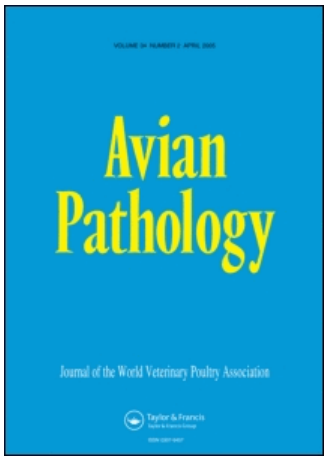

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Preliminary antigenic characterization of an avian pneumovirus isolated from commercial turkeys in Colorado, USA

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An avian pneumovirus (APV) isolated from turkeys showing respiratory disease in Colorado, USA, shared some characteristics with earlier subgroup A and B APV strains. This virus, designated the Colorado isolate (Colorado), when used after either seven passages in chick embryo fibroblasts (CEF), or seven passages in CEF followed by six turkey passages, induced clinical signs in turkeys that were similar to those caused by earlier APV strains. Although it induced an antibody response in specific pathogen free chickens, clinical signs were not seen. Unlike subgroups A or B, Colorado did not cause ciliostasis in tracheal organ cultures, but produced a cytopathic effect in chick embryo fibroblasts typical of that seen with other APV strains. Monospecific antisera to A or B strains did not neutralize Colorado and *vice versa;* nor did monoclonal antibodies, which neutralize subgroup A or B strains, neutralize Colorado. However, it was partially neutralized by a subgroup A hyperimmune serum. A homologous enzyme-linked immunosorbent assay (ELISA) antigen was essential for the detection of Colorado antibodies, since ELISAs in which subgroup A or B strains were used detected antibody to Colorado very poorly. Subgroup A and B vaccines protected turkeys against challenge with Colorado. However, while Colorado protected turkeys, and to some extent chickens, against subgroup A strains, protection against a subgroup B challenge was less good in both species.

These results indicate that Colorado should be classified as an APV, but the antigenic differences suggest that it does not belong to subgroups A or B, and represents a separate subgroup (subgroup C) or possibly a separate serotype.

Introduction

The first report of the existence of an avian pneumovirus (APV) was by Buys & Du Preez (1980), who demonstrated the virus to be a major cause of respiratory disease in turkey flocks in South Africa. Subsequently, the virus was reported as the cause of turkey rhinotracheitis (TRT) in turkeys in the UK (McDougall & Cook, 1986; Wilding *et al.*, 1986; Wyeth *et al.*, 1986), where the virus was first characterized (Cavanagh & Barrett, 1988). APV is now known to be a major cause of disease in turkey flocks of all ages (Naylor & Jones, 1993).

Only one serotype of APV has so far been described (Cook *et al.*, 1993a). However, two subgroups, A and B, have been reported within that serotype, based on both sequence differences within the G glycoprotein (Juhasz & Easton, 1994; Naylor *et al.*, 1997) and patterns of neutralization by monoclonal antibodies that recognize the G glycoprotein (Collins *et al.*, 1993; Cook *et al.*, 1993a).

Infections caused by APV have now been reported from many parts of the world, including mainland Europe (Giraud *et al.*, 1986; Hafez, 1990; 1998); Israel (Weisman *et al.*, 1988), Japan (Tanaka *et al.*, 1995) and South America (Jones, 1996). However, there have been no reports of the

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Table 1. *Scoring system used to assess clinical signs in turkeys and chickens following inoculation with avian pneumoviruses*

Turkeys		Chickens	
Score	Clinical sign[a]	Score	Clinical sign
1	Nasal exudate	1	Slight nasal exudate
2	Cloudy nasal exudate	2	Moderate nasal exudate
3	Copious nasal exudate	3	Copious nasal exudate
4	Copious, cloudy nasal exudate	4	Cloudy nasal exudate
5	Ocular discharge	5	Ocular discharge
5	Swollen sinus, slight	5	Swollen sinus
6	Swollen sinus, moderate	6	Swollen head
7	Swollen sinus, severe		

[a] Clinical sign scores are cumulative.

presence of APV in the US. In May 1996, a respiratory infection, characterized by coughing, rhinitis and sinusitis, was described in commercial turkeys in the state of Colorado, USA (Senne *et al*., 1997). Serum samples collected from these turkeys were examined in the UK for APV antibodies by enzyme-linked immunosorbent assay (ELISA), using a subgroup A strain of TRT as antigen (Cook *et al*., 1996), and low antibody titres to APV were detected in some of the sera. A virus was subsequently isolated from these turkeys (Senne *et al*., 1998a,b) and shown to have some antigenic relationship with existing TRT isolates (Cook *et al*., 1998a,b). This paper describes preliminary studies performed in an attempt to characterize this virus.

Materials and Methods

Virus strains

USA isolate. Respiratory tract material from turkeys in Colorado showing signs of respiratory infection were received at the National Veterinary Services Laboratories, Ames, IA, USA. This material was passaged twice via the yolk sac of 6-day-old embryonated eggs from a specific pathogen free (SPF) chicken flock, after which haemorrhages were observed on the embryos. A yolk sac membrane homogenate from the second embryo passage was then inoculated onto monolayers of chick embryo fibroblasts (CEF). A cytopathic effect (CPE), typical of that described for APV (Buys *et al*., 1989), was observed by the second CEF passage. Preliminary identification as an APV was based on electron microscopic examination and indirect immunofluorescence using an antiserum to a subgroup A strain of APV. This virus, which had received two yolk sac and seven CEF passages and is designated the Colorado isolate, was the starting material for this study.

