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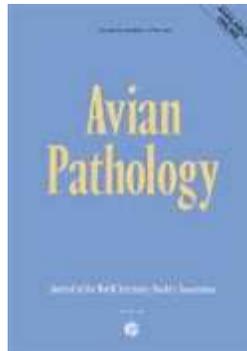
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A seroprevalence investigation of chicken astrovirus infections

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## Abstract

Two genetically different isolates of chicken astrovirus (CAstV), named CAstV612 and CAstV11672, which share low levels of antigenic relatedness in cross-indirect immunofluorescence (IIF) tests, have been identified recently. In this study, separate IIF tests for detecting antibodies to the CAstV612 and CAstV11672 isolates have been used to determine the seroprevalences of CAstV infections in 4 generations of flocks involved in broiler chicken production. CAstV antibodies were detected in 78% (73% CAstV612; 46% CAstV11672) of serum samples from UK broiler flocks and in all 10 flocks tested, indicating that infections were very common. Twenty-three (96%) of 24 and 26 (93%) of 28 broiler parent flocks, aged 23-26 weeks from 3 UK organisations, were positive for antibody to CAstV612 and CAstV11672 respectively. Of 718 samples tested from these parent flocks, 415 (53%) were positive for either CAstV612 or CAstV11672 antibody. CAstV infections were also widespread in parent flocks, with screening of pooled serum samples showing that antibodies to both CAstVs were detected in flocks from 7 other UK poultry organisations and in flocks from 8 other European countries. The seropositivities for CAstVs were substantially less in grandparent (28%) and great grandparent (21%) flocks. Overall, higher seropositivities were observed for CAstV612 than for CAstV11672 in broiler, parent, Grandparent and Great grandparent flocks. A limited study of 99 sera from 10 turkey breeder flocks showed low level seropositivities for CAstV612 (9%) and CAstV11672 (2%), indicating that turkeys were infected with CAstVs or antigenically related viruses.

## Introduction

To date two different astrovirus species have been detected in chickens. Avian nephritis virus (ANV), originally considered to be a picornavirus, was characterised as an avian astrovirus on the basis of its nucleotide sequence (Imada *et al.*, 2000), while Baxendale & Mebatsion (2004) reported the isolation of a different astrovirus, named chicken astrovirus (CAstV), from broiler chickens affected by runting syndrome. We have recently identified a number of small spherical viruses, previously designated enterovirus-like viruses (ELVs), as chicken astroviruses on the basis of sequence identities shared with CAstV (Todd *et al.*, 2009). On the basis of indirect immunofluorescence (IIF) testing and sequence comparisons involving partial ORF 1b (RNA polymerase) sequences, the 612 isolate of CAstV (CAstV612), originally described by McNeilly *et al.* (1994), shares a close antigenic and genetic relationship with the CAstV characterised by Baxendale & Mebatsion (2004), but differs antigenically and genetically from the 11672 and FP3 CAstV isolates (Todd *et al.*, 2009). The CAstVFP3 isolate, which was originally isolated from the meconium of dead-in-shell chicks during an investigation of early broiler mortality in the UK (Spackman *et al.*, 1984), is closely related antigenically and genetically to the CAstV11672 virus, which was isolated in 2005 in our laboratories from 1-day-old chicks as part of an investigation of hatchability problems in the UK.

Our interest in avian astroviruses relates to their possible involvement in causing enteritis and growth depression in chickens (Guy *et al.*, 2009). CAstVs and ANVs were detected in a recent USA investigation of gut and faecal samples from healthy broiler chicken flocks and from flocks affected by enteritis and growth problems (Pantin-Jackwood *et al.*, 2007). CAstVs have also been detected in gut

content samples from UK flocks with growth problems (V.J. Smyth & D. Todd, unpublished results). In addition, CAstVs, originally recognised as ELVs, have also been shown to cause varying degrees of growth retardation following experimental infections of 1-day-old SPF or broiler chicks (McNulty *et al.*, 1990; 1994). However, the nature and extent of the disease problems caused by CAstVs have not been determined, largely due to the absence of convenient, sensitive and specific diagnostic tests.

The detection of virus-specific antibodies in serum samples provides a convenient way of determining the extents of virus infection within animal populations. Earlier small-scale studies by McNeilly *et al.* (1994) and by Baxendale & Mebatsion (2004) showed that infections with the CAstV612 and with first-characterized CAstV were common in broiler and broiler parent flocks from the UK, Europe and the USA. However, no information has been published on the seroprevalence of CAstVs that are antigenically closely related to CAstV11672. The lack of cell culture growth systems for CAstV612 and CAstV11672 has prevented the development of serum neutralization tests with which the serotypic relationship of these isolates could be determined. In this paper we report the development and application of IIF tests for detecting serum antibodies to the CAstV612 and CAstV11672 isolates. The seroprevalences of CAstV infections have been determined for broiler, broiler parent, grandparent (GP) and great grandparent (GGP) flocks from within the UK. In addition, we report the results of serological testing of breeder flocks from other European countries and a small number of turkey flocks.

