MARIA ANGELA ORSI

CARACTERIZAÇÃO BIOLÓGICA, MOLECULAR, IMUNOLÓGICA E ESTABILIDADE TÉRMICA DAS ESTIRPES VACINAIS E DE ISOLADOS DA DOENÇA DE NEWCASTLE DE AVES DE PRODUÇÃO INDUSTRIAL E MIGRATÓRIAS NO BRASIL.

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências Básica da Faculdade de Ciências Médicas da Universidade Estadual de Campinas para obtenção do título de Doutor em Clínica Médica.

ORIENTADORA: PROFª Drª CLARICE WEIS ARNS
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CAMPINAS

Unicamp

2010

FICHA CATALOGRÁFICA ELABORADA PELA
BIBLIOTECA DA FACULDADE DE CIÊNCIAS MÉDICAS DA UNICAMP
Bibliotecário: Sandra Lúcia Pereira – CRB-8ª / 6044

Orsi, Maria Angela
Or8c Caracterização Biológica, Molecular, Imunológica e estabilidade térmica das estirpes vacinais e de vírus da Doença de Newcastle de aves de produção industrial e migratória no Brasil / Maria Ângela Orsi. Campinas, SP : [s.n.], 2010.

Orientador : Clarice Weis Arns
Tese (Doutorado) Universidade Estadual de Campinas. Faculdade de Ciências Médicas.

1. Vírus da doença de Newcastle. 2. Vacinas. 3. Ave - migração. 4. Gallus gallus. 5. Aves migratórias. 6. Variabilidade genética. I. Arns, Clarice Weis. II. Universidade Estadual de Campinas. Faculdade de Ciências Médicas. III. Título.

Título em inglês : Biological, molecular, immunological and thermostability characterization of Newcastle Disease vaccine strains and isolates from migratory and industrially raised birds in Brazil

Keywords:
- Newcastle disease virus
- Vaccines
- Migration, bird
- Chickens
- Migratory birds
- Genetic variability

Titulação: Doutor em Clínica Médica
Área de concentração: Ciências Básicas

Banca examinadora:

Profa. Dra. Clarice Weis Arns
Profa. Dra. Liana Maria Cardoso Verinaud
Profa. Dra. Leonilda Maria Barbosa dos Santos
Prof. Dr. Edison Luiz Durigon
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Data da defesa: 20-04-2010

Banca examinadora da tese de Doutorado

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Curso de pós-graduação em Clínica Médica da Faculdade de Ciências Médicas da Universidade Estadual de Campinas.

Data: 20/04/2010

DEDICATÓRIA

Para meus queridos pais Olga e Oswaldo

Que me incentivaram no caminho da educação, mostrando que devia seguir o que queria e que com garra tudo é possível, sempre presentes em minha vida.

A meu esposo Anthony por seu amor e companheirismo e pela super ajuda em todos os momentos de minha vida e agora no doutorado.

E minha filha Giulia Andrea, *que com sua presença encanta a minha vida e de quem muito me orgulha.*

AGRADECIMENTOS

A Deus, por nos ter me dado forças para persistir no meu ideal e colocar pessoas maravilhosas na minha vida que sempre me ajudaram,

As minhas irmãs Silvia Regina, Ana Tereza, Maria Estela e Carmen Lucia pelo apoio recebido nesta fase da minha vida. Em especial a Silvia Regina pelos cuidados com os meus pais na minha ausência.

A minha sogra Dona Fumiko pela grande força relacionado o meu aprimoramento profissional.

À minha querida orientadora e amiga Profa Dra Clarice, pelo carinho, confiança, incentivo, ensinamentos e pela maneira eficiente como me conduziu na elaboração da tese.

Ao Professor Fernando Rosado Spilki pela sua colaboração nos trabalhos, na realização das análises filogenéticas e sugestões, foram fundamentais.

A amiga Dra Maria da Gloria por acreditar e colaborar com o nosso crescimento.

A Coordenação Geral de Apoio Laboratorial (CGAL) do Ministério da Agricultura, Abastecimento e Reforma Agrária, e aos Srs Coordenadores do Lanagro/SP que com seu aval foi possível dar continuidade ao meu crescimento profissional. Ao Ministério da Agricultura, Pecuária e Abastecimento- MAPA por autorizar o uso de dados laboratoriais da área de avicultura e desse modo fazer uma coisa inédita que é divulgar dados governamentais.

A minha amiga Luciana H. Antoniassi da Silva, pelas longas conversas, gargalhadas, minha companheira pela amizade e carinho, além da preciossíma ajuda na parte molecular.

Aos professores Dra Liana Maria Cardoso Verinaud, Dra Adriana Gut Lopes Ricetto, pelas críticas e sugestões ao presente trabalho, por ocasião de sua análise prévia (qualificação)

Aos amigos da Aviárias-Lanagro/ SP onde os trabalhos foram desenvolvidos, e amigos pessoais, pelo companheirismo, horas de prazer e pelos deliciosos momentos de convivência.

Agradeço a amizade, carinho e ajuda técnica dos colaboradores do Lanagro/SP: Luciano, Soraya, Jaíra, Margarida, Berlina, Dilmara, Maria do Livramento e Luiz Carlos .

Aos velhos amigos do Lanagro/SP (Dr. Deak, Simone, Adriana, Amiris, Soraya, Fátima, Tio Zé, Gonçala, Anali, Shirlei, Roseni, Lili, Dilmara, André, Fúlvia, Emília, Carla e Zuca) que me apoiaram incondicionalmente nesta nova fase da minha vida, me ajudando com seu companheirismo e carinho.

Aos novos amigos do Lanagro/SP (Fernanda, Marcus, Wanderson, Cristhian, Adilson, Eluana, Ana Maria, Juliana,) pelo carinho.

Aos meus amigos e colegas do LVA, os que já não se encontram aqui: Fernando, Renata, Lia, Regis, Rods dos quais me lembro com carinho, pois me proporcionaram muitos momentos agradáveis.

A estes maravilhosos meninos do laboratório: Luciana Helena, Matheus, Luciana, Paula, Márcia, Judite, Paulo Anselmo, Daniel, Alyne, Marina, Sonia, Nuri, Marcela, Fernanda e Flávio que tive a prazer de conviver e que me ajudaram na parte da informática, design e pelo seu companheirismo, pelo apoio e pelos deliciosos momentos de convivência, os quais moram em meu coração.

Ao amigo Geneci pelo carinho e pelo seu jeitinho de trabalhar com "células e meio de cultura" que não resistem a seu encanto.

A coordenação do Curso de pós-graduação - Clinica Médica pelo apoio recebido.

Á Universidade Estadual de Campinas agradeço pela formação científica.

A CAPES, CNPq, FUNCAMP e MAPA pelo suporte financeiro.

A você que direta e indiretamente me ajudou e que não foi citado aqui - Obrigada.

BONS AMIGOS

Abençoados os que possuem amigos, os que os têm sem pedir.
Porque amigo não se pede, não se compra, nem se vende.
Amigo a gente sente!

Benditos os que sofrem por amigos, os que falam com o olhar.
Porque amigo não se cala, não questiona, nem se rende.
Amigo a gente entende!

Benditos os que guardam amigos, os que entregam o ombro pra chorar.
Porque amigo sofre e chora.
Amigo não tem hora pra consolar!

Benditos sejam os amigos que acreditam na tua verdade ou te apontam a realidade.
Porque amiga é a direção.
Amigo é a base quando falta o chão!

Benditos sejam todos os amigos de raízes, verdadeiros.
Porque amigos são herdeiros da real sagacidade.
Ter amigos é a melhor cumplicidade!

Há pessoas que choram por saber que as rosas têm espinho
Há outras que sorriem por saber que os espinhos têm rosas!

MACHADO DE ASSIS

SUMÁRIO

LISTA ABREVIATURAS

VDN	Vírus da Doença de Newcastle
DN	Doença de Newcastle
ET	Estabilidade térmica
AF	A Fresco
L	RNA Polimerase
HN	Proteína Hemaglutinina-Neuraminidase
M	Proteína de Matriz
N	Nucleoproteína
P	Fosfoproteína
F	Proteína de Fusão
APMV-1	Paramixovírus Aviário tipo1
MAPA	Ministério da Agricultura, Abastecimento e Reforma Agrária
IPIC	Índice de Patogenicidade Intracerebral
AcMo	Anticorpo Monoclonal
IPIV	Índice de Patogenicidade intravenoso
NT	Nucleotídeos
RNA	Ácido Ribonucléico
aa	Aminoácidos
TMME	Tempo médio de Mortalidade Embrionária
IM	Injeção Intramuscular
HA	Teste de Hemaglutinação
HI	Teste de Inibição de Hemaglutinação
SPF	Livre de patógenos específicos
PCR	Reação em cadeia da polimerase
RT-PCR	Transcriptase Reversa
OIE	Organização Mundial de Saúde Animal
ELISA	Teste imunoenzimático
PBS	Salina fosfatada tamponada
bp	Pares de base
M	Marcador de peso molecular
$DIOE_{50}$	Dose Infectante 50% em ovos embrionados

RESUMO

O vírus da doença de Newcastle (VDN) é o agente causador de uma das mais importantes doenças em aves e representa uma ameaça para a indústria avícola. O VDN é um membro da família Paramyxoviridae, subfamília Paramyxovirinae, gênero Avulavirus. São vírus envelopados, não-segmentados dotados de genoma RNA de fita simples sentido negativo, associado à doença do trato respiratório, digestivo e nervoso das aves. O Controle da DN se baseia em biossegurança, uso de vacinas e detecção precoce de lotes infectados.

No presente estudo, examinamos dez vacinas vivas comercializadas no Brasil quanto à sua estabilidade térmica (ET), virulência e imunogenicidade. Em outra etapa do trabalho, investigou-se a soroprevalência perante o VDN em regiões de produção avícola voltada à exportação ou não de produtos aviários, seguido da detecção do VDN em aves migratórias. Os estudos são complementados pela análise filogenética de VDN isolado de aves comerciais, dos resultados alcançados, cumpre ressaltar: i) os testes de ET revelam elevada estabilidade para as vacinas utilizadas no país, mesmo após dois anos de sua fabricação; ii) o grau de proteção conferido por vacinas vivas contra a DN não depende da virulência residual conforme testes de inoculação intracerebral; iii) a soroprevalência contra VDN em aves nas regiões produtoras e exportadoras, foi de 39,1% e foram isoladas 77 amostras virais, sempre com perfil não-patogênico; iv) sorologia realizada em uma segunda oportunidade detectou-se uma soroprevalência de 28,8% e isolamento de 15 amostras virais que também foram caracterizadas como não-patogênicas; v) observou-se numa soropositividade de 41,7 a 84,3% dependendo da região e isolamento de 12 VDN na região Nordeste, caracterizados como não-patogênicas, indicando que nas áreas não exportadoras circulam vírus de baixa patogenicidade; vi) o genoma dos vírus isolados e das vacinas vivas, atesta que os vírus circulantes em aves comerciais são de provável origem vacinal e pertencem à classe II sendo 71,8% do genótipo II ou La Sota-like e 28,2% do genótipo I ou Ulster-like; vii) por último, a caracterização biológica dos isolados de aves migratórias mostram que há circulação de vírus de baixa e alta patogenicidade em nosso território. Portanto, este conjunto de trabalhos evidencia o status do Brasil como país livre da Doença de Newcastle em aves comerciais.

ABSTRACT

Newcastle disease virus (NDV) is the agent that causes one of the most important diseases in birds and represents a threat to industrial aviculture. NDV is a member of the *Paramyxoviridae* family, *Paramyxovirinae* subfamily, Avulavirus genus.

In the present study, live vaccines commercialized in Brazil were examined in regard to their thermostability (TS), virulence and immunogenicity. In another stage of this work, soroprevalence was investigated, with viral isolation and characterization carried out in poultry production regions focused on production for exportation or domestic commercialization, followed by the detection of the virus in migratory birds. The studies were complemented by phylogenic analysis carried out on the isolates from the commercial birds. From the results obtained, it is important to underscore that i) the tests of TS revealed a high stability of the vaccines used in Brazil, even two years after their manufacture; ii) the level of protection given by live vaccines against NDV does not depend on residual virulence, as confirmed by tests of intracerebral inoculation iii) soroprevalence against NDV in regions with production for exportation was 39.1% and it was possible to isolate 77 samples, always with a non-pathogenic profile; iv) on a second opportunity, a soroprevalence of 28.8% was detected, with isolation of 15 samples, also classified as non-pathogenic; v) in the Brazilian Northeast, a seropositivity of 41.7 to 84.3% was observed depend of region, with isolation of 12 NDVs, characterized as non-pathogenic, indicating that virus of low pathogenicity circulates in those areas that do not export; vi) the genome of the isolated virus and vaccine implies that the circulating virus in commercial birds probably originates from the vaccine and belongs to class II, being 71.8% from genotype II or La Sota–like and 28.2% from genotype I or Ulster-like; vii) finally, the biological characterization of isolates from migratory birds showed that there is circulation of virus of low and high pathogenicity in Brazil. However, this set of studies agrees with the status of Brazil as being a country free of Newcastle disease in commercial birds.

1- INTRODUÇÃO

O agente etiológico da Doença de Newcastle, o vírus da Doença de Newcastle (VDN), pertence ao gênero *Avulavirus* membros da família Paramyxoviridae, subfamília *Paramyxovirinae e ordem* Mononegavirales (van Regenmortel et al., 2000, Mayo et al., 2002 a, 2002b). Os membros da família Paramyxoviridae são vírus RNA envelopados com fita simples e genoma não segmentado de polaridade negativa. Possui morfologia esférica às vezes pleomórfica, com 150 a 200 nm de diâmetro, um nucleocapsídeo em forma de espiral, de 12 a 17 nm de diâmetro. As partículas virais apresentam projeções típicas, de dois tamanhos: A maior, uma simples glicoproteína (HN), associada à atividade de hemaglutinação e neuraminidase; a espícula menor é formada pela glicoproteína F, que está associada à fusão do envelope viral com a membrana celular da célula hospedeira, resultando no efeito citopático característico – formação de sincício. O VDN tem um RNA genômico de 15 Kb e codifica seis proteínas virais: RNA polimerase (L), proteína Hemaglutinina-neuraminidase (HN), proteína de fusão (F), proteína matrix (M), fosfoproteína (P) e nucleoproteína (NP). Elas estão dispostas na ordem 3'-NP-P-M-F-HN-L-5' (Obërdorfer & Werner, 1998; de Leeuw & Peeters, 1999).

O VDN é também referido como paramixovírus aviário-1 (APMV-1), que é um dos nove sorotipos identificados por infectar aves. Os isolados de APMV-1 podem ser classificados como lentogênica, mesogênica ou velogênica (baixa, moderada e alta virulência para aves, respectivamente).

Algumas reações cruzadas foram demonstradas entre os paramixovírus e as mais significativas foram entre APMV-3 e APMV-7 e entre APMV-3 e APMV-1 (Box et al., 1988). Somente os vírus dos sorotipos APMV-1, APMV-2 e APMV-3 foram descritos dando efeitos patológicos em galináceos. Já o APMV-6 foi isolado em perus.

A doença de Newcastle (DN) é uma doença viral listada no Código Zoosanitário Internacional da Organização de Saúde Animal (OIE) causada por uma estirpe virulenta do paramixovírus aviária tipo 1 e a notificação dos focos desta doença é compulsória.

O VDN afeta muitas espécies de aves causando uma severa perda econômica no setor avícola (Cattoli et al., 2009) sendo, portanto uma constante ameaça para as aves criadas comercialmente, e considerada enzoótica na maior parte dos países. Lotes comerciais de aves sofrem medidas de controle da doença e da disseminação do vírus. Em aves comerciais a maioria dos países utiliza a vacinação para manter a Doença de Newcastle monitorada constantemente, sabendo que esta doença é o maior fator limitante para aumentar a produção avícola. As perdas causadas por surtos da DN têm sido significativas**,** resultando na eliminação de milhares de aves em diversos países e restrições nas importações/exportações. É considerada como sendo uma das mais devastadoras doenças de galinha e de outras aves. Os prejuízos e danos que o VDN pode causar não se limitam apenas às aves infectadas, com mortalidade de lotes, mas

também o impacto econômico negativo devido a restrição do comércio de animais e subprodutos com embargos de áreas e países com focos da doença (Aldoux et al., 2001).

O impacto desta doença também é sentido na criação doméstico ou fundo de quintal (Alexander, 2008), sendo que para muitos países este tipo de criação significa o único acesso a fonte de proteína animal apresentada na forma de carne e ovos acessível às populações de baixa renda. Spradbrow (1992) relatou que a DN é responsável por 90% da morte ocorrida na criação de galinha doméstica do Nepal. O controle DN nestas aves em países em desenvolvimento é dificultado, afetando o desenvolvimento da produção comercial avícola.

A primeira descrição da doença**,** no Brasil, data de 1953, em Belém e Macapá (Santos et al., 1954), atribuindo-se o surto da doença à importação de carcaças de frangos congelados contaminados com o vírus procedente dos Estados Unidos (Vaistsman & Moussatché, 1954). O primeiro isolamento do vírus da doença de Newcastle no Brasil foi realizado por Cunha & Silva (1955). Essa amostra de vírus foi designada de M33 e caracterizada biologicamente (Oliveira et al., 2000). Posteriormente, foram assinalados surtos esporádicos nos estados do Rio de Janeiro e Minas Gerais, que foram rapidamente controlados através de vacinação e medidas profiláticas complementares (Santos et al., 1954). Após o 1º impacto na década de 50 no Brasil, embora endêmica, foi observada apenas esporadicamente, acometendo plantéis de pequena expressão, controlando-se rapidamente os focos através de vacinação e medidas profiláticas complementares. A enfermidade da Doença de Newcastle ressurgiu na década de 70, a sob a forma Newcastle viscerotrópica (altamente patogênica). No período de 1970 a 1975 a DN teve uma prevalência de 10.7% (1350 focos) na indústria avícola de Minas Gerais e catorze estados do Brasil. Nos próximos vinte anos vários focos da VDN ocorreram no país porem com menor intensidade. O baixo número de aves notificadas por foco indica que ocorreu em aves de criação doméstica. Após o ano de 1995, não foram notificados focos da DN em criações comerciais, no Brasil.

O Ministério da Agricultura em 1994-1995 montou um laboratório com estrutura adequada de biossegurança para caracterização viral. Este laboratório tornou-se referência para o Brasil e para o Mercosul. Até 1995, na maioria dos focos da doença de Newcastle notificados ao MAPA, foram relatados apenas com base nas suspeitas clínicas, sem o respectivo isolamento e caracterização do agente, portanto, perfeitamente possível que em alguns dos focos notificados o VDN patogênico poderia não estar presente.
Em 1997, houve o primeiro isolamento do vírus de Newcastle em avestruzes (Struthio camelus) importadas pelo Brasil (Doretto et al., 1999a). No mesmo ano, Doretto et al. (1999b) isolaram o VDN em dois lotes de pintos de avestruzes. Neste período, também foram isoladas 3 estirpes de vírus vacinais de frango de corte, matriz de corte e postura comercial. Em 1998, o VDN foi isolado também em flamingos (Phoenicopterus ruber) importados pelo Brasil (Doretto et al., 1999c). No entanto, os testes de patogenicidade mostraram que

as cepas não eram patogênicas. De acordo com a bibliografia pesquisada este foi o primeiro isolamento do APMV-1 em avestruzes. Neste mesmo período foram isolados vírus vacinais de frango de corte provenientes de Pernambuco e de pinguins capturados na região praiana que apresentavam problemas respiratórios, aerossaculite e ataxia. Da mesma forma, em 1999, no estado do Paraná, o VDN foi isolado em aves exóticas importadas, principalmente dos grupos de psitacídeos e palmípedes. Em ambas as ocorrências, todas as aves importadas foram sacrificadas e destruídas para evitar a disseminação do VDN. O foco da ND diagnosticado no Rio de Janeiro, no ano de 1999, foi o único caso de isolamento oficial do VDN no Brasil, a partir de uma suspensão de fezes de patos doentes oriundos do município de Japeri, RJ. O novo isolado viral foi denominado JAP99 (Portz et al., 2000; Oliveira Jr. et al., 2000, 2005) e o foco ficou restrito a uma fazenda, acometendo apenas aves caipiras, onde as ações emergenciais foram facilmente realizadas e a difusão do VDN evitada. Em 2000, no estado do Rio de Janeiro, foram isolados vírus patogênicos e vacinais em uma propriedade, mostrando a possibilidade de isolamentos de vírus com diferentes virulências simultaneamente. O foco da DN registrado em 2001, em Goiânia, ocorreu em aves caipiras em um assentamento e foi rapidamente controlado. Também foi isolado vírus vacinal de tucano no Rio de Janeiro. Após alguns anos sem ocorrer a doença, em 2006 foi isolada uma estirpe do APMV-1 no Rio Grande do Sul em aves de fundo de quintal (consumo familiar). Medidas de controle (quarentena, sacrifício das aves, barreira sanitária, isolamento e desinfecção) das áreas acometidas, impediram a propagação do agente (Flores et al., 2006).

Não há tratamento para a doença de Newcastle, entretanto uma política de eliminação (baseada em sacrifício de aves potencialmente infectadas, quarentena, limpeza e desinfecção) ou vacinação ou uma combinação pode ser usada para controlar a DN. Em situação de emergência em países previamente livres da DN, erradicação pela eliminação é a opção preferida. Alguns países possuem estirpes de DN não patogênicas (Nova Zelândia), a maioria dos outros países tem experimentado surtos da estirpe virulenta, como nos continentes africanos, asiáticos e americanos que possuem VDN enzoótica (Spradbrow 1988; Rweyemamu et al., 1991). Em países onde a DN virulenta é considerada enzoótica, a vacinação é uma prática rotineira. Esquemas de vacinação e tipo de vacinas usadas variam dependendo do potencial e virulência dos vírus de desafio, assim como o tipo de produção e esquema de produção de aves. Vacinas vivas atenuadas e vacinas inativadas têm sido desenvolvidas, bem como vacinas recombinantes. A 1ª estirpe vacinal atenuada de VDN, "Hertfordshire", foi obtida por passagem seriada em ovos embrionados (SPF) de um vírus de campo virulento isolado na Inglaterra em 1933. Depois esta estirpe virulenta foi chamada como Herts 33 e tornou-se um padrão de desafio e vírus de referência (OIE, 1996).

As vacinas vivas usadas no controle do VDN ao redor do mundo são com raras exceções, as estirpes lentogênicas. As estirpes vacinais mais utilizadas são: La Sota/46 (conhecida por La Sota), B1/47

(conhecida como Hitchner/B1 ou B1), F (Asplin), V4, Ulster, C2 e VG/GA. As estirpes La Sota/46 e B1/47 foram isoladas e desenvolvidas na década de 40 nos Estados Unidos da América (Goldhaft, 1979) e segundo Hitchner & Johnson, (1948), as vacinas lentogênicas são as mais usadas hoje no mundo. Elas replicam no trato digestório e respiratório com indução de uma resposta imunológica humoral local e sistêmica. A estirpe vacinal La Sota é geralmente mais virulenta e induz resposta imune mais evidente que estirpe vacinal B1 (Glisson & Kleven, 1993), mas ambas podem ser administradas na água de bebida e em spray através das vias nasal e ocular. Pintinhos saudáveis são vacinados precocemente com 1 a 4 dias de idade.

Em 1966, uma estirpe "lentogênica entérica suave" do VDN foi isolada de galinhas saudáveis em Queensland na Austrália (Simmons, 1967) e denominada de V4.

A estirpe vacinal VG/GA foi isolada no começo dos anos 90 de perus comerciais na Georgia nos USA (Beard et al., 1993). Esta estirpe replica também os tratos gastrointestinal e respiratório de galinha, produzindo uma resposta imune semelhante àquela induzida pela estirpe vacinal B1, porém sem nenhum sinal respiratório reconhecido (Glisson & Kleven, 1993). A estirpe vacinal Ulster, considerada "avirulenta", é uma estirpe de laboratório isolada de perus com afinidade entérica, causando o mínimo de reação vacinal em galinha. Estudos conduzidos por Franzo et al, (2009) mostraram que patos imunizados por esta estirpe apresentaram o epitélio traqueal íntegro semelhante ao controle, sendo, portanto indicada para primeira vacinação.

Embora seja possível identificar pelo menos três sistemas de produção no setor avícola brasileiro – como o integrado, o cooperativo e o independente – o sistema que tem se tornado mais presente é o sistema de integração vertical com mais de 80% da produção avícola. Este sistema é firmado através de um contrato de parceria entre empresas e o avicultor, o que proporciona um maior controle sanitário em toda a cadeia produtiva. O destino da produção brasileira em 2008 foi de 67% para o consumo interno e 33% para a exportação. Em 2008, os estados do Paraná (26,8%), Santa Catarina (27%) e Rio Grande do Sul (21,3%) foram responsáveis por 75% das exportações brasileiras (ABEF, 2008).

A capacidade produtiva da avicultura nacional tem oferecido condições vantajosas para que a mesma possa competir em mercados internacionais, mesmo frente a países que fornecem subsídios para seus produtores. Deste modo a presença do produto brasileiro nestes mercados só pode ser diminuída através de alegadas dificuldades ou barreiras sanitárias, já que as diversas taxações e impostos que incidem sobre o produto não consegue diminuir o seu custo relativo.

De qualquer forma, é imperativo que o Brasil se adiante aos obstáculos sanitários e, para tanto, uma importante iniciativa foi a criação do Programa Nacional de Sanidade Avícola (PNSA), instituído em 19 de dezembro de 1994 através da Portaria Ministerial Número 193. Entre as atribuições do PNSA (regulamentado pelo artigo 1º da Portaria 115, de 04 de outubro de 1995) se destacam a confirmação e o

controle de focos de duas enfermidades: a doença de Newcastle e a Influenza Aviária, sendo que a segunda não existe nos plantéis comerciais brasileiros.

A doença de Newcastle pode ser controlada sem vacinação, ou somente com vacinação de reprodutoras e poedeiras ou pelo uso de ambas as medidas. Outras medidas são os controles sanitários através de medidas de higiene, quarentena e vacinação nacional ou talvez regional. Monitoramento e sistema de relato nacional ou internacional são essenciais para a eficiência destas medidas.

A quantidade de vacina viva produzida e usada no Brasil, conforme dados relatados através do controle de qualidade Oficial por Orsi et al., 1999; 2001, aliado ao baixo reisolamento do vírus vacinal no território nacional e a grande variação na virulência dos diferentes isolados do VDN, indicam a necessidade de mais pesquisa sobre esta temática.

Fatores que promovem a ocorrência da doença em aves vacinadas podem ser divididos em dois grupos: 1) Fatores que diminuem a resistência do hospedeiro a infecção; e 2) Mudança do vírus que permite tornar-se uma barreira imunológica (Panshin et al., 2002).

Apesar da redução gradual da DN no país nos últimos anos para alguns autores (Seal, et al, 1998; Clavijo et al., 2000) a doença de Newcastle continua endêmica no Brasil, servindo de fonte de disseminação de vírus, principalmente pelo tráfico de aves silvestres. Estudo de prevalência realizada em aves ornamentais do Rio de Janeiro com 11% de isolamento viral foi relatado por Couceiro et al, (1999), outro estudo no mesmo estado mostrou cerca de 3,2% de soropositividade para VDN em aves de cativeiro (Bellucci et al., 1999). A soropositividade de 85,7% foi encontrada na cidade de São Paulo em Irerês apesar do genoma do VDN não ter sido detectado (Demétrio, 2002).

As principais áreas produtivas da avicultura brasileira estão localizadas nas regiões Sul e Sudeste do país, com principais produtores e exportadores de material genético avícola, ovos de consumo, produtos de aves e frangos de corte. Nos estados de São Paulo e Minas Gerais estão localizados a produção dos bisavozeiros e avozeiros. Nestes estados, além da produção avícola comercial tem ocorrido um expressivo desenvolvimento da criação de aves ornamentais, perus, silvestres como as ratitas (emas e avestruzes), psitacídeos, passariformes, entre outros.

O controle dessa doença é de importância vital para o Brasil, país que é o maior exportador e o 3º maior produtor mundial de frango de corte. Para aprimorar a eficiência, uma nova proposta de vigilância epidemiológica a "vigilância ativa" foi elaborada e implantada nas maiores áreas produtoras. O diagnóstico convencional da DN está baseado no isolamento viral em ovos embrionados, conjugado a testes sorológicos para as nove hemaglutininas do VDN e determinação de patogenicidade. Esta vigilância ativa se baseava em soroprevalência aliado ao diagnóstico da DN. Atualmente o teste de inibição da hemaglutinação é o mais usado no mundo e reconhecido pelos órgãos internacionais (OIE, 2004). Porém o teste de ELISA quando

aliado ao isolamento é utilizada como ferramenta de monitoramento, pois esta técnica pode ser automatizada ou semi automatizada, facilitando assim o diagnóstico.

A metodologia de isolamento convencional (ovos embrionados) é trabalhosa e relativamente demorada, limitando o monitoramento da doença. Para tanto, outra abordagem diagnóstica, reconhecida atualmente pela OIE são os testes moleculares como RT-PCR (reação em cadeia pela polimerase), com resultados rápidos, que associada ao sequenciamento de DNA permite a análise filogenética e identificação do agente. Jestin & Jestin desenvolveram em 1991, a 1º RT-PCT para a identificação dos VDN. Usando primer universal amplificando segmento de 238 pb do gene da proteína de fusão, positivo para 30 diferentes isolados testados, e confirmados através de enzima de restrição. O RNA viral foi extraído do fluído alantóide infectivo. A reação foi considerada altamente específica, não houve amplificação com outros vírus aviários. Mais recentemente foi desenvolvido por Gohm et al. (2000) um RT-PCR para a detecção do VDN em tecidos e fezes infectadas, descrevendo um estudo para determinar a sensibilidade de sua técnica. Usando um par de primers universal, amplificando uma região de 182 pb do gene F incluindo o sítio de clivagem. Este método barra no tipo amostras a ser colhida (tropismo nos tecidos das diferentes), a amostra deve ser colhido de uma vasta quantidade órgãos, evento esse já relatado (Wilde et al., 1990).

