied as feed-added vaccine. They showed good efficacy in controlling ND as they conferred adequate flock immunity in low-risk exposed areas, in countries free from velogenic viruses like Australia and New Zealand and in some tropical countries where the rearing rural poultry is certainly an important popular activity.

The V4-HR and I_2 are two vaccine strains with enhanced thermostability developed by the Australian Centre for International Agricultural Research (ACIAR) since 1984. The V4-HR strain was successfully tested in some Asian and African countries (Young *et al.*, 2002). The I_2 strain is similar to V4-HR but free from commercial ownership.

The I_2 strain showed the best thermostability out of 18 isolates selected from 45 avirulent Australian ND viruses (Spradbrow 1993/4). Generally, this strain fulfils the standards of production required and quality control aspects of the master seed concerning virulence, spread, induction of Immunity, growth in embryonated chicken eggs and chicken embryo kidney cells monolayer (Bensink and Spradbrow, 1999). This strain could be administrated safely as eye drops, water-added or feed-added vaccines (Alders and Spradbrow 2001).

Although, I_2 strain was tested in Sudan under laboratory conditions (Wegdan 2002) but, at the moment, it may not be satisfactory in the field because circulating virulent viruses could not be masked by such strain. Hence, an easily handled and durable vaccine of mesogenic origin is greatly needed under such situation. Freeze dried I_2 vaccine stored at 4^oC retained a titre greater than 10^6EID50/dose for at least 12 months, 8 weeks at 28^oC and for 2 weeks at 37^oC. Liquid I_2 vaccine diluted 1 in 4with 2% gelatin and antibiotic solution retained a high titre for at least 6 months at 4^oC and for 7-14 days at 28^oC.

Fortunately, some mesogenic ND virus strains may respond to thermal selection (Spradbrow 1992a, Young *et. al*. 2002). In this research work the response of Komarov strain to selection for heat resistance was studied and the stability of the residual (heat stable) virus with and without stabilizers was evaluated and compared with parent strain.

Materials and Methods:

The candidate strain:

The mesogenic Komarov strain of NDV was used in the experiments. Twelve vial of lyophilized allantoic harvest were kindly supplied by the CVRL. Each vial contains 400 doses and each dose of about 10^7EID_{50}. No stabilizer was added to this vaccine strain.

Primary selection for heat resistance:

The repeated short exposure to $56^\circ C$ and harvesting residual infective particles was the protocol adopted in selection for heat resistance. Grant water bath SS40-2 (Grant Instruments Ltd., Cambridge, Barrington, England) was used in the experiments (Figure

3-1). According to Ideris *et al.*, (1990), and with some modifications, four heat selection cycles were carried out.

The 12 lyophilized vials of vaccine were reconstituted by sterile double distilled water and submerged in a water bath set constantly at 56^{o}C. Two vials were removed after one, two, three, five, seven and nine hours. Every two vials were pooled and immediately cooled in ice bath. Before inoculation, a brief clarification was carried by cold centrifugation at 1500 rpm for 3 minutes.

The undiluted clear supernatant was used to inoculate five embryonated chicken eggs (ECEs) 9-10 days of incubation. Each ECE received 0.1 ml inoculum volume. Five other ECEs were inoculated by the diluent alone as control. All ECEs were incubated at 37^{o}C and candled daily for 7 days.

Allantoic fluids of dead embryos were tested for virus multiplication by haemagglutination activity test. Positive harvests were involved in microtitre haemagglutination test (see general materials and methods) to estimate their titre. Further three cycles of heat treatment were subsequently done. The residual virus obtained from one-hour exposure at 56^{o}C from each cycle was used for the next cycle.

Terminal limiting dilution:

The heat-adapted virus obtained from the last cycle was subjected to three consecutive purification steps by terminal limiting dilution in ECEs according to De Boer and Barber (1964). Briefly, the harvest of the last cycle was 10 fold serially diluted in sterile normal saline and each dilution was used to inoculate 5 ECEs. The least positive harvest of each step was 10 fold serially diluted and subsequently involved in the next

step. Finally a virus lot was prepared from the last step by inoculating 50 ECEs. The harvest collected at the third day of infection was cooled and clarified.

Addition of stabilizers:

Separate 10% solutions of lactalbumen hydrolysate (LAH), lactose (Lac), sucrose (Suc) and skimmed milk powder (SMP) in 100 ml double distilled water were prepared, sterilized by autoclaving at 15 lb/in for 20 minutes (121^{o}C) and then cooled to 4^{o}C. Equal volumes of stabilizer solutions and the clarified harvest of the virus were mixed so as to prepare 5% concentration of stabilizer in the final product. The mixture was dispensed in 1.5ml aliquots in robber-capped vials for lyophilization. Some vials were filled with the clarified harvest alone.

Lyophilization:

Normal lyophilization was carried out in Edwards's lyophilizer as follows:

- Shelves were chilled to -40^{o}C or lower.
- Trays of vaccines were placed and chilling continued for further two hours.
- The condenser was set on. When temperature reached -45^{o}C…
- The vacuum pump was set to draw full vacuum (till reach about 100mTorr).
- Constant shelves' temperature was maintained at-30^{o}C.
- While keeping linear adjustment between pressure and temperature, the shelves temperature was gradually raised to-20^{o}C for 2 hours, to -18^{o}C for 3 hours, to 15^{o}C for 3 hours then at 0^{o}C overnight.
- Temperature was raised to 15^{o}C for 4 hours.
- Vacuum was drawn to maximum and rubber stoppers were applied to the vials.

- Finally, all valves were opened and vials were removed.

Stability tests on the lyophilized products:

Tests for stability of haemagglutinin were carried out as follows; a number of vials from each group of stabilizer-free and stabilizer-added products were left at room temperature (28-32^{o}C). Other groups were kept under refrigeration (4-8^{o}C). The stability of infectivity was carried only for the stabilizer-free product. Control vials from the parent vaccine were incorporated with each group.

