Juliana Fortes Vilarinho Braga

ETIOLOGY OF VERTEBRAL OSTEOMYELITIS IN BROILERS

Belo Horizonte
Escola de Veterinária of UFMG
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To my husband, mom and grandma, with all my love and gratitude.

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- Chico Xavier

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LIST OF ABBREVIATIONS

AA amyloidosis	Amyloid A protein amyloidosis
AMC	Amoxicillin + clavulanic acid
AMX	Amoxicillin
APEC	Avian Pathogenic *Escherichia coli*
APR	Apramycin
ATCC	American Type Culture Collection
BA	Blood agar
BCO	Bacterial condronecrosis with osteomyelitis
BHI	Brain and heart infusion
BP	Base pairs
CC	Clonal complex
CCCD	Culture Collection of CEFAR Diagnóstica
CEF	Cephalotin
CETEA	Committee for Ethics in Animal Experimentation
CFU	Colony forming unit
CLSI/NCCLS	Clinical and Laboratory Standards Institute (Former NCCLS)
CST	Colistin
DNA	Deoxyribonucleic acid
E. cecorum	*Enterococcus cecorum*
E. coli	*Escherichia coli*
E. durans	*Enterococcus durans*
E. faecalis	*Enterococcus faecalis*
E. faecium	*Enterococcus faecium*
E. hirae	*Enterococcus hirae*
ECOR	*Escherichia coli* Reference Collection
EDTA	Ethylenediaminetetraacetic acid
ENR	Enrofloxacin
ESBL	Extended spectrum β-lactamases
ExPEC	Extraintestinal pathogenic *Escherichia coli*
FFC	Florfenicol
FOX	Cefoxitin
GEN	Gentamicin
HE	Hematoxylin-eosin
HLAR	High-level aminoglycoside resistance
HLGR	High-level gentamicin-resistance
INRA	Institut National de la Recherche Agronomique
LB	Luria-Bertani
MCK	MacConkey
MH	Mueller-Hinton
MLST	Multilocus Sequence Typing
NAL	Nalidixic acid
NEO	Neomycin
OD	Optical density
PCR	Polymerase Chain Reaction
PFGE	Pulsed-field gel electrophoresis
PFIE	Plateforme d'Infectiologie Expérimentale

PMSF	Phenylmethylsulfonyl fluoride
RNA	Ribonucleic acid
RPM	Rotations per minute
S. aureus	*Staphylococcus aureus*
SDS	Sodium dodecyl sulfate
SPF	Specific pathogen free
ST	Sequence type
T4	4th thoracic vertebra of chicken vertebral column
TE	Tris-EDTA
TET	Tetracycline
TMP	Trimethoprim
TmpStx	Trimethoprim + sulfamethoxazole
TOC	Turkey osteomyelitis complex
UB	Flumequine
UFMG	Universidade Federal de Minas Gerais
VO	Vertebral osteomyelitis
VRE	Vancomycin-resistant enterococci
XNL	Ceftiofur

ABSTRACT

Locomotor disorders represent a major challenge in modern poultry production worldwide and they may be related to non-infectious and infectious etiologies. Vertebral osteomyelitis is a bacterial disease described in outbreaks in many countries, characterized by infection of the mobile thoracic vertebra (T4), which results in the compression of the spine, reduced mobility and death of affected broilers. The objective of this study was to determine the frequency of vertebral osteomyelitis in broilers in the state of Minas Gerais, and to determine the bacterial etiologies involved in disease and their molecular characteristics. For this, we analyzed 608 broilers with locomotor disorders, which had their clinical signs recorded and then necropsied. Vertebral column samples and joints with gross changes were collected for bacterial isolation, molecular and histopathological analysis. Vertebral osteomyelitis was found in 5.1% (31/608) of the birds, which had different degrees of limited mobility, related to the level of spinal cord compression. The bacteria most frequently isolated from lesions were: *Enterococcus* spp. (53.6%), *E. faecalis* (32.1%) and *E. hirae* (7.1%); *Escherichia coli* (42.8%) in co-infection with *E. faecalis* in two cases; *Staphylococcus aureus* (14.3%) in co-infection with *Enterococcus* spp. or *E. hirae* in two cases. *E. coli* strains harbored different genetic pattern as assessed by PFGE, regardless of flock origin and lesion site (vertebral osteomyelitis or arthritis). The *E. coli* strains belonged to seven sequence types (STs) described previously (ST117, ST101, ST131, ST371 and ST3107) or newly described in this study (ST5766 and ST5856). Most strains belonged to ECOR phylogenetic group D (66.7%) and diverse serogroups (O88, O25, O12 and O45), some of worldwide importance. The antimicrobial susceptibility profile also showed the diversity of the strains and revealed a high proportion of multidrug-resistant strains (73%), mainly to quinolones and beta-lactams. Multilocus sequence typing (MLST) analysis of *E. faecalis* revealed that the strains belonged to eight different STs, being six (ST49, ST100, ST116, ST202, ST249, and ST300) previously described and ST708 and ST709 first identified in this study. ST49 was the most frequently isolated from vertebral osteomyelitis lesions. *E. faecalis* strains showed the highest resistance to aminoglycoside antibiotics, mainly to gentamicin (40.0%), and low resistance to vancomycin (10%). The results indicated that, in Brazil, vertebral osteomyelitis in broilers may not be caused by a single infectious agent and suggested geographical differences concerning the frequency and etiology of the disease, as comparing our region in Brazil with reports in other countries. Furthermore, our results showed the diversity of *E. faecalis* STs involved with this disease and high frequency of aminoglycoside resistance and low frequency of vancomycin-resistance. Also, vertebral osteomyelitis and arthritis could be associated with highly diverse *E. coli*, which were often multidrug-resistant. Some *E. coli* strains belonged to STs described also in humans, which may represent a concern to public and animal health.

Keywords: broiler; locomotor disorders; histopathology, bacterial infections; *Enterococcus faecalis*; *Escherichia coli*; *Enterococcus hirae*; *Staphylococcus aureus*; molecular characterization.

RESUMO

As alterações locomotoras representam um desafio na produção avícola moderna em todo o mundo e podem ter origem não infecciosa e infecciosa. A osteomielite vertebral é uma doença bacteriana descrita em surtos em diversos países caracterizada por infecção da vértebra torácica móvel (T4) que resulta em compressão da medula espinhal, dificuldade de locomoção e morte das aves acometidas. O objetivo deste trabalho foi determinar a frequência da doença em frangos de corte do estado de Minas Gerais, além de conhecer e caracterizar molecularmente os agentes etiológicos envolvidos na doença. Para isso, foram analisados 608 frangos de corte com problemas locomotores que tiveram os sinais clínicos registrados e foram submetidos à necropsia. Amostras de corpo vertebral e articulações com alterações macroscópicas foram coletadas para isolamento bacteriano, histopatologia e análise molecular. Osteomielite vertebral foi encontrada em 5,1% (31/608) das aves, as quais apresentaram diferentes graus de dificuldade locomotora relacionados ao nível de compressão da medula espinal. As bactérias mais frequentemente isoladas das lesões foram: Enterococcus spp. (53,6%), E. faecalis (32,1%) e E. hirae (7,1%); Escherichia coli (42,8%) em co-infecção E. faecalis em dois casos; Staphylococcus aureus (14,3%) em dois casos em co-infecção com Enterococcus spp. ou E. hirae. Os isolados de E. coli apresentaram diferentes padrões genéticos por PFGE, independentemente do lote estudado e tipo de lesão (osteomielite vertebral ou artrite). Os isolados pertenciam a sete sequence types (STs) descritos anteriormente (ST117, ST101, ST131, ST371 e ST3107) ou descritos pela primeira vez neste estudo (ST5766 e ST5856). A maioria dos isolados pertenciam ao grupo filogenético D (66,7%) e diversos sorogrupos (O88, O25, O12 e O45), alguns de importância mundial. O perfil de susceptibilidade antimicrobiana também refletiu a diversidade dos isolados e revelou alta frequência de cepas multirresistentes (73%), principalmente às quinolonas e beta-lactâmicos. A análise do Multilocus sequence typing (MLST) revelou que os isolados de E. faecalis pertenciam a oito STs distintos. Desses, seis (ST49, ST100, ST116, ST202, ST249 e ST300) foram previamente descritos, enquanto ST708 e ST709 foram descritos pela primeira vez nesse estudo. E. faecalis ST49 foi o mais frequentemente isolado das lesões vertebrais. Os isolados da bactéria apresentaram maior percentual de resistência antimicrobiana aos aminoglicosídeos, principalmente à gentamicina (40.0%), e baixa resistência à vancomicina (10%). Os resultados desse estudo demonstram que, no Brasil, osteomielite vertebral em frangos de corte pode não ser causada por um único agente infeccioso e sugere diferenças geográficas relativas à frequência e etiologia da doença entre esta região do Brasil e outros países. Além disso, nossos resultados demonstraram a diversidade de STs de E. faecalis envolvidos na doença com alta frequência de isolados resistentes a aminoglicosídeos e baixa frequência de E. faecalis resistentes à vancomicina. Nossos resultados demonstram, ainda, que osteomielite vertebral e artrite podem estar associadas à E. coli altamente diversas, as quais são frequentemente resistentes a múltiplas drogas antimicrobianas. Alguns isolados de E. coli pertencem a STs descritos também em seres humanos, o que representa uma preocupação para a saúde pública e animal.

Palavras-chave: frangos de corte; problemas locomotores; histopatologia; infecções bacterianas; Enterococcus faecalis; Escherichia coli; Enterococcus hirae; Staphylococcus aureus; caracterização molecular.

INTRODUCTION

Poultry products, meat and eggs, are a major source of animal protein available on the market due to its excellent quality, easy access to all sections of society and great variety of products at lower cost to the consumer (Amaral, 2003). Due to its popularity as a food and short production cycle, the poultry represent one of the animals most selected for production worldwide (Emmans and Kyriazakis, 2000) and the numbers of Brazilian poultry demonstrate the excellent performance of the sector in the country. Currently, Brazil is the largest exporter and second largest producer of poultry meat in world rankings (ABPA, 2015a). In 2014, the state of Minas Gerais accounted for 7.1% of the slaughtered poultry, occupying the fifth position among the Brazilian states (ABPA, 2015b).

Most broilers are created using modern intensive production systems worldwide, where birds are confined in high-density warehouses (FAO, 2007) and raised from birth to slaughter in approximately 40 days. However, there is evidence that the optimization of the production for these systems, though producing meat at low cost, results in birds with reduced viability and welfare with limited locomotion capacity (Kestin et al., 1992; Bessei, 2006).

Locomotor pathologies or "leg problems" represent a major concern in commercial flocks of broilers, particularly those that lead to limitations in mobility or lameness (Scahaw, 2000). They are responsible for significant economic losses and decrease in animal welfare in the poultry industry (Araújo et al., 2011). These losses occur for carcasses condemnations in slaughterhouse due to fractures, hematoma and lesions on the skin, as well as by the decrease in the growth and performance of affected broilers. Once these birds can not have adequate access to food and water, they become weak and lighter, presenting worst zootechnical results (Silva et al., 2001; Almeida-Paz, 2010).

The development of many locomotor diseases are related to genetic selection and the rapid growth of broilers, which is demonstrated by their frequency in broilers, broiler breeders, ducks and turkeys raised in confinement (Kestin et al., 1992). These diseases have been a problem since the beginning of intensive poultry production and has been linked to numerous causes as nutrition (poisoning, deficiencies or imbalances), genetics, management practices and others that can affect directly the growth and development of the locomotor system (Silva et al., 2001), such as infections and trauma (Julian, 1998).

Among the infectious causes that lead to locomotor disorders in broilers is vertebral osteomyelitis, also known as enterococal spondylitis. Vertebral osteomyelitis is an infectious bacterial disease that usually affects the free thoracic vertebra leading to vertebral necrosis and inflammation with consequent compression of the spinal cord, resulting in limited mobility and mortality of affected birds (Martin et al., 2011). Vertebral osteomyelitis has been reported and studied in several countries of relevant poultry. In Brazil, however, there are no studies about the disease, despite the locomotor problems are common in most broiler farms, with field reports indicating the occurrence of vertebral osteomyelitis in the state of Minas Gerais.

OBJECTIVES

General

This study aimed to determine the frequency of vertebral osteomyelitis, and provides data on the etiology of the disease in poultry with locomotor disorders in the state of Minas Gerais.

Specific

1. To establish the frequency of vertebral osteomyelitis in broilers with locomotor disorders in the state of Minas Gerais;

2. To describe the clinical and pathological changes in broiler with vertebral osteomyelitis in the state of Minas Gerais;

3. To identify the etiologic agents involved in the cases of vertebral osteomyelitis in broilers with locomotor disorders in the state of Minas Gerais;

4. To investigate the antibiotic susceptibility and genetic relationships among *Enterococcus faecalis* isolates from vertebral osteomyelitis in broilers with locomotor disorders in the state of Minas Gerais;

5. To determine the molecular and phenotypic characteristics of *Escherichia coli* isolated from broiler lesions with locomotor disorders in the state of Minas Gerais; and

6. To perform a clinical and pathological characterization of lesions associated with *Escherichia coli* isolated from broilers with locomotor disorders in the state of Minas Gerais.

CHAPTER 1

Vertebral osteomyelitis in broilers: a review

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Abstract: Vertebral osteomyelitis is an emerging disease in broilers worldwide. The inflammatory process in the affected thoracic vertebra (T4) and spinal cord compression leads to clinical signs related to locomotor impairment and death of birds. The pathogenesis of the disease is poorly understood and *Enterococcus cecorum* is the bacterium frequently associated with the disease. However, *E. faecalis*, *E. durans*, *Escherichia coli* and *Staphylococcus aureus* were recently detected in cases of the disease, raising questions about its etiopathogenesis. An important aspect related to these bacteria is their role as source virulence and antibiotic resistance genes and its possible dissemination to other bacteria, animals and humans. Since there are still many questions about vertebral osteomyelitis in broilers, the knowledge on its prevention, control and treatment are limited. In this review, we compile and discuss the current knowledge on vertebral osteomyelitis in broilers and raise relevant aspects concerning the disease.

Keywords: locomotor diseases, bacterial infections, *Enterococcus* spp., *Enterococcus cecorum*, *Escherichia coli*.

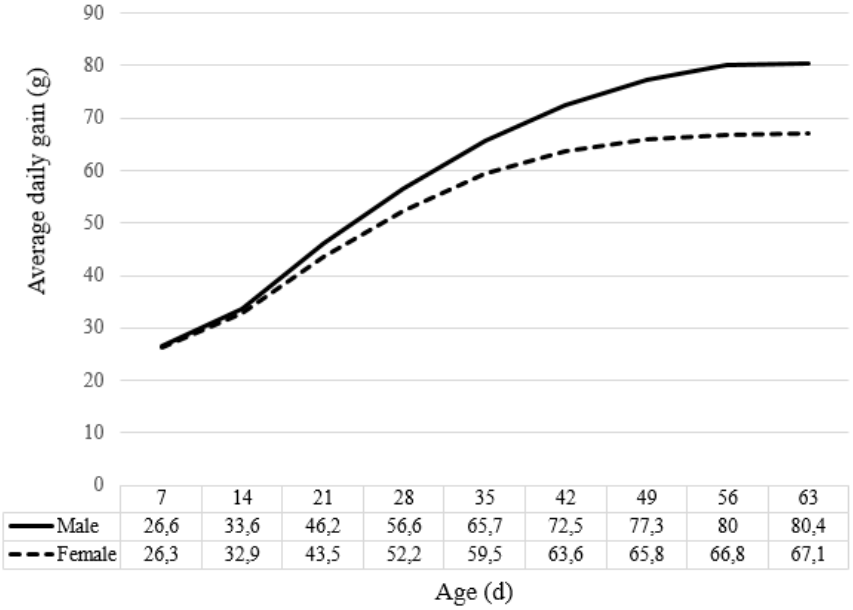
Introduction

Vertebral osteomyelitis is an emerging disease that affects broilers worldwide (Devriese *et al.*, 2002; Wood *et al.*, 2002; Herdt *et al.*, 2009; Aziz and Barnes, 2009; Gingerich *et al.*, 2009; Stalker *et al.*, 2010; Kense and Landman, 2011; Boerlin *et al.*, 2012). The disease has been mainly described causing outbreaks in broilers and broiler breeders associated with infection by *Enterococcus cecorum*, which is a normal inhabitant of the chicken intestinal tract. Vertebral osteomyelitis is characterized by infection with inflammation and necrosis of the free thoracic (T4) vertebral body. The infection results in spinal cord compression and impaired mobility of affected broilers, which often die by dehydration or starvation (Aziz and Barnes, 2007). The pathogenesis of vertebral osteomyelitis is still poorly understood in this species (Martin *et al.*, 2011). In recent years, Enterococci have emerged as an important cause of nosocomial infections, with resistant microorganisms largely involved in these cases (McGaw, 2013). This review aimed to compile and discuss the current knowledge on vertebral osteomyelitis in broilers, as well as to raise relevant aspects concerning the disease.

