Distribution of chicken anaemia virus in the reproductive tissues of specific-pathogen-free chickens

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The specific-pathogen-free (SPF) flocks of chickens maintained by the Department of Microbiology and Immunology at Cornell University became infected, inadvertently, with chicken anaemia virus (CAV), as demonstrated by seroconversion. Chickens from five flocks representing three different strains were examined for the presence of CAV using nested PCR. Virus was detected in ovaries, infundibula, vas deferentia, testes and spleens. Ovaries were positive in 38 to 72% of the hens in four flocks with 13 to 56 birds examined per flock. Interestingly, the ovaries were often the only positive tissues, while a few hens had only positive spleens. In roosters, the vas deferens was positive in 30 to 79% of the birds with 5 to 19 birds examined per flock; the vas deferens was the only positive tissue in 20 to 37%. Individual cells in the theca externa and rare epithelial cells in the infundibular epithelium were positive for CAV by *in situ* PCR. Positive cells were not detected in testes or vas deferentia. The SH-1 strain of CAV was isolated from these tissues and partially sequenced. Only minor sequence differences were found compared to CIA-1 and Cux-1. Embryos from matings between persistently infected dams and sire had CAV-positive cells in mesenchyme near the developing vertebral column. The data show that CAV persists in the reproductive tissues far longer than previously thought, and that it can be vertically transmitted from persistently infected birds.

Introduction

The *Circoviridae* is a family of small non-enveloped viruses, 14 to 25 nm in diameter, with a single-stranded, circular DNA genome (Noteborn & Koch, 1995; Studdert, 1993). The genus *Circovirus* includes porcine circovirus (PCV) (Tischer *et al.*, 1982), psittacine beak and feather disease virus (PBFDV) (Ritchie *et al.*, 1989) and chicken anaemia virus (CAV) (Yuasa *et al.*, 1979). CAV differs from PCV and PBFDV, lacking DNA sequence identity or similarity and having a different genomic organization (Bassami *et al.*, 1998; Niagro *et al.*, 1998). Recently, human TT virus (TTV) has been fully sequenced and

found to have a similar genomic organization to CAV, including a GC-rich stretch of 36 nucleotides with 80·6% identity to the Cux-1 strain of CAV (Miyata *et al.*, 1999).

CAV was first isolated in 1979 in Japan (Yuasa *et al.*, 1979). Since its first description, it has been detected in chickens all over the world and is considered to be ubiquitous (Von Bülow & Schat, 1997). All characterized isolates of CAV belong to a single serotype (Von Bülow & Schat, 1997). However, there are differences between isolates that affect virus cell tropism *in vitro* (Renshaw *et al.*, 1996). All known strains cause a similar disease syndrome, chicken infectious anaemia (CIA).

Clinical disease, CIA, occurs only in chicks infected before 3 weeks of age or in older immunosuppressed chickens. Susceptible chicks develop a severe anaemia (Goryo *et al.*, 1985; Taniguchi *et al.*, 1983; Yuasa *et al.*, 1979) and immunosuppression that may lead to secondary infections (Goryo *et al.*, 1987; Vielitz & Landgraf, 1988; Von Bülow & Schat, 1997). Chicks experimentally infected at 1 day of age develop lesions 8 days post-inoculation (p.i.) and anaemia 12 to 16 days p.i. (Von Bülow & Schat, 1997). Histologically, there is complete atrophy of lymphoid cells in a wide variety of

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tissues. In the bone marrow, cells of all haematopoietic lineages are depleted during active infection (Goryo *et al.*, 1989; Pope, 1991; Smyth *et al.*, 1993). Most chicks completely recover by 32 to 36 days p.i., when neutralizing antibodies are present (Von Bülow & Schat, 1997). Virus can be isolated from most tissues except serum up to 28 days p.i. and can be detected in rectal contents up to 49 days p.i.

Hoop (1993) reported that experimentally infected males transmitted CAV through their semen until they developed antibodies to CAV. Similarly, experimentally infected hens also transmitted CAV to their offspring until the hens developed CAV antibodies between 8 to 14 days p.i. (Hoop, 1992). In both cases the percentage of offspring infected was less than 10%. In natural outbreaks, vertical transmission may occur from 3 to 9 weeks after exposure, but vertical transmission has not been reported after development of immunity (Chettle *et al.*, 1989; Engström & Luthman, 1984; Vielitz & Landgraf, 1988). The tissues or cells involved in the vertical transmission of CAV have not yet been identified.

