

Incidental *Mycobacterium*-induced granulomatous inflammation of the follicular pharyngeal tonsils in a South African farmed ostrich (*Struthio camelus*)

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Avian mycobacteriosis (AM) is a zoonotic disease caused by *Mycobacterium avium* complex (MAC), which can spread from avians to other farmed animals such as cattle and pigs as well as to humans. This study is the first report of granulomatous inflammation, as a result of avian mycobacteriosis, in the follicular pharyngeal tonsils of a farmed ostrich. The head of an apparently healthy farmed adult ostrich was obtained after slaughter. Each pharyngeal fold displayed a large tissue mass. This tissue was routinely prepared for light microscopy and stained with haematoxylin and eosin, periodic acid Schiff, Grocott methenamine silver, Gram and Ziehl-Neelsen. Immunohistochemistry (IHC) and polymerase chain reaction (PCR) were performed to identify *Mycobacterium* spp. and *Mycobacterium tuberculosis* complex, respectively. Histologically, the tissue masses consisted of confluent mature micro-granulomata that were characterised by central caseous necrosis surrounded by multinucleated giant cells, macrophages and lymphoid cells and an outer mature fibrous connective tissue capsule. Within some foci of caseous necrosis were variably sized colonies of small, Gram-negative, acid-fast bacilli, which showed positive IHC labelling for *Mycobacterium* spp., leading to a presumptive diagnosis of AM. PCR thus proved useful in excluding the presence of notifiable *Mycobacterium* spp. The significance and role of the pharyngeal tonsils of ratites in diseases such as AM warrant specific attention. Moreover, as ratites are known to present with AM infections with apparently no visible loss in body condition, as presumably occurred in the present case, it is imperative that unusual masses in apparently healthy ratites be thoroughly investigated.

Introduction

Mycobacterium avium complex (MAC) is the causative agent of a bacterial infection known as avian mycobacteriosis (AM) (García, LeClear & Gaskin 2001; Tell, Woods & Cromie 2001; Thornton *et al.* 1999) or avian tuberculosis (ATB) (Cooper 2005; Thornton *et al.* 1999). However, as classical tuberculous lesions are not the only manifestation of mycobacterial infections in birds, it is advocated that the term avian mycobacteriosis be preferentially used (Tell *et al.* 2001). Avian mycobacteriosis is a zoonotic disease that can spread from avians to other farmed animals such as cattle and pigs (García *et al.* 2001; Tully & Shane 1996) as well as to humans (Kincade 1959). The disease has been extensively researched in many avian species and a thorough review is provided by Tell *et al.* (2001). The many subspecies of *M. avium* are grouped together under the umbrella term *Mycobacterium avium* complex (Cromie *et al.* 2000; Pavlik *et al.* 2000; Thornton *et al.* 1999) and *M. avium* subspecies *avium* is reported to be the most common cause of AM (Gerlach 1994; Gyimesi *et al.* 1999).

Avian mycobacteriosis mainly affects the digestive system (Bowes 1993; Cooper 2005; Cromie *et al.* 2000; García *et al.* 2001; Shivaprasad 2002; Tully & Shane 1996) with secondary spread of infection to other visceral organs (Bowes 1993) where the typical nodular lesions are easily detected (Doneley *et al.* 1999; Huchzermeyer 1997). MAC bacilli are usually shed in the faeces of affected birds (Thornton *et al.* 1999) and ingestion of contaminated faeces is the most common mode of infection (Bowes 1993; Cooper 2005; García *et al.* 2001; Tully & Shane 1996).

Avian mycobacteriosis, although of less importance to the commercial poultry industry (Cromie *et al.* 2000; Sanford, Rehmtulla & Josephson 1994), still plagues poultry production in developing countries, causes losses in other commercially farmed birds such as ratites and also affects wild, endangered species in captive breeding programmes (Cromie *et al.* 2000). Mycobacteriosis in ratites (Sanford, Rehmtulla & Josephson 1994), including the ostrich (*Struthio camelus*), is considered to be rare (Bowes 1993; Cooper 2005; Huchzermeyer 1997; Kazda, Pavlik & Hruska 2009; Montali *et al.* 1976; Shane *et al.* 1993) and is restricted mainly to birds kept in zoological

collections (Huchzermeyer 1997). However, AM has recently been diagnosed in ostriches in Canada (Bowes 1993), the Czech Republic (Levkut *et al.* 1998), Slovakia (Ševčíková *et al.* 1999), Australia (Doneley *et al.* 1999), the Netherlands (Pavlik *et al.* 2000), Poland (Wozikowski 2001), the USA (García *et al.* 2001) and in Japan (Ryo, Hiroaki & Yoshihiro 2006). Avian mycobacteriosis has also been described in the emu (*Dromaius novaehollandiae*) (Cromie *et al.* 2000; Krishna Mohan Roa & Chowdary 1980; Pocknell *et al.* 1996; Thornton *et al.* 1999), the greater rhea (*Rhea americana*) (Sanford *et al.* 1994; Tacconi & Valente 1981) and the kiwi (*Apteryx mantelli*) (Davis, Watson & Billing 1984). Mycobacteriosis caused by *M. bovis* has been reported in a cassowary (*Casuarius casuarius*) in the Czech Republic (Pavlik *et al.* 2002) but this can be considered a rare event (Kazda *et al.* 2009).

The normal morphology of the ostrich oropharynx has been documented (Porchescu 2007; Tadjalli, Mansouri & Poostpasand 2008; Tivane 2008) and, more recently, structures that can be classified as follicular tonsils have been described in the oropharynx of both the ostrich and emu (Crole & Soley 2012a). It is clear that the pharyngeal folds typically found in these birds and the associated follicular and non-follicular pharyngeal tonsils form an extensive and immunologically active region in the ostrich oropharynx (Crole & Soley 2012a). There also appear to be no reports in the literature on any clinical conditions affecting the pharyngeal folds or tonsils of the ostrich, or those of any other ratite species. When compared with the wealth of information, including numerous case reports, available on AM in various bird species, the body of literature covering AM in ratites is small, reflecting its rarity in this group of birds. However, it is clear that this disease does occur in ratites (Sanford *et al.* 1994), is a global phenomenon and requires specific investigation in these commercially important birds.

