

ISSN 1682-8356
ansinet.org/ijps



INTERNATIONAL JOURNAL OF POULTRY SCIENCE

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Genetic Grouping of Nephropathogenic Avian Infectious Bronchitis Virus Isolated in Morocco

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Abstract: Thirty Moroccan isolates of infectious bronchitis virus (IBV) recovered from unvaccinated broiler chickens flocks originated from different regions of Morocco between 1997 and 2002, and two references strains Massachusetts and 4/91 (vaccine strains) were classified by reverse transcriptase-polymerase chain reaction (RT-PCR) and restriction fragment length polymorphism (RFLP) analysis using two restriction enzymes Hae III and AluI. RFLP patterns of the amplified S1 gene of IBV (1720 bp) digested by two restriction enzymes HaeIII and AluI showed that Moroccan isolates of IBV were classified into five genotypes with Three genotypes different from vaccine strains, vaccine protection test showed that Massachusetts type vaccine could not protect chickens against challenge with this three new genotypes.

Key words: Nephropathogenic infectious bronchitis virus, RT-PCR, RFLP, massachusetts vaccine

Introduction

Infectious bronchitis, the prototype of the coronaviridae family in new order Nidovirales (Cavanagh *et al.*, 1997b), causes an acute and highly contagious disease of the respiratory and urogenital tract of Chickens worldwide distribution (Collisson *et al.*, 1992; Cavanagh and Naqi, 1997a) causing severe economic losses to the poultry industry (Maiti *et al.*, 1985; Muneer *et al.*, 1986; King, 1988; Ambali and Jone, 1990; Lucio and Fabrikant, 1990; Parson *et al.*, 1992; Wang *et al.*, 1996; Di Fabio *et al.*, 2000). IBV has a single stranded positive sense RNA genome that is 27.6 kilobase long (Boursnell *et al.*, 1987; Collisson *et al.*, 1992), encoded three major structural proteins, the nucleocapsid protein (N), the membrane protein (M) and the spike glycoprotein (S) which post-translationally cleaved in two subunits designated S1 and S2 (Cavanagh *et al.*, 1983 a, b). The S1 subunit is biologically the most important because contains epitomes for virus neutralization, cell attachment, cell tropism and serotype specificity (Mockett *et al.*, 1984; Niesters *et al.*, 1987; Collisson *et al.*, 1992; Wang *et al.*, 1997; Moore *et al.*, 1997, Casais *et al.*, 2003). The molecular characterization of IBV is based mainly on analysis of the S1 protein gene (Meir *et al.*, 2004; Huang *et al.*, 2004; Zhou *et al.*, 2004; Liu and Kong, 2004; Lee *et al.*, 2004; Ziegler *et al.*, 2002; Li and Yang, 2001; Cavanagh *et al.*, 2001a; Cavanagh, 2001b, De Wit, 2000; Kingsham *et al.*, 2000; Handberg *et al.*, 1999; Wang *et al.*, 1996; Kwon *et al.*, 1993). IBV undergo a high frequency of mutation resulting in the emergence of different serotypes (Gelb *et al.*, 1991; Cook and Huggins, 1986). Vaccination with one serotype does not ensure complete protection against heterologous

serotypes (Lambrechts *et al.*, 1993; Hofstad, 1981) which emerge by changes in the IBV genome by point mutation, deletions, insertions or recombination (Lee and Jackwood, 2001, Jia *et al.*, 1995; Wang *et al.*, 1994; King, 1988). Generation of genetic variants is created in nature resulting from few amino acid changes in the spike glycoprotein of IBV (Cavanagh *et al.*, 1992; Kant *et al.*, 1992). In Morocco, Massachusetts type vaccine was applied to prevent and control the incidence of the disease since 1960. IBV was first isolated in 1986 (EL Houadfi *et al.*, 1986); at that time, no one nephropathogenic case of IBV has been reported and outbreaks were easily controlled by vaccination. The first outbreak of nephropathogenic (NIB) was recorded in 1991 following by some cases in 1993 and 1995; since 1997-98 NIBV increased markedly with rate of mortality reaching between 10 to 60% in Massachusetts vaccinated and no vaccinated chicken flocks. 4/91 vaccine United Kingdom variant strain designated 4/91 (Parsons *et al.*, 1992) or 793/B (Gough *et al.*, 1992) was used but not extensively from the year 2001, but the disease persists in spite of the vaccination.

The objective of this work is to classify genotypically Moroccan avian nephropathogenic IBV field isolates recovered from unvaccinated broiler chickens and to investigate the ability of the Massachusetts type live attenuated vaccine to protect chickens against challenge by their isolates.