The Department of Microbiology and Immunology at our Institute maintains three genetic strains of specific-pathogen-free (SPF) chickens in two filtered-air, positive-pressure (FAPP) houses. These SPF flocks were considered to be free of CAV infection based on periodic serologic testing of randomly selected birds (B. Lucio & K. A. Schat, unpublished data) and chick susceptibility tests (Lucio *et al.*, 1990). However, these flocks became accidentally infected with CAV (SH-1 strain), most likely in July 1996 based on the development of antibodies to CAV. Interestingly, the seroconversion was associated with the development of sexual maturity and genetic background of the chicken strain. Thus, not all chickens became antibody positive, even while the three strains were maintained in the same rooms (Cardona *et al.*, 2000). Producers of SPF flocks often report that chickens become seropositive after the onset of sexual maturity, but the source of infection is seldom identified (McNulty, 1991).

The objective of this work was to determine the location of CAV in chronically infected chickens. Initial studies focussed on the detection of CAV in the reproductive tracts of breeder birds and determining the cell types that harbour virus. Once the virus was detected in selected birds, further studies determined that the virus could be transmitted to embryos.

Methods

■ **Chickens.** Tissue and serum samples were obtained from several SPF breeding flocks of P2a [major histocompatibility complex (MHC): $B^{19}B^{19}$], N2a (MHC: $B^{21}B^{21}$) and S13 (MHC: $B^{13}B^{13}$) White Leghorn chickens maintained by the Department of Microbiology and Immunology at Cornell University (CU) (Table 1). Each flock was hatched from eggs collected from the previous SPF flock of the corresponding strain. Chickens were maintained in an FAPP house under strict shower-in biosecurity throughout their lives. All of the flocks are numbered sequentially and the chickens used for this work came from flocks identified in Table 1. All flocks were euthanized and necropsied at 14

months of age except for flock 96-1 (P2a), which was euthanized at 20 months of age when they were retired as breeders. Tissues were collected under sterile conditions from each chicken and stored at −20 °C or in 10% neutral buffered formalin at room temperature.

A small group of chickens was removed from the 97-2 flock and placed in an isolation unit where they were housed in individual cages for up to 18 months (flock 97-2a). One male was used 18 months after natural infection with CAV to artificially inseminate three hens. Fertile eggs were collected and incubated at 41 °C for 9 days, after which time they were harvested, placed in 10% neutral buffered formalin and stored at room temperature. These birds were also euthanized, necropsied and tissues collected as described.

■ **DNA extraction.** DNA was extracted from each tissue sample using standard techniques (Moore, 1988) with some modifications. Briefly, tissues were minced and incubated overnight at 37 to 41 °C in digestion buffer. Each sample was extracted with a mixture of phenol–chloroform–isoamyl alcohol (25:24:1) once and the DNA precipitated at −20 °C with 1 vol. of isopropyl alcohol and 0·1 vol. 3 M NaCl overnight. The DNA was pelleted by centrifugation at 14000 *g* at 4 °C for 20 min. The DNA was resuspended in TE pH 7·4, and quantified using a DU-50 Beckman spectrophotometer at 260 and 280 nm.

■ **PCR.** A nested PCR method was used for tissue screening. Two μg of tissue DNA was amplified with primers O3F (CAAGTAATTTCA-AATGAACG) and O3R (TTGCCATCTTACAGTCTTAT). The first PCR reaction consisted of 5 min at 94 °C followed by 35 cycles consisting of 1 min at 94 °C, 2 min at 45 °C and 1 min at 72 °C for 1 min, followed by 10 min at 72 °C. One μl of the first PCR reaction was amplified with primer N3 (CCACCCGGACCATCAAC) and primer N4(GGTCCTCAAGTCCGGCACATTC). After an initial denaturation for 5 min at 94 °C, the nested PCR reaction was performed for 30 cycles using the following program: two cycles of 30 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C, followed by two cycles each with the annealing temperature decreasing by 1 °C down to 56 °C, and ending with 20 cycles of 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C, followed by one extension step of 10 min at 72 °C. Testis DNA from the two P2a flocks was amplified using *β*-actin primers (primer 5′ actin, CCCCCGTGCT-GTGTTCCCATCTATCG; primer 3′ actin, GGGTGCTCCTCAGGGG-CTACTCTCAG). A 5 min denaturation step was followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C followed by a final step of 7 min at 72 °C.