This study is the first report of granulomatous inflammation, as a result of AM, in the follicular pharyngeal tonsils of an ostrich and represents the first report of AM in this species in South Africa in a period of almost 20 years (Anon 1985).

Materials and methods

Specimen collection

The head of an apparently healthy (but displaying proliferative masses of the pharyngeal folds), farmed adult ostrich was obtained after slaughter as part of a routine collection of research material for a morphological study of the oropharynx. The bird was approximately 14 months old and of unknown sex. The head had been immersion fixed in 10% neutral-buffered formalin and stored for approximately 18 months in the Department of Anatomy and Physiology, Faculty of Veterinary Science, University of Pretoria. The head was incised to reveal the oropharynx (Crole & Soley 2010). The masses were described, measured and photographed *in situ* using a Canon EOS 5D digital camera (Canon, Japan) with a 28 mm – 135 mm lens before being excised, together with the pharyngeal folds.

Histology

Appropriate longitudinal and transverse sections representing different areas of the masses were excised. The tissue samples were routinely prepared for light microscopy using a Shandon model 2LE Automatic Tissue Processor (Shandon, Pittsburgh, PA, USA). Sections were stained with haematoxylin and eosin (H&E), periodic acid Schiff stain (PAS) (McManus 1946), Grocott methenamine silver (GMS), Gram and Ziehl-Neelsen (ZN) and viewed and micrographed using an Olympus BX50 equipped with the analySIS CC12 Soft Imaging System (Olympus Corporation, Tokyo, Japan) and an Olympus BX63 light microscope (Olympus Corporation, Tokyo, Japan) equipped with a DP72 camera and Olympus cellSens imaging software.

Immunohistochemistry

Sections submitted for immunostaining were mounted on positively-charged microscope slides (SuperFrost® Plus, Menzel-Glasser®), and dried overnight in a 38 °C – 40 °C oven. Routine dewaxing in xylol, followed by rehydration through a graded ethanol and distilled water series, took place inside a fume hood. Sections were subsequently incubated with 3.0% hydrogen peroxide in methanol for 15 min in a humidified chamber at room temperature, then rinsed in distilled water, followed by 0.1 M phosphate buffered saline (PBS), pH 7.6 containing 0.1% bovine serum albumin (BSA) for 5 min per rinse. Heat-induced epitope retrieval (HIER) was performed via microwave heating (96 °C) in citrate buffer (pH 6) for 14 min. Thereafter the test sections (still in buffer) were allowed to cool on the bench before rinsing in distilled water and then in PBS/BSA buffer for 5 min. Sections were incubated with normal goat serum (catalogue no: G9023, Sigma) and diluted 1:5 with PBS/BSA buffer, pH 7.6 for 20 min in a humidified chamber at room temperature. The blocking serum was drained and replaced by the primary polyclonal rabbit anti-*Mycobacterium bovis* antibody (pAbBCG) (BCG strain; catalogue no: B 0124, DakoCytomation), at a dilution of 1:1000 in PBS/BSA buffer for 90 min in a humidified chamber at room temperature. The biotinylated polyclonal goat-anti-rabbit link antibody (catalogue no: EO432, DakoCytomation) was used at a dilution of 1:500 in PBS/BSA buffer. The link antibody was incubated with the tissue sections for 30 min in a humidified chamber at room temperature, followed by rinsing in distilled water and then in PBS/BSA buffer as before. Peroxidase conjugated avidin (Vectastain ABC Kit, Elite PK6100 Standard, Vector Laboratories) was applied and incubated on the sections for 30 min in a humidified chamber at room temperature. The sections were rinsed twice, as before. The sections were immersed in a NovaRED substrate (catalogue no: SK-4800, Vector Laboratories) in a humidified chamber at room temperature. During this time, the validated positive tissue control section (see below) with the same substrate was examined carefully for signs of positive staining at 100 × magnification, using a light microscope. As soon as there was clear evidence of positive staining (focal or multifocal red granular cytoplasmic positivity in macrophages) in the positive tissue control section, the

test sections were immediately rinsed in a distilled water bath to halt the substrate reaction. The sections were then counterstained with Mayer's haematoxylin for 1 min – 2 min and rinsed under running tap water for 10 min to remove excess substrate. Sections were routinely dehydrated through increasing alcohol concentrations and xylol, mounted and coverslipped. In the negative reagent control section, the primary antibody was replaced by normal goat serum. No background staining was observed in the negative control section (not shown). The formalin-fixed, paraffin-embedded, positive control tissue was from an 18-month-old male buffalo with typical mycobacterial lesions in the tonsils. The tissue cultured positive for *M. bovis* (PM 71.12/S3776.12; Section of Pathology, Department of Paraclinical Sciences, University of Pretoria). Formalin-fixed tissue from a sheep with a confirmed case (via PCR) of Johne's disease (paratuberculosis) (Western Cape State Laboratory; case number 13.044) stained strongly with the pAbBCG, indicating that this antibody also recognises non-tuberculous bacteria.