Materials and Methods

Viruses: Seventy IBV isolates were recovered from natural outbreaks of IB in different types of commercial chicken flocks unvaccinated and Massachusetts

vaccinated, between 1997 and 2002 originates from different regions in Morocco. Most poultry flocks had been experiencing kidney damages associated with high mortality in broilers and severe drop eggs production in layers. The viruses were isolated in Specific-pathogen-free (SPF) embryonated chicken eggs (ECE), propagated and titrated in Vero cell line cultures. The isolates were previously identified as IBV by RT-PCR and nested PCR using universal primers within 3' untranslated region (3'UTR) as described by Adzhar *et al.*, (1997). Only thirty isolates recovered from unvaccinated broiler chickens and two IBV reference strains, were used to classify genotypically Moroccan IBV isolates by RT-PCR-RFLP analysis.

Genotyping by RT-PCR-RFLP analysis: To perform further molecular characterization of strains such a genotyping classification by RT-PCR-RFLP, the whole S1 gene of strains was amplified by RT-PCR and digested by two restriction endonucleases Hae III and Alu I (Promega).

Viral RNA extraction: The RNA extraction was performed according to the method described by Lin *et al.*, 1991. Briefly, 20 to 30 ml of allantoic fluid obtained from inoculated eggs by stock viruses was centrifuged at 40.000 rpm for 120mn, the pellet was resuspended in a lysis buffer containing 10mM Tris-HCl pH 7.4, 0.1 mM EDTA, 1% w/v Sodium Dodecyl Sulfate (SDS) and 50 mM NaCl. Viral RNA was then extracted with phenol: chloroform: IsoAmyl Alcohol (IAA) (50:48:2), Ethanol precipitated and redissolved in 20µl of ultrapure water free of DNase and RNase.

Primers: The primers described by Kwon *et al.* (1993) were used for reverse transcription reaction and for amplification of the S1 glycoprotein gene. S1 OLIGO3': 5'CATAACTAA CATAAGGGCAA3' is complementary to a region at the 5'end of the S2 glycoprotein gene, and S1 OLIGO5' 5'TGAAAAGTGAACAAAAGACA3' is identical to a sequence near the 5' end of the S1 glycoprotein gene (Promega).

RT-PCR: The RT-PCR was conducted as described by Kwon *et al.* (1993), with slight modification. Briefly the reverse transcription reaction was performed in 20µl of a mixture containing 2µl of 10x PCR buffer (500mM KCl, 200mM Tris-HCl (pH 8.4), 0.5mg/ml nuclease free bovine serum albumin (BSA), 2µl of 10mM each dNTP, 250ng primer S1 OLIGO3', 20U RNasin (Promega), 1.5µl of 80 mM MgCl₂, 5µl RNA solution, a reaction volume of 20µl was obtained by adding sterile DEP treated ultrapure water, after heating at 65°C for 10mn, 200U of M.MLV-RT (Promega) was added and the mixture was incubated for 1h at 45°C, after incubation the reaction was stopped by heating to 95°C for 5mn.

The PCR was performed in 100µl of a mixture containing 8µl of 10x PCR buffer, 250ng of the S1 OLIGO5' primer, 3.5 µl of 80 mM MgCl₂, 5 Units Taq DNA polymerase (Promega) were added to the reaction mixture, a 100µl total reaction volume was obtained by adding sterile DEP treated ultrapure water. PCR was performed by 35 cycles of denaturation at 94°C for 1mn, annealing at 47°C for 1mn and polymerization at 72°C for 3mn. The initial denaturation step was conducted at 95°C for 5mn and the final elongation step at 72°C for 10mn. The PCR products were analyzed on a 1% agarose gel containing Ethidium bromide (0.5µg / ml).

RFLP analysis: The S1 products with a predicted size of 1720 bp were digested with two restriction endonucleases Hae III (Amersham Pharmacia biotech) and AluI (Promega) according to the manufacturers specifications. The restriction fragment patterns were observed following electrophoresis in a 2% agarose gel at 80V constant voltage.

Protection test: Three experiments were conducted to examine the protection afforded by live MA5 (Massachusetts type) vaccination, against challenge with three strains representing three genotypes different from vaccine strains type Massachusetts.

Protocol design: Sixty 1 day-old Specific-pathogen-free (SPF) chicks were placed in HEPA-filtered positive pressure isolation unit. Every experimental group is composed of 12 birds. The vaccination and the challenge of birds were administered by oculonasal route in a volume of 100µl. Three groups designed (1, 2, 3) of birds three week-old were vaccinated with two doses (about 2.10^3 EID₅₀) of Massachusetts type vaccine: MA5 (Intervet, Morocco). Three weeks later (at six weeks of age), the birds were challenged respectively with 10^4 CTID₅₀ of one of the three field isolates A, B, C of IBV which represent respectively genotype I, II, and III.