Results:

Response of the Komarov strain to selection for heat resistance:

Residual (heat resistance) viral particles were detected only after one-hour exposure at 56^{o}C. Haemagglutination titre of the residual virus rose from 7 log2 after the first cycle to 10 log2 after the fourth cycle. Figure (3-2).

Haemagglutinin stability of the selected virus:

Stability of haemagglutinin of the selected virus particles at room temperature and at refrigeration compared with the parent virus was expressed in log2 and shown in table (3-1).

Stability of infectivity of the selected virus:

Table 3-2 showed HA titre of the harvests obtained from the stable viruses grown in ECEs after days of storage at refrigeration and at room temperature.

Effect of stabilizers on stability of the selected Komarov strain:

Haemagglutinin thermostability of selected Komarov strain to which stabilizers were added and stored at two different temperatures was shown in table (3-3). Table (3-4) shows the differences in pellet characteristics between different stabilizers added.

Figure (3-1):

Water bath used in thermostability experiments

Grant SS40-2 water bath.

Figure (3-2):

Haemagglutination titres of allantoic fluid harvests of the residual virus from Komarov strain after four heat selection cycles

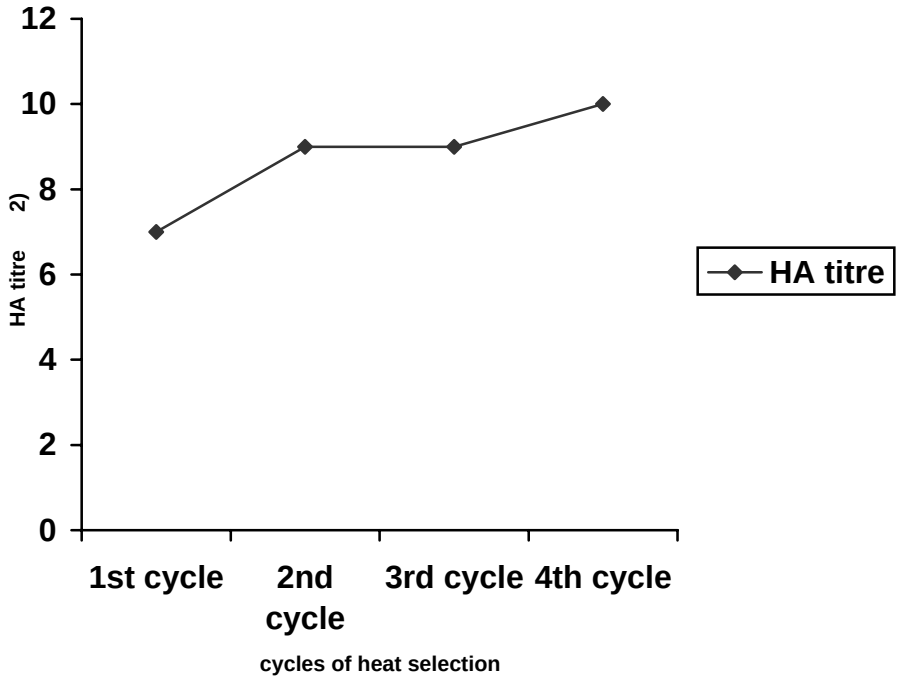


Table (3-1):

The remaining virus Haemagglutination titres of selected and parent vaccine strains stored at two different temperature

Virus	Temp	Log 2 HA titre after days at specific temperature								
		1	**4**	**7**	**10**	**15**	**20**	**25**	**30**	**37**
Selected K strain	**+4°C**	10	10	9	9	8	8	7	7	7
	28-32°C	9	8	8	7	4	-	1	1	1
Parent vaccine	**+4°C**	9	8	7	6	-	3	2	2	-
	28-32°C	7	4	4	2	1	1	1	-	-

Table 3-2:

Infectivity titres of the residual stable virions from selected and parent strains stored lyophilized without stabilizer at two different temperatures.

Virus	**Temp**	**Days and HA titre of infectivity**									
		1	**2**	**5**	**9**	**12**	**15**	**20**	**30**	**37**	**50**
Selected K strain	**+4°C**	10	10	10	10	10	9	9	8	8	8
	28-32°C	9	9	8	8	8	7	4	3	3	3
Parent vaccine	**+4°C**	9	9	7	7	7	3	3	2	2	2
	28-32°C	8	8	3	2	2	1	1	-	-	-

Figure (3-3):

Haemagglutinin thermostability of heat selected and parent Komarov strains stored at two different temperatures