Polymerase chain reaction

Preparation of template DNA and PCR amplification for *Mycobacterium tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *Mycobacterium canettii*, *Mycobacterium africanum*, *Mycobacterium microti*, *Mycobacterium pinnipedii*, *Mycobacterium caprae*, *M. bovis* BCG) (Tuberculosis Laboratory, Zoonotic Diseases Section, Onderstepoort Veterinary Institute, South Africa) were performed according to the method described by Miller *et al.* (1997) with minor modifications. Paraffin sections were placed in a centrifuge tube together with 200 μ L of water with 0.5% Tween-20. The tube contents were boiled for 10 min and followed by snap freezing (2 min at -20°C). This step was repeated and samples were subjected to boiling for a third time for 10 min. The tube was centrifuged at $3000 \times g$ for 10 min to pellet the tissue. Ten μ L of the sample was tested by PCR using primers to identify *M. tuberculosis* complex. *Mycobacterium bovis* and *Mycobacterium* species, other than *M. tuberculosis* isolates, as well as water, were included as controls. Boiled cell suspension from a field strain of *M. bovis* was used as a positive control. The reaction contained 20 mm of each primer, 125 U of Taq polymerase and 0.2 mm of nucleotides. Following an initial cycle of 10 min at 94°C and 135 s at 72°C , amplification was continued for 50 cycles of 45 s at 94°C and 75 s at 72°C , followed by a final 10 min extension at 72°C . Amplification products were analysed by electrophoresis in 2.0% agarose gel stained with ethidium bromide. A band size of 123 base pairs (bp) was considered positive for *M. tuberculosis* complex and the absence of a band or bands at any other position was considered negative. Polymerase chain reaction for MAC was not performed on the samples as the primers were not available at the time and the test was never validated in the laboratory.

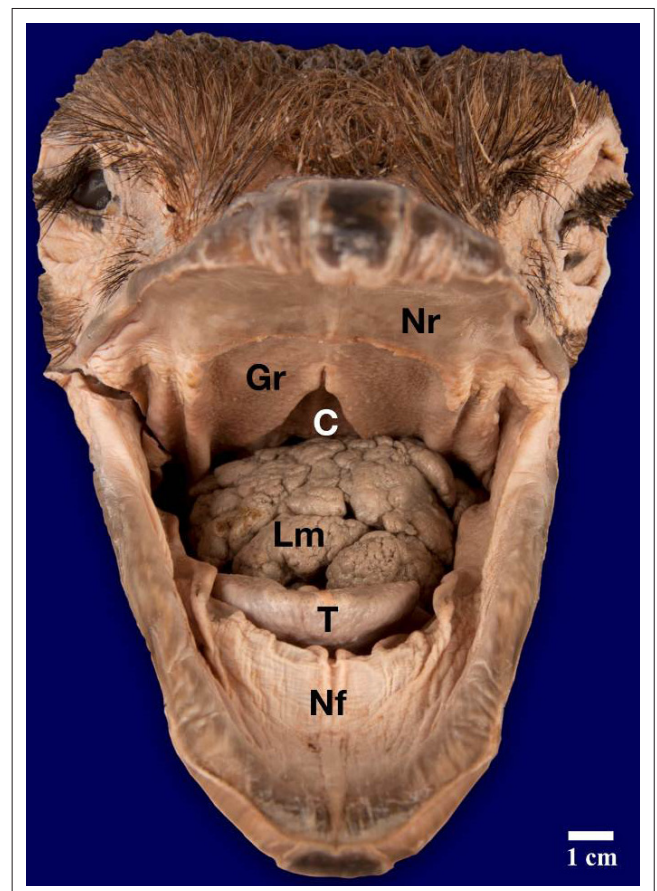
Results

Gross pathology

The only outward evidence of pathology of the head examined was distension of the gular region (the external region of skin between the arms of the mandible) and

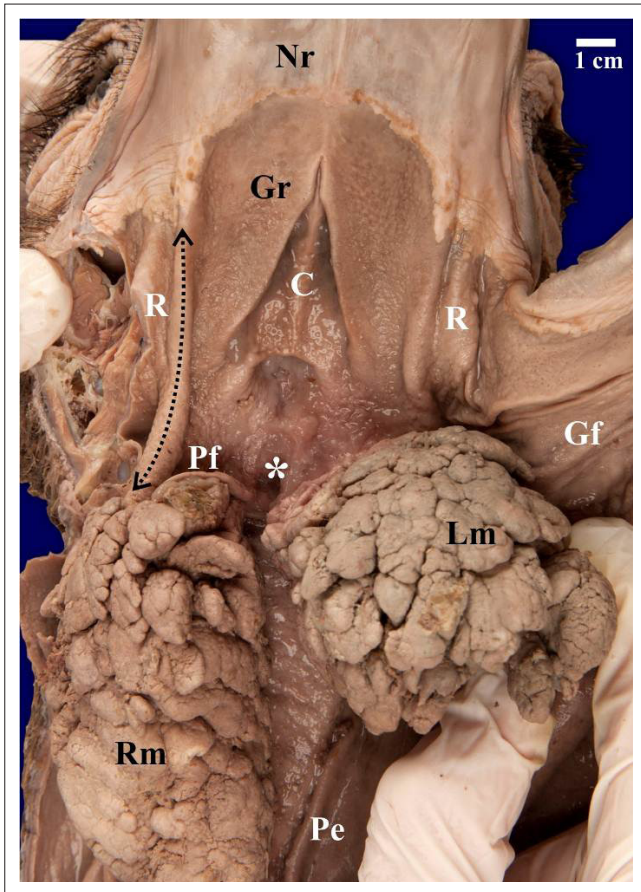
proximal oesophagus. Examination of the oropharynx revealed a large, round, cauliflower-like, cream-coloured (in the formalin-fixed state), firm tissue mass associated with the left side of the oropharynx, although it occupied the entire oropharynx caudal to the tongue body (Figure 1). This tissue mass was interposed between the glottis and choana (Figure 1 and Figure 2) and measured 7.0 cm \times 5.0 cm with a circumference of 17.5 cm. A second tissue mass emanating from the right side of the oropharynx was visible in the proximal oesophagus in the openly displayed oropharynx (Figure 2). The right tissue mass was similar in appearance to the left tissue mass but was elongated (Figure 2 and Figure 3) and measured 8.0 cm \times 4.0 cm with a circumference of 14.0 cm. The tissue masses originated from and were intimately attached to the dorsal surface of the pharyngeal folds, with the rostral, left tissue mass originating from the left pharyngeal fold and the caudal, right tissue mass originating from the right pharyngeal fold (Figure 2).

