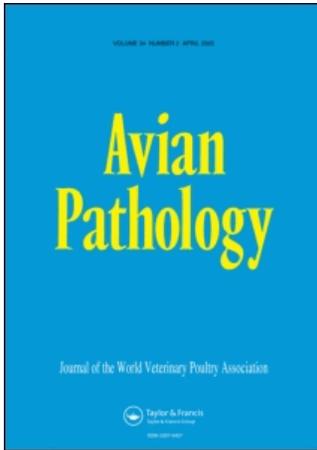


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Cloning, expression and immunogenicity of the avian pneumovirus (Colorado isolate) F protein

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The F protein of the Colorado isolate of avian pneumovirus (APV), expressed from a DNA plasmid, was recognized by antiserum to both A and B subgroup APVs. After two intramuscular injections of turkeys with this plasmid, a homologous antibody response was detected by enzyme-linked immunosorbent assay. This antibody also recognized subgroup A APV. However, there was no neutralization of the Colorado isolate or of subgroup A or B viruses. Although no significant clinical protection was detected following homologous challenge of poults, an anamnestic serological response was seen, suggesting that a systemic antibody response but no local mucosal immunity was induced.

Introduction

Avian pneumovirus (APV) is a major cause of disease of turkeys. Until recently, two subgroups (A and B) were recognized within a single serotype, based on the G glycoprotein sequence and neutralization tests with G-specific monoclonal antibodies (mAbs) (Collins *et al.*, 1993; Cook *et al.*, 1993; Juhasz & Easton, 1994). However, there is good cross-protection between the subgroups (Cook *et al.*, 1995; Etteradossi *et al.*, 1995). In 1997 a new APV, the Colorado isolate (Colorado), was isolated from turkeys in Colorado (Senne *et al.*, 1997) and subsequently in Minnesota, USA (Jirjis *et al.*, 2000). Although vaccines developed to the A and B subgroups protect well against challenge with Colorado, molecular sequencing has revealed major differences from the A and B subgroups (Seal, 1998). Colorado is not neutralized by any of the mAbs (Cook *et al.*, 1993) that recognize the A and B subgroups, or by monospecific antisera to these two subgroups (Cook *et al.*, 1999). It is, however, partially neutralized by a hyperimmune subgroup A antiserum. These findings suggest that it represents a third subgroup, or possibly a new serotype of APV (Cook *et al.*, 1999). Recently, non-A, non-B APVs have been described in France, suggesting further antigenic diversity (Toquin *et al.*, 2000).

The F gene sequences of Colorado (Dar *et al.*, 1999) and the Minnesota isolate (Seal *et al.*, 2000) have been published. The F protein is one of the major immunogens expressed on the surface of pneumoviruses and, as such, is important in inducing protective immunity (Yu *et al.*, 1994). In this paper, we report the expression of the F gene of Colorado from a DNA plasmid and the induction of an immune response in turkeys following intramuscular injection of this plasmid.

Materials and methods

Messenger RNA (mRNA) was isolated from secondary chick embryo fibroblasts (CEF) 72 h post-infection with \log_{10} 6.0 median tissue culture infective doses (TCID₅₀) of Colorado, using the polyAtract 1000 kit (Promega), and was subjected to a reverse transcriptase polymerase chain reaction (RT-PCR) using the Access RT-PCR system (Promega). The sequences of the primers used were: CCCGGGA-CAAGTG (based on the conserved sequences between APV genes) and ACTGGAAGAATTCGCGGCCGAGGAATTTTTTTTTTTTTTTTTT.

A 1.6 kb RT-PCR product was cloned into the pGEM T-easy vector (Promega) and two independent clones selected for sequencing. There was a single base substitution between our F clones and that reported for Colorado (Dar *et al.*, 1999) and the Minnesota isolate (Seal *et al.*, 2000), causing an amino acid change at position 192 (S to G). The significance of this change remains to be established.