■ **Southern blotting.** PCR fragments were separated in 1·5% agarose gels, exposed to ultraviolet light for 2 min, denatured and transferred to a nylon membrane (Nytran; Schleicher and Schuell), using standard Southern blotting techniques (Sambrook *et al.*, 1989). The DNA was cross-linked to the membrane in a Spectolinker XL-1500 UV cross-linker (Spectronics). The Genius probe labelling and detection kit (Boehringer Mannheim) was used for hybridization and detection according to the manufacturer's instructions. The probe used was a nick-translation labelled clone of the CAV genome. Briefly, pCIA-AB (Soiné *et al.*, 1993) was digested with *Eco*RI and fractionated on a 0·75% agarose gel; the smaller band (2·3 kb) was cut out from the gel and the DNA was purified using the Concert gel purification kit (GibcoBRL) according to the manufacturer's instructions.

■ **Sequencing.** Primers to CIA-1 were used to amplify the hyper-variable region of VP-1 (Renshaw *et al.*, 1996) of the CAV present in the SPF flocks (SH-1 strain). Two μg of ovarian tissue DNA from a 97-0 hen was amplified with primers O1F (AGGTGTATAAGACTGTAAG) and PshA1R (GAACAGGTGCCAGCCCCCAAACAT). The PCR reaction

Table 1. Tissue distribution of chicken anaemia virus in hens from five breeding flocks of SPF chickens at 14 months of age

| Flock ID | Strain | MHC | No. of CAV positive/total no. of hens (%) | | | | | Distribution of CAV in hens with positive ovaries [No. CAV positive/total no. of hens (%)] | | | |
| | | | | Spleen | | Reproductive organs | | | | | |
			Negative	Total	Spleen only	Ovary	Infundibulum	Ovary alone	Spleen + ovary	Infundibulum + ovary	Spleen + ovary + infundibulum
96-1	P2a	$B^{19}B^{19}$	8/56 (14)	22/56 (39)	5/56 (9)	40/56 (72)	9/56 (16)	21/56 (38)	13/56 (23)	3/56 (5)	3/56 (5)
97-0	P2a	$B^{19}B^{19}$	12/49 (24)	18/49 (37)	5/49 (10)	22/49 (45)	19/49 (39)	10/49 (20)	3/49 (6)	6/49 (12)	3/49 (6)
97-1	N2a	$B^{21}B^{21}$	18/40 (45)	13/40 (33)	7/40 (18)	15/40 (38)	NT	9/40 (23)	6/40 (15)	NT	NT
96-2	S13	$B^{13}B^{13}$	0/13 (0)	5/13 (39)	0/13 (0)	9/13 (69)	NT	4/13 (31)	5/13 (38)	NT	NT
97-2a	S13	$B^{13}B^{13}$	0/3 (0)	2/3 (67)	0/3 (0)	3/3 (100)	3/3 (100)	0/3 (0)	0/3 (0)	1/3 (33)	2/3 (67)

NT, Not tested.

Table 2. Tissue distribution of chicken anaemia virus in roosters from four breeding flocks of SPF chickens at 14 months of age

| Flock ID | Strain | MHC | No. CAV positive/total no. of roosters (%) | | | | | Distribution of CAV in roosters with positive vas deferens (VD) [No. CAV positive/total no. of roosters (%)] | | | |
| | | | | Spleen | | Reproductive organs | | | | | |
			Negative	Total	Spleen only	Testis*	VD	VD alone	Spleen + VD	Testis + VD	Testis + VD + spleen
96-1	P2a	$B^{19}B^{19}$	1/9 (11)	3/9 (33)	0/9 (0)	1/9 (11)	6/9 (67)	2/9 (22)	3/9 (33)	0/9 (0)	1/9 (11)
97-0	P2a	$B^{19}B^{19}$	4/19 (21)	6/19 (32)	0/19 (0)	3/19 (16)	15/19 (79)	7/19 (37)	5/19 (33)	1/19 (5)	2/19 (11)
97-1	N2a	$B^{21}B^{21}$	3/10 (30)	5/10 (50)	3/10 (30)	1/10 (10)	3/10 (30)	2/10 (20)	0/10 (0)	0/10 (0)	1/10 (10)
96-2	S13	$B^{13}B^{13}$	1/5 (20)	3/5 (60)	0/5 (0)	3/5 (60)	2/5 (40)	1/5 (20)	1/5 (20)	0/5 (0)	0/5 (0)
97-2a	S13	$B^{13}B^{13}$	0/1 (0)	1/1 (100)	0/1 (0)	1/1 (100)	1/1 (100)	0/1 (0)	0/1 (0)	0/1 (0)	1/1 (100)

* In none of the birds was the testis the only positive tissue.