Removal of the tissue masses, together with the pharyngeal folds and proximal oesophagus revealed a highly vascularised area of supporting connective tissue in the retropharyngeal region dorsal to the attachment of the pharyngeal folds. Due to the extent of the vascularisation, this region resembled a haematoma. When sectioned in the midline, the cut



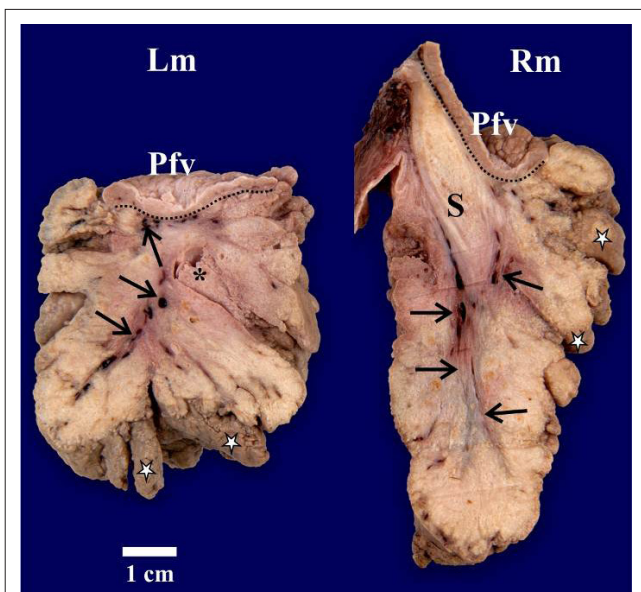
Note: the mass fills the oropharynx caudal to the tongue body (T), and lies between the glottis (not shown) and choana (C). Nr, Non-glandular region of the roof; Gr, glandular region of the roof; Nf, non-glandular region of the floor.

FIGURE 1: Cranial view of the open oropharynx showing the left tissue mass (Lm) *in situ*.



Note: The masses emanate from the dorsal surfaces of the left and right pharyngeal folds (Pf), respectively. Note the exaggerated cord-like fold (dotted arrow) formed by the lateral aspect of the glandular roof (Gr), adjacent to the rictus (R), to support the elongated right mass which is positioned in the proximal oesophagus (Pe). The left mass has been reflected caudally to show the underlying structures. Nr, Non-glandular region of the roof; Gf, glandular region of the floor; C, choana; *, infundibular cleft.

FIGURE 2: The oropharynx openly displayed to demonstrate the positioning of the separate left (Lm) and right (Rm) tissue masses.



Note: The masses are attached to, and originate from, the dorsal surface (black dotted line) of the pharyngeal folds. The masses are lobulated (stars) and well vascularised (black arrows). A thick stalk (S) suspends the right mass and the left mass displays a small area of tissue (*) which macroscopically resembles tonsillar tissue. Ventral surface of the pharyngeal folds (Pfv).

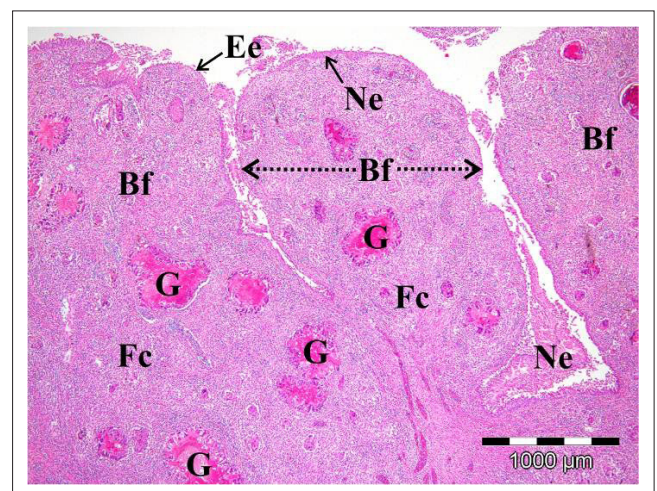
FIGURE 3: Excised left (Lm) and right (Rm) tissue masses, sectioned in the midline.

surface of the tissue masses clearly demonstrated the vast proliferation of tissue emanating from the dorsal aspect of each pharyngeal fold (Figure 3). The tissue masses were well vascularised, lobulated and cream-coloured (Figure 3). The right tissue mass was suspended by a prominent mucosal fold of the glandular roof (Figure 2) which, when sectioned, was seen to be supported by a thick inner stalk (Figure 3). The left tissue mass contained a small, isolated tract of tissue that macroscopically resembled normal tonsillar tissue (Figure 3). The follicular pharyngeal tonsils (Crole & Soley 2012a) were absent and replaced by the tissue masses.