- One group of birds vaccinated with two doses of Massachusetts strain has kept as vaccine control.
- One group of birds no vaccinated and no challenged has kept as negative control.
- All groups were placed in separate rooms to avoid cross contamination.
- The birds were inspected daily for clinical respiratory signs and signs indicative of nephritis such as hunched posture, depression, reluctance to move, and diarrhea (Cumming, 1963; Siller and Cumming 1974).
- Clinical signs and mortality were recorded daily until 21 days post challenge.
- The serum antibody titers of birds were measured with commercial Kit (Kits IDEXX) for antibody of IBV, ND and IBD, at the age of 20 days (before

vaccination) and three weeks after vaccination (before challenge).

- g. Two birds from infected groups and no infected groups were necropsied at 7, 12, 15 and 21 days pi. Small portions from kidneys were collected aseptically, from each bird had died or experiencing damages of kidneys and fixed in neutral buffered Formalin for histological examination. The protection level was evaluated by assessing mortality rate, severity of clinical signs, macroscopic and microscopic changes in kidneys.

Histopathological examination: Fixed kidney tissues were embedded in paraffin, sectioned at 4 to 5µm by conventional methods and stained with Haematoxylin-Eosin prior to be examined microscopically for histopathological changes for typical lesions of avian nephropathogenic infectious bronchitis.

Results

Amplification of S1. The amplified S1 glycoprotein gene of 30 IBV Moroccan isolates and two vaccine strains Massachusetts type (MA5) and 4/91 had the predicted size of 1720 bp, Fig. 1.A-B, excepted the RT-PCR product of strain Mo/83/99 which had a product size of approximately 600bp, as shown in lane 8 of Fig. 1B.

Genotyping by RFLP. The 29 Moroccan isolates of IBV were classified into five genotypes according to their RFLP patterns, using two restriction enzymes HaeIII and AluI. Representative cleavage profiles with HaeIII and AluI digestion are shown in Fig.1.C-D. Five of the 30 isolates (17%) belonged to genotype I and showed different RFLP patterns than vaccine strains Massachusetts and 4/91 when digested with HaeIII or AluI; these isolates have been recovered from poultry flocks between 1997 and 2001. Eight of the 30 isolates (27%) belonged to genotype II, the RFLP patterns of this group isolates was similar to vaccine strain Massachusetts type when digested with HaeIII, but differed from those when digested with AluI, These isolates have been recovered from the poultry flocks between 1998 and 2001. Four of the 30 isolates (13%) belonged to genotype III, the RFLP patterns of this group isolates was similar to vaccine strain 4/91 (Intervet) when digested with AluI, but differed from those when digested with HaeIII, these isolates have been recovered from the poultry flocks between 2000 and 2001. Eight of the 30 isolates (27%) recovered from the poultry flocks between 1997 and 2001, belonged to genotype IV, the RFLP patterns of this group isolates was similar to vaccine strain Massachusetts type digested with Hae III or AluI. Four of the 30 isolates (13%) recovered from the poultry flocks between 2000 and 2002 belonged to genotype V, the RFLP patterns of this group isolates was similar to vaccine strain 4/91 (Intervet) digested with Hae III or AluI.

ELISA test: There were no detectable antibodies against the three viruses of the diseases of Bronchitis Infectious (BI), Newcastle Disease (ND) and Infectious Bursal Disease (IBD), at three weeks of age before vaccination. All sera collected from chickens three weeks after vaccination (at six weeks of age) were positive in ELISA test for antibodies anti-IBV, and negative for antibodies anti-NDV and anti-IBDV.

Protection test: The results obtained are summarized in Table 1. The vaccine Massachusetts type (MA5) didn't protect the chicken against the challenge with the three new genotypes I, II and III, to the level of the kidney, but apparently it could protect the chicken to the level of the respiratory tract since no respiratory sign has been observed to the level of the all chickens. Rate mortality is maintained very elevated either 50% and 30% for genotype I and II respectively in spite of vaccination.

Histopathology

All strains induced histological lesions of challenged bird kidneys. The lesions consisted of a focal proliferation of the inflammatory cells mainly heterophils, around proximal and distal tubules, massive infiltration of inflammatory cells heterophiles and lymphocytes in interstitium (diffuse interstitial nephritis) nephritis with severe degeneration of parenchyma tissue, necrosis in epithelial cells and their desquamation of the basement membrane of the collecting tubules and the collecting ducts, dilatation of lumen of some renal tubules and collecting ducts with deposits of urates and debris of necrotic cells in their lumina (Fig. 2).