Epidemiology of the disease

Vertebral osteomyelitis has been reported in poultry in different countries of Europe, such as United Kingdon (Wood *et al.*, 2002), Netherlands (Devriese *et al*., 2002; Kense and Landman, 2011), Belgium (Herdt *et al.*, 2009), Hungary (Makrai *et al*., 2011), Norway (Kolbjørnsen *et al.*, 2011), and Bulgaria (Dinev, 2013). The disease was also described in North and South America, such as in Canada (Stalker *et al.*, 2010), several US states (Pennsylvania, Washington, North Carolina, South Carolina, Arkansas, Mississippi, Alabama, and California) (Aziz and Barnes, 2009; Gingerich, 2009) and Brazil (Braga *et al.*, 2016c).

The disease occurs more frequently in males and several lineages can be affected (Wood *et al*., 2002; Gingerich, 2009). It is interesting to note that the higher body weight normally observed in males (Fig. 1) implies an increase in the weight supported by the joints and a greater chance of trauma, which is suggested for another locomotor condition known as bacterial condronecrosis with osteomyelitis (BCO), more frequent in male broilers (Wideman and Prisby, 2013).



	7	14	21	28	35	42	49	56	63
Male	26,6	33,6	46,2	56,6	65,7	72,5	77,3	80	80,4
Female	26,3	32,9	43,5	52,2	59,5	63,6	65,8	66,8	67,1

Figure 1. Average daily weight gain (g) of male and female broilers from seven to 63 days-old. Adapted from Cobb manual (2015).

Affected broilers are usually older than 30 days-old, with reported outbreaks of the disease ranging from three to 18 week-old (Herdt *et al.* 2009; Armour *et al.*, 2011; Robbins *et al.*, 2012). However, there is a report in a flock older than 15 days. Initially, the percentage of affected birds in a flock was relatively high, ranging from 5% to 10%, and then following reports of 2% to 4% (Gingerich, 2009).

Predisposing factors for vertebral osteomyelitis are not well defined (Kense and Landman, 2011; Robbins *et al*., 2012), however, immunosuppression and environmental conditions have been identified as factors that contribute for the occurrence of the disease (Stalker *et al.*, 2010; Armour *et al.*, 2011). Any immunosuppressive condition can naturally predispose to an opportunistic infection by *E. cecorum*, which is a normal intestinal commensal. A study demonstrated differences in the pathogenicity among isolates from clinical cases and

intestinal commensal *E. cecorum*, raising the question whether the emergence of clones is most likely the cause for the increased occurrence of infections (Boerlin *et al.*, 2012).

Some evidences suggest that the higher incidence of Enterococci-associated diseases in poultry may be due to horizontal spread of dominant clones of *E. cecorum* which exhibit increased pathogenicity (Kense and Landman, 2011; Boerlin *et al.*, 2012). Strains with genotypes similar to those isolated from vertebral osteomyelitis cases were rarely recovered from the cecum of birds with vertebral osteomyelitis and the presence of these isolates was not statistically associated with a higher risk of disease (Borst *et al.*, 2012). These findings suggest that long-term cecal transport of pathogenic clones may not be necessary in the pathogenesis of vertebral osteomyelitis caused by *E. cecorum*. However, as the disease has a chronic character, requiring weeks from the time of infection to the onset of clinical signs, pathogenic strains may be transient in the gastrointestinal tract and therefore not recoverable in the moment of clinical presentation (Borst *et al.*, 2012). Field observations showed that the disease occurred in successive flocks, suggesting persistence of *E. cecorum* on the farm (Herdt *et al.*, 2009; Kense and Landman, 2011).

Despite the worldwide distribution, the way in which pathogenic clones of *E. cecorum* spread remains undetermined. Epidemiological studies on distinct outbreaks of vertebral osteomyelitis suggest that mechanical spreading by biological vectors or inadequate biosafety can contribute to disease transmission, although horizontal transmission between geographically distant locations was considered unlikely (Borst *et al.*, 2012).

Kense and Landman (2011) demonstrated that vertical transmission does not occur. Recently, Borst *et al.* (2014) showed that SPF and non-SPF chicken embryos inoculated with *E. cecorum* isolated from vertebral lesions had lower survival rate when compared to embryos inoculated with *E. cecorum* isolated from the intestines of healthy birds. The embryos infected with pathogenic strains had lesions of septicemia, such as hemorrhage and edema. In embryos inoculated with non-pathogenic strains, these lesions were observed only 48 hours later.

Etiologic agents

Most inflammatory diseases of bones are caused by bacterial infections, although other agents can also infect bones (Craig *et al.*, 2016). The bacterial agents of greatest importance in the etiology of vertebral osteomyelitis in poultry are described.

The genus *Enterococcus*

Enterococcus spp. are gram-positive and spherical bacteria, which occur alone, in pairs or short chains. They are non-motile, non-spore-forming, facultative anaerobic with diverse biochemical properties (Wages, 1998). However, the relationship between biochemical characteristics and pathogenicity of the species remains unknown (Thayer *et al.*, 2008). *Enterococcus* spp. are ubiquitous in nature with worldwide distribution in avian species. They are considered part of the normal intestinal microbiota of chickens and commonly found in poultry environments. The frequency that different species of *Enterococcus* spp. are isolated from the intestinal tract of healthy birds can vary according to the age, but only a limited number of species is isolated more often. *E. faecium*, *E. cecorum*, *E. faecalis*, *E. hirae* and *E. durans* were the species regularly isolated in at least one of three different age groups (1 day-old, 3 to 4 week-old, and more than 12 week-old) examined by Devriese *et al.* (1991).

Minimal spinal cord lesions and low mortality due to vertebral and joint changes were previously associated with Enterococci (Devriese *et al.*, 2002; Wood *et al.*, 2002; Landman *et*

al., 2003; Perez, 2004). However, since 2002, *E. cecorum* was more frequently recognized as cause of outbreaks of non-vertebral and vertebral osteomyelitis (the last one also known as spondylitis) and arthritis in broiler and broiler breeders (Aziz and Barnes, 2007; Herdt *et al.*, 2009; Aziz and Barnes, 2009; Gingerich, 2009; Stalker *et al.*, 2010; Martin *et al.*, 2011; Boerlin *et al.*, 2012; Aitchison *et al.*, 2014). Jung and Rautenschlein (2014) described *Enterococcus cecorum* isolation from a broiler flock with pericarditis, hepatitis, femoral head necrosis and/or vertebral osteomyelitis and concluded that bacteremia and generalized infection seem to be important steps in the pathogenesis of infection caused by this bacterium in broilers.

E. cecorum occurs more frequently in intestines of chickens older than 12 weeks of age (Devriese *et al.*, 1991) and was rarely associated with clinical disease in these birds (Devriese *et al.*, 2002; Wood *et al.*, 2002; Chadfield *et al.*, 2004; Thayer *et al.*, 2008). This reflects on the limited number of publications regarding its role in disease and its pathogenicity (Makrai *et al.*, 2011). Two main hypotheses were proposed to explain the recent increase in the incidence of infections with *E. cecorum*: 1) changes in the host or environmental factors; and 2) emergence of individual clones with increased pathogenicity (Boerlin *et al.*, 2012). To prove the second hypothesis, the authors analyzed *E. cecorum* isolates recovered from the cecum of healthy birds and of birds with vertebral osteomyelitis by pulsed-field gel electrophoresis (PFGE). Genotypes of *E. cecorum* isolated from vertebral lesions were significantly more similar to each other than the *E. cecorum* isolated from the cecum of healthy birds and of birds with vertebral osteomyelitis, regardless the affected flock.

Infections by *E. hirae* are relatively frequent in broilers, but its importance is not as understood as the infections caused by other bacteria, such as *Escherichia coli* and *Staphylococcus aureus*. Diseases caused by *E. hirae* have increasing incidence in some countries, such as Norway, where the bacterium was isolated from cases of osteomyelitis in broilers (Kolbjørnsen *et al.*, 2011). In addition, there are reports of focal cerebral necrosis in chicks (Devriese *et al.*, 1991; Randall *et al.*, 1993) and cases of diarrhea in one week-old chicks (Kondo *et al.*, 1997). Velker *et al.* (2011) described endocarditis associated with *E. hirae* in different broiler flocks, with co-isolation of *E. faecalis*, *E. coli*, and a mixture of several other opportunistic bacteria from lesions. Although the meaning of this finding was considered unknown by the authors, they assumed these as opportunistic infections or resulting from tissue autolysis. Recently, Braga et al. (2016c) reported *E. hirae*, *E. faecalis*, *E. coli* and *S. aureus* in single or mixed culture from vertebral osteomyelitis cases in broilers. The molecular analysis and histopathology with special stains allowed the confirmation of concomitant agents in the lesion and discarded the possibility of contamination.

In addition to vertebral osteomyelitis, Enterococci are often associated with other diseases in poultry. In day-old chicks, Enterococci are generally responsible for infection in the yolk sac (Deeming, 2005). *E. faecalis* has been associated with hepatic granulomas in turkeys (Hernandez *et al.*, 1972) and pulmonary hypertension syndrome in broilers (Tankson *et al.*, 2001). Cases of arthropathy associated to AA amyloid and concomitant systemic amyloidosis caused by arthropathic and amyloidogenic *E. faecalis* was described in laying hens (Landman *et al.*, 1994) and broiler breeders (Steentjes *et al.*, 2002). In domestic ducks, *E. faecalis* have been isolated from cases of arthritis (Bisgaard, 1981), whereas *E. faecium* (Sandhu, 1988) and *E. cecorum* (Jung et al., 2013) have been associated with acute septicemia in Pekin ducks. *E. durans* was isolated from young chickens with bacteremia and encephalomalacia (Cardona *et al.*, 1993; Abe *et al.*, 2006).

Enterococci are lactic acid forming bacteria with an important role in food due to its deterioration and fermentation, as well as their use as probiotics in humans and production animals. They are also important as nosocomial pathogens that cause bacteremia, endocarditis and other infections. Some strains are resistant to many antibiotics and own virulence factors,

such as adhesins, invasins, hemolysin, and pili. Specific genetic lineages of hospital-adapted strains emerged and some *E. faecalis* are considered high-risk Enterococci, such as the clonal complexes CC2, CC9, CC28 and CC40. These are characterized by the presence of antibiotic resistance determinants and/or virulence factors usually located on pathogenicity islands or plasmids, highlighting a major role for bacteria mobile genetic elements in establishing problematic strains (Franz *et al.*, 2011).

Some studies showed little phylogenetic diversity of *E. faecalis* isolates, with nucleotide identity of 97.8% to 99.5%. However, the sequence identity of shared genetic content among isolates ranged from 70.9% to 96.5%. In general, most of *E. faecalis* diversity can be attributed to the inclusion of mobile genetic elements into a widely conserved genome, with these mobile elements supporting the exchange of chromosomally encoded characteristics (Palmer *et al.*, 2012). Studies that compared *E. faecalis* isolates obtained from different lesions in eight broiler breeder flocks and *E. faecalis* isolated from healthy birds revealed 12 different sequence types (STs) and lack of correlation between ST and lesion type, although ST82, ST174 and ST177 represented 81% of the strains associated with lesions (Gregersen *et al.*, 2010).

Escherichia coli

E. coli has been also isolated from cases of vertebral osteomyelitis in poultry (Dinev, 2013; Braga *et al.*, 2016c), which is part of normal intestinal microbiota of humans and many animal species. *E. coli* are Gram-negative non-spore-forming bacillus, with 2-3 x 0.6 µm in size, and most strains are motile with peritrichous flagella (Barnes *et al.*, 2008).

Several *E. coli* strains are able to express virulence factors and cause intestinal or extra-intestinal diseases (Ambrozic *et al.*, 1998). Currently, *E. coli* is considered the most important Gram-negative bacterium due to its different mechanisms of pathogenicity and described diseases (Nakazato *et al.*, 2009). In avian species, pathogenic strains are named Avian Pathogenic *Escherichia coli* (APEC) (Ewers *et al.*, 2004), which are responsible for extra-intestinal diseases known generically as colibacillosis.

Colibacillosis has a worldwide occurrence and leads to significant economic losses in all types of poultry (Dziva and Stevens, 2008). The most common lesions associated with colibacillosis are perihepatitis, pericarditis and airsacculitis, although other syndromes such as osteomyelitis/arthritis, yolk sac peritonitis, salpingitis, coligranuloma, omphalitis and cellulitis can also be found (Barnes *et al.*, 2008). According to Barnes *et al.* (2008), the presence of *E. coli* in bone and synovial tissues is a common sequel of colisepticemia and the affected birds could probably not completely eliminate the bacterial infection.

Several virulence factors are associated with APEC, such as: F1 and P fimbrial adhesins, aerobactin iron acquisition system, k1 capsular antigen, complement resistance and many proteins, such as Tsh autotransporter (Dho-Moulin and Fairbrother, 1999). Although APEC strains are the major pathogens for commercial poultry, the knowledge on virulence factors are still incomplete. Considering that avian colibacillosis occurs worldwide in its various forms, it is believed that the phylogenetic analysis of clonal relationships among *E. coli* isolates from different countries and regions may provide a greater understanding about its pathogenesis (Schouler *et al.*, 2004).

At present, there is no single gene or specific virulence genes set systematically associated with APEC, complicating the diagnosis and development of drugs that target all APEC strains. This diversity and the fact that most *E. coli* are non-pathogenic, hamper the diagnosis of an avian *E. coli* isolate as causal agent (Guabiraba and Schouler, 2015). Despite this, it has been shown that five genes (iroN, ompT, hlyF, iss and iutA) located on the large virulence plasmid ColV are associated with APEC strains (Johnson *et al.*, 2008). Moreover,

Schouler *et al.* (2012) showed that four combinations of genes allowed the diagnosis of more than 70% of the APEC strains.

Phylogenetic studies based on *E. coli* Reference Collection (ECOR), a set of 72 *E. coli* strains isolated from various animal hosts and different geographic origins (Ochman and Selander, 1984), showed that there are four main phylogenetic groups for *E. coli* designated A, B1, B2 and D (Selander *et al.*, 1987; Herzer *et al.*, 1990). However, no avian strain was included in the ECOR collection and no APEC strains were placed in the *E. coli* phylogenetic tree. Epidemiological molecular studies showed that most APEC strains can be grouped into a limited number of clones (Ngeleka *et al.*, 1996; Da Silveira *et al.*, 2002; La Ragione and Woodward, 2002; Ewers *et al.*, 2004). The clonal nature of APEC has been demonstrated by phylogenetic analysis (Whittam and Wilson, 1988; White *et al.*, 1990; White *et al.*, 1993; Da Silveira *et al.*, 2002). Many studies have also revealed the prevalence of several serogroups and particular combinations of genes associated with virulent strains of APEC. These observations suggested that only a limited number of virulent genotypes exist (Blanco *et al.*, 1998; Ngeleka *et al.*, 2002; Ewers *et al.*, 2004; Rodriguez-Siek *et al.*, 2005a; Rodriguez-Siek *et al.*, 2005b).

