Inclusion body hepatitis associated with an outbreak of fowl adenovirus type 2 and type 8b in broiler flocks in South Africa

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Inclusion body hepatitis is an acute disease of chickens ascribed to viruses of the genus *Aviadenovirus* and referred to as fowl adenovirus (FAdV). There are 12 FAdV types (FAdV1 to FAdV8a and FAdV8b to FAdV11), classified into five species based on their genotype (designated FAdVA to FAdVE). A total of 218 000 chickens, 2–29 days of age, were affected over a 1-year period, all testing positive by microscopy, virus isolation and confirmation with polymerase chain reaction (PCR). Affected birds were depressed, lost body weight, were weak and had watery droppings. Pathological changes observed during necropsy indicated consistent changes in the liver, characterised by hepatomegaly, cholestasis and hepatitis. Lesions were also discernible in the spleen, kidney and gizzard wall and were characterised by splenomegaly, pinpoint haemorrhages, nephritis with haemorrhage, visceral gout and serosal ecchymosis of the gizzard wall. Histopathological lesions were most consistently observed in the liver but could also be seen in renal and splenic tissue. Virus isolation was achieved in embryonated eggs and most embryos revealed multifocal to diffuse hepatic necrosis, with a mixed cellular infiltrate of macrophages and heterophils (necro-granulomas), even in the absence of macroscopic pathology. Virus isolation results were verified by histopathology and PCR on embryonic material and further characterised by nucleotide sequence analysis. Two infectious bursal disease virus isolates were also made from the Klerksdorp flock. Nucleotide sequence analysis of the L1 hexon loop of all the FAdV isolates indicated homology (99%) with prototype strains P7-A for FAdV-2, as well as for FAdV-8b.

Introduction

Fowl adenoviruses (FAdV) are ubiquitous infectious agents of poultry (McFerran & Adair 2003) and have been isolated, not only from healthy avian hosts but also as primary pathogens of various species, including chicken, turkey and quail. Diseases associated with FAdV are involved in economically important conditions such as egg drop syndrome, turkey haemorrhagic enteritis, quail bronchitis and inclusion body hepatitis (McFerran & Adair 2003). Inclusion body hepatitis (IBH) is a disease of chickens caused by viruses of the genus *Aviadenovirus* (McFerran & Adair 2003), more specifically FAdV. They are classified into five species based on genotype (designated FAdVA to FAdVE) and into 12 types (FAdV1 to FAdV8a and FAdV8b to FAdV11) (Benkö *et al.* 2005; McFerran & Adair 2003). Differences in the virulence of types and even strains of the same type have been reported (Erny, Barr & Fahey 1991; McFerran & Adair 2003). Inclusion body hepatitis is characterised by sudden onset chick mortalities of short duration (generally 5 days) and in meat-producing birds (3–7 weeks of age) (McFerran & Adair 2003). Initial outbreaks are often associated with mortalities of ± 10%, but mortalities of up 60% – 70% have also been reported (Dahiya *et al.* 2002; Gomis *et al.* 2006). Hepatitis with intranuclear inclusion bodies in affected hepatocytes is typically found upon histopathological examination (McFerran & Adair 2003). Other pathological conditions commonly associated with FAdV include immunosuppression, hydropericardium, growth retardation and gizzard erosion (Marek *et al.* 2010; McFerran & Adair 2003; Schade *et al.* 2013; Schonewille *et al.* 2010). Although both vertical and horizontal transmission has been described, vertical transmission is of particular importance. Vertical transmission occurs when sero-negative breeding stock is exposed to aviadenoviruses during production. Typically, clinical signs of disease are only seen in offspring after hatching. The first case of IBH was reported from the USA in 1963 (Helmbolt & Frazier 1963). Since then, the disease has been described with increasing frequency and severity in poultry-producing countries worldwide. This article is the first published record of IBH in South Africa and describes the method of isolation and the pathological and histopathological lesions seen in laboratory hosts. Significant production losses occurred in broilers (10% – 20% mortalities) in an integrated broiler production enterprise.