A base molecular da patogenicidade se baseia na replicação do VDN em célula infectada, as partículas são produzidas com um glicoproteína precursora de fusão (FO). Para ser funcional, o precursor tem que ser clivado em dois peptídeos, F1 e F2, os quais permanecem ligados por ponte dissulfeto (Rott & Klenk, 1988). A tripsina é capaz de clivar o FO para todas as amostras do VDN (Nagai et al., 1976). Estudos que compararam a sequência de aminoácidos deduzidos do precursor FO do vírus da ND, variando na virulência para galinha, tiveram a seqüência de aminoácidos ^{112}R/K-R-Q-K/R-R^{116} no C-terminal da proteína F2 e F (fenilalanina) no resíduo 117 do N-terminal da proteína F1. Entretanto os vírus de baixa patogenicidade tiveram sequência na mesma região de ^{112}G/E-K/R-Q-G/E-R ^{116} e L(leucina) no resíduo 117 (Collins et al., 1993). Alguns vírus variantes de pombos (PPMV-1) examinados tiveram a sequência ^{112} G-R-Q-K-R-F^{117,} e mostraram altos valores de IPIC (Collins et al., 1993,1994). A presença de aminoácidos básicos adicionais em amostras virulentas indica que a clivagem pode ser efetuada pelas enzimas do hospedeiro presente em uma grande variedade de tecidos e órgãos. Ao contrário, vírus de baixa patogenicidade a clivagem do FO pode ocorrer somente com protease reconhecendo-se uma simples arginina, isto é enzimas tipo tripsina. Vírus de baixa patogenicidade estão restritos a áreas no hospedeiro, onde enzimas tipo tripsinas estão presentes, tais como no trato digestivo e respiratório, ao passo que vírus patogênico pode replicar em uma variedade de tecidos e órgãos, resultando em infecção sistêmica (Rott, 1979).

O uso da dedução de aminoácidos no sítio de clivagem F0 como um prognóstico de virulência tem sido aceito pela OIE. O que tem levado a um aumento de grupos que usam esta ferramenta em suas pesquisas, realizado 1º por Collins et al, 1993; outros pesquisadores têm usado para patotipar vírus (Jestin et al., 1994; Collins et al., 1994; Seal et al., 1995; Marin et al., 1996; King & Seal, 1997).

Usando enzimas de restrição foi possível definir seis linhagens do VDN (I a VI) e através do sequenciamento foram confirmados os 6 grupos e adicionados mais 2 linhagens VII e a VIII (Ballagi-Pordany et al., 1996). Diversas sublinhagens têm sido descritas dentro destas oito linhagens (Lomniczi et al., 1998; Seal et al., 1998; Herczeg et al., 1999; Yang et al., 1999; Herczeg et al., 2001; Ke et al., 2001; Yu et al., 2001).

Considerável diversidade genética tem sido detectada nos vírus da DN, os quais podem partilhar parâmetros temporal, geográfico, antigênico e ou epidemiológico. A classificação se dá dentro de linhagens ou cluster e isto tem se mostrado valioso na epidemiologia global bem como na difusão local do ND (Aldous et al., 2003).

A classificação do VDN é bastante diversificada e muitas vezes os autores se referem a classificação como linhagens e ou genótipos. Análises filogenéticas classificam os vírus da DN em duas classes, Classe I dividida em nove genótipos e Classe II também em nove genótipos; sendo que a maioria dos isolados velogênicos pertencem à classe II, enquanto que os lentogênicos pertencem à classe I (Czegledi et al., 2006; Kim et al., 2008).

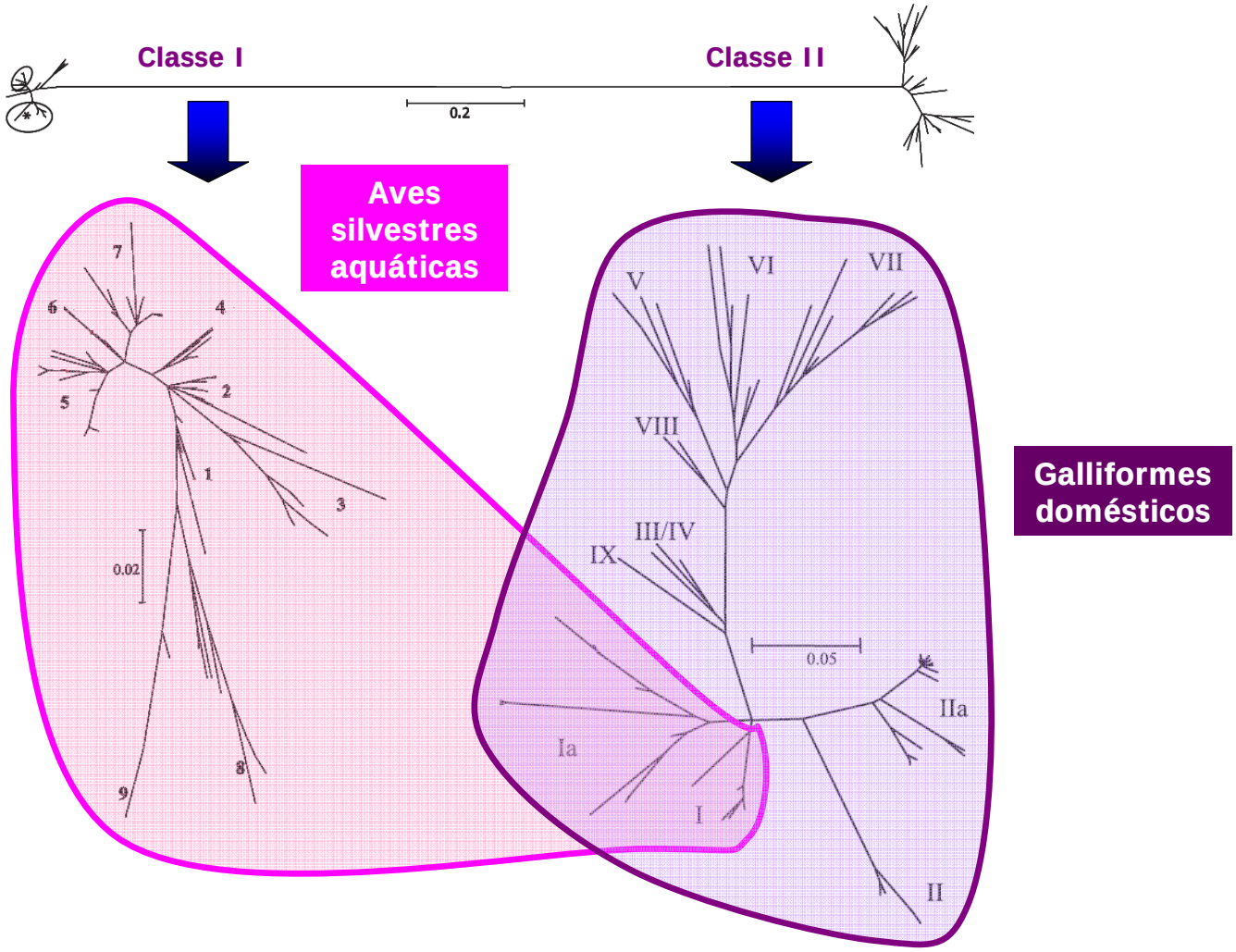

Figura 1: Análises filogenéticas classificam os VDN em duas classes: I e II. A classe I é dividida em 9 genótipos denominados de 1 a 9 e os vírus são encontrados principalmente em aves silvestres aquáticas. A classe II também é dividida em 9 genótipos, I ao IX, e os vírus encontrados principalmente em galliformes (Adaptado de Kim et al., 2007).

O papel das aves silvestres na epidemiologia da doença de Newcastle não é suficientemente entendido, embora existam indicações que as aves silvestres infectadas têm o potencial de propagação da doença e de forma muito rápida por longas distâncias (Abolnik, 2007). As aves silvestres, especialmente aves aquáticas migratórias são muitas vezes consideradas como um reservatório do VDN e um risco potencial para a introdução do VDN em países livres. Esta preocupação é corroborada pelo surgimento de focos DN ter ocorrido em 1991 (Finlândia, Reino Unido, Holanda, República Tcheca e na Áustria) onde as aves silvestres foram presumidamente as principais fontes de infecção (OIE 1997, 1996). A transmissão primária do VDN é dependente dos órgãos onde os vírus se multiplicam. Aves com sinais da doença

respiratória espalham os vírus por aerossóis e é facilmente transmitido entre as aves. Já os vírus que possuem a replicação restrita ao tecido intestinal podem disseminar através de alimento e água; ingestão de fezes contaminadas ou inalação de partículas infectantes produzida pelas fezes secas. Na Grã Bretanha em 1984 em uma epizootia em aves foi demonstrada esta forma de disseminação (Alexander, 1985). A propagação ocorreu provavelmente através de uma forma variante do VDN diferenciada por Anticorpos monoclonais, introduzido no país por pombos extraviados (Alexander et al., 1984). O vírus propagou-se entre pombos britânicos a partir deste para os selvagens (Lister et al., 1986).

A propagação de VDN e seu sucesso são devido à capacidade do vírus de sobreviver em excreções e hospedeiros mortos. O vírus pode sobreviver por diversas semanas em calor de ambiente úmido e pode sobreviver em material congelado. Entretanto eles podem ser destruídos rapidamente pela desidratação dos raios ultravioletas da luz solar (OIE, 2004).

O VDN tem sido frequentemente isolado de aves migratórias, principalmente das aquáticas selvagens e de outras aves aquáticas não migratórias (Alexander et al., Stallknecht et al., 1991; Bannerjee et al., 1994). Dez amostras do VDN foram isoladas de 321 suabes de cloaca e traquéia de patos-reais capturados em Nova Zelândia em 1997. Estes isolados apresentaram baixos índices de patogenicidade com IPIC variando até 0,0 a 1,6. Nove deles foram semelhante às estirpes originalmente isoladas no Japão e ao vírus Queensland/66 da Austrália pela sua análise filogenética, porém uma amostra isolada foi muito similar ao vírus isolado de patos na França (Stanislawek et al., 2002). Em 2000 cinco VDN lentogênicos foram isolados de gansos durante o inverno no oeste do Japão (Shenqing et al., 2002), também em recente levantamento em aves selvagens na Austrália dois isolados do VDN não patogênicos foram obtidos de suabes traqueal e cloacal de patos, sendo um dos vírus semelhante a estirpe vacinal V4 e o outro pertencente aos grupo H de anticorpos monoclonais (genótipo 6), Peroulis & O´Riley, 2004.

O processo de reinfecção e preservação dos vírus em patos foram elucidados por Takakuwa et al., 1998. Foi verificada a movimentação das aves aquáticas no processo de migração do hemisfério norte para o hemisfério sul no outono, voltando para o hemisfério norte na primavera, botando os ovos no verão nos ninhos em territórios nórdicos como Sibéria, Alaska e Canadá. O vírus é preservado no inverno quando os patos estão ausentes e as aves são re-infectadas na primavera no seu retorno do sul. Entretanto as áreas de parada e invernada de aves aquáticas migratórias são importantes sítios de transmissão intra e interespécies além da soropositividade para VDN de gansos selvagens que foi mostrado por Muller et al., 1999.

A maioria dos isolados do VDN são de aves silvestres principalmente aquáticas e são classificadas como lentogênicas, não causando doença clínica, entretanto comparação genética recente entre os vírus isolados de galinha e aves aquáticas silvestres sugere que os vírus velogênicos surgiram a partir da estirpe avirulenta originada de aves silvestres. Esta hipótese é mostrada em dois casos bem conhecidos na Irlanda

em 1990 onde dois surtos em galinha apresentavam os vírus isolados altamente virulento e aparentemente idêntico, mas estes vírus possuíam uma relação antigênica e genética muito próxima aos vírus de baixa patogenicidade normalmente isolados de aves aquáticas selvagens (Alexander et al., 1992; Collins et al., 1993). Molecularmente estes vírus virulentos tinham 4 nucleotídeos diferentes nos sítios de clivagem da proteína F0 quando comparado aos vírus associados a baixa virulência, o que pode explicar a alta virulência para galinha (Collins et al., 1993).

No 2º caso, entretanto na década de 60 foi reconhecido que a Austrália possuía seu próprio vírus avirulento endêmico, caracterizado como Queensland/66 ou estirpe V4 (Simmons, 1967), a caracterização molecular no sítio de clivagem mostrava uma sequência ^{112} GKQGRL^{117,} e uma extensão da proteína de HN de 45 aminoácidos (Toyoda et al., 1989). Algumas amostras destes vírus estão envolvidas com a forma respiratória da doença (Hooper et al., 1999).

2- OBJETIVOS

2.1- Objetivos gerais:

- Isolar e caracterizar estirpes do vírus da doença de Newcastle (VDN) a partir de amostras cloacal e traqueal de aves de produção industrial e de aves migratórias capturadas em diferentes regiões do Brasil;

- Estudar sob o ponto de vista biológico, molecular, imunológico e de estabilidade térmica as vacinas vivas (B1, La Sota, Ulster 2C, VG-GA) usadas no país

2.2- Objetivos específicos:

- Estudar a estabilidade térmica das estirpes de vacinas vivas usadas no Brasil;

- Caracterizações biológicas das vacinas vivas usando técnicas convencionais (Tempo médio de mortalidade embrionária e índice de patogenicidade intracerebral);

- Detecção do VDN por meio de técnicas convencionais (isolamento em ovos embrionados, identificação e caracterização biológica) em áreas produtoras e exportadoras e em áreas não exportadoras, assim como em aves migratórias;

- Análise filogenética baseada no gene da proteína F do VDN através do sequenciamento, com vistas a caracterizar os genótipos circulantes em aves nas regiões produtoras exportadoras ou não exportadoras, assim como, as principais vacinas usadas no Brasil.

3- CAPÍTULOS

3- CAPÍTULOS (Trabalhos publicados e submetidos)

CAPÍTULO 1 - LONG-TERM STABILITY STUDIES ON PROTECTION AGAINST NEWCASTLE DISEASE BY COMMERCIAL LIVE VACCINE USED IN BRAZIL.
Estudo de estabilidade térmica de longa duração e proteção conferida pela vacinas comerciais brasileiras usadas no Brasil. (Publicado na Biologicals em 2009-37: 252-258).

CAPÍTULO 2 - NEWCASTLE DISEASE VIRUS VACCINE STRAINS: IMMUNOGENICITY IS NOT INFLUENCED BY ICPI.
*Estirpes vacinais do vírus da doença de Newcastle: Imunogenicidade não é influenciada pelo IPIC. (*Publicado no Brazilian Journal of Poultry Science em 2009- v11, n.2:73-77).

CAPÍTULO 3 - PREVALENCE OF NEWCASTLE DISEASE VIRUS IN BROILER CHICKENS (*Gallus* gallus) IN BRAZIL
Prevalência do vírus da Doença de Newcastle em frango de corte (Gallus gallus) no Brasil.
(Publicado no Brazilian Journal of Microbiology em 2010 – 41:349-357).

CAPÍTULO 4 - A SURVEY FOR MAINTENANCE OF VIRULENT NEWCASTLE DISEASE VIRUS-FREE AREA IN POULTRY PRODUCTION IN BRAZIL
Um levantamento para manutenção de área livre do vírus virulento da Doença de Newcastle em áreas produtoras de aves no Brasil. **(**Publicado no Brazilian Journal of Microbiology em 2010- 41: 368-375).

CAPÍTULO 5 - ABSENCE OF HIGHLY PATHOGENIC NEWCASTLE DISEASE VIRUS IN SOME DOMESTIC COMMERCIALIZATION REGIONS OF BRAZIL *Ausência de vírus da Doença de Newcastle altamente patogênico em algumas regiões não exportadoras de aves no Brasil. (*A ser submetido a publicação).

CAPÍTULO 6 - MOLECULAR CHARACTERIZATION OF FIELD ISOLATES OF NEWCASTLE DISEASE VIRUS AND VACCINE STRAINS USED IN BRAZIL
Caracterização molecular de isolados de campos do vírus da Doença de Newcastle e estirpes vacinais usadas no Brasil. (A ser submetido a publicação).

CAPÍTULO 7 - ISOLATION AND BIOLOGICAL CHARACTERIZATION OF NEWCASTLE DISEASE VIRUS IN MIGRATORY BIRDS IN BRAZIL
Isolamento e caracterização biológica do vírus da Doença de Newcastle em aves migratórias no Brasi.
(Submetido a publicação no Journal of Wildlife Disease).

CAPÍTULO 1

LONG-TERM STABILITY STUDIES AND PROTECTION CONFERRED BY COMERCIAL LIVE VACCINE OF NEWCASTLE DISEASE USED IN BRAZIL

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Biologicals

Available online at www.sciencedirect.com
ScienceDirect
Biologicals 37 (2009) 252–258
IABS BIOLOGICALS
www.elsevier.com/locate/biologicals

Long-term stability studies on protection against Newcastle disease by commercial live vaccine used in Brazil

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Received 16 August 2008; revised 17 March 2009; accepted 6 April 2009

Abstract

The thermostability (TS) and efficacy offered by live vaccines against Newcastle disease strains B1, La Sota, VG-GA and Ulster, produced or imported by four Brazilian laboratories, were evaluated during their validity period. Kinetic profiles were obtained from samples conserved in refrigerators during 0, 4, 8, 12, 16, 20 and 24 months after their manufacturing. The statistical analysis of the vaccine titre effect obtained by the fresh air (FA) method showed that the vaccine profiles were parallel and coincident, presenting a significant descending trend. The vaccine titres and efficiency proofs at the end of the validity period were above the level of legislation requirements and showed an average loss in titre of 0.40 and 0.66 $\log_{10}$ within the first and second validity years, respectively. The titre obtained by TS, within the month after manufacturing, had no significant difference from the titre obtained by FA within 24 months after manufacturing, being their pairs of observations positively correlated ($r = 0.49$, $p = 0.0003$), showing that the TS method, which anticipates the vaccines' performance at the end of the validity period, can substitute the FA method 24 months after manufacturing.

Keywords: Thermostability; Protection; Newcastle disease vaccine; Commercial live vaccines; Virus vaccine

1. Introduction

The commercial live vaccines of Newcastle disease (ND) were developed in the 1940s, with the discovery of strains bearing low pathogenicity Newcastle Disease virus (NDV) [1–3] and, for more than 50 years, an attenuated NDV vaccine has been used successfully in chickens to prevent disease [4]. There are three types of vaccines used for ND: live lentogenic, heat-tolerant, live mesogenic and inactivated [5]. Some countries have specific legislation on the use and control of quality of such vaccines. However, the policy varies greatly depending on the "enzootic status" or ND perception as a threat for poultry production. The success of the vaccination depends on several factors inherent to the vaccine: the immunogenic virus quality employed in its manufacturing, antigen stability, and the amount of virus used per dose, as well as the virus profile, in order to become an immunological barrier [6]. Veterinary vaccines should be controlled by the manufacturer and, in several countries, also by independent analysts, before they are approved for commercial use [7].

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1045-1056/09/$36.00
doi:10.1016/j.biologicals.2009.04.001

Despite of the organ, which is enrolled on the quality control measures, it is generally accepted that the product quality is a responsibility of the manufacturer. The vaccines should be tested for potency and efficacy, identity, safety and purity, using both *in vitro* and/or *in vivo* assays. Based on international standards, each country has specific requirements for approval of NDV vaccine batches, and in Brazil these requirements are defined by the Ministry of Agriculture [8–10].

Efforts for ND prophylaxis in broiler chickens in Brazil are focused on the active immunization by the use of live lentogenic vaccines, being La Sota, B1, Ulster and VG-GA the most often used strains. Reports on the use of live vaccines prepared from the B1 strain are abundant in the literature [11–14], as well as a considerable efficacy of B1 and La Sota strains [13,15–17]. Many commercial vaccines are extremely thermolabile. All conventional live vaccines have the disadvantage of needing to be kept at low temperatures to maintain their efficacy (the vaccine antigens are kept in appropriate conditions to maintain the integrity antigenic, which is of fundamental importance to have an appropriate immune response). This is not a problem for intensive poultry production in an industrial setting; however, the cold chain maintenance during distribution can be very difficult in tropical countries, particularly where there is high ambient temperature [18]. The Malaysian thermostable variant of V4 lost less than 1 $\log_{10}$ unit for 1 h at 50 °C and less than 3 $\log_{10}$ units after 6 h [19]. The V4 vaccine was stable in freeze-dried form for 3 months at 18–22 °C and lost only about 1 $\log_{10}$ on storage for three months further [20]. The I2 vaccine freeze-dried lost about 1 $\log_{10}$ of infectivity when stored for six days at 26–32 °C [21]; when reconstituted, after storage for 24 days at 30–35 °C, it still produced substantial protection in vaccinated chickens. Thermostability is defined as the ability of a substance to retain its activity at a particular level after treatment at a defined temperature for a specified time [22], for example, a virus is exposed to a constant temperature of 56 °C and samples are taken at given time intervals. The infectivity of the virus is then measured and considered thermostable if the decrease in the infectivity titre is less than 1 $\log_{10}$, following exposure for 1 h [23]. The vaccine stability is then defined by the length of time the vaccine will retain an infectivity titre sufficient to induce a protective immune response, at a particular temperature [24,25]. In their study about thermostability of Newcastle disease life vaccine, Simi et al. [25] noted that the vaccines kept at a temperature of 37 °C for seven days showed equal conservation at temperatures between 2 and 8 °C for two years. There are several methods to measure the vaccine efficacy, varying from sophistication to cost effectiveness. Chickens successfully vaccinated with ND vaccines produce antibodies that are present in the plasma and that will react specifically with Newcastle disease virus, using the hemagglutination inhibition test-HI and immunoenzymatic assays (ELISA, circulating antibody) or artificial challenge protocol for avian vaccines, usually including a challenge procedure in which vaccinated and unvaccinated hosts receive a defined dose of virulent challenge organism by injection.

Brazil has become the biggest broiler chicken exporter and the third producer in the world. Considering that Newcastle disease is one of the most serious poultry diseases in developing countries, it has become a worldwide poultry pathogen of significant economic importance. The ND prophylaxis in broiler chickens in Brazil is focused on the active immunization by using live lentogenic vaccines. There is much information about the results achieved by using the FA method, while the information on the TS method results is rather sparse.

The purpose of this study is to assess the ability of the TS method after manufacturing to be used as a substitute for the FA method 24 months after manufacturing, which was confirmed by the vaccine's protection test study.

2. Materials and methods

2.1. Facilities

The experiment was conducted by the Committee of Poultry Sanity of Lanagro/SP. The efficiency test using challenge with pathogenic virus was conducted in the biosecurity area, level 3.

2.2. Vaccine viruses

The ten commercial lyophilized vaccines were prepared using the strains B1, La Sota, Ulster 2C, VG-GA, from four main producers (Table 1), manufactured in Brazil or imported.

2.3. Thermostability titration (TS)

The titration was performed as described by [25]. Three vials of vaccine in each test were maintained at 37 °C for seven days. Vial contents were reconstituted as recommended by the manufacturers, diluted log 10 until $10^{4.0}$ before interval of $10^{-0.7}$ with dilutions $10^{-4.7}$ $10^{-5.4}$, $10^{-6.1}$ and $10^{-6.8}$ and inoculated into the allantoic cavity of 9–11-day-old embryonated fowl eggs, using seven eggs in each dilution. The embryos found dead until 24 h were discarded and the others were sampled and submitted to hemagglutination assay soon after the end of the observation period. The EID_{50} calculation was performed in accordance standard methods [26].

2.4. Fresh air titration (FA)

The titration was performed until $10^{-5.0}$ with interval of $10^{-0.7}$, with the dilutions $10^{-5.7}$, $10^{-6.4}$, $10^{-7.1}$ and $10^{-7.8}$, and inoculated into the allantoic cavity of 9–11-day-old

Table 1
Identification of the vaccine strains used in laboratories experiments.

Laboratories	I			II			III		IV	
Codes	A	B	C	D	E	F	G	H	I	J
Strains	B1	La Sota	Ulster	B1	La Sota	VG-GA	B1	La Sota	B1	La Sota

embryonated fowl eggs, using seven eggs in each dilution. The eggs were incubated during six days at 37 °C, and observed daily by candle. The embryos found dead until 24 h were discarded and the other samples were submitted to hemagglutination assay soon after the end of the observation period. The EID_{50} calculation was performed in accordance with the methodology by [26].

2.5. Chickens

Two hundred-fifteen-day-old unsexed chickens used in the experiments were from specific-pathogen-free (SPF) eggs (Granja Rezende, Brazil), flocks known to be free of antibodies against NDV. The chickens were housed in ten isolation units — twenty in each unit — operated under negative pressure with intake and exhausted air. Isolation units were placed on the high containment facility in the biosecurity area of LANAGRO/SP, a level 3 biosafety facility. In addition, ten one-day-old chickens using the same condition above were maintained by control. Five one-day-old chickens were used as source of sera to confirm the negative NDV serological status of the chickens at the beginning of the experiment.

2.6. Experimental design

2.6.1. Planning the experiments

The ten vaccine titres were obtained using the TS and FA methods within the month after manufacturing, and the same vaccines were conserved in refrigerators and again their titres were obtained by FA within periods of 4, 8, 12, 16, 20, and 24 months after manufacturing, using three vials for each titration. There were five consecutive batches for each vaccine selected as soon as they arrived in lab for the official test, with the exception of A, B and E with four batches.

2.6.2. Statistical analysis

The profile's FA titres were studied by the repeated measures analysis [30] under split-plot design. The vaccine was the principal effect and the period considered the secondary. The regression analysis was used to study the period's trend. Considering the FA loss of titre in 24 month ($D = FA_{period\ 0} - FA_{period\ 24}$) and it difference between TS in period 0 after manufacturing ($D = FA_{period\ 24} - TS_{period\ 0}$), the null hypothesis H_0: $D = 0$ was tested using Student's t-test for paired samples: $t = (\overline{d}/s_d)\sqrt{n}$, where $\overline{d}$ is the mean of difference/loss, s_d the standard deviation of the difference/loss and n the number of pair of observed samples. The hypothesis was not significantly to the level of $p = 0.05$ if $t \geq t\,(n-1, p/2)$.

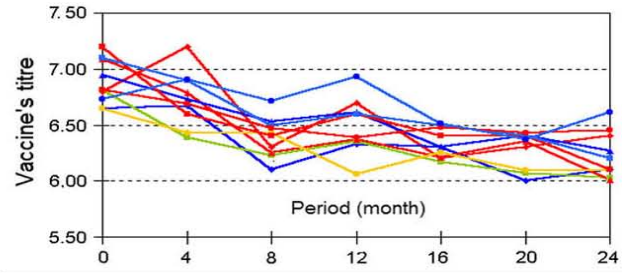
2.6.3. Protection test

Two hundred chickens in 10 isolation units were vaccinated only once by eye-drop administration of different vaccines at 7 days of age. Ten chickens in one unit remained as unvaccinated controls. The lyophilized vaccine at the end of validity was reconstituted using PBS, pH 7.2 in the proportion of 30 mL/1000 dose vaccine, a dose vaccination eye drop as methodology used by [27,28]. On day 21, after vaccination, all groups (vaccinated and unvaccinated) were challenged by intramuscular injection (IM), with one pathogenic strain of Newcastle disease virus "São João do Meriti" [29]. The titre was determined by the same technique described for the fresh air titration. Hundred milliliters of suspension of NDV with the titre $10^{6.0}$ ELD_{50} were administered by intramuscular technique described in [8]. After the challenge, the birds were observed daily for 10 days, to record clinical signs and mortality. The resistance to the challenge was expressed as percentage of total protection and refers to the absence of clinical signs (morbidity) and death in 90% of vaccinated and challenged birds, and the presence of symptoms and/or mortality in less than 90% of the group challenged testimony.

3. Results

The laboratories were designed from I to IV, and the identification of the vaccine strains La Sota, B1, Ulster; VG-GA is shown in Table 1.

The mean FA titre profile at seven observed intervals of time up to twenty-four months (two years) of maintenance in refrigerator of ten vaccines is shown in Fig. 1. The H—F conditions were not satisfied ($p = 0.0284$) and we used the multivariate analysis of variance that showed that the profiles are parallel ($P_{period \times vaccines} = 0.8708$) and coincident ($P_{vaccines} = 0.1439$), that is, we could consider that vaccine



n	42	42	42	42	42	42	42
min	5.93	5.93	5.43	5.43	5.53	5.43	5.13
LL	6.78	6.58	6.29	6.36	6.26	6.17	6.10
FA mean	6.90	6.73	6.43	6.49	6.35	6.30	6.23
UL	7.01	6.87	6.56	6.63	6.44	6.43	6.35
max	7.63	7.63	7.43	7.63	7.13	7.43	7.43

Legend:
n=number of observations
min =minimum value of vaccine's FA titre
max=maximum value of vaccine's FA titre
LL=lower limit of 95% confidence interval for true FA mean
UL=upper limit of 95% confidence interval for true FA mean

Vaccines against Newcastle disease:
A B C D
E F G H
I J

Fig. 1. Kinetic of the mean FA titre profile of ten vaccines against Newcastle disease utilized in four Brazilian laboratories — number of observations (n), minimum and maximum values, lower and upper limits of 95% confidence interval. Source: National Agricultural Laboratory — Lanagro/SP — Campinas — SP, Brazil.

Table 2
The relationship between FA periods' titre mean.

Parameter	Estimate	t Value	Prob($\|t\| > 0$)	Standard error of estimate
Intercept	7.10	72.45	0.0001	0.10
Period	−0.23	−4.09	0.0001	0.06
Period^2	0.02	2.27	0.0241	0.01

profiles are similar and the differences in vaccines titres do not change with respect to periods. Whereas the profiles were parallel and coincident and the difference in the mean FA titre of the 10 vaccines was not significant, we represent them with the mean FA titre within 0, 12 and 24 months of maintenance in refrigerator $10^{6.90}$, $10^{6.49}$ and $10^{6.23}$, respectively. Furthermore, the difference between periods was significant ($p_{\text{period}} = 0.0001$), as well as the linear ($p = 0.0001$) and quadratic ($p = 0.0167$) relationship, suggesting by its descending aspect (Table 2) that there were losses of titres during the periods. Those vaccines at 12 months of maintenance in refrigerator showed a significant mean loss of titre of 0.40 $\log_{10}$ (with 95% confidence limits between 0.27 and 0.54, data not shown).

The description of the mean loss of FA titre of ten vaccines during twenty-four months of maintenance in refrigerator is shown in Fig. 2. The vaccine titre within 24 months of maintenance in refrigerator showed a significant mean loss of titre of 0.66 $\log_{10}$ (with 95% confidence limits between 0.50 and 0.81) corresponding to 9.71% of the initial titre. There was a maximum loss of 2.10 and minimum of −0.30. The vaccines B, C, D, E, G and H had losses significant.

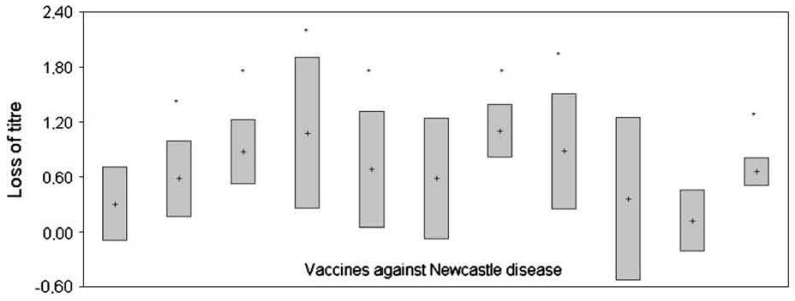
The values of the titres obtained by TS method are drawn in Fig. 3. The mean was 6.08 of $\log_{10}$ (with limits of 95% of confidence between 6.00 and 6.18). There was a maximum of 6.63 and minimum of 5.43.