Table 3. *In situ* PCR of selected tissues that are positive or negative for CAV by nested PCR

Chicken strain*	Nested PCR	No. of tissues positive/no. tested by *in situ* PCR assay				
		Spleen	Ovary	Infundibulum	Testis	Vas deferens
S13	Pos.	1/1	1/2	1/2	ND	0/1
	Neg.	0/4	0/1	0/1	0/2	0/1
P2a	Pos.	1/3	2/7	1/1	ND	0/6
	Neg.	ND	ND	ND	ND	ND
N2a	Pos.	ND	0/4	ND	0/2	0/3
	Neg.	ND	ND	ND	ND	ND

* Tissues were selected from flocks as needed.
ND, Not done.

was performed for 35 cycles using the same protocol as used in the first step of the nested PCR. The hypervariable regions of VP-1 and VP-3, generated with the O3F and O3R primers, were cloned into pCR TOPO 2.1 vector using the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. DNA sequencing was carried out at the BioResource Center at CU on a Perkin Elmer Biosystems model 377-XL DNA sequencer using dye-terminator chemistry. The thermocycling was done in a Perkin Elmer Biosystems model 9600 thermocycler.

■ **Sequence analysis.** Sequences were aligned with published CAV sequences using the program Multiple Sequence Alignment version 1.0.1.2 (Informax Vector NTI suite) software. GenBank accession numbers of the aligned sequences are as follows: CIA-1 is L14767, L-028 is U69549, Cux-1 is M55918, ConnB is U69548, 82-2 is D31965. The sequences for the virus isolated from the SPF flocks, SH-1, are AF21564 and AF21563 for VP1 and VP3, respectively.

■ **Virus isolation.** Semen samples were obtained from the 97-2a male on 6 days over a 3 month period. Tissues were obtained from selected chickens from the 96-1 and 96-2 flock, finely minced, frozen and thawed three times and extracted with chloroform. The supernatant fluids were passed through a 0.2 μm filter. A one-quarter volume of each sample was added to 2.5×10^5 CU-147 cells (Calnek *et al.*, 2000) in 3 ml of Leibovitz–McCoy medium containing 10% foetal bovine serum. The cells were incubated at 41 °C and split every third day to a density of 1×10^6 cells/ml. Cells were checked daily for cytopathic effect and at 6, 10, 14 and 19 days p.i. examined for the presence of CAV protein VP3 using monoclonal antibody 51.3 in an indirect immunofluorescence assay (Chandratilleke *et al.*, 1991).

■ ***In situ* PCR.** Sections of formalin-fixed tissues were embedded in paraffin, cut to 3 μm and placed on poly-L-lysine coated slides. The sections were deparaffinized and re-hydrated in graded ethanols. Methods for *in situ* PCR are a modification of techniques from Fares *et al.* (1998) and Nuovo *et al.* (1993). Briefly, tissues were permeabilized with 6 mg/ml proteinase K and 0.01% Triton X-100. After permeabilization, the PCR master mix (with primers N3 and N4) was added to the slides and the tissue DNA amplified for 50 cycles in a PTC-100 thermocycler (MJ Research) using the following protocol: 1 min at 95 °C, 2 min at 45 °C, 1 min at 72 °C and a final extension step for 10 min at 72 °C.