Discussion

The result of RT-PCR-RFLP showed the presence in Morocco of five field IBV genotypes with three new IBV genotypes different from vaccine strains Massachusetts type used in Morocco since 1960 and 4/91 used since 2000, the fourth genotype was similar to vaccine strain MA5 (Massachusetts type) and the fifth genotype was similar to vaccine strain 4/91. Concerning chicken Mo/83/99 isolate, the primers didn't amplify the complete sequence of the S1 gene; this could be due to a mutation, a deletion or an insertion of basis to the level of the S1 sequence. The field isolates of genotype II have RFLP fragments similar to those of the Massachusetts vaccine after digestion by the HaeIII enzyme; suggest that the strains of this genotype are originated of the Massachusetts vaccine. Whereas the isolates belonging to genotype III have RFLP fragments similar to those of the 4/91 vaccine after digestion by the AluI enzyme; suggest that the strains of this genotype are originated of the 4/91 vaccine, indeed several study suggested that the prevalent strains may have originated by recombination with the vaccine strains (Kusters *et al.*, 1987). However the isolates of genotype I have a RFLP patterns completely different from current vaccine strain

Table 1: Clinical and necropsic signs in challenged SPF chickens six week old with three IBV strains representing three genotypes different from vaccines strains Massachusetts

Days post challenge	Group 1				Group 2				Group 3			
	Resp	Neph	Diar	Mort	Res	Neph	Diar	Mort	Res	Neph	Diar	Mort
7	-	+	-	1	-	+	-	-	-	+	-	-
12	-	+++	++	3	-	++	+	2	-	++	+	-
15	-	+++	++	2	-	++	+	1	-	+	-	-
21	-	++	-	-	-	+	-	1	-	-	-	-

Resp: Respiratory signs, Neph: Nephritic signs, Diar: Diarrhoea, Mort: mortality. + : mild, ++ : moderate, +++: severe, - : non signs (or no Mortality).

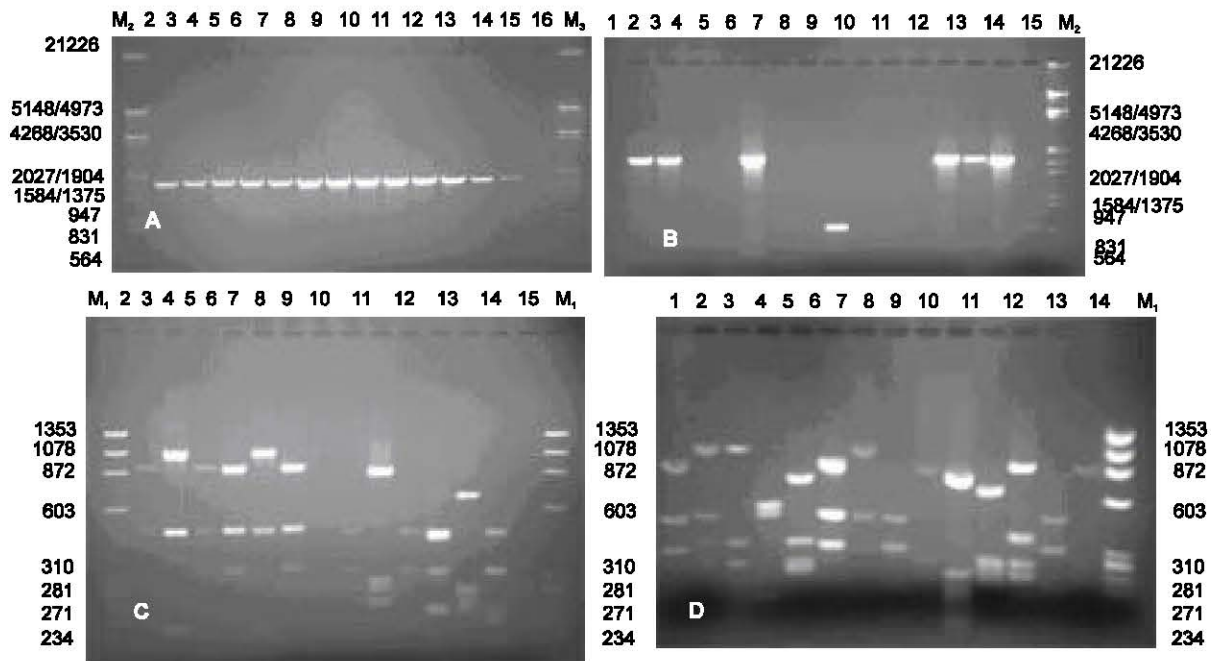


Fig. 1: A. Agarose gel electrophoresis of RT-PCR amplified S1 gene from Moroccan IBV isolates, lane 3 to 14 and two reference strains of IBV (Vaccine strains) lane 2: Massachusetts (MA5), lane 3: 4/91. Lane 15: water control, Lane 1 and 16 M2: molecular weight marker III. B. Agarose gel electrophoresis of PCR amplified S1 gene from Moroccan IBV isolates, lane 1 to 14, Lane 8: Mo/83/99* isolate, Lane 15: water control, Lane 16: M2: molecular weight marker III. C. RFLP patterns of Moroccan IBV isolates, lane 2 : Mass, lane 3: 4/91, the lanes 4, 5, 6, 7, 8 represent the isolates of IBV I, II, III, IV and V, respectively digested with HaeIII, the lanes 9, 10, 11, 12, 13, 14, 15 represent the same isolates digested with AluI. M1: Phi X 174 Hae III digested. D. RFLP patterns of Moroccan IBV isolates., lane 1: Mass, lane 2: 4/91, the lanes 3, 4, 5, 6, 7, 8 represent the isolates of IBV VI, VII, VIII, IX and X respectively, digested with HaeIII, the lanes 9, 10, 11, 12, 13, 14, 15 represent the same isolates digested with AluI. M1: Phi X 174 Hae III digested.