Histopathology and immunohistochemistry

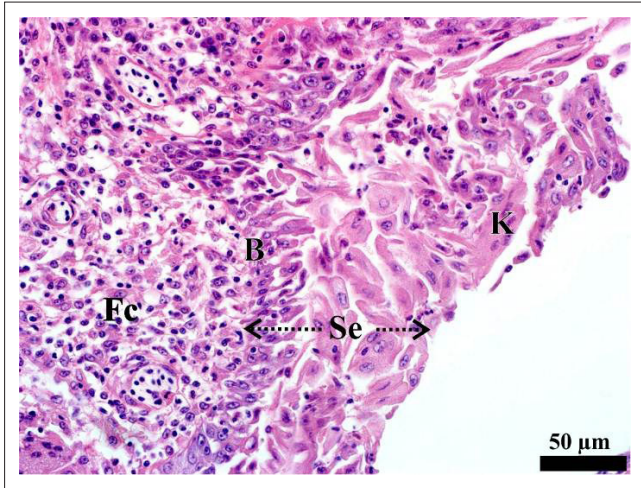
Histology revealed that the tissue masses consisted of confluent micro-granulomata (Figure 4). The surface of each mass was partly covered by a nondescript epithelium (Figure 4), that, when present, appeared to be stratified (Figure 5) and that was extensively eroded and multifocally ulcerated. The epithelium was pushed into blunt papillary folds by the underlying proliferating tissue (Figure 4). In some places, the stratified epithelium displayed signs of keratinisation (Figure 5) evidenced by dead keratinocytes and keratin flakes. Gram-negative bacteria were present, in some regions, on the surface of the epithelium (Figure 6), including areas of ulceration. The right tissue mass was suspended by a thick stalk (Figure 3) composed of dense regular connective tissue with embedded micro-granulomata and blood vessels (Figure 7). Some parts of the stalk displayed more compact regular connective tissue with thick-walled arteries and fewer micro-granulomata than in the surrounding tissue, thus indicating advanced maturity. The tissue masses were both richly supplied with blood vessels of which some were thick-walled (Figure 8).

Both tissue masses consisted of numerous variably sized, coalescing micro-granulomata with mature surrounding and intervening fibrous connective tissue (Figure 4). The micro-granulomata were generally characterised by a central



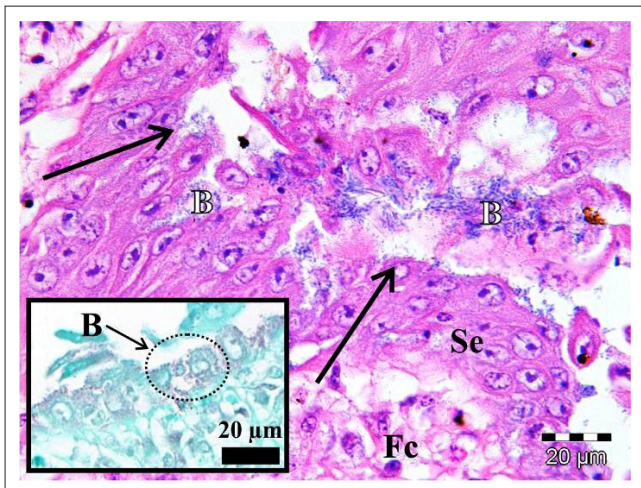
Note: The folds are covered by a nondescript epithelium (Ne) which in places is eroded (Ee). Note the numerous micro-granulomata (G) interspersed in the matrix of fibrous connective tissue (Fc). Haematoxylin and eosin stained section.

FIGURE 4: Blunt papillary folds (Bf) forming the surface of the tissue masses.



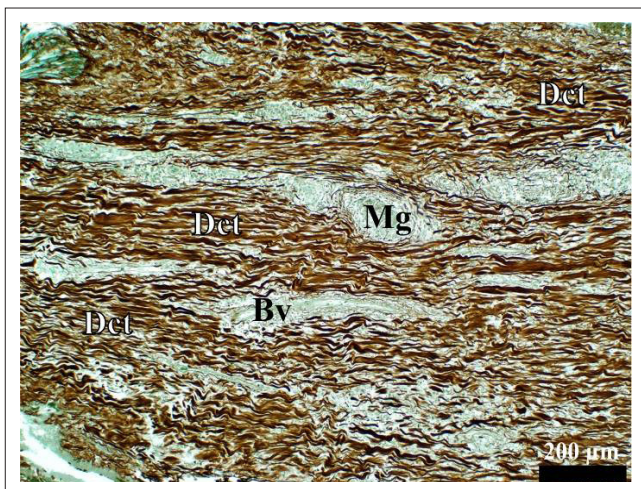
Note: the obvious disruption of the epithelium with only a *Stratum basale* (B) being clearly distinguishable. Fibrous connective tissue (Fc) and keratinocytes (K). Haematoxylin and eosin stained section.

FIGURE 5: Stratified epithelium (Se) on the surface of the granuloma.



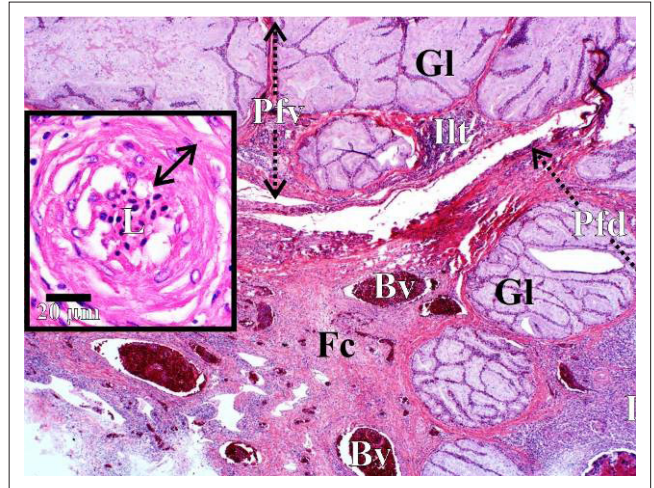
Note: the bacteria, together with desquamated cells, lie on the epithelial surfaces (arrows). Inset, gram stain showing the colonies of bacteria (B) to be Gram negative. Fc, Fibrous connective tissue haematoxylin and eosin stained section.

FIGURE 6: Stratified epithelium (Se) at the distal end of the right tissue mass demonstrating colonies of bacteria.