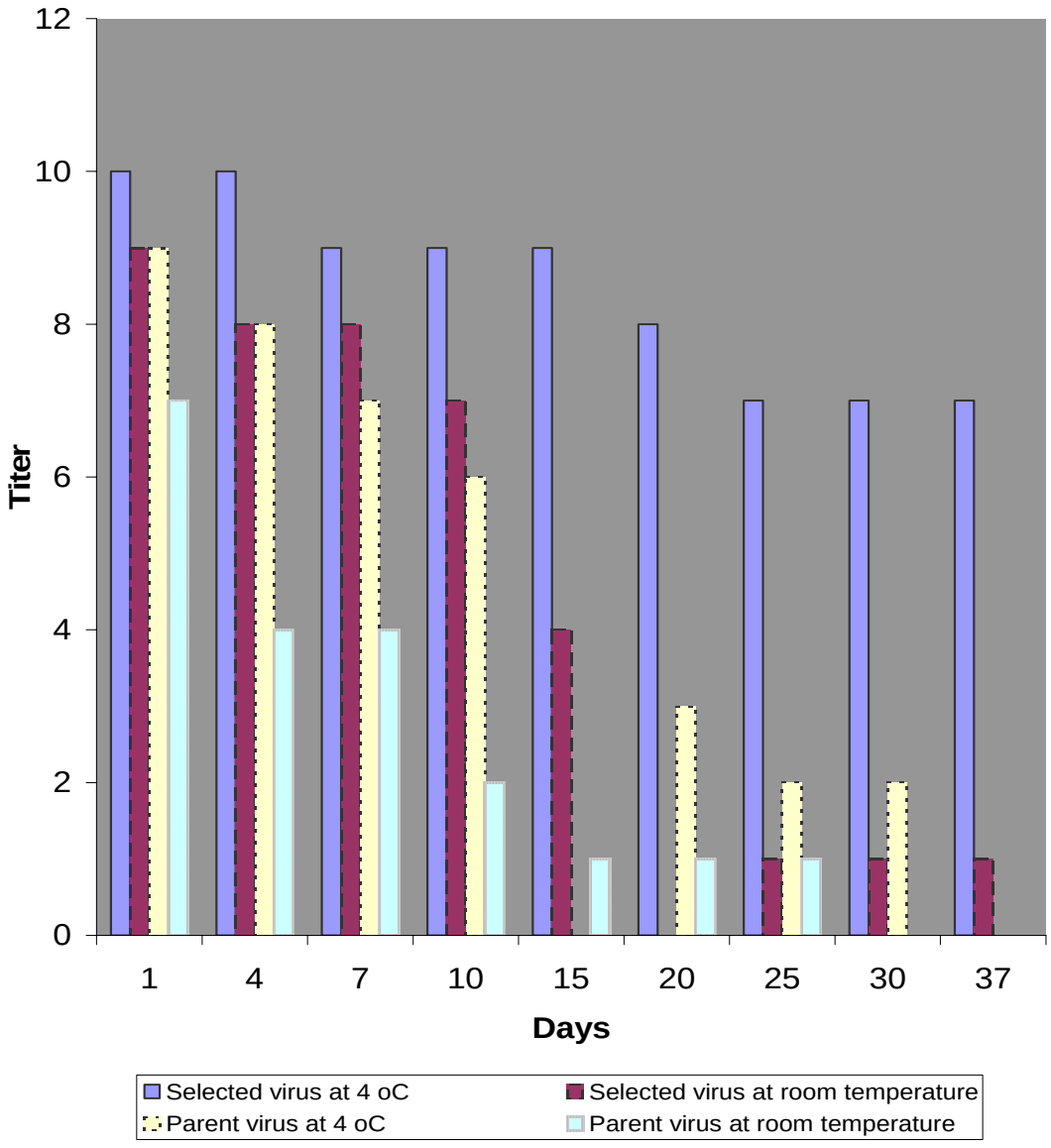


Table (3-3):

Detectable haemagglutination titres of stabilizer-added selected vaccine strain during nine weeks of storage at two different temperatures

Stabilizer	Temp	HA titres after weeks of storage								
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Lactalbumen hydrolysate	+4°C	8	8	8	8	8	8	8	8	8
	28-32°C	8	8	7	7	7	7	6	6	6
Lactose	+4°C	8	8	8	8	8	8	8	8	8
	28-32°C	8	8	8	8	8	7	7	6	6
Sucrose	+4°C	8	8	8	8	8	8	8	7	7
	28-32°C	8	8	8	8	7	7	7	7	6
Skimmed milk powder	+4°C	6	6	-	-	-	-	-	-	-
	28-32°C	6	5	5	-	-	-	-	-	-
No stabilizer	+4°C	9	9	8	7	7	7	7	7	7
	28-32°C	8	4	1	1	1	-	-	-	-

Table (3-4):

Characteristics of stabilizer-added lyophilized vaccine pellets after one-month storage at room temperature

Stabilizer	Pellet size	Pellet colour	Reconstitution	HA performance
Lactalbumen hydrolysate	N	N	Easily	Good
Lactose	S	N	Easily	Good
Sucrose	F	N	Not easily	Good
Skimmed milk powder	N	N	Easily	Not good
No additive	S	B	Easily	Good

N: normal. **S:** shrinkaged. **F:** foamy appearance. **B:** brownish.

Discussion

In this study, we have made selection for heat resistance on the Komarov strain. As to our knowledge there is no information available regarding subjection of this strain for heat selection. This may be considered the first report of such selection.

Practically the prolonged exposure to 56^{o}C without selection at lower temperatures is a method of heat selection that we found unsuitable for Komarov strain as no residual virus recovered after exposure for 2hours or longer. This may be due to the very small proportion of heat resistance virions in the parent vaccine. Although Ideris *et al.,* 1990 obtained a residual infectivity after 9 hours exposure on subsequent selection for V4 strain but this is probably because they do selection for enhanced thermostability that is known to be natural character of this strain (Spalatin and Hanson 1976).

The protocol used in this study centers around using short periods of exposure to 56^{o}C and harvesting residual virus (the method advocated by Goldman and Hanson 1955). Fortunately, the candidate strain showed a good response to this heat selection protocol. The titre of residual heat-stable virus was successfully raised from 7 log2 by the first cycle to 10 log2 after the forth cycle.

The strategy of initial selection for heat resistance in a candidate virus seems to be more accurate than plaque or terminal dilution selection because these particles differ from the parent virus in the time needed to produce plaques or cytopathic effects. Accordingly we did primary heat selection followed by purification by terminal limiting dilution. Such strategy is adopted according to the Australian experience but the

difference is that they did it in a known heat stable virus for enhanced thermostability (Ideris *et al,* 1990). Moreover, the selected heat-stable virus would not be expected to be antigenically different from the parent virus as King (2001) demonstrated that a more thermostable isolates could be selected from heterogeneous ND virus populations in vaccine strains with minor antigenic changes. This is in agreement with the statement that heat resistance is not truly a mutant or genotypic character of an organism and that virus resistance to heat inactivation is just a trait that can readily be augmented by selection involving heat shock (Spradbrow, 1992).

Without heat selection the stability of live virus vaccines could be enhanced by lyophilization (Mariner *et al.,* 1991) or by addition of stabilizers (Ezeifeka and Onunkwo 2004). In our study addition of four types of stabilizers greatly enhanced stability of the lyophilized product. Lactalbumen hydrolysate and lactose showed comprehensive good results but addition of sucrose showed physical change on storage at room temperature despite the fact that there was no significant difference in its stability compared with LAH and lactose. The skimmed milk powder-added product showed some difficulty in haemagglutinin test performance. This may be due to the large molecular weight of the powder that interfered with the capacity of the virus to bind chicken red blood cells.