Avian pneumovirus strains. Viruses used in these experiments have all been described previously (Cook *et al*., 1993a, 1995):

Subgroup A strains: 3B, #8544, 82/90, 91/78, 2381/88;

Subgroup B strains: 11/94, 2178/90, NL C/90[2], 1062, 182/88.

TRT vaccines. A commercially available live-attenuated vaccine developed from a subgroup A strain (TRT Nobilis, Intervet International, Boxmeer, The Netherlands), and an experimental live-attenuated vaccine based on a subgroup B strain, 11/94, were used.

Growth and assay of viruses. Both secondary CEF monolayers and chick embryo tracheal organ cultures (TOC) (McDougall & Cook, 1986) were used as detailed under individual experiments.

Serology

ELISA. This was performed as described previously (Cook *et al*., 1996), using a subgroup A strain, a subgroup B strain, or the Colorado isolate as antigen. Antibody titres of $\geq \log_2 7.0$ were considered to be positive.

In vitro *cross-neutralization tests.* As detailed in individual experiments, these were performed in either TOC as described previously (Cook *et al*., 1989) or in secondary CEF cultures in microtitre plates. Two assay systems were used because not all the APV isolates have been adapted to grow in CEF and, conversely, the Colorado isolate of APV does not cause ciliostasis in TOC (Anon, 1998). Monospecific antisera to different APV strains, or APV monoclonal antibodies (mAbs) that recognize the G glycoprotein, were used. The preparation of both these types of antibodies has been described previously (Cook *et al*., 1993a). The mAbs used were numbers 1, 3, 4, 6, 7, and 8 (Cook *et al*., 1993a). In the neutralization tests, they were tested against 100 median infectious doses (ID_{50}) of virus. Monospecific antiserum to the Colorado isolate of APV was prepared in SPF chicks inoculated occulonasally (o.n.) with $\log_{10} 5.3$ median tissue culture infective doses ($TCID_{50}$) of virus, and bled 3 weeks later.

Hyperimmune antisera to a subgroup A, a subgroup B strain and the Colorado isolate were raised in SPF chickens, inoculated once o.n., then boosted 3 weeks later by intravenous inoculation and bled after a further 2 weeks.

In vivo *studies in chickens and turkeys*

Experimental animals.

Chickens. Eggs obtained from a SPF flock were incubated and the chickens hatched in the research facilities of Intervet UK.

Turkeys. One-day-old mixed sex poults were obtained from commercial breeding flocks. Except where indicated, turkeys were free from maternally derived antibodies (MDA) to APV.

Throughout these experiments, both chickens and turkeys were housed in negative pressure isolators. They were inoculated o.n. at the ages shown in individual experiments. In each case, the inoculum volume was 100 μl. Clinical signs were recorded for each bird individually using a scoring system, which was slightly different for the two species, and is shown in Table 1.

In vivo *protection studies*

These were performed similarly in the two species. One- or 7-day-old birds were inoculated o.n. with the appropriate virus strain (see individual experiments). Three weeks later, the birds were bled, then

Table 2. *Clinical signs and antibody response of turkeys and chickens to the Colorado isolate of avian pneumovirus*

Species	Age (days)	Number	Passage level	Inoculum ($TCID_{50}$/bird)	Mean clinical sign score/bird (day postinoculation) 3	4	5	6	7	10	Antibody response (ELISA) GMT[d] (log_2)	Range (log_2)
Turkey	16[a]	15	CEF7[b]	5.6	0.5	1.47	1.7	1.1	0.3	0	9.7[e]	7.6 to 11.6
	7	10	CEF7	4.7	0.7	2.7	3.0	5.0	1.7	0.1	8.6[e]	7.6 to 9.6
	16	9	TP6[c]	1.7	2.1	9.2	6.4	8.8	4.7	0	9.5[e]	7.6 to 11.6
Chicken	18	10	CEF7	5.3	0	0	0	0	0	0	10.2[f]	6.6 to 11.6
	21	9	TP6	1.7	0	0	0	0	0	0	<4.6[e]	—

[a] Poults with maternally derived TRT antibodies; all other groups free from APV antibodies when inoculated.
[b] Seven passages in chick embryo fibroblasts.
[c] Seven passages in CEF followed by six passages in turkey poults.
[d] Geometric mean titre.
[e] Bled 11 days postinoculation.
[f] Bled 3 weeks postinoculation.

challenged o.n. with virulent virus. Each bird was observed individually for clinical signs until 10 days postchallenge, when the birds were again bled and the experiment terminated. The serum samples collected pre- and postchallenge were tested for APV antibodies by ELISA.

Results

Growth of the Colorado isolate of APV in vitro

The Colorado isolate grew well in CEF, achieving titres of a least $log_{10}6.5$ $TCID_{50}$ /ml within seven passages in CEF, and producing a syncytial type of CPE, typical of that seen following inoculation of CEF monolayers with subgroup A or B strains of APV. Chick embryo TOCs in either tubes or small flasks, each containing multiple rings, were inoculated with the seventh CEF-passaged material. A total of six passages in TOC was given. No ciliostasis was observed. When the supernatant from each harvest was inoculated onto CEFs, virus could be recovered from the first and second TOC passages (titres $log_{10}2.7$ and $log_{10}1.6$ $TCID_{50}$/ml, respectively), but no virus was recovered and no CPE was observed in CEFs inoculated with supernatant from the subsequent TOC passages.