*: named as suggested by Cavanagh 2001c, "country / isolate number / year of isolation". Mo: Morocco.

and the others isolates and seemed to be newly introduced in the poultry flocks in Morocco. The clinical manifestations, the gross kidney lesions, the high mortality rate and the histopathological lesions to the level of the kidneys of the chickens vaccinated with Massachusetts type and challenged with the new three genotypes of IBV, were similar to those described in previous outbreaks of cases of nephropathogenic IBV (Lee *et al.*, 2004; Zhou *et al.*, 2004; Ignjatovic *et al.*, 2003; Ignjatovic *et al.*, 2002; Ziegler *et al.* 2002; Butcher *et al.*,

1989 and Brown *et al.*, 1987; Siller and Cumming, 1974), this result showed also that Massachusetts type vaccine does not protect chickens against this three new genotypes, this finding is in agreement with the result obtained by (Cook *et al.*, 2001; Lambrechts *et al.*, 1993) which reported that vaccines of the Massachusetts type not protect against challenge with nephropathogenic IBV strains. The results of the protection test permit to conclude that the outbreaks of the IB observed lately in Morocco were due to these three new genotypes of

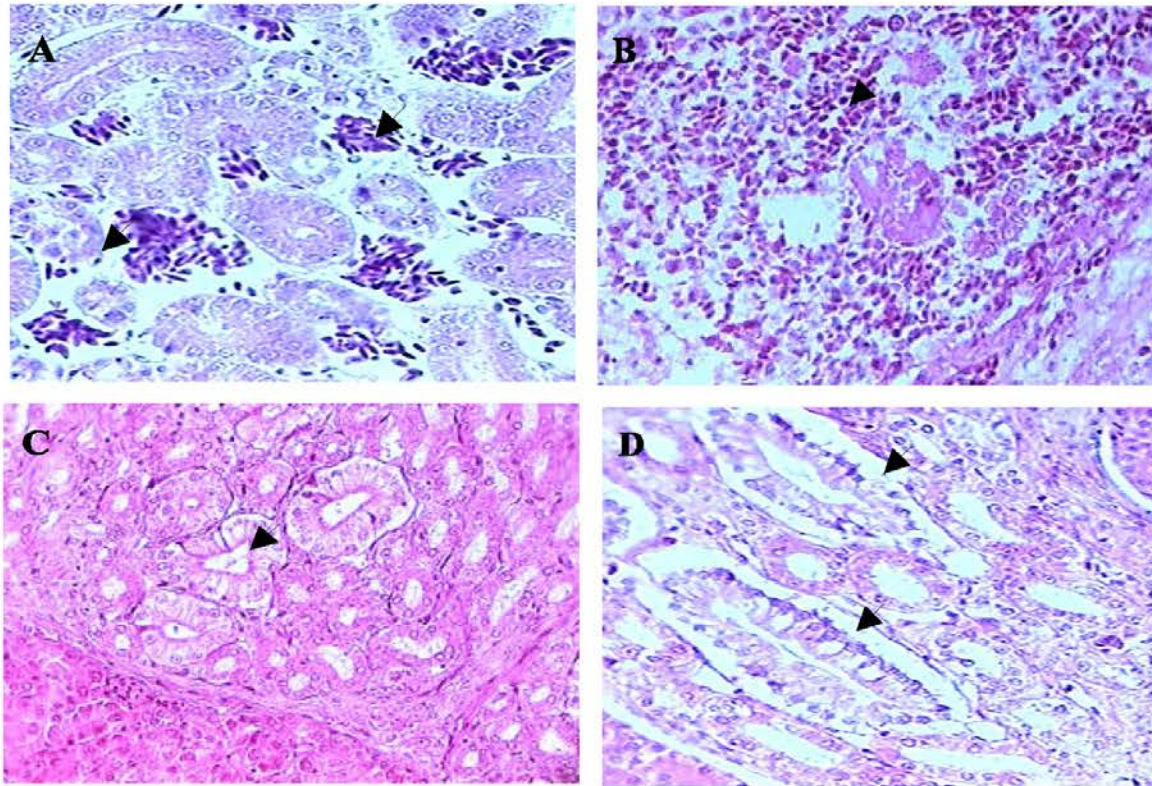


Fig. 2: Histological lesions of kidneys of chickens challenged at six weeks of age with genotypes I, II, III. A. Focal interstitial nephritis (focus of inflammatory cells infiltration: monocytes and heterophiles) B. Diffuse interstitial nephritis with severe degeneration of parenchyma tissue. C. Epithelial cells degeneration of collecting ducts with deposits of urates crystals (note arrows). D. Epithelial cells degeneration of collecting ducts and epithelial cells detachment of the basal membrane.