## Methods

**Virus and virus growth.** Isolate 612 of CAstV (CAstV612) was previously regarded as an enterovirus-like virus (McNeilly *et al.*, 1994). It was originally isolated in South Africa during an investigation of respiratory distress in broiler chickens (McNeilly *et al.*, 1994). The virus was initially propagated in SPF chick embryos using yolk sac inoculation. For this investigation, virus that had been passaged twice in chorioallantoic membranes (CAMs) of 10-day-old SPF embryos was used. Primary chick embryo kidney (CEK) cells that had been grown on glass coverslips, were inoculated with the 612 virus pool, diluted 1:1000, and incubated for 48h at 37°C before the infected coverslip cultures were harvested.

The 11672 isolate of CAstV (CAstV11672) was originally isolated by yolk sac inoculation of SPF chick embryos using tissue homogenates prepared from weak 1-day-old broiler chickens that were submitted to our laboratory as part of a 2004-5 investigation into reduced hatchability problems. The virus received additional passages in SPF chick embryos (yolk sac route) and clarified homogenates of whole embryos harvested between 3 and 5 days after inoculation with 6<sup>th</sup> passage virus were used. This 11672 virus pool, diluted 1:1000, was used to infect coverslip cultures of primary chick embryo liver (CEL) cells. These were incubated for 48h at 37°C before harvesting.

**Experimental antisera.** Antiserum to CAstV11672 was prepared by collecting serum from 5-week-old SPF chickens that had been experimentally infected at 1-day-old with embryo-grown virus using the oral route of inoculation. Antiserum specific to CAstV612 was prepared by experimental infection of 6-week-old SPF chickens using oral inoculation followed by an intravenous boost as described previously (McNeilly *et al.* 1994).

**Field serum samples.** The majority of serum sets, comprising from 10 to 34 samples, were obtained from 3 UK poultry organisations, designated A, B and C to preserve confidentiality. These were collected in 2005-2007 as individual samples from broiler, parent, GP and GGP flocks. Pooled serum samples prepared by generating 3 pools (each comprising 4 sera) from 12 individual serum samples were obtained from additional UK and European parent flocks. Details of the serum numbers and age of bird at testing are contained within Tables 1-4. Ninety-nine turkey serum samples collected from 10 breeder flocks (aged 30-50 weeks) were obtained from organisation B.

**Indirect immunofluorescence tests.** Indirect immunofluorescence tests were performed with coverslip cultures infected with either CAstV612 or CAstV11672 as described previously (Todd *et al.*, 2009). Briefly, acetone-fixed coverslip cultures were incubated for 1h at 37°C with dilutions of chicken serum samples. After washing, bound chicken antibody was reacted with FITC-conjugated goat anti-chicken Ig (usually 1:100) for 1h at 37°C. Immunofluorescent staining was detected using a UV microscope.

**Statistics.** Differences in flock seropositivities at point of lay and at mid lay were statistically compared with Barnard's Unconditional Exact Hypothesis Test of Superiority using the statistical software package StatXact. StatXact was again used when the proportions of serum samples that were seropositive for both CAstV612 and CAstV11672 were compared with the proportions that would be expected if the

infections with these CAstVs were unrelated (Table 4). In this case an exact version of the One-Sample Binomial Test was used.

## Results

**Optimisation of IIF tests.** In the case of both CAstV11672 and CAstV612, 1:1000 dilutions of the virus pools were chosen to infect their respective cell cultures, as this dilution produced sufficient numbers of positively staining cells using the homologous antisera. Reaction of CAstV11672-infected CEL cells and CAstV612-infected CEK cells with either the homologous or the heterologous experimental antisera produced intracytoplasmic immunofluorescent staining. With some infected cells, brightly-staining, granular inclusions were observed, whereas other infected cells on the same coverslip displayed more evenly dispersed staining patterns. Similar staining patterns were observed when field serum samples were used. Preliminary IIF testing identified broiler parent flocks with substantial proportions of samples that were positive for antibody to CAstV11672 and CAstV612. Fifty serum samples, 5 from each of 10 different parent flocks, were tested by IIF for CAstV11672 antibodies using 3 different serum dilutions (1:25, 1:100, 1:500). Forty-one (82%) positives were obtained using the 1:100 dilution, whereas 37 (74%) and 33 (66%) were obtained using the 1:25 and 1:500 dilutions respectively. When tested at the 1:25 dilution, some samples produced high levels of non-specific staining of uninfected cells, which made the detection of virus-specific staining difficult or impossible to detect. However, with 2 samples, positive signals were detected using the 1:25 dilution, which were not detected using 1:100. For flock testing purposes, it was decided to use a 1:100 dilution. A similar preliminary investigation was

performed with CEK coverslip cultures that had been infected with CAstV612. In this case a total of 28 serum samples, 4 from each of 7 flocks, were evaluated using 3 different serum dilutions (1:20, 1:100 and 1:500). Results indicated that 21, 21 and 16 samples were positive at 1:20, 1:100 and 1:500 dilutions respectively. A dilution of 1:100 was selected for flock testing purposes on the basis that 1:20 dilutions produced high levels of non-specific staining.