Replication of the Colorado isolate of APV in vivo

One group of SPF chickens and two groups of commercial turkeys were inoculated o.n. with the seventh CEF passage virus (Table 2). One group of turkeys was from a flock where no TRT vaccines had been used (MDA-negative) and the other was hatched from a flock that had been given live-attenuated and inactivated TRT vaccines; this group had a mean MDA titre of $log_2 8.0$ at the time of inoculation. Further groups of MDA-negative turkeys and SPF chickens were inoculated with virus that had been given six passages in turkeys after the six passages in CEF cells (see later). No clinical signs of respiratory infection were seen in either group of chickens. However, the CEF-passaged virus had replicated, since all chickens inoculated with that virus showed a good antibody response, measured by ELISA using the Colorado isolate as antigen, by 3 weeks postinoculation (p.i.). The virus that had received six passages in turkeys failed to induce an antibody response in chickens by 11 days p.i. (Table 2).

Clinical signs were seen in both groups of turkeys inoculated with the virus at CEF passage seven level, the signs being more marked in the MDA-negative poults. The clinical signs seen following inoculation with the turkey-passaged virus were much more severe. Sera collected 11 days p.i. were tested for APV antibodies by ELISA, using the Colorado isolate as antigen. A good antibody response was seen in all three groups of turkeys.

Passage of the Colorado isolate of avian pneumovirus in turkeys

The CEF-passaged virus was given six passages in 2- to 3-week-old turkeys. During each passage, poults were swabbed from the buccal cavity daily between 3 and 6 days p.i., and virus content determined by assay in CEF and TOC. This swab fluid was used as inoculum for subsequent passages without any *in vitro* culture in between. The amount of virus recovered daily at each passage level is shown in Table 3. Although the titre of the viral inoculum decreased with passage level, the amount of virus recovered in the buccal cavity swabs, following assay in CEFs, increased with passage; greatest amounts being recovered during the final (sixth) passage. It is noteworthy that virus was detected only in CEF. Virtually no ciliostatic virus was detected in swab fluids at any passage level.

The amount of virus in respiratory tract tissues was also determined during passage 4. Two birds were sampled each time and the results are sum-

Table 3. *Recovery of the Colorado isolate of avian pneumovirus in buccal cavity swab fluid collected from turkeys*

		Amount of virus in buccal cavity swab fluid (day postinoculation)							
Passage number	Inoculum ($TCID_{50}$)	3		4		5		6	
		CEF[a]	TOC[b]	CEF	TOC	CEF	TOC	CEF	TOC
1	5.6	2.3	$\leqslant 1.0$	2.3	$= 1.0$	1.9	< 1.0	< 1.0	< 1.0
2	1.6	3.0	< 1.0	2.2	< 1.0	2.3	< 1.0	< 1.0	< 1.0
3	2.2	2.3	1.2	2.8	1.3	1.8	2.2	1.6	1.2
4	1.6	2.8	ND[c]	2.7	ND	2.7	ND	ND	ND
5	1.7	2.4	< 1.0	2.3	< 1.0	2.7	< 1.0	ND	ND
6	1.5	3.4	ND	3.1	ND	3.4	ND	3.4	ND

[a] In chicken embryo fibroblasts, expressed as $TCID_{50}$/ml.
[b] In tracheal organ cultures, expressed as CD_{50} /ml.
[c] Not done.

marized in Table 4. Virus replicated well in sinus and nasal tissue but, interestingly, not in the trachea. No virus was recovered beyond 5 days p.i.

In vitro neutralization tests

A pool was prepared from the serum samples collected at 3 weeks p.i. of the SPF chickens inoculated with the CEF-passaged Colorado isolate of APV (see earlier) and tested in a neutralization test in CEF against 100 $TCID_{50}$ of the homologous virus. It had a neutralizing antibody titre of 1:800. This antiserum was then tested in neutralization tests in TOC against 100 $TCID_{50}$ of three subgroup A (3B, #8544 and 2381/88) and three subgroup B (11/94, 2178/90 and NL C/90[2]) APV strains. In each case, the neutralization titre was $<1:10$.

In addition, monospecific antisera raised previously to four subgroup A and four subgroup B APV strains (3B, 82/90, 91/78 and 2381/88, and 11/94, 2178/90, 1062 and 182/88, respectively) were tested against 100 $TCID_{50}$ of the Colorado isolate in neutralization tests in CEF. In each case, the neutralization titre was $<1:25$. Similarly, the mAbs, which recognize the G glycoprotein of subgroups A and B, failed to neutralize the Colorado isolate, even at a dilution of 1:10.

Cross-neutralization tests were also performed in which a subgroup A (3B) strain, a subgroup B (11/94) strain and the Colorado isolate were tested against hyperimmune antisera raised in SPF chickens to each of the three viruses (Table 5). There was good neutralization between the subgroup A and B strains. The hyperimmune antiserum to the Colorado isolate neutralized the homologous virus well. However, the Colorado isolate was neutralized poorly by the subgroup A, and not at all by the subgroup B hyperimmune antiserum.