TABLE 1: Summary of the cases of inclusion body hepatitis during 2008 and 2009.

Sample date	Age (days)	Area	Province	Number of birds in flock	Number of birds affected with IBH†	Percentage of birds affected	Macropathology‡	FAdV isolation	PCR positive	FAdV type¶
2008, Nov.	~18	Klerksdorp	North West	49 909	6530	13.08	(+)	(+)	(+)	8b
2008, Nov.	18	Klerksdorp	North West	49 801	9100	18.27	(+)	(+)	(+)	2
2008, Nov.	~11	Klerksdorp	North West	49 949	6800	13.61	(+)	(+)	(+)	8b
2008, Nov.	14	Klerksdorp	North West	49 980	8690	17.39	(+)	(-/+)‡	(+)	8b
2008, Nov.	14	Klerksdorp	North West	49 921	10 100	20.23	(+)	(+)	(+)	8b
2008, Dec.	9	Klerksdorp	North West	49 798	5975	12.00	(+)	(-/+)‡	(+)	8b
2008, Nov.	~11	Klerksdorp	North West	48 997	8980	18.33	(+)	(+)§	(+)	8b
2008, Dec.	9	Wesselsbron	Free State	20 961	2812	13.42	(+)	(-/+)‡	(+)	8b
2008, Nov.	14	Klerksdorp	North West	49 870	5580	11.19	(+)	(+)	(+)	2
2008, Nov.	14	Klerksdorp	North West	49 760	8950	17.99	(+)	(+)	(+)	8b
2008, Nov.	~9	Klerksdorp	North West	49 850	6869	13.78	(+)	(+)	(+)	8b
2009, Feb.	18	Ventersdorp	North West	20 980	3116	14.85	(+)	(+)	(+)	n.d.
2009, Feb.	8	Klerksdorp	North West	49 820	9690	19.45	(+)	(-/+)‡	(+)	n.d.
2009, Apr.	19	Klerksdorp	North West	49 910	8314	16.66	(+)	(-/+)‡	(+)	n.d.
2009, Apr.	19	Klerksdorp	North West	49 858	10 890	21.84	(+)	(+)	(+)	n.d.
2009, Aug.	28	Wesselsbron	Free State	19 000	3180	16.74	(+)	(+)	(+)	n.d.
2009, Aug.	24	Wesselsbron	Free State	47 968	7689	16.03	(+)	(+)	(+)	n.d.
2009, Oct.	14	Klerksdorp	North West	49 946	9667	19.35	(+)	(+)	(+)	n.d.
2009, Nov.	14–17	Syferfontein	North West	18 950	3389	17.88	(+)	(-/+)‡	(+)	n.d.
2009, Nov.	14–17	Syferfontein	North West	18 970	3465	18.27	(+)	(+)	(+)	n.d.
2009, Nov.	14	Wesselsbron	Free State	44 890	4550	10.14	(+)	(-/+)‡	(+)	n.d.
2009, Dec.	~12	Wolmaransstad	North West	26 992	4378	16.22	(+)	(-/+)‡	(+)	n.d.
2009, Dec.	~12	Wolmaransstad	North West	26 914	4663	17.33	(+)	(+)	(+)	n.d.
2009, Dec.	~12	Wolmaransstad	North West	26 990	5340	19.79	(+)	(+)	(+)	n.d.
2009, Dec.	~12	Wolmaransstad	North West	19 891	3390	17.04	(+)	(+)	(+)	n.d.
2009, Dec.	15	Botshabelo	North West	25 980	3980	15.32	(+)	(+)	(+)	n.d.
2009, Dec.	17	Klerksdorp	North West	49 987	8907	17.82	(+)	(+)	(+)	2
2009, Dec.	17	Klerksdorp	North West	49 730	10 030	20.17	(+)	(+)	(+)	2
2009, Dec.	20	Wesselsbron	Free State	43 000	6970	16.21	(+)	(+)	(+)	2
2009, Dec.	20	Wesselsbron	Free State	19 000	2980	15.68	(+)	(-/+)‡	(+)	2
2009, Dec.	20	Wesselsbron	Free State	43 000	7680	17.86	(+)	(-/+)‡	(+)	2
2009, Dec.	20	Wesselsbron	Free State	48 000	6657	13.87	(+)	(+)	(+)	2