Some sequences of APEC strains genome was determined, still requiring extensive comparative genomic analysis of APEC strains of different serogroups (Johnson *et al.*, 2007; Dziva *et al.*, 2013; Mangiamele *et al.*, 2013; Huja *et al.*, 2015). The comparative genomic study of APEC serogroup O78 revealed that genetic variability occurs even within a single serogroup (Huja *et al.*, 2015). According to Rasko *et al.* (2008), that studied different pathogenic *E. coli*, the pangenome of the bacterium has a reservoir consisting of more than 13,000 genes. This has great implication on diversity and pathogenesis of *E. coli* strains and their ability to colonize and cause disease in the human host. Approximately half of the genome content of any *E. coli* represents the core-conserved genome and the open pangenome of *E. coli* species indicates that continuous genetic sequencing should result in the identification of approximately 300 new genes per genome.

Other agents involved in vertebral osteomyelitis

Staphylococcus pyogenes was isolated from vertebral osteomyelitis cases in seven to 16 week-old chickens (Carnaghan, 1966). Nairn (1973) reported the isolation of *Staphylococcus aureus* of vertebral lesions in turkeys naturally affected with locomotor disorder. The experimental inoculation in turkeys resulted in osteomyelitis in the vertebral body and long bones. Van Veen (1999) reported the involvement of *Aspergillus fumigatus* in vertebral osteomyelitis outbreaks in two flocks of 17-19 week-old broilers.

Pathogenesis

Bacterial inflammatory processes of bones can be originated from hematogenous, local extension, and implantation routes, being the first the most common in animals. When the inflammation is originated from vascular areas of the medullary cavity or periosteum is referred as osteomyelitis or periostitis, respectively. A more general but less frequently used term for inflammation of bones is osteitis (Craig *et al.*, 2016).

The pathogenesis of vertebral osteomyelitis in birds remains largely unknown (Kensi and Landman, 2011; Robbins *et al.*, 2012). There is a limited number of publications about the pathogenicity of *E. cecorum* (Makrai *et al.*, 2011) and the genetic basis for the recently acquired pathogenicity of certain *E. cecorum* clones and the pathogenesis of vertebral lesions characteristic of the disease remain unknown (Borst *et al.*, 2012).

The disease was reproduced experimentally by Martin *et al.* (2011), through the inoculation of *E. cecorum* by oral and intravenous routes. Gross lesions were observed five weeks after the experimental infection in 6.1% and 2.9% of broilers inoculated orally or intravenously, respectively. However, histologic lesions were observed in 30.3% of broilers inoculated orally, and the macroscopic evidence of disease was suggested to be higher if the broilers were older.

The free thoracic (T4) vertebral body is singly affected in vertebral osteomyelitis and the reasons for this predilection are unknown. The single free thoracic vertebral articulation is located between the immediately anterior fused thoracic vertebrae and the posterior synsacrum (Fig. 2), enabling body position adjustments and flexibility during walk and flight, and is subject to greater biomechanical stress and microtraumas than any other vertebra. Excessive stress may lead to changes in vascular flow with development of micro-thrombi, sequestrum and multiplication of bacteria, if present in blood (Aziz and Barnes, 2007; Stalker *et al.*, 2010; Wideman and Prisby, 2013; Aitchison *et al.*, 2014).

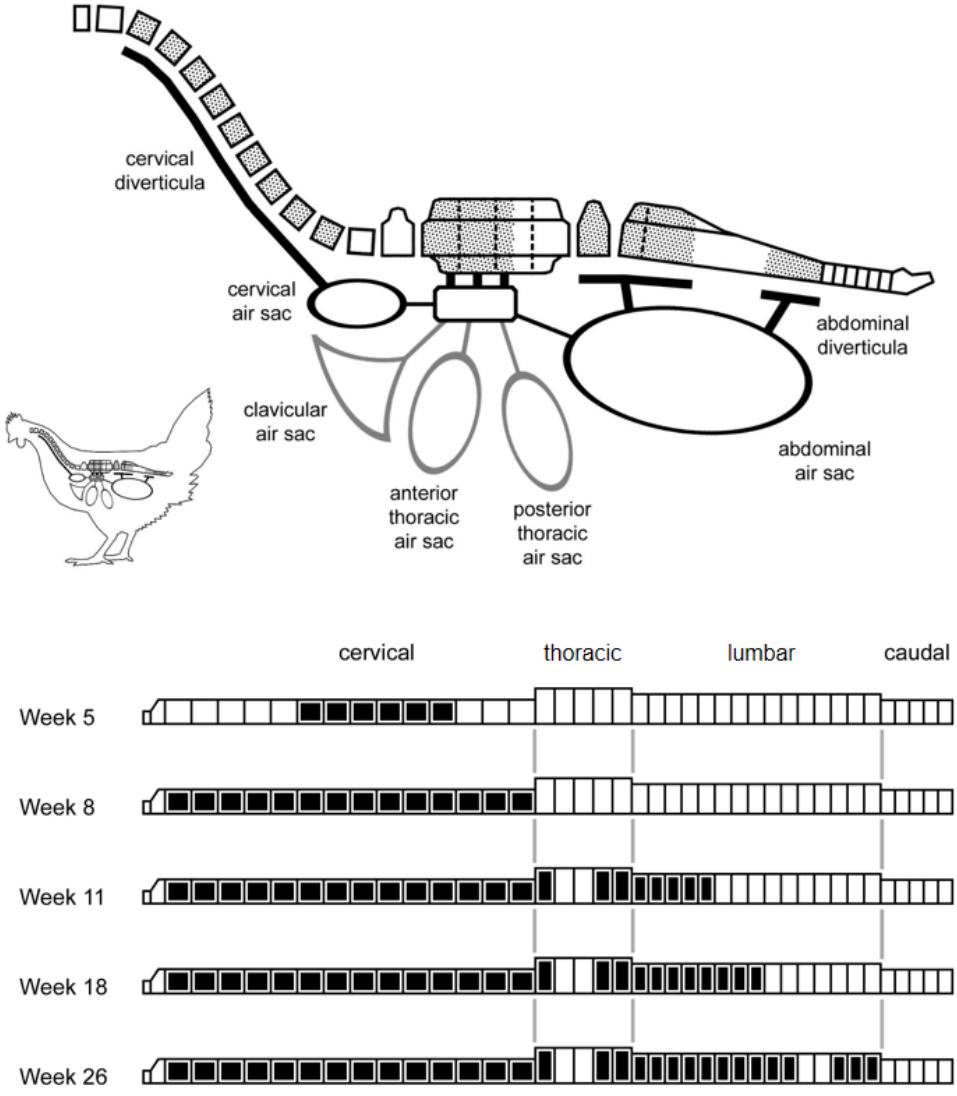


Figure 2. Pneumatization of the vertebral column in the chicken (*Gallus gallus*). Pneumatic vertebrae are represented in dotted (upper diagram) or black (lower

diagram). The vertebral column is pneumatized by diverticula of cervical and abdominal air sacs and lungs. Adapted from King (1957) and Hogg (1984) apud Wedel (2008).

According to Stashak and Mayhew (1984), vertebral osteomyelitis is usually secondary to hematogenous dissemination of a microorganism. However, other theories have been proposed to explain how the bacteria reach the mobile thoracic vertebra. Currently, the most accepted theory suggests that the agent has access to the bones via bloodstream due to rupture of the intestinal mucosal barrier (Stalker *et al.*, 2010; Martin *et al.*, 2011), as in coccidiosis or bacterial enteritis (Gingerich, 2009). According to Armour *et al.* (2011) and Martin *et al.* (2011), any factor that interferes negatively with intestinal health or disturbs the balance of intestinal microbiota could possibly predispose to systemic dissemination of *E. cecorum*.

A possible link of the vertebral osteomyelitis with air sacs and pneumatic vertebra could exist (Aziz and Barnes, 2007), as shown in Fig. 2. However, it is interesting to note that the pneumatization of the vertebra where the disease occurs (T4) begins only after eight weeks of age. The experimental inoculation of *E. cecorum* in two week-old broilers by air sac route did not result in vertebral osteomyelitis (Martin *et al.*, 2011), suggesting that for experimental studies older broilers should be inoculated. It is worth mentioning that, in a study conducted by Tankson *et al.* (2002), *E. faecalis*, *E. durans*, and *E. coli* were isolated from the heart and lung of healthy birds in 15% of cases, however, there are no studies that provide this information for *E. cecorum*.

It is interesting to note some aspects in the pathogenesis of BCO that could assist in the understanding of the pathogenesis of vertebral osteomyelitis. This disease affects more often the femur and tibiotarsus, but it can also occur in the free thoracic vertebra. BCO initiates with the degeneration and necrosis of the cartilage followed by bacterial invasion, mainly associated to *S. aureus*, *E. coli* and *E. cecorum*, often in mixed culture, and with other bacteria (Wideman and Prisby, 2013).

It is believed that the BCO begins with mechanical damage to the columns of chondrocytes poorly mineralized present mainly in the proximal growth plate of fast-growing bones, such as the femur and tibia, followed by colonization of the chondronecrotic clefts by opportunistic bacteria spread through the blood. Terminal BCO presents itself as degeneration, necrosis and bacterial infection at the proximal ends (epiphyseal and metaphyseal growth plates) of the femur and tibiotarsus. A similar process may occur in the growth plates of other bones that are subject to severe torque and shear stresses, as occur in the fourth thoracic vertebra, which functions as a flexible pivot between the cranially fused vertebrae of notarium and caudally fused vertebrae of synsacrum (Carnaghan, 1966; McNamee and Smyth, 2000; Dinev, 2009; Wideman *et al.*, 2012).

According to Barnes *et al.* (2008), the involvement of *E. coli* in infectious processes of bone and synovial tissues is a common sequel of colisepticemia. Osteomyelitis caused by hematogenous spread of *E. coli* after infection by the hemorrhagic enteritis virus was experimentally reproduced in turkeys (Droual *et al.*, 1996). Some authors report that, although intravenous inoculation of *E. coli* promoted hematogenous spread to bones and joints and reproduction of lesions, bird mortality caused by initial sepsis is usually high (Bayyari *et al.*, 1997). Thus, the inoculation of lower counts of *E. coli* into the air sacs after pretreatment with dexamethasone has been the indicated as a method for the reproduction of the disease (Huff *et al.*, 2000). Other studies demonstrated that several sites of infection are usually involved in turkeys and the bones most frequently affected are tibiotarsus, femur, humerus, and thoracolumbar vertebrae (Muttalib *et al.*, 1996). According to Bayyari *et al.* (1997), the bacterium colonizes the vascular branches that invade the growth plate of growing bones, causing an inflammatory response that results in osteomyelitis. The transphyseal vessels in birds

may possibly serve as conduits for the process of bacterial spread to the joint and surrounding soft tissues.

Clinicopathological changes

Clinical signs

The clinical signs are similar in all the vertebral osteomyelitis reports (Gingerich, 2009), although with variable onset age of clinical presentation. In cases of osteomyelitis and arthritis caused by *E. cecorum*, Herdt *et al.* (2009) reported that the clinical signs started during the first and second weeks of age with mortality rate of 7%. In the osteomyelitis cases studied by Makrai *et al.* (2011), the clinical signs started between the 5th and 9th week of age up to the 10th to 13th week, with a mortality rate ranging from 8% to 30%, which was higher than previously reported (Wood *et al.*, 2002; Herdt *et al.*, 2009).

The main clinical sign observed is the limited mobility, with lameness that can range from mild to severe. The affected birds frequently acquire the posture described as "sitting on their hocks", characterized by legs extended cranially and support given by tibiotarsus-metatarsus joints (Fig. 3a) (Gingerich, 2009; Braga *et al.*, 2016c). This is considered the classic clinical presentation of the disease, which is similar to that observed in birds with spondylolisthesis (Wood *et al.*, 2002; Gingerich, 2009).

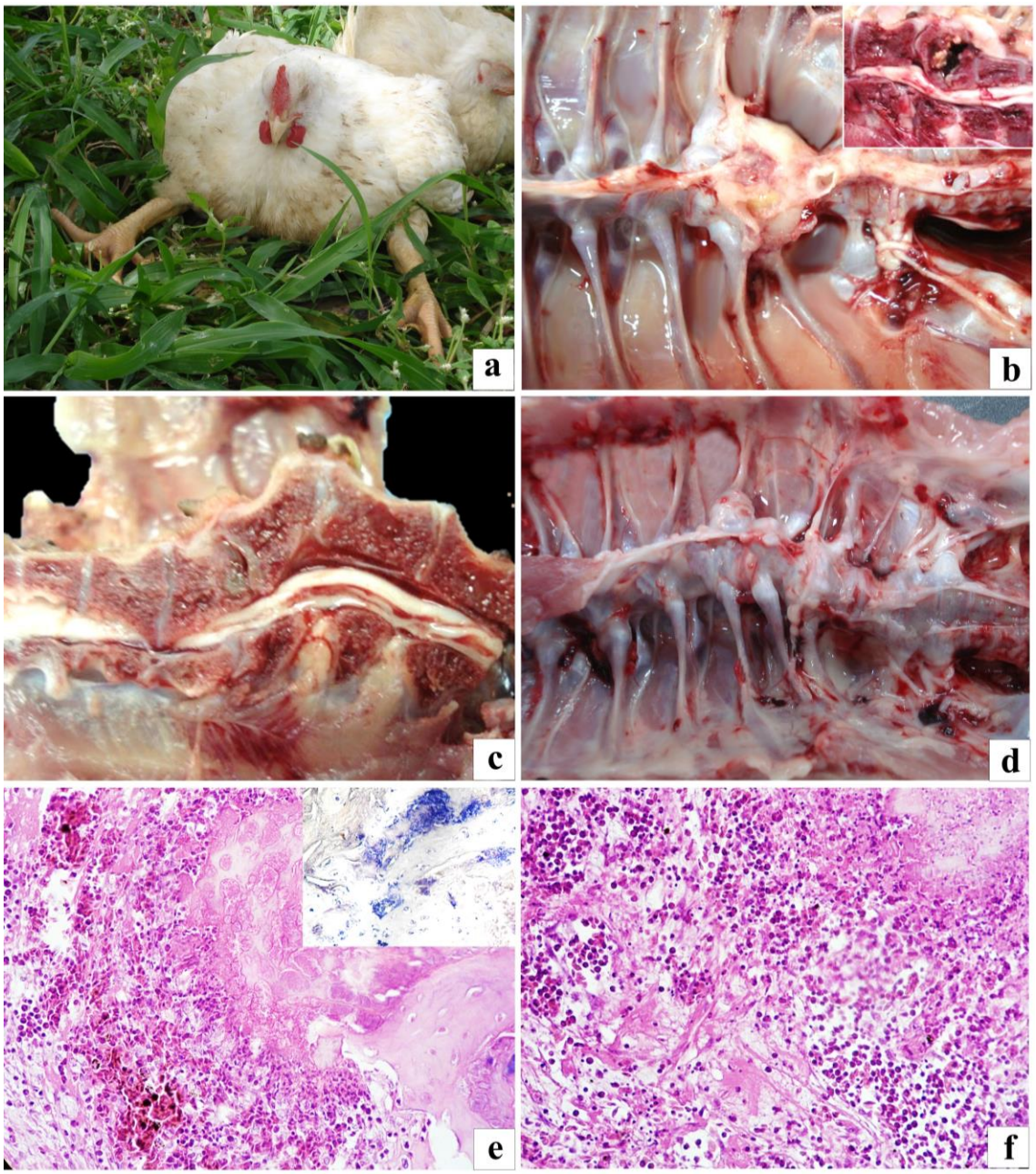


Figure 3. Clinicopathological changes of vertebral osteomyelitis and differential diagnosis in broilers. (a) Broiler showing the classical clinical sign of vertebral osteomyelitis. (b) Gross changes of vertebral osteomyelitis revealing enlargement of affected vertebral body (T4). Inset: sagittal section with caseonecrotic material in the T4 vertebra and spinal cord compression. (c) Vertebral body displacement of T4 vertebra characteristic of spondylolisthesis with spinal cord compression. (d) Scoliosis characterized by lateral deviation of vertebral column. (e, f) Histological changes of vertebral osteomyelitis. There are necrotic tissue, cell debris, heterophils, hemorrhage and fibrin. HE, 400x. Inset: Gram positive bacteria associated to vertebral lesion. Goodpasture, 400x.