Detection of antibodies to different APVs by ELISA

Antisera raised in groups of 10 chickens or 10 turkeys to each of the three APV strains were tested in ELISAs in which a subgroup A, a subgroup B strain or the Colorado isolate were each used as antigen. The results summarized in Figure 1 show that antigens prepared from subgroup A or B strains detect antibodies to those two subgroups, raised in each species. Although, in this particular study, the use of the subgroup A antigen appeared to give higher titres, it is clear that each antigen would detect antibodies to each subgroup. However, for the adequate detection of antibodies to the Colorado isolate, it was necessary to use the homologous antigen. Antibodies raised to the A or B subgroups in turkeys were not detected by the Colorado antigen; however, that antigen did detect antibodies raised to subgroups A or B in chickens, although rather poorly.

Protection studies

Protection provided by subgroup A and B vaccines in turkeys against challenge with the Colorado isolate. Groups of 10 1-day-old MDA-positive poults were vaccinated o.n. with $\log_{10}2.7$ $TCID_{50}$ of the subgroup A vaccine or $\log_{10}2.8$ $TCID_{50}$ of the subgroup B vaccine. Three weeks later, they were bled, then challenged, together with a previously unvaccinated group, with $\log_{10}5.0$ $TCID_{50}$ of the Colorado isolate, that had received seven passages in CEF. Clinical signs were assessed daily following challenge. The results summarized in Table 6 show that both vaccines protected fully against the heavy challenge with the Colorado isolate. Sera collected 3 weeks postvaccination and 10 days postchallenge were tested for APV antibodies by ELISA using a subgroup A strain and the Colorado isolate as antigen. The antibody titres are summarized in Table 7. When the subgroup A

Table 4. *Replication in respiratory tract tissues of turkeys of the Colorado isolate of avian pneumovirus that had been given four passages in turkeys*

Day post inoculation	Amount of virus ($TCID_{50}$ per gm of tissue) from		
	Nasal tissue[a]	Sinus	Trachea
3	1.6, 2.5	$\leqslant$1.0, 1.0	$\leqslant$1.0, $<$1.0
4	1.6, 2.8	1.0, 2.5	$\leqslant$1.0, $<$1.0
5	3.0, $<$1.0	2.1, $<$1.0	$<$1.0, $<$1.0
7	$<$1.0, $<$1.0	1.0, $<$1.0	$<$1.0, $<$1.0

[a] Two birds sampled each time.

strain was used as antigen, low levels of MDA were still detectable in the unvaccinated group at 3 weeks of age. An antibody response to both vaccines was detected using this antigen. Neither MDA nor a response to either vaccine was detected when the Colorado isolate was used as ELISA antigen. The Colorado antigen detected a response to the homologous challenge strain in all three groups. However, the response was significantly lower (Student's *t* test) in the group vaccinated with the subgroup B strain, compared with the unvaccinated challenge control group. The strong antibody response, detected using the subgroup A strain, in both vaccinated groups following challenge, suggests an anamnestic response.

Protection provided by the Colorado isolate against challenge with subgroup A or B APV strains.

Two experiments were performed, one in SPF chickens and one in MDA-negative turkeys. In the first experiment, two groups of 10 1-day-old SPF chickens were inoculated o.n. with $\log_{10}5.1$ $TCID_{50}$ of seventh CEF-passaged Colorado isolate. Three weeks later, one vaccinated group and one unvaccinated control group were challenged o.n. with $\log_{10}4.2$ CD_{50} of subgroup A strain, 2381/88. The other vaccinated group and a control group were challenged o.n. with $\log_{10}4.8$ CD_{50} of subgroup B strain, 11/94. The clinical sign scores recorded following challenge are summarized in Table 8. Although the clinical signs caused by the APV strains, particularly by the subgroup A strain in SPF chickens, were rather mild, these results indicate that the Colorado isolate protected SPF chickens poorly against challenge with either the subgroup A or subgroup B APV strain.

Table 5. *Neutralization of the Colorado isolate by hyperimmune antiserum to subgroups A and B APV*

Hyperimmune serum	Reciprocal neutralization titre against 100 $TCID_{50}$ of virus strain		
	Subgroup A	Subgroup B	Colorado
Subgroup A	**80**[a]	160	20
Subgroup B	320	**160**	$<$20
Colorado	40	$<$20	**320**

[a]Homologous titre in bold.

The chickens were bled 3 weeks after the primary inoculation and 10 days after the second inoculation, and the sera were tested by ELISA using a subgroup A strain or the Colorado isolate as antigen. A poor antibody response was detected 3 weeks after inoculation, even in the two APV-inoculated groups (Table 9). APV antibodies were detected with each ELISA antigen following challenge with either a subgroup A or a subgroup B strain. Titres detected using the subgroup A antigen were similar in all groups. When the Colorado isolate was used as antigen, titres were higher in the groups that had been inoculated twice, particularly in the group inoculated initially with the subgroup A strain, where highest titres to the first inoculation had been seen. Very low titres of antibody were seen using Colorado as antigen in the groups inoculated only with the subgroup A or B strains (challenge control groups).