Indeed, the present results could furnish a promising line of research in thermostable vaccinology in Sudan and could be considered a base to develop an affordable thermostable ND vaccine.

CHAPTER IV

RT-PCR

Evaluation of RT-PCR for Rapid Detection of Sudanese Isolates and Vaccine Strains of Newcastle Disease Virus

Introduction:

Newcastle disease virus (NDV) is an important pathogen of domestic fowl, pigeon and other birds (Alexander, 1991). The virus can be readily grown in the allantoic cavity of embryonated chicken eggs (ECE) and in many cell culture systems of chicken and mammalian origins (Liu and Bang, 1953; Chanock, 1955, Brandt 1961, Wheelock and Tam, 1961). Its complete genome is composed of six genes separated by conserved non-coding regions (Phillips *et al.*, 1998). The Fusion (F) gene codes for a precursor protein (Alexander, 1990). Routine laboratory diagnoses of ND necessitates primary isolation of the virus in embryonated chicken eggs (ECE) or cell culture and subsequent characterization and identification by HI or serum neutralization tests. However, these conventional methods are time consuming and cumbersome. The surge of new techniques in molecular biology has revolutionized rapid detection and pathotyping of NDV worldwide.

Several molecular studies were conducted on NDV whole genome. However, nucleic acid sequence analysis of the F gene played an important role in understanding virus infectivity and disease expression (Horvans *et al.*, 1992 and Hu *et al.*, 1992). Reverse transcriptase (RT) polymerase chain reaction (RT-PCR) for detection of NDVs

was applied for the first time by Jestin and Jestin (1991). Subsequently, several studies targeting other regions of the genome were conducted (Makkay *et al.,* 1999). Ballagi-Pordany *et al.,* (1996) and Kant *et al.,* (1997) used restriction enzyme endonucleases to differentiate between pathotypes. Jorecki Black *et al.,* (1992) and Aldous *et al.,* (2001) on the other hand used a probe hybridization assay. Sequence analysis of the amplified region was done by Toyoda *et al.,* (1989), Collins *et al.,* (1993) and King and Seal (1997). Confirmation of the specificity of the PCR product by amplification of a region, internal to the annealing site of the first primers, was also described using nested RT-PCR (Nanthakumar *et al.,* 2000).

The objective of the present study was to evaluate RT-PCR for detection of Sudanese isolates and vaccine strain of NDV in cell culture and ECE.

Materials and Methods:

Cell culture and virus propagation:

The Sudanese isolates of NDV; OB, KU, GR, A12 and A105, vaccine strains; Komarov, B1, LaSota Clone30 and Clone 79 were obtained from the Central Veterinary Research Laboratory (CVRL), Soba, Khartoum, as lyophilized allantoic harvest. All viruses were propagated in monolayers of Vero cell line as described in chapter II (Figure 4-1). The infectious harvests were collected and centrifuged at 3000 rpm for 10 minutes and the cell free supernatant was used for ssRNA extraction.

Extraction of viral nucleic acid:

The QIAamp viral RNA kit (QIAGEN 1999) was used to extract ssRNAs of the Sudanese isolates and vaccine strains following the mini spin protocol as discussed in chapter II. Briefly, 140 ul of virus suspension were added to 560 ul AVL buffer

containing carrier RNA into a 1.5 ml microcentrifuge tube and mixed by pulse-vortexing for 15 seconds followed by incubation at room temperature for 10 minutes. 560 ul of absolute ethanol were added and mixed by pulse-vortexing for 15 seconds. The mixture was passed through the QIAamp spin column where the viral RNA molecules were restrained. The column then washed twice by 500ul of 2 washing buffers WB1 and WB2, respectively. Finally, ssRNAs were carefully eluted by 60 ul of buffer AVE equilibrated to room temperature.

Primer selection:

The first pair of primers (nd1 and nd2), as shown in chapter II, was used to amplify 356bp long fragment from the F gene including the fusion protein cleavage site from all vaccine strains and field isolates of ND virus. The nd1 primer has the sequence of: 5'-GCAGCTCGAGGGATTGTGGT-3' nucleotide position 158-177 in F gene, while nd2 has the reverse sequence of: 5'-TCTTTGAGCAGGAGGATGTTG-3' nucleotide position 513-493.

Reverse transcription polymerase chain reaction:

For the one step RT-PCR the reaction mixture was prepared as follows; 18 ul of mixture 1 and 25 ul of mixture 2 (see chapter II) were mixed in a PCR tube then 1 ul from each primer and 5 ul of the target RNA were added. Reactants were mixed thoroughly by pulse vortexing for few seconds and briefly centrifuged.

Thermal profiles were done on Techne PHC-2 thermal cycler as described in chapter II. After amplification, 12 ul from each RT-PCR product was stained with 3 ul bromophenol blue and loaded onto 1% SeaKem agarose in TBE buffer and

electrophoresed at 88mV/A for 40 minutes. The PCR products were visualized under UV light and documented.

Results:

The NDV RT-PCR based assay afforded sensitive and specific detection of all isolates of ND viruses and vaccine strains. The specific 356 bp PCR products obtained from allantoic and vero cell culture extracts were visualized on ethidium-bromide stained gel (Figure 4-2). The specificity studies indicated that viral nucleic acid extract of IBD virus or total nucleic acid from Vero cell controls or embryonated chicken egg (ECE) fluids failed to produce the specific 356 bp PCR product (Figure 4-3).

Discussion:

In Sudan, most of ND viruses were isolated from extensive disease outbreaks (Fadol, 1991). Attempts to isolate viruses of low virulence by a survey are very rare.