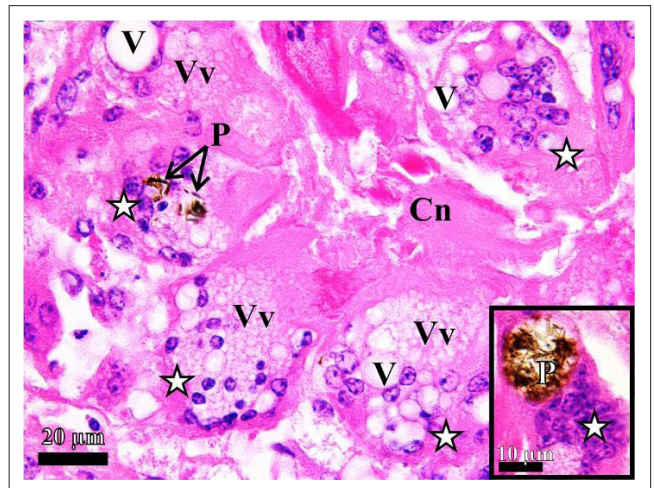
Note: the wavy pattern of the collagen fibres forming the dense regular connective tissue (Dct). Micro-granulomata (Mg) and blood vessels (Bv) are embedded within the stalk. Grocott methenamine silver stain.

FIGURE 7: The connective tissue stalk suspending the right tissue mass.



Note: the ventral surface consists of the normal components, including simple, branched tubular mucus-secreting glands (GI) and patches of inter-nodular lymphoid tissue (Ilt). The dorsal surface, from where the tissue masses originate, displays some normal features (glands and lymphoid tissue); however, these structures are invaded by tracts of fibrous connective tissue (Fc) and a rich network of blood vessels (Bv). Inset, a thick-walled (double-headed arrow) blood vessel displaying the lumen (L) filled with blood cells. Haematoxylin and eosin stained sections.

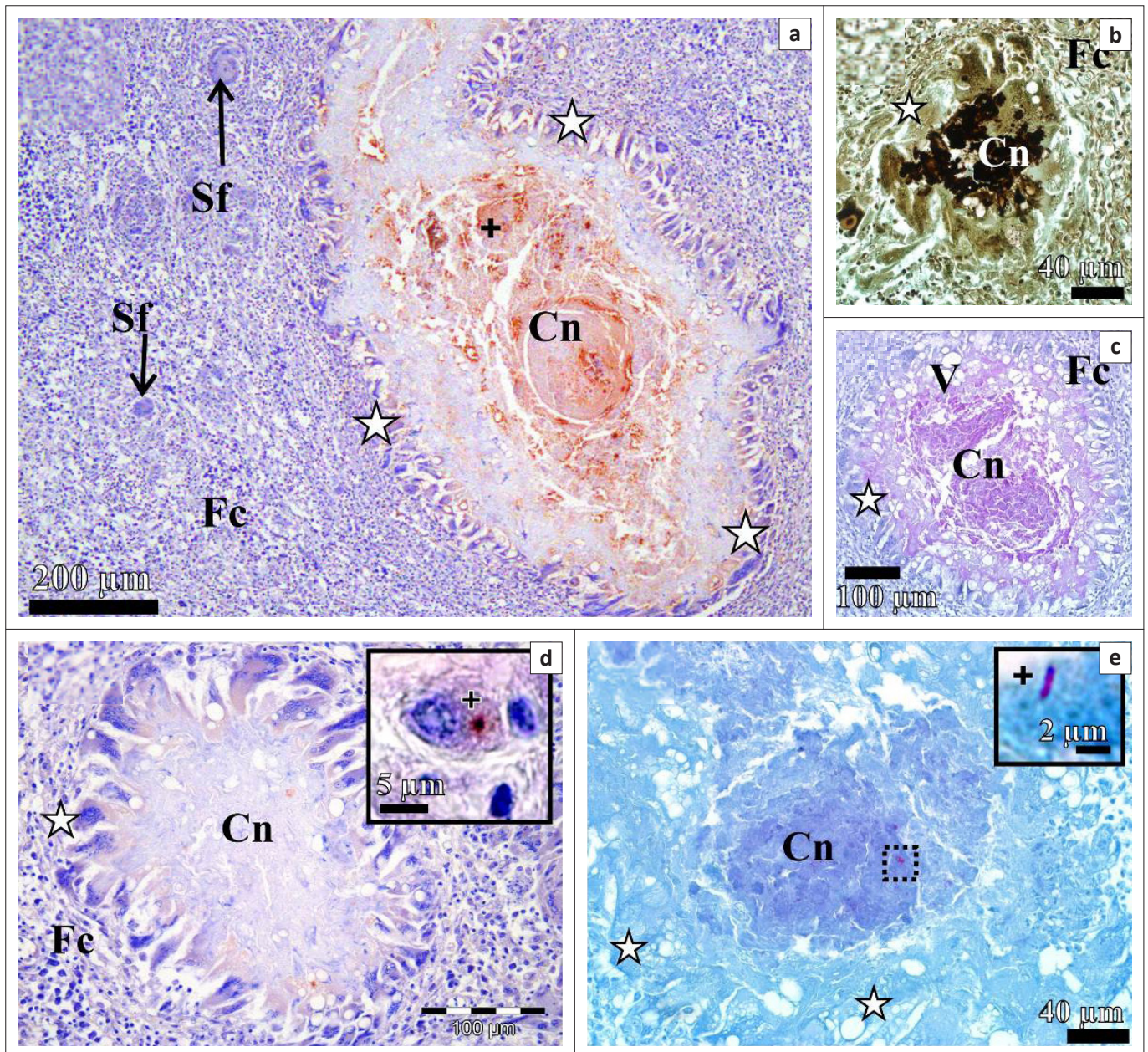
FIGURE 8: Junction between the ventral (Pfv) and dorsal (Pfd) surfaces of the pharyngeal fold.



Note: the micro-granuloma is characterised by an outer rim of multinucleated giant cells (stars) displaying single large vacuoles (V) and/or small, coalescing intracytoplasmic vacuoles (Vv). Some vacuoles contain phagocytosed (P) foreign material. A region of caseous necrosis (Cn) defines the centre of the micro-granuloma. Inset, a multinucleated giant cell (star) containing a large amount of phagocytosed (P) foreign material. Haematoxylin and eosin stained sections.