The F gene was sub-cloned into the pUC-derived eukaryotic expression vector pI.18, bringing it under the transcriptional control of the human CMV promoter and intron A sequence. Secondary CEF were transfected with the F/pI.18 plasmid, fixed, and stained using goat

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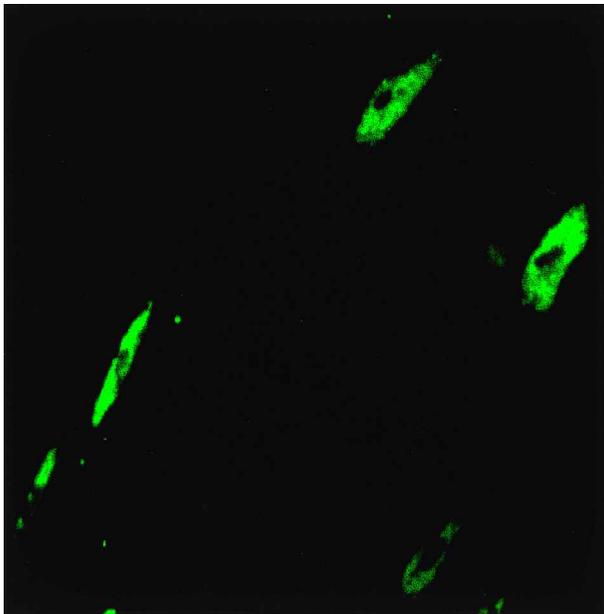


Figure 1. Expression of the F protein of the Colorado isolate in chick embryo fibroblasts. CEFs were transfected with the F/pI.18 plasmid, and expression of the F protein detected with chicken serum specific for the Colorado isolate and goat anti-chicken IgG-FITC conjugate.

anti-chicken immunoglobulin (Ig)G (H + L) FITC conjugate (KPL) after incubation with the following antisera: a monospecific and a hyperimmune antiserum raised in specific pathogen free (SPF) chickens to Colorado (Cook *et al.*, 1999); a monospecific antiserum raised in SPF chickens and a hyperimmune serum raised in turkeys against the subgroup A strain 8544; and a monovalent and a hyperimmune antiserum raised in SPF chickens to subgroup B strain 11/94 and a hyperimmune antiserum raised in SPF chickens against the Minnesota isolate (Cook *et al.*, 1999).

Results and discussion

Expression of the F protein in the cytoplasm of CEF is shown in Figure 1 and is the first report on the

expression of the F gene of Colorado. Both monospecific and hyperimmune antisera raised to subgroups A and B recognized the expressed F protein, as did antisera raised against both the Colorado and Minnesota isolates.

Three experiments were performed in commercial turkeys, obtained as 1-day-old poults from a commercial breeding company, and shown to be APV-negative serologically.

In Experiment 1, 10 poults immunized once at 5 days of age by intramuscular (i.m.) injection of 100 µg F/pI.18 plasmid were bled 7 weeks later. No APV antibody was detected by enzyme-linked immunosorbent assay (ELISA) (Cook *et al.*, 1996) using either A or B subgroup or Colorado as antigen.

In Experiment 2, groups of 10 poults were immunized once, at 5 days of age, by i.m. injection of 100 µg of either the F/pI.18 plasmid or a control plasmid (pI.18). All poults were bled at 29 days of age and the sera tested as already described. No APV-specific antibody was detected. The poults were challenged oculonasally (o.n.) at 42 days with log₁₀ 2.5 TCID₅₀ of virulent APV (Colorado) (Cook *et al.*, 1999). Clinical signs were assessed (Cook *et al.*, 1999) between 3 and 10 days post-challenge, and the results are summarized in Table 1. The single immunization provided no priming (no anamnestic response) and no protection against challenge.

In Experiment 3, groups of 8 poults were injected with 100 µg of either the F/pI.18 or pI.18 plasmid at 5 and 26 days of age. All poults were bled at 26 and 42 days, and the sera tested for APV antibodies. No antibodies were detected at 26 days of age, but at 42 days (using Colorado as antigen) APV antibodies were detected in 4/8 poults that had received the F/pI.18 plasmid (Table 2). Although titres were not high, they were

Table 1. Protection provided by DNA vaccination of turkeys against challenge with the Colorado isolate of avian pneumovirus