Diagnosis of Newcastle disease includes conventional virus isolation and identification. If there is no history of vaccination, serology is useful to identify a previous infection but is known to be laborious and time consuming (Alexander, 1991). The serological tests commonly used for detection of infection include, haemagglutination inhibition (HI) and enzyme linked immunosorbent assay (ELISA). However, these tests are less valuable as tools for detecting ND virus infection as they are complicated by cross-reactions between other viruses (Alexander, 1991). Laboratory isolation of the virus from suspected field outbreaks necessitate the presence of sophisticated facilities, which are not available in most field diagnostic units.

All viruses used in the present studies were isolated during outbreaks of the disease in large poultry farms over a period of 27 years from different geographical

location in the Sudan. Three of these viruses were pathotyped as velogenic viruses. The remaining 2 virus isolates have not yet been pathotyped. The viral RNA of the vaccine strain (Komarov strain) was incorporated in each RT-PCR assay as a positive control.

The specificity of the test indicated that the primers did not amplify any region in the culture extracts nor the genome of the IBD virus under the same stringency conditions described in this study.

In conclusion, this one tube RT-PCR technique could provide a means of rapid detection of NDV. In addition, the described RT-PCR assay could be used as a supportive or complementary method to the conventional methods currently used for diagnosis of the disease.

Figure (4-1):

Vero cell line showing growth of Newcastle disease virus

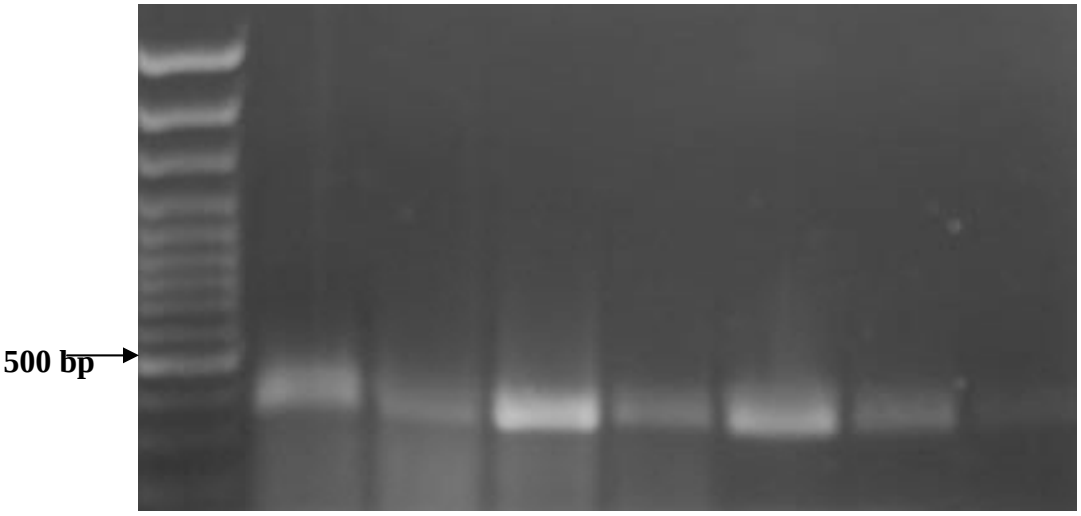
A

B

C

A: Vero cell line showing syncytia.
B: 90% cytopathic effect due to ND virus growth in Vero cell line.
C: Uninfected Vero cell line monolayer (Control).

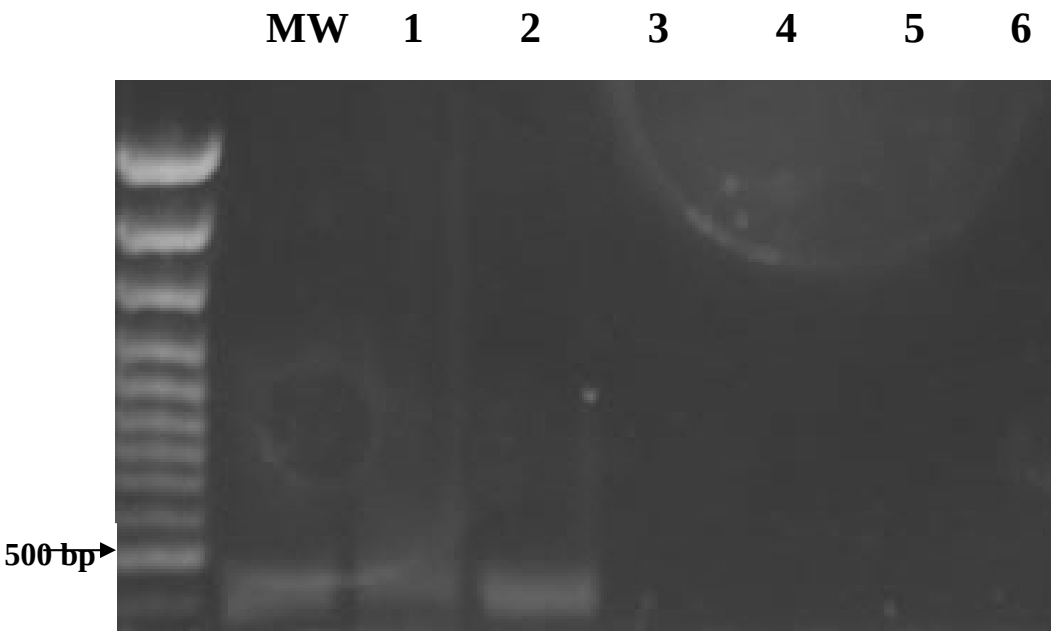
Figure (4-2):
RT-PCR amplification of the specific 356 bp PCR products from field isolates and vaccine strains of Newcastle disease virus.



Lane MW: 100 base pair (bp) molecular weight marker; **Lane 1-5**: NDV field isolate, OB, KU, GR, A12 and A105, respectively; **Lane 6-7**: Vaccine strains, Komarov and B1, respectively.

Figure (4-3):

Specificity of the RT-PCR assay for detection strains of Newcastle disease virus.



Lane MW: Molecular weight marker; **Lane 1-3**: LaSota, Clone 30 and Clone 79 respectively. **Lane 4**: Vero cell extract; **Lane 5**: Viral nucleic acid extracts from IBD; **Lane 6**: Un-infected allantoic fluid extract.