FIGURE 9: Morphology of a typical micro-granuloma.

area of caseous necrosis with vacuolation and a peripheral rim of multinucleated giant cells (MNGCs), some of which displayed small coalescing intra-cytoplasmic vacuoles and some large, single vacuoles (Figure 9 and Figure 10). Occasional smaller foci were formed by clusters of MNGCs (Figure 10a). Within occasional foci of caseous necrosis were variably sized colonies of bacteria (Figures 10a–c). The bacteria were small, Gram-negative, acid-fast bacilli (Figure 10c) that showed positive IHC labelling for *Mycobacterium* spp. (Figure 10a and Figure 10b). MNGCs occasionally contained the above-mentioned bacteria or foreign material (Figure 9). Foreign particles were brown, globular, slightly refractive (Figure 9) and stained strongly with GMS (Figure 10d). There was no evidence of fungi (PAS and GMS) (Figure 10d and Figure 10e).



Note: The micro-granulomata are characterised by a peripheral ring of multinucleated giant cells (MNGCs) (stars) and a central area of caseous necrosis (Cn). Fibrous connective tissue (Fc) surrounds the micro-granulomata. Small foci (Sf) of micro-granulomata formed by a collection of MNGCs are also present; no fungal hyphae are apparent and vacuoles (V) within the MNGC's cytoplasm.

FIGURE 10: Micro-granulomata imaged using special stains and immunohistochemistry (IHC) labelling. (a) Strong, positive IHC labelling for *Mycobacterium* spp. within the region of caseous necrosis of a micro-granuloma; (b) Foreign material contained within MNGCs and areas of caseous necrosis react strongly with Grocott methenamine silver stain; (c) Micro-granuloma stained with periodic acid Schiff, again showing no evidence of fungal hyphae; (d) Positive IHC labelling for *Mycobacterium* spp. within an epithelioid macrophage of a micro-granuloma; (e) Acid-fast bacillus within the zone of caseous necrosis of a micro-granuloma.

Surrounding the MNGCs was a variable admixture of epithelioid macrophages, numerous plasma cells, lymphocytes and occasional heterophils. Intermingled with the inflammatory cells were fibroblasts, collagen fibres and numerous congested blood vessels, forming peripheral granulation tissue. The fibroblasts and intervening collagen fibres occasionally formed concentric layers around the MNGCs and inflammatory cells, creating an onion-skin effect. Lymph nodules and inter-nodular lymphoid tissue were present nearer the pharyngeal folds (Figure 8); however, this tissue was markedly atrophic.

The ventral surface of the pharyngeal folds presented the normal histological structure (Figure 8) previously described for the ostrich (Crole & Soley 2012a). However, the dorsal surface of the folds was severely affected by the intrusion of micro-granulomata, with only small regions exhibiting a normal structure. The structure of both the follicular and non-follicular tonsil associated with the dorsal aspect of the pharyngeal folds (Crole & Soley 2012a) was obliterated by the proliferating zone of granulomatous inflammation (Figure 8).

Polymerase chain reaction

Polymerase chain reaction (PCR) for the detection of *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. canettii*, *M. africanum*, *M. microti*, *M. pinnipedii*, *M. caprae*, *M. bovis* BCG) was negative.

Discussion

This study is the first report in 20 years of AM in a farmed ostrich in South Africa. It is also the first report of AM affecting the pharyngeal tonsils in a ratite species. Mycobacteriosis is a chronic, progressive disease resulting in systemic granulomatous inflammation and in ratites usually presents as unthriftiness, loss of weight and diarrhoea (Shivaprasad 2002). Granulomatous inflammation is characteristic of AM reported in ostriches (Cooper 2005; Doneley *et al.* 1999; García *et al.* 2001; Hood 1977; Ševčíková *et al.* 1999) as well as in other ratite species such as the greater rhea (Sanford *et al.* 1994), emu (Pocknell *et al.* 1996; Shane *et al.* 1993) and kiwi (Davis, Watson & Billing 1984). It was noted that lesions observed in the greater rhea were also typical of those described in other birds (poultry, psittacines and other captive exotic birds) with AM (Sanford *et al.* 1994). On histopathology, the lesions seen in the present study resembled the typical granulomatous inflammatory response seen in many avian infections (Shivaprasad 2002). In the ostrich, this response has been reported for infectious agents such as *Pseudomonas aeruginosa* (Momotani *et al.* 1995), *Aspergillus* sp. (Fitzgerald & Moisan 1995), *Moraxella phenylpyruvica* (Gürel, Gülçubuk & Turan 2004) as well as for non-infectious causes, such as chronic granulomatous conjunctivitis (Saroglu, Yucel & Aktas 2000). Although the reported case of granulomatous conjunctivitis was caused by *M. phenylpyruvica*, the authors noted the resemblance between these lesions and those seen in tuberculosis (Gürel, Gülçubuk & Turan 2004). However, in the current study, there was no evidence of fungi with either PAS or GMS staining, whereas the Gram stain indicated the presence of Gram-negative bacteria. ZN staining subsequently revealed low numbers of acid-fast bacilli, prompting a presumptive diagnosis of AM (Allen 1992; Gyimesi *et al.* 1999), which was further supported by positive IHC labelling for *Mycobacterium* spp. Immunostaining with pAbBCG (as in the present study) has proved to be a reliable ancillary method for the detection of mycobacterial organisms (Ulrichs *et al.* 2005). Crossed immunoelectrophoresis showed that the polyclonal antibody reacted with approximately 100 different BCG antigens, many of which were common to other *Mycobacteria* species (DakoCytomation information sheet B0124/RUO/OS/13.05.05). It would appear from previous studies on AM in ostriches (Bowes 1993; Doneley *et al.* 1999; Ševčíková *et al.* 1999) that low numbers of acid-fast bacilli, as observed in the present study, are typical for this condition. The distribution of bacteria, both free within the central concentration of necrotic debris and locked within the cytoplasm of the MNGCs of the micro-granulomata has also previously been reported (Doneley *et al.* 1999; Pocknell *et al.* 1996; Shane *et al.* 1993).