Severely affected broilers may remain in lateral recumbency (Gingerich, 2009). They occasionally use their wings to help in locomotion, which may result in laceration and

hematoma in the wings (Makrai *et al.*, 2011). One of the consequences of impaired locomotion is the difficulty to have access to water and food, resulting in lower growth rate and death due to dehydration or starvation. In addition, sick broilers are more likely to cannibalism (Barnes *et al.*, 2008).

Gross changes

The macroscopic examination of the vertebral column thoracolumbar region of affected broilers reveals gross changes in the free thoracic vertebra (T4), which shows a palpable whitish to yellowish enlargement (Fig. 3b). The sagittal section of these lesion shows caseonecrotic material inside the vertebral body characterized by yellow to gray exudate, granular and friable, which is surrounded by a thick whitish capsule of fibrous connective tissue (Fig. 3b) (Gingerich, 2009; Martin *et al.*, 2011; Robbins *et al.*, 2012, Braga *et al.*, 2016c). Marked lesion characterized by increased volume of the vertebral body due to the infection results in narrowing of the overlying spinal canal, which would cause compression of the spinal cord (Makrai *et al.*, 2011; Aitchison *et al.*, 2014, Braga *et al.*, 2016c). For early stages of disease, there is no large increase of the vertebral body and mild or no spinal compression that can be seen in the sagittal section. Body condition of affected birds is variable, from good nutritional to cachectic condition. According to Makrai *et al.* (2011), some broilers may have subcutaneous edematous and green-brownish lesions in the region of tibiotarsus-metatarsal joint.

In some cases, the involvement of bone and joint can occur, process named osteoarthritis. In these cases, the most commonly affected bones are tibiotarsus, femur, thoracolumbar vertebral column and humerus (Muttalib *et al.*, 1996). In long bones, the lesion occurs more frequently in the proximal growth plate. The injuries usually occur where endochondral ossification is developing and extends to the cartilage of adjacent growth plate (McNamee and Smyth, 2000). Stalker *et al.* (2010) described an outbreak of typically unilateral lesions of osteomyelitis and arthritis associated with *E. cecorum*. These were characterized by fibrinous exudate into the articular space of tibiotarsus-metatarsal or coxo-femoral joints, extending to the tendon sheath. Rasheed (2011) reported that the joints with arthritis were increased in volume, swollen and hyperemic with purulent yellowish-white exudate inside the joint space.

Histopathology

The microscopic changes in cases of vertebral osteomyelitis were detailed after the experimental reproduction of the disease with *E. cecorum* (Martin *et al.*, 2011) and were similar to those reported in natural cases of the disease (Stalker *et al.*, 2010; Robbins *et al.*, 2012; Aitchison *et al.*, 2014, Braga *et al.*, 2016c). On the histopathologic examination, the free thoracic vertebral body and the adjacent vertebrae of notarium and synsacrum present necrotic tissue and exudate composed of fibrin, hemorrhage and heterophils (Fig. 3e and 3f). The bone tissue that forms the basis of the spinal canal is replaced by fibrous connective tissue and exudate, leading to spinal canal stenosis and spinal cord compression. In addition, there are fibrous connective tissue proliferation and bone remodeling in the surrounding areas of the lesion. Also, there are areas of bone and cartilage tissue sequestrum within the exudate. When bacterial colonies are present (Fig. 3e, inset), they are numerous and associated to the sequestrated areas (Aitchison et al., 2014; Braga et al., 2016c). In addition to these changes, Aitchison *et al.* (2014) and Braga *et al.* (2016c) described reactive osteoid formation and cartilaginous metaplasia in the areas where there was severe thickening of the vertebral body, resulting in areas of spinal cord compression. In these areas, there was axonal loss and

degeneration, and the neuropil was disorganized and vacuolated, indicating a compressive effect.

Martin *et al.* (2011) reported histologic changes in broilers in the absence of macroscopic lesions, with mild histologic lesions in the subchondral vertebral areas, with no extension to the articular cartilage or adjacent vertebrae. A moderate to severe infiltration of lymphocytes and diffuse fibroplasia in the affected vertebra with intralesional bacteria was confirmed in half (4/8) of the cases. Braga *et al.* (2016c) also observed lesions in adjacent vertebrae, which were characterized by the degeneration and necrosis of the articular cartilage (T4/T5), and occasional presence of clefts associated or not with hemorrhages and bacterial colonies. In the study performed by Martin *et al.* (2011), osteochondrosis was observed in all birds, some of them with different degrees of subluxation on the free thoracic vertebra.

Stalker *et al.* (2010) reported the occurrence of concomitant osteomyelitis and arthritis, The arthritis was characterized by severe inflammation with heterophilic infiltration into the synovium and tendon sheaths of tibiotarsus-metatarsus joints. According to Craig *et al.* (2016), suppurative arthritis is characterized by greater amount of heterophils in the synovial fluid and membrane, and occasionally in adjacent structures. When the etiologic agent is a bacterium, heterophils are usually abundant and may be degenerated, which is frequently considered a septic arthritis. Most of young broilers with septic arthritis of hematogenous origin may also have osteomyelitis, possibly to the concomitant localization of the microorganism in the bone and synovial membrane, or a result of the close vascular relationship between epiphyseal bone and synovial membrane in young animals, with the spread of infection from one location to another. Foci of osteomyelitis originating in endochondral ossification sites of epiphysis below the articular cartilage may penetrate the cartilage, spreading the infection directly into the synovial fluid. In the joints that the capsule is inserted beyond the growth plate, inflammatory foci in the metaphysis can contaminate the synovial fluid by penetration in the cortical region near to the growth plate order. This region is relatively porous in young animals due to the intensive structural remodeling that occurs in the cortex of the metaphysis during rapid growth.

Diagnosis

Vertebral osteomyelitis may be suspected in birds presenting signs of sitting on the hocks (McNamee and Smyth, 2000). For the macroscopic diagnosis, lungs and kidneys must be removed to provide the visualization and careful examination of the vertebral column. A sagittal section of vertebral column should be performed in order to allow the evaluation of the vertebral body and the degree of spinal cord compression (Gingerich, 2009, Braga *et al.*, 2016c).

The differential diagnosis of vertebral osteomyelitis includes other pathologies that may cause spinal cord compression or changes in nerves with impaired mobility. One of these conditions is spondylolisthesis ("kinky back"), characterized by subluxation of the free thoracic vertebrae (Armour *et al.*, 2011; Robbins *et al.*, 2012). Grossly, these cases shows varying extents of ventral dislocation of the 4^{th} thoracic vertebra, whose posterior end raises the 5^{th} thoracic vertebra. The dislocation can produce kyphotic angulation of the spinal canal and varying degrees of spinal cord compression (Fig. 3c). Necrotic and inflammatory lesions of the vertebral body in broilers with spondylolisthesis may not be present (Dinev, 2012). The scoliosis characterized by lateral deviation of the spine (Fig. 3d) should also be considered for differential diagnosis of the conditions aforementioned (Droual *et al.*, 1991). Proper monitoring of the flock may help in the early detection of these conditions, facilitating their diagnosis (Gingerich, 2009). Some birds with paralytic or the neurological form of Marek's disease may present clinical signs similar to vertebral osteomyelitis and should be among the differential

diagnoses. In Marek's disease, no changes in the vertebral column are observed, but in the peripheral nerves, which become yellow-gray with loss of striations, acquiring an edematous appearance in some cases (Schat and Nair, 2008).

Prevention, treatment and control

Information on prevention, treatment and control of the disease are limited, in view that the origin and pathogenesis of vertebral osteomyelitis remain unclear, and most studies are related to infections caused by *E. cecorum* (Kense and Landman, 2011). For the prevention of vertebral osteomyelitis, recommendations on management practices have been made to reduce the risk of developing the disease, such as: 1) avoiding excessive food restriction; 2) following the suggested weight gain patterns and nutritional recommendations; 3) promoting adequate control of coccidiosis; 4) avoiding high density of poultry; 5) ensuring adequate access to feeders; and 6) preventing respiratory diseases. All practices to prevent bacterial infections that could produce bacteremia would probably help to avoid bone and articular inflammation.

Antibiotics have been used to treat the bacterial infection in vertebral osteomyelitis. Although several antibiotics have shown efficacy against the commonly described bacteria, the difficulty is to achieve adequate concentrations of the antibiotics in the vertebral column. In the reported outbreaks of the disease, antibiotics have been ineffective in reducing mortality possibly due to antimicrobial resistance of *E. cecorum* or the inability of the antibiotic to effectively penetrate the anatomical areas where the bacteria is located (Kense and Landman, 2011). The antimicrobial susceptibility profiles of *E. cecorum* isolated from outbreaks in different countries were similar (Herdt *et al.*, 2009; Aitchison *et al.*, 2014). Aitchison *et al.* (2014) reported that, after isolation and identification of *E. cecorum*, it was difficult to perform the antibiotic susceptibility test due to the growth conditions. The test was performed on tryptose blood agar and nevertheless the bacteria showed poor growth. Makrai *et al.* (2011) reported that, after the onset of the outbreak, broilers showing clinical signs were separated from those clinically normal and the clinically normal were treated with different antibiotics (amoxycillin, amoxycillin with clavulanic acid, lincomycin or doxycycline), resulting in no new clinical case of the disease in the flock.

After the occurrence of the disease, the elimination of subsequent cases will require repeated cycles of disinfection and usually would not occur after a single cleaning and disinfection. Increased efforts in subsequent flocks are required to eliminate the disease. Some practices that can reduce the risk of vertebral osteomyelitis in the subsequent flocks include: 1) emptying and completely disinfecting the aviary; 2) changing or composting the litter bed; 3) adequate cleaning of water lines; and 4) continuously sanitizing the water (Gingerich, 2009; Stalker *et al.*, 2010; Armour *et al.*, 2011; Martin *et al.*, 2011).

Antimicrobial resistance and public health

As previously mentioned, Enterococci are normal bacteria in the gastrointestinal tract of animals and humans, often seen as beneficial commensal organisms (Tannock, 1995). However, they may also be opportunistic pathogens, responsible for serious systemic infections and spread of antimicrobial resistance and virulence determinants (Wisplinghoff *et al.*, 2004; Heuer *et al.*, 2006). In recent years, Enterococci have emerged as a major cause of nosocomial infections, particularly *E. faecalis* (Kola *et al.*, 2010), causing extraintestinal infections in humans (Creti *et al.*, 2004). These bacteria have intrinsic resistance to many antibiotics and have acquired new

resistance genotypes, with special concern on vancomycin-resistant Enterococci (VRE) (Cetinkaya *et al.*, 2000; Willems and Bonten, 2007). The VRE have become a major problem in nosocomial infections. A retrospective study of 10 human patients with osteomyelitis showed that eight of these cases were due to infection by *Enterococcus faecalis* resistant to vancomycin with one death reported due to bacteremia (Holtom *et al.*, 2002).

It is interesting to note that, generally, antibiotics target basic bacterial physiology and biochemistry, causing cell death or inhibiting its growth. Bacterial targets that are different or nonexistent in eukaryotic cells (including human) are: bacterial cell wall; cell membrane; protein synthesis; DNA and RNA synthesis; and metabolism of folic acid (vitamin B9) (Fig. 4). Some examples are β-lactams, such as penicillins, cephalosporins and carbapenases that block the synthesis of the cell wall that is essential for bacterial survival. Furthermore, bacterial ribosomes are the target of tetracyclines, aminoglycosides, macrolides and other antibiotics (Wright, 2010).

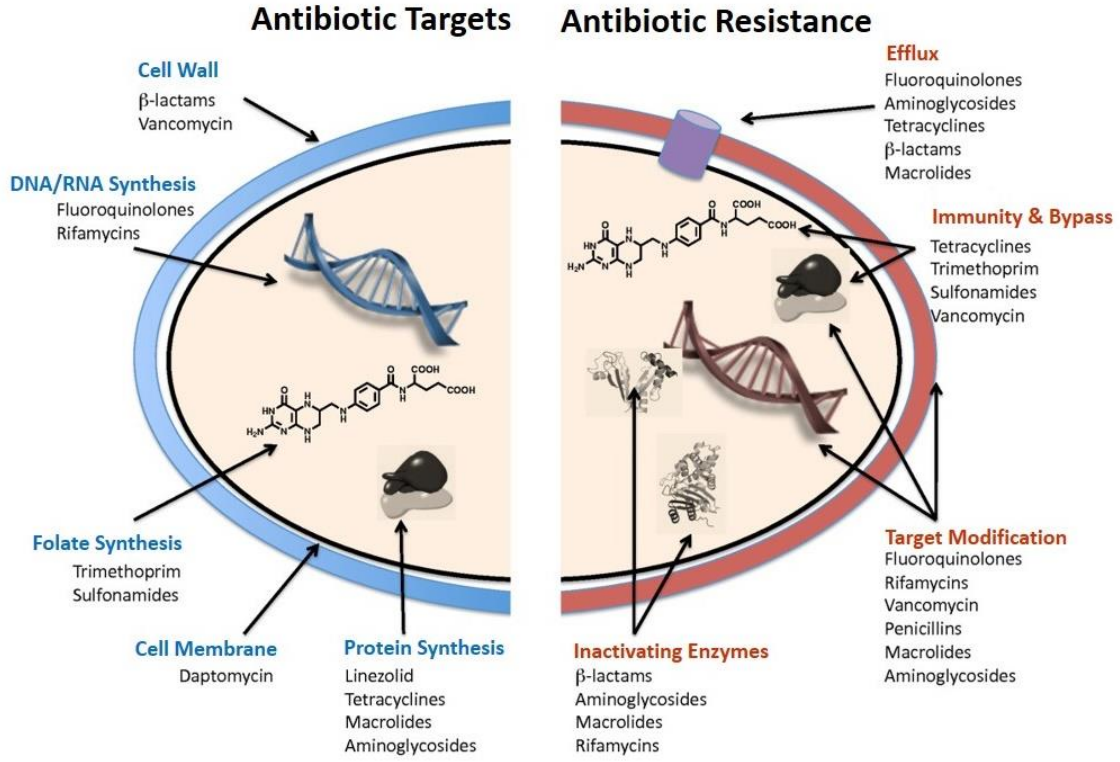


Figure 4. Antibiotic targets and mechanisms of resistance in bacteria (Adapted from Wright, 2010).

On the other hand, bacteria can display antibiotic resistance using four general mechanisms: 1) target modification; 2) efflux; 3) immunity and bypass; and 4) inactivating enzymes (Fig. 4). The target modification occurs by mutation of the genetic code of the targets (e.g. topoisomerases which are the target for fluoroquinolones antibiotics) or the production of enzymes that modify the antibiotic targets. Resistance to vancomycin, which represents a major concern in Enterococci, is version of the target modification where new biosynthetic machinery is engaged in changing the cell wall structure. Efflux occurs through a large family of pump proteins that eject the antibiotic from the interior to the exterior of the bacterial cell. Considering bacterial immunity, antibiotics or their targets are linked to proteins that prevent the connection to its target. One of the most specific mechanism involved in antibiotic resistance is given by

enzymes that recognize and modify antibiotics, resulting in the elimination of the functional characteristics that allow the interaction with their targets (e.g., β-lactamase cleaves the central β-lactam ring, which is characteristic of the class and essential for antibiotic activity) (Wright, 2010).

Resistant bacteria in animals and their by-products and the possible transmission to humans through contamination of carcasses represent a concern in animal and public health (Moreno *et al.*, 2006). Enterococci contaminate not only raw meat, but may also be associated with processed meat products, such as fermented raw sausages or cooked products (Martin *et al.*, 2005; Barbosa *et al.*, 2009; Ruiz-Moyano *et al.*, 2009). Although there is no description of food intoxication in humans associated with *E. faecalis*, a recent study performed in Brazil showed the presence of these bacteria in 42% of chicken carcasses tested. All these strains were resistant to at least one antibiotic tested, with detection of the antimicrobial resistance genes *erm(B), vanC-1, aph(3')-llla, ant(6)-la, vanB, vanA, aac(6')-le-aph(2'')-la, erm(A)*e *tet(M)*. This highlights the role of *E. faecalis* in public health, once these microorganisms may have the ability to transmit antimicrobial resistance genes to other organisms present in the intestinal tract of humans and animals, resulting in limited use of these drugs for clinical treatments (Campos *et al.*, 2013). Hayes *et al.* (2003) analyzed 981 raw meat samples available commercially from various species (chicken, turkey, swine and bovine) and isolated 1,357 *Enterococcus* spp. strains, which included *E. faecalis* (29%) and *E. hirae* (5.7%). These authors also detected high level of gentamicin resistance in 4% of the strains, most of them isolated from chicken meat. Braga *et al.* (2016b), analyzing *E. faecalis* isolates from vertebral osteomyelitis in broilers, demonstrated that the highest level of antibiotic resistance was for aminoglycosides, mainly gentamicin (40%).