CHAPTER V

NESTED RT-PCR

Detection of Newcastle Disease Virus in Clinical Samples from Experimentally Infected Birds using Nested RT-PCR Assay

Introduction:

Newcastle disease is caused by avian paramyxovirus type-1 (an RNA virus) which belonged to the order Mononegavirale (Pringle 1997). Different pathotypes or clinical forms of the disease were reported in different countries including Sudan (Alexander 1992, Spradbrow 1988, Fadol 1991, Mahasin *et al*., 1980, Elamin *et al.,* 1993, Ballal *et al.*, 1996,). This wide range of variation in virulence and pathogenicity of virus strains was responsible for the great losses encountered among commercial and rural flocks with bad economical sequences.

Control of the disease depends mainly upon correct and accurate diagnosis. In this respect the virological and serological diagnostic procedures are known to be expensive, time consuming and cumbersome. However, the advanced molecular biological approaches of diagnosis became reliable alternatives (Jarecki Black and King, 1993, Collins *et al.*, 1994, Kant *et al.*, 1997 and Ali and Reynolds, 2000). The reverse transcriptase polymerase chain reaction (RT-PCR) had been applied for the first time by Jestin and Jestin (1991). The virus could be detected directly from the clinical samples without previous isolation (Kant *et al.*, 1997). They performed a hemi-nested RT-PCR assay for detection and subsequent differentiation of virus isolates. Nanthakumar *et al.*, (2000) detected the virus from suspected clinical samples by RT-PCR but they differentiated the virus by restriction enzyme analysis of the PCR product. Kho *et al*.,

(2000) performed a nested RT- PCR enzyme linked immunosorbent assay (RT-nPCR-ELISA) technique for rapid and sensitive detection of ND viruses. The technique has been developed to work directly on tissue samples. The authors calculated their method of PCR product detection to be 10 folds more sensitive than electrophoresis and they claimed the nested PCR to be 100 folds more sensitive than standard PCR or 1000 folds as described by Aradaib *et al*., (1998).

In this research work a nested RT-PCR assay will be evaluated for direct detection of NDV in clinical and postmortem samples from chicks experimentally infected with virulent virus.

Materials and Methods:

Experimental infection of birds:

A local isolate of NDV designated KU-p2 was used to induce experimental infection in six weeks old white leghorn chicks. Twenty chicks were randomly selected and were divided into 2 groups (A and B). Group A (10 chick) received two drops of undiluted infective allantoic fluid through nasal and ocular routes. Group B (10 chicks) were kept in another facility and left as controls. The experimental birds were fed a balanced ration with free access to water. The birds were then observed daily for onset of clinical signs.

Collection of samples:

On day five post infection, blood samples were collected from the wing vein in sterile tubes containing EDTA. The blood was washed twice with sterile PBS and kept at 4°C till used for virus isolation and viral RNA extraction.

Tracheal and cloacal swabs were taken in sterile PBS containing antibiotics (5000 units/ml penicillin, 4mg/ml streptomycin and 2000units/ml mycostatin) and kept at -20°C.

In the same day chicks were slaughtered and parts from internal organs were collected separately. Liver, heart, spleen, lung, kidney and brain tissues were removed in sterile Petri dishes and homogenized by sterile pair of scissors. PBS containing antibiotics was used to make 20% w/v of the homogenized tissues. The homogenates were then kept at -20^{o}C till used.

Virus isolation in Embryonated chicken eggs (ECE):

Virus re-isolation was performed in embryonated chicken eggs (ECE) 9-10 days of incubation. ECE were kindly supplied by the Viral Vaccine Production Department of the CVRL, Khartoum, Sudan. Each sample was briefly centrifuged and the clear supernatant fluid was used to inoculate five ECEs via the allantoic cavity with 0.1 ml.

Extraction of viral nucleic acids:

The QIAamp extraction kit (QIAamp, Hamburg, Germany) was used to extract viral nucleic acids. RNAs were extracted from supernatant of infected cell cultures and homogenate from infected tissues using QIAamp viral RNA kit (QIAamp, Hamburg, Germany) as per manufacturer's instructions discussed in chapter II.

Primers design:

Two pairs of primers (nd1, nd2) and (nd3, nd4) were synthesized by Roth (Carl Roth GmbH+Co., Karlsrube, Germany) according to the published F gene sequence of the velogenic NDV Miyadera strain (see chapter II). These pairs produced 356bp and 216bp PCR products respectively (Nanthakumar *et al.*, 2000).

RT-PCR amplification:

Titan one tube enzyme mix system was used to prime synthesis of first strand cDNA and to perform PCR in one step. The reaction mixture (5ul of the sample RNA extract, 1ul

from each primer of the first pair, 2ul of dNTPs, 2.5ul DDT, 0.5 ul Rnase inhibitor, 10ul of 5x PCR buffer, 4ul magnesium chloride, 1ul titan enzyme mix and 23ul of dd H2O) were pipetted in 0.5 PCR tube. The tube was then incubated at 50^{o}C for 30 minutes for reverse transcription, then cycled 40 times at 94^{o}C for one minute, 52^{o}C for one minute, at 68^{o}C for one minute and finally, incubated at 68^{o}C for 10 minutes.

Nested RT-PCR:

For nested amplification, 2 ul of the first amplified 356 bp PCR product were transferred to a PCR tube containing amplification buffer which consist of (10 µl of 5X PCR buffer; 10 ul Mg Cl_2 of 1.5 mM concentration; 2 µl of primers (nd3 and nd4) at a concentration of 20 picogram; 8 ul of dNTPs including ATP, TTP, GTP, CTP; 1 µl of Taq DNA polymerase (Perkin Elmer Corporation, Norwork, CT) at a concentration of 5.0 units/ ul. Double distilled water was added to each PCR tube to obtain a total volume of 50 µl. The PCR tubes were placed in the thermal cycler for another 40 cycles at the same temperature per cycles described above.