IBH, inclusion body hepatitis; FAdV, fowl adenovirus; PCR, polymerase chain reaction; n.d., not done.
†, birds were considered positive if they showed typical lesions of IBH, such as liver necrosis and haemorrhage upon necropsy; ‡, some of the birds in these flocks were negative on virus isolation, but only 25% of the samples that yielded negative results on virus isolation originated from chickens with typical necrohaemorrhagic liver lesions; §, very virulent infectious bursal disease positive; ¶, PCR for confirmation of FAdV is done as a routine diagnostic assay and typing of the isolates is only done if requested by the client.

Research method and design

Although previous outbreaks of IBH in South Africa might have been undetected or incorrectly diagnosed, this specific outbreak commenced with sudden acute mortalities in a broiler production flock near Klerksdorp in North West Province, South Africa (Table 1). Thereafter, several other broiler flocks in the country displayed the same pattern of sudden acute mortalities.

Macroscopic examination

Liver samples were collected during necropsy from broiler production birds that died or were depressed, lost body weight, were weak and had watery droppings. Samples were collected from broiler carcasses with macroscopic hepatic lesions and all macroscopic lesions were noted.

Virus isolation

Small blocks of liver tissue with macroscopic lesions (25 mm² – 100 mm²) were homogenised in a mortar and pestle and diluted 1:5 in sterile phosphate-buffered saline (PBS). After centrifugation (400 g for 5 min), the supernatant was collected and treated with 5% chloroform for 30 min to eliminate bacterial and fungal contamination. Samples were diluted 1:1000 before inoculation into eggs (see below).

The method for virus isolation described for bluetongue virus isolation (Foster & Luedke 1968) was adapted. Briefly, specific pathogen-free embryonated chicken eggs were incubated at 37 °C for 11–12 days. Eggs were candled and suitable chorio-allantoic blood vessels marked. Windows (4 mm × 8 mm) were drilled in the shells overlying the marked blood vessels. Prepared samples (0.1 mL) were inoculated intravenously into the exposed chorio-allantoic blood vessels. The windows were sealed with a drop of non-toxic polyvinyl acetate dispersion or Ponal glue (Henkel Pty Ltd, Alberton, South Africa).

After inoculation, the eggs were incubated at 37 °C for a maximum of 7 days and candled daily. Embryo mortalities within 48 h were regarded as non-specific and discarded. Embryos that had died after 48 h were screened for bacterial contamination on blood tryptose agar and incubated under micro-aerophillic conditions for 48 h. Four to six eggs were used per field sample and a maximum of two passages per sample were performed.

All embryos that died from 48 h post-inoculation until the study termination, as well as all embryos that survived until termination (at 7 days post inoculation), were harvested and necropsied. After evaluation, one liver lobe was harvested and stored at 4 ºC for future passage, whilst the other lobe was harvested and fixed in 10% phosphate-buffered formalin for histological examination. In addition, any developing organs showing gross abnormalities (such as the spleen, mesonephros and metanephros) were also harvested and fixed.

Electron microscopy

Small blocks of liver tissue (1.0 mm × 1.0 mm) were collected during embryo necropsy from selected cases (38-08RD, 55-08 and 58-08) with classical liver lesions and fixed in 2.5% glutaraldehyde. Fixed samples were sent to the Electron Microscopy Unit of the Faculty of Veterinary Science, University of Pretoria, Onderstepoort, for transmission electron microscopy.