The design of the second experiment was similar. Two groups of 7-day-old commercial turkeys, free from MDA to APV, were inoculated o.n. with $\log_{10}4.6$ $TCID_{50}$ of seventh CEF-passaged Colorado isolate. Three weeks later, together with unvaccinated control groups, they were challenged with $\log_{10}4.3$ CD_{50} of the subgroup A strain, 3B, or with $\log_{10}5.0$ CD_{50} of the subgroup B strain, 11/94. The clinical sign scores following challenge are summarized in Table 8. The Colorado isolate was shown to protect turkeys better than SPF chickens against challenge with a subgroup A strain. However, protection was still poor against subgroup B challenge. The antibody response to the primary and secondary APV inoculation is summarized in Table 9. The turkeys had responded better to primary inoculation with the Colorado isolate than had the SPF chickens, but no antibody response was detected using the subgroup A antigen. Following challenge, a strong antibody response was detected in all groups, using a subgroup A antigen. The Colorado antigen detected a good response to challenge in the groups primed with that virus, and a poor response in the groups that were only challenged with subgroup A or B strains.

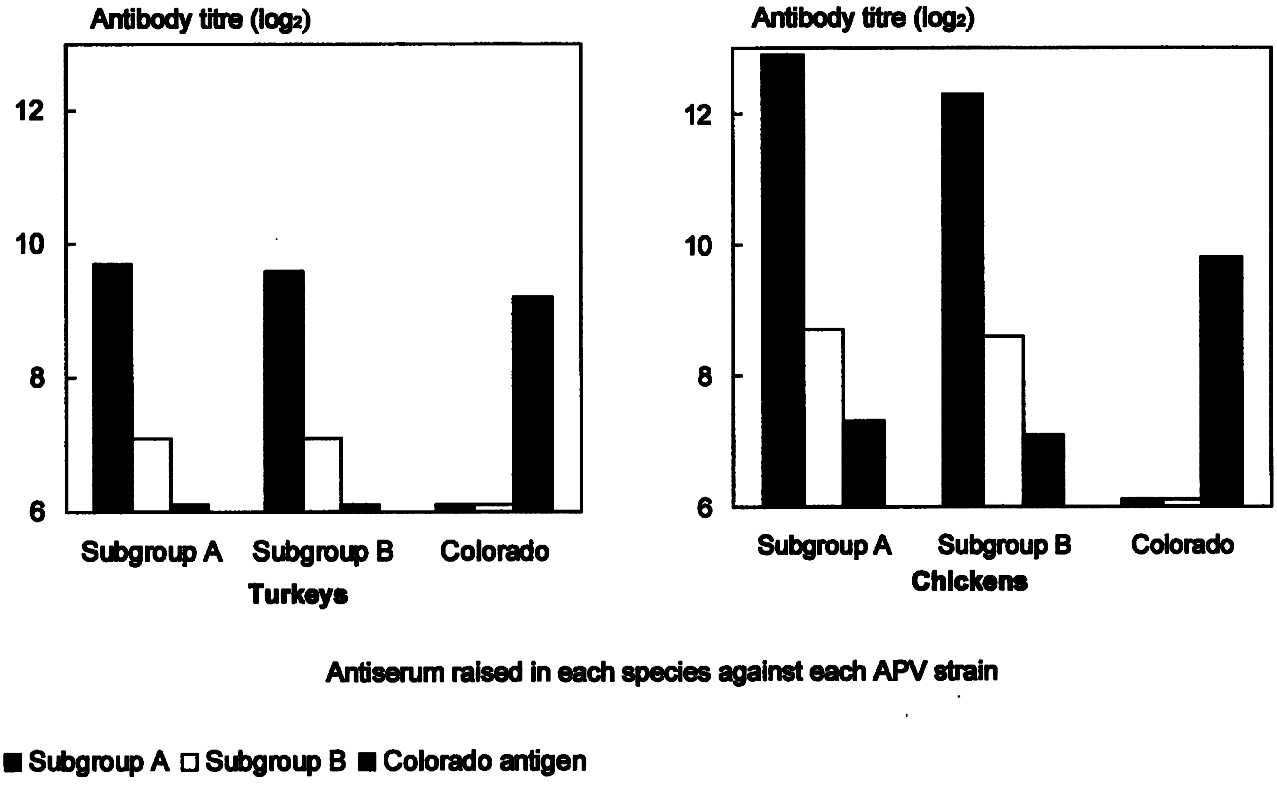

Figure 1. *Antibody response to different subgroups of APV using each subgroup as antigen in the ELISA.*

Discussion

The results reported in this paper provide interesting and important information regarding APVs. First, they shed new light of the epidemiology of the virus by providing evidence for its presence in the US. Until now, the US had been free from APV infection, although antibodies to the subgroups of APV found in Europe and elsewhere were known to be present in other countries on the American continent (Jones, 1996). The APV strain studied here was isolated from turkeys in the state of Colorado. What appears to be a very closely related virus antigenically (unpublished observation) is reported as a continuing problem in turkeys in the state of Minnesota (Anon, 1998). Therefore, a type of APV, antigenically distinct from APVs described elsewhere, has now been reported in two separate states in the US, but has not been reported from other countries. Interestingly, the infection does not, as yet, appear to have spread to other parts of the US; nor have there been reports of the presence of this virus in chickens. This may not be surprising, however, as it has been shown previously (Cook *et al.*, 1993b) that APV strains may have different tropism for chickens and turkeys.