Following amplification, 20 microliters from each nested PCR reaction containing amplified product were loaded onto gels of 1.5% SeaKem agarose (FMC Bioproduct, Rockland ME) and electrophoresed. The gels were stained with ethidium bromide, and the nested PCR products were visualized under UV light.

Results:

The experimentally infected birds developed inappetance, greenish diarrhea, depression, respiratory embarrassment followed by onset of nervous signs by day six post infection. NDV was recovered in allantoic fluid from all tissues collected from experimentally infected chicks except the blood, liver and spleen.

The specific 356 bp PCR products, visualized on ethidium bromide-stained agarose gel, were obtained from all NDV RNA samples tested. The specific 356 bp PCR product was visualized from a variety of tissue samples from the experimentally infected chick including, lung, kidney, heart, brain, cloaca, and trachea. The blood, liver and spleen failed to demonstrate the primary 356 bp PCR product (Figure 5-1). The brain tissue showed the highest intense band. However, the nested amplification increased the sensitivity of the RT-PCR and the nested 216 bp PCR product was detected from all tissue samples including blood, liver and the spleen (Figure 5-2). Application of RNA from infectious bursal disease (IBD) virus and infectious bronchitis (IB) virus and blood samples from non infected chicks or total nucleic acid extracts from Vero cell controls failed to demonstrate the specific 356 bp PCR product (Figure 5-3). Table (5-1) showed comparative sensitivity between conventional virus isolation, RT-PCR and nested PCR in detection of ND virus.

Discussion:

Newcastle disease (ND) is a worldwide veterinary problem in poultry industry (Waterson, 1963; Ali, 1978; Zakia et al., 1983; Ideris et al., 1990; Alexander et al., 1999; King and Seal 1997). Very little information is available about field isolates originally recovered in Sudan. Further studies on these field isolates are necessary to determine their biology, ecology and molecular epidemiology. The NDV field isolates used in this study represented a range of topotype viruses, isolated from diverse geographic locations in Sudan including western, eastern, northern and central Sudan. The isolates were recovered from different avian species including chicken, pigeon and wild bird.

The described NDV RT-PCR assay using primers derived from F gene of the virus reproducibly and specifically detected NDV RNA in infected cell cultures and clinical

samples. Selection of the primers was based on the observation that the designed region of the F gene is highly conserved. The specific 356 bp PCR product was visualized from a variety of tissue samples from the experimentally infected chicks including, lung, kidney, heart, brain, cloaca, and trachea. The blood, liver and spleen failed to demonstrate the primary 356 bp PCR product. The brain tissue showed the highest intense band compared to tissues from other organs. This is probably due to the neurotropic nature of this virus strain. However, the nested amplification increased the sensitivity of the RT-PCR and the nested 216 bp PCR product was detected from all tissue samples including blood, liver and the spleen. The nested NDV RT-PCR assay was a simple procedure that efficiently detected all NDV isolates under the stringency condition used in this study. It is well documented that nested amplification increases the sensitivity of the PCR assay and confirms the identity of the primary PCR product (Aradaib *et al.*, 1998; Aradaib *et al.*, 2003). In the present study, the use of nested amplification removes the hazardous hybridization assay with radiolabled cDNA probes. In addition, hybridization confirmation assay is tedious, laborious and usually takes overnight.

The specificity studies indicated that the specific 356 bp PCR product was not amplified from 1.0 pg of RNA from infectious bursal disease (IBD) and infectious bronchitis; total nucleic acid extracts from Vero cell controls; or total nucleic acid extract from blood of non infected chicks under the same stringency condition described in this study. Temperature and time for denaturation, primer annealing and extension, enzyme and $MgCl_2$ concentration, and number of cycles of the three temperatures per time segments were very important for maintaining sensitivity and specificity of the PCR reaction.

The NDV RT-PCR assays provide supportive diagnostic techniques to the lengthy cumbersome conventional virus isolation procedures. The QIAamp kit provided a simple procedure that takes only one hour for viral RNA extraction. The thermal cycling profiles for reverse transcription and RT-PCR assay, including the primary and nested amplifications, required 6 hours. The time required from sample submission to interpretation of the final results was consistently 7 hours. This means that confirmatory diagnosis of submitted samples, from NDV suspected bird could be made within the same working day. The rapidity, sensitivity and specificity of the RT-PCR assay would greatly facilitate detection of NDV infection in an outbreak among susceptible birds.

This RT-PCR-based assay for detection of NDV field isolates provides the basis for future diagnosis of NDV. Further studies are in progress to determine the capability of the described NDV RT-PCR assay to detect additional isolates of NDV, and to evaluate its potential as a sensitive and specific diagnostic assay through comparison with current diagnostic techniques used for detection of NDV infection.

In conclusion, the described NDV RT-PCR assay, using primers derived from F gene of NDV, should provide rapid detection of NDV infection during an epizootic of the disease among susceptible Birds.

Table (5-1):

Comparative sensitivity between conventional virus isolation, RT-PCR and nested PCR in detection ND virus directly from tissue samples.

Clinical sample	Virus isolation in ECE	Detection by RT-PCR	Detection by Nested PCR
Blood 1	-	-	+
Blood 2	-	-	+
Liver	-	-	+
Lung	+	+	+
Spleen	-	-	+
Kidney	+	+	+
Heart	+	+	+
Cloaca	+	+	+
Trachea	+	+	+
Brain	+	+	+

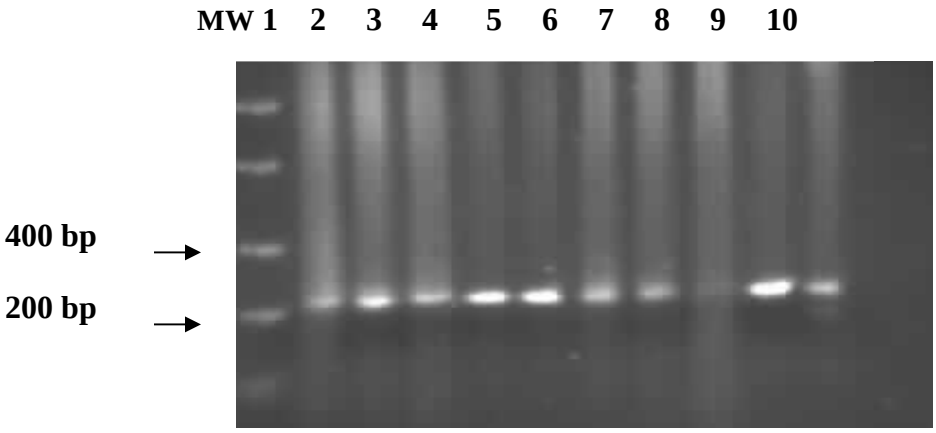
Figure (5-1):

Visualization of the specific 356 bp PCR product from tissue samples from experimentally infected chicks.