It is shown in Fig. 4 the description of the difference between the FA titre within 24 months of maintenance in refrigerator (FA period$_{24}$) and TS within 0 month after manufacturing (TS period$_0$). The mean difference of 10 vaccines 0.13 $\log_{10}$ (with 95% confidence limits close to 0.03 and 0.24) was significant. For each vaccine, the titre difference between FA and TS was not significant, with exception of the vaccine J.

In summary, considering the level demanded for respecting the legislation, the TS titre mean within 0 month was less than the FA titre within 24 months – and those titres were positively correlated ($r = 0.49$, $p = 0.0003$) – these results indicate the TS method within the month after manufacturing test to anticipate the behavior of the vaccine against Newcastle Disease within 24 months of maintenance in refrigerator.

The challenge results of each vaccine at the end of the validity period are shown in Table 3. Clinical signs of transient reaction vaccine were not observed at the clinical examination of vaccinated birds. The challenge results of pathogenic viruses of Newcastle Disease in the 28th day of age of the experimental SPF birds showed satisfactory result of efficiency for all vaccines.

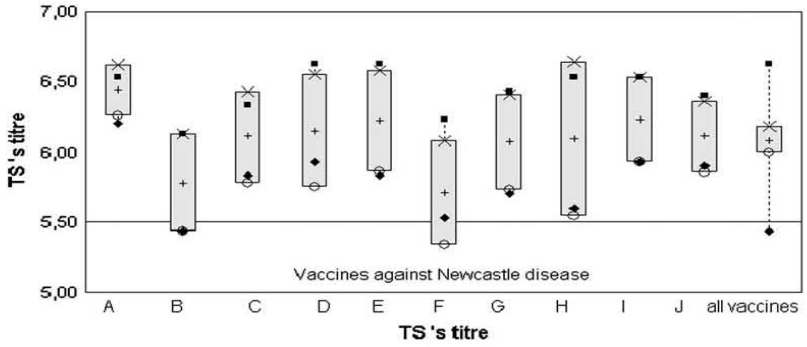
The experimental data show protection varying between 90 and 100%; therefore, the vaccines were protected from



Values	Loss of titre (FA period 0 - FA period24)										
minimum	0.10	0.20	0.60	0.50	0.10	0.10	0.90	0.17	-0.30	-0.20	-0.30
LL95%	-0.10	0.16	0.52	0.25	0.04	-0.09	0.81	0.24	-0.53	-0.22	0.50
+ **mean**	**0.30**	**0.58**	**0.88**	**1.08**	**0.68**	**0.58**	**1.10**	**0,88**	**0.36**	**0.12**	**0.66**
UL95%	0.71	1.00	1.23	1.91	1.32	1.25	1.39	1.51	1.25	0.46	0.81
maximum	0.67	1.10	1.10	2.10	1.30	1.50	1.50	1.57	1.50	0.40	2.10
standard error	0.13	0.15	0.11	0.30	0.23	0.24	0.10	0.23	0.32	0.12	0.08
coefficient of variation	84.21	57.70	25.34	62.12	75.27	92.37	21.32	58,56	198,96	231,24	80,42
n	4	5	5	5	5	5	5	5	5	5	49

Legend:
LL 95% = lower limit of 95% confidence interval for the mean loss of titre
UL 95% = upper limit of 95% confidence interval for the mean loss of titre
n=number of observations
* Significant loss, p-value=0.05

Fig. 2. The mean FA loss of titre of ten vaccines during twenty-four months of maintenance in refrigerator, limits of 95% confidence interval, standard error, coefficient of variation and number of observations (n). Source: National Agricultural Laboratory – Lanagro/SP – Campinas – SP, Brazil.



	A	B	C	D	E	F	G	H	I	J	all vaccines
◆ minimum	6,20	5,43	5,83	5,93	5,83	5,53	5,70	5,60	5,93	5,90	5,43
× LL95%	6,26	5,43	5,78	5,75	5,86	5,34	5,73	5,54	5,93	5,85	6,00
+ mean	6,44	5,78	6,11	6,15	6,22	5,71	6,07	6,09	6,23	6,11	6,08
○ UL95%	6,62	6,13	6,43	6,55	6,58	6,08	6,41	6,64	6,53	6,36	6,18
▪ maximum	6,53	6,13	6,33	6,63	6,63	6,23	6,43	6,53	6,53	6,40	6,63
standard error	0,06	0,13	0,10	0,14	0,13	0,13	0,12	0,20	0,11	0,09	0,05
coefficient of variation	4,34	8,98	2,19	6,33	5,13	1,26	3,06	3,73	8,27	3,48	5,38
n	5	5	4	5	5	5	5	5	5	5	48

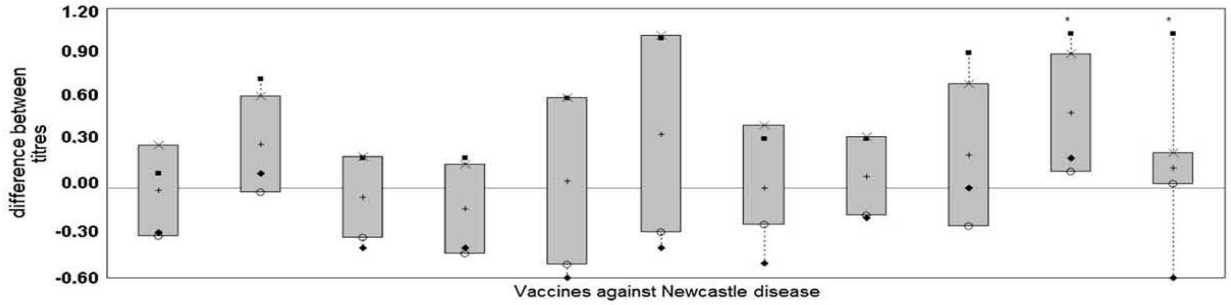
Legend:
LL95% =lower limit of 95% confidence interval for the mean
UL95% =upper limit of 95% confidence interval for the mean
n=number of observation

Fig. 3. The mean TS of titre of ten vaccines after manufacturing, limits of 95% confidence interval, standard error, coefficient of variation, number of observations (*n*). Source: National Agricultural Laboratory — Lanagro/SP — Campinas — SP, Brazil.

challenge and negative SPF chickens developed clinical signs of ND. The death and/or clinical signs of ND appeared on the 3rd day after challenge in birds that were not protected (the protection was 0%).

4. Discussion

The product quality control standard used in biological poultry of Requirements of the Brazilian Ministry of Agriculture



Values	A	B	C	D	E	F	G	H	I	J	All vaccines
	Difference between titres FA period 24 - TS period 0										
◆ minimum	-0,30	0,10	-0,40	-0,40	-0,60	-0,40	-0,50	-0,20	0,00	0,20	-0,60
○ LL 95%	-0,32	-0,03	-0,33	-0,44	-0,51	-0,30	-0,25	-0,19	-0,26	0,11	0,03
+ mean	-0,02	0,29	-0,06	-0,14	0,05	0,36	0,00	0,08	0,22	0,50	0,13
× UL 95%	0,29	0,61	0,21	0,16	0,61	1,02	0,42	0,34	0,70	0,90	0,24
▪ maximum	0,10	0,73	0,20	0,20	0,60	1,00	0,33	0,33	0,90	1,03	1,03
standard error	0,10	0,12	0,10	0,11	0,20	0,24	0,15	0,09	0,17	0,14	0,05
n	4	5	5	5	5	5	5	5	5	5	49

Legend:
LL 95%= lower limit of 95% confidence interval for the mean difference between titres
UL 95% upper limit of 95% confidence interval for the mean difference between titres
n=number of observations
* Significant difference , p-value =0.05

Fig. 4. The differences between the vaccine's FA titre in twenty-four months of maintenance in refrigerator and the vaccine's TS titre 0 month after manufacturing, limits of 95% confidence interval, standard error and number of observations (*n*). Source: National Agricultural Laboratory — Lanagro/SP — Campinas — SP, Brazil.

Table 3
Results of challenge with pathogenic strains of ND, from SPF birds, 28th day of live, vaccine at 7 day.

Laboratories	Code	Vaccine strains	Vaccination methods	N	MM[a]	Protection[b] %
I	A	B1	Eye drop	20	0/20	100%
	B	La Sota	Eye drop	20	2/20	90%
	C	Ulster	Eye drop	20	0/20	100%
II	D	B1	Eye drop	20	1/20	95%
	E	La Sota	Eye drop	20	1/20	95%
	F	VG-GA	Eye drop	20	0/20	100%
III	G	B1	Eye drop	20	0/20	100%
	H	La Sota	Eye drop	20	0/20	100%
IV	I	B1	Eye drop	20	0/20	100%
	J	La Sota	Eye drop	20	0/20	100%
Control	—	—	—	10	10/10	0%

[a] Mortality and morbidity — number of birds displaying clinical signs or death of NDV.
[b] Percentage of surviving birds that did not display clinical signs of disease.

(Regulation # 186/97) [31] consign that the minimum viral titre accepted at the time of manufacturing of ND vaccines is $10^{6.5}$ EID_{50} and the minimum viral titre in the final term of validity is $10^{5.5}$ EID_{50}. In this study, it was observed that, overall vaccines, the mean viral titre obtained by the FA method in the two-year-period was $10^{6.23}$, with a significant average loss of 0.66 log 10, corresponding to 9.71% of the initial titre, though the loss has been significant ($p < 0.05$) for each vaccine, except for vaccines A, E, H, I and J. For that reason, these results changed the minimum viral titre in the beginning of the validity test to $10^{6.20}$ EID_{50} in the new regulation # 07/06 [8]. Furthermore, it was shown the significant correlation between the titres obtained by the FA method at the end of the validity period and by the TS method in the beginning; there was no significant difference between their means, since their results were similar to that found by [25]. Accelerated degradation studies on the international reference preparation (IRP) for Newcastle disease vaccine live are reported by [32], showing that the virus content is stable when stored at 4 °C for two years, with data similar to ours. Therefore, within the month after manufacturing, the thermostability test indicates the live vaccine efficiency against Newcastle disease, anticipates its performance during 24 months of storage at a temperature between 2 and 8 °C, and can be used as substitute of the FA method 24 months after manufacturing.

All vaccines researched showed efficacious, facing the challenge as the pathogenic virus of Newcastle disease at the end of its validity period. The results of the challenge with pathogenic viruses of Newcastle Disease in the 28th day of age of the SPF experimental birds showed satisfactory efficiency of all vaccines or results equal to or above the minimum required by the Brazilian legislation. As proof of efficacy, the data showed that 100% of the non-vaccinated control birds died.

Acknowledgments

We would like to thank the Brazilian Ministry of Agriculture for authorizing this paper publication, in particular, the coordinations of Lanagro/SP, the Laboratory Support and the Veterinary Products. We would also like to thank the civil servant of the Service for Poultry Disease, in special, Dilmara Reischak and Sandra Regina Zarpelon, for their technical help; Luiz Carlos da Silva Porto, for his animal care assistance. We thank Dr. Orlando J.F.G. Bordoni, for his English support and Etna Lima Macário, translator at the Research Commission (FCM-Unicamp), for collaborating with the English review.

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CAPÍTULO 2

NEWCASTLE DISEASE VIRUS STRAINS: IMMUNOGENICITY IS NOT INFLUENCED BY ICPI

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Brazilian Journal of Poultry Science

Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola

ISSN 1516-635X Apr - Jun 2009 / v.11 / n.2 / 73 - 77

Newcastle Disease Virus Vaccine Strains: Immunogenicity is not Influenced by ICPI

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■ Keywords

Newcastle disease virus (NDV) vaccines, intracerebral pathogenicity index (ICPI), mean death time (MDT).

■ Acknowledgments

We are thankful to the Brazilian Ministry of Agriculture for authorizing publication of this paper. We would like to thank the coordinating bodies of Lanagro/SP, the Animal Laboratory, as well as the coordination of Veterinary Products. We also thank the Poultry Disease Sector staff, particularly Maria do Livramento de Jesus Nascimento and Jaira Alves dos Santos, for their expert technical help, and Luiz Carlos da Silva Porto, for assisting in animal care. C.W.A. and F.R.S. acknowledge the funding provided by CNPq (National Council of Scientific and Technological Development, Brazil).

Arrived:
Approved:

ABSTRACT

Intracerebral pathogenicity index (ICPI) and mean death time (MDT) were determined using commercial live vaccines against Newcastle disease available in Brazil. The ICPI profiles obtained for B1 vaccine strains were nonvirulent and varied from 0 to 0.19, and their MDT was 104-116 hours. The LaSota strains had an ICPI varying between 0.02 and 0.37 and MDT from 92 to 116 hours. ICPI and MDT for the Clone 30 were 0.11 and 104 hours, respectively. For Ulster vaccines, ICPI and MDT were 0 and >150 hours; for VG-GA was 0.03 and 140 hours; and for C2, 0.04 and >144 hours. Eye drop vaccination and IM challenge, at the 1st week and the 4th week, respectively, resulted in highest protection for B1 (95-100%) and LaSota (90-100%) strains. The variability in vaccine ICPI did not interfere with immune response and all vaccines provided similar protection. All vaccines were considered non virulent and were classified as lentogenic according to the immunobiological product standards.

INTRODUCTION

The Newcastle disease virus (NDV) or avian paramyxovirus serotype 1 (APMV-1) is a RNA virus belonging to the genus Avulavirus of the family *Paramyxoviridae* (Mayo MA. 2002a; Mayo MA 2002b). It is an important pathogen that can affect commercial poultry producers worldwide by producing outbreaks, and resulting in trade barriers (Alexander *et al.*, 1997). The disease is economically important, since it causes high morbidity and mortality, reduces egg production, deteriorates egg quality, and impairs live performance.

The importance and impact of a NDV isolate is directly related to its virulence. Laboratory tests were developed by Hanson and Brandly (1955) proposed the classification of NDV isolates upon allantoic inoculation using mean dead time (MDT) as "velogenic", "mesogenic", and "lentogenic", based on chicken embryo mortality at <60 hours, 60-90 hours, or >90 hours, respectively. Other tests designed to differentiate strains directly assess clinical signs or death in infected birds, calculating a pathogenicity index. The most widely used test is the intracerebral pathogenicity index (ICPI) (Hanson, 1980). NDV infection is currently defined as a notifiable disease if the virus has an ICPI of 0.7 in day-old chicks (*Gallus gallus domesticus*) (OIE, 2000b). An APMV-1 virus that does not meet the OIE definition for causing ND is referred to as a low-virulence APMV-1 or NDV.

Efforts for ND prophylaxis in broiler chickens in Brazil are focused on the active immunization by the use of live lentogenic vaccines. The virus strains most commonly used in vaccines are the La Sota (Goldhaft, 1980) and the B1 strains (Hitchner & Johnson, 1948), as well as viruses from the asymptomatic enteric pathotype, which are usually based on the

V4, VG-GA or Ulster 2C viruses. These viruses are selected by manufacturers in order to improve vaccine immunogenicity or to enable their use by a particular method of application (Alexander *et al*., 2004). Some lentogenic vaccines have been cloned (Clone 30), selecting a virus which produces less vaccine reactions than a La Sota-like virus, with superior immunogenicity as compared to HB1-like viruses (Alexander *et al*., 2004).

In Brazil, as well as in the European Union, legislation defines the pathogenicity of viruses allowed to be used in vaccines. The virus seed from live vaccines must be tested, and their ICPI must have values lower than 0.4, while the seed of the inactivated virus used in vaccines must have an ICPI value lower than 0.7 (CEC, 1993). In Brazil, only lentogenic strains with ICPI <0.4 are allowed in commercial vaccines. A similar legislation was adopted by the Organization for Animal Health (OIE, 2004) and also in Brazil, as described in Regulation # 07/06 (BRAZIL, 2006). Brazilian law requires that vaccines used to immunize poultry against ND are produced from strains with low pathogenicity. The quality antigen of the viruses used in vaccines is of paramount importance to provide adequate immune response against NDV.

This study was carried out to determine if the profile of the Brazilian vaccines is in accordance with that described by the Organization for Animal Health - OIE (2004), and if there is a correlation between different ICPI/MDT and protection conferred by the same strains.

MATERIALS AND METHODS

Facilities. The experiment was carried out at the Poultry Health Sector of Lanagro/SP. The efficiency test, using pathogenic virus challenge, was conducted at biosafety level 3.

Virus vaccines. The commercial lyophilized vaccines, manufactured in Brazil or imported, were prepared with the strains (B1, La Sota, Ulster 2C, Clone 30, VG-GA, and C2). The titer was determined using the technique described for thermostability titration (Simi *et al*., 1970; Orsi *et al*., 2009, in press), and varied between 105.50 and 106.70. The vaccine vials were reconstituted in proper dilution, as recommended by the manufacturers, and strains were propagated in embryonated SPF chicken eggs.

Vaccine virus propagation in embryonated SPF chicken eggs. The vaccine and reference strains were replicated by inoculating embryonated SPF eggs (9-11 days old), via allantoic cavity. Embryos that died within 24 hours were discarded, and the remaining were tested for hemagglutination (HA) activity in the allantoic fluid, according to the technique described in Regulation # 07/06 (Brazil, 2006). The fluids containing HA activity were pooled, and stored at -80ºC until use.

Birds. One hundred and seventy-five unsexed day-old chickens derived from specific pathogen-free (SPF) eggs (Granja Rezende, Brazil), from flocks known to be free from antibodies against NDV were used in the experiments. Birds were housed in nine isolation units (20 per unit), operated under negative pressure with filtered air intake and exhausted air. Isolation units were placed in a high containment facility (biosafety level 3) of Lanagro/SP. In addition, ten one-day-old chickens, housed under similar conditions, were maintained as controls. Five one-day-old chickens were used as serum source to confirm the negative NDV serological status of the chickens at the beginning of the experiment.

Experimental design. Tests were carried out with the most common NDV strains used by the poultry industry (La Sota and B1), produced by four different manufacturers. One hundred sixty chickens were distributed into eight groups according to vaccine manufacturer and vaccine strain, housed in isolation units, and vaccinated once at seven days of age via eye drop. Each group (n=20) was vaccinated with one given commercial vaccine. Four vaccines were manufactured with La Sota strain and four with B1 strains by 8 different manufacturers.

One unit with ten birds was kept as unvaccinated control. The lyophilized vaccine was reconstituted using PBS at pH 7.2 in the proportion of 30 mL/1000 dose vaccine, and administered via eye drop dose, according to the methodology of Paulillo (1984, 1989). Twenty-one days after vaccination, all groups (vaccinated and unvaccinated) were challenged by intramuscular injection (IM). After the challenge, birds were observed for clinical signs and daily mortality for 10 days. Resistance to challenge was expressed as percentage of total protection, and refers to the absence of clinical signs (morbidity) and death in 90% of the vaccinated and challenged birds, and to the presence of clinical signs and/or mortality in less than 90% of the challenged group.

Challenge study. Three weeks after vaccination, each bird (28 days of age) was challenged with the pathogenic of Newcastle disease virus strain "São João do Meriti", ICPI=1.75, IVPI=2.33, and MDT=48 hours (Doretto Júnior, 2003). The titer was determined using the technique described for fresh air titration (Orsi *et al*., in press). One hundred milliliters of NDV suspension

containing $10^{6.0}$ EID50 was administered by intramuscular injection (Brazil, 2006).

Biological characterization: Intracerebral pathogenicity index (ICPI). ICPI was obtained using the in vivo test described in Regulation # 182/94 (Brazil, 1994). The World Organization for Animal Health (OIE, 2004) defines pathogenic Newcastle disease virus strains as those presenting an IPIC ≥0.70, and Newcastle disease is an infection of birds caused by the avian paramyxovirus serotype 1 (APMV-1) that meets the following virulence criterion: it has an ICPI ≥ 0.70 in day-old chicks (*Gallus gallus*).

Mean death time (MDT). Fresh, infective bacteria-free allantoic fluids were used for this test, which was assessed as described by Hanson & Brandly (1955), Alexander (1988), and Brazil (1994, 2006).

RESULTS

Pathogenicity test of the ND vaccine strains. Seven B1 strain vaccines, six La Sota strain vaccines, and the strains Ulster, VG-GA, Clone 30, and C2 were tested.

The biological characterization of the live vaccine strains showed that the ICPI of B1 strains ranged from 0.0 to 0.19 and its MDT, between 104 and 116 h (Table 1). The ICPI of the La Sota strain ranged from 0.02 to 0.37, and MDT varied between 92 and116 h (Table 2). The ICPI and the MDT were 0.11 and 104h for the Clone 30 strain, respectively; 0 and > 150h for the Ulster strain,0.03 and 140h for the VG-GA strain, and 0.04 and >144h for the C2 strain (Table 3).

Table 1 - Pathogenicity index values obtained for commercial NDV B1 vaccine strains.

Laboratory	ICPI*	MDT**	References
1	0.03	104	***
2	0.13	104	***
3	0	116	***
4	0.11	116	***
5	0.19	116	***
6	0.19	116	***
7	0.09	104	***
Standards	0.20	120	****

* ICPI, intracerebral pathogenicity index in day-old chicks. *** Present study. ** MDT, mean death time (hours) of chick embryos infected with one minimum lethal dose. **** Allan et al, 1978; Alexander & Allan, 1974.

The biological characterization of the ND vaccine strains includes ICPI and MDT determinations. All vaccine viruses were classified as lentogenic strains, according to their MDT values, and the embryos remained alive for more than 90 hours. ICPI values ranged from 0.0 to 0.37, characterizing the vaccine virus are lowly virulent or as nonpathogenic strains.

Table 2 - Pathogenicity index values obtained for commercial NDV La Sota vaccine strains.

Laboratory	ICPI*	MDT**	References
1	0.23	92	***
2	0.37	96	***
3	0.10	116	***
4	0.02	116	***
5	0.37	104	***
8	0.32	116	***
Standards	0.40	103	****

*ICPI, intracerebral pathogenicity index in day-old chicks, ***Present study. **MDT, mean death time (hours) of chick embryos infected with one minimum lethal dose. ****Allan *et al.*, 1978; Alexander & Allan, 1974.

Table 3 - Pathogenicity index values obtained for commercial NDV vaccine strains.

Laboratory	Strains	ICPI*	MDT**	References
1	Ulster	0	>150	***
2	VG-GA	0.03	140	***
4	Clone 30	0.11	104	***
4	C2	0.04	>144	***
Standards	Ulster	0	>150	****

* ICPI, intracerebral pathogenicity index in day-old chicks, *** Present study. ** MDT, mean death time (hours) of chick embryos infected with one minimum lethal dose. **** Allan *et al.*, 1978; Alexander & Allan, 1974.

Protective efficacy of the different ND vaccine strains. The B1 vaccine strain groups had higher numbers of survivors and, independently from manufacturer, the protective efficacy varied between 95 and 100 %. Regarding the La Sota vaccine strains, the protective efficacy varied from 90 to 100 % (Table 4).

Table 4 - Challenge results for NDV "São João do Meriti"* strain of SPF birds vaccinated at 7 7 days of a 28th days olddays, use of eye drop by vaccination methods.

Laboratories	Vaccine Strains	N° of chickens		% Protective Efficacy***
		Immunized	MM**	
L1	B1	20	0	100
L2	B1	20	1	95
L3	B1	20	0	100
L4	B1	20	0	100
L1	La Sota	20	2	90
L2	La Sota	20	1	95
L3	La Sota	20	0	100
L4	La Sota	20	0	100
Control	---	10	10	0

* Newcastle disease virus strain "São João do Meriti", ICPI=1.75, IVPI=2.33, MDT=48 hours; **MM: Mortality and morbidity: Number of birds displaying NDV clinical signs or death. ***Percentage of surviving birds that did not display clinical signs of disease. Calculated as: 1-(n. of affected chickens + n. of dead chickens)/n. of immunized chickens X 100.

Considering that their protection ranged from 90

to 100%, all vaccines protected against the challenge. Unvaccinated SPF chickens developed clinical ND clinical signs and/or death, , and 0% protection against ND appeared by the 3rd day after challenge

Relationship between efficacy test and ICPI. The relationship between the efficacy test and the intracerebral pathogenic test (ICPI) of two vaccine strains (La Sota and B1) showed that B1 vaccine strains had an ICPI varying from 0 to 0.19 and its protective efficacy varied between 95 and 100%. La Sota vaccine strains presented an ICPI between 0.02 and 0.37 and protective efficacy from 90 to 100% (Table 5).

Table 5 - Protective efficacy and ICPI of different vaccine strains.

Laboratories	Vaccine strains	Protective Efficacy	ICPI
L1	B1	100	0.03
L2	B1	95	0.13
L3	B1	100	0
L4	B1	100	0.11
L1	La Sota	90	0.23
L2	La Sota	95	0.37
L3	La Sota	100	0.10
L4	La Sota	100	0.02

DISCUSSION

There is little information on the typical biological characteristics (ICPI and MTD) of vaccines used in Brazil. According to Nunes *et al.* (2002), who performed a comparative morphometric analysis of vaccine virulence of NDV lentogenic strains (La Sota, Ulster, and VG-GA), La Sota and Ulster presented the same virulence on the third day after vaccination, and both caused higher swelling of tracheal mucosa than the VG-GA strain. The results obtained with chickens vaccinated with La Sota strains in the present study are consistent with a previous experiment quantifying tracheal swelling (Jorge *et al.*, 1998). Newcastle disease is defined by the OIE (OIE, 2000a, 2004) as an avian infection caused by the avian paramyxovirus serotype 1 (APMV-1), which meets the following criterion for virulence: intracerebral pathogenicity index (ICPI) of 0.7 or greater in day-old SPF chicks (*Galllus gallus domesticus*).

The use of vaccines in the European Community is allowed when the seed vaccine to be tested shows an ICPI <0.4 ($10^7 EID_{50}$ / bird), or <0.5 ($10^8 EID_{50}$ /bird).

The OIE Standard Committee recommends that a vaccine must have an ICPI <0.7 in order to meet the estimated interlaboratory variability. A safety margin is allowed, and the seed strains used for vaccination must have an ICPI ≤ 0.4 (CEC, 1993). These guidelines were adopted by OIE (2000b). The ICPI results for all vaccines used in Brazil, as observed in the present study, complied with the OIE requirements (ICPI ≤ 0.37) relative to nonpathogenic strains.

The main goal of live vaccines is to establish an infection status in a flock, preferably in each bird at the time of application. Individual bird treatments, such as intranasal instillation, eye drop, and beak dipping, are often used with lentogenic vaccines.

The vaccine administration method via eye drop is probably the most effective for live lentogenic vaccines. It ensures that the vaccine reaches individual birds and, consequently, the obtained titers are usually uniform throughout the flock.

The results obtained with the pathogenic NDV strain in 28-day-old SPF birds in the present experiment showed that the challenge was efficient, with results equal to or above the minimum required by the Brazilian legislation (Brazil, 2006).

This study confirms that all live vaccine strains used in Brazil are lentogenic, based on chicken embryo mortality at > 90 hours (Hanson and Brandly, 1955).

In conclusion, it was confirmed that the vaccine strain B1 (ICPI between 0 and 0.13) and the vaccine strain La Sota (ICPI between 0.02 and 0.37) are efficient up to their validity period of 24 months, producing results equal to or above the minimum required by the Brazilian legislation.

The results obtained in the present study showed that the intracerebral pathogenicity index differences among Newcastle disease virus strains do not interfere with the immune response in *Gallus gallus domesticus*.

CONCLUSION

All vaccines used in Brazil were considered nonvirulent, and were classified as lentogenic according to the standards established for immunobiological products. The differences in intracerebral pathogenicity index among Newcastle disease virus strains do not interfere with the immune response in Gallus gallus domesticus.

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CAPÍTULO 3

PREVALENCE OF NEWCASTLE DISEASE VIRUS IN BROILER CHICKENS (Gallus gallus) IN BRAZIL

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Brazilian Journal of Microbiology

Brazilian Journal of Microbiology (2010) 41: 349-357
ISSN 1517-8382

PREVALENCE OF NEWCASTLE DISEASE VIRUS IN BROILER CHICKENS (*Gallus gallus*) IN BRAZIL

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Submitted: June 06, 2009; Returned to authors for corrections: July 23, 2009; Approved: February 17, 2010.

ABSTRACT

This study was carried out during 2002/2003, aiming to determine the prevalence of virulent Newcastle disease virus strains (NDV) in Brazilian commercial poultry farms. Clinical samples were obtained from the Southeastern, Southern and Central-Western regions, which comprise the main area of the Brazilian poultry production. Serum samples and tracheal and cloacal swabs of 23,745 broiler chickens from 1,583 flocks, including both vaccinated chickens and those with no vaccination information, were tested for NDV using a diagnostic ELISA kit. The seropositivity was 39.1%, and the isolation percentage by flock varied from 1.0 to 7.6%, and by region from 6.5 to 58.4%. Higher isolation rates (74.3-83.3%) were obtained after three passages in embryonated chicken eggs. All isolates preliminarily identified as NDV were characterized as nonpathogenic strains, as their Intracerebral Pathogenicity Index (ICPI) was below 0.7. Based on results of this study, Brazil can claim a virulent NDV-free status for commercial flocks.

Key words: Newcastle Disease Virus, NDV-free status, pathogenicity, broiler chickens, biological characterization.

INTRODUCTION

Avian paramyxovirus-1 (APMV-1), the causative agent of the Newcastle Disease (NDV), is classified as a member of genus *Avulavirus* in the family *Paramyxoviridae* (14, 15). Newcastle disease (ND), one of the most important viral diseases in industrial aviculture (3), affects domestic poultry and wild birds and may cause acute mortality marked by hemorrhagic lesions, respiratory and apparent or unapparent enteric infections, among others. Therefore, the etiopathogenic diagnosis should be conventionally based on isolation and biological characterization of field samples (29).

NDV detection and pathotyping of avian isolates are extremely important because the appearance of virulent virus has significant economic consequences related to vaccination and eradication, impairing the ability of a given geographic region to export poultry products (24).

The widespread presence of lentogenic strains in feral

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birds and the use of such viruses as live vaccines mean that isolation of NDV is not enough to confirm a disease diagnosis and compliance with statutory requirements that may be in place (8). Viral characterization using the pathogenicity test or nucleotide sequencing are also required, as the importance and impact of a given NDV isolate are directly related to its virulence. Once analysis of a given field disease solely may be an unreliable measurement of pathogenicity due to the possibility of concurrent infections and bad technical management, laboratory assessments of the virus pathogenicity are necessary. For this purpose, currently three "*in vivo*" tests are available, which include determination of ICPI (Intracerebral Pathogenicity Index), IVPI (Intravenous Pathogenicity Index) and MDT (Mean Death Time) (3).