A definitive diagnosis of AM requires culture of the mycobacterium. However, this method is both slow and difficult (Gyimesi *et al.* 1999) and was not possible in the present study as no fresh tissue was available. The identification of *Mycobacterium* spp. by PCR has been used on formalin-fixed tissue in cattle and elk, where *M. bovis* was the causative organism (Miller *et al.* 1997). This technique has also been used for the diagnosis of *M. avium* in a duck (Thornton *et al.* 1999). However, this method holds its own challenges, especially if the period of formalin fixation of the tissue is lengthy (Gyimesi *et al.* 1999). The negative result of the PCR for the *M. tuberculosis* complex on the formalin-fixed tissue in the present study must be interpreted with caution due to the lengthy fixation of the tissue. Therefore, although a notifiable species was not detected, one cannot be excluded as the causative agent and nor can any of the organisms of the *M. avium* complex.

The association of the twin tissue masses with the pharyngeal folds raises the question of their origin and the source of inflammation. A recent study of the pharyngeal folds (*Plica pharyngea*) and associated lymphoid tissue in the ostrich revealed the presence of follicular pharyngeal tonsils (*Tonsilla pharyngea*) (located retropharyngeally), small non-follicular pharyngeal tonsils (on the dorsal surface of the pharyngeal folds) and pharyngeal lymph nodules (*Lymphonoduli pharyngeales*) (present on the dorsal and ventral surfaces of the pharyngeal folds) (Crole & Soley 2012a). Based on this information, it was evident that the tissue masses originated specifically from the follicular pharyngeal tonsils due to their location as well as by the almost complete obliteration of the tonsillar tissue. The ventral surface of the pharyngeal folds remained structurally normal whereas the dorsal surface and associated non-follicular pharyngeal tonsils were partially involved in the granulomatous reaction, most likely due to their proximity to the follicular pharyngeal tonsils. The location of the tissue masses raises an interesting question concerning the ability of the ostrich to ingest food and water. The feeding method of paleognathous birds has been described as cranioinertial (Bonga Tomlinson 2000) or 'catch-and-throw' (Gussekkloo & Bout 2005) whereby food is accelerated from the bill tips through the oropharynx to land caudal to the laryngeal mound, in the proximal oesophagus. Simultaneously the linguo-laryngeal apparatus (Crole & Soley 2012b) would prevent food from entering the glottis. Although the bird in question would have been able to feed in this manner (Bonga Tomlinson 2000; Gussekloo & Bout 2005) while the granulomas were small, it would not have been possible near the time of slaughter due to the position and size of the granulomas, especially taking into consideration that the left granuloma occupied almost the entire caudal oropharynx and the right granuloma the proximal oesophagus. There is little doubt that the twin granulomas, had they continued to grow, would eventually have inhibited feeding, drinking and respiration.

A report of a mycotic (*Aspergillus*) granuloma of the nasal passages in an ostrich suggested that the inciting cause

was local damage due to the inhalation of plant material (Fitzgerald & Moisan 1995). Similarly, the presence of pollen grains in the *Mycobacterium*-positive granulomatous reaction in the conjunctiva of an ostrich brought into question which factor (pollen or *Mycobacterium*) was the inciting cause and it could not be determined which factor was responsible for the initial insult (Ševčíková *et al.* 1999). The inciting cause of the granulomatous inflammation of the tonsils in the present study is also ambiguous as it could not be determined whether the initial reaction was caused by the impaction of feed or the mycobacterium. The tonsillar crypts of the ostrich are deep and branching (Crole & Soley 2012a), which may predispose them to trapping fine particulate matter. The foreign material had the appearance of plant matter and stained strongly with GMS, similar to that reported for AM in another ostrich (Ševčíková *et al.* 1999). Circumstantial evidence (foreign material and acid-fast bacilli within the micro-granulomata) points to the possible role of contaminated feed. An unusual feature in the present study was the bilateral nature of the granulomatous inflammation. The feed may have carried a high load of infection and both tonsils may have become infected due to direct exposure.

Conclusion

True pharyngeal tonsils have recently been identified in birds and their structure has been described in the ostrich and emu (Crole & Soley 2012a). Tonsils have been identified in mammals as very important tissues for diagnostic sampling for infectious diseases (Liebler-Tenorio & Reinhard 2006) and in birds the pharyngeal tonsil is prone to the formation of micro-abscesses after infection (Casteleyn *et al.* 2010). The significance and role of the pharyngeal tonsils of ratites in diseases such as AM warrant specific attention. It is noteworthy that although AM in ratite species is considered to be rare, many cases, like the present study, do not present as typical AM (Bowes 1993; García *et al.* 2001; Hood 1977; Nouvel & Leclerc-Cassan 1970; Pocknell *et al.* 1996; Ševčíková *et al.* 1999). In the present study, there were presumably no noticeable signs of loss of body condition, as the ostrich was deemed fit for slaughter. Such an asymptomatic adult bird may become a source of infection to the remainder of the flock (Bowes 1993; Doneley *et al.* 1999). Similarly, other ratite species (Bowes 1993; García *et al.* 2001; Hood 1977; Pocknell *et al.* 1996; Ševčíková *et al.* 1999; Shane *et al.* 1993) have presented with AM infections with apparently no visible loss in body condition (Pocknell *et al.* 1996). This is an important consideration when investigating unusual masses in apparently healthy ratite species.

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Competing interests

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

Authors' contributions

M.R.C. (University of Pretoria) located the specimen and drafted the manuscript. S.J.C. (University of Pretoria) carried out the histopathology and IHC preparation, staining, analysis and interpretation. J.T.S. (University of Pretoria) and M.R.C. assisted in the interpretation of the material. J.T.S. supervised the study, participated in the design and was responsible for the final editing. All authors read and approved the final manuscript.

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