E. coli strains also have a major importance because of their role in public health. Most serotypes of the bacterium isolated from chickens are pathogenic only for avian species and will not cause infection in humans or in other mammals (Meno *et al.*, 2002). However, some *E. coli* strains isolated from poultry lesions have genetic similarities to those that cause diseases in humans, a close relationship subject of research as may constitute a risk to the consumer health (Andrade, 2005). A few studies have suggested the possibility of APEC be related to extraintestinal infections in humans (Ewers *et al.*, 2007; Johnson *et al.*, 2007).

The multiple antimicrobial resistance characteristics of APEC strains also show genetic diversity of isolates, which are often resistant to the following antibiotics: tetracycline, chloramphenicol, sulfonamides, aminoglycosides, fluoroquinolones, β-lactam and extended spectrum β-lactam (Mellata, 2013; Braga *et al.*, 2016a). Genes encoding resistances are often located in the large transmissible plasmids R (Koh and Kok, 1984). It is not surprising that multidrug-resistant APEC often carry conjugative plasmids (Caudry and Stanisich, 1979). In addition, ColV plasmids are often found in APEC strains and seem linked to virulence (Johnson *et al.*, 2006; Johnson *et al.*, 2008). Plasmids can serve as a reservoir of antimicrobial resistance genes and are horizontally transferable to the same and other species of bacteria of potential risk to human health (Johnson *et al.*, 2005).

It worth to note that there are two general strategies for the acquisition of resistance. One comprises mechanisms that transfer resistance vertically from one bacterium to their offspring, such as mutations in chromosomal genes which give rise to products insensible to drugs, such as point mutations in genes encoding DNA gyrase and topoisomerase IV resulting in resistance to fluoroquinolones antibiotics, such as ciprofloxacin. The second strategy includes actions of genes located on mobile genetic elements, such as plasmids, that can be vertically or horizontally transmitted to other bacteria, even those of different genera (Wright, 2010). Acquisition of new genetic material by antimicrobial-susceptible bacteria from resistant strains of bacteria may occur through conjugation, transformation, or transduction, with transposons

often facilitating the incorporation of the multiple resistance genes into the genome or plasmids (Tenover, 2006).

Conclusions

Vertebral osteomyelitis is an emerging disease demanding a diversity of studies for its understanding. Many aspects on the etiology and pathogenesis of the disease remain unclear, which limits the knowledge on its prevention and control. Most reports associate the disease to infection by *Enterococcus cecorum*, probably emerging clones with higher pathogenicity. However, other Enterococci and *Escherichia coli* have been isolated from vertebral osteomyelitis in broilers, raising questions on the role of any specific bacterium in the development of the disease, once its occurrence is related to meat type chicken. Many of the bacteria isolated from cases of the disease are often multidrug-resistant and the possible transmission of these bacteria or their antibiotic resistance encoding genes are a major concern for animal and public health.

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References

Abe, Y.; Nakamura, K.; Yamada, M.; Yamamoto, Y., 2006. Encephalomalacia with *Enterococcus durans* infection in the brain stem and cerebral hemisphere in chicks in Japan. Avian. Dis. 50, 139-41.

Aitchison, H.; Poolman, P.; Coetzer, M.; Griffiths, C.; Jacobs, J.; Meyer, M.; Bisschop, S., 2014. Enterococcal-related vertebral osteoarthritis in South African broiler breeders: A case report. J. S. Afr. Vet. Assoc. 85, 01-05.

Ambrozic, J.; Ostroversnik, A.; Starcic, M.; Kuhar, I; Grabnar, M; Zgur-Bertok, D., 1998. *Escherichia coli* CoIV plasmid pRK100: genetic organization, stability and conjugal transfer. Microbiol. 144, 343–352.

Andrade, C. L., 2005. Histopatologia e identificação da *Escherichia coli* como agente causal da celulite aviária em frangos de corte. Dissertação de Mestrado. Universidade Federal Fluminense. 62p.

Armour, N.K.; Collet, S.R.; Williams, S.M., 2011. *Enterococcus cecorum*-related arthritis and osteomyelitis in broilers and broiler breeders. Poult. Inform. Profession. 117, 1–7.

Aziz, T.; Barnes, H.J., 2007. Is spondylitis an emerging disease of broilers? World Poult. 23, 44–45.

Aziz, T.; Barnes, H.J., 2009. Spondylitis is emerging in broilers. World Poult. 25, 14.

Barbosa, J.; Ferreira, V.; Teixeira, P., 2009. Antibiotic susceptibility of Enterococci isolated from traditional fermented meat products. Food Microbiol. 26, 527–532.

Barnes, H.J.; Vaillancourt, J.P.; Gross, W.B., 2008. Colibacillosis. In: Saif, Y.M. (Ed.), Diseases of Poultry, Blackwell Publishing, Ames (Iowa), pp. 631-656.

Bayyari, G.R.; Huff, W.E.; Rath, N.C.; Balog, J.M.; Newberry, L.A.; Villines, J.D.; Skeeles, J.K., 1997. Immune and physiological responses of turkeys with green-liver osteomyelitis complex. Poult. Sci. 76, 280-288.

Bergmann, V.; Heider, G.; Vogel, K., 1980. Mycotic spondylitis as a cause of locomotor disorders in broiler chicken. Monatshefte fur Veterinarmedizin 35, 349-351.

Bisgaard, M., 1981. Arthritis in ducks: aetiology and public health aspects. Avian Pathol. 10, 1121.

Blanco, J.E.; Blanco, M.; Mora, A.; Jansen, W.H.; García, V.; Vázquez, M.L.; Blanco, J., 1998. Serotypes of *Escherichia coli* isolated from septicaemic chickens in Galicia (northwest Spain). Vet. Microbiol. 61, 229–235.

Braga, J.F.V.; Chanteloup, N.K.; Trotereau, A.; Baucheron, S.; Guabiraba, R.; Ecco, R.; Schouler, C., 2016a. Diversity of *Escherichia coli* strains involved in vertebral osteomyelitis and arthritis in broilers in Brazil. BMC Vet. Res. (Article in process).

Braga, J.F.V.; Leal, C.A.G.; Silva, C.C.; Fernandes, A.A.; Martins, N.R.S.; Ecco, R., 2016b. Molecular characterization and antibiotic susceptibility of *Enterococcus faecalis* isolated from vertebral osteomyelitis in broilers in Brazil. Vet. Res. Commun. (Article in process).

Braga, J.F.V.; Silva, C.C.; Teixeira, M.P.F.; Martins, N.R.S.; Ecco, R., 2016c. Vertebral osteomyelitis associated with single and mixed bacterial infection in broilers. Avian Path. In press.

Boerlin, P.; Nicholson, V.; Brash, M., Slavic, D.; Boyen, F.; Sanei, B.; Butaye, P., 2012. Diversity of *Enterococcus cecorum* from chickens. Vet. Microbiol. 157, 405–411.

Borst, L.B.; Suyemoto, M.M.; Robbins, K.M.; Lyman, R.L.; Martin, M.P.; Barnes, H.J., 2012. Molecular epidemiology of *Enterococcus cecorum* isolates recovered from enterococcal spondylitis outbreaks in the southeastern United States. Avian Pathol. 41, 479-485.

Borst, L.B.; Suyemoto, M.M.; Keelara, S.; Dunningan, S.E.; Guy, J.S.; Barnes, H.J., 2014. A chicken embryo lethality assay for pathogenic *Enterococcus cecorum*. Avian Dis. 58, 244-248.

Campos, A.C.F.B.; Souza, N.R., Silva, P.H.C.; Santana, A.P., 2013. Resistência antimicrobiana em *Enterococcus faecalis* e *Enterococcus faecium* isolados de carcaças de frango. Pesq. Vet. Bras. 33, 575-580.

Cardona, C.J.; Bickford, A.A.; Charlton, B.R.; Cooper, G.L., 1993. *Enterococcus durans* infection in young chickens associated with bacteremia and encephalomalacia. Avian Dis. 37, 234-239.

Carnaghan, R.B.A., 1966. Spinal cord compression in fowls due to spondylitis caused by *Staphylococcus pyogenes*. J. Comp. Pathol. 76, 9-14.

Caudry, S.D.; Stanisich, V.A., 1979. Incidence of antibiotic-resistant *Escherichia coli* associated with frozen chicken carcasses and characterization of conjugative R plasmids derived from such strains. Antimicrob. Agents Ch. 16, 701-709.

Cetinkaya, Y.; Falk, P.; Mayhall, C.G., 2000. Vancomycin-resistant Enterococci. Clin. Microbiol. Rev. 13, 686-707.

Chadfield, M.S.; Christensen, J.P.; Christensen, H.; Bisgaard, M., 2004. Characterization of streptococci and Enterococci associated with septicaemia in broiler parents with a high prevalence of endocarditis. Avian Pathol. 33, 610-617.

Cobb, 2015. Cobb500: Broiler Performance & Nutrition Supplement. Disponível em:<http://www.cobb-vantress.com/docs/default-source/cobb-500-guides/Cobb500_Broiler_Performance_And_Nutrition_Supplement.pdf> Consultado em:11/01/2016.

Craig, L.E.; Dittmer, K.E.; Thompson, K.G., 2016. Bones and Joints. In: Maxie, M.G. (ed.), Jubb, Kennedy, and Palmer's Pathology of Domestic Animals. 6th ed., vol.1, St Louis, MO: Elsevier, pp.16-163.

Creti, R.; Imperi, M.; Bertuccini, L.; Fabretti, F.; Orefici, G.; Di Rosa, R.; Baldassarri, L., 2004. Survey for virulence determinants among *Enterococcus faecalis* isolated from different sources. J. Med. Microbiol. 53, 13–20.

Da Silveira, W.D.; Ferreira, A.; Lancellotti, M.; Barbosa, I.A.; Leite, D.S.; de Castro, A.F.; Brocchi, M., 2002. Clonal relationships among avian *Escherichia coli* isolates determined by enterobacterial repetitive intergenic consensus (ERIC)-PCR. Vet. Microbiol. 89, 323–328.

Deeming, D.C., 2005. Yolk sac, body dimensions and hatchling quality of ducklings, chicks and poults. Brit. Poult. Sci. 46, 560-564.

Devriese, L.A.; Cauwerts, K.; Hermans, K.; Wood, A.M., 2002. *Enterococcus cecorum* septicemia as a cause of bone and joint lesions resulting in lameness in broiler chickens. Vlaams Diergen. Tijds. 71, 219–221.

Devriese, L.A.; Hommez, J.; Wijfels, R.; Haesebrouck, F., 1991. Composition of the enterococcal and streptococcal intestinal flora of poultry. J. Appl. Bacteriol. 71, 46–50.

Dho-Moulin, M.; Fairbrother, J.M., 1999. Avian pathogenic *Escherichia coli* (APEC). Vet. Res. 30, 299-316.

Dinev, I., 2009. Clinical and morphological investigations on the prevalence of lameness associated with femoral head necrosis in broilers. Brit. Poult. Sci. 50, 284–290.

Dinev, I., 2012. Pathomorphological investigations on the incidence of clinical spondylolisthesis (kinky back) in different commercial broiler strains. Revue Méd. Vét. 163, 511-515.

Dinev, I., 2013. Pathomorphological investigations on the incidence of axial skeleton pathology associated with posterior paralysis in commercial broiler chickens. Poult. Sci. 50, 283-289.

Droual, R.; Bickford, A.A.; Farver, T.B., 1991. Scoliosis and tibiotarsal deformities in broiler chickens. Avian Dis. 35, 23-30.

Droual, R.; Chin, R.P.; Rezvani, M., 1996. Synovitis, osteomyelitis, and green liver in turkeys associated with *Escherichia coli*. Avian Dis. 40, 417-424.

Dziva, F.; Hauser, H.; Connor, T.R.; van Diemen, P.M.; Prescott, G.; Langridge, G.C.; Eckert, S.; Chaudhuri, R.R.; Ewers, C.; Mellata, M.; Mukhopadhyay, S.; Curtiss, R. 3rd.; Dougan, G.; Wieler, L.H.; Thomson, N.R.; Pickard, D.J.; Stevens, M.P., 2013. Sequencing and functional annotation of avian pathogenic *Escherichia coli* serogroup O78 strains reveal the evolution of *E. coli* lineages pathogenic for poultry via distinct mechanisms. Infect. Immun. 81, 838–849.

Dziva, F.; Stevens, M.P., 2008. Colibacillosis in poultry: unraveling the molecular basis of virulence of avian pathogenic *Escherichia coli* in their natural hosts. Avian Pathol. 37, 355–366.

Ewers, C.; Janssen, T.; Kiessling, S.; Philipp, H.C.; Wieler, L.H., 2004. Molecular epidemiology of avian pathogenic *Escherichia coli* (APEC) isolated from colisepticemia in poultry. Vet. Microbiol. 104, 91–101.

Ewers, C.; Li, G; Wilking, H.; Kiessling, S.; Alt, K.; Antão, E.M.; Laturnus, C.; Diehl, I.; Glodde, S.; Homeier, T.; Böhnke, U.; Steinrück, H.; Philipp, H.C.; Wieler, L.H., 2007. Avian pathogenic, uropathogenic, and newborn meningitis-causing *Escherichia coli*: how closely related are they? Int. J. Med. Microbiol. 297, 163-176.

Franz, C.M.A.P.; Huch, M.; Abriouel, H.; Holzapfel, W.; Gálvez, A., 2011. Enterococci as probiotics and their implications in food safety. Int. J. Food Microbiol 151, 125–140.

Gingerich, E.N.; Barnes, J.H.; Owen, R.L.; Rankin, S.C., 2009. Spinal abscesses due to *Enterococcus cecorum* in broiler chickens: an emerging disease? American Association of Avian Pathologists Conference, Seattle. Proceedings.

Gregersen, R.H.; Petersen, A.; Christensen, H.; Bisgaard, M., 2010. Multilocus sequence typing of *Enterococcus faecalis* isolates demonstrating different lesion types in broiler breeders, Avian Pathol. 39, 435-440.

Guabiraba, R.; Schouler, C., 2015. Avian colibacillosis: still many black holes. FEMS Microbiol. Lett. 362, 1–8.

Hayes, J.R.; English, L.L.; Carter, P.J.; Proescholdt, T.; Lee, K.Y.; Wagner, D.D.; White, D.G., 2003. Prevalence and antimicrobial resistance of enterococcus species isolated from retail meats. Appl. Environ. Microbiol. 69, 7153-7160.

Herdt, P.; Defoort, P.; Van Steelant, J.; Swam, H.; Tanghe, L.; Van Goethem, S.; Vanrobaeys, M., 2009. *Enterococcus cecorum* osteomyelitis and arthritis in broiler chickens. Vlaams Diergen. Tijds. 78, 44–48.

Hernandez, D.J.; Roberts, E.D.; Adams, L.G.; Vera, T., 1972. Pathogenesis of hepatic granulomas in turkeys infected with *Streptococcus faecalis* var. *liquefaciens*. Avian Dis. 16, 201-216.

Herzer, P.J.; Inouye, S.; Inouye, M.; Whittam, T.S., 1990. Phylogenetic distribution of branched RNA-linked multicopy single-stranded DNA among natural isolates of *Escherichia coli*. J. Bacteriol. 172, 6175–6181.

Heuer, O.E.; Hammerum, A.M.; Collignon, P.; Wegener, H.C., 2006. Human health hazard from antimicrobial-resistant Enterococci in animals and food. Clin. Infect. Dis. 43, 911-916.

Hogg, D.A., 1984. The distribution of pneumatisation in the skeleton of the adult domestic fowl. J. Anat. 138, 617-629.