The emergence of the Colorado isolate of APV poses interesting questions regarding the epidemiology of APV infections. It is also known that APVs can infect pheasant and guinea fowl (Gough *et al.*, 1988). It has also been suggested that migratory birds may play a role in the transmission of APV (Jones, 1996) and, recently, Heffels-Redmann *et al.* (1998) demonstrated APV-neutralizing antibodies in sera from sea gulls sampled in Northern Germany. However, the epidemiology of APV infections is still poorly understood, and further studies are required to elucidate the method of transmission of this virus and the possible role of wild birds in its spread.

A further interesting difference between the Colorado isolate of APV and the two previously reported subgroups is that the Colorado isolate did not cause ciliostasis in TOCs, even after six back passages in turkeys. Furthermore, although the virus was recovered from buccal cavity swabs during this turkey passaging, virus that had been given four passages in turkeys failed to replicate in the trachea, although it had replicated in nasal and sinus tissue (Table 4). It is unlikely that this is a feature unique to this particular isolate, since a second isolate of APV from the US was also not ciliostatic (not shown). Until now, all APV strains examined have been found to be ciliostatic, yet this recent isolate is not. TOCs have previously been found to be an excellent system, possibly the best, for the primary isolation of APV from field material. The emergence of the Colorado isolate suggests that this is no longer the case. The original isolate of APV in South Africa was obtained following passage of tissue from infected birds via the yolk sac of SPF embryos, followed by adaptation to cell culture (Buys & Du Preez, 1980). A similar technique was used to isolate the APV from turkeys in Colorado (Senne *et al.*, 1998b).

Table 6. *Protection provided by live-attenuated subgroup A and B strains of APV against challenge with the Colorado isolate*

	Clinical signs following challenge of turkeys vaccinated 3 weeks earlier with:					
	Subgroup A		Subgroup B		Unvaccinated	
Day post challenge	Number[a]	Score[b]	Number	Score	Number	Score
3	1	0.1	0	0	2	0.5
4	0	0	0	0	4	0.7
5	0	0	0	0	10	2.9
6	0	0	0	0	7	3.0
7	0	0	0	0	2	1.1
10	0	0	0	0	0	0

[a]Number showing clinical signs of 10 in group.
[b]Mean score for all poults in group.

Table 7. *Antibody response, measured by ELISA, in turkeys bled 3 weeks after vaccination with live-attenuated subgroup A or B strains of APV and 10 days after challenge with the Colorado isolate*

	Geometric mean titre (log_2) by ELISA ($\pm$ s.d.)			
	3 weeks after vaccination ELISA antigen		10 days after challenge ELISA antigen	
Vaccination	Subgroup A	Colorado	Subgroup A	Colorado
Subgroup A	10.0 ($\pm$1.5)	$<$7.0	13.3 ($\pm$1.9)	10.5 ($\pm$1.5)
Subgroup B	9.7 ($\pm$1.2)	$<$7.0	13.0 ($\pm$2.1)	9.4 ($\pm$0.9)[a]
Unvaccinated	8.6 ($\pm$0.9)	$<$7.0	8.7 ($\pm$0.8)	10.8 ($\pm$0.8)[a]

[a]Significantly different (Students *t* test).

Table 8. *Protection provided in chickens and turkeys by the Colorado isolate against challenge 3 weeks later with APV subgroup A or B*

	Clinical sign scores in Colorado-inoculated (vaccinated) chickens or turkeys challenged with APV subgroup A or B							
	SPF chickens				APV-free turkeys			
	Subgroup A challenge		Subgroup B challenge		Subgroup A challenge		Subgroup B challenge	
Day post challenge	'Vaccinated'	No vaccine	'Vaccinated'	No vaccine	'Vaccinated'	No vaccine	'Vaccinated'	No vaccine
3	0[a]	0.1	0.8	0.9	1.7	4.5	1.5	2.5
4	0.6	1.4	1.9	2.0	1.7	8.8	2.9	2.9
5	0.8	1.4	1.9	2.6	0.9	11.8	2.2	5.3
6	1.2	1.4	1.0	2.3	0	11.4	0.1	7.1
7	0.5	0	0.7	1.4	0	5.7	0.6	7.7
10	0.3	0.3	0	0	0	0	0	0

[a] Mean score for all birds in group.

These findings suggest that the use of yolk-sac inoculation of embryos should be at least one of the methods considered when attempting APV isolation.

Earlier studies (Cook *et al.*, 1995) have shown that a subgroup A strain of APV protects well against challenge with subgroup B strains. The present results, while demonstrating the excellent protection provided by subgroup A and B strains against challenge with the Colorado isolate, indicate that the protection provided by the Colorado isolate against challenge with strains of the A and B subgroups in either chickens or turkeys was less convincing. In these protection studies, the Col-

Table 9. *Antibody titres, measured by ELISA, in chickens or turkeys bled 3 weeks after inoculation with the Colorado isolate of APV and 10 days after challenge with subgroup A or B strains*