**Serological testing of broiler and broiler parent flocks.** Sets of serum samples (n = 25 to 38), collected at slaughter (approximately 40 days) from 10 broiler flocks (organisation A) were separately tested for antibody to CAstV612 and CAstV11672 using IIF. All 10 flocks were antibody-positive for both viruses, with CAstV612 seropositivities ranging from 50 to 95% (mean 73%) and CAstV11672 seropositivities ranging from 26 to 64% (mean 46%). Closer examination of the results indicated that 238 (78%) of 306 samples contained antibody to either CAstV612 or CAstV11672, and that 110 (36%) of 306 samples were positive for antibody to both CAstV612 and CAstV11672.

Testing of serum samples (n=10) from 10 parent flocks (organisation A) during rear (aged 9-14 weeks) showed that all flocks were positive for CAstV11672 antibody, with seropositivities ranging from 10-100% (mean 60%) (Table 1). When samples (n~30) from parent flocks from organisation A were tested at point of lay (23-25 weeks), 15 of 15 and 18 of 18 flocks were positive for antibody to CAstV612 and CAstV11672 respectively. Seropositivity for CAstV612 ranged from 10 to 90% (mean 46%), and seropositivity for CAstV11672 antibody ranged from 0 to 77% (mean 40%). Close examination of the results showed that houses on the same site

varied considerably in seropositivity. For example, 77, 23 and 20% of the samples from 3 houses on farm A/18 were positive for CAstV11672 antibody (Table 1).

Serum samples (n= ~10 to 30) from parent flocks from 2 other organisations (B and C) were also tested at ages that corresponded to point of lay or early in lay. Results showed that 4 of 5 farms from organisation B were seropositive for both CAstV612 (range 0 to 83%; mean 30%) and CAstV11672 (range 0 to 57%; mean 33%). With organisation C, 4 of 4 flocks and 3 of 4 flocks were positive for CAstV612 (range 20 to 60%; mean 41%) and CAstV11672 (range 0 to 40%; mean 20%) antibodies respectively (Table 1).

Four of the parent flocks (A/14.1, A/19.2, A/20.2, A/21.2) from organisation A, which had been tested at point of lay, were re-tested at 46 weeks of age, within the second half of the laying period. With CAstV612, 3 of the 4 flocks showed similar or reduced seropositivity, whereas with one flock (A/19.2) the seropositivity increased significantly ( $p < 0.05$ ) from 67 to 100%. With CAstV11672, the seropositivity also increased significantly in flock A/19.2 (from 33 to 70%), while those of the other 3 flocks remained at a similar level (A/14.1) or diminished (A/20.2 and A/21.2) respectively. Testing of serum sets (n = 7 to 10) from organisation C at 25-26 weeks of age demonstrated that 4 of 4 parent flocks were seropositive for CAstV612 (range 20 to 56%; mean 41%) and CAstV11672 (range 0 to 40%; mean 20%) antibody. It was noted that the CAstV612 seropositivity was substantially higher at 47-50 weeks (88%) than at 25-26 weeks (41%) (Table 1). Taking the serum samples from broiler parent flocks aged 20 weeks and over as a whole, 348 (45%) of 772 samples were positive for CAstV612 antibody and 304 (35%) of 880 samples were positive for CAstV11672 antibody. Overall, 415 (53%) of 781 samples were positive for antibody to either CAstV612 or CAstV11672.

**Serological testing of grandparent and great grandparent flocks.** Testing of serum samples (n = up to 30) from GP flocks (aged 20-26 weeks) from organisation A showed that 10 of 13 (77%) and 5 of 13 (38%) were positive for CAstV612 and CAstV11672 antibody respectively (Table 2). With regards to CAstV612, seropositivity ranged from 0 to 100% (mean 29%), whereas with CAstV11672, seropositivity ranged from 0 to 80% (mean 13%). Overall, 68 (33%) of 208 serum samples were positive for antibody to either CAstV612 or CAstV11672.