After PCR amplification, coverslips were removed and the slides were washed in 1 × SSC for 5 min. A digoxigenin-labelled probe was heated for 10 min at 100 °C and placed on the section. The slide was then hybridized for 4 to 12 h at 50 °C. After hybridization the slide was washed in 1 × SSC containing 0.05% BSA for 10 min at 42 °C. Anti-digoxigenin antibody conjugated with alkaline phosphatase (Roche) was diluted 1:5000 in a dilution buffer (0.1 M Tris, 0.1 M NaCl), added to the slide, and incubated for 30 min at 37 °C for 30 min. The secondary antibody was removed by two 5 min washes in wash buffer (0.1 M Tris, 0.1 M NaCl, 0.05 M MgCl₂). The colour substrate (NBT/BCIP) was diluted at 20 μl/ml in detection buffer (0.1 M Tris, 0.1 M NaCl, 0.05 M MgCl₂) and incubated at room temperature in the dark until colour development was observed after 1 to 4 h. The reaction was stopped in distilled water and the slides were counterstained with haematoxylin for 2 min followed by three washes of 2 min in distilled water. The sections were dehydrated in graded ethanols followed by xylenes, allowed to air dry, and covered permanently with a cover-slip.

A CAV-specific probe was generated by amplification of pCIA-1 with the O3F and O3R primers (Soiné *et al.*, 1993) as previously described. A negative control probe was generated by amplification of purified duck enteritis virus (DEV) DNA using primers in the UL6 region as described (Plummer *et al.*, 1997).

Serial sections were amplified with CAV-specific primers as described above. One slide was hybridized with the CAV probe and one was hybridized with the DEV probe. Both slides were washed and exposed to the colour substrate as described.

Results

Detection of CAV in spleens by nested PCR

Between 32 and 60% of the spleens were positive in flocks 96-1, 96-2, 97-1 and 97-0. In only a few instances was the spleen the only positive tissue. The data are summarized in Table 1 for hens and Table 2 for roosters.

Detection of CAV in the reproductive tract of female chickens by nested PCR

CAV was detected in the reproductive tissues of chickens from all flocks using the nested PCR assay (Table 1). The

percentage of positive ovaries ranged from 38 to 100%, although the data for the latter flock (97-2a) were based on very few birds. Anatomically distinct parts of the oviduct were analysed separately for the 96-1 flock and were positive in 16% (infundibulum) to 40% (magnum) of the chickens tested. The infundibulum of the hens of flock 97-0 was the only part of the oviduct examined: 39% were positive. The oviducts from the 97-1 and 96-2 flocks were not tested.

CAV was often detected in the ovaries while virus could not be detected in the other organs. Excluding flock 97-2a, the percentage of hens with only ovaries positive ranged from 20% in flock 97-0 to 38% in flock 96-1. In 5 and 12% of the hens with positive ovaries in flocks 96-1 and 97-0, CAV was also detected in the infundibulum. All remaining hens with positive ovaries had positive spleens also (Table 1). Two of the three hens from flock 97-2a were positive in all three tissues tested and one was positive in infundibulum and ovary only.

Detection of CAV in the reproductive organs of roosters by nested PCR

The results for the detection of CAV in the reproductive tracts of roosters from flocks 96-1, 96-2, 97-1 and 97-0 are summarized in Table 2. Interestingly, the incidence in the vas deferentia was higher than in the testes in all flocks but 96-2.

The vas deferens was most frequently the positive tissue. It was the only positive tissue in 20 to 37% of the CAV-positive males in flocks 96-1, 97-0, 97-1 and 96-2 (Table 2). The testes were never positive in the absence of a positive spleen or vas deferens. Spleens and vas deferentia were both positive in 0 (flock 97-1) to 33% in flocks 96-1 and 97-0.

Detection of CAV in tissues from hens and roosters by *in situ* PCR

Selected samples from chickens positive for CAV were used to characterize the cells harbouring virus. The results are summarized in Table 3. In the spleen a few CAV-positive cells were detected. These positive cells were morphologically similar to small lymphocytes and were located in the white pulp. Positive cells were not detected when serial sections were hybridized with the DEV probe. Spleens that were negative in the nested PCR were consistently negative in the *in situ* PCR.