Lane MW: Molecular weight marker. **Lane 1**: Blood. **Lane 2**: Blood. **Lane 3**: Liver. **Lane 4**: Lung. **Lane 5**: Spleen. **Lane 6**: Kidney. **Lane7**: Heart. **Lane8**: Cloaca. **Lane 9**: Trachea. **Lane 10**: Brain.

Figure (5-2):

Visualization of the nested 216 bp PCR products from all tissue samples tested.



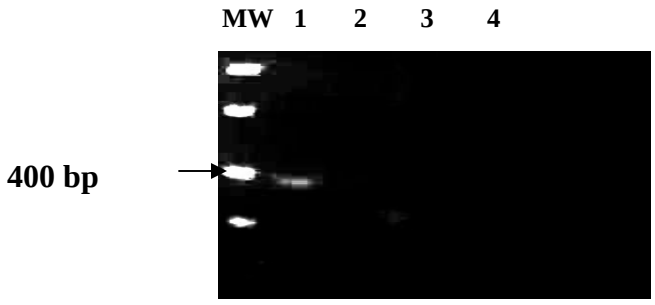
Lane MW: Molecular weight marker. **Lane 1**: Blood. **Lane 2**: Blood.

Lane 3: Liver. **Lane 4**: Lung. **Lane 5**: Spleen. **Lane 6**: Kidney.

Lane7: Heart. **Lane 8**: Cloaca. **Lane 9**: Trachea. **Lane 10**: Brain.

Figure (5-3):

Specificity of the RT-PCR for detection the specific 356 bp PCR product from NDV RNA.



Lane MW: Molecular weight marker. **Lane 1**: NDV RNA (Positive control). **Lane 2**: Infectious bursal disease virus extract. **Lane 3**: Infectious bronchitis virus extract. **Lane 4**: Blood sample from non-infected chick (negative control).

CHAPTER VI

GENERAL DISCUSSION

Investment in poultry industry in Sudan is still suffering from some technical problems concerning rearing systems, diagnosis of disease outbreaks and control programming. The role of rural poultry and wild birds in the epidemiology of ND is carefully studied in many countries (Young *et al.*, 2002), yet such studies in the Sudan are still inadequate. However, the suspicion that enzootic viruses circulating among rural and backyard small-size flocks are responsible for the great losses in commercial poultry could not be ruled out. Rearing of scavenging poultry in mixed farms commonly practiced in Sudan, i.e. for control of ticks in cattle or sheep, exposes commercial poultry reared nearby to infection by most disastrous viral infections. Though, special attention should be paid to the control diseases in local chicken. The harsh environmental temperature represents persistent constraint for attempting application of vaccination protocol with standard vaccines for rural poultry in the Sudan. Consequently, the need for a durable and affordable vaccine to be used efficiently under such conditions is a must.

The Australian Centre for International Agricultural Research (ACIAR) had devoted abundant research work over along period of time to develop ND thermostable vaccines for rural poultry in the tropics. The idea is based chiefly upon selection of an Australian avirulent strain (V4) for enhanced heat resistance. The original research work was carried in Malaysia by Ideris *et al*. (1990) and they designated a variant as V4-UPM. Other two heat resistant strains I2 and I8 had been nominated later (Bensink and Spradbrow 1999). These lentogenic thermostable ND vaccines were used in most tropical countries. They were available as food-added vaccines. Therefore, they could be supplied

at any time and anywhere and persistently compete with virulent field viruses and confer protective level of immunity against them. However, development of a mesogenic thermostable vaccine may become more advantageous and could be used by different routes of application once or twice a year. It is well known that better and long lasting immunity could be induced by mesogenic vaccine strains rather than lentogenic ones.

The Komarov strain as one of mesogenic strains of ND virus commonly used as vaccine was supposed to respond positively to selection for heat resistance (Spradbrow 1992a). Fortunately, our results showed that this supposition is true. The arduous training method of selection by stepwise exposure to higher temperature and harvesting of the residual virus followed by selection at 56^{o}C was proposed but it was found unnecessary (Spradbrow 1992b). In parallel this method was replaced, in this study, by the short exposure to 56^{o}C and harvesting residual virus and was fruitful. However, further studies to select this strain for enhanced heat resistance are certainly needed.

The stabilizers tried in this research work were those available at the moment. Concentration of these stabilizers is fixed through the experiment due to time and funding constraints. Nevertheless, economical and concentration manipulation studies should be carried out along the way to this vaccine development. Moreover, molecular biological studies to characterize selected viruses are also needed.

The cumbersome and time-consuming conventional laboratory diagnostic methods including isolation and characterization of the virus are no longer suitable. Molecular diagnostic procedures manipulated in this study proved that they could be reliable alternatives. The RT-PCR results showed that this technique is specific and

sensitive if performed to distinguish ND from other similar viruses, while the nested PCR assay results obviously indicated that it is highly sensitive even if no virus isolation took place. However, further analysis by restriction enzymes, probe hybridization or sequencing of the PCR products obtained by these techniques is essential to be used for virus characterization.

Generally, the present research results paved the way for more research regarding diagnosis and control of livestock viral diseases in the Sudan.

CHAPTER VII

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