Serum samples (n = up to 10) were also tested from GP and GGP flocks from organisation C. None of 5 GP flocks, aged 24 weeks, was positive for CAstV11672 antibody, whereas 4 of 5 GP flocks were positive for antibody to CAstV612 (range 0 to 40%; mean 18%). Testing the same GP flocks at 48-54 weeks of age showed that 4 of the 5 remained seropositive for CAstV612 (range 0 to 60%; mean 20%) while 2 of the 5 flocks were positive for CAstV11672 antibody (range 0 to 20%; mean 6%). When tested at 23-30 weeks, 2 of 5 GGP flocks were positive for CAstV612 antibody (range 0 to 50%; mean 12%) whereas none of the 5 flocks were seropositive to CAstV11672. Subsequent testing when the same flocks were aged 48-57 weeks showed that 3 of 5 were seropositive for CAstV612 (range 0 to 40%; mean 12%) and CAstV11672 (range 0 to 50%; mean 18%) respectively. With one GGP flock (C14), the increase in CAstV11672 seropositivity with age was significant ( $p < 0.05$ ). Taken together, these results indicated that the seroprevalences of CAstV612 infections (15%) of GP and GGP flocks from organisation C were higher than those of CAstV11672 infections (6%).

**Serological screening of parent flocks from other UK poultry producers and producers within other Europe.** A screening test in which 12 serum samples were tested as 3 separate pools (with each pool comprising 4 serum samples) was used to assess the prevalence of CAstV612 and CAstV11672 antibodies in 36 parent flocks sourced from 10 different UK poultry organisations and 25 parent flocks sourced from poultry organisations located in 7 other EU countries (Table 3). Using this testing approach, 21 (58%) and 23 (64%) of the 36 UK flocks were seropositive for CAstV612 and CAstV11672 respectively. Of the 10 UK organisations surveyed, flocks from 9 (90%) organisations were positive for CAstV612 antibody and flocks from 8 (80%) organisations were positive for CAstV11672 antibody.

Parent flocks from 8 European countries including Croatia, the Czech Republic, Denmark, Germany, Ireland, the Netherlands, Norway and Sweden were seropositive for both CAstV612 and CAstV11672. Of 25 flocks tested, 15 (60%) and 20 (80%) were positive for antibody to CAstV612 and CAstV11672 respectively.

**Serological testing of turkey flocks.** Sets of serum samples (n=~10) from 10 turkey breeder flocks aged 20-50 weeks and belonging to one UK organisation, were also tested for the presence of antibody to CAstV612 and CAstV11672. Of 99 samples tested, 9 (9%) derived from 2 flocks were positive for CAstV612 antibody and 2 (2%) samples from a single, different flock were positive for CAstV11672 antibody.

## **Discussion**

This is the first paper to report the seroprevalences of chicken astrovirus infections in 4 generations of flocks involved in commercial chicken production. Overall, testing

indicated that CAstV infections were very common in broiler and broiler parent flocks within the UK and appeared to be widespread also within European breeder flocks. Not unexpectedly, seroprevalences were less in GP and GGP flocks, where higher levels of biosecurity are generally maintained. There is also serological evidence that turkey flocks are infected with CAstVs or viruses that are antigenically related to CAstVs.

This study relates to the detection of antibodies to 2 antigenically different CAstV isolates, which were identified recently as astroviruses in our laboratory (Todd *et al.*, 2009). In cross-indirect immunofluorescence tests using antisera raised in experimentally infected chickens, heterologous titres of 1:32 and 1:64 were obtained for CAstV612 and CAstV11672 isolates respectively and differences of >64-fold and 32-fold were observed between the homologous and heterologous IIF reactivity titres with the antisera to CAstV612 and CAstV11672 respectively (Todd *et al.*, 2009). Based on recent sequencing work, which showed that the capsid proteins of the 612 and 11672 CAstV isolates shared approximately 40% amino acid identity (V.J. Smyth & D. Todd; unpublished results), it is very likely that CAstV612 and CAstV11672 isolates are representative of 2 different serotypes of CAstV. It remains to be determined whether the low level IIF cross-reactivity is due to antigenic similarity shared by the capsid, RNA polymerase or viral protease proteins of these CAstV isolates. So far their poor growth in cell culture, has prevented us from developing serum neutralization tests with which their serotypic relationship could be investigated and serotype-specific antibodies could be detected in field sera. Therefore, our approach has been to detect serum antibodies to the two CAstV types using IIF tests based on chicken embryo derived cell cultures separately infected with each of the isolates. On the basis of the low levels of antigenic relatedness and