Day post-challenge	Clinical sign scores in DNA-vaccinated poults challenged with Colorado											
	Experiment 2 (one vaccination at 5 days) ^a						Experiment 3 (two vaccinations at 5 and 26 days) ^b					
	F/pI.18 plasmid		pI.18 plasmid		Not vaccinated		F/pI.18 plasmid		pI.18 plasmid		Not vaccinated	
Number ^c	Score ^d	Number	Score	Number	Score	Number	Score	Number	Score	Number	Score	
3	3	0.3	1	0.1	4	0.9	0	0	0	0	1	1.38
4	6	1.7	5	0.7	9	4.9	4	0.50	1	0.38	3	3.38
5	6	1.6	4	3.3	9	6.8	2	2.63	3	1.38	5	3.00
6	4	1.5	4	3.9	7	6.3	2	0.25	2	0.50	4	3.13
7	4	1.4	3	0.3	3	3.0	0	0	0	0	3	3.38
10	0	0	0	0	0	0	0	0	0	0	0	0

^a Groups of 10 poults.

^b Groups of eight poults.

^c Number of poults in the group showing clinical signs.

^d Mean clinical sign score for all poults in the group.

Table 2. Homologous avian pneumovirus antibody response, measured by ELISA (Colorado antigen), following two intramuscular injections of plasmids expressing, or not expressing, the F gene of Colorado and challenge with virulent APV (Colorado) (Experiment 3)

Bird number	Antibody titre (log ₂) at different ages following injection of the plasmids at 5 and 26 days of age								
	F/pI.18 plasmid			PI.18 plasmid			Not inoculated		
	26 days	42 days	52 days ^a	26 days	42 days	52 days ^a	26 days	42 days	52 days ^a
1	4.6	6.6	14.6	<4.6	<4.6	11.6	<4.6	<4.6	12.6
2	<4.6	5.6	15.6	<4.6	<4.6	11.6	<4.6	<4.6	9.6
3	<4.6	<4.6	13.6	<4.6	<4.6	10.6	<4.6	<4.6	9.6
4	<4.6	8.6	15.6	<4.6	<4.6	11.6	<4.6	<4.6	11.6
5	<4.6	<4.6	11.6	<4.6	<4.6	10.6	<4.6	<4.6	10.6
6	<4.6	<4.6	15.6	<4.6	<4.6	11.6	<4.6	<4.6	11.6
7	<4.6	4.6	14.6	<4.6	<4.6	11.6	<4.6	<4.6	9.6
8	<4.6	7.6	15.6	<4.6	<4.6	9.6	<4.6	<4.6	10.6
GMT (SD) ^b	<4.6	5.2 (2.0)	14.5 (1.4)	<4.6	<4.6	11.1 (0.8)	<4.6	<4.6	10.7 (1.1)

^a Bled 10 days post-challenge.

^b Geometric mean titre (standard deviation).

significantly different from those in the pI.18-vaccinated group ($P = 0.0007$, Kruskal-Wallis test). These sera also cross-reacted with subgroup A antigen, but only very poorly with the subgroup B antigen (Table 3). However, in a neutralization test (Cook *et al.*, 1999) they did not neutralize either Colorado or the A or B subgroup viruses (not shown). The poult were challenged o.n. at 42 days with log₁₀ 1.6 TCID₅₀ of virulent Colorado, and substantial clinical signs were seen in the

Table 3. Heterologous antibody response after two intramuscular injections of plasmids expressing, or not expressing, the F gene of Colorado and measured by ELISA using subgroup A or B avian pneumovirus as antigen (Experiment 3)

Bird number	Antibody titre (log ₂) by ELISA at 42 days of age after two injections ^a with			
	F/pI.18 plasmid ^b		PI.18 plasmid ^c	
	Subgroup A antigen	Subgroup B antigen	Subgroup A antigen	Subgroup B antigen
1	6.6	6.6	4.6	4.6
2	5.6	5.6	<4.6	4.6
3	4.6	<4.6	4.6	5.6
4	7.6	5.6	4.6	4.6
5	5.6	<4.6	4.6	<4.6
6	4.6	<4.6	<4.6	4.6
7	4.6	4.6	<4.6	4.6
8	8.6	4.6	4.6	5.6

^a Poults were injected at 5 and 26 days of age, and bled at 42 days of age.

^b Plasmid expressing the Colorado F gene.