A few positive cells were detected in the theca externa of the ovaries from three chickens (Fig. 1A). The positive cells were small and fusiform, consistent with either the fibrocytes that compose most of the theca externa or the luteal cells that are scattered throughout the layer (Hodges, 1974). These positive cells could not be further differentiated. Two samples of the infundibulum had CAV-positive mucosal epithelial cells (Fig. 1B). The morphology of these CAV positive cells was consistent with the non-secreting, ciliated cells of the surface epithelium (Hodges, 1974). Positive cells were not detected in
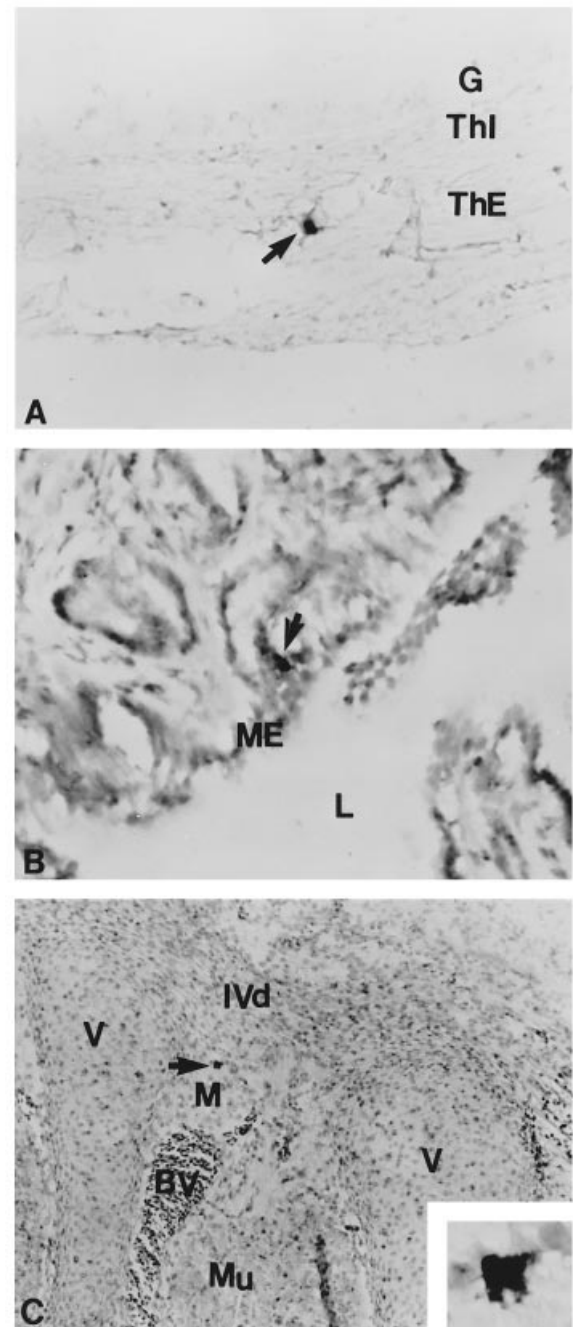

Fig. 1. *In situ* PCR for CAV on female reproductive tract tissues. (A) A positive cell in the theca externa of a developing follicle in the ovary of a hen from flock 97-0 is indicated with an arrow (250 ×). ThE, theca externa; ThI, theca interna; G, granulosa cell layer. (B) Two adjacent mucosal epithelial cells in the infundibulum of a hen from flock 97-2 are indicated with an arrow (250 ×). ME, mucosal epithelium; L, infundibular lumen. (C) A positive cell in the mesenchyme between forming vertebrae is indicated with an arrow (100 ×) and is shown at higher magnification (625 ×) in the inset. V, vertebra; IVd, intervertebral disc; Mu, muscle; BV, blood vessel; M, mesenchyme.

sections hybridized with the DEV probe. Positive cells were not observed in any of the samples of the testis or vas deferens sections by *in situ* PCR.

Table 4. Detection of CAV by nested PCR assay and by virus isolation (VI) in MDCC-CU147

ID	Strain	MHC	Assay	Ovary	Infundibulum	Vas deferens	Testis
		Flock		No. positive/no. tested			
96-1	P2a	$B^{19}B^{19}$	PCR	9/10	3/4	2/2	1/2
			VI	3/10	0/4	0/2	1/2
96-2	S13	$B^{13}B^{13}$	PCR	2/2	3/4	2/2	2/3
			VI	2/2	1/4	0/3	2/3