because a serum dilution of 1:100 was used in each of the IIF tests, it is likely that most positive results recorded using the IIF tests based on CAstV612 and CAstV11672 will be with sera from chickens infected with CAstVs that are antigenically similar to the CAstV612 and CAstV11672 isolates respectively. However, it is acknowledged that high levels of antibody induced by infection with one of these CAstV “antigenic types” will be detected by the IIF test based on the other CAstV antigenic type. That this may be occurring is supported in part by the detection in some flock types of higher proportions of serum samples that are seropositive to both CAstV isolates than would have been predicted if the detections were non-related (Table 4). For example, the occurrence of antibodies to both CAstV isolates in 20.8% broiler samples is significantly different ( $p = 0.0065$ ) than the value that would have been expected (9.7%) on the basis of the seropositivities for CAstV612 (29.5%) and CAstV11672 (32.9%). Significant differences were also observed for other flock types and together they provide support for the view that with some serum samples antibodies to one CAstV isolate are being detected in the IIF test based on the other CAstV isolate. Although it is likely that flocks are infected with both CAstV antigenic types, we cannot exclude the possibility that antibodies induced by infection with other antigenically different, but yet cross-reactive CAstV isolates could be detected by both tests. Taken as a whole, the use of two IIF tests based on antigenically different CAstV isolates broadens the range of CAstVs that are serologically detected.

CAstV antibody detected at slaughter (approximately 6 weeks) in broiler chickens is actively-acquired, indicating that broiler chicks are exposed to CAstVs in the first few weeks of life. Although performance data was unavailable for the broiler flocks that were serologically tested, no clinical growth retardation problems were

reported for these flocks. At present our knowledge of CAstV pathogenicity is very limited. Therefore, it is not possible to predict the clinical effects of the CAstV infections on broilers in the field. However, CAstV-related factors that are likely to affect broiler flock performance include variation in the pathogenicity of CAstV isolates present, the size of infectious challenge, the age at which most chicks become infected, interactions with other enteric viruses and the levels of maternal antibody. The results from broiler parent flocks, where substantial proportions of individual adults have no detectable antibody, indicated that the maternal antibody levels in progeny chicks are likely to be variable. Enteric infections occur in chicks with and without maternal antibody, as is the case with the astrovirus ANV (Takase *et al.*, 1994). It remains to be determined whether the presence of maternal antibody reduces the severity of pathogenic effects such as reduced weight gain and virus spread to internal organs such as the kidney, which is known to occur with the FP3 isolate of CAstV (Smyth *et al.*, 2007).

The CAstVs that cause infections in young chicks may be “carried over” from one broiler crop to another, particularly if cleaning and disinfection of the house have been inadequate. Astrovirus contamination of the environment within the house may be high since these viruses can be excreted in large numbers in faeces and astrovirus infectivity shows relatively high levels of resistance to inactivation (Guy *et al.*, 2009). Although most infections are likely to occur as a result of horizontal transmission involving the faecal-oral route, it is also possible that some chicks will be infected with virus that has been vertically transmitted virus from infected in-lay parents. The isolation of the CAstVFP3 from the meconium of dead-in-shell chicks (Spackman *et al.*, 1984) and the more recent detection of other CAstVs, including the 11672 isolate, in 1-day-old chicks and dead-in-shell chicks during an investigation of hatchability

problems (Todd, unpublished results) are consistent with the view that isolates of this astrovirus can be vertically transmitted. Although the incidence of vertical transmission is unknown, the detection of increased seropositivity to both CAstVs after point of lay in 1 of 4 broiler parent flocks investigated from organisation A indicated that CAstV infections can occur in adult chickens during lay, thereby creating the opportunity for vertical transmission. A significant increase in seropositivity during lay to CAstV11672 was also observed in one GGP flocks from organisation C. However, our results from broiler chickens tested at slaughter (78% seropositivity to either CAstV isolate) and from broiler parent flocks tested at 9-14 weeks (60% seropositivity to CAstV11672) suggested that most infections occur in young chickens. Failure to detect complete seroconversion as seen in the majority of parent, GP and GGP flocks tested at point of lay and after may be because the IIF test is not sufficiently sensitive to detect lower levels of antibody. Alternatively, it is possible that virus spread may be less efficient in older birds due to reduced susceptibility, and/ or lower levels of virus replication and excretion. The reductions in CAstV11672 seropositivity from 60% to 40% detected in broiler parent flocks (organisation A) tested at 9-14 weeks and at 23-25 weeks, and in 2 of 4 parent flocks tested at 25 weeks and 46 weeks suggest that the levels of astrovirus-specific antibody may decline with time. Reduced seropositivity in older chickens supports the view that CAstV re-infections, which would be expected to increase antibody levels, were not common and suggests that chickens may be protected against re-infections.