The World Organisation for Animal Health (OIE) is recognized by the World Trade Organization for standardization of matters related to animal health that may affect international trade. NDV infection is defined as a notifiable disease if the virus in day-old chick (*Gallus gallus*) has ICPI of 0.7 or above, or contains certain multiple basic amino acids at the fusion (F) protein cleavage site (19). APMV-1 that does not meet the OIE definition for causing ND is referred to as a low-virulence APMV-1 or NDV.

The first description of this disease in Brazil occurred in Belém and Macapá in 1953, (22). The outbreak was a consequence of the importation of NDV-contaminated frozen chicken carcasses from the United States (28). The first NDV isolation in Brazil was accomplished by Cunha and Silva (10). This NDV strain has been designated as M33 and its biological characterization was performed by Oliveira et al. (20). After the first impact in the 1950's decade, the Newcastle disease, although endemic, has been seen only sporadically, attacking breeding stocks of small economic expression. Outbreaks have been quickly controlled by vaccination and complementary prophylactic measures.

Poultry is the Brazilian leading export product in the meat sector. The performance of this sector in 2006 consolidated Brazil in the first-position, conquered in 2004, as the world's biggest exporter both in volume and revenue. Behind the soybean complex, poultry ranks second in the Brazilian agribusiness exporting rank (2).

The broiler chicken production in the country is concentrated in the Southern and Southeastern regions (1), where the main producers and exporters of genetic material from chickens are located. Lately the Central-Western region has also experienced a significant expansion.

The nationwide poultry production efficiency makes Brazil a competitive nation in international markets, even in the absence of economic subsidies. Aiming to guarantee better sanitary conditions to Brazilian avian products, an epidemiological project, in agreement with the National Program for Poultry Sanity (PNSA), was implemented for the control of Newcastle Disease in the country.

This study evaluated the prevalence of Newcastle Disease in commercial birds in poultry producing areas in Brazil and the occurrence of the virus in this aviary segment. When pathogenic viruses are absent, the country can be declared free of virulent Newcastle Disease.

MATERIALS AND METHODS

Sample calculation and source

Samples were collected weekly from apparently healthy birds in by official service, in slaughterhouses located in selected areas of nine states of the Southeastern, Southern and Central-Western states. Blood serum of 15 birds per flock and pools of eight cloacal swabs and eight trachea swabs were placed separately in a buffered saline solution (PBS), with antibiotics (10,000 U/ml penicillin, 10 mg/ml streptomycin, 0.25 mg/ml gentamicin and 5,000 IU/ml nystatin), adjusted to pH 7.0-7.4, and cold-stored .

Collected samples were sent to a screening centre in each state to ensure analysis viability, and insertion of data into a computerized information system. The material was sealed up and sent in the thermal ice boxes to the National Agricultural Laboratory (Lanagro/SP) in Campinas, São Paulo, Brazil.

The calculation of the number of samples for the study was based on the total population of birds in each federative state, from a total of 410,729,182 birds in the country, estimated by the Brazilian Ministry of Agriculture. Federative states were selected based on their importance for the Brazilian poultry industry, and comprised three regions: Southeastern (Minas Gerais and São Paulo states) Southern (Paraná, Santa Catarina and Rio Grande do Sul states) and Central Western (Goiás, Distrito Federal, Mato Grosso and Mato Grosso do Sul states).

Assuming a prevalence of at least 1% and a sensitivity of 99% for the detection of at least one infected flock, a minimum of 485 flocks were planned to be analyzed per region. The determination of the number of birds to be sampled by flock took into account the 95% sensitivity of the diagnostic test (ELISA), with an expected minimum prevalence of 30% of infected flocks, with confidence degree of 99%, resulting in 15 chickens per flock.

The number of samples in each region was calculated using the following formula:

$[1 - (1-C)\ 1/\ (D*SENS)]* [M - (/2\ D*SENS-1)]$, where:

C= reliability degree

M = n. of units (animal/flocks) at risk

D = n. of ill/infected units

SENS = sensitivity test

The calculated number of samples was 23,745 broiler chickens (1,583 flocks) being 8,880 birds from 592 farms of the Southeastern region, 7,530 birds from 502 farms in the Southern region and 7,335 birds from 489 farms in the Central-Western region. This project was carried out during 2002/2003, and the period of sample receipt was April 10th to December 30th 2002.

An epidemiological enquiry was performed in all regions where the viral isolation was made, including the identification of counties and their properties. An epidemiological survey was conducted in each positive case to determine the possible sources of NDV isolates.

Detection of NDV antibodies

Chicken serum samples were diluted 1:500 and examined for NDV antibodies by indirect enzyme-linked immunosorbent assay (ELISA), using a commercial ELISA test kit (Flockscreen - Guildhay Laboratories Inc., Guilford, England), run in 96-well microtiter plates containing NDV antigen. The ELISA test was performed according to the manufacturer's recommendations. When at least one bird from a flock was ELISA positive, the whole flock was considered positive.

Virus isolation

Cloacal and tracheal swabs from all ELISA seropositive birds and from 2.5% of the seronegative flocks were submitted to viral isolation. The swabs, stored in transport media composed of phosphate-buffered saline solution (PBS) containing antibiotics, were sent to the National Agricultural Laboratory (Lanagro/SP), Campinas, São Paulo within 48 hrs after collection, in a refrigerated container (2-8ºC). In the laboratory the samples were stored at -80ºC until analyzed. The swabs were pooled and inoculated into five specific-pathogen-free embryonated chicken eggs (9-11 days old), and processed according to standard NDV isolation procedures described by the Regulation #182/94 of the Brazilian Ministry of Agriculture (5). The samples were submitted to three trials in embryonated chicken eggs, before considered negative.

Haemagglunation (HA) and haemagglutination inhibition (HI) tests were carried out according to the technique described in the Regulation # 182/94 of the Brazilian Ministry of Agriculture (5).

Viral identification

NDV was determined using reference antisera APMV-1 to APMV9 by haemagglutination inhibition (HI) test, carried out according to Allan et al (4) and Regulation # 182/94 of the Brazilian Ministry of Agriculture (5). As APMV-5 does not produce haemagglutination, this antiserum was not used in the analyses. The antisera were produced and kindly provided by the Veterinary Laboratory Agency – VLA, located in Weighbridge, Surrey, United Kingdom.

Biological characterization

The pathogenicity of the NDV isolates was determined by measurement of the Intracerebral Pathogenicity Index (ICPI), as described in the Regulation # 182/94 (5). According to the World Organisation for Animal Health (OIE), an IPIC ≥ 0.70 indicates that NDV is pathogenic.

Statistical analysis

Statistical analysis was performed using the Chi-square test or Fisher's exact test (11,23), Differences were considered statistically significant when p≤ 0.05.

RESULTS

The highest prevalence of seropositive samples occurred in Southeastern region (66.4%), followed by Central Western (23.3%) and Southern (22.3%) regions. In the country, the prevalence was 39.1%. The seropositivity and frequency of virus isolation results are shown in Table 1. There was a significant difference in the percentage of seropositive samples in the three regions (p= 0.0001). The average percentage of isolation per flock was 4.9%, being 7.6% in the Southeastern region, 5.4% in the Southern region and 1.0% in the Central-Western region. The Southeastern region presented the highest percentage when compared to the other regions. Considering the total number of isolates, 58.4% of the flocks were from the Southeastern region, followed by 35.1% in Southern region and 6.5% in the Central-Western region.

The number of passages in embryonated eggs required for isolation of NDV is shown in Tables 2 and 3. The isolation percentage in each passage in relation to the total samples is summarized in Table 2, showing that 14.3% of the samples were positive in the 1st passage, 6.5% in the 2nd passage and 79.2% in the 3rd passage. The isolation percentage in each passage of vaccinated birds in relation to total isolates is presented in Table 3, showing 9.5% positivity in 1st passage, 7.1% in 2nd passage and 83.3% in 3rd passage. The isolation percentages from flocks with no information on vaccinal status, in relation to total isolates, were as follows: 20% positivity in 1st passage, 5.7% in 2nd passage and 74.3% in 3rd passage. There was no significant difference in the percentage of isolation by vaccinal status (p=0.4793).

Table 1. Relationship between seropositivity and NDV isolation

Region	Flocks (n)	ELISA Seropositive* flocks %	Isolation		
			(n)	Flocks %	Region %
Southeastern	592	393 (66.4)*	*45*	*7.6*	*58.4*
Southern	502	112 (22.3)	*27*	*5.4*	*35.1*
Central-Western	489	114 (23.3)	*05*	*1.0*	*6.5*
TOTAL	1.583	619 (39.1)	*77*	*4.9*	*100.0*

* The percentage of seropositive flocks in the Southeastern region was significantly higher than that in the two other regions

Table 2. NDV isolates in each passage in embryonated chicken eggs (SPF) in relation to the total number of samples

Passage	NDV Isolates	
	N	%
1st	11	14.3
2nd	05	6.5
3rd	61	79.2
Positive	77	100.0

Table 3. NDV isolates by passages in embryonated chicken eggs, according to vaccination

Passages	NDV isolates (n)*		Isolation / Total	
	Vaccinated	Not informed	% vaccinated	% Not informed
1st	4	7	9.5	20.0
2nd	3	2	7.1	5.7
3rd	35	26	83.3	74.3
Total	42	35	100.0	100.0

*There was no significant difference in the percentage of isolation by vaccinal status (p=0.4793).

The state with highest viral isolation percentage per flock was São Paulo (8.8%), followed by Paraná (7.9%), Minas Gerais (5.3%), Santa Catarina (4.7%), Rio Grande do Sul (2.8%), Mato Grosso do Sul (1.8%) and Mato Grosso (1.0%). Among isolates, 44.2% were from São Paulo state, 19.5% from Paraná, 14.3% from Minas Gerais state, 10.4% from Santa Catarina, 5.2% from Rio Grande do Sul and Mato Grosso do Sul, and 1.3% from Mato Grosso. As shown in Table 6, no virus isolation was obtained in the Federal District and state of Goiás. Figure 1 shows the isolation of NDV by geographic region.

In the pathogenicity characterization of NDV isolates (Table 4), the ICPI ranged between 0 and 0.66. In 41.5% of the NDV isolates, ICPI varied from 0 to 0.10, in 35.1% varied from 0.11 to 0.30, in 18.2% varied from 0.31 to 0.50 and in 5.2% varied from 0.51 to 0.66. These data indicate that none of the isolates was pathogenic, as their ICPI was lower than 0.70. The correlation between ICPI and samples region was not significant (p=0.6792), nor was significant the correlation between ICPI and birds vaccination status.

The grouping of the viruses by ICPI and region is shown in the Table 5. ICPI of vaccine strains are also shown in this table, for comparison. The 1st group, with 41.5% isolates, presented ICPI between 0 and 0.10, similar to vaccinal strains Ulster, VG-GA and V4. The isolates in the 1st group were mainly in the Southeastern regions (68.7%), followed by the Southern (25%) and Central-Western (6.3%) regions. The 2nd group, represented by B1 vaccinal strain (ICPI from 0.11 to 0.30), comprised 35.1% of NDV isolates, being 46.1% in the Southeastern region, 44.4% in the Southern region and 7.4% in the Central-Western region. In the 3rd group, represented by the La Sota and Clone 30 vaccinal strains. comprised 18.2% of isolates (ICPI from 0.31 to 0.50), being 50% in the Southeastern region, 42,9% in the Southern region and 7.1% in the Central-Western region. The 4th group, with no vaccine strain used in Brazil, comprised 5.2% of isolates (ICPI from 0.51 to 0.66), being 75% in the Southeastern region and 25% in the Southern region.

Table 4. Biological characterization of NDV

Region	Vaccination status	Number of isolates	Range of ICPI
Southeastern	+	20	0-0.1
	+	12	0.11 – 0.30
	+	07	0.31-0.50
	+	03	0.51-0.66
Southeastern	-	02	0-0.1
	-	01	0.11-0.30
Southern	-	08	0-0.1
	-	12	0.11-0.30
	-	06	0.31-0.50
	-	01	0.51-0.66
Central-Western	-	02	0-0.1
	-	02	0.11-0.30
	-	01	031-050
Total		77	

Table 5. Grouping of NDV isolates and vaccine strains by ICPI and region

Group	Range of ICPI	NDV isolates		Region %	Vaccine strains
		N	%		
(1)	0.00 to 0.10	32	41.5	68.7 ST 25.0 S 6.3 CW	Ulster, VG-GA, V4, C2
(2)	0.11 to 0.30	27	35.1	46.1 ST 44.4 S 7.4 CW	B_1
(3)	0.31 to 0.50	14	18.2	50.0 ST 42.9 S 7.1 CW	La Sota, Clone 30
(4)	0.51 to 0.66	04	5.2	75.0 ST 25.0 S	Other virus

ST= Southeastern S= Southern CW= Central-Western

Table 6. Percentage of NDV isolates by state

State	Number of flocks surveyed	NDV/flock %	NDV isolates	
			n	%
Minas Gerais	206	5.3	11	14.3
São Paulo	386	8.8	34	44.2
Paraná	190	7.9	15	19.5
Santa Catarina	171	4,7	08	10.4
Rio Grande do Sul	141	2.8	04	5.2
Mato Grosso do Sul	226	1,8	04	5,2
Mato Grosso	98	1.0	01	1.3
Goiás	108	0	0	0
Distrito Federal	57	0	0	0

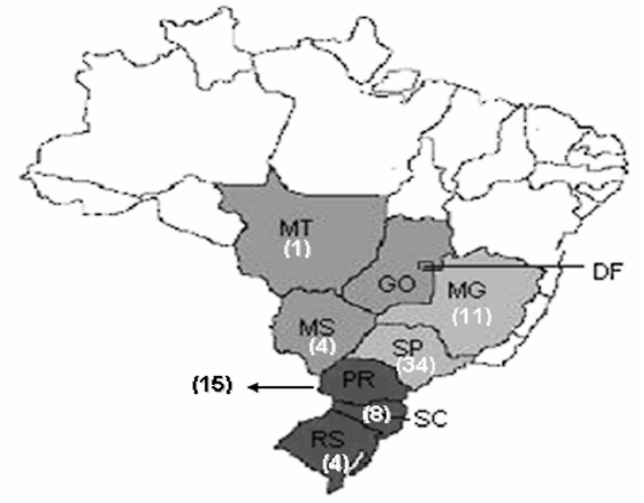

Figure1. Number of Newcastle Disease Virus isolates according to the state (number of isolates in white).

DISCUSSION

The main objective of the present study was to investigate the presence of NDV in commercial healthy birds in the most important geographic areas for Brazilian poultry production and export. Birds vaccination efficacy can be monitored by means of serological tests (18) and a number of serological techniques can be used to detect specific antibodies to NDV. The haemagglutination inhibition (HI) test is the method of choice, but in recent years, several enzyme-linked immunosorbent assays (ELISA) were developed (16,30). Many studies on the sensitivity, specificity and correlation between HI test and ELISA indicate that results may not agree (9,17,28). Commercially available NDV antibody ELISA kits are more sensitive than the HI test and, for diagnostic laboratories, the major advantages of ELISA kits are the assay standardization, the enhanced effectiveness due to semi-automation, and the speed for rapid screening for multiples agents (26).

The percentages of positive samples detected by ELISA were 66.4%, 23.3% and 22.3% in Southeastern, Central-Western and Southern regions, respectively. These results confirm that poultry in the Southeastern region is vaccinated and that in Minas Gerais and São Paulo states and the northern region of Paraná state all categories of commercial birds are vaccinated against Newcastle disease, including broiler chickens, explaining the large number of sample serology reagents of this disease in these states. In addition, these results suggest virus movement from one region to others where no information on vaccination is available. This movement may be a consequence of the high density of birds in some regions, proximity to other species of birds, and also the coexistence of subsistence farms, side-by-side to well developed poultry husbandry.

ND vaccination in broiler chicken is not a practice in the states of Rio Grande do Sul, Santa Catarina, Mato Grosso, Mato Grosso do Sul, Goiás and the Federal District. However, in these states, birds for long life purposes, such as reproduction or egg production, are vaccinated. The vaccine selling maps available in the vaccine producing laboratories (data not shown), and the amount of vaccines sold for veterinary stores during the sampling period suggest that vaccination of broiler chickens was done, including non-industrial husbandry exploration and subsistence birds.

In this study, the frequency of NDV in healthy birds varied from 1.0 to 7.6% per flocks, varying from 6.5% to 58.4% according to the geographic region. The isolation was higher in regions where vaccination is widely used, confirming results reported by Alexander (3), who observed that vaccine protects birds from clinical disease where virus replication and excretion may occur, even though in low levels. Kapcyznski et al., (12), studying exotic Newcastle disease (END) viruses that caused a major outbreak among commercial and backyard poultry in California (USA), observed that vaccines protected chickens against morbidity and mortality and significantly reduced the incidence and viral titers shed from chickens in comparison with sham controls, but did not prevent infection and virus shedding. Vaccinated commercial broilers exhibited 66% mortality and shed significantly more virus than broiler breeders.

A serological study was also conducted in Benin-Africa, in three different regions (Southern, Central and Northern regions), and 56%, 75% and 69% of the chickens were seropositive, respectively, (6, 7). The African results were similar to those obtained in this work. The presence of virus already in the first week of life of the birds, observed in the most regions due to litter reuse, led to the stimulation of the immune system

The highest virus isolation was observed in Southeastern region, followed by Southern and Central-Western regions. This is the first report indicating presence of NDV in regions where vaccinal status is not informed. Results also indicated a higher virus circulation in Southern region than in Central-Western region. The high number of vaccination reported in the Southeastern region certainly correlates with the high number of isolates in the area. Similar prevalence, between 5 and 29%, was found in one small chicken flock and purebred poultry flocks In Switzerland (25).

Seropositivity and virus isolation in states with no vaccination against ND in broiler chickens can be explained by the high density of birds in some regions, the proximity with distinct categories of birds, and the coexistence of subsistence and low-technology farms along with highly technified poultry farms.

A better viral isolation was reached when samples were submitted to three passages in embryonated eggs. Contradictorily, Kouwenhoven (13) observed that 85% of the positive samples were positive in the 1st passage and that only 10% needed a second blind passage. In exceptional cases, three blind passages were needed. The nature of the sample plays an important role in these tests. The two main sites of NDV replication in infected poultry appear to be the respiratory and intestinal tracts; therefore, specimens should always include cloacal and tracheal swabs (3). As pools from both tracheal and cloacal samples were taken from healthy birds with no sign of the disease, it is possible that some harvests occurred during a period of very small elimination of the virus. This may be observed by the low number of isolations in the first and second passages. In most samples, the embryos were not killed in the initial two passages and no lesions were observed, which could be due to low virus content of the inoculum. Therefore, a third passage seems to be necessary for virus adaptation to embryonated eggs.

In 94.8% of the isolates, the ICPI varied from 0.0 to 0.50, which is the range where the Ulster 2 C, V4 Queensland, B1 and La Sota vaccine strains are located (4). This explained by the fact that the most frequently used vaccines in Brazil are La Sota and B1 (21). Based on these results, the isolated NDV strains can be classified as avirulent (lentogenic), although the virus genome has not been sequenced yet.

The isolation of virus with ICPI varying from 0.51 to 0.66 indicates the circulation of non-virulent/apathogenic strains in regions where vaccination status was not informed.

Yongolo (31) found similar results as the author also isolated lentogenic strains from healthy carrier birds.

The results in the present study show that the industrial poultry produced in the nine studied Brazilian states is free of Newcastle disease, which is in accordance to requirements of the International Animal Health Code.

ACKNOWLEDGEMENTS

Authors are grateful to the Brazilian Ministry of Agriculture for providing financial and technical resources and authorization for the publication of this paper. The authors also thank Lanagro/SP, in particular the civil servants at the Poultry Disease Sector and Administration Sectors (computer assistance). Cleide A. M. Silva and Andrea F. Semolini (FCM/Unicamp) for their statistical support. C.W.A. and F.R.S. acknowledge the support from CNPq (National Council of Scientific and Technological Development, Brazil).

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CAPÍTULO 4

A SURVEY FOR MAINTENANCE OF VIRULENT NEWCASTLE DISEASE VIRUS-FREE AREA IN POULTRY PRODUCTION IN BRAZIL

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Brazilian Journal of Microbiology

Brazilian Journal of Microbiology (2010) 41: 368-375
ISSN 1517-8382

A SURVEY FOR MAINTENANCE OF VIRULENT NEWCASTLE DISEASE VIRUS-FREE AREA IN POULTRY PRODUCTION IN BRAZIL

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Submitted: February 03, 2009; Returned to authors for corrections: March 16, 2009; Approved: February 17, 2010.

ABSTRACT

In 2003, Brazil was recognized as a pathogenic Newcastle Disease Virus (NDV) strain-free country for commercial poultry. This research was conducted in Brazil between December 2003 and March 2005 to verify the maintenance of this virulent NDV-free status. Serum samples from 5,455 flocks for commercial poultry farms were collected, comprising 81,825 broiler chickens. The farms were located in nine states of the country, grouped in three geographic regions. Serological evidence of NDV infection was detected in 28.8% of the surveyed farms. However, all fifteen viruses isolated and identified as Newcastle Disease Virus (NDV) were characterized as nonpathogenic strains, based on the Intracerebral Pathogenicity Index. These results showed that Brazil preserves the virulent NDV-free status for commercial flocks.

Key words: Newcastle Disease Virus, pathogenicity, poultry, biological characterization.

INTRODUCTION

Newcastle disease virus (NDV) is a negative-stranded RNA virus of the *Avulavirus* genus within the *Paramyxoviridae family* of the Mononegavirales order (17,18). NDV is also referred to as avian paramyxovirus-1 (APMV-1), one of the nine identified paramyxovirus serotypes known to infect birds, representing one of the most important threats to the poultry industry. Infection in birds can be acute, chronic or asymptomatic and may affect wild and domestic birds, a significant source of protein in developing countries (14). Newcastle Disease (ND) is frequently responsible for devastating losses in poultry production. Spradbrow (23) estimated that in Nepal 90% of poultry dies each year as a result of this disease. NDV infection in poultry ranges from unapparent to rapidly fatal, depending on the virus pathotype (6). Therefore, the ethiopathogenic diagnosis should be based on isolation and biological characterization of field samples (26). Nowadays, the disease has a worldwide distribution with a large rank of hosts. Alexander (3) reported that natural or

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experimental infection comprises over 250 different bird species, although many existing species have never yet been diagnosed. The widespread presence of lentogenic strains in feral birds and the use of such viruses as live vaccines mean that the isolation of NDV is not enough to confirm a disease diagnosis. For confirmation the virus needs to be characterized by pathogenicity tests or nucleotide sequencing. The importance and impact of a given NDV isolate are directly related to the virulence of the viral strain. As field investigation of the disease can be an unreliable measure, laboratory assessment of the virus pathogenicity by the determination of the Intracerebral Pathogenicity Index (ICPI) in day-old chicks *Gallus gallus* should be done. The World Organization for Animal Health-OIE (30) defines ND as a notifiable disease when the virus has an ICPI of 0.7 or above or presents multiple basic amino acids at the F protein cleavage site. An APMV-1 virus which does not meet the OIE definition for causing ND is referred to as a low-virulence APMV-1 or NDV.

In 2003, Brazil was recognized as a country free of pathogenic NDV strains in commercial poultry (19, 31). However, despite the rigorous biosecurity measures adopted by the poultry industry, the risk of reintroduction of viruses into domestic poultry is always present. The present work was conducted to verify whether Brazil maintains the non-virulent Newcastle disease status for commercial flocks.

MATERIALS AND METHODS

Sample calculations and sources

Samples were collected weekly during seven consecutive production cycles of birds in slaughterhouses located in selected areas of nine states of the Southeastern, Southern and Central Western regions of Brazil, corresponding to those where the Brazilian poultry industry is concentrated. Blood serum of 15 birds per flock and pools of eight tracheas and eight cloacae swabs were placed separately in a buffered saline solution (PBS) with antibiotics (10,000 U/ml penicillin, 10 mg/ml streptomycin, 0.25 mg/ml gentamicin and 5,000 IU/ml nystatin), adjusted to pH 7.0-7.4, and cold-stored.

Collected samples were sent to a screening centre in each state to ensure analysis viability, and insertion of data into an computerized information system. The material was sealed up and sent in the thermal ice boxes to the National Agricultural Laboratory (Lanagro/SP) in Campinas, São Paulo, Brazil.

The calculation of the number of samples for the study was based on the total population of birds in each federative state, from a total of 410,729,182 birds in the country, according to the Brazilian Ministry of Agriculture. Federative states were selected based on their importance for the Brazilian poultry industry, and comprised three regions: Southeastern (Minas Gerais and São Paulo states) Southern (Paraná, Santa Catarina and Rio Grande do Sul states) and Central Western (Goiás, Distrito Federal, Mato Grosso and Mato Grosso do Sul states).

The number of samples in each region was calculated using the following formula:

[1- (1-C) 1/(D*SENS)]* [M- (/2 D*SENS-1)], where:

C= Reliable degree

M = n. of units (animal/flocks) at risk

D = n. of ill/infected units

SENS = Sensitivity test

The calculated number of samples was 81,825 broiler chickens, being 27,165 birds in 1,811 farms of the Southeastern region, 28,905 birds of 1,927 farms in the Southern region and 25,755 birds of 1,717 farms in the Central Western region.

Detection of NDV antibodies

Chicken serum samples were diluted 1:500 and examined for NDV antibodies by indirect enzyme-linked immunosorbent assay (ELISA), using a commercial ELISA test kit (Flockscreen - Guildhay Laboratories Inc., Guilford, England), run in 96-well microtiter plates containing NDV antigen. The ELISA test was performed according to the manufacturer's recommendations. When at least one bird from a flock was ELISA positive, the whole flock was considered positive.

Virus isolation

Cloacal and tracheal swabs from all ELISA seropositive

birds and from 30.3% of ELISA seronegative birds were submitted to isolation of virus. The swabs, stored in transport media composed of phosphate-buffered saline solution (PBS) containing antibiotics, were sent to the National Agricultural Laboratory (Lanagro/SP), Campinas, São Paulo within 48 hrs after collection, in a refrigerated container (2-8ºC). In the laboratory the samples were stored at -80ºC until analyzed. The swabs were pooled and inoculated into five specific-pathogen-free embryonated chicken eggs (9-11 days old), and processed according to standard NDV isolation procedures described by Alexander *et al.* (4) and by the Regulation #182/94 of the Brazilian Ministry of Agriculture (13). The samples were submitted to three trials in embryonated chicken eggs, before considered negative.

Virus identification

NDV was identified using reference antisera APMV-1 to APMV-9 by the haemagglutination inhibition (HI) test, carried out according to Alexander (2) and Regulation # 182/94 of the Brazilian Ministry of Agriculture (13). APMV-5 was not used in the analyses as it does not produce haemagglutination. Antisera were produced by the Veterinary Laboratory Agency (VLA) in Weybridge, Surrey, United Kingdom.

Biological pathotyping of the NDV

Pathotyping was based on measurement of the Intracerebral Pathogenicity Index (ICPI) in one day-old chicks from specific-pathogen-free (SPF) parents. In the ICPI test, birds are inoculated intracerebrally and then examined every day for eight days. The birds are scored at each observation: 0- if normal, 1- if sick and 2- if dead. The ICPI is the mean score per bird per observation over the eight-day period. According to the World Organization for Animal Health-OIE (30) an isolate with an ICPI≥ 0.70 should be classified as pathogenic, and the ICPI for live vaccines should not exceed 0.4 (14). These guidelines have also been adopted by OIE (27). ICPI was determined following procedures of the World Organization for Animal Health (30) and the Regulation 182/94 of the Brazilian Ministry of Agriculture (13).

Statistical analysis

Statistical analysis was performed using the Chi-Square or Fisher's Exact test (25). Differences were considered statistically significant when $p \leq 0.05$.

RESULTS

The serology and virus isolation results are shown in Table 1. The highest prevalence of seropositive samples occurred in Southeastern region (44.8%), followed by Southern (23%) and Central Western regions (18.3%). In the country, the prevalence was 28.8%. The average percentage of isolation per flock was 0.27%, being 0.44% in the Southeastern region, 0.23% in the Central Western region and 0.15% in the Southern region. There was a significant difference in the percentage of seropositive samples in the three regions ($p< 0.0001$). The Southeastern region presented the highest percentage when compared to the other regions.

Table 1. Relationship between NDV-seropositivity and NDV isolation

Region	Flocks (n)	Birds (n)	ELISA seropositive flocks %	NDV isolation			
				(n)	ELISA positive %	Flocks %	Region %
Southeastern	1,811	27,165	812 (44.8)*	08	1.0	0.44	53.3
Southern	1,927	28,905	444 (23,0)	03	0.4	0.15	20.0
Central Western	1,717	25,755	315 (18.3)	04	0.9	0.23	26.7
TOTAL	5,455	81,825	1,571(28.8)	15	0.8	0.27	100.0

*The percentage of seropositive flocks in the Southeastern region was significantly higher than that in the other two regions ($p< 0.05$).

The NDV isolation percentages in ELISA positive flocks by region were 1.0%, 0.9% and 0.4% in Southeastern, Central Western region and Southern regions, respectively. Considering the total number of isolates, 53.3% of the flocks were from the Southeastern region, 26.7% from the Central Western region and 20.0% from the Southern region.

The states with higher viral isolation percentage per flock were São Paulo (0.58%) and Mato Grosso do Sul (0.50%), followed by Rio Grande do Sul (0.37%), Minas Gerais (0.16%) and Paraná (0.14%), as shown in Table 2. Among isolates, 46.7% were from São Paulo state, 26.7% from Mato Grosso do Sul state, 13.3% from Rio Grande do Sul state and 6,7% from Minas Gerais and Paraná states. Four states (Mato Grosso, Distrito Federal, Goiás and Santa Catarina) did not present any viral isolate. Figure 1 shows the isolation of NDV by geographic region.

Table 2. Percentage of NDV positive flocks according to region and state

Region	States	Number of flocks surveyed	ELISA positive flocks (%)	Isolation positive flocks (%)
Southeastern	Minas Gerais	620	0.16	6.7
	São Paulo	1,191	0.58	46.7
Central Western	Mato Grosso do Sul	796	0.50	26.7
Southern	Paraná	688	0.14	6.7
	Rio Grande do Sul	542	0.37	13.3

Figure 1. Number of Newcastle Disease Virus isolates according to the geographic region (number of isolates in black)

In the characterization of pathogenic NDV isolates (Table 3), the ICPI ranged between 0.0 and 0.47. In 53.3% of the NDV isolates, ICPI varied from 0 to 0.10, and in 26.6% varied from 0.11 to 0.30. In 20% of the isolates, the ICPI values varied from 0.31 to 0.47. These data indicate that none of the isolates was pathogenic, as their ICPI was lower than 0.70.