Polymerase chain reaction

Polymerase chain reaction (PCR)-assay was performed on embryo liver homogenates originating from experimentally infected embryonated chicken eggs, because PCR-assays performed on submitted samples were unsuccessful. To confirm the presence of *Aviadenovirus*, viral DNA was extracted from 200 µL homogenised embryonic liver tissue using the High Pure Viral Nucleic Acid extraction kit (Rochè, Johannesburg, South Africa). Degenerate primers hexon A and hexon B were used to PCR-amplify position from 144 to 1041 of the L1 encoding region of the hexon protein, using conditions described by Meulemans *et al.* (2001). The PCR amplifications were performed in a total volume of 50 µL and contained 2 µL viral DNA, 125 pmoles of each primer, 2.5 U Pyrobest polymerase (Takara, Otsu, Japan), 5 µL 10× supplied Pyrobest buffer and 7.5 mM deoxynucleotide triphosphates. Amplification products were separated on 2% agarose gels stained with cybergold (Invitrogen; Life Technologies, Johannesburg, South Africa) and visualised with a darkreader. All PCR amplification products were gauged by co-loading the middle range Fastruler (Thermo Scientific, Waltham, USA) and 50 bp ladder (Promega, Madison, USA) onto agarose gels.

Samples of embryonic liver, spleen and bursa were collected during embryo necropsy, pooled and homogenised after addition of 1 mL PBS and sent to Deltamune Diagnostic Laboratory for infectious bursal disease virus (IBDV) reverse transcription (RT)-PCR using the method described by Zierenberg, Raue and Müller (2001).

Sequencing of the polymerase chain reaction products

Sequencing of the PCR products were performed in both directions (Inqaba Biotechnical Industries Pty Ltd, Pretoria, South Africa), using the hexon A and hexon B primers described by Meulemans *et al.* (2001). Electropherograms

of the sequences generated were inspected with FinchTV (Geospiza, Seattle, USA). Sequences obtained were assembled and conflicts resolved using CLC bio version 5 software (CLC bio, Aarhus, Denmark). Sequences were copied and aligned using the Basic Local Alignment Search Tool with the *blastn* algorithm for highly similar sequences (Altschul *et al.* 1997).

Results

Virus detection

Of the field samples inoculated into embryonated eggs, 60% yielded positive virus isolation results. Livers from these broiler carcasses (94%) showed typical lesions of IBH, such as liver necrosis and haemorrhage (Figure 1), whilst the other samples originated from broilers with pericarditis, hepatomegaly and ascites.

Only 25% of the samples that yielded negative results on virus isolation originated from chickens with typical necro-haemorrhagic liver lesions. The remaining negative samples were collected from either broiler carcasses exhibiting hepatomegaly, pericarditis and ascites, or from cases with haemorrhage in the proventriculus and caecal tonsils.

Transmission electron microscopy

Samples of embryonic liver tissue from selected embryos with typical liver pathology revealed icosahedral structures 70 nm in diameter, consistent with adenovirus-like particles, in intra-nuclear crystalline arrays (data not shown).

Amplification reaction

The PCR product was successfully amplified in the samples. Nucleotide sequence analysis demonstrated 99% sequence homology to the prototype strain for FAdV-2 and 99% sequence homology to the prototype strain for FAdV-8b.

Pathology in chicken embryos

Macropathological and histopathological changes after experimental infection of embryonated chicken eggs are demonstrated in Figures 1 and 2. A large percentage of embryos showed macroscopic pathological changes (Figure 2). The liver was most consistently affected, with changes characterised by hepatomegaly, cholestasis and hepatitis (Figures 2a–2c). Lesions were also present in the spleen, kidney and gizzard wall, characterised by splenomegaly and pinpoint haemorrhages (Figure 2a), nephritis with haemorrhage (Figure 2b), visceral gout and serosal ecchymosis of the gizzard wall.