The detection of CAstV infections in some GP and GGP flocks, which maintain very high levels of biosecurity, highlights the potential difficulties in eradicating this virus. However, the probability that some CAstVs can be vertically transmitted and have the potential to cause pathogenic effects in chicks as

demonstrated experimentally (Smyth *et al.*, 2007) suggests that organisations producing SPF eggs should take steps to eliminate CAstV infections so that potential contamination of chick embryo-derived poultry vaccines can be avoided. Serological screening provides the tool by which infected flocks can be identified and freedom of infection demonstrated. The IIF tests described in this paper are not suited to flock screening involving large sample numbers on the basis that they are labour-intensive, time-consuming and require expertise when the immunofluorescent staining is being read. Tests such as ELISA will be required if large-scale flock testing programmes are to be implemented. At present, the poor cell culture growth of CAstVs makes the production of virus antigen difficult and has restricted the development of antibody-detecting ELISA tests. Recombinant antigens may be of use if virus yields from cell culture cannot be improved.

On the basis of earlier experimental infection studies (McNeilly *et al.*, 1994; Smyth *et al.*, 2007) and recent investigations, which demonstrated the presence of CAstVs in samples from broiler flocks with severe uneven growth problems (V.J. Smyth & D. Todd; unpublished results), it is likely that CAstVs contribute to enteritis and growth depression problems. The control of these disease problems is desirable. The high prevalence of infections in breeder and broiler flocks indicates that virus eradication is not a practical option. However, it may be possible to reduce the severity of the clinical effects by vaccinating parent birds during rear to achieve uniformly high levels of maternal antibody and to reduce the possibility of vertical transmission occurring. Results presented in this paper indicate that the antibody levels in breeders at point of lay vary considerably (0 to 100%), and this will result in variable levels of maternal antibody. Work with ANV, the first characterized astrovirus of chickens, has shown that the presence of maternal antibody does not

prevent enteric infections, but can limit the spread of the virus to internal organs (Takase *et al.*, 1994). Work is underway to investigate the protective effects of maternal antibody and increasing age against the pathogenic effects of experimental CAstV infections.

In summary, the serological results presented in this paper contribute to the understanding of CAstV epidemiology and highlight potential problems for controlling infections in commercial broiler and SPF flocks.

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**Table 3.** *Indirect immunofluorescence detection of antibody to CAstV612 and CAstV11672 isolates in pooled serum samples from UK and European broiler parent flocks*

Org./ farm	Country	Positive for CAstV612 antibody	Positive for CAstV11672 antibody	Org./ farm	Country	Positive for CAstV612 antibody	Positive for CAstV612 antibody
A/22 <sup>a</sup>	UK	0 <sup>b</sup>	0 <sup>b</sup>	L/8	UK	0 <sup>b</sup>	2 <sup>b</sup>
A/23	UK	1	0	L/9	UK	1	0
A/24	UK	0	2	L/10	UK	0	1
A/25	UK	0	1	L/11	UK	0	1
D/1	UK	1	1	L/12	UK	0	0
E/1	UK	3	2	M/1	Croatia	3	3
E/2	UK	2	2	M/2	Croatia	2	1
E/3	UK	0	1	N/1	Czech Rep.	3	2
E/4	UK	2	2	N/2	Czech Rep.	2	1
E/5	UK	3	2	N/3	Czech Rep.	1	1
E/6	UK	2	0	O/1	Denmark	2	1
E/7	UK	1	0	O/2	Denmark	0	0
F/1	UK	3	2	P/1	Germany	0	0
F/2	UK	0	1	P/2	Germany	0	3
G/1	UK	1	2	P/3	Germany	1	2
G/2	UK	0	1	P/4	Germany	0	1
H/1	UK	1	0	P/5	Germany	2	2
H/2	UK	3	2	P/6	Germany	3	1
I/1	UK	3	3	Q/1	Ireland	1	1
I/2	UK	3	1	Q/2	Ireland	0	3
J/1	UK	1	0	Q/3	Ireland	3	2
J/2	UK	0	0	Q/4	Ireland	0	2
J/3	UK	1	0	Q/5	Ireland	0	1
K/1	UK	0	0	R/1	Netherlands	1	3
L/1	UK	0	0	R/2	Netherlands	1	2
L/2	UK	2	1	S/1	Norway	0	0
L/3	UK	2	1	S/2	Norway	1	1
L/4	UK	1	1	T/1	Sweden	1	0
L/5	UK	0	3	T/2	Sweden	0	0
L/6	UK	0	3	T/3	Sweden	0	2
L/7	UK	3	0				

<sup>a</sup>: Organisations are coded alphabetically.

<sup>b</sup>: Number positive for CAstV antibody by IIF out of 3 tested.

**Table 4.** Summary of indirect immunofluorescence test results for CAstV612 and CAstV11672 antibody obtained with different flock types.