The grouping of the viruses by ICPI and region is shown in the Table 4. The ICPI of vaccinal strains is also shown in Table 4 for comparison. The 1st group, with 53.3% of the isolates, presented ICPI 0.0 to 0.10, similar to vaccinal strains Ulster, VG-GA, V4 and C2. The isolates in the 1st group were mainly from Southeastern and Central Western regions (37.5%) followed by Southern region (25%). The 2nd group, represented by B1 strain (ICPI from 0.11 to 0.30), comprised 26.6% of the NDV isolates – 75% in the Southeastern and 25% in the Central Western regions. The 3rd group represented by La Sota and Clone 30 strains, comprised 20% of isolates (ICPI-0.31 to 0.47) – 66.6% in the Southeastern and 33.4% in the Southern regions.

Table 3. Intracerebral pathogenicity index (ICPI) of NDV isolates

Region	Vaccination	Number of isolates	Range of ICPI
Southeastern	+	03	0-0.1
	+	02	0.11 – 0.30
	+	01	0.47
Southeastern	-	01	0.15
	-	01	0.42
Southern	-	02	0-0.1
	-	01	0.36
Central West	-	03	0-0.1
	-	01	0.14
Total		15	

Table 4. Grouping of the NDV isolates and Vaccinal Strains by ICPI

Group	Range of ICPI	Number of isolates	NDV %	Region %	Vaccinal Strain
(1)	0.00 to 0.10	8	53.3	37.5 ST 37.5 CW 25 S	Ulster, VG-GA, V4, C2
(2)	0.11 to 0.30	4	26.6	75 ST 25 CW	B_1
(3)	0.31 to 0.47	3	20,0	66.6ST 33.4S	La Sota, Clone 30

ST= Southeastern, S= Southern CW= Central Western

DISCUSSION

The present study confirms results of a previous report indicating that poultry in the Southeastern region of Brazil is vaccinated against NDV (19), although there are flocks of this region with no information about vaccination. In addition, in the Northern part of state of Paraná, all categories of commercial poultry were vaccinated against Newcastle disease, leading to a large number of serology positive samples in this state. On the other hand, the present study demonstrated that NDV was circulating in regions with no information on vaccination. An important point to consider is the "spreadability" of the live vaccines, given their capacity to immunize individuals other than those individually vaccinated (11). The risk factors associated with seropositive NDV were identified by East *et al.* (16), who demonstrated that age of the flock, proximity to neighbor poultry farms and location of the farm (either in increase Sydney Basin or Eastern Victoria, Australia) were the most important. Several other risk factors in the slaughterhouse were postulated: security level, sanitation of chicken water supply and exclusion of wild and free range birds from chicken housing (12, 16, 25). The risk of seropositive NDV may be attributed to the occurrence and frequency of breaches in security and hygiene rather than to the adopted levels of biosecurity and hygiene (15).

Results show that vaccination against ND in broiler chicken does not occur in the states of Rio Grande do Sul, Santa Catarina, Mato Grosso, Mato Grosso do Sul and Goiás, nor in the Federal District. However, it is known that in these states, birds are vaccinated only when used for long-life purposes, such as egg production.

In this study, NDV was isolated from healthy birds in a frequency varying from 0.15% to 0.44% per flock. The isolation varied from 20.0 to 53.3% according to the geographic region. In a previous study, Orsi *et al* (19) reported a higher frequency of 1.0 to 7.6% per flock, varying from 6.5% to 58.4% according to the geographic region. The isolation was higher in regions where vaccination was widely used. These data are in accordance with Alexander and Gough (6), who suggested that the vaccine protects birds from clinical diseases but replication and virus excretion may still occur, even in lower levels.

The decrease in the number of NDV isolation is evident when results are compared to those reported by Orsi *et al* (19). This decrease in positivity may be attributed to improvements in biosecurity and prohibition of transit of people in farms given the fear/preoccupation caused by avian influenza during this period. Between 2002 and 2005, several outbreaks of influenza virus were extensively reported in the world through the newspapers, TV and OIE communications, leading towards a change in posture and better control of the Newcastle disease virus in Brazil.

A serological study was also conducted in Benin, Africa, in three ecologically different regions (Southern, Central and Northern regions), and 56%, 75% and 69% of the chickens were seropositive, respectively (9; 10). The African results were higher than those obtained in Brazil. Litter reuse observed in most regions can explain the presence of the virus as early as the first week of life of the bird, leading to the stimulation of the immune system.

The highest virus isolation in Southeastern region in Brazil, followed by the Central Western and Southern regions, were also reported in a previous study of Orsi *et al* (19), who detected more virus circulation in the Central Western region if compared to the Southern region. The more effective vaccination in the Southeastern area may correlate with the high number of isolates in this area. These results are in accordance with the history of vaccination, as a prevalence ranging from 5 to 29% was found in one small chicken flock and pure-bred poultry flocks (22).

Serological evidence and viral isolation in states that do not use vaccine against Newcastle Disease in broiler chickens can be explained by the high amount of birds in some regions, by the proximity with distinct categories of birds, and the coexistence low technology farms along with highly technified poultry farms.

The virus isolates in this study presented ICPI below 0.47. This can be attributed to the most frequent use of vaccines B1

and La Sota in Brazil, which present ICPI 0,2 and 0.4, respectively (1,8). The World Organization for Animal Health (30) recommends that vaccine should have an ICPI below 0.7, in order to meet the estimated interlaboratory variability and the required safety margin. Thus, the master seed of live vaccines should not present ICPI exceeding 0.4 (14).

Orsi *et al*. (20) verified that the ICPI values for all vaccines used in Brazil varied from 0 to 0.37. The ICPI values of the isolates in this study are similar to those obtained for vaccines.

Yongolo (32), in Tanzania , also isolated lentogenic and mild virulent NDV from birds with clinical Newcastle disease, as well as from healthy carrier birds.

The results in the present study indicate that biosecurity measures associated with vaccination programs as postulated by the International Animal Health Code are crucial for the preservation of the virulent NDV-free status for industrial poultry in Brazil.

ACKNOWLEDGMENTS

Authors would like to thank the Brazilian Ministry of Agriculture for providing financial and technical resources, and authorization for publication this work coordinated by LANAGRO/SP. They also thank Cleide Aparecida Moreira Silva, from FCM/UNICAMP, for statistical analysis. C.W.A. and F.R.S. acknowledge the support from CNPq (National Council of Scientific and Technological Development, Brazil).

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CAPÍTULO 5

ABSENCE OF HIGHLY PATHOGENIC NEWCASTLE DISEASE VIRUS IN SOME DOMESTIC COMMERCIALIZATION REGIONS OF BRAZIL

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A ser submetido à publicação

ABSENCE OF HIGHLY PATHOGENIC NEWCASTLE DISEASE VIRUS IN SOME DOMESTIC COMMERCIALIZATION REGIONS OF BRAZIL

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ABSTRACT

Newcastle disease virus (NDV) surveillance programs have been conducted in Brazilian geographic areas not involved in chicken meat export. During 2004-2005, clinical samples of 17,265 broiler chickens from 1,151 flocks in the Northern, Northeast, and Southeastern regions were collected. The results showed 84.3% in Northeast, 57.7% in Northern and 41.4% in the Southeastern region. Twelve viruses were found in biological samples from the Northeast region, and they were preliminarily identified as NDV. Complementary, they were characterized as nonpathogenic strains by the intracerebral pathogenicity index (ICPI). These results showed that, at least from samples collected during 2004/2005, Brazil may remain with the virulent NDV-free status.

Keywords: Newcastle disease virus, isolation, broiler chickens, biological characterization, domestic commercialization regions

Short title: NDV in commercial birds in domestic commercialization regions in Brazil

INTRODUCTION

Newcastle disease virus (NDV) is the causative agent of Newcastle disease (ND), being classified in the *Avulavirus* genus of the *Paramyxoviridae* family (13,14). It is also referred to as avian paramyxovirus type 1 (APMV-1), one of the nine identified serotypes of paramyxoviruses known to infect birds. NDV is one of the most important etiological agents of poultry diseases in the world, with great economic importance to the poultry industry (4). It has a wide host range, comprising at least 241 species from 27 of the 50 orders of birds (2). Domestic fowl, pigeons, quail, turkey, pheasants, and guinea fowl are highly susceptible to infection. Many species of wild birds are also susceptible. NDV have also been reported to infect animals other than birds, ranging from reptiles to humans (12).

The increasing disease concern has led to trade restrictions and embargos placed on countries and areas where there are outbreaks. ND is a highly contagious disease that may result in 100% morbidity and mortality in chicken flocks (4). The symptoms seen in infected birds vary widely and are dependent on factors, such as virus pathogenicity, age of the host, coinfection with other organisms, environmental stress, immune status, host species and virus dose (15). The symptoms may include respiratory and/or nervous signs, and lesions are usually observed in the respiratory, digestive and nervous systems; however, sometimes they are seen in other tissues and organs, depending on the tropism of the particular strain.

Detection of NDV and avian pathotype of isolates is extremely important because the appearance of virulent virus has significant economic consequences of vaccination, eradication, in addition to affect the ability of a given geographic region to export poultry products (23).

NDV infection is defined as a notifiable disease if the virus has an ICPI of 0.7 or greater in day-old chick (*Gallus gallus*) or by the presence of certain multiple basic amino acids at the F protein cleavage site by the Animal Health World Organization-OIE (16). APMV-1 strains, which do not meet the OIE definition for causing ND, are referred to as a low-virulence APMV-1 or NDV. Brazil is considered free from NDV infection (18), and serological tests are mainly conducted on commercial poultry flocks in areas which are intended to support chicken meat export, namely Southern and Southeastern regions. The objective of this research was to verify which are the pathotypes of NDV circulating in poultry raised in commercialization areas in Brazil.

MATERIALS AND METHODS

Sample calculation and sample source

Samples were collected weekly during seven consecutive production cycles of birds in slaughterhouse located in selected areas of four states of States of the Northern, Northeast and Southeastern regions of Brazil, which do not bear the main activity of Brazilian poultry industry by official federal meat inspection service. Blood serum of 15 birds per flocks and pools of eight tracheal and eight cloacal swabs were placed separately in a buffered saline solution (PBS) with antibiotics, (10,000 U/ml penicillin, 10 mg/ml streptomycin, 0.25mg/ml gentamicin and 5,000 IU/ml nystatin), adjusted to pH 7.0-7.4, and cold-stored.

Collected samples were sent to a screening center in each state to ensure analysis viability, and insertion of data into an computerized information system. The material was sealed up and sent in iced thermal boxes to the National Agricultural Laboratory (Lanagro/SP).

The calculation of the number of samples for the study was based on the total population of birds in each federative state, from a total of 410,729,182 birds in the country, estimated by the Brazilian Ministry of Agriculture. Federative states were selected based on their importance for the increase to Brazilian poultry industry, and comprised in three regions: Northern region (Tocantins-TO), Northeast region (Bahia and Sergipe), and Southeastern region (Espírito Santo).

Assuming a prevalence of at least 1% and a sensitivity of 99% for the detection of at least one infected flock, a minimum of flocks were planned to be analyzed per region depend of the size of the local aviculture.

The determination of the number of birds to be sampled by flock took into account the 95% sensitivity of the diagnostic test (ELISA), with an expected minimum prevalence of 30% of infected flocks, with confidence degree of 99%, resulting in 15 chickens per flock.

The number of samples in each region was calculated using the following formula:

[1- (1-C) 1/ (D*SENS)]* [M- (/2 D*SENS-1)], where:

C= reliability degree

M = n. of units (animal/flocks) at risk

D = n. of ill/infected units

SENS = sensitivity test

The calculated number of samples was 17,265 broiler chickens (1,151 flocks), being 390 birds of 26 flocks in the Northern region (Tocantins-TO), 12,750 birds of 850 flocks in the Northeast region (Bahia and

Sergipe), and 4,125 birds of 275 flocks in the Southeastern region (Espírito Santo). This project was carried out during May 21st 2004 and January 5th 2005).

An epidemiological enquiry was performed in all regions where the viral isolation was made, including the identification of counties and their properties. An epidemiological survey was conducted in each positive case to determine the possible sources of NDV isolates.

Detection of NDV antibodies

Chickens serum samples were diluted 1:500 and examined for NDV antibodies by indirect enzyme-linked immunosorbent assay (ELISA), using a commercial ELISA test system (Flockscreen-Guildhay Laboratories Inc. Guilford, England), run in 96-well microtiter plates containing NDV antigen. The ELISA test was performed according to the manufacturers' recommendations. When at least one bird from flocks was ELISA positive, the whole flocks was considered positive

Virus isolation

Tracheal and cloacal swabs from all ELISA seroposive birds were submitted to isolation of virus. The swabs, stored in transporte media composed of phosphate- buffered saline solution (PBS) with antibiotics, were sent to the National Agriculture Laboratory (Lanagro/SP),Campinas, São Paulo within 48 hrs after collection , in a refrigerated container (2-8º C). In the laboratory the samples were stored at -80º C until analyzed. The swabs were pooled and inoculated into five embryonated specific-pathogen-free chicken eggs (9-11 days old), and processed according to standard NDV isolation procedures described by Regulation # 182/94 of the Brazilian Ministry of Agriculture (6), following international standards. The samples were submitted to three trials in embryonated chickens eggs, before considered negative

Haemagglunation (HA) and haemagglutination (IH) inhibition tests

The HA and HI assays were completed by microtiter methods. The HA assay of allantoic fluids harvested from inoculation of embryonated eggs was used to identify NDV-positive embryos. The initial characterization of isolates was performed using the hemagglutination inhibition test with NDV-specific polyclonal antisera (3, 6).

Viral identification

NDV was identified using reference antisera APMV-1 to APMV-9 by haemagglutination inhibition (HI) test, carried out according Regulation # 182/94 of Brazilian Ministry of Agriculture (6). Since APMV-5 was not used in the analyses as it does not produce haemagglutination. Antisera were produced by the Veterinary Laboratory Agency-VLA, located in Weybridge, Surrey, United Kingdom.

Biological characterization of NDV (“*in vivo* test”)

For pathotyping, the Intracerebral Pathogenicity Index (ICPI) in one day-old chicks from specific pathogen free (SPF), parents was measured. In the ICPI test, birds are inoculated intracerebrally and then examined every day for eight days. The birds are scored at each observation: 0-if normal, 1-if sick and 2-if dead. The ICPI is the mean score per bird per observation over the eight-day period. According to the manual of standards by the World Organization for Animal Health (3, 4, 6). The World Organization for Animal Health (OIE) defines pathogenic Newcastle disease viruses with ICPI≥ 0.70.

Statistical analysis

Statistical analysis was performed using the Chi-square test (21). Differences were considered statistically significant when $p \leq 0.05$. The estimates of the common relative risk were obtained following Severino, Richard, 2000 (22).

RESULTS

The serology and virus isolation results are shown in Table 1. The highest prevalence of seropositive samples occurred in Northeast region (84.3%), followed by Northern (57.7%) and Southeastern region (41.4%). There was a significant difference of percentage for positivity among regions ($p< 0.0001$).
The common relative risks were estimated only for two states (Espírito Santo-ES and Sergipe-SE), which have completed data, are shown in the Table 2. In the ES state the odds ratio of occurring flocks seropositive in the no vaccined flocks is 3.0 (0.34-26.8) times greater than the vaccined

Table 1- Relationship between NDV-seropositivity, Vaccinal status and NDV isolation

Region	Flocks (n)	Number of birds	ELISA Seropositive * Flocks	Status		Vaccina		NDV Isolation	
				Yes	No	NI	N	Flocks	Region
Northern	26	390	15 (57.7)	0	0	26	0	0	0
Northeast	850	12,750	717(84.3)*	825	4	21	12	1.4	100
Southeastern	275	4,125	114(41.4)	223	4	48	0	0	0
	1,151	**17,265**		**1,048**	**8**	**95**	**12**	**1.0**	**100**

* The percentage of seropositive flocks in the Northeast region was significantly higher than that in the two other regions. Yes –Vaccined, No- Not vaccined , NI- not informed.

flocks, the relative risk seropositive incidence given the flocks was not vaccined is 1.5 (0.67-3.38) times of the outcome in the vaccined flocks and the ratio of the incidence of seropositive flocks given the vaccined flocks is 0.50 (0.13-1.96) times of the outcome in the no vaccined flocks.

In the SE state the odds ratio of occurring flocks seropositive in the vaccined flocks is 2.4 (0.22-25,94) times greater than the no vaccined flocks, the ratio of the incidence of seropositive flocks given the flocks was vaccined is 1.24 (0.68-22.45) times of the outcome in the no vaccined flocks. The ratio of the incidence of seropositive flocks given the vaccined flocks is 0.52 (0.09-3,18) times of the outcome in the no vaccined flocks.

Every seropositive flock was submitted to viral isolation. Twelve viral isolates from the Northeast region – only in the State of Bahia – showed 100% of positivity. The percentage of isolates by sampled flocks was 1.4%, and in the whole country was 1.0%.

Table 3 shows virus isolation percentages in regions and states; Bahia was the state which all isolates showed viruses (100%), and the percentage of NDV by flocks was 1.7%. Three states (Tocantins, Sergipe and Espírito Santo) did not present any viral isolate. Twelve viruses were isolated from seven municipal districts of Bahia, as follows: four isolates from one municipal district, three from another one and one from each of the other districts.

The virus was identified as Newcastle disease virus using the Intracerebral Pathogenicity Index (Table

Table 2 - Estimates of the Common Relative Risk of Southeastern and Northeast (Sergipe)

Vaccinal status	Southeastern (ES)			Vaccinal status	Northeast – Sergipe (*)		
	Elisa(+)%	Elisa(-)%	Total%		Elisa(+)%	Elisa(-)%	Total%
No	3(75.0)	1(25.0)	4(100.0)	Yes	85(82.5)	18(17.5)	103(100.0)
Yes	94(49.7)	95(50.3)	189(100.0)	No	2(66.7)	1(33.3)	3(100.0)
total	97(50.3)	96(49.7)	193(100.0)	Total	87(82.1)	19(17,9)	106(100.0)

Estimates of the Common Relative Risk	value	95% Confidence Bounds		Estimates of the Common Relative Risk	value	95% Confidence Bounds	
		Lower limit	Upper Limit			Lower limit	Upper Limit
OR	3,00	0,34	26,81	OR	2,36	0,22	25,94
RR1	1,51	0,67	3,38	RR1	1,24	0,68	22,45
RR2	0,50	0,13	1,96	RR2	0,52	0,09	3,18

OR=odds ratio
RR1=relative risk seropositive
RR2=relative risk seronegative

4), the ICPI ranged between 0.0 and 0.54. In 91.7% of the NDV isolates, ICPI varied from 0 and 0.40, and 8.3% varied from 0.40 to 0.54. These data indicate that none of the isolates was pathogenic, as their ICPI was lower than 0.70. The virus was identified as Newcastle disease virus using the Intracerebral Pathogenicity Index (Table 4), the ICPI ranged between 0.0 and 0.54.

Table 3- Percentage of NDV positive flocks (isolation) according to region and States

Region	States	Number of Flocks surveyed	NDV/Flocks%	Isolation Positive flocks (%)
Northern	Tocantins	26	0	0
Northeast	Bahia	723	1.7	100
	Sergipe	127	0	0
Southeastern	Espírito Santo	275	0	0
Total		**1,151**	**1.0**	

In 91.7% of the NDV isolates, ICPI varied from 0 and 0.40, and 8.3% varied from 0.40 to 0.54. These data indicate that none of the isolates was pathogenic, as their ICPI was lower than 0.70.

Table 4- Intracerebral pathogenicity index (ICPI) of NDV isolates

Region	Vaccination	Number of Isolate	Range of ICPI
Northeast	+	02	0,0-0,10
	+	06	0,11-0,20
	+	02	0,21-0,30
	+	01	0,31-0,40
	+	01	0,41-0,54

+Vaccined

DISCUSSION

The Brazil consolidated the first-position conquered in 2004 as the world's biggest exporter both in volume and revenue, and the third-position in production of chicken meat. In Brazilian agribusiness, the chicken meat also occupies the second place in export ranking, behind soybean complex.

The country has wide arable land, favorable weather conditions and workmanship more accessible than in other countries. In addition, its technical and management conditions allow the development of the agricultural activity, making the country the world's biggest producer of broiler chickens, and responsible for helping to solve the animal protein hunger in the world. Newcastle disease outbreaks usually interrupt the international trade of poultry meat and byproducts; therefore, Newcastle disease outbreaks may a severe economic impact on the Brazilian poultry production and the national economy.

In 2003 and March 2005, Brazil was recognized as a country free of pathogenicity NDV strains in commercial poultry (18, 26). However the level of seropositivity and the presence of Newcastle disease virus in areas poultry raised in domestic commercialization areas was unknown. The main objective of the present study was to investigate which are the pathotypes of NDV circulating in poultry raised in domestic commercialization areas of Brazilian poultry production.

Serological tests for NDV may be used to demonstrate virus infection or for monitoring vaccination (17). A number of serological techniques used for the detection of antibodies have been applied to NDV. The HI test is the method of choice; however, in recent years, several enzyme-linked immunosorbent assays (ELISA) were developed (25). Studies on the sensitivity, specificity and correlation between HI test and ELISA test produced variable results (9).

Positive samples were detected by ELISA test, with a percentage of 84.3%, 57.7% and 41.4% in Northeast, Northern and Southeastern, respectively. Similar results were found by Bell (7, 8), when conducting serological study in Benin, Africa, and by Orsi et al. (20), when verifying the maintenance of virulent NDV-free status for commercial poultry farms – the prevalence occurred in Southeastern was similar (44.8%); however, lower result was observed in the Southern (23%) and 18.3% in the Central Western regions. Another study (24) reported lower prevalence (between 5 - 29%) in small chicken flocks and pure-bred poultry flocks; higher prevalence (66.4%) in the Southeastern was obtained in another research (18). This result confirms that Southeastern poultries were vaccinated, independent of the State (Minas Gerais, São Paulo or Espírito Santo).

The early stimulation of the immune system can be obtained by the bed reuse observed in the regions, and this may justify virus already present in the first week of life of the birds. This confirms that all categories of commercial birds were vaccinated against Newcastle disease, justifying a larger number of seropositive samples. This fact can also be justified by the proximity to other species of birds, and also the existence of subsistence farms, side by side with well-developed poultry husbandry.

In this study, we isolated NDV in healthy birds with a frequency from 0 to 1.7% for flocks and from 0% to 100% by geographic region; similar results were obtained by Orsi et al (20) with the percentage of isolation per flocks of 0.44% and 53.3% in the Southeastern region. However, another research by Orsi et al (18) resulted in 7.6% per flock and 58.4% in the Southeastern region. This study was conducted before the dawn of the Influenza H5N1 era and biosafety restriction measures related , when was frequent the occurrence of breaches in security and hygiene rather than the underlying levels of biosecurity and hygiene used (10).

The isolation rate was higher in regions where vaccination is widely used. According to Alexander et al. (4), vaccination protects birds against clinical disease; however, the virus replication and excretion may still occur, even though in lower levels. In study conducted by Kapcyznski & King (11) using live and inactivated vaccines showed that those immunogens may reduce but not prevent infection and spread of exotic NDV – vaccinated commercial broiler presenting exotic NDV infections exhibited 66% mortality, and a significant increase in virus shedding may be observed in broiler-breeders.

Viral isolates were identified as Newcastle disease virus and their biological characterization showed intracerebral index range between 0.0 and 0.54, as shown in Table 3. From the isolates obtained, 91.7% had ICPI from 0.0 to 0.40, identifying the vaccine strains - Ulster 2 C, V4 Queensland, B1 and La Sota (5), this result is similar to that obtained by Orsi et al (20). The most frequently used vaccines in Brazil are La Sota and B1, which present ICPI levels of 0.4 and 0.2, respectively (1, 5). It was observed that 16.7% of the isolated viruses varied from 0 to 0.10. The higher amount of isolated viruses was found in the range of ICPI=0.11 to 0.20 (50%), identifying B1 vaccine strains, which indicate reisolation of B1 vaccine strain,

confirming vaccine history in Bahia. These results showed 25% of reisolation of other types of strains, with ICPI values from 0.21 to 0.40 , identifying La Sota vaccine strains.

Strains (8.3%) with ICPI range from 0.40 to 0.54 showed that in regions with vaccine history there was circulation of non-virulent / apathogenic strain with different ICPI. According to a study performed by Orsi et al. (19) observed that the ICPI profiles obtained for all vaccine strains used in Brazil varied from 0 to 0.37.

The data presented in this study show that none of the 12 isolated samples were pathogenic, with ICPI < 0.70; similar results were obtained by Orsi et al (18, 20). The results obtained in the present study showed that non-export industrial poultry from the four studied Brazilian federative units nonpathogenic Newcastle disease virus was found, as established in the standard postulated by the International Animal Health Code.

ACKNOWLEDGEMENTS

We would like to thank the Brazilian Ministry of Agriculture – Livestock and Food Supply – for authorizing publication of this paper. We are especially grateful to the coordinations of Lanagro/SP, General Laboratory Support, Sanitary Programs and Animal Sanitary Defense Animal. We would also like to thank the civil servants for the Poultry Disease Sector, in special to Adriana M. R. Catharino, Fulvia Di Pillo, Maria L. J. Nascimento and Carla V. O. Bronzato (for their expert technical support), Luiz Carlos da S. Porto for animal care assistance, and Berlina M. S. Bordoni for IT assistance. Finally, our sincere thanks to the employees at the Research Commission (FCM-Unicamp), Cleide A. M. Silva, for her statistical support, and Etna Lima Macário, for collaborating with the English review.

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CAPÍTULO 6

MOLECULAR CHARACTERIZATION OF FIELD ISOLATES OF NEWCASTLE DISEASE VIRUS AND VACCINE STRAINS USED IN BRAZIL

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À ser submetido

MOLECULAR CHARACTERIZATION OF FIELD ISOLATES OF NEWCASTLE DISEASE VIRUS AND VACCINE STRAINS USED IN BRAZIL

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Keywords: Newcastle disease virus, live vaccine, fusion gene, NDV circulating in Brazil

Short title: Molecular characterization of NDV and vaccine in Brazil

Abstract

Isolates of Newcastle disease virus (NDV) obtained during an official surveillance, conducted on different geographic areas of poultry production were genotyped, as well as NDV live vaccines marketed in Brazil, Genomic fragments were amplified by reverse transcription polymerase chain reaction (RT-PCR), followed by partial sequencing of the fusion protein gene. Deduced amino acid sequences were inferred and sequences were phylogenetically compared with those from strains previously reported in other geographic regions. Our results showed that all Brazilian isolates collected during 2002-2005 (n=39) have amino acid motifs ^{112} GKQ↓GRL^{117} (11) and ^{112}GRQ↓GRL^{117} (26 conserved, thus belonging to the non-virulent NDV type.. There were two virus (n=2) (BR-1568, BR-6225) from which a substitution at position 116 – from arginine (R) to alanine (A) ^{112}G-K-Q↓G-A-L^{117} – and another one at position 114 – from glutamine(Q) to threonine (T) ^{112}G-R-T↓G-R-L^{117} were observed. Phylogenetic analysis showed that all isolates (39) and vaccines (11) used in Brazil clustered within I and II genetic groups; thus being classified as avirulent viruses, according to the amino acid sequence at the fusion protein cleavage site. The main findings include the absence of virulent NDV circulating in our country and the fact that isolates are highly similar to the live vaccine strains currently marketed. One may consider the solely circulation of live vaccine strains or derived strains from either La Sota or Ulster type vaccines.

1. Introduction

Newcastle disease (ND) caused by Newcastle disease virus (NDV) is one of the most serious poultry disease causing large economic impact in many developed countries (Leslie, 2000). ND outbreaks are not only extremely costly, but control measures like vaccination, and have a major negative economic impact, since they lead to trade restriction of animals and their products (Alexander, 2003) and embargos on countries and areas where outbreaks occur (Aldous et Alexander, 2001).

NDV is a member of the *Paramyxoviridae* family and has been placed in the *Avulavirus* genus (Mayo, 2002) The viral single-stranded-RNA genome has approximately 15.586 kb in length (Phillips et al, 1998). Six proteins are produced from RNA genome: hemagglutinin-neuraminidase (HN), fusion (F), nucleocapsid (N), matrix (M), phosphoprotein (P) and large RNA-directed RNA polymerase (L) (Alexander, 2003). The primary molecular determinant for NDV pathogenicity is the presence of multiple basic amino acid residues at the F protein cleavage site; therefore, the sequence analysis of the F protein cleavage site can aid in pathotype prediction and prediction of viral virulence (Liu et al., 2007).

Various methods used to identify and analyze NDV include examining differences in pathogenicity, antigenicity, and genome sequences (Liu et al, 2007), but differential monoclonal antibody binding tests may fail when viruses are closely antigenic similar (Aldous et al, 2003). However, nucleotide sequence analysis may allow the differentiation of highly related isolates, thus resulting in a promising epidemiological tool (Alexander et al, 1999).

The *Paramyxoviridae* fusion protein is mainly secreted as a non-functional precursor (F_0), which is post-transcriptionally cleft on the functional subunits F1 and F2 by host proteases, then allowing the virus particles to penetrate after fusion of the viral envelope with the cell membrane (Glickman et al., 1988).

The World Organisation for Animal Health (OIE) defines the virulence of NDV or Newcastle disease based on the intracerebral pathogenicity index ($\geq$0.70) or presence of multiple basic amino acids at the F protein cleavage site (OIE, 2006). Virulent strains have a multibasic amino acid, and viruses of low virulence have a singular amino acid sequence (^{112}GRQGRL^{117}) at the F0 cleavage site (Aldoux et al., 2003).

Phylogenetic analysis revealed two distinct classes; class I and class II viruses, consisting of at least nine genotypes (Kim at al, 2007, Liu et al., 2007). The class II viruses have been predominantly isolated from domestic fowl (Alexander, 1995; Aldous, et al, 2003) and responsible for several major panzootics of ND in poultry worldwide (Alexander, 1988). The lentogenic viruses from classes I and II isolated

from waterfowl, live bird markets and shorebirds (Kaleta and Baldauf, 1988; Seal et al, 2005 and Kim et al, 2007).

Phylogenetic studies point to at least eight genogroups (I-VIII) of NDV that were described according to the isolation host of, geographical or/and temporal restrictions, and virulence (Lomniczi et al., 1998; Alexander et al, 1999; Herczeg et al,1999; Yang et al., 1999; Herczeg, 2001; Ke et al., 2001; Yu et al., 2001, Liang et al.,2002). However, Alexander et al (1997) classified the lineages using the corresponding monoclonal antibody-binding groups. Recently, the genetic grouping of ND was revised, and six lineages (1 to 6) were described. The first two genetic groups (I-II) are genetically associated with the widely used vaccine strains La Sota and Ulster.