Holtom, P.D.; Zamorano, D.; Patzakis, M.J., 2002. Osteomyelitis attributable to vancomycin-resistant Enterococci. Clin. Orthop. Relat. Res. 403, 38-44.

Huff, G.R.; Huff, W.E.; Rath, N.C.; Balog, J.M., 2000. Turkey osteomyelitis complex. Poult. Sci. 79, 1050-1056.

Huja, S.; Oren, Y.; Trost, E.; Brzuszkiewicz, E.; Biran, D.; Blom, J.; Goesmann, A.; Gottschalk, G.; Hacker, J.; Ron, E.Z.; Dobrindt, U., 2015. Genomic avenue to avian colisepticemia. mBio 6(1):e01681-14.

Johnson, T.J.; Kariyawasam, S.; Wannemuehler, Y.; Mangiamele, P.; Johnson, S.J.; Doetkott, C.; Skyberg, J.A.; Lynne, A.M.; Johnson, J.R.; Nolan, L.K., 2007. The genome sequence of avian pathogenic *Escherichia coli* strain O1:K1:H7 shares strong similarities with human extraintestinal pathogenic *E. coli* genomes. J. Bacteriol. 189, 3228-3236.

Johnson, J.R.; Kuskowski, M.A.; Smith, K.; O'Bryan, T.T.; Tatini, S., 2005. Antimicrobial resistant and extraintestinal pathogenic *Escherichia coli* in retail foods. J. Infect. Dis. 191, 1040-1049.

Johnson, T.J.; Siek, K.E.; Johnson, S.J.; Nolan, L.K., 2006. DNA sequence of a colV plasmid and prevalence of selected plasmid-encoded virulence genes among avian *Escherichia coli* strains. J. Bacteriol. 188, 745–758.

Johnson, T.J.; Wannemuehler, Y.; Doetkott, C.; Johnson, S.J. Rosenberger, S.C.; Nolan, L.K., 2008. Identification of minimal predictors of avian pathogenic *Escherichia coli* virulence for use as a rapid diagnostic tool. J. Clin. Microbiol. 46, 3987–3996.

Jung, A.; Metzner, M.; Köhler-Repp, D.; Rautenschlein, S., 2013. Experimental reproduction of an *Enterococcus cecorum* infection in Pekin ducks. Avian Pathol. 42, 552-556.

Jung, A.; Rautenschlein, S., 2014. Comprehensive report of an *Enterococcus cecorum* infection in a broiler flock in Northern Germany. BMC Vet. Res. 10, 311.

Kense, M.J.; Landman, W.J.M., 2011. *Enterococcus cecorum* infections in broiler breeders and their offspring: molecular epidemiology. Avian Pathol. 40, 603–612.

King, A.S., 1957. The aerated bones of *Gallus domesticus*. Acta Anat. 31, 220–230.

Koh, C.L.; Kok, C.H., 1984. Antimicrobial resistance and conjugative R plasmids in *Escherichia coli* strains isolated from animals in peninsular Malaysia. Southeast Asian Trop. Med. Public Health 1, 37-43.

Kola, A.; Schwab, F.; Barwolff, S.; Eckmanns, T.; Weist, K.; Dinger, E.; Klare, I.; Witte, W.; Ruden, H.; Gastmeier, P., 2010. Is there an association between nosocomial infection rates and bacterial cross transmissions? Crit. Care Med. 38, 46–50.

Kolbjørnsen, Ø.; David, B.; Gilhuus, M., 2011. Bacterial osteomyelitis in a 3-week-old broiler chicken associated with *Enterococcus hirae*. Vet. Pathol. 48, 1134-1137.

Kondo, H.; Abe, N.; Tsukuda, K.; Wada, Y., 1997. Adherence of *Enterococcus hirae* to the duodenal epithelium of chicks with diarrhoea. Avian Pathol. 26, 189-194.

La Ragione, R.M.; Woodward, M.J., 2002. Virulence factors of *Escherichia coli* serotypes associated with avian colisepticaemia. Res. Vet. Sci. 73, 27–35.

Landman, W.J.M.; Gruys, E.; Dwars, R.M., 1994. A syndrome associated with growth depression and amyloid arthropathy in layers: a preliminary report. Avian Pathol. 23, 461-470.

Landman, W.J.M.; Veldman, K.T.; Mevius, D.J.; van Eck, J.H., 2003. Investigations of *Enterococcus faecalis*-induced bacteraemia in brown layer pullets through different inoculation routes in relation to the production of arthritis. Avian Pathol. 32, 463–471.

Makrai, L.; Nemes, C.; Simon, A.; Ivanics, E.; Dudás, Z.; Fodor, L.; Glávits, R., 2011. Association of *Enterococcus cecorum* with vertebral osteomyelitis and spondylolisthesis in broiler parent chicks. Acta Vet. Hung. 59, 11–21.

Mangiamele, P.; Nicholson, B.; Wannemuehler, Y.; Seemann, T.; Logue, C.M.; Li, G.; Tivendale, K.A.; Nolan, L.K., 2013. Complete genome sequence of the avian pathogenic *Escherichia coli* strain APEC O78. Genome Announc. 1(2):e0002613.

Martin, B.; Garriga, M.; Hugas, M.; Aymerich, T., 2005. Genetic diversity and safety aspects of Enterococci from slightly fermented sausages. J. Appl. Microbiol. 98, 1177–1190.

Martin, L.T.; Martin, M.P.; Barnes, H.J., 2011. Experimental reproduction of Enterococcal spondylitis in male broiler breeder chickens. Avian Dis. 55, 273–278.

McNamee, P.T.; Smyth, J.A., 2000. Bacterial chondronecrosis with osteomyelitis ("femoral head necrosis") of broiler chickens: a review. Avian Pathol. 29, 253–270.

Mellata, M., 2013. Human and avian extraintestinal pathogenic *Escherichia coli*: infections, zoonotic risks, and antibiotic resistance trends. Foodborne Pathog. Dis. 10, 916–932.

Menão, M.C.; Ferrreira, C.S.A. Castro, A.G.M.; Knöbi, T.; Ferreira, A.J.P., 2002. Sorogrupos e *Escherichia coli* isolados de frangos com doença respiratória crônica. Arq. Inst. Biológico 69, 15-17.

Moreno, M.R.F.; Sarantinopoulos, P.; Tsakalidou, E.; De Vuyst, L., 2006. The role and application of Enterococci in food and health. Int. J. Food. Microbiol. 106, 1-24.

Mutalib, A.; Miguel, B.; Brown, T.; Maslin, W., 1996. Distribution of arthritis and osteomyelitis in turkeys with green liver discoloration. Avian Dis. 40, 661-664.

Nairn, M.E., 1973. Bacterial osteomyelitis and synovitis of the turkeys. Avian Dis. 17, 504-517.

Nakazato, G.; Campos, T.A.; Stehling, E.G.; Brocchi, M.; da Silveira, W.D., 2009. Virulence factors of avian pathogenic *Escherichia coli* (APEC). Pesq Vet Bras 29, 479-486.

Ngeleka, M.; Kwaga, J.K.; White, D.G.; Whittam, T.S.; Riddell, C.; Goodhope, R.; Potter, A.A.; Allan, B., 1996. *Escherichia coli* cellulitis in broiler chickens: clonal relationships among strains and analysis of virulence-associated factors of isolates from diseased birds. Infect Immun 64, 3118-3126.

Ochman, H.; Selander, R.K., 1984. Standard reference strains of *Escherichia coli* from natural populations. J. Bacteriol. 157, 690–693.

Palmer, K.L.; Godfrey, P.; Griggs, A.; Kos, V.N.; Zucker, J.; Desjardins, C.; Cerqueira, G.; Gevers, D.; Walker, S.; Wortman, J.; Feldgarden, M.; Haas, B.; Birren, B.; Gilmorea, M.S., 2012. Comparative genomics of Enterococci: variation in *Enterococcus faecalis*, clade structure in *E. faecium*, and defining characteristics of *E. gallinarum* and *E. casseliflavus*. MBio. 3, e00318-11.

Perez, S., 2004. Spinal cord lesions in broiler chickens. Vet. Rec. 155, 536.

Randall, C.J.; Wood, A.M.; MacKenzie, G., 1993. Encephalomalacia in first-week chicks. Vet. Rec. 132, 419.

Rasheed, B.Y., 2011. Isolation and identification of bacteria causing arthritis in chickens. Iraqi J. Vet. Sci. 25, 93-95.

Rasko, D.A.; Rosovitz, M.J.; Myers, G.S.; Mongodin, E.F.; Fricke, W.F.; Gajer, P.; Crabtree, J.; Sebaihia, M.; Thomson, N.R.; Chaudhuri, R.; Henderson, I.R.; Sperandio, V.; Ravel, J., 2008. The pangenome structure of *Escherichia coli*: comparative genomic analysis of *E. coli* commensal and pathogenic isolates. J. Bacteriol. 190, 6881-6893.

Riddell, C.; Topp, R., 1972. Mycotic spondylitis involving the first thoracic vertebra in chickens. Avian Dis. 16, 1118-1122.

Robbins, K.M.; Suyemoto, M.M.; Lyman, R.L.; Martin, M.P.; Barnes, H.J.; Borst, L.B., 2012. An outbreak and source investigation of enterococcal spondylitis in broilers caused by *Enterococcus cecorum*. Avian Dis. 56, 768-773.

Rodriguez-Siek, K.E.; Giddings, C.W.; Doetkott, C.; Johnson, T.J.; Fakhr, M.K.; Nolan, L.K., 2005a. Comparison of *Escherichia coli* isolates implicated in human urinary tract infection and avian colibacillosis. Microbiol 151, 2097–2110.

Rodriguez-Siek, K.E.; Giddings, C.W.; Doetkott, C.; Johnson, T.J.; Nolan, L.K., 2005b. Characterizing the APEC pathotype. Vet Res 36, 241–256.

Ruiz-Moyano, S.; Martin, A.; Benito, M.J.; Aranda, E.; Casquette, R.; Cordoba, G., 2009. Safety and functional aspects of preselected Enterococci for probiotic use in Iberian dry-fermented sausages. J. Food Sci. 74, M398–404.

Sandhu, T.S., 1988. Fecal streptococcal infection of commercial white Pekin ducklings. Avian Dis. 32, 570-573.

Schat, K.A.; Nair, V., 2008. Marek´s Disease. In: Saif, Y.M.; Fadly, A.M.; Glisson, J.R.; McDougald, L.R.; Nolan, L.K.; Swayne, D.E. (Eds.), Diseases of Poultry, 12th ed., Ames, IA: Blackwell Publishing, pp.452-514.

Schouler, C.; Koffmann, F.; Amory, C.; Leroy-Sétrin, S.; Moulin-Schouleur, M., 2004. Genomic subtraction for the identification of putative new virulence factors of an avian pathogenic *Escherichia coli* strain of O2 serogroup. Microbiol 150, 2973–2984.

Schouler, C.; Schaeffer, B.; Brée, A.; Mora, A.; Dahbi, G.; Biet, F.; Oswald, E.; Mainil, J.; Blanco, J.; Moulin-Schouleur, M., 2012. Diagnostic strategy for identifying avian pathogenic *Escherichia coli* based on four patterns of virulence genes. J. Clin. Microbiol. 50, 1673–1678.

Selander, R.K.; Caugant, D.; Whittam, T.S., 1987. Genetic structure and variation in natural populations of *Escherichia coli*. In: Neidhardt, F.C.; Ingraham, J.L.; Low, K.B. et al. (Eds.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. Washington, D.C.: American Society for Microbiology, pp. 1625–1648.

Stalker, M.J.; Brash, M.L.; Weisz, A.; Ouckama, R.M.; Slavic, D., 2010. Arthritis and osteomyelitis associated with *Enterococcus cecorum* infection in broiler and broiler breeder chickens in Ontario, Canada. J. Vet. Diagn. Invest. 22, 643–645.

Stashak, T.S.; Mayhew, I.G., 1984. The nervous system. In: Jennings, P.B.; Saunders, W.B. (Eds.), The practice of large animal surgery. Philadelphia: W.B. Saunders, pp.1013-1016.

Steentjes, A.; Veldman, K.T.; Mevius, D.J.; Landman, W.J.M., 2002. Molecular epidemiology of unilateral amyloid arthropathy in broiler breeders associated with *Enterococcus faecalis*. Avian Pathol. 31, 3139.

Tankson, J.D.; Thaxton, J.P.; Vizzier-Thaxton, Y., 2001. Pulmonary hypertension syndrome in broilers caused by *Enterococcus faecalis*. Infect. Immun. 69, 6318-6322.

Tankson, J.D.; Thaxton, J.P.; Vizzier-Thaxton, Y., 2002. Bacteria in heart and lungs of young chicks. J. Appl. Microbiol. 92, 443-450.

Tannock, G.W., 1995. Normal microflora: An introduction to microbes inhabiting the human body, 1st ed. London: Chapman and Hall, 116p.

Tenover, F.C., 2006. Mechanisms of antimicrobial resistance in bacteria. Americ. J. Med. 119(6A), S3–S10.

Thayer, S.G.; Waltman, W.D.; Wages, D.P., 2008. *Streptococcus* and *Enterococcus*. In: Saif, Y.M.; Fadly, A.M.; Glisson, J.R.; McDougald, L.R.; Nolan, L.K.; Swayne, D.E. (Eds.), Diseases of Poultry. 12th ed. Ames, Iowa: Blackwell Publishing, pp. 900–908.

Van Veen, L.; Dwars, R.M.; Fabri, T.H.F., 1999. Mycotic spondylitis in broilers caused by *Aspergillus fumigatus* resulting in partial anterior and posterior paralysis. Avian Pathol. 28, 487-490.

Velkers, F.C.; Graaf-Bloois, L.V.; Wagenaar, J.A.; Westendorp, S.T.; van Bergen, M.A.P.; Dwars, R.M.; Landman, W.J.M, 2011. *Enterococcus hirae*-associated endocarditis outbreaks in broiler flocks: clinical and pathological characteristics and molecular epidemiology. Vet. Quart. 31, 3-17.

Wages, D.P., 1998. Streptococcosis. In: Swayne, D.E.; Glisson, J.R.; Jackwood, M.W.; Person, J.E.; Reed, W.M. (Eds.), Isolation and identification of Avian Pathogens, 4th ed. American Association of Avian Pathologists: Kennett Square, PA, pp.58-60.

Wedel, M.J., 2008. Evidence for bird-like air sacs in Saurischian Dinosaurs. J. Exp. Zool. 311A:[1-18].

White, D.G.; Wilson, R.A.; Emery, D.A.; Nagaraja, K.V.; Whittam, T.S., 1993. Clonal diversity among strains of *Escherichia coli* incriminated in turkey colisepticemia. Vet Microbiol 34, 19–34.

White, D.G.; Wilson, R.A.; Gabriel, A.S.; Saco, M.; Whittam, T.S., 1990. Genetic relationships among strains of avian *Escherichia coli* associated with swollen-head syndrome. Infect Immun 58, 3613–3620.

Whittam, T.S.; Wilson, R.A., 1988. Genetic relationships among pathogenic strains of avian *Escherichia coli*. Infect Immun 56, 2458–2466.

Wideman, R.F.; Hamal, K.R.; Stark, J.M. et al. A wire-flooring model for inducing lameness in broilers: Evaluation of probiotics as a prophylactic treatment. Poult Sci, v.91, p.870–883, 2012.

Wideman, R.F.; Prisby, R.D. Bone circulatory disturbances in the development of spontaneous bacterial chondronecrosis with osteomyelitis: a translational model for the pathogenesis of femoral head necrosis. Front Endocrinol (Lausanne), v.3, p.1-14, 2013.

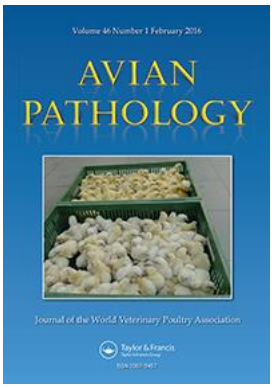
Willems, R.J.L.; Bonten, M.J.M., 2007. Glycopeptide-resistant Enterococci: deciphering virulence, resistance and epidemicity. Curr. Opin. Infect. Dis. 20, 384-390.