Flock type/ Organisation Age	Positive for CAstV612 antibody (%)	Positive for CAstV11672 antibody (%)	Positive for both CAstV612 and CAstV11672 antibody (%)	Percentages expected if infections non-related	Significance <sup>b</sup>
Broiler /A	221/306 <sup>a</sup> (72.2%)	139/306 <sup>a</sup> (45.4%)	110/306 <sup>a</sup> (35.9%)	32.8%	0.2668
Parent/ A 23-25 wk	209/449 (46.5%)	187/449 (41.6%)	111/449 (24.7%)	19.3%	<b>0.0065</b>
Parent /A 46 wk	49/100 (49.0%)	22/ 100 (22.0%)	19/100 (19.0%)	10.8%	<b>0.0205</b>
Parent/ B 20 wk	44/149 (29.5%)	49/149 (32.9%)	31/149 (20.8%)	9.7%	<b>0.0001</b>
Parent/ C 25-26 wk	16/39 (41.0%)	8/39 (20.5%)	3/39 (7.7%)	8.4%	1.000
Parent /C 47-50 wk	30/34 (88.2%)	9/34 (26.5%)	9/34 (26.5%)	23.4%	0.7992
GP/ A 20-26 wk	60/208 (28.8%)	32/208 (15.4%)	24/208 (11.5%)	4.4%	<b>0.0000</b>
GP/ C 24 wk	8/50 (16.0%)	0/50 (0%)	0/50 (0%)	0%	N/A
GP/ C 48-56 wk	10/48 (20.8%)	3/48 (6.3%)	2/48 (4.2%)	1.3%	0.2581
GGP/ C 23-30 wk	6/50 (12.0%)	0/50 (0%)	0/50 (0%)	0%	N/A
GGP/ C 48-57 wk	6/49 (12.2%)	9/49 (18.4%)	0/49 (0%)	2.2%	0.6724

<sup>a</sup>. Number positive for CAstV antibody by IIF/ number tested.

<sup>b</sup> Significance relates to the differences that exist between the percentages expected if the infections with each CAstV type were unrelated and the proportion obtained.

**Table 1.** Indirect immunofluorescence detection of antibody to CAstV612 and CAstV11672 in serum samples from broiler parent flocks from 3 UK organisations A, B and C.

Organisation/ Farm. house	Age week	Positive for CAstV612 antibody (%) <sup>a</sup>	Positive for CAstV11672 antibody (%) <sup>a</sup>	Positive for either CAstV612 or CAstV11672 antibody (%) <sup>a</sup>	Organisation/ Farm. house	Age week	Positive for CAstV612 antibody (%) <sup>a</sup>	Positive for CAstV11672 antibody (%) <sup>a</sup>	Positive for either CAstV612 or CAstV11672 antibody (%) <sup>a</sup>
A/1.2	14	NT <sup>b</sup>	2/10 (20)	NA <sup>c</sup>	A/20.1	23	6/30 (20)	13/30 (43)	18/30 (60)
A/2.1	14	NT	4/10 (40)	NA	A/20.2	23	13/30 (43)	7/30 (23)	15/30 (50)
A/3.1	14	NT	9/10 (90)	NA	A/21.1	25	11/30 (37)	15/30 (50)	17/30 (57)
A/4.1	9	NT	10/10 (100)	NA	A/21.2	25	15/30 (50)	9/30 (30)	18/30 (60)
A/5.1	14	NT	4/10 (40)	NA	A/14.1	46	3/26 (12)	6/30 (20)	6/26 (23)
A/6.1	14	NT	8/10 (80)	NA	A/19.2	46	19/19 (100)	14/20 (70)	19/19 (100)
A/7.1	14	NT	7/10 (70)	NA	A/20.2	46	14/29 (48)	1/30 (3)	14/29 (48)
A/8.1	9	NT	5/10 (50)	NA	A/21.2	46	13/26 (50)	2/30 (6)	13/26 (50)
A/9.1	9	NT	1/10 (10)	NA					
A/10.1	14	NT	10/10 (100)	NA	B/1	20	25/30 (83)	16/30 (53)	25/30 (83)
					B/2	20	6/29 (21)	13/30 (43)	14/29 (48)
A/11.1	25	20/30 (67)	15/30 (50)	26/30 (87)	B/3	20	11/30 (37)	17/30 (57)	19/30 (63)
A/12.1	23	7/30 (23)	9/30 (30)	14/30 (47)	B/4	20	0/30 (0)	0/30 (0)	0/30 (0)
A/12.2	23	6/30 (20)	16/30 (53)	19/30 (63)	B/5	20	2/30 (7)	3/30 (10)	4/30 (13)
A/13.1	25	19/30 (63)	14/30 (47)	20/30 (67)					
A/14.1	25	15/30 (50)	8/30 (27)	16/30 (53)	C/1	26	3/10 (30)	1/10 (10)	4/10 (40)
A/15.1	24	9/30 (30)	0/30 (0)	9/30 (30)	C/2	25	6/10 (60)	0/10 (0)	6/10 (60)
A/15.2	24	3/30 (10)	11/30 (37)	12/30 (40)	C/3	26	2/10 (20)	4/10 (40)	5/10 (50)
A/16.1	24	27/30 (90)	20/30 (67)	27/30 (90)	C/4	25	5/9 (56)	3/10 (30)	6/9 (67)
A/17.2	25	NT	14/30 (47)	NA					
A/18.1	25	16/29 (55)	23/30 (77)	24/29 (83)	C/5	48	9/10 (90)	2/10 (20)	9/10 (90)
A/18.2	24	NT	7/30 (23)	NA	C/6	47	8/8 (100)	5/10 (50)	8/8 (50)
A/18.3	24	NT	6/30 (20)	NA	C/7	48	7/9 (78)	1/10 (10)	7/9 (78)
A/19.1	25	22/30 (73)	17/30 (57)	28/30 (93)	C/8	50	6/7 (86)	2/10 (20)	6/7 (86)
A/19.2	25	20/30 (67)	10/30 (33)	23/30 (77)					