IBV, because the chicken was not protected against the challenge with these three new nephropathogenic IBV strains, in spite of the presence of anti-IBV antibody at the chicken vaccinated with the Massachusetts type. Indeed vaccination with one serotype does not ensure complete protection against heterologous serotypes (Lambrechts *et al.*, 1993; Hofstead, 1981) which emerge by changes in the IBV genome by point mutation, deletions, insertions or RNA recombination (Lee and Jackwood, 2001; Jia *et al.*, 1995; Wang *et al.*, 1993, 1994; King, 1988; Cavanagh *et al.*, 1988) which were responsible for outbreaks of IB in the vaccination chicken flocks. In addition to serotype changes, the genetic variation may result in changes of the tissue tropism and pathogenicity of the virus which lead to the generation of new IBV pathotype. Sequencing, comparison with reference IBV strains and cross protection tests are necessary to know the origin of these new isolates and for future avian infectious bronchitis disease control in Morocco.

Acknowledgements

We wish to thank the Moroccan National Research

Council (PARS and PROTAS) and Direction Elevage Agricultural Ministry Morocco for the financial support.

References

- Adzhar, A., R.E. Gough, D. Haydon, K. Shaw, P. Britton, and D. Cavanagh, 1997. Molecular analysis of the 793/B serotype of infectious bronchitis virus in Great Britain. *Avian Path.*, 26: 625-640.
- Ambali, A.G. and R.C. Jone, 1990. Early pathogenesis in chicks of infection with an enterotropic strain of infectious bronchitis virus. *Avian Dis.*, 34: 865-870.
- Boursnell, M.E., T.D. Brown, I.J. Foulds, P.F. Green, F.M. Tomley and M.M. Bins, 1987. Completion of the sequence of the genome of the coronavirus avian infectious bronchitis virus. *J. Gen. Virol.*, 68: 57-77.
- Brown, T.P., J.R. Gilsson, G. Rosales, P. Villegas and R.B. Davis, 1987. Studies of avian urolithiasis associated with an infectious virus. *Avian Dis.*, 31: 629-636.
- Butcher, G.D., R.W. Winterfield and D.P. Shapiro, 1989. An outbreak of nephropathogenic H13 Infectious bronchitis in commercial broiler. *Avian Dis.*, 33: 823-826.