Histopathological lesions were most consistently observed in the liver although lesions could also be seen in renal and splenic tissue (Figure 1). Necrogranulomas charcterised by multifocal to diffuse hepatic necrosis with a mixed cellular infiltrate of macrophages and heterophils were found in most embryos, even in the absence of macroscopic pathology (Figure 1a). Basophilic intra-nuclear inclusion bodies were
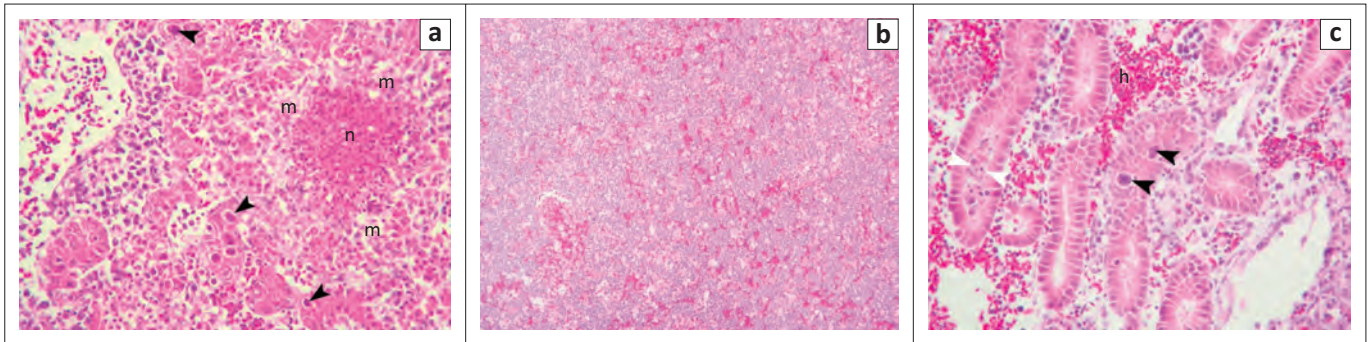
Source: Taken by Louis Maartens

Hepatitis is histologically characterised by large basophilic intranuclear inclusions (a; black arrows) and necrotic areas (a; n) surrounded by an indistinct cuff of macrophages (a; m) and heterophils. Single cell necrosis is present in the spleen (b), whilst the lesions in affected renal tissues (c) are characterised by intra-nuclear inclusions in tubular epithelium (c; black arrows), interstitial haemorrhage (c; h) and renal tubular epithelium injury (c; white arrows). All slides were stained with haematoxylin and eosin.

FIGURE 1: Histological changes seen in the (a) liver, (b) spleen and (c) kidney of infected chicken embryos.
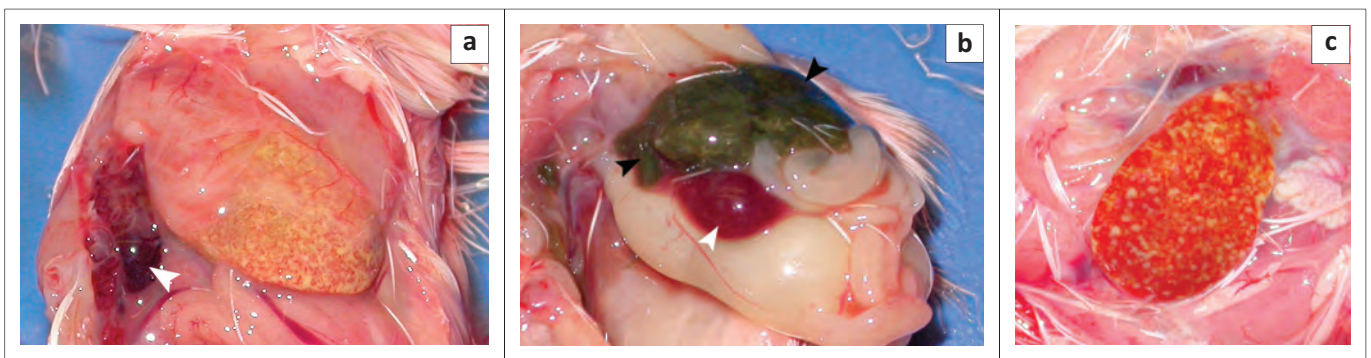
Source: Taken by Louis Maartens

Pathological changes in the liver include hepatomegaly (a, b, c), cholestasis (b; black arrows) and focally extensive to diffuse hepatitis (a, b, c). Splenomegaly (b; white arrow) and nephritis (a; white arrow) can occasionally be seen.