<sup>a</sup> Number positive for CAstV antibody by IIF/ number tested (percent positive).

<sup>b</sup> Not tested.

<sup>c</sup> Not applicable.

**Table 2.** Indirect immunofluorescence detection of antibody to CAstV612 and CAstV11672 isolated in serum samples from grandparent and great grandparent flocks from UK organisations A and C

Organisation/ Farm. house	Age week	Positive for CAstV612 antibody (%) <sup>a</sup>	Positive for CAstV11672 antibody (%) <sup>a</sup>	Positive for either CAstV612 or CAstV11672 antibody (%) <sup>a</sup>	Organisation/ Farm. house	Age Week	Positive for CAstV612 antibody (%) <sup>a</sup>	Positive for CAstV11672 antibody (%) <sup>a</sup>	Positive for either CAstV612 or CAstV11672 antibody (%) <sup>a</sup>
A/22.1	20	10/10 (100)	8/10 (80)	10/10 (100)	C/9	54	0/9	0/10 (0)	0/9 (0)
A/22.2	20	1/7 (14)	0/10 (0)	1/7 (14)	C/10	48	1/10 (10)	0/10 (0)	1/10 (10)
A/22.3	20	1/8 (13)	0/10 (0)	1/8 (13)	C/11	54	6/10 (60)	1/10 (0)	6/10 (60)
A/23.1	24	0/16 (0)	0/20 (0)	0/16 (0)	C/12	54	1/9	0/10 (0)	1/9 (11)
A/23.3	24	6/10 (60)	0/10 (0)	6/10 (60)	C/13	54	2/10 (20)	2/10 (20)	3/10 (30)
A/24.2	24	8/26 (31)	10/30 (33)	13/26 (50)	C/14 <sup>b</sup>	23	1/10 (10)	0/10 (0)	1/10 (10)
A/25.1	24	1/26 (4)	0/30 (0)	1/26 (4)	C/15 <sup>b</sup>	24	5/10 (50)	0/10 (0)	5/10 (50)
A/26.2	24	6/19 (32)	0/29 (0)	6/19 (32)	C/16 <sup>b</sup>	24	0/10 (0)	0/10 (0)	0/10 (0)
A/27.2	24	3/28 (11)	1/30 (3)	4/28 (14)	C/17 <sup>b</sup>	30	0/10 (0)	0/10 (0)	0/10 (0)
A/28.1	23	14/28 (50)	6/30 (20)	16/28 (57)	C/18 <sup>b</sup>	30	0/10 (0)	0/10 (0)	0/10 (0)
A/29.1	26	10/10 (100)	7/10 (7)	10/10 (100)					
A/30.1	25	0/10 (0)	0/10 (0)	0/10 (0)					
A/31.1	23	0/10 (0)	0/10 (0)	0/10 (0)	C/14 <sup>b</sup>	56	0/10 (0)	5/10 (50)	5/10 (50)
					C/15 <sup>b</sup>	51	4/10 (40)	0/10 (50)	4/10 (40)
C/9	24	0/0 (0)	0/10 (0)	0/10 (0)	C/16 <sup>b</sup>	48	1/9 (11)	2/10 (20)	3/9 (33)
C/10	24	1/10 (10)	0/10 (0)	1/10 (10)	C/17 <sup>b</sup>	54	0/10 (0)	2/10 (20)	2/10 (20)
C/11	24	4/10 (40)	0/10 (0)	4/10 (40)	C/18 <sup>b</sup>	57	1/10 (0)	0/10 (0)	1/10 (10)
C/12	24	2/10 (20)	0/10 (0)	2/10 (20)					
C/13	24	1/10 (10)	0/10 (0)	1/10 (10)					

<sup>a</sup> Number positive for CAstV antibody by IIF/ number tested (percent positive).

<sup>b</sup> Great grandparent flocks.