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A                                          B
   82-2 ADGSKSEAAENWPNCCVPL      82-2 VKYSSDHQNRWRKGGQPMTGGIAYATGKMRPDEQQYPAMPPDPPIITSTT
  CIA-1 ADGSQSQAAQNWPNCWLPL     CIA-1 VKYSSDHQNRWRKGEQPMTGGIAYATGKMRPDEQQYPAMPPDPPIITATT
  ConnB ADGSKSQAGNNWPNGCVPL     ConnB VKYSSDHQNRWRKGGQPMTGGIAYATGKMRPDEQQYPAMPPDPPIITATT
  Cux-1 ADGSKSQAADNWPNCWLPL     Cux-1 VKYSSDHQNRWQKGGQPMTGGIAYATGKMRPDEQQYPAMPPDPPIITATT
  LO28  ADGSQSQAAQNWPNCWLPL     LO28  VKYSSDHQNRWRKGEQPMTGGIAYATGKMRPDEQQYPAMPPDPPIITATT
  SH-1  ADGSQSQAAQNWPNCWLPL     SH-1  VKYSSDHQNRWRKGEQPMTGGIAYATGKMRPDEQQYLVMPPDPPIITSTT
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Fig. 2. Alignment of CAV VP-1 with published sequences. (A) Alignment of amino acids 135 to 153. Arrows indicate residues 139 to 144. (B) Alignment of amino acids 240 to 290. Arrows indicate residues 277 and 278.

Virus isolation

Virus isolation was attempted from tissues from 21 PCR-positive chickens. Virus was isolated from nine chickens (Table 4). Two tissues were used from seven chickens, but virus was never isolated from the two tissues from the same bird. There were several tissues positive by nested PCR from which virus could not be isolated. Infundibulum and vas deferens were difficult to use for virus isolation. This may be due to their small sizes and tendency to dry out when stored at −20 °C for an extended period. In addition, virus was isolated from five of the six semen samples collected from the single rooster from flock 97-2a.

Sequence analysis

The 410 amino acids of the 5′ end of VP-1 from several CAV strains were compared. The American CAV isolates, CIA-1, L-028 and ConnB, differ from SH-1 by 2, 6 and 14 amino acids respectively, while Cux-1, the European strain, differs by 11 amino acids and the Japanese isolate, 82-2, differs from SH-1 in 15 positions. The glutamine residues in positions 139 and 144, which determine differences in *in vitro* cell tropism between CIA-1 and Cux-1 (Renshaw *et al.*, 1996), were present in both CIA-1 and SH-1 (Fig. 2 A). However, there are two amino acid residues unique to SH-1, leucine at position 277 and valine at position 278 (Fig. 2 B). The sequence of VP-3 of SH-1 was compared to the same CAV strains with the exception of L-028 and ConnB, for which VP-3 sequences are

not available. SH-1 has a unique alanine at position 20 and differs from CIA-1 by one amino acid, and from Cux-1 and 82-2 by two amino acids.

Detection of CAV in embryos by *in situ* PCR

Two embryos from matings between the sire and dams from flock 97-2a had CAV-positive cells in the vertebral column. In both cases, the positive cells were located in mesenchymal cells surrounding the developing cartilaginous vertebrae, outside the region of the intervertebral disks. The positive cells were large with round to oval nuclei with an open, reticulated chromatin pattern consistent with mesenchymal cells (Fig. 1 C). Positive cells were not observed in the serial sections hybridized with the control DEV probe.

Discussion

The results add new and important information toward a more complete understanding of the pathogenesis of infection with CAV. First, the finding that virus can persist in chickens long after seroconversion is important, especially in view of the second new finding that CAV can persist in reproductive tissues. It was generally believed that clearance of CAV and subsequent life-long resistance to infection coincides with the development of neutralizing antibodies. For example, Yuasa *et al.* (1983) reported that CAV could be isolated from experimentally infected chickens in the presence of neutralizing antibodies but only up to 49 days p.i. However, the flocks that were used for the detection of CAV in the reproductive organs

in the present study were infected at least 12 months prior to tissue sampling based on flock seroconversion data (Cardona *et al.*, 2000). Interestingly, the three strains of chickens examined for the presence of virus did not differ in the distribution of CAV in their tissues, but considerable differences were noted in antibody responses between N2a (96 to 100% antibody positive) and S13 ($<$ 30% antibody positive) (Cardona *et al.*, 2000). The reasons for the persistence of the virus in either scenario are not clear and need further investigation.