Wisplinghoff, H.; Bischoff, T.; Tallent, S.M.; Seifert, H.; Wenzel, R.P.; Edmond, M.B., 2004. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clin. Infect. Dis. 39, 309-317.

Wood, A.M.; Mackenzie, G.; Mcgillveray, N.C.; Brown, L.; Devriese, L.A.; Baele, M., 2002. Isolation of *Enterococcus cecorum* from bone lesions in broiler chickens. Vet. Rec. 150, 27.

Wright, G.D., 2010. Q&A: Antibiotic resistance: where does it come from and what can we do about it? BMC Biol 8:123.

CHAPTER II



Vertebral osteomyelitis associated with single and mixed bacterial infection in broilers

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Abstract: Vertebral osteomyelitis (VO) is a worldwide emerging disease that affects broilers. Once in Brazil, there are no studies concerning the frequency of VO, the objective of this study was to determine the frequency and etiology of VO in broilers in a highly productive broiler region in the country. For this, we analyzed 608 broilers with locomotor problems from 18 commercial farms, which had his clinical signs recorded and then euthanized for necropsy. Samples from vertebral body with gross changes were collected for molecular and histopathological analysis and bacterial isolation. From broilers with locomotor alteration, 5.1% (31/608) had vertebral osteomyelitis and, of these, 93.5% were 40 days-old or older and 89.7% were males. Broilers with VO had different degrees of limited mobility, which were related to the level of compression to the spinal cord. Bacteria of the genus *Enterococcus* spp. (DNA detected in 53.6%) were the etiological agents involved in most VO cases. *Enterococcus faecalis* was detected most frequently (35.7%), but *Enterococcus hirae* was also present in some lesions (7.1%). *Escherichia coli* was detected in 35.7% of vertebral lesions and co-infection with *Enterococcus faecalis* was confirmed in 7.1% cases. *Staphylococcus aureus* was involved in 14.3% of the cases, being 7.1% in co-infection with *Enterococcus* spp. or *Enterococcus hirae*. Our study showed that, in Brazil, VO in broilers may not be caused by a single infectious

agent. Also, has lower frequency compared to recent reports in other countries. These findings provide information regarding the disease in this country and suggest geographical differences, considering Brazil and other countries, concerning the frequency and etiology of the disease.

Keywords: broilers, vertebral osteomyelitis, *Enterococcus* species, *Escherichia coli,* molecular pathology, histopathology

Introduction

Vertebral osteomyelitis (VO) is a disease that affects broilers and broiler breeders worldwide (Devriese *et al.*, 2002; Wood *et al.*, 2002; de Herdt *et al.*, 2008; Aziz & Barnes, 2009; Gingerich *et al.*, 2009; Stalker *et al*., 2010; Kense & Landman, 2011; Boerlin *et al*., 2012). Outbreaks of the disease reported until this moment have been associated with *Enterococcus cecorum* infection (Martin *et al*., 2011).

Broilers affected often showed the posture described as "sitting on their hocks", characterized by legs extended cranially and support given by tibiotarsus-metatarsus joints, which is considered the classic clinical sign of the disease. However, this signal is similar to other conditions as spondylolisthesis (Wood et al., 2002; Gingerich *et al*., 2009), tibial dyschondroplasia (Sauveur & Mongin, 1978) and femoral head necrosis (condronecrosis) (Wiseman & Prisby, 2013). Especially regarding to spondylolisthesis, confusion between the two diseases may have caused underestimation of the prevalence of VO for years (Wood *et al*., 2002; Gingerich *et al*., 2009).

Although the pathogenesis of VO has not been fully elucidated, the disease was reproduced experimentally (Martin *et al*., 2011), despite the description of milder clinical signs and gross lesions. Reasons for the tropism of the infection, precisely in the body of the free thoracic vertebra (T4), are unknown. However, as this is the only vertebra of the thoracic vertebral column that moves freely, it is possible that changes in vascular flow or microtrauma could play a role (Aziz & Barnes, 2007).

Enterococcus cecorum occurs in the intestinal tract, and may have access to the bloodstream via rupture of the intestinal mucosa, caused by previous enteric diseases, such as coccidiosis or bacterial enteritis (Gingerich *et al.*, 2009). In addition to this hypothesis, some authors speculate whether the bacteria could reach the vertebrae through the air sacs, as some vertebrae are pneumatic bones (Aziz; Barnes, 2007). Neverteless, pneumatisation of the free fourth thoracic vertebra and synsacrum by abdominal air sac occurs later, at 77 days posthactching (Hogg, 1984).

In recent years, the genus *Enterococcus* has emerged as a significant cause of nosocomial infections, particularly *Enterococcus faecalis* (Kola *et al.*, 2010), which causes extraintestinal infections in humans (Creti *et al.*, 2004). Resistant microorganisms, largely involved in these cases, are selected by the large indiscriminate use of antibiotics in human and veterinary medicine (McGaw, 2013). These resistant microorganisms, when present in animals and animal by-products, may be able to be transmitted to human beings (Foulquié *et al.*, 2006). In addition, gowns worn by patients and health care workers, medical equipment, microsphere beds, and environmental surfaces (Gould & Freeman, 1993) would be sources of nosocomial infection. Regarding animal and public health this is very important (Foulquié *et al.*, 2006) and demonstrates the need for studies on diseases related to these bacteria.

In Brazil, there are no studies concerning the frequency of vertebral osteomyelitis, although previous field observations have indicated the occurrence of the disease. The objective

of this study was to determine the frequency and etiology of vertebral osteomyelitis in broilers in a highly productive broiler region in Brazil.

Materials and methods

Samples. In order to determine the frequency of vertebral osteomyelitis, we evaluated 608 broilers with locomotor problems, between the years of 2012 and 2014. The broilers were from the largest area of poultry meat production in Minas Gerais state, which is the fifth largest poultry meat producer and exporter in Brazil. The sample size was defined considering a prevalence of 4% observed in a previous pilot project, with a confidence interval of 95% and margin of error of 15% (Pan American Zoonoses Center, 1973).

The birds were from 38 flocks of 18 commercial farms localized in nine different municipalities in the state of Minas Gerais. The broilers were grouped in less than 40 days-old (n=122) or 40 days-old or older (n=486), with minimal age of 21 days-old and maximum age of 56 days-old. Regarding to the gender, most were males (n=479), with fewer females (n=91) and undetermined for some birds (this information was not recorded) (n=38). Broilers were clinically assessed and were then euthanized and necropsied. The procedures in this study were performed in accordance with the recommendations by the Animal Experimentation Ethics Committee of Universidade Federal de Minas Gerais (Protocol 205/2011).

Clinical signs. Broilers presenting locomotor disorders were placed in station and encouraged to move for change in gait and posture assessment, which were individually registered, as well as through image record.

Necropsy. After recording clinical signs, the broilers were euthanized by cervical dislocation (Oliveira, Alves; Rezende, 2002) for necropsy, during which all the observed changes in the locomotor system (axial skeleton - sagittal section - and appendage), related to size, shape, color and consistency were assessed and recorded. The free thoracic vertebra was considered as T4, since there are five thoracic vertebrae in fowls (Hogg, 1984) and the last (T5) vertebrae fuses with the lumbosacral vertebrae to form the synsacrum (Baumel, 1979). The vertebral column of all broilers was sectioned along the longitudinal midline to analyze the vertebral body and spinal cord. Samples for bacterial culture and isolation were collected aseptically from broilers presenting enlarged free thoracic vertebra. Vertebral samples and fecal contents of the large intestine of broilers with osteomyelitis were also collected in sterile microtubes and frozen at -20 °C for DNA extraction and subsequent PCR for the etiologic agents described below (see subsection *Polymerase Chain Reaction*). The vertebral columns with gross lesions were fixed in 10% neutral buffered formalin for 48 to 56 hours for further processing and histopathological analysis.

Histopathology. Vertebral columns corresponding to lumbar and thoracic segments were decalcified in 24% formic acid. For slide preparation, tissues were dehydrated in increasing ethanol concentrations, diaphanised in xylene, embedded in paraffin to obtain 4-µm thick serial sections, which were stained with hematoxylin-eosin (HE) and Goodpasture (Luna, 1968) and analysed under a light microscope.

Bacteriology. Aseptically collected swabs of the vertebral lesions were inoculated onto two blood agar (BA) plates and one MacConkey agar (MCK) plate. One BA plate was incubated in

microaerophilic conditions at 37 °C for 24 to 72 hours, while the other plates were incubated at the same temperature and time, but under aerobic conditions. After the initial growth, the morphology of isolated colonies was characterized and subcultured, Gram stained and submitted to catalase and oxidase tests. Bacterial isolates were subject to automatic bacterial identification by VITEK 2 system (bioMérieux, Inc. Hazelwood, MO, USA), using commercially available identification cards for Gram-positive and Gram-negative bacteria, in accordance to the manufacturer's recommendations. After bacterial identification, the colonies were plated on Mueller-Hinton agar (MH) for growth and then inoculated into microtubes containing BHI (brain and heart infusion) broth and 30% glycerol and stored at - 80 °C.

DNA extraction. For DNA extraction from vertebral lesions, tissue samples were ground in a mortar and pestle, and combined with three volumes of 6M sodium iodide. The DNA was subsequently recovered on silicon dioxide microspheres, as described previously by Vogelstein & Gillespie (1979) and Boom *et al.* (1990). Extraction of DNA from reference bacterial strains was performed by boiling (Marques & Suzart, 2004) with modifications. Briefly, a colony was taken directly from the MH plate and transferred with a 10 µL calibrated loop into a microtube containing 300 µL of sterile deionized water and homogenized for 10 seconds by vortexing. Then, the microtube was placed in a dry bath at 100 °C for 30 minutes and centrifuged at 14,000 x g for two minutes. The supernatant was placed in a new microtube and stored at -80 °C. The extraction of DNA from feces was performed by boiling with Chelex® 100 (Bio-Rad, Richmond, California). Briefly, approximately five milligrams of feces were added to 300 µL of Chelex® 100, homogenized by vortexing, centrifuged at 14,000 x g for 15 seconds and incubated at 95 °C. Then, the samples were centrifuged and the supernatant frozen at -20 °C. The concentration and purity of DNA extracted from vertebral samples and bacterial colonies were assessed by spectrophotometry.

Polymerase Chain Reaction (PCR). The DNA extracted from vertebral lesions and feces was subjected to PCR for *Enterococcus* spp., *E. faecalis*, *E. cecorum*, *E. hirae*, *E. durans* and only vertebral lesions for *Escherichia coli* using specific primers and amplification protocols previously described (Tab. 1). The primers used for the detection of *Enterococcus* spp. amplified a product of 112 base pairs (bp) from the *tuf* gene region (Ke *et al.*, 1999). For the detection of *E. faecalis*, *E. cecorum*, *E. hirae* and *E. durans*, primers were used to amplify a region of the *sodA* gene (manganese dependent superoxide dismutase), generating products of 360 bp, 371 bp, 187 bp and 295 bp, respectively (Jackson *et al.*, 2004). The primers used for the detection of *Escherichia coli* amplified a product of 585 bp from the *malB* promoter gene (Wang *et al.*, 1996). To assess viability and quality of extracted DNA, all samples were subjected to PCR to detect *β-actin* (endogenous control gene).

Table 1. The oligonucleotide sequences and amplified product sizes used for the detection of selected etiological agents involved in cases of vertebral osteomyelitis.

Target	Name of primer	Oligonucleotides sequence	Product (bp)	Reference
Enterococcus spp.	ENT-1 ENT-2	TACTGACAAACCATTCATGATG AACTTCGTCACCAACGCGAAC	112	Ke *et al.* (1999)
Enterococcus faecalis	FL-1 FL-2	ACTTATGTGACTAACTTAACC TAATGGTGAATCTTGGTTTGG	360	Jackson *et al.* (2004)
Enterococcus cecorum	CE-1 CE-2	AAACATCATAAAACCTATTTA AATGGTGAATCTTGGTTCGCA	371	Jackson *et al.* (2004)
Enterococcus hirae	HI-1 HI-2	CTTTCTGATATGGATGCTGTC TAAATTCTTCCTTAAATGTTG	187	Jackson *et al.* (2004)
Enterococcus durans	DU-1 DU-2	CCTACTGATATTAAGACAGCG TAATCCTAAGATAGGTGTTTG	295	Jackson *et al.* (2004)
Escherichia coli	ECO-1 ECO-2	GACCTCGGTTTAGTTCACAGA CACACGCTGACGCTGACCA	585	Wang *et al.* (1996)

PCR reactions were performed using a final volume of 25 µL (PCR Master Mix Promega, Madison, WI, USA) and 200 to 300 ng of DNA template, in a thermocycler (Veriti® Thermal Cycler, Applied Biosystems, Inc., Foster City, CA, USA). The reference strains of *E. faecalis* (CCCD-E006, Cefar Diagnostica), *E. hirae* (INCQS 00036; ATCC 8043), *E. durans* (INCQS 00552; ATCC 6056), *E. cecorum* (courtesy Poultry Diagnostic and Research Center, University of Georgia, USA) and *Escherichia coli* (courtesy prof. Dr. Marcos Bryan Heinemann, Universidade Federal de Minas Gerais) were used as positive controls. As negative control, reactions were performed with all reagents except for template DNA. The final product of each reaction was subjected to electrophoresis in 1.5% agarose gel containing ethidium bromide along with molecular weight marker of 100 bp (LowRanger100bp DNA Ladder Norgen®).

Results

History. Broilers were from the municipalities of Belo Horizonte, Bom Jesus do Amparo, Curvelo, Igarapé, Itabira, Igaratinga, Pará de Minas, Prados and São Sebastião do Oeste, which are all in the state of Minas Gerais (Brazil). The broiler farms had different management and biosecurity practices, with the number of broilers per flock ranging from 20,000 to 40,000. Broiler farms usually raise birds up to approximately 42 to 45 days before processing. Although information regarding the utilization of growth promoters was not available for all farms, antibiotics such as zinc bacitracin and colistin were confirmed to be of frequent use. The historic uses of antibiotics in the sampled farms include enrofloxacin, fosfomycin, amoxicillin, and trimethoprim sulfa, which were most commonly used to treat respiratory or enteric diseases.

Frequency of the disease. Of the broilers with locomotor alteration evaluated in this study, 5.1% (31/608) had vertebral osteomyelitis. Of these, 93.5% (29/31) were 40 days-old or older (average of 44.1 days), while 6.5% (2/31) were less than 40 days-old (average of 35 days). Regarding the gender of affected broilers, 89.7% (26/29) were male and 10.3% (3/29) female. Other causes for locomotor alterations, such as spondylolisthesis, scoliosis, arthritis, tibial dyschondroplasia, bumblefoot and femoral head necrosis, were also found (unpublished data).

Clinical signs. Broilers with vertebral osteomyelitis presented different degrees of limited mobility, which were related to the level of compression of the spinal cord (Figure 1). In cases of mild clinical signs (6.4%, 2/31), often associated with mild compression, broilers presented impaired ability to stand up and move, with hunched posture and displacement of their center of gravity (Figure 1a). In cases of moderate clinical signs (58.1%, 18/31), usually associated with moderate spinal cord compression, broilers rested on their tibiotarsus-metatarsus joints and pectoral muscles, but did not present cranially extended legs (Figure 1b). Only in the cases of severe clinical signs (35.5%, 11/31) with marked spinal cord compression, broilers presented the classic clinical signs of the disease, namely, legs extended cranially and support provided mainly by the pectoral muscles, making the locomotion movements extremely impaired, this is known as "sit on the hocks" (Figure 1c). A few broilers had become dehydrated and were in poor body condition.