	Geometric mean titre (log_2) by ELISA (± standard deviation) in							
	SPF chickens				APV-free turkeys			
	3 weeks after first inoculation ELISA antigen		10 days after second inoculation ELISA antigen		3 weeks after first inoculation ELISA antigen		10 days after second inoculation ELISA antigen	
APV strains inoculated[a]	Subgroup A	Colorado	Subgroup A	Colorado	Subgroup A	Colorado	Subgroup A	Colorado
Colorado/A	< 7.0	7.7 (± 1.6)	13.7 (± 1.7)	12.0 (1.8)	< 7.0	9.1 (± 0.70)	> 15	15.0 (± 1.1)
Colorado/B	< 7.0	< 7.0	12.8 (± 2.3)	9.8 (3.3)	< 7.0	9.0 (± 1.2)	> 15	> 15
None/A	< 7.0	< 7.0	12.9 (± 0.7)	7.2 (1.9)	< 7.0	< 7.0	12.2 (± 0.98)	7.0 (± 0.84)
None/B	< 7.0	< 7.0	12.3 (± 0.8)	7.1 (0.7)	< 7.0	< 7.0	11.5 (± 0.62)	7.0 (± 1.2)

[a] First virus inoculated at 1-day-old, second at 3 weeks of age.

orado isolate used had received seven passages in CEFs. It is possible that this virus may, therefore, have been partially attenuated by this passaging. However, when turkeys were inoculated with virus that had been given either seven CEF passages, or seven CEF plus six passages in turkeys, both inocula caused clinical signs and induced similar antibody responses (Table 2). Therefore, it seems unlikely that the material used to 'vaccinate' the chickens and turkeys was over-attenuated.

It has been reported previously (Cook *et al*., 1993b) that different APV strains may show different virulence for chickens and turkeys, therefore different challenge strains were used in each study in an attempt to select a strain that would cause substantial clinical signs in each unvaccinated challenge control group. However, the subgroup A challenge strain used in chickens caused only poor clinical signs in the unvaccinated control group and it is unfortunate that no other more virulent subgroup A strain was available for use in chickens. Also, the poor antibody response suggested that the Colorado isolate might not have taken well in the chickens. However, there was an indication in the studies in both chickens and turkeys that the Colorado isolate provided better protection against challenge with subgroup A than against challenge with subgroup B. This finding would support the *in vitro* studies reported here, which indicated that a hyperimmune subgroup A antiserum neutralized the Colorado isolate to some extent, while a hyperimmune subgroup B serum did not.

The serological studies reported here using the ELISA show that antibodies to A or B subgroup strains could be detected equally well using either subgroup A or B antigens. However, they do indicate clearly the importance of using the homologous antigen when testing sera for evidence of infection with the Colorado isolate. When the original disease outbreak in Colorado was being investigated, we found APV antibodies in a small number of serum samples from the affected flocks using the subgroup A antigen. However, these samples were ones which, when we subsequently retested them with the Colorado isolate as antigen, were found to have very high antibody titres, in excess of $\log_2 11.0$. The results above presented show that positive flocks are likely to remain undetected unless the Colorado isolate is used as antigen in the ELISA.

From the time that APVs were first described in South Africa in the late 1970s (Buys & Du Preez, 1980) until the mid-1990s, all the available evidence indicated that there was only one known serotype of APV (Cook *et al*., 1993a). The isolation of an APV strain from turkeys in Colorado, USA (Senne *et al*., 1998a,b) changes that situation. Seal (1998) showed major differences between the Colorado isolate and subgroups A or B strains based on sequence analysis of the M protein gene. The results reported here also indicate that this isolate shows significant differences from APV strains described previously and suggest that it might be the first report of a second serotype of APV. The results of the *in vitro* cross-neutralization tests, using monospecific antisera to subgroup A and B strains, and mAbs specific for the G glycoprotein, indicate major differences between the Colorado isolate and subgroup A and B strains of APV. However, a hyperimmune antiserum to a subgroup A strain did partially neutralize the Colorado isolate, and the original isolate was partially identified by an indirect immunofluorescence test using a hyperimmune serum raised to a subgroup A strain (Senne *et al*., 1998b). Furthermore, the antibody responses found in the challenge studies following re-inoculation with different APVs suggest the sharing of some common antigens between these viruses. These results, together with the excellent protection provided by subgroup A or B strains against challenge with the Colorado isolate, do indicate some antigenic relationship between the isolate from the US and those from elsewhere. However, full analysis of the relationship between these APV strains must await the results of sequencing studies.

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RÉSUMÉ

Caractérisation antigénique préliminaire d'un pneumovirus aviaire isolé chez des dindes commerciales au Colorado, USA

Au Colorado, USA, un pneumovirus aviaire (APV) a été isolé chez des dindes présentant des symptômes respiratoires. Ce virus a présenté des caractéristiques communes avec les souches d'APV appartenant aux sous-groupes A et B.