- Casais, R., B. Dove, D. Cavanagh and P. Britton, 2003. Recombinant avian infectious bronchitis virus expressing a heterologous spike gene demonstrates that the spike protein is a determinant of cell tropism. *J. Virol.*, 2003 Aug., 77: 9084-9089.
- Cavanagh, D., 1983a. Coronavirus IBV: further evidence that the surface projections are associated with two glycopolypeptides. *J. Gen. Virol.*, 64: 1787-1791.
- Cavanagh, D., 1983b. Coronavirus IBV: structural characterisation of the spike protein. *J. Gen. Virol.*, 64: 2577-2583.
- Cavanagh, D., P.J. Davis and A. Mockett, 1988. Amino acids within hypervariable region 1 of avian coronavirus IBV (Massachusetts serotype) spike glycoprotein are associated with neutralization epitopes. *Virus Res.*, 11, 141-150.
- Cavanagh, D., P.J. Davis and J.K.A. Cook, 1992. Infectious bronchitis virus: evidence for recombination within the Massachusetts serotype. *Avian Path.*, 21: 401-408.
- Cavanagh, D. and S.A. Naqi, 1997a. Infectious Bronchitis, In: B.W. Calnek, H.J. Barnes, C.W. Beard, L.R. McDougald & Y.M. Saif, Diseases of Poultry, 10th Eds, Ames, Iowa State University Press. pp: 511-526.
- Cavanagh, D., D.A. Brian, M.A. Brinton, L. Eujananes, K.V. Homles, M.C. Horzink, M.M.C. Lai, H. Laude, P.J.W. Plageman, S.G. Siddell, W. Spaan, F. Taguehi and P.G. Talbot, 1997b. Nidovirales: a new order comprising Coronaviridae and arteriviridae. *Arch. Virol.*, 142: 629-633.
- Cavanagh, D., K. Mawditt, M. Sharma, S.E. Drury, H.L. Ainsworth, P. Britton and R.E. Gough, 2001a. Detection of a coronavirus from turkey poult in Europe genetically related to infectious bronchitis virus of chickens. *Avian Path.*, 30: 365-378.
- Cavanagh, D., 2001b. Innovation and discovery: the application of nucleic-based technology to avian virus detection and characterisation. *Avian Path.*, 30: 581-598.
- Cavanagh, D., 2001c. A nomenclature for avian Coronavirus isolates and the question of species status. *Avian Path.*, 30: 109-115.
- Collisson, E.W., R.L. Parr, L. Wang and A.K. Williams, 1992. An overview of the molecular characteristics of avian infectious bronchitis virus. *Poult. Sci. Rev.*, 4: 41-55.
- Cook, J.K.A. and M.B. Huggins, 1986. Newly isolated serotypes of infectious bronchitis virus disease. *Avian Path.*, 15: 129-138.
- Cook, J.K., J. Cheshier, W. Baxendale, N. Greenwood, M.B. Huggins and S.J. Orbell, 2001. Protection of chickens against renal damage caused by a nephropathogenic infectious bronchitis virus, *Avian Path.*, 30: 423-426.
- Cumming, R.B., 1963. Infectious avian nephrosis (uraemia) in Australia. *Aust. Vet. J.*, 39: 145-147.
- De Wit, J.J., 2000. Detection of infectious bronchitis virus. *Avian Path.*, 29: 71-93.
- Di Fabio, J., L.I. Rossini, S.J. Orbell, G. Paul, M.B. Huggins, A. Malo, B.G. Silva and J.K. Cook, 2000. Characterisation of infectious bronchitis viruses isolated from outbreaks of disease in commercial flocks in Brazil. *Avian Dis.*, 44: 582-589.
- EL Houadfi, M.D., R.C. Jones, J.K.A. Cook and A.G. Ambali, 1986. The isolation and characterisation of six avian infectious bronchitis virus isolated in Morocco. *Avian Path.*, 15: 93-105.
- Gelb, J.J., J.B. Wolff and C.A. Moran, 1991. Variant serotypes of infectious bronchitis virus isolated from commercial layer and broiler chickens. *Avian Dis.*, 35, 82-87.
- Gough, R.E., C.J. Randall, M. Dagless, D.J. Alexander, W.J. Cox and D. Parsons, 1992. A new strain of infectious bronchitis virus infecting domestic fowl in Great Britain. *Vet. Rec.*, 130: 493-94.
- Handberg, K.J., O.L. Nielsen, M.W. Pedersen and P.H. Jorgensen, 1999. Detection and strain differentiation of infectious bronchitis virus in tracheal tissue from experimentally infected chickens by reverse transcription-polymerase chain reaction. Comparison with an immunohistochemical technique. *Avian Path.*, 28: 327-335.
- Hofstad, M.S., 1981. Cross immunity in chickens using seven isolates of avian infectious bronchitis virus. *Avian Dis.*, 25: 650-654.
- Huang, Y.P., H.C. Lee, M.C. Cheng and C.H. Wang, 2004. S1 and N gene analysis of avian infectious bronchitis viruses in Taiwan. *Avian Dis.*, 48: 581-589.
- Ignjatovic, J., R. Reece, F. Asthon, P. Scott and P. Hooper, 2002. Pathogenicity of Australian Strains of Avian Infectious Brochitis Virus. *J. Comp. Path.*, 126, 115-123.
- Ignjatovic, J., R. Reece and F. Asthon, 2003. Susceptibility of Three Genetic Lines of Chicks to Infection with a Nephropathogenic T Strain of Avian Infectious Bronchitis Virus. *J. Comp. Path.*, 128:92-98.
- Jia, W., K. Karaca, C.R. Parrish and S.A. Naqi, 1995. A novel variant of avian infectious bronchitis virus resulting from recombination from three different strains. *Arch. Virol.*, 140: 259-271.
- Kant, A., O. Koch, D.J. Van Roozelaar, J.O. Kusters, F.A.J. Poelwijk and B.A.M. Van Der Zeijst, 1992. Location of antigenic sites defined by neutralizing monoclonal antibodies on the SI avian infectious bronchitis virus glycopolypeptide. *J. Gen. Virol.*, 73: 591-596.
- King, D.J., 1988. Identification of recent infectious bronchitis virus isolates that are serologically different from current vaccine strains. *Avian Dis.*, 32: 362-364.