FIGURE 2: Macroscopic pathological changes in the livers of infected chicken embryos.

present on the periphery of some necrogranulomas. Similar basophilic intra-nuclear inclusions were occasionally observed in the renal tubular epithelium (Figure 1c). Furthermore, congestion and sugillations were present in the renal interstitium, with desquamated cellular debris present in the tubules. Diffuse single cell necrosis was observed in the enlarged spleens of most affected embryos (Figure 1b).

Ethical considerations

This study was approved by the Animal Ethics Committee of Deltamune (Pty) Ltd and the Ethics Committee of the University of Pretoria, project number V004-11.

Discussion

In the present study, two FAdV types were successfully isolated in embryonated chicken eggs inoculated by the intravascular route. The presence of IBDV in one of the Klerksdorp flocks could also be confirmed by isolation and PCR using this isolation technique.

It was concluded that, although embryo mortality was seen between the fourth and the 7th day of virus isolation in the majority of cases, embryonal death could not be used as a reliable indicator of successful FAdV isolation. A 1:100 dilution of the organ suspension contained enough virus to kill all the embryos that survived the initial 24 h after

inoculation, but an unacceptably high proportion of embryos were lost as a result of non-specific deaths most probably related to shock reactions. The non-specific deaths were eliminated by the dilution of the organ suspension 1:500, but this dilution failed to kill 100% of the inoculated embryos. Further dilution of the inoculum resulted in the death of a progressively smaller proportion of the embryos.

Macroscopically visible hepatitis and hepatic necrosis evident at necropsy of dead birds is not a pathognomic indicator of FAdV infection. Similarly, the presence of liver lesions in embryos was not a 100% sensitive indicator of successful FAdV isolation, as liver lesions were not entirely specific for FAdV isolation; embryos inoculated with material from samples that contained IBDV strains developed similar lesions.

Histological examination of liver, spleen and kidney tissues from the embryos used for virus isolation yielded valuable information on the aetiology of the isolates. The large basophilic intra-nuclear inclusions in hepatocytes and renal tubular epithelium that were observed in a number of affected embryos were highly characteristic of FAdV infection. In this study, it was found that in the absence of the characteristic inclusions, multifocal hepatic necrogranulomas could be used as an indicator of successful FAdV isolation, because it correlated 100% with PCR results, even in the

absence of macroscopic hepatic lesions. Histopathology of the embryonic liver and spleen, however, also successfully differentiated between the IBDV and FAdV isolates.

Conclusion

Two fowl adenovirus types (FAdV-2 and FAdV-8b) and strains of IBDV were successfully isolated in embryonated chicken eggs inoculated by the intravascular route. The combination of embryo mortality and the presence of macroscopic liver pathology can be used to screen for successful isolation of FAdV and IBDV, but it must be emphasised that some FAdV isolates may replicate in chicken embryos without causing mortality or enough tissue damage to enable macroscopic detection of positive embryos during necropsy. More sophisticated methods such as histopathology or PCR are needed to differentiate between FAdV and IBDV isolates and to detect successful isolation of those isolates that do not result in embryonic death or macroscopic liver lesions.

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Competing interests

The authors declare that they have no financial or personal relationship(s) which may have inappropriately influenced them in writing this article.

Authors' contributions

H.W.J. (Deltamune [Pty] Ltd) prepared the manuscript and performed the molecular analysis and typing of the isolates. H.A. (Avimune [Pty] Ltd) identified the outbreak in the field and provided the field data. L.H.M. (Deltamune [Pty] Ltd) isolated the virus and performed the gross and histopathological examinations. E.H.V. (University of Pretoria) assisted in writing the manuscript.

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