Ce virus, désigné isolat du Colorado (Colorado), après avoir subi sept passages sur des fibroblastes d'embryons de poulet (CEF) ou sept passages sur CEF suivis de six passages sur dinde a induit chez les dindes des symptômes similaires à ceux entraînés par les premières souches d'APV. Ce virus a induit des anticorps chez les poulets SPF mais n'a pas entraîné de symptômes. A la différence des sous-groupes A et B, le Colorado n'a pas entraîné de ciliostase dans les cultures d'anneaux de trachée, mais sur CEP a produit un effet cytopathogène typique de celui observé avec les autres souches d'APV. Les sérums monospécifiques anti souches A et B n'ont pas neutralisé le Colorado et vice versa, il en est de même des anticorps monoclonaux qui neutralisent les souches des sous-groupes A et B, et qui ne neutralisent pas la souche Colorado. Cependant, cette souche a été partiellement neutralisée par un sérum hyperimmun anti sous-groupe A. Un antigène ELISA homologue a été indispensable pour la détection des anticorps anti-Colorado, vu que les ELISAs contenant la souche A ou la souche B détectent très mal les anticorps anti-Colorado. Les vaccins de sous-groupes A ou B protègent les dindes éprouvées avec le virus Colorado. Alors que le virus Colorado a protégé les dindes et à un moindre degré les poulets contre les souches de sous-groupe A, la protection contre une épreuve de sous-groupe B a été moins bonne pour les deux espèces.

Ces résultats indiquent que le virus Colorado devrait être classé comme un APV, mais les différences antigéniques suggèrent qu'il n'appartienne pas aux sous-groupes A ou B et représente un sous-groupe différent (sous-groupe C) ou peut être un sérotype différent.

ZUSAMMENFASSUNG

Vorläufige Antigencharakterisierung eines aus kommerziellen Puten in Colorado, USA, isolierten aviären Pneumovirus

Ein aviäres Pneumovirus (APV), das in Colorado, USA, aus Puten mit einer Atemwegserkrankung isoliert wurde, teilte einige Charakteristika mit früheren APV-Stämmen der Untergruppen A und B. Dieses Virus wurde als das Colorado-Isolat (Colorado) bezeichnet. Es verursachte bei Puten ähnliche klinische Symptome wie frühere APV-Stämme, wenn es entweder nach 7 Passagen in Hühnerembryofibroblasten (CEF) oder nach 7 CEF-Passagen und anschließenden 6 Putenpassagen benutzt wurde. Bei SPF-Küken verursachte es zwar eine Antikörperreaktion, aber keine erkennbaren klinischen Symptome. Anders als die Untergruppen A und B verursachte Colorado keine Ziliostase in Trachea-Organkulturen, aber es bewirkte einen ZPE in CEF-Kulturen, der für den mit anderen APV-Stämmen festgestellten ZPE typisch war. Monospezifische Immunseren gegen A- oder B-Stämme neutralisierten nicht Colorado und umgekehrt; auch monoklonale Antikörper, die A- und B-Stämme neutralisieren, neutralisieren nicht Colorado. Das Virus wurde jedoch durch ein Hyperimmunserum gegen APV der Untergruppe A teilweise neutralisiert. Für den Nachweis von Colorado-Antikörpern mittels ELISA war ein homologes Antigen erforderlich, da sich Colorado-Antikörper mit APV der Untergruppen A und B sehr schlecht nachweisen ließen. Untergruppe-A- und B-Vakzinen schützten Puten gegen die Testinfektion mit Colorado. Während jedoch Colorado Puten und in gewissem Umfang auch Hühner gegen Stämme der Untergruppe A schützte, war der Schutz gegen eine Testinfektion mit APV der Untergruppe B bei beiden Spezies weniger gut.

Diese Ergebnisse zeigen, dass Colorado als ein APV klassifiziert werden sollte, aber die Antigenunterschiede lassen darauf schließen, dass es nicht zu den Untergruppen A oder B gehört und eine gesonderte Untergruppe (Untergruppe C) oder vielleicht einen gesonderten Serotyp repräsentiert.

RESUMEN

Caracterizacion antigenica preliminar de un pneumovirus aviar aislado a partir de pavos comerciales en Colorado, USA

Un pneumovirus aviar (APV) aislado a partir de pavos con enfermedad respiratoria en Colorado, USA, compartía algunas características con cepas de los subgrupos iniciales A y B de APV. Este virus, denominado aislado Colorado (Colorado), cuando se inoculaba a pavos [después de siete pases en fibroplastos de embrión de pollo (CEF) o siete pases en CEF y seis pases en pavo] daba lugar a una sintomatología clínica similar a la producida por las cepas de APV. Aunque daba lugar a formación de anticuerpos en pollos SPF, no inducía en éstos un cuadro clínico. A diferencia de los subgrupos A o B, Colorado no daba lugar a ciliostasis en cultivos traqueales aunque producía, en fibroblastos de embrión de pollo el CPE característico de las cepas de APV. Antisueros monoespecíficos frente a las cepas A o B no neutralizaban Colorado y viceversa, tampoco los anticuerpos monoclonales que neutralizaban las cepas de los subgrupoos A y B, neutralizaban Colorado. Sin embargo éste era parcialmente neutralizado por un suero hiperimmune del subgrupo A. Un ELISA de antígeno homólogo fue esencial para la detección de anticuerpos frente a Colorado, mientras que los ELISA en los que se utilizaron cepas de los subgrupos A y B detectaban escasamente Colorado. Las vacunas frente a los subgrupos A y B protegían a los pavos frente a un *challenge* con Colorado. Sin embargo, mientras que Colorado protegía a pavos y hasta cierto punto, a pollos contra las cepas del subgrupo A, la protección frente al subgrupo B era menor en ambas especies.

Estos resultados indican que Colorado debería ser clasificado como un APV, pero las diferencias antigénicas sugieren que éste no pertenece a los subgrupos A o B y que representa un subgrupo aparte (subgrupo C) o posiblemente un serotipo diferente.