^c Control plasmid.

non-immunized, challenge control group (Table 1). Although the F/pI.18 plasmid appeared to provide some protection against challenge, this was not statistically different from that provided by the pI.18 plasmid ($P = 0.29$, Kruskal-Wallis test). However, the antibody response in the F/pI.18-vaccinated group was boosted anamnesticly following challenge (Table 2), suggesting that at least 7/8 of these poult had been primed by the vaccination.

The upper respiratory tract is the primary target for APV infections (Cook *et al.*, 1991; Catelli *et al.*, 1998). Khehra (1998) showed that locally produced APV-specific IgA and IgG following APV inoculation is important in protecting against challenge. Possibly, in the present work, no local immunity was induced in the respiratory tract, although this requires confirmation.

Optimization of our DNA plasmid might improve protection. Li *et al.* (1998) demonstrated, in a murine challenge model, that i.m. delivery of a DNA vector encoding an extracellular form of the RSV F protein induced neutralizing antibodies and higher levels of protection than the membrane bound form. Furthermore, if the rabbit beta globin intron II sequence, thought to stabilize F mRNA transcripts, was inserted upstream of the truncated RSV F gene, complete protection against challenge could be achieved. An alternative method for delivery of the F gene is by recombinant fowlpox virus vector. Previous work showed that such a recombinant vaccine expressing the subtype A F gene induced neutralizing antibodies and provided partial protection against homologous mucosal challenge (Yu *et al.*, 1994). Further experiments are required to fully explore the efficacy of DNA vaccines against APV challenge.

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RÉSUMÉ

Clonage, expression et immunogénicité de la protéine F du pneumovirus aviaire (souche Colorado)

La protéine F de la souche Colorado de paramyxovirus aviaire (APV) exprimée 'a partir d'un plasmide ADN est reconnue par les antisérum des sous groupes A et B d'AVPs. Apr'es deux injections intramusculaires de ce plasmide 'a des dindes, une réponse en anticorps homologues a été détectée par ELISA. Ces anticorps reconnaissent également le sous groupe A d'AVP. Cependant, ces anticorps ne neutralisent pas la souche Colorado ni les virus des sous groupes A et B. Bien qu'aucune protection clinique significative n'ait été observée apr'es une épreuve homologue, une réponse sérologique anamnétique a été notée, suggérant qu'une réponse systémique en anticorps ait été induite et non une réponse immunitaire locale mucoale.

ZUSAMMENFASSUNG

Klonierung, Exprimierung und Immunogenität des F-Proteins des aviären Pneumovirus (Stamm Colorado)

Das von einem DNA-Plasmid exprimierte F-Protein des Colorado-Isolats des aviären Pneumovirus (APV) wurde durch Immuneserum gegen APVs sowohl der Untergruppe A als auch der Untergruppe B erkannt. Nach zwei intramuskulären Injektionen von Puten mit diesem Plasmid wurde mit dem ELISA eine homologe Antikörperantwort nachgewiesen. Dieser Antikörper erkannte auch APV der Untergruppe A. Es gab jedoch keine Neutralisation des Colorado-Isolates oder von Virusstämmen der Untergruppen A oder B. Obwohl nach homologer Testinfektion von Putenküken kein signifikanter klinischer Schutz nachweisbar war, wurde eine anamnestiche serologische Reaktion festgestellt, was darauf schließen ließ, dass eine systemische Antikörperreaktion, aber keine lokale Schleimhautimmunität verursacht wurde.

RESUMEN

Clonaje, expresión e inmunogenicidad de la proteína F de pneumovirus aviar (cepa Colorado)

La proteína F de la cepa Colorado del pneumovirus aviar (APV), expresada a partir de un plásmido de ADN, fue reconocida por antisueros frente a los subgrupos A y B de APVs. Tras dos inoculaciones intramusculares de pavos con este plásmido, se detectó, mediante ELISA, una respuesta homóloga de anticuerpos. Este anticuerpo también reconoció el subgrupo A de APV. Aun así, no se observó neutralización de la cepa Colorado o de los virus del subgrupo A o B. Aunque no se detectó una protección clínica significativa después de la inoculación experimental homóloga de pavos jóvenes, se observó una respuesta serológica anamnésica, lo que sugirió que se indujo una respuesta humoral sistémica, pero no una inmunidad local de mucosas.