The finding that ovaries and to a lesser degree the infundibulum are sites for persistence of CAV in hens is surprising. CAV replicates mostly in lymphoid tissues after infection of susceptible chickens (Adair *et al.*, 1993; Hoop & Reece, 1991; Smyth *et al.*, 1993). The spleen was therefore selected as a tissue representing target cells for lymphocytic infection in adult birds. Unexpectedly, CAV was often detected in ovaries in the absence of splenic virus. Examination of a limited number of positive ovaries by *in situ* PCR showed that very few cells were virus positive. The presence of CAV in intrathecal cells in ovaries and epithelial cells in the infundibulum suggests strongly that CAV, although lymphotrophic during the early pathogenesis, can infect other cell types.

Interestingly, the testes of chickens with CAV detectable in the vas deferentia were frequently negative (56 and 63% in flocks 96-1 and 97-0, respectively). The presence of PCR inhibitors in testis DNA was eliminated as a possible explanation because actin was amplified from these DNA samples. The vas deferens is the major storage organ for sperm in the chicken. The spermatozoa progress from efferent ductules to the epididymis and finally to the vas deferens and seminal vesicle and in the process mature and become motile (Etches, 1996). It is possible that CAV is present in precursor spermatozoa in the testes but at levels below the detection limits of the nested PCR. CAV may begin replication during the maturation process leading to detectable levels in the vas deferens. Alternatively, CAV may be found in the buffers and proteins added to the seminal fluid as spermatozoa progress through the male reproductive tract (Etches, 1996).

It is not clear how CAV persists in the cells of the reproductive tract. The tissues have not been examined for the presence of viral proteins, but such studies are likely to be inconclusive in view of the few positive cells detected by *in situ* PCR. Therefore, it cannot be excluded that CAV establishes a chronic persistent or latent infection in the female reproductive tract. Alternatively, it is possible that CAV is present as episomal DNA or that it is integrated in chromosomal DNA. Independent of the question of the form of CAV in the reproductive organs these results differ dramatically from previous reports on the persistence of CAV in infected chickens and demonstrate clearly that CAV specifically persists in the reproductive tissues of males and females.

The detection of virus in embryos from matings using a positive sire and positive dams indicates that CAV can be vertically transmitted to embryos long after infection of immunocompetent dams and sires. These findings contrast with earlier reports in which the appearance of CIA lesions in chicks and virus isolation in MSB-1 cells were used to detect vertical transmission (Hoop, 1992, 1993; Hoop *et al.*, 1992; Yuasa & Yoshida, 1983). The nested PCR method used in this work to detect CAV in breeder birds is substantially more sensitive than virus isolation. It may be that the presence of maternal antibodies reduced the titre of virus transmitted to chicks to levels that were undetectable by virus isolation in MSB-1 cells. In addition, the presence of maternal antibodies may have reduced virus replication thus preventing expansion of virus and decreasing the possibility of isolation.

The localization of the positive cells in 11-day-old embryos is of considerable interest. The origin of the mesenchymal cells near the developing vertebrae is not clear, but it is likely that these cells are still pluripotential and able to migrate to several tissues including gonads, bone marrow and liver. CAV could be isolated from whole embryos, embryonal liver, yolk sac and chorioallantoic membranes after inoculation in the yolk sac at 5 days of incubation, but other tissues were not examined (Von Bülow & Witt, 1986). K. A. Schat & K. A. Ealy (unpublished data) examined tissues of CAV-positive embryos using the nested PCR and found that most tissues including gonads, bone marrow and liver were positive. However, VP3 could not be detected in bone marrow smears even when positive with PCR, suggesting that virus replication was either limited and below the detection limits of the assay or absent. In view of the limited evidence for virus replication in embryos and the observation by Cardona *et al.* (2000) that seroconversion occurred during sexual development it is proposed that viral DNA is transferred to the embryo in the absence of virus replication followed by activation of the virus during sexual development.

Partial sequence information on the PCR products indicates that CAV strain SH-1 has minor differences from two strains, Cux-1 and CIA-1, for which extensive pathogenesis studies have been reported (Hu *et al.*, 1993*a*, *b*; Smyth *et al.*, 1993). However, these studies did not use nested PCR assays for the detection of CAV and did not determine if virus persisted in gonad tissues. Although we cannot exclude the possibility that the pathogenesis of infection by SH-1 is different from that by Cux-1 or CIA-1, it is more likely that there are no differences. Based on these findings it is postulated that (1) vertical transmission of CAV may occur far more frequently and over a longer period of time than previously indicated, and (2) that the presence of antibodies will neither eliminate the virus from reproductive tissues nor prevent vertical transmission.

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