- Kingsham, B.E., C.L. Keeler, Jr., W.A. Nix, B.S. Landman and J. Gelb Jr., 2000. Identification of avian infectious bronchitis virus by direct automated cycle sequencing of the S-1 gene. *Avian Dis.*, 44: 325-335.
- Kusters, J.G., H.G.M. Niesters, N.M.C. Bleumink-Plym, F.G. Davelaar, M.C. Horzinek and B.A.M. Van Der Zeijst, 1987. Molecular epidemiology of infectious bronchitis virus in the Netherlands. *J. Gen. Virol.*, 68: 343-352.
- Kwon, H.M., M.W. Jackwood and J. Gelb, 1993. Differentiation of infectious bronchitis virus serotypes using polymerase chain reaction and restriction fragment length polymorphism analysis. *Avian Dis.*, 37: 194-202.
- Lambrechts, C., M. Pensaert and R. Ducatelle, 1993. Challenge experiments to evaluate cross-protection induced at the trachea and kidney level by vaccine strain and Belgian nephropathogenic isolates of infectious bronchitis virus. *Avian Path.*, 22: 577-590.
- Lee, C.W. and M.W. Jackwood, 2001. Spike gene analysis of the DE072 strain of infectious bronchitis virus: origin and evolution. *Virus Gene*, 22: 85-91.
- Lee, S.K., H.W. Sung and H.M. Kwon, 2004. S1 glycoprotein gene analysis of infectious bronchitis viruses isolated in Korea. *Arch. Virol.*, 149: 481-494.
- Li, H. and H. Yang, 2001. Sequence analysis of nephropathogenic infectious bronchitis virus strains of the Massachusetts genotype in Beijing. *Avian Path.*, 30: 535-541.
- Lin, Z., A. Kato, Y. Kudou and S. Ueda, 1991. A new typing method for the avian infectious bronchitis virus using polymerase chain reaction and restriction enzyme fragment length. *Arch Virol.*, 116: 19-31.
- Liu, S. and X. Kong, 2004. A new genotype of nephropathogenic infectious bronchitis virus circulating in vaccinated and non-vaccinated flocks in China. *Avian Path.*, 33: 321-327.
- Lucio, B. and J. Fabrikant, 1990. Tissue tropism of three cloacal isolates and Massachusetts strain of infectious bronchitis virus. *Avian Dis.*, 34: 865-870.
- Maiti, N.K., S.N. Sharma and D.S. Sambyal, 1985. Isolation of infectious bronchitis virus from intestine and reproductive organs of laying hens with dropped eggs production. *Avian Dis.*, 29: 509-513.
- Meir, R., E. Rosenblut, S. Perl, N. Kass, G. Ayali, S. Perk, and E. Hemsani, 2004. Identification of a novel nephropathogenic infectious bronchitis virus in Israel. *Avian Dis.*, 48: 635-641.
- Mockett, A., D. Cavanagh and T. Brown, 1984. Monoclonal antibodies to the S1 spike and membrane proteins of avian infectious coronavirus strain Massachusetts M41. *J. Gen. Virol.*, 65: 2281-2286.
- Moore, K.M., M.W. Jackwood and D.A. Hilt, 1997. Identification of amine acid involved in a serotype and neutralization specific epitope within the S1 subunit of avian infectious bronchitis virus. *Arch. Virol.*, 142: 2249-2256.
- Muneer, M.A., D.A. Halvorson, V. Sivanandan, J.A. Newman and C.N. Coon, 1986. Effects of infectious bronchitis virus (Arkansas strain) on laying chickens. *Avian Dis.*, 30: 644-647.
- Niesters, H.G., N.M. Bleumink-Pluym, A.D. Osterhaus, M.C. Horzinek and B.A. Van Der Zeijst, 1987. Epitopes on the peplomer protein of infectious bronchitis virus strain M41 as defined by monoclonal antibodies. *Virol.*, 161: 511-519.
- Parsons, D., M.M. Ellis, D. Cavanagh and J.K. Cook, 1992. Characterisation of an infectious bronchitis virus isolated from vaccinated broiler breeder flocks. *Vet Rec.*, 131: 408-411.
- Siller, W.G. and R.B. Cumming, 1974. The Histopathology of an interstitial nephritis in the fowl produced experimentally with infectious bronchitis virus. *J. Path.*, 114: 163-173.
- Wang, L., D. Junker and E.W. Collisson, 1993. Evidence of natural recombination within the S1 gene of infectious bronchitis virus. *Virol.*, 192: 710-716.
- Wang, L., D. Junker, L. Hock, E. Ebiary and E.W. Collisson, 1994. Evolutionary implication of genetic variations in the SI gene of infectious bronchitis virus. *Virus Res.*, 14: 327-338.
- Wang, C.H., M.C. Hsieh and P.C. Chang, 1996. Isolation, pathogenicity, and H120 protection efficacy of infectious bronchitis virus isolated in Taiwan. 1996. *Avian Dis.*, 40: 620-625.
- Wang, H.N., Q.Z. Wu, Y. Huang and P. Liu, 1997. Isolation and identification of infectious bronchitis virus from chickens in Sichuan, *Avian Dis.*, 41: 279-282.
- Ziegler, A.F., B.S. Ladman, P.A. Dun, A. Schneider, S. Davison, P.G. Miller, H. Lu, D. Weinstock, M. Salem, R.J. Eckroade and J. Gelb, Jr., 2002. Nephropathogenic infectious bronchitis in Pennsylvania chickens 1997-2000. *Avian Dis.*, 46: 847-858.
- Zhou, J.Y., D.Y. Zhang, J.X. Ye and L.Q. Cheng, 2004. Characterization of an avian infectious bronchitis virus isolated in China from chickens with nephritis. *J. Vet. Med. B.*, 51: 147-152.