Many countries perform vaccination with live and killed vaccines as a prevention practice. The prophylaxis of Newcastle disease in broiler chickens in Brazil is focused on the surveillance of ND in domestic and wild birds and active immunization by using live lentogenic vaccines, such as LaSota, B1, Ulster, Clone 30 , VG-GA and C2 strains, all considered non-virulent and classified as lentogenic (Orsi et al.,2009a).

The first description of this disease in Brazil was in 1953 (Santos et al., 1954) and the first isolation was accomplished by Cunha and Silva (1955). A serological and virological survey conducted in samples collected in 2002 showed that Brazil remains with the virulent NDV-free status for commercial flocks (World Trade Organization, 2005, Orsi et al, 2010a). From December 2003 to March 2005, the same condition was observed in a second survey (Orsi et al, 2010b), and similar results were obtained for non-export geographical regions during the years 2004 and 2005 (Orsi et al, 2010c; in press).

A complete epidemiological analysis of NDV isolates in Brazil has not been conducted and the genetic relatedness between the Brazilian NDV isolates and the domestic or wild bird isolates in other countries is unknown so far. In order to define the molecular epidemiology of NDV circulating in Brazil, NDV isolated from healthy broiler chickens from different geographic regions of export and non-export areas during 2002 to 2005 were characterized molecularly, and its possible relationship to different types of live vaccines used is discussed at the present work.

2. Materials and methods

2.1. Virus Isolates

APMV-1 viruses strains used in this research were obtained by the National Agricultural Laboratory in Campinas (Lanagro/SP), the National Reference Laboratory for Poultry in Brazil. RT/PCR were

performed and thirty-nine positive samples were selected to be sequenced out of the one-hundred-four viruses (37.5%), (This virus coming from three surveyed programs was conducted in Brazil in order to determine the prevalence of virulent Newcastle disease virus strains (NDV) in commercial poultry farms. The **1st** was realized in 2002/2003, samples of 23,745 broiler chickens from 1,583 flocks was used and taken from the Southeastern, Southern and Central-Western regions, 39.1% of seropositivity was detected and were isolated 77 viruses, identified as NDV with intracerebral pathogenicity index (ICPI) varying between 0.0 and 0.66 by Orsi et al, 2010a. The **2nd** was conducted between Dec 2003 and March 2005 the samples from 5,455 flocks were collected, comprising 81,825 broiler to verify the maintenance of virulent NDV-free status for same states and regions of the 1st surveyed, Serological evidence of NDV infection was found in 28.8% of the farms surveyed and fifteen viruses were isolated and identified as NDV and ICPI showing the index 0.0 and 0.47 by Orsi et al, 2010b. The **3rd** NDV surveillance programs have been conducted 2004-2005, and samples of 17,265 broiler chickens from 1,151 flocks in the Northern, Northeastern, and Southeastern regions were collected, in Brazilian geographic areas not involved in chicken meat export, the results showed 73.3% of seropositivity on overall poultry flocks. Twelve viruses were found and ICPI varying between 0.0 and 0.54 by Orsi et al., 2010c (in press). These viruses presented different ICPI (ranging from 0 to 0.66), and represented all the different states and regions sampled where the viruses were isolated. The details of Newcastle disease virus isolates in broiler chickens used in this study were shown in table 1.

Table 1. Details of Newcastle disease virus isolates in Broiler chickens used in this study.

NDV strain*	Region of Brazil	ICPI	Vaccine used
BR-728	Southeastern	0.05	B1
BR-730, BR-732	Southeastern	0	B1
BR-733	Southeastern	0.06	B1
BR-734	Southeastern	0	B1
BR-766	Central-Western	0	WI
BR-941	Southern	0.22	WI
BR-942	Southern	0	WI
BR-943	Southern	0.30	WI
BR-944	Southern	0.12	WI
BR-945	Southern	0.66	WI
BR-982	Central-Western	0.08	WI
BR-1035	Southeastern	0.14	B1
BR-1052	Southeastern	0.58	B1
BR-1103	Southern	0.48	WI
BR-1220, BR-1223	Southeastern	0.50, 0.48	+
BR-1321	Southeastern	0.48	B1
BR-1374	Southern	0.14	WI
BR-1524	Southeastern	0.61	B1
BR-1545	Southeastern	0.10	B1
BR-1547	Southeastern	0.48	B1
BR-1568	Southeastern	0.15	B1
BR-1582	Southeastern	0.60	B1
BR-1612	Southern	0.15	WI
BR-4961	Southeastern	0	B1
BR-4980	Central-Western	0.10	-
BR-5070	Southeastern	0.15	-
BR-5100	Southeastern	0.02	WI
BR-5818	Southern	0.04	-
BR-6424	Southern	0.36	WI
BR-6425	Southeastern	0.47	B1
BR-7538, BR-7549	Northeast	0.20 , 0.25	B1
BR-7540	Northeast	0.06	B1
BR-7550	Northeast	0.37	B1
BR-7958, BR-8486	Northeast	0.14, 0.11	B1
BR-8487	Northeast	0.21	B1

Orsi et al., 2010 a, b; Orsi et al., c (in press)

. Strains =B1, - not vaccined; WI- without information; + vaccined

The original infective allantoic fluids were diluted 10^{-1} to 10^{-2} and passed once again in embryonated eggs in this laboratory, before they were used in molecular studies.

2.2. Virus vaccines

Eleven commercial lyophilized vaccines, manufactured in Brazil or imported, prepared with strains (B1, La Sota, Ulster 2C, Clone 30, VG-GA and C2) were used. The titre was determined by the same technique described for thermostability titration (Simi et al, 1970; Orsi et al., 2009b) and varied from $10^{5.50}$ to $10^{6.70}$, following manufacture information. Vaccine vials were reconstituted according to the manufacturer's instructions and strains were propagated in SPF embryonated chicken eggs.

2.3. Vaccine virus propagation in SPF embryonated chicken eggs

Vaccine strains were replicated by inoculation of SPF embryonated eggs (9-11 days old) via allantoic cavity. Embryos dead until 24 hours were discarded and the others sampled and submitted to hemagglutination assay (HA) soon after the end of the observation period was carried out, according to the technique described in Regulation # 07/06 (Brazil, 2006). The virus containing fluids were pooled and conserved at – 80ºC until use.

2.4. Viral RNA extraction and RT-PCR

For RNA preparation, 200 ul of infected allantoic fluids were used and viral RNA was extracted using a high pure viral nucleic extraction kit™ (Roche Diagnostic™, Manheim, Germany), as the manufacturer's recommendations. cDNA was synthesized using the high-capacity cDNA kit (Applied Biosystenms™, Foster City, USA), according to the manufacturer's instructions. NDV viral genomic fragments were amplified using primers (MSF1: 5´-GACCGCTGACCACGAGGTTA-3´, 2: 5´-AGTCGGAGGATGTTGGC AGC- 3´) reported by Aldous et al (2003) for the amplification of 723 pb fragment F protein gene. The RT-PCR reaction mixture was prepared and contained 1X reaction buffer, 0.2 mM each of dATP, dCTP, dGTP, dTTP, 5 pmol of sense and antisense primer, 1mM of MgSO4, 1U (5U/µl) of reverse transcriptase. The RT-PCR was performed with uninterrupted thermal cycling, using a Gene Amp PCR system 9700 (Applied Biosystems, USA), with the following program: 94ºC for 2 min, (35 cycles of 94ºC for 30 seconds, 58ºC for 30 seconds, 72ºC for 1 minute), and a final extension at 72ºC for 7 minutes. Two microliters of RNA from each sample were used for RT-PCR amplification. The PCR

products were analyzed by electrophoresis on a 1% agarose gel stained with ethidium bromide and viewed under UV light. .

2.5. Sequencing of PCR products

PCR products were sequenced three times each, both on forward and reverse direction, using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems™, Foster City, USA). The selection was made by different ICPI and represented all the different states, regions, and time in which the viruses were isolated. Thirty-nine virus sequences were obtained with enough quality for further analysis.

2.6. Phylogenetic relationship

Phylogenetic analyses of NDV isolates characterized in this study, showing in table 1 nucleotide sequences from NDV strain F gene reference, were retrieved from the GenBank database (http://www.ncbi.nlm.n/ih.gov/genbank/ index.html), including sequences obtained from viruses circulating in the USA (AF309418), Taiwan/China (AY372142, AY372141, AY372143), China (DQ195265), Slovakia (EF364036, EF035485), Barbados (EF110539), Russia (AY972103, AY972102, AY972101), N. Ireland (AY562991), (Argentina (AY734535, AY734536), United Kingdom (AY471835), Italy (AJ880277), Hungary (AY471834), China (EF589137, DQ417113, AY635814), South Africa (EF030959, EF030960, EF030961) in table2.

Bioedit package, version 7.0.5.2 (Hall, 1999), was used to manipulate nucleotide and amino acid retrieved sequences. Sequence alignments were performed using the Clustal W software, version 1.83 (Thompson et al., 1994).

The evolutionary history was inferred using the neighbor-joining method (Saitou & Nei, 1987). The optimal tree with the sum of branch length = 1.75758047 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches (Felsenstein, 1985). Evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980), and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 206 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

3. Results

3.1. Proteolytic cleavage site of F_0 protein

RT-PCR using primers (Aldous et al, 2003) resulted in amplification of 723 bp products (figure 1). Nucleotide sequences were available in GenBank with the accession numbers (table 2). The 723 bp fragment between nucleotides of the fusion gene was amplified, including the fusion protein cleavage site, and the sequence was analyzed (fig. 1). Nucleotide similarities of 723 bp and predicted amino acid sequence of residues of Brazilian isolates were compared with corresponding sequences of representative strains.

Cleavage site motifs of 39 NDV strains are listed in table 3. The cleavage site of Brazilian viruses showed motifs ^{112}GKQ↓GRL^{117,} ^{112}GKQ↓G**A**L^{117}, ^{112} GRQ↓GRL^{117} and ^{112} GR**T**↓GRL^{117}. Among cleavage-site motifs of low virulence and avirulent strains, twenty-six showed ^{112}GRQ↓GRL^{117}, eleven showed ^{112}GKQ↓GRL^{117,} one showed ^{112}GKQ↓G**A**L^{117} and ^{112} GR**T**↓GRL^{117}.The results showed that low-virulence and avirulent NDV strains exhibit sequence motif ^{112} GR (K)-Q(**T)**-GR**(A)**/L^{117}. Among genotype II viruses, two viruses in their cleavage sites ^{112GKQ}↓G**A**L^{117}, ^{112}GR**T**↓GRL^{117} were different. The motif ^{112} GRQ↓GRL^{117} was present in most isolates.

3.2. Alignment of the deduced amino acid sequences

Genomic regions coding for the F protein cleavage site were amplified by RT-PCR to confirm NDV identity and to achieve molecular characterization analysis. The amplification products of 723 bp for the F protein cleavage site and a nuclear localization signal were obtained (fig. 1). The deduced aa sequence at the F protein cleavage site was characteristic of non-virulent viruses (table 3). Twenty-nine isolates showed the amino acid sequence ^{112}G-R-Q-G-R-L^{117} at the C-terminus of the F2 protein and ten isolates 112G-K-Q-G-R-L^{117}. The Brazilian-1568 isolate had substitution at position ^{116} from arginine (R) to alanine (A) ^{112}G-K-Q-G-A-L^{117}, the other Brazilian-6425 isolate ^{112}G-R-T-G-R-L^{117} had substitution at position ^{114} from glutanine (Q) to threonine (T); these amino acids are equivalent to basic amino acids.

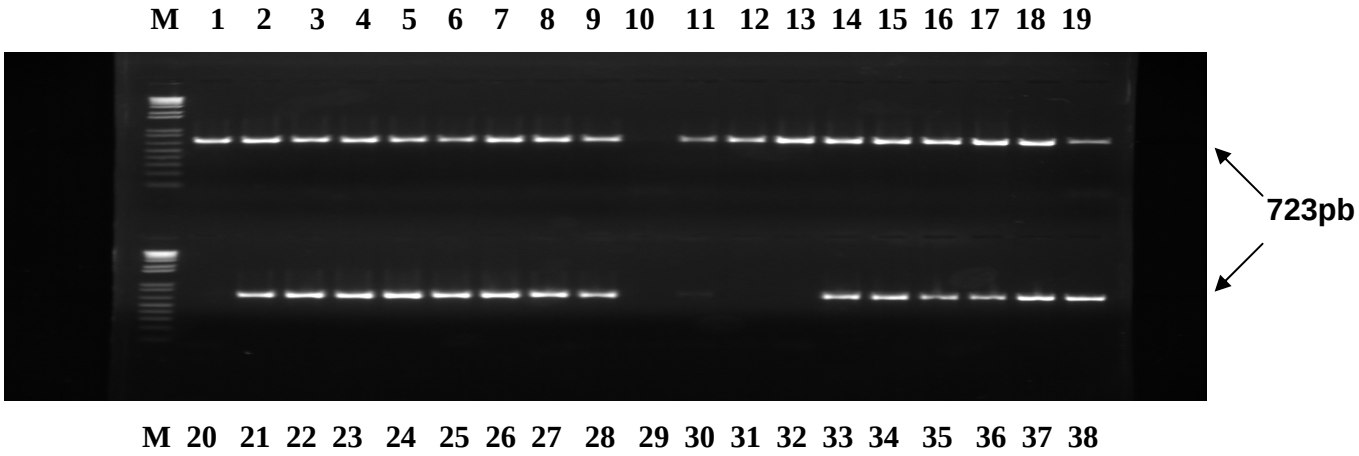

Fig.1. Analysis of RT-PCR products of different NDV isolates by gel electrophoresis M-Mol. Weight marker; (1) 728, (2) 732, (3) 733, (4) 734, (5) 766, (6) 941, (7) 943, (8) 944, (9) 945, (10) Liq. Allantoic (Neg), (11) 982, (12) 1052, (13) 1103, (14) 1220, (15) 1321, (16) 1374, (17) 1524, (18) 1612, (19) 4961, (20) White1, (21) 4980, (22) 5100, (23) 5818, (24) 5958, (25) 6424, (26) 7550, (27) 8486, (28) 8487, (29) Liq. Allantoic (Neg), (30) CER_1 (31) CER_2, (32)-White, (33)-45/Vaccine Clone 30, (34) Standard Ulster, (35) 47/06Vaccine La Sota, (36) 61/Vaccine C2, (37) 65/Vaccine B1, (38) 71/Vaccine VG-GA.

3.3. Phylogenetic Analyses

The region of F genes between nucleotides 112 and 117 was compared by phylogenetic analysis. The 39 NDV isolates collected in Brazil from 2002 to 2005 were classified into two clusters, based on the tree topology, and they belonged to two (I and II) of the six known genotypes (fig.2, table 3). Phylogenetic analysis of isolated genotype showed that the sequence obtained in the present study falls into two clusters, namely I and II, which co-circulate during the period analyzed.

Among these, 28 belong to APMV-1 genotype II or La Sota Like (fig .2), and 11 belong to APMV-1 genotype I. Most NDVs isolated in Brazil before 2002 corresponded to genotype II (71.8%),

Table 2. Reference NDV strains from GenBank used in the phylogenetic analysis of F gene

Gen Bank Accession nº	**Isolates**	**Species**	**Country**	**Year**	**Reference**
AF309418	B1	----	USA	2000	Unpublished
AY372142	P95-2-17	Pigeon	Taiwan/ China	1996	Vet. Microbiol.104 (1-2), 19-30 (2004)
DQ195265	LaSota	----	China	2004	Zhongguo Jiaqin 26, 11-14 (2004)
EF364036	3154/1981	Domestic fowl	Slovakia	2005	Virology Submitted (Jan/2007)
EF110539	-----	Blue-winged teal	Barbardos	2004	Avian Dis. 51 (3), 781-787 (2007)
EF035485	28/2003	Domestic fowl	Slovakia	1981	Virology
AY972103	2687/2001	Duck	Russia	2001	Molecular Genetic Submitted mar/2005
AY972102	3638/2002	Duck	Russia	2002	Molecular Genetic/ Submitted Mat/2005
AY972101	3652/2002	Anas	Russia	--	Molecular Genetic Submitted Mar/2005
AY562991	Ulster/67	Chicken	N. Ireland	2004	Unpublished
AY734535	Tigre 6/99	Pigeon	Argentina	1999	Unpublished
AY372141	P84-1-5	Pigeon	Taiwan/ China	1996	Vet. Microbiol. 104 (1-2), 19-30 (2004)
AY372143	P95-3-22	Pigeon	Taiwan/ China	1996	Vet. Microbiol. 104 (1-2), 19-30 (2004)
AY734536	Capital 3/97	Pigeon	Argentina	1997	Unpublished
AY471835	PUKPI94401	Pigeon	United Kingdom	1994	Avian Pathol. 33 (2), 258-269 (2004)
AJ880277	IT-227/82	Pigeon	Italy	1982	Virus Genes 32 (1), 49-57 (2006)
AY471834	PHUPI84357	Pigeon	Hungary	1984	Avian Pathol. 33 (2), 258-269 (2004)
EF589137	Guizhou	Pigeon	China	--	Unpublished
DQ417113	STP96	Pigeon	China	--	Direct Submission
EF030960	DOZA06N621	Dove	South Africa	--	Direct Submission
AY635814	Gxp22	Pigeon	China	--	Unpublished
EF030961	DOZA06UP470	Dove	South Africa	--	Direct Submission
EF030959	DOZA06N591	Dove	South Africa	--	Direct Submission

Brazilian isolates belonging to one cluster (II) (genotype II) closed virus (AY372142) of pigeon from Taiwan/China, virus (DQ192565) isolate in chickens from China and virus (EF364036) isolate of domestic fowl from Slovakia. Brazilian isolates belonging to another cluster (I) (genotype I) closed only virus isolate of chickens from N. Ireland, which was designed as Ulster/67.

The results are shown in fig. 2. The viruses BR-733, BR-982, BR-728, BR-944, BR-766, BR-734, BR-942, BR-730, BR-1545, BR-732, BR-4961 one separate cluster (77% bootstrap) are closely related to viruses of genotype I or Lineage 1- Ulster-like (81% bootstrap).

The viruses BR-5100, BR-943, BR-7540, BR-6424, BR-1582, BR-1374, BR-5070, BR-7958, BR-7538, BR-1612, BR- 8487, BR-941,BR-1052, BR-7550, BR-1220, BR-4980, BR-945, BR-1547, BR-1223, BR-1103, BR-1321,BR-7549, BR-8486, BR-5818, BR-1035, BR-1524 and BR-1568 constituted a separate cluster (75% bootstrap) and are closely related to viruses of genotype II or Lineage 2- Lasota - like (89% bootstrap). The BR-1524 was closed with isolate AY372142 from pigeon or Taiwan China.

The isolate BR-6425 was closely related to virus La Sota – like and different La Sota live vaccine strains used in Brazil (74/06, 77/06, 47/06,017/06, 67/06 and 015/06) (98% bootstrap), and 47-La Sota and other strains used 61-C2 , 65-B1 and 45-Clone 30 with 75% bootstrap. BR-1568 closed isolates (DQ195265,) strains La Sota from China and the virus EF364036 isolate of domestic fowl in Slovakia (75% of bootstrap). From pathotype prediction based on the fusion protein cleavage site, thirty-nine strains were placed in the avirulent/nonpathogenic group; among them, twenty-six had ^{112}GRQGR/L^{117} sequence and eleven had ^{112}GKQGR/L^{117} sequence. One had ^{112}GKQG**A**L ^{117} and another ^{112}GR**T**GRL^{117} sequences. These changes did not alter their structure.

Table 3. Cleavage site and Genotype of NDV isolates in Brazil

NDV strain	Cleavage site	Genotype
BR-728	GKQ↓GRL	I
BR-730	GKQ↓GRL	I
BR-732	GKQ↓GRL	I
BR-733	GKQ↓GRL	I
BR-734	GKQ↓GRL	I
BR-766	GKQ↓GRL	I
BR-941	GRQ↓GRL	II
BR-942	GKQ↓GRL	I
BR-943	GRQ↓GRL	II
BR-944	GKQ↓GRL	I
BR-945	GRQ↓GRL	II
BR-982	GKQ↓GRL	I
BR-1035	GRQ↓GRL	II
BR-1052	GRQ↓GRL	II
BR-1103	GRQ↓GRL	II
BR-1220	GRQ↓GRL	II
BR-1223	GRQ↓GRL	II
BR-1321	GRQ↓GRL	II
BR-1374	GRQ↓GRL	II
BR-1524	GRQ↓GRL	II
BR-1545	GKQ↓GRL	I
BR-1547	GRQ↓GRL	II
BR-1568	GKQ↓GAL	II
BR-1582	GRQ↓GRL	II
BR-1612	GRQ↓GRL	II
BR-4961	GKQ↓GRL	I
BR-4980	GRQ↓GRL	II
BR-5070	GRQ↓GRL	II
BR-5100	GRQ↓GRL	II
BR-5818	GRQ↓GRL	II
BR-6424	GRQ↓GRL	II
BR-6425	GRT↓GRL	II
BR-7538	GRQ↓GRL	II
BR-7540	GRQ↓GRL	II
BR-7549	GRQ↓GRL	II
BR-7550	GRQ↓GRL	II
BR-7958	GRQ↓GRL	II
BR-8486	GRQ↓GRL	II
BR-8487	GRQ↓GRL	II

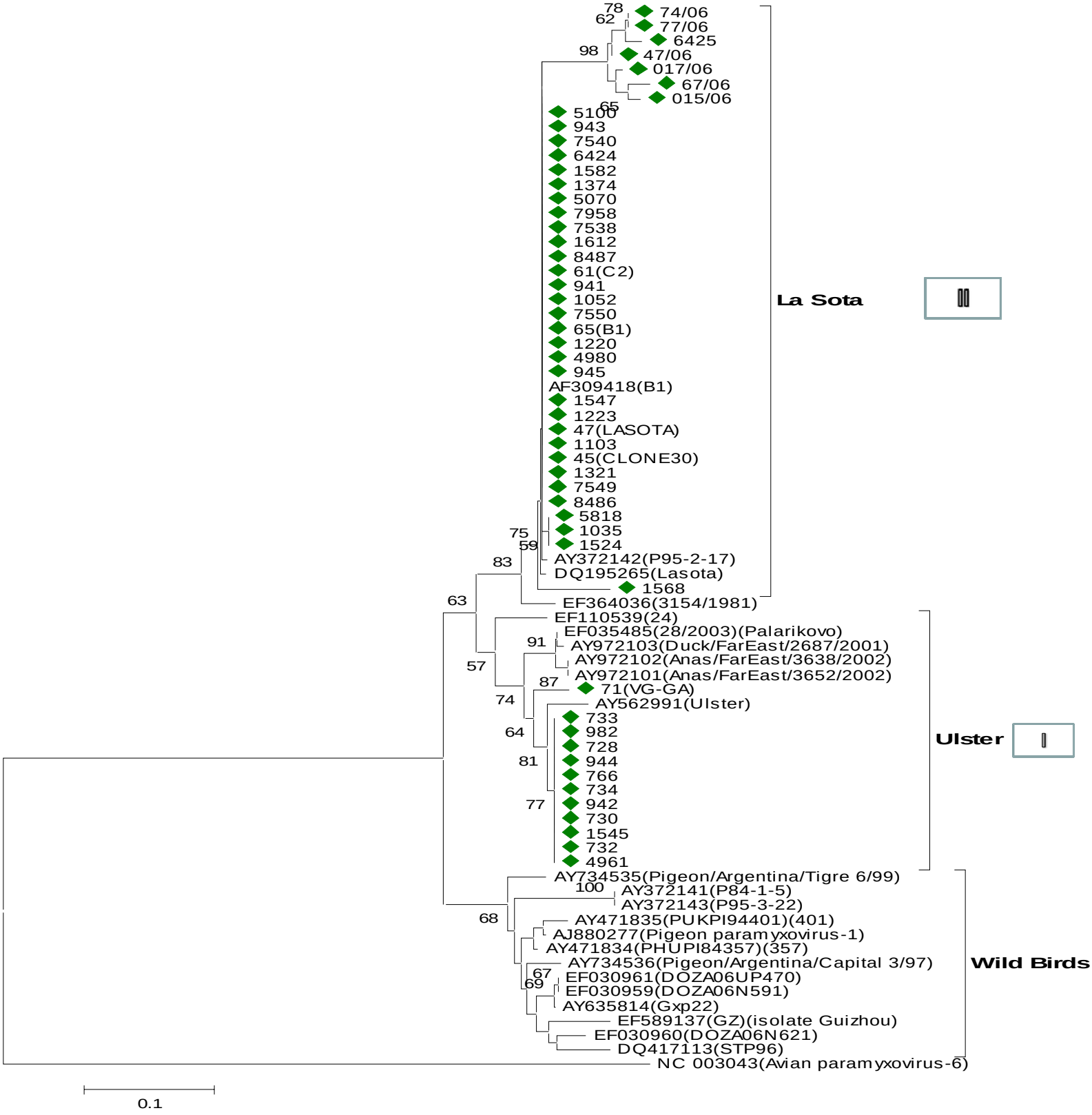

Fig. 2. Phylogenetic tree prepared with the partial F protein gene nucleotide sequences of Brazilian isolates of genotypes I (I) and isolates of genotypes II (II) and worldwide distributed strains of NDV. For the parameters used to measures genetic relationship please refer to the text. GenBank accession numbers are related on table 2. The isolates characterized in the present work are assigned with a green lozenge; the tree was rooted using a NDV sequence.

4. Discussion

Brazil is the biggest exporter and the 3rd producer of broiler chickens in the world. In 2003, Brazil was declared free of virulent NDV for commercial flock areas (Orsi et al., 2010a; World Trade Organization, 2005). Between December 2003 and March 2005, Orsi et al. (2010b) verified the maintenance of virulent NDV-free status for the same areas. The surveillance programs were conducted by Orsi et al. (2010c-in press), during 2004/2005, in geographic areas not involved in chicken meat export in Brazil, remaining with the virulent NDV-free status. The conventional diagnosis methods were used in the researches above cited.

Intracerebral pathogenicity index was determined by Orsi et al. (2009a), using the commercial live vaccines against Newcastle disease used in Brazil, showing that the ICPI varied from 0 to 0.37. There were viruses with ICPI above 0.37 from field isolates; therefore, there was circulation of non-virulent/apathogenic strain with different ICPI of vaccine strains. Respecting the results obtained only by biological characterization (ICPI), it could be interpreted that these isolates were of low virulence or apathogenic.

Until recently, any attention was given if non-pathogenic field isolates from chickens were direct derivatives of vaccine seeds of natural route (Wehmann et al, 1999). In order to solve this question, it was necessary to use a method to specifically identify the isolate type (Seal et al., 2005).

As described in the OIE diagnostic manual, determination of the nucleotide sequence (and the deduced amino acid sequence) of specific regions of genomes of these viruses allows an estimation of their pathogenicity; basically, any virus that has two basic residues at positions 115 and 116 should be considered at high risk, and the one which has a phenylalanine at position 117 should be considered a high risk virus. The virus which has both should be considered virulent. The virus that has leucine at position 117, and a single basic residue at 116 proceeded by any other non-basic residue is considered avirulent.

The partial F gene sequence analysis of Brazilian NDV isolates showed to be a powerful tool to elucidate the diversity of NDV strains circulating in the poultry industry.

The F cleavage activation sites of the viruses isolates were GKQ↓GR, GRQ↓GR, GKQ↓GA and GRT↓GR, lacking dibasic amino acids at positions 112 and 115 along with an L rather than F at residue 117, indicating that all broiler chickens isolates were avirulent or of low-virulence, according to OIE standards (Alexander, 2003).

Phylogenic analysis revealed that all isolated viruses used in this study and two of the six genetic lineages proposed by Aldous et al (2003) belonged to class II**.** Among them, 71.8% to APMV-1 genotype II

or La Sota –Like (28) and 28.2% belonged to genotype I or Ulster-like (11). However, while the study by Whemann et al. (1999), showed that the viruses collected in Hungary were only La Sota–type, the Canadian collection predominantly included B-1. Conversely, in accordance with the results obtained by Whenmann et al, (1999), the present study also showed that field isolates in different regions are clone derivatives of vaccine strains used in mass vaccinations. This fact was also reinforced by Alexander et al. (1997), who reported that 18% of all isolates received by the reference laboratory of Newcastle disease from OIE in England (Weybridge) were identified as La Sota or B1.

In this study, only non-pathogenic viruses of genotype II were isolated; however, Miller et al (2009) cited that there are representatives of low-virulence viruses (loNDV) and virulent viruses (vNDV) in this genotype (II) – 75.5% (n=59) and 24.5% (n=19), respectively.

Lomniczi et al. (1998) proposed that three different genotypes (II, III and IV) were responsible for epizootics during the first panzootic (before 60s). Our data show that 71.8% of the isolates belong to genotype II; therefore, most NDV are isolated in Brazil. Apathogenic NDV strains have been isolated from chicken with previous vaccination history or not (Lancaster & Alexander, 1975). The data obtained in this research confirm data of previous studies, adding the fact that viruses were isolated in health broiler chickens.

Contrarily, Perozo et al (2008), in molecular studies of viruses isolated in Mexico, found only virulent viruses belonging to genotype V; Liu et al (2008, China), isolated pathogenic viruses from genotypes VIId and III from outbreaks of infected chicken flocks. The result obtained by Snoeck et al (2009), in their study about Newcastle disease in West Africa, was in part similar to ours – the sequences clustered with representative viruses of genotypes 1, 2, 3, 4 and 5. Conversely, Lien et al (2007, Taiwan) showed that the viruses isolated in their country belonged to genotype VIId; however, since they were postulated as genotype 2, emerged in genotype VIId.

The results obtained using amino acid sequences of fusion protein cleavage site region in pathotyping of NDV showed similar results to those obtained by conventional diagnosis results, as data shown by Orsi et al (2010 a, b, c) (in press); however, Tan et al (2008) found different results from biological and molecular characterization.

Avian flocks are inoculated regularly with attenuated vaccines (avirulent) to control this disease in Brazil, and the strains most used are La Sota, B1, Clone 30, Ulster VG-GA, C2, and V4.

This finding also confirmed the presence and circulation of ND viruses with the same genotype, but different pathogenicity index of vaccines used in Brazil (Orsi et al, 2009a), showing that both vaccinated and unvaccinated birds can suffer nonpathogenic virus replication similar to vaccine virus.

Although this study has presented one reasonable number of NDV sequences, it was shown that there were only two changes in NDV circulating in these geographic regions. However, these substitutions did alter yet the virus structure, since they did not change protein. The fusion peptide sequence in F was highly conserved among NDV isolates.

Two nucleotide changes can result in an emergent virulent form of NDV from a low virulence, but this occurrence is not documented (Miller et al, 2009). Evidence suggesting that low virulence can become highly pathogenic and this type of mutation were documented in Australia, by Gould et al (2001), and in Ireland, by Alexander et al (1992).

Most genomic changes in nonsegmented RNA viruses are result of recombination or intrinsic error rate of polymerase (it generates a big number of genetic variants called quasispecies). The presence of section pressures at specific amino acids within proteins is recognized as adaptive evolutions (Miller et al., 2009).

Recombination infrequently reports nonsegmented negative strand RNA viruses (Spann et al, 2003). The events of recombination identified in Gen Bank are questioned by some researchers regarding the role of recombination in NDV evolution (Afonso, 2008).

The phylogenetic analysis in the present study evidenced that during 2002-2005, in Brazil, isolated viruses were low-virulent; therefore, there was not virulent virus in commercial birds during the study periods and isolated viruses were genetically identical or similar to commercial vaccines used in Brazil; differently, Seal et al (2005) found low-virulent NDV isolate not related to commonly utilized commercial vaccine strains in North America.

Genetic variability is a strong indicator of virus evolution, and the ability of a genotype to persist in a given population was shown as a phenomenon for other members of the Paramyxovidae family (Ludewick et al., 2005), which is of the same family of NDV. Further studies, including a larger sample size and more nucleotide sequences, should be conducted to determine the significance of the few amino acid substitutions reported in this study.

Acknowledgements

We would like to thank the Brazilian Ministry of Agriculture – Livestock and Food Supply. We are grateful to the Poultry Disease Sector staff of Lanagro/SP, in special to Maria do Livramento de Jesus Nascimento and Jaíra Alves dos Santos, for their expert technical help. We would also like to thank the Animal Laboratory Virology – Unicamp – to make possible this study. This work was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). Finally, we sincerely thank the

employee for the Research Commission (FCM-Unicamp), Etna Lima Macário, for collaborating with the English review.

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CAPÍTULO 7

ISOLATION AND BIOLOGICAL CHARACTERIZATION OF NEWCASTLE DISEASE VIRUS IN MIGRATORY BIRDS IN BRAZIL

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Submetido, Journal of Wildlife Disease

ISOLATION AND BIOLOGICAL CHARACTERIZATION OF NEWCASTLE DISEASE VIRUS FROM MIGRATORY BIRDS IN BRAZIL

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ABSTRACT

This study reports the isolation of NDV in migratory birds, investigating 4,487 birds combined into 202 pools, collected from different migratory routes points during 2003–2008 in Brazil. The results of this investigation showed 23 NDV isolates, of which 13 were categorized as pathogenic (pathogenicity indexes ranging from 1.68 and 1.82) and 10 as non-pathogenic. The pathogenic NDV isolates showed that migratory birds were obtained from four Calidris pusilla, three of the family Hirundinidae, two from Arenaria interpres, and one each of Sterna hirundo, Tringa melanoleuca, Sunda and Haematopus Minutillo Calidris. No virus circulation was detected in the states of Amapá, Pará or Alagoas. The nonpathogenic viruses were isolated in the states of Tocantins, Pernambuco and Rio Grande do Sul. Pathogenic viruses were isolated in the states of Amazonas, Maranhão and Rio Grande do Norte. In Bahia, there were both pathogenic and nonpathogenic viruses. In regard to the types of birds involved, 78.3% of the NDV isolates were obtained from the order Charadriiformes, 13% from the order Passeriformes, and 4.3% each from the orders Anseriformes and Columbiformes. The complementary surveillance investigations, involving the sampling done from migratory, resident-wild and domestic poultry birds in the same and nearby areas showed the isolation of 19 ND viruses (pathogenic and non-pathogenic). As the first report of the presence of NDV in migratory birds, this study should raise concerns in regard to role of these birds as reservoirs and transmitters of NDV in Brazil.

Keywords: biological characterization, isolation, migratory birds, Newcastle disease virus, pathogenicity.

INTRODUCTION

Newcastle disease virus (NDV), a member of the Paramyxoviridae family and designated avian paramyxovirus serotype 1 (APMV-1), is the causative agent of one of the most serious poultry diseases worldwide. There has been increased concern and vigilance in regard to the potential for certain pathogenic microorganisms to be transported and disseminated by migratory birds, particularly in light of the occurrence and unprecedented spread of West Nile virus, since 1999, in North America, where free-living birds have played a significant role (Rappole and Hubálek, 2000; Male, 2003).

Migratory birds could be involved in the spread of microbial pathogens, playing a role as biological or mechanical vectors or carrying infected hematophagous ectoparasites (Húbalek, 2004). There is a long history of NDV isolated from wildlife (Rosenberger et al., 1975; King, 1996; Zanetti et al., 2005; Douglas et al., 2007), and it has been frequently isolated from migratory feral waterfowl and other aquatic birds (Alexander et al., 1979**;** Stallknecht et al., 1991**,** Bannerjee et al., 1994). The introduction of NDV in flocks of commercial birds by migratory birds and wild birds has been documented. Wild birds, especially migratory waterfowl, are therefore often regarded as a reservoir of NDV infection, and are considered a potential risk for the introduction of NDV into NDV-free countries (Kouwnhoven, 1993; Muller et al., 1999). Alexander et al. (1998a), reported the epidemiology of Newcastle disease outbreaks in Great Britain during 1997, suggesting that migratory birds may have been responsible for the primary introduction of the disease.

The potentially virulent strains of NDV are maintained in migratory waterfowl populations in nature, and some of these may be transmitted to domestic poultry and acquire pathogenicity after serial passages in the chicken population (Takakuwa et al.,1998). The majority of NDVs isolated from wild birds, especially waterfowl, are lentogenic, and do not cause apparent clinical disease; however, recent genetic comparisons between NDVs in domestic poultry and feral waterfowl have suggested that velogenic viruses may arise from avirulent strains originated from wild birds. Considering that it was not clear whether the acquisition of virulence took place in feral bird populations, with subsequent introduction of the virulent mutant into poultry, or whether an avirulent virus was transmitted to chickens and then became virulent, Shengqing et al. (2002) made in vivo passages in chickens of an avirulent strain isolated from wild goose. After passages by air-sac inoculation, followed by passages in chick brain, the virus became highly virulent in chickens, demonstrating velogenic properties in pathogenicity tests (MDT > 120 hrs→56 hrs; ICPI 0.00→1.88; IVPI 0→2.67). The original isolate showed the typical avirulent type, which progressed incrementally to a typical virulent type during repeated passages in chickens. These results clearly demonstrate that avirulent

viruses, maintained in wild waterfowl in nature, have the potential to become velogenic after transmission and circulation in chicken populations. Brazil is regularly visited by thousands of migratory birds that arrive from the Arctic region to South America with the proximity of the boreal winter. These birds reside in Brazil from September through May, during hot or mild temperature months, which include spring, summer and the first two months of autumn often stopping or wintering in or at the shores of bodies of water. This condition facilitates the transmission of NDV by fecal-oral route, since the virus can be passed from migratory birds through untreated water and soil to the same migratory birds or to domestic fowls.

The northern portion of Brazil is the gateway to northern migrants in the country (Figure 1). Thus, the Amazon and coastal region north and northeast are sites with many records of migratory species in the northern hemisphere. These birds arrive in the country between August and October and return to their breeding between March and May (Nunes et al., 2006).

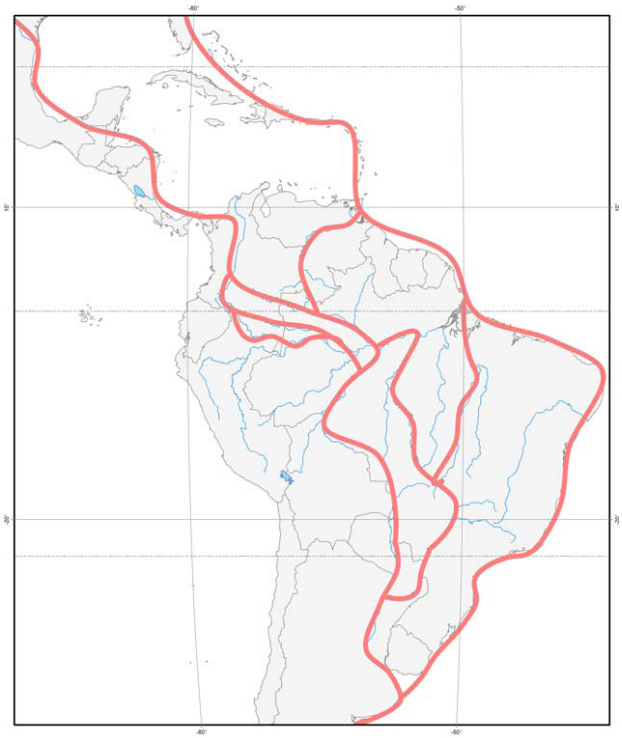

Figure 1: Key migratory routes that pass through Amazon. Coastal routes follow the coastline, while inland routes follow major Amazonian rivers (Nunes et.al, 2006)

In 2003 Brazil was declared free of Newcastle disease in industrial poultry flocks by the World Trade Organization (WTO, 2005), in agreement with a recent study which found areas in Brazil free of the disease (Orsi et al., 2010). However, despite the rigorous biosecurity measures adopted by the poultry industry, there is always the risk of reintroduction of viruses into domestic poultry.

In the present study, samples from migratory birds were sent to the National Agropecuary Laboratory (Lanagro/SP), Campinas, São Paulo, Brazil. From 2003 through January 2008, were analyzed by virological methods to determine whether APMV-1 was present in this population, in order to verify whether the birds play a role as a potential reservoir of Newcastle disease in Brazil.

MATERIALS AND METHODS

Sample collection

1. Migratory Birds

Tracheal/cloacal swabs and organs of dead migratory birds were collected on 21 occasions in different locations throughout Brazil (the states of Amazonas/AM, Amapá/PA, Alagoas/AL, Bahia/BA, Maranhão/MA, Pará/PA, Pernambuco/PE, Tocantins/TO, Rio Grande do Norte/RN and Rio Grande do Sul/RS). The samples (swabs and organs of 4,487 birds) were grouped and analyzed into 202 pools (Table 1). Location and places for sampling were chosen on the basis of previous observations that constitute points of stopping/wintering and intermediary points along the migration route of birds or places where migratory birds died (Figure 2).

2. Complementary Surveillance

After the above-described sample collection in various places throughout Brazil, those same areas as well as nearby areas were submitted to an active complementary surveillance in regard to migratory, resident-wild and domestic poultry birds. In this surveillance, tracheal and cloacal swabs were collected on 16 occasions.

Virus Isolation

Swabs and organ tissues were stored in transport media composed of phosphate-buffered saline solution (PBS) containing antibiotics (10,000 U/ml penicillin, 10 mg/ml streptomycin, 0.25 mg/ml gentamicin and 5000 U/ml mycostatin), adjusted to pH 7.0–7.4. Following collection, the samples were placed into a refrigerated container (2–8º C) and sent to the diagnostic laboratory, where they were received within 48

hours post-sampling and stored at –80ºC until being analyzed. The analyses were performed at the National Agropecuary Laboratory (Lanagro/SP), Campinas, São Paulo, Brazil. Pools samples were inoculated into 5 embryonated specific pathogen free chicken eggs (9–11 days old). The virus isolation was carried out according to the standard procedure established by Regulation #182/94 of the Brazilian Ministry of Agriculture. The samples were submitted to three trials by passage in embryonated chicken eggs, before considered negative.

Hemagglutination (HA) and Hemagglunation Inhibition (HI) Assays

The HA and HI assays were carried out by microtiter methods. The HA assay of allantoic fluids harvested from inoculated embryonating eggs was used to identify NDV-positive embryos. The initial characterization of isolates was performed using the hemagglutination inhibition test with NDV-specific polyclonal antisera (Alexander, 1998 b); Regulation #182/94 of the Brazilian Ministry of Agriculture.

Biological Characterization (in vivo test)

Pathotyping was performed using standard procedures to determine the intracerebral pathogenicity index (ICPI) of day-old chickens, according to the manual of standards by the World Organization for Animal Health (Alexander, 1998; Alexander, 2004) and Regulation #182/94 (Brazilian Ministry of Agriculture). The World Organization for Animal Health (OIE) defines as pathogenic Newcastle disease viruses with ICPI $\geq$ 0.70.

RESULTS

A total of 23 isolates were obtained from the samples taken from the 4,487 migratory birds (Table 1). The breakdown by pools and regions was: 16 isolates from the Northeast region, from 1,958 birds combined into 94 pools; 3 from the North region, from 2,164 birds combined into 84 pools; and 3 from the Southern region, from 365 birds combined into 24 pools. The states of Amapá, Pará and Alagoas did not show any viral isolates.

Isolated viruses were identified as Newcastle disease virus and then characterized using the ICPI, according to the World Organization for Animal Health standard (Anonymous, 2008), which defines virulent viruses as those with an ICPI of $\geq$ 0.70.

Thirteen isolates were considered pathogenic and 10 non-pathogenic. Of the 13 pathogenic isolates 10 were from the Northeast region and 3 from the North region (there being no pathogenic isolates from the South region). Of the 10 non-pathogenic virus isolates, 6 were from the Northeast region, 1 was from the North

region and 3 were from the South region. Thirteen pathogenic isolates had ICPIs in the range from 1.68 and 1.82, while the 10 non-pathogenic isolates had ICPI values ranging between 0.0 and 0.50 (Table 1).

The breakdown by bird species shows there were 9 NDV isolates from *Calidris ssp* (these being 6 from *Calidris pusilla* and 1 each from *Calidris minutilla, Calidris alba, Calidris fuscicollis*), 3 from *Arenaria interpres*, 3 from the family Hirundinidae, 2 from *Charadrius semipalmatus*, and 1 each from *Tringa melanoleuca, Haematopus palliatus, Sterna hirundo, Larus dominicanus, Columba livia* and the family Anatidae (Table 2).

Considering only the pathogenic NDV isolates, 4 were obtained from *Calidris pusilla*, 3 from the Hirundinidae family, 2 from *Arenaria interpres,* and 1 each from *Sterna hirundo, Tringa melanoleuca*, *Haematopus palliatus* and *Calidris minutilla* (Table 2 and Figure 3).

From the non-pathogenic NDV isolates, 2 were obtained from *Calidris pusilla*, 2 from *Charadrius semipalmatus*, and 1 each from *Larus dominicanus, Calidris fuscicollis, Calidris alba, Arenaria interpres, Columba livia* and the family Anatidae.

The breakdown by taxonomic order was: 78.3% from birds of the Charadriiformes order, 13% from birds of the Passeriformes order, while 4.3% each were from birds of the Anseriformes and Columbiformes orders. Of the ND viruses isolated from the order Charadriiformes, 55.5% were pathogenic and 44.5% were nonpathogenic.

Table 1. Number of NDV isolates APMV serotype and ICPI range from migratory birds by Brazilian regions/states, during 2003 to 2007.

Regions	States	Isolation years	Sample types	Number of birds	Number of samples (pools)	Number of isolates	Virus serotype (n)	ICPI Range
	TO	2003	S	405	27	0	- - -	- - -
	AM	2003	S	297	8	0	- - -	- - -
North	AM	2004	S	203	7	3	APMV-1 (3)	1.76-1.77
	AP	2005	C	316	8	0	---	---
	PA	2006	S, O	942	33	0	---	---
	TO	2006	O	1	1	1	APMV-1	0.50
Subtotal				**2,164**	**84**	**4**		
	RN	2003	C	388	22	5	APMV-1 (5)	1.68-1.72
	PE	2003	C	41	4	0	- - -	- - -
	PE	2003	S	85	7	0	- - -	- - -
Northeast	MA	2004	C	475	21	3	APMV-1 (3)	1.75-1.82
	PE	2005	C	32	5	0	- - -	- - -
	PE	2005	C	15	6	0	- - -	- - -
	AL	2006	O	02	1	0	- - -	- - -
	AL	2006	O	01	1	0	- - -	- - -
	BA	2006	C	814	14	4	APMV-1 (4)	0.0-0.12; 1.75-1.77
	PE	2006	C	71	6	4	APMV-1 (4)	0.04;0.06;0.09;0.42
	PE	2006	O	01	1	0	- - -	- - -
	PE	2007	S	33	6	0	- - -	- - -
Subtotal				**1,958**	**94**	**16**		
	RS	2004	C	196	9	0	- - -	- - -
South	RS	2006	C	149	14	2	APMV-1 (2)	0.0–0.15
	RS	2006	C	20	1	1	APMV-1 (1)	0.0
Subtotal				**365**	**24**	**3**	**---**	**---**
Total				**4,487**	**202**	**23**		

C= only cloacal swabs; S= cloacal and tracheal swabs; O= organs
APMV: Avian paramyxovirus

Figure 2. Locations and corresponding Federal State of Brazil that constitute stopping/wintering and intermediary points along the migratory route of birds.

With the exception of *Larus dominicanus,* which comes from the Southern Hemisphere, all the other species are migratory birds coming from the Northern Hemisphere.

The results of the complementary surveillance, involving the collections made from migratory, resident-wild and domestic poultry birds on 16 occasions after the initial sampling, in the same areas and in nearby areas. These collections yielded 19 ND viruses (Table 3). The samples from the North Region (Manaus/AM) yielded 15 ND viruses (14 pathogenic and 1 non-pathogenic), all from domestic birds. Of the 14 pathogenic viruses, the breakdown by species was 7 from ducks (*Anas platyrhynchos*), with ICPI values ranging from 1.27 to 1.88 and 7 from chickens (*Gallus gallus*), and with ICPI values ranging from 1.59 to 1.79. One non-pathogenic virus (ICPI 0.51) was from *Gallus gallus*. In the Northeast Region, only 3 ND

viruses were isolated, all non-pathogenic, from migratory birds (*Calidris pulsilla*, *Calidris alba* and *Arenaria interpres*), with ICPI values ranging from 0.01 to 0.09.

Table 2. Locations and names of migratory birds in which NDV was isolated

Cities or locations/States	NDV/ number of samples	ICPI range	Scientific names/ number of isolates	Migration types	Common names
			North Region		
Manaus/Amazonas	3/7	1.76 to 1.77	Family Hirundinidae (3)	VN, VS	swallows
Alvorada/Tocantins	1/1	0.50	*Columba livia*	C	pigeon
			Northeast Region		
Ilha de Campechá/ Maranhão	3/21	1.75 to 1.82	*Calidris pusilla* *Calidris minutilla* *Tringa melanoleuca*	VN VN VN	semipalmated sandpiper least sandpiper greater yellowlegs
Galinhos/ Rio Grande do Norte	5/22	1.68 to 1.72	*Calidris pusilla (3)* *Arenaria interpres (2)*	VN VN	semipalmated sandpiper ruddy turnstone
Mangue Seco/ Bahia	4/14	0.0 to 0.12 1.75 to 1.77	*Calidris pusilla* *Charadrius semipalmatus Haematopus palliatus* *Sterna hirundo*	VN VN VN VN	semipalmated sandpiper semipalmated plover american oystercatcher common tern
Coroa do Avião/ Pernambuco	4/6	0.04 to 0.42	*Calidris pusilla* *Calidris alba* *Arenaria interpres* *Charadrius semipalmatus*	VN VN VN VN	semipalmated sandpiper sanderling ruddy turnstone semipalmated plover
			South Region		
Lagoa do Peixe/ Rio Grande do Sul	2/14	0.01 to 0.15	Calidris fuscicollis Larus dominicanus	VN VS	white-rumped sandpiper kelp gull
Lagoa do Peixe/ Rio Grande do Sul	1/1	0.0	Family Anatidae	VN, VS	teal

VS=Seasonal visiting from the southern hemisphere;
VN=Seasonal visiting from the northern hemisphere;
R=Resident (evidence of reproduction in the available country);
C=Cosmopolitan
p://www.cbro.org.br/CBRO/pdf/avesbrasil_jul2006.pdf

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Table 3. NDV isolates from the active complementary surveillance in regard to migratory, resident- wild and domestic poultry birds from different Brazilian Regions in the period from 2003 to 2007.

Cities/States	Year	Bird types (n)	Sample types	NDV/ n. of samples	ICPI Range	Scientific names/ number of isolates (n)	Common names	
North Region								
Manaus/Amazonas	2005	ducks	S	0/6	---	---		---
Manaus/Amazonas	2006	chicken, duck parrot, widgeon pheasant	S	1/44	1.88	*Anas platyrhynchos*	Duck	
Manaus/Amazonas	2006	chicken, duck, quail, goose	S	14/58	1.27 - 1.82 1.59 - 1.79 0.51	*Anas platyrhynchos (6)* *Gallus gallus (7)* *Gallus gallus (1)*	Duck Chicken Chicken	
Manaus/Amazonas	2006	duck, chicken	S	0/8	---	---		---
Northeast Region								
Galinhos/ Rio Grande do Norte	2003	chicken, duck, pigeon, goose	S	0/13	---	---		---
Galinhos/ Rio Grande do Norte	2004	chicken, duck widgeon, pigeon	S	0/14	---	---		---
Barra do Cunhaú/ Rio Grande do Norte	2004	chicken	S	0/10	---	---		---
Jandaira, Vera Cruz, Itubera, Prado, Porto Seguro/Bahia	2006	backyard	S	0/48	---	---		---
Jandaira/Bahia	2006	chicken	S	0/20	---	---		---
Galinhos/ Rio Grande do Norte	2006	chicken, duck, turkey, guinea hen	S	0/14	---	---		---
Galinhos/ Rio Grande do Norte	2006	backyard	S	0/3	---	---		---
Nova Viçosa/Bahia	2006	duck, chicken	S	0/20	---	---		---
Recife/Pernambuco	2006	backyard	S	0/34	---	---		---
Coroa do Avião/Pernambuco	2007	migratory, subsistence birds	S	3/4	0.025 0.09 0.015	*Calidris alba* *Calidris pusilla* *Arenaria interpres*	Sanderling Semipalmated sandpiper Ruddy turnstone	
South Region								
Lagoa do Peixe/ Rio Grande do Sul	2006	migratory birds	S	1/8	0.06	*Larus domenicanus*	Kelp gull	
Lagoa do Peixe/ Rio Grande do Sul	2006	migratory birds	S	0/8	---	---		---

S=cloacal and tracheal swabs

In the South Region, only 1 ND virus (non-pathogenic) was isolated, from a pool of migratory birds *(Larus domenicanus*), with an ICPI value of 0.06. All these ND viruses isolated from migratory birds were obtained from birds of the Charadriiformes order.

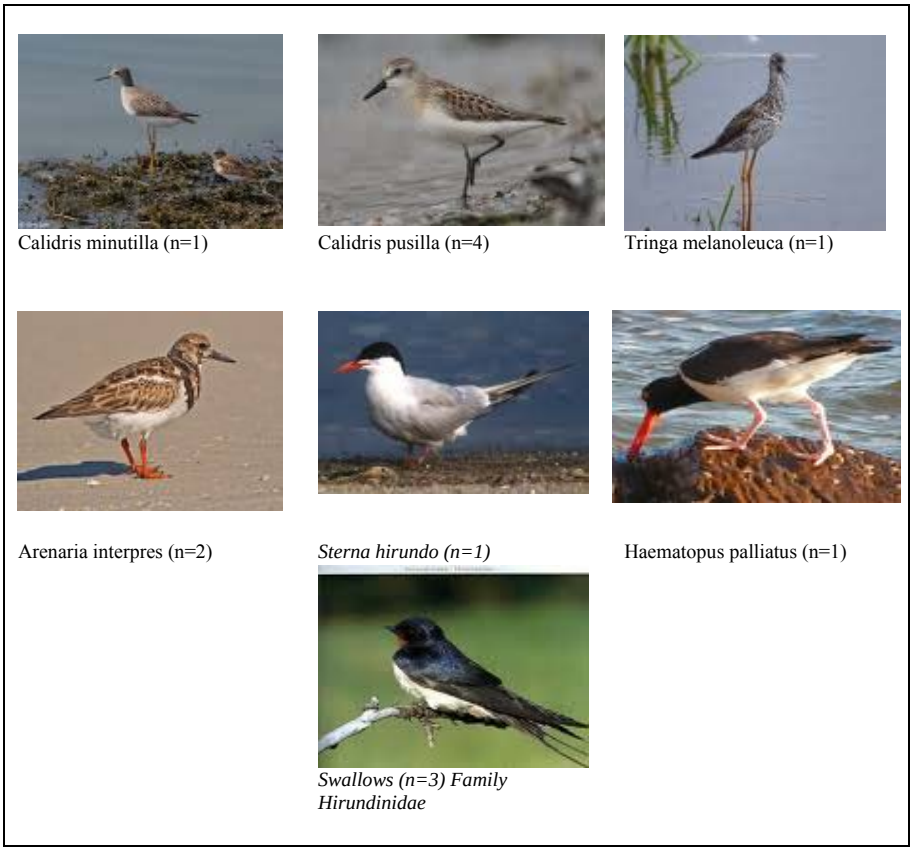

Figure 3. Migratory birds (n=13) isolated with pathogenic NDV strains.

DISCUSSION

The data obtained here represent the first isolation and biological characterization of NDV from migratory birds in Brazil. According to the literature, the majority of NDVs isolated from migratory birds and wild birds, especially waterfowl, are nonvirulent. Conversely, in the present study, over half (56.5%) of the viruses isolated were pathogenic. Recent genetic comparisons between NDV in domestic poultry and feral waterfowl have suggested that viruses circulating among various wild species could cause ND outbreaks in poultry. Mutation and host selection may play a crucial role because, apparently, the main prerequisite that determines the virulence of strains is the presence of two aa substitutions at the F cleavage motif and the presence of a phenylalanine at position 117. However, the mechanisms involved are still unknown (Zanetti et al., 2005).

In the present study, 13 pathogenic ND viruses were obtained and characterized. One NDV strain isolated from *Sterna hirundo* presented an ICPI value of 1.77, similarly to the results obtained by Usachev et al. (2006), from a specimen collected at the Volga River Delta in 2001, where the sequence of this virus indicated that it was velogenic.

In the present study, 1 nonpathogenic ND virus was isolated from a pigeon population. The pigeon paramyxovirus serotype 1 (PPMV-1) affects doves and pigeons, being known to infect poultry (Capua et al., 2002; Kommer et al., 2002), and the virulence of this virus has been reported as variable (Meulemans et al., 2002). The increased pathogenicity index after serial passage in chickens or embryonated eggs indicates that the viruses that circulate among pigeon populations could lead to ND outbreaks (Kommers et al., 2003; Kim et al., 2008).

Eight nonpathogenic NDVs were isolated, similarly to what was obtained by Zanetti et al. (2005), in a study with wild healthy birds in Argentina. These results are cause for concern, since there are several reports suggesting that velogenic viruses arise from lentogenic ones in nature (Gould et al., 2001). The changes of virulence from wild to domestic transmission and from lentogenic to velogenic waterfowl isolates after passages in chickens were demonstrated by Shengqing et al. (2002).

Considering the ICPI results, our findings are in agreement with those obtained by Liu et al. (2008), in China; however, the same situation was not found by Lee et al. (2009), who reported the isolation, in Korea, of 13 nonpathogenic viruses and 1 pathogenic NDV.

Duck species produce few clinical symptoms of disease after infection, even after infection with NDV strains, which are highly virulent in chickens, as shown by the in vivo test of the present study. Concerning the percent of NDV isolated from birds belonging to the Charadriiformes order, the present findings are in agreement with those obtained by Hanson et al. (2008) concerning avian influenza.

By showing the presence of pathogenic and nonpathogenic NDV in migratory birds, as well as in resident-wild birds and domestic fowls close to the relevant migration areas, the present study shows that migratory birds are viable NDV carriers and a reservoir of the virus in regard to its spread to domestic fowls. The isolation of NDV from domestic chickens with a pathogenicity index (ICPI) similar to that of NDV from migratory birds was especially significant in this regard, since in the places where the samples were obtained there were no large-scale poultry farms, suggesting that the virus was being spread by migratory birds.

ACKNOWLEDGMENTS

We thank all the people who contributed to the capture and identification of the birds included in this study, and we are especially grateful to the Brazilian Ministry of Health, Ministry of Environment and Ministry of Agriculture through its Animal Laboratory, Sanitary Programs and Sanitary Defense for providing financial and technical resources and authorization to publish this paper, under coordination of Lanagro/SP. We are also grateful to Luiz Carlos da S. Porto, for providing us with animal-care assistance.

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4- DISCUSSÃO GERAL

4.1- Estudos da estabilidade térmica e proteção conferida pelas vacinas comerciais vivas da Doença de Newcastle usadas no Brasil

O título viral mínimo aceito na data de fabricação e o título mínimo ao final do período de validade da vacina da DN são de $10^{6,5}$ $DIOE_{50}$ e $10^{5,5}$ $DIOE_{50}$ respectivamente, segundo normas para o controle de qualidade dos produtos biológicos usados na avicultura (Portaria n º 186/97-MAPA). As vacinas em geral apresentavam o título viral médio pelo método "a fresco" (AF) no final de validade de dois anos de $10^{6,2}$ $DIOE_{50}$, com uma significativa perda média de título de 0,66 log10, correspondente a 9,71% da faixa inicial. Com base nos resultados obtidos neste estudo foi possível mudar o título mínimo exigido no início do teste de validade para $10^{6,20}DIOE_{50}$ na nova Portaria nº 07/06 (Brasil, 2006).

Houve uma correlação significativa entre os títulos obtidos pelo método AF e aqueles observados no final do período de validade e pelo método de "estabilidade térmica" (ET) no início, não houve diferença significativa entre eles, com resultados foram semelhantes aos encontrados por Simi et al. (1970). Estudos de degradação acelerada de preparação de referência para a vacina viva da DN no mercado internacional foram relatados em um passado distante por Frerichs & Herbert, (1974), mostrando que o conteúdo de vírus é estável quando armazenado a 4°C por 2 anos, com dados semelhantes ao nosso. Portanto, dentro do mês após a fabricação, o teste ET antecipa o desempenho da vacina durante 24 meses de armazenamento a uma temperatura entre 2 a 8ºC, e este teste pode ser usado como substituição do método AF 24 meses após a fabricação. Todas as vacinas pesquisadas mostraram-se eficazes, frente ao desafio com o vírus patogênico da DN ao final do seu período de validade com resultados iguais ou superiores ao exigido pela legislação brasileira.

4.2- Estirpes Vacinais da DN: Imunogenicidade não é influenciada pelo IPIC

Há pouca informação sobre as características biológicas de vacinas utilizadas no Brasil, medidas por metodologias clássicas (índice de patogenicidade intracerebral-IPIC e tempo médio de mortalidade embrionária-MTD). Até o momento somente uma análise comparativa morfométrica da virulência das estirpes vacinais contra a doença de Newcastle (La Sota, Ulster e VG-GA) foi realizada (Nunes et al., 2002), onde as estirpes La Sota e Ulster apresentaram a mesma virulência no 3º dia após a vacinação, levando a maior inchaço da mucosa traqueal que a da estirpe VG-GA. Os resultados obtidos a partir de aves vacinadas com a estirpe La Sota são consistentes com um experimento prévio que quantificava o inchaço traqueal (Jorge et al., 1998). A DN é definida pela OIE segundo critérios para a virulência específicos, (OIE, 2000a, 2004) listados a seguir: (IPIC) de 0,7 ou mais, em pintinhos SPF de 1 dia de

idade (Galllus gallus domesticus). O uso de vacinas na Comunidade Européia é permitido quando a semente vacinal a ser testada tem um IPIC <0,4 (10^7DIOE$_{50}$/ave), ou <0,5 (10^8DIOE$_{50}$/ave). O comitê de Padrões da OIE recomenda que a vacina deva ter um IPIC <0,7, a fim de atender a variabilidade interlaboratorial. Uma margem de segurança é permitida e as sementes das estirpes utilizadas para a vacinação devem ter um IPIC ≤ 0,4 (CEC, 1993), adotadas pela OIE (2000b). Os resultados IPIC para todas as vacinas utilizadas no Brasil determinados no presente estudo cumprem as exigências da OIE (IPIC ≤ 0,37).

O objetivo principal de vacinas vivas é o de estabelecer um estado de infecção controlada por uma cepa atenuada em um lote no momento da aplicação. Na formulação de tais imunógenos, há um tênue balanço entre a capacidade de replicação do agente vacinal (e consequente manutenção de uma virulência residual) e o grau de proteção conferida por esta vacina. O método de administração da vacina por via ocular é mais eficaz para vacinas vivas lentogênicas, garantindo que a vacina atinja aves individuais e, conseqüentemente, os títulos obtidos são geralmente uniformes em todo o lote. Os resultados de desafio aqui realizados demonstraram eficácia satisfatória (Brasil, 2006). Este estudo confirma que todas as estirpes de vacinas vivas utilizadas no Brasil são lentogênicas, com base no tempo médio de mortalidade embrionária (MDT) superior a 90 horas (Hanson e Brandly, 1955). Os resultados obtidos no presente estudo mostram que diferenças nos índices de patogenicidade intracerebral entre as estirpes do VDN mais usadas em vacinas, independente do grau de virulência residual, não interferem com a resposta imune.

4.3. Prevalência do vírus da Doença de Newcaslte em frangos de corte (*Gallus gallus*) no Brasil

O principal objetivo do presente estudo foi investigar a prevalência do VDN em aves comerciais nas áreas geográficas mais importantes para a produção e exportação avícola brasileira. A eficácia da vacinação em aves pode ser monitorada no plantel por meio de exames sorológicos (OIE 2007), tendo destaque a inibição da hemaglutinação (HI), sendo este o método de escolha, ainda que nos últimos anos vários ensaios imunoenzimático do tipo ELISA tenham sido descritos (Miers et al.,1983; Wilson, et al., 1984). A soropositividade foi de 66,4%, 23,3% e 22,3% no Sudeste, Centro-Oeste e Sul, respectivamente. Os anticorpos encontrados na região Sudeste são provavelmente oriundos de imunização ativa, já que esta prática é comum nesta região. A vacinação da DN em frangos de corte não é uma prática nos estados do RS, SC, MT, MS, GO e DF. No entanto, nesses estados, as aves de vida longa (reprodução ou produção de ovos) são vacinadas. Neste estudo, a freqüência do VDN em aves saudáveis variou de 1,0 a 7,6% por lotes, e variando de 6,5% para 58,4% dos plantéis pesquisados, de acordo com a região geográfica. O

isolamento foi maior nas regiões onde a vacinação é amplamente utilizada, em semelhança aos resultados relatados por Alexander & Gough (2003), que observaram que a vacina protege as aves da doença clínica, porém a replicação do vírus e excreção pode ocorrer. Kapcyznski & King (2005), estudando o VDN em aves exóticas observaram que vacinas protegiam os frangos de corte contra morbidade e mortalidade e reduziu significativamente a excreção viral em comparação ao grupo controle, mas não impediu a infecção e disseminação do vírus. Um estudo sorológico foi realizado também em Benin-África, em três diferentes regiões (Sul, Central e Norte), e 56%, 75% e 69% dos frangos foram soropositivos, respectivamente, (Bell, 1991; Bell, 1992), com resultados semelhantes aos obtidos neste trabalho. A presença do vírus, já na primeira semana de vida das aves observada na maioria das regiões, devido à reutilização de cama, levou à estimulação do sistema imunológico. A maior taxa de isolamento do vírus foi observada na região Sudeste, seguido pelo Sul e Centro-Oeste. Este é o primeiro relato indicando a presença de VDN em regiões onde o ”status vacinal” não é informado. Os resultados também indicaram uma maior circulação do vírus vacinal na região Sul do que na região Centro-Oeste. O elevado número de vacinação relatados na região Sudeste, certamente se correlaciona com o alto número de isolados na área. Prevalências semelhantes, entre 5 e 29%, foram encontradas em um pequeno lote de frango e de lotes de reprodutoras na Suíça (Schelling et al., 1999).

Soropositividade e isolamento do vírus em estados sem vacinação contra a DN em frangos de corte podem ser explicados pela elevada densidade de aves em algumas regiões, a proximidade com categorias distintas de aves, e a coexistência de explorações agrícolas de subsistência e de baixa tecnologia. O melhor resultado do isolamento viral foi alcançado quando as amostras foram submetidas a três passagens em ovos embrionados. Contraditoriamente, Kouwenhoven (1993) observaram que 85% das amostras positivas foram positivas já na 1ª passagem e que apenas 10% necessitaram de uma segunda passagem. Em casos excepcionais, três passagens foram necessárias. A natureza da amostra desempenha um papel importante nestes testes. Os dois principais locais de replicação do VDN parecem ser as vias respiratórias e intestinais de aves, portanto, as amostras devem incluir sempre amostras de suabes cloacal e traqueal (Alexander e Gough, 2003).

”Pools” tanto da traquéia e cloaca foram colhidas de aves saudáveis sem qualquer sinal da doença, é possível que algumas colheitas ocorressem durante um período de eliminação muito pequena de vírus. Isso pode ser observado pelo baixo número de isolamentos nas 1ª passagens e 2ª passagem. Portanto, uma 3ª passagem parece ser necessária para a adaptação do vírus aos ovos embrionados. Em 94,8% dos isolados, o IPIC variou de 0,00 para 0,50; que é o intervalo onde as estirpes vacinas Ulster 2 C, V4 Queensland, B1 e La Sota estão caracterizadas (Allan et al., 1978). Estes achados podem ser explicados pelo fato das vacinas mais utilizadas no Brasil serem baseadas em La Sota e B1, e o que foi isolado

provavelmente é vírus vacinal (Orsi et al., 2001). Com base nestes resultados, todas as cepas de VDN aqui isoladas podem ser classificadas como avirulentas (lentogênicas). O isolamento de vírus com IPIC variando de 0,51 a 0,66 indica a circulação de cepas não patogênicas/apatogênicas em regiões onde também a vacinação não foi informada, mas presume-se que deva ter sido realizada. Yongolo (1996) encontrou resultados semelhantes, ou seja, o isolamento de estirpes lentogênicas a partir de aves saudáveis. Os resultados deste estudo mostram que as aves industriais produzidas nos nove estados brasileiros estudados estão livres de amostras virulentas da Doença de Newcastle, em conformidade com o disposto no Código Internacional de Saúde Animal.

4.4. Levantamento para a manutenção de área livre de vírus virulento da doença de Newcastle em áreas de produção de frango no Brasil

Este estudo confirma os resultados do trabalho anterior, indicando que frangos de corte na região Sudeste do Brasil foram vacinados contra a DN (Orsi et al., 2010a). O presente estudo demonstrou também que o VDN estava circulando em regiões com nenhuma informação sobre vacinação. Um ponto importante a considerar é a difusão do vírus em consequência das vacinas vivas (Bell, 2001).

Os fatores de risco associados à soropositividade da DN foram identificados por East et al. (2006), que demonstraram que a idade dos lotes, na proximidade de granjas de aves vizinhas e localização da granja foram as mais importantes. Vários outros fatores de risco no matadouro foram postulados: o nível de segurança, saneamento do abastecimento de água de frango e de exclusão de aves selvagens e vida livre (Bojeson et al., 2003; Gibbens et al., 2001; Tablante et al., 2002). O risco de aves soropositivas ao VDN pode ser atribuído à ocorrência e brechas na segurança e higiene mais do que os níveis de biossegurança (East et al., 2006). Os resultados mostram que a vacinação contra a DN em frangos de corte não ocorre nos estados do RS, SC, MT, MS e GO, nem no DF. Mas sabe-se que nestes estados as aves são vacinadas apenas quando utilizados para fins de longa-vida, como a produção de ovos. Neste estudo, VDN foi isolado em uma freqüência que varia de 0,15 para 0,44% no lote e de 20,0 a 53,3% de acordo com a região geográfica. Em um estudo anterior, Orsi et al, 2010a relataram uma maior freqüência de 1,0 a 7,6% por lote, e de 6,5% para 58,4%, na região geográfica. O isolamento foi maior nas regiões onde a vacinação era amplamente utilizada. Estes dados estão de acordo com Alexander e Gough (2003), que sugeriram que a vacina protege as aves de doenças clínicas, mas a replicação e excreção do vírus pode ainda ocorrer, mesmo em níveis mais baixos.

A diminuição no número de isolamento do VDN é evidente quando os resultados são comparados com aqueles relatados por Orsi et al (2010a). Esta redução na positividade pode ser atribuída à melhoria da

biossegurança e proibição do trânsito de pessoas nas granjas dado o medo e preocupação causada pela gripe aviária durante este período. Entre 2002 e 2005, vários surtos de vírus da gripe foram amplamente divulgados em todo o mundo através dos jornais, televisão e comunicações OIE, e que provavelmente conduziu a uma mudança de postura e melhor controle do vírus da doença de Newcastle no Brasil. Os resultados Africanos foram superiores aos obtidos no Brasil. Reutilização de cama observadas na maioria das regiões pode explicar a presença do vírus logo na 1ª semana de vida da ave, levando à estimulação do sistema imunológico. O maior número de isolamento do vírus na região Sudeste do Brasil, seguido pelo Centro-Oeste e Sul, também foram relatados em um estudo prévio de Orsi et al (2010a), que detectou maior circulação do vírus na região Sul comparado à região Centro Oeste. A vacinação mais eficaz na região Sudeste pode estar correlacionada com o elevado número de isolados nesta área. Evidência sorológica e isolamento viral em estados que não usam a vacina contra a doença de Newcastle em frangos de corte pode ser explicada pela grande quantidade de aves em algumas regiões, pela proximidade com categorias distintas de aves, e a coexistência granjas de baixa tecnologia de produção. Os vírus isolados no presente estudo apresentaram IPIC abaixo de 0,47. Isto pode ser atribuído ao uso mais freqüente de vacinas B1 e La Sota no Brasil, que apresentam IPIC 0,2 e 0,4, respectivamente (Alexander & Allan, 1974; Allan et al., 1978). A Organização Mundial de Saúde Animal (WOAH, 2008) recomenda que a vacina deva ter um IPIC inferior a 0,7, a fim de satisfazer à variabilidade interlaboratorial estimado a margem de segurança exigido. Assim, as sementes das vacinas vivas não devem apresentar IPIC superior a 0,4 (Council of the European Communities, 1993). Orsi et al. (2009) verificaram que os valores IPIC para todas as vacinas utilizadas no Brasil variavam de 0,0 a 0,37. Os valores IPIC dos isolados neste estudo são semelhantes aos obtidos para as vacinas comerciais. Os resultados deste estudo indicam que as medidas de biossegurança associadas aos programas de vacinação como postulado pelo Código Zoossanitário Internacional são cruciais para a preservação do status de livre do VDN virulento para avicultura industrial no Brasil.

4.5. Ausência de vírus altamente patogênico da Doença de Newcastle em regiões avícolas não exportadoras no Brasil

Em 2003 e março de 2005, o Brasil foi reconhecido como um país livre do VDN patogênico em aves comerciais (Orsi et al., 2010a; WTO, 2005). A manutenção do status de livre em áreas exportadoras foi mantida conforme relatado por Orsi et al. (2010b). Contudo, o nível de soropositividade e presença de VDN em áreas não relacionadas com a exportação de frango de corte eram completamente desconhecidos. O principal objetivo deste estudo foi investigar a presença de DN em aves comerciais em áreas não ligadas a exportação avícola brasileira.

Os testes sorológicos para VDN podem ser utilizados para demonstrar a infecção por vírus ou por acompanhamento de vacinação (OIE, 2007). O teste de HI é o método de escolha, no entanto, nos últimos anos, várias ensaios imunoenzimáticos (ELISA) foram desenvolvidos (Wilson et al., 1984), a correlação entre HI e teste ELISA produziu resultados variáveis (Cvelic-Cabrilo et al., 1992). Neste estudo as amostras soropositivas foram detectadas pelo teste ELISA, com uma percentagem de 84,3%, 57,7% e 41,4% no Nordeste, Norte e Sudeste, respectivamente. Resultados semelhantes foram encontrados por Bell (1991, 1992), na realização de estudos sorológicos em Benin, na África, e por Orsi et al. (2010b) ao verificar a manutenção do status livre para VDN virulento nas explorações avícolas. Este resultado confirma que aves do Sudeste foram vacinadas, independentes do Estado (MG, SP ou ES). A estimulação precoce do sistema imunológico pode ser obtida pela reutilização de cama observadas nas regiões, e isso pode justificar a presença do vírus já na primeira semana de vida das aves. Isto confirma que todas as categorias de aves comerciais foram vacinadas contra a doença de Newcastle, justificando um maior número de amostras soropositivas. Neste estudo, foram isoladas estirpes vacinais VDN em aves saudáveis com um frequências entre 0 e 1,7% para lotes e de 0% a 100% por região geográfica; resultados semelhantes foram obtidos por Orsi et al, 2010b com o percentual de isolamento por lotes de 0,44% e 53,3% na região Sudeste. Vale a pena ressaltar que este estudo foi realizado antes do aparecimento da Influenza H5N1, quando foi frequente a ocorrência de falhas na segurança e higiene, mais que nos níveis de biossegurança e de higiene utilizadas (East et al., 2006). A taxa de isolamento foi maior nas regiões onde a vacinação é muito utilizada. De acordo com Alexander et al. (2003), a vacinação protege as aves contra a doença clínica, no entanto, a replicação do vírus e excreção ainda pode ocorrer embora em níveis mais baixos. Em estudo realizado por Kapcyznski & King (2005) com vacinas vivas e inativadas mostrou que os imunógenos podem reduzir, mas não impedir a infecção e disseminação do VDN exóticos.

Os isolados virais foram identificados como o VDN e sua caracterização biológica mostrou que índice de patogenicidade intracerebral variava de 0,0 a 0,54.

Os resultados obtidos neste estudo mostraram que somente vírus não patogênicos foram detectados nas amostras estudadas conforme postulado pelo Código Zoossanitário Internacional.

4.6. Caracterização molecular de isolados de campo de vírus da DNe e das estirpes das vacinais usadas no Brasil do VDN

Brasil é o maior exportador e o 3º maior produtor de frangos de corte do mundo. Em estudo realizado no periodo 2002 a 2005 no Brasil por Orsi et al. (2010 a, b e d) em áreas produtoras e

exportadoras e não ligadas a exportação mostraram que somente vírus de baixa patogenicidade foram detectados através de métodos convencionais de diagnóstico. Orsi et al. (2009), utilizando as vacinas comerciais vivas da DN utilizados no Brasil, mostrando que o IPIC variou de 0,0 a 0,37. Havia vírus com IPIC acima de 0,37 de isolados de campo e, portanto, havia circulação de VDN não virulento /apatogenico com IPIC diferentes cepas vacinais. Com os resultados obtidos pela caracterização biológica (IPIC), interpretamos que esses isolados eram de baixa virulência ou apatogénica. Até recentemente, nenhuma atenção era dada se um isolado de campo era não-patogênico e provável derivados da vacinação com vacinas vivas atenuadas (Wehmann et al., 1999). A fim de resolver esta questão, foi necessário usar um método para identificar especificamente o tipo do isolado, conforme descrito anteriormente (Seal et al., 2005). Por determinação da OIE (2005), a seqüência de nucleotídeos de regiões específicas do genoma permite uma estimativa da sua patogenicidade. Ou seja, qualquer vírus da DN que tem dois resíduos de base nas posições 115 e 116 deve ser considerado de alto risco, e a única que tem uma fenilalanina na posição 117 deve ser considerado um vírus de alto risco. O vírus que possuem ambos deve ser considerado virulento. O vírus que tem leucina na posição 117, e um resíduo de base única de 116 seguida por qualquer outro resíduo de base é considerado avirulento. A análise da seqüência parcial do gene F dos VDN isolados no Brasil mostrou ser uma ferramenta poderosa para elucidar a diversidade de VDN que circulam na indústria avícola. Os sítios de clivagem de ativação da proteína dos vírus isolados estavam faltando aminoácidos dibásico nas posições 112 e 115, juntamente com um L ao invés de F no resíduo 117. Este resultado indica que todos os isolados eram avirulentos ou de baixa virulência, de acordo com as normas da OIE (Alexander, 2003). Análise filogenética revelou que todos os vírus isolados utilizados neste estudo pertencem a duas das seis linhagens genéticas proposto por Aldous et al. (2003) pertenciam à classe II. Entre eles, 71,8% eram APMV-1 genótipo II ou La Sota-Like e 28,2% pertenciam ao genótipo I ou Ulster-like. Por outro lado, de acordo com os resultados obtidos por Whenmann et al. (1999), o presente estudo também mostrou que isolados de campo em diferentes regiões são clones derivados de cepas de vacinas utilizadas nas vacinações em massa. Este fato também foi reforçado por Alexander et al. (1997), que relataram que 18% de todos os isolamentos recebidos pelo laboratório de referência da DN, na Inglaterra (OIE-Weybridge) foram identificados como B1 ou La Sota. Neste estudo, apenas os vírus apatogênicos do genótipo II foram isolados, no entanto, Miller et al. (2009) citou que existem representantes de vírus de baixa virulência (loNDV) e vírus virulento (vNDV) neste genótipo (II) 75,5 % (n = 59) e 24,5% (n = 19), respectivamente. Nossos dados mostram que 71,8% dos isolados pertencem ao genótipo II, portanto, os mais isolados no Brasil. VDN apatogénica foram isoladas de frango com histórico de vacinação anterior ou não (Lancaster & Alexander, 1975). Contrariamente, Perozo et al. (2008), em estudos moleculares de vírus isolados no México, encontraram apenas os vírus virulentos

pertencentes ao genótipo V; Liu et al. (2008, na China), isolaram vírus patogênicos de genótipos VIId e III a partir de surto de lote de aves infectadas. Os resultados obtidos por Snoeck et al. (2009), em seu estudo sobre a doença de Newcastle na África Ocidental foi em parte semelhante à nossa. Inversamente, Lien et al. (2007, Taiwan) mostraram que o vírus isolado em seu país pertencia ao genótipo VIId, no entanto, uma vez que foram postuladas como genótipo 2, surgiu no genótipo VIId. Os resultados obtidos molecularmente através da análise das seqüências de aminoácidos de proteína de fusão (sitio de clivagem) do VDN mostraram resultados semelhantes aos obtidos pelos diagnósticos convencionais aliados a caracterização biológica, conforme dados apresentados por Orsi et al. (2010 a, b, d). Porém, Tan et al. (2008) encontraram resultados diferentes na caracterização biológica e molecular. Lotes de aves são inoculados regularmente com vacinas vivas atenuadas (avirulenta) para controlar esta doença no Brasil, e as cepas mais utilizadas são La Sota, B1, Clone 30, Ulster VG-GA, C2 e V4. Este achado também confirmou a presença e circulação dos vírus da DN com o mesmo genótipo, mas índice de patogenicidade diferente de vacinas utilizadas no Brasil (Orsi et al., 2009e), mostrando aves vacinadas e não vacinadas podem replicar vírus apatogênico, similar ao vírus da vacina. Embora este estudo apresentasse um número razoável de seqüências do VDN, demonstrou-se que havia apenas duas mudanças nos vírus que circulam nestas regiões geográficas. A seqüência de peptídeo de fusão em F foi altamente conservada entre VDN. Duas mudanças de nucleotídeos podem resultar em uma forma virulenta de VDN emergente de uma baixa virulência, mas esta ocorrência não está documentada (Miller et al., 2009). Evidências que sugerem que vírus de baixa virulência possa se tornar altamente patogênica, e este tipo de mutação foram documentados na Austrália por Gould et al (2001) e na Irlanda por Alexander et al. (1992).

A análise filogenética do presente estudo evidenciou que no período 2002 a 2005, no Brasil, foram isolados vírus de baixa virulência e, portanto, não havia vírus virulento em aves comerciais durante os períodos de estudo e vírus isolados eram geneticamente idênticas ou semelhantes às vacinas comerciais utilizados no Brasil. Estes achados foram diferentes de Seal et al. (2005) que encontraram VDN de baixa virulência e não relacionados com cepas vacinais utilizados na América do Norte. Outros estudos, incluindo um tamanho de amostra maior e mais seqüências de nucleotídeos devem ser realizados para determinar o significado das poucas substituições de aminoácidos relatados neste estudo.

4.7 Isolamento e caracterização biológica dos vírus da Doença de Newcastle em aves migratórias no Brasil.

Os dados obtidos representam o primeiro isolamento e caracterização biológica do VDN em aves migratórias no Brasil. Segundo a literatura, a maioria dos VDN isoladas de aves migratórias e aves

selvagens, especialmente aves aquáticas, são não virulentos. No presente estudo, mais da metade (56,5%) dos vírus isolados foram patogênicos. Recentes comparações genéticas entre VDN em aves domésticas e aves aquáticas silvestres têm sugerido que os vírus que circulam entre várias espécies selvagens podem causar surtos em galinhas. Mutação e seleção do hospedeiro podem desempenhar um papel crucial, pois, aparentemente, o pré-requisito principal que determina a virulência das cepas é a presença de substituições de dois aa no sítio de clivagem da proteina F e a presença de um aminoácido fenilalanina na posição 117. No entanto, os mecanismos envolvidos ainda são desconhecidos (Zanetti, et al., 2005). No presente estudo, 13 estirpes patogênicas da DN foram obtidas e caracterizadas. Uma amostra isolada de Sterna hirundo apresentou IPIC com o valor de 1,77, semelhante aos resultados obtidos por Usachev et al. (2006) a partir de um espécime coletado no rio Volga Delta em 2001, onde a sequência deste vírus indicara que era velogênica.

No presente estudo, uma amostra do VDN apatogênica foi isolada de uma população de pombos. O paramixovírus sorotipo 1 (APMV-1) afeta pombos, sendo conhecido também por infectar aves (Capua et al., 2002; Kommer et al., 2002) e a virulência destes vírus tem sido relatada como variável (Meulemans et al., 2002). O índice de patogenicidade aumenta após passagens cegas em galinhas ou ovos embrionados, indicando que os vírus que circulam entre as populações de pombos podem levar a surtos da DN (Kommers et al., 2003; Kim et al., 2008). Oito estirpes VDN não patogênicos foram isolados, semelhante ao obtido por Zanetti et al. (2005) em um estudo com aves silvestres saudáveis na Argentina. Estes resultados são motivos de preocupação, pois há vários relatos sugerindo que amostras velogênicas surgem a partir de lentogênicas na natureza Gould et al., 2001). As alterações de virulência na transmissão de aves silvestres para domésticas e de isolados lentogênicos para velogênicos após passagem em galinha foram demonstrados por Shengqing et al. (2002). Considerando os IPIC encontrados, os resultados estão de acordo com aqueles obtidos por Liu et al. (2008), na China. No entanto, a mesma situação não foi encontrado por Lee et al. (2009), que relataram o isolamento na Coréia de 13 vírus não patogênicos e 1 patogênico. Espécies de patos produziram poucos sinais clínicos da doença após a infecção experimental, mesmo após a infecção com cepas do VDN que são altamente virulentas para galinhas, como mostrada pelo ensaio *in vivo* do presente estudo. No que diz respeito à porcentagem do VDN isolados de aves pertencentes a ordem dos Charadriiformes, os resultados estão de acordo com aqueles obtidos por Hanson et al. (2008), relativa à gripe aviária. Ao mostrar a presença do VDN patogênicos e não patogênicos em aves migratórias, silvestres residentes e domésticas, o presente estudo mostrou que as aves migratórias são portadoras viáveis do VDN e provável reservatório e potencial propagador às aves domésticas. O isolamento do VDN em aves domésticas com um índice de patogenicidade (IPIC) semelhante ao VDN encontrado em aves migratórias foi especialmente significativo. As amostras virais foram obtidas de

regiões onde não havia explorações avícolas em larga escala, sugerindo que os vírus estejam sendo transmitidos por aves migratórias ou silvestres autóctones. Estudos complementares deverão ser explorados para melhor entender estes aspectos.

5- CONCLUSÕES GERAIS

5. CONCLUSÕES GERAIS

5.1. Estudo da estabilidade térmica e proteção conferida pelas vacinas comerciais vivas da Doença de Newcastle usadas no Brasil:

- O presente estudo mostrou que o teste de termo estabilidade antecipa o seu desempenho durante 24 meses de armazenamento a uma temperatura entre 2 e 8º C e pode ser usado como substituição do método "A Fresco" com 24 meses, após a fabricação;
- Os resultados do desafio com vírus patogênico da doença de Newcastle mostraram que todas as vacinas apresentavam eficiência satisfatória;
- Resultados obtido deram suporte científico para mudança de titulo mínimo aceito pós fabricação de $10^{6,5}$ para $10^{6,2}$.

5.2. Estirpes vacinais da Doença de Newcastle versus imunogenicidade:

- Os resultados obtidos neste trabalho mostraram que a diferença no índice de patogenicidade intracerebral não interfere com a resposta imune em *Gallus gallus domestico.*
- As vacinas vivas usadas no país foram consideradas não virulentas e classificadas como lentogênicas de acordo com o padrão estabelecido para produtos imunobiológicos;

5.3. Prevalência do vírus da DN em frango de corte no Brasil:

- Trata-se do primeiro estudo para determinar a soroprevalência do VDN nas principais áreas de produção avícola brasileira foi de 39,1%;
- Foram isolados e caracterizados 77 vírus da doença de Newcastle em ovos embrionados SPF provenientes de 23.745 aves pertencentes a 1.583 lotes, com IPIC variando de 0,0 a 0,66;
- O isolamento viral por lote apresentou o percentual de 1,0 a 7,6% e por região de 6,5-58,4%;
- As maiores taxas de isolamento (74,3-83,3%) foram obtidas após três passagens em ovos embrionados de galinha (SPF);
- Todos os isolados foram identificados como VDN e caracterizadas como cepas não patogênicas (ICPI≤ 0,7);

- O resultado deste trabalho levou o Brasil reivindicar o status "livre do VDN virulento em aves comerciais" e facilitar o comércio internacional.

5.4- **Um levantamento para a manutenção de área livre de vírus virulento da Doença de Newcastle em áreas de produção de frango no Brasil**:

- A soroprevalência da infecção VDN foi detectada em 28,8% dos lotes pesquisados;
- Foram isolados e caracterizados, por meio do IPIC (0,0 a 0,47), 15 estirpes de VDN provenientes de 81.825 aves pertencentes a 5.455 lotes, com base em tais dados, o Brasil mantém o status "livre do VDN virulento nas aves comerciais".

5.5. Ausência de vírus altamente patogênico da Doença de Newcastle em regiões avícolas não exportadoras no Brasil:

- Somente VDN de baixa patogenicidade circula nas regiões avícolas não exportadoras.
- Foram isoladas 12 amostras VDN em amostras biológicas da região Nordeste, proveniente de 17.265 aves e pertencentes a 1.151 lotes; os VDN identificados foram caracterizados como cepas não patogênicas através do IPIC e apresentou valores de 0,0 a 0,54. Os estudos sorológicos revelaram, 84,3% de soropositividade nos lotes avaliados na região nordeste, 57,7% na região norte e 41,4% na região sudeste;

5.6. Caracterização molecular de isolados de campo do vírus da doença de Newcastle e das estirpes das vacinas usadas no Brasil:

- Os VDN isolados no período de 2002 a 2005 foram caracterizados como estirpes não patogênicas em aves comerciais;
- A análise filogenética mostrou que os isolados e vacinas utilizadas foram classificados nos genótipos I e II, sendo que 71,8% pertencem ao genótipo II, ou La Sota, e 28,2% ao genótipo I, ou Ulster–like;
- Em dois vírus isolados foram observadas modificações na sequencia de aa, sendo estas sinônimas sem potenciais para modificações nos produtos peptídicos. São eles: BR1568 →

mudança de nucleotídeo (nt) 116 - Argenina (R) → Alanina (A), mudança de aa. BR6425 → nt 114 - Glutamina (Q) →Treonina (T);

- Por meio do sequenciamento parcial do gene F ficou demonstrado que estas amostras virais são geneticamente idênticas ou semelhantes às estirpes vacinais utilizadas no Brasil, havendo circulação apenas de estirpes vacinais ou cepas derivadas de vacinas La Sota ou vacinas tipo Ulster.

5.7 Isolamento e caracterização biológica dos vírus da doença de Newcastle em aves Migratórias no Brasil:

- Foram isolados e caracterizados 23 VDN, sendo 13 patogênicas e 10 não patogênicas, com índice de patogenicidade intracerebral (IPIC) variando de 0,0 a 1,82;
- Não foi detectado o vírus nos estados do AP, PA e AL;
- Vírus não-patogênicos foram isolados nos estados de TO, PE e RS;
- Vírus patogênicos foram isolados no AM, MA e RN;
- Na BA, houve isolamento tanto de vírus patogênico como não patogênico;
- 78,3% dos VDN isolados foram obtidos a partir de aves da ordem Charadriiformes, 13% da ordem Passeriformes, e 4,3% de cada uma das ordens Anseriformes e Columbiformes;
- Na vigilância complementar 07 amostras do VDN patogênicos foram isoladas em patos e galinhas, 01 amostra não patogênica em galinhas e 03 não patogênicas em aves migratórias da ordem Charadriiformes, com IPIC similar ao obtido anteriormente nas aves migratórias.

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7- ANEXO

UNICAMP

CEUA/Unicamp

Comissão de Ética no Uso de Animais
CEUA/Unicamp

CERTIFICADO

Certificamos que o Protocolo nº **2059-1**, sobre **"Estudo dos virus e das estirpes vacinais da doença de Newcastle no Brasil"**, sob a responsabilidade de **Profa. Dra. Clarice Weis Arns / Maria Angela Orsi**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética no Uso de Animais – CEUA/Unicamp em **08 de fevereiro 2010**.

CERTIFICATE

We certify that the protocol nº **2059-1**, entitled **"Study of virus and vacinal strains of Newcastle disease"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - Unicamp) on **February 8, 2010**.

Campinas, 08 de fevereiro 2010.

Profa. Dra. Ana Maria A. Guaraldo
Presidente

Fátima Alonso
Secretária Executiva