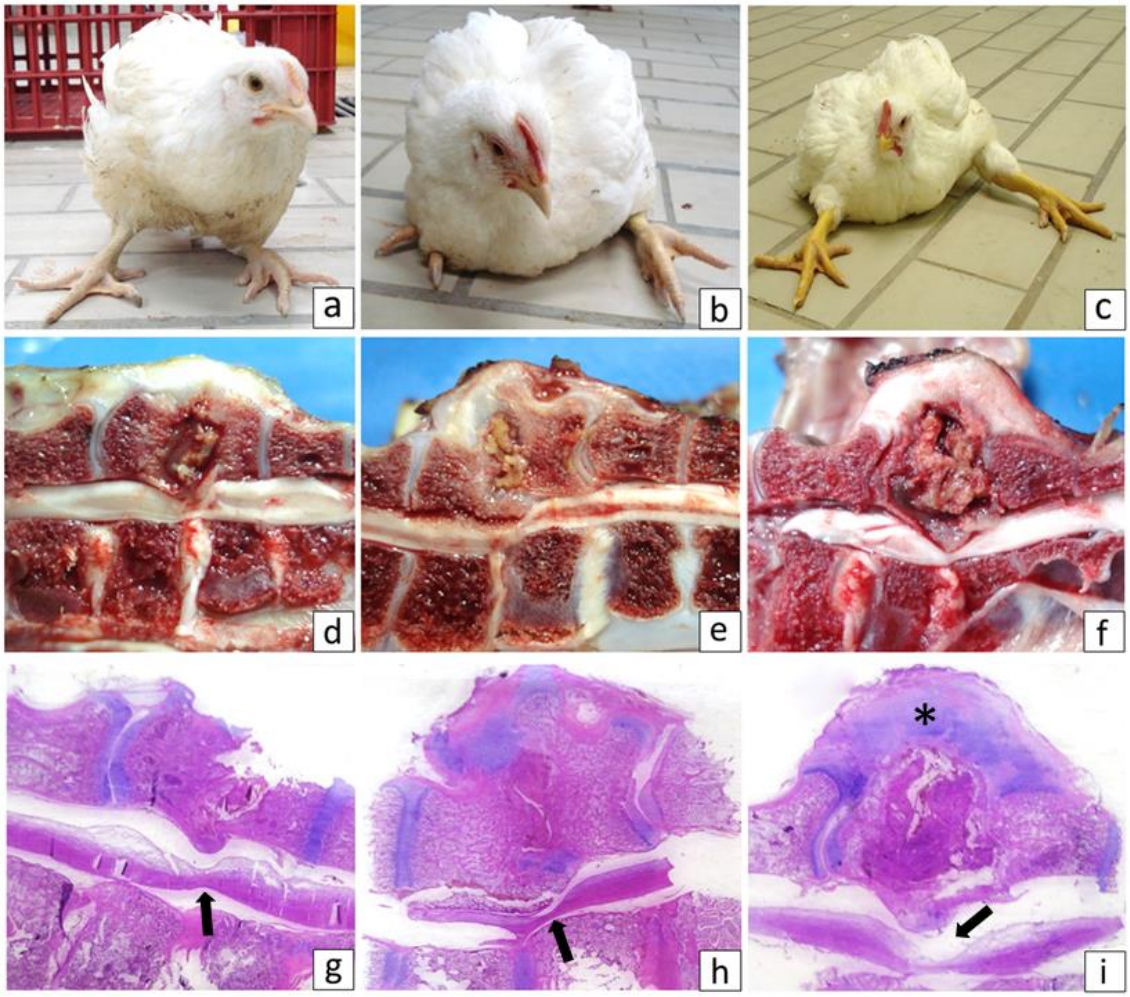

Figure 1. Broilers with different degrees of vertebral osteomyelitis. In broiler with (a) mild, (b) moderate and (c) marked signs. Sagittal section of the vertebral column with variable amounts of caseonecrotic material in the T4 vertebral body (d), (e) and (f). The necrotic tissue in the region of the vertebral bodies is projecting into the spinal canal leading to spinal cord compression. In the submacroscopic images of vertebral lesions (d), (e) and (f), note the increased volume of vertebral body projecting into the vertebral canal and compressing the spinal cord (arrow) to different degrees (g), (h) and (i). A thick layer of fibrous

tissue and disorganized neocartilage (arrows) connecting both vertebral bodies are observed on the necrotic area. HE.

Necropsy. Various degrees of enlargement of the T4 vertebra (mobile) and the adjacent vertebrae (T3 and T5) were observed. Gross lesions were classified according to intensity as mild, moderate and marked, based on the lesion extension and compression strength of the spinal cord, since the macroscopic appearance did not show great variation. Most broilers had moderate lesions (54.8%, 17/31), followed by marked (29.1%, 9/31) and mild (16.1%, 5/31). The enlargement was yellow to yellowish-grey and firm, due to a fibrous capsule surrounding the vertebral body. On the cut surface, there was a variable amount of yellow and friable material, which fully replaced the vertebral body and intervertebral space in more advanced lesions. The presence of the caseonecrotic material led to the enlargement of the vertebral body and its protrusion into the vertebral canal, leading to the compression and malacia of the spinal cord to differing degrees (Figure 1d, 1e, 1f, 1g, 1h and 1i). In addition to the T4 vertebral lesions, some broilers showed necrotizing dermatitis and myositis in the pectoral region, characterized by several degrees of necrosis and hemorrhages in the pectoral muscles, which were due to the broilers resting on their chests. In cases with no correlation between clinical signs and lesions, i. e., severe clinical signs and mild vertebral lesion (22.6%, 7/31), the broilers usually presented concomitant diseases, as femoral head necrosis (19.4%, 6/31), coccidiosis (9.7%, 3/31), arthritis (6.4%, 2/31), necrotic hepatitis (6.4%, 2/31), tibial osteomyelitis (3.2%, 1/31), or airsacculitis (3.2%, 1/31).

Twelve other broilers with locomotor disorders had lesions only in the cranial part of the T5 vertebra, which involved the articular cartilage and growth plate. These lesions were characterized by a focal, or focally extensive, and moderately friable gray area, which was occasionally associated with clefts.

Histopathology. The lesions ranged from mild to marked, considering a descriptive classification regarding the lesion extension. The necrotic tissue from the vertebral body protruded into the vertebral canal causing compression of the superjacent spinal cord, which lead to ischemia and subsequent degeneration and loss of axons in the white matter (Figure 2a). In broilers with marked lesions, the damage also extended to the gray matter, characterized by neuronal and neuropil necrosis. Major changes in the vertebral body included areas of bone necrosis, which presented fragmented and intensely eosinophilic trabeculae that contained pyknotic osteocytes and were accompanied by several degrees of osteoclasia, characterized by the presence of osteoclasts in Howship´s lacuna (Figure 2b). Marked inflammatory infiltrate, composed predominantly of heterophils and some macrophages, was observed in areas of bone necrosis. In the most acute cases there was intense heterophilic infiltration and fibrin, while in those with the largest population of macrophages, an intense proliferation of fibrous connective tissue was observed in the marrow, characterizing myelofibrosis. In some cases, there was formation of neocartilage peripheral to the lesions and/or certain bacteria associated with areas of necrosis (Figure 2c). Bacterial colonies were detected in 35.5% (11/31) of lesions and were identified as Gram-positive (25.8%, 8/31), Gram-negative (16.1%, 5/31) or both in the same lesion (6.4%, 2/31) by Goodpasture staining (Figure 2d). In all these cases, PCR identified the bacteria as *Enterococcus* spp., *Enterococccus faecalis* and/or *Escherichia coli*.

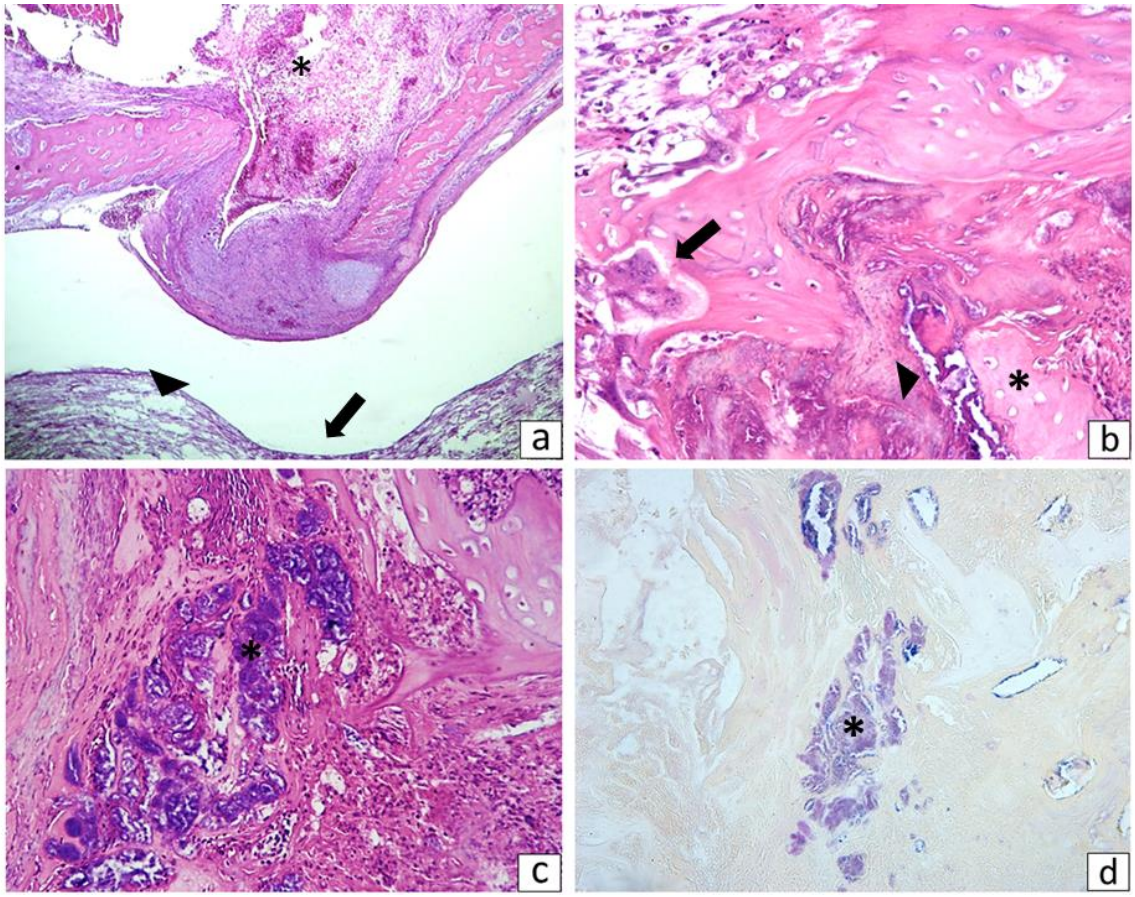

Figure 2. Broilers, vertebral osteomyelitis. (a) Inflammatory and necrotic processes, which modify the vertebral body morphology (*) and compress the spinal cord (arrow), lead to axonal loss (arrow head). HE, 40x. (b) necrotic bone tissue (*) with resorption areas (osteoclasts in Howship´s lacuna) (arrow). Observe the necrotic debris with intralesional bacterial colonies (arrow). HE, 400x. (c) In the vertebral body there are numerous intralesional bacterial colonies (*), which are surrounded by necrotic bone tissue and cellular debris (i.e. heterophils, erythrocytes and fibrin). HE, 400x. (d) Gram-negative and Gram-positive bacteria (*) were observed in the vertebral lesions. Goodpasture, 200x.

Histologic changes of those broilers that had macroscopic lesions in the T5 vertebra only were characterized by areas of degeneration and necrosis of articular cartilage (T4/T5), and occasionally by the formation of clefts, which were associated, or not, with hemorrhages that extended to metaphysis. Bacterial colonies were observed in several clefts.

Bacterial isolation and identification. The major bacterial agent identified by VITEK 2 was *Enterococcus faecalis* (Tab. 2). In 44.0% (11/25) of the cases the bacteria was the single agent involved in the vertebral lesions and in 16.0% (4/25), *E. faecalis* was in co-infection with *E. coli* or *S. aureus*. All Enterococci species in single infection were identified as *E. faecalis* by VITEK 2 and by PCR, and in the cases of co-infection with *S. aureus* the bacteria were not *E. faecalis* by PCR, but other Enterococci (Tab. 2, see PCR results section). Another frequently isolated bacterium was *Escherichia coli*, present in single infection in 32.0% (8/25) of the cases and in 8.0% (2/25) in co-infection with *E. faecalis*, as described previously. *Staphylococcus aureus* was detected in single infection in 8.0% (2/25) of the cases, and in additional 8.0% (2/25) of the cases, the bacterium was associated with *Enterococcus faecalis*, which were

identified by PCR as *Enterococcus* spp. or *Enterococcus hirae*. For three samples there was no bacterial growth on agar medium and for a further three samples, the bacteriological sampling was not possible.

Table 2. Etiologic agents in single infection and co-infection assessed by bacterial isolation and PCR involved in vertebral osteomyelitis in broilers

	Etiologic agente	Bacterial isolation % (n/N)	PCR % (n/N)
Single infection	*Enterococcus* spp.	44.0 (11/25)	42.8 (12/28)
	E. faecalis	100.0 (11/11)	58.3 (7/12)
	E. hirae	0.0 (0/11)	8.3 (1/12)
	E. cecorum	0.0 (0/11)	0.0 (0/12)
	UI species	0.0 (0/11)	33.3 (4/12)
	Escherichia coli	32.0 (8/25)	35.7 (10/28)
	Staphylococcus aureus	8.0 (2/25)	ND
Co-infection	*E. coli* + *E. faecalis*	8.0 (2/25)	7.1 (2/28)
	S. aureus[1] + *Enterococcus* spp.[2]		3.6 (1/28)
	S. aureus[1] + *E. hirae*[2]		3.6 (1/28)

[1]Identified by bacterial isolation; [2]Identified by PCR as *Enterococcus* spp. and *E. hirae*, but as *E. faecalis* by bacterial isolation. UI species = Unidentified species of Enterococci. ND = not done.

PCR. Bacteria of the genus *Enterococcus* spp. were the etiological agents involved in most cases of vertebral osteomyelitis in this study, with DNA from this genus detected in single infection in 42.8% (12/28) of the cases. *Enterococcus faecalis* was detected solely in 58.3% (7/12) and *Enterococcus hirae* in 8.3% (1/12) of the cases. In 33.3% (4/12), the species of *Enterococcus* involved in single infection were not determined by PCR. *Escherichia coli* DNA was detected in single infection in 35.7% (10/28) of the vertebral injuries and co-infection with *Enterococcus faecalis* was confirmed in 7.1% (2/28) of the cases, based on the detection of both bacterial DNA in the same sample. Three cases were PCR negative for all the etiological agents studied. In other three cases with mild vertebral lesions, the necrotic material was scarce and the collection of samples both for DNA extraction and histopathology was unfeasible. All samples were negative for *Enterococcus cecorum* and positive for β-actin, confirming the viability of the extracted DNA and the absence of this etiological agent in the studied cases. All feces samples were positive for *Enterococcus* spp., one was positive for *E. cecorum*, 25.0% (7/28) for *E. durans* and 89.3% (25/28) for *E. coli*. The samples were considered positive for *Staphylococcus aureus* on basis of bacterial isolation results, once the PCR for this species could not be performed.

The comparison between bacterial isolation and PCR was possible in 25 cases and were in agreement in 64% (16/25) of results. In 36% (9/25) of the cases differences between results mainly related to *Enterococcus* species identification were noted. Considering five cases in

which the isolated bacteria was identified as *E. faecalis* by VITEK 2, results by PCR indicated *Enterococcus* genus without discriminating species for three isolates and identified *E. hirae* for other two isolates. In the other four results, there was a divergence between bacterial isolation and DNA detection. In four cases, although the bacteriology enabled single *E. coli* isolation, DNA analysis revealed a co-infection of *E. coli* and *E. faecalis* by PCR. In these cases, the agreements of the DNA detection and histopathology (Goopasture staining of intralesional bacteria) were considered conclusive results. It is important to emphasize that PCR was performed in duplicate using DNA from isolated characterized colonies and from exudate collected aseptically of the vertebral lesions, to confirm the intralesional etiologic agent.

Discussion

This study provides the first insight regarding the frequency and etiology of vertebral osteomyelitis in broilers in Brazil, and demonstrates different aspects of the disease in relation to other countries where the disease has been reported (Devriese *et al.*, 2002; Wood *et al.*, 2002; de Herdt *et al.*, 2008; Aziz & Barnes, 2009; Gingerich *et al.*, 2009; Stalker *et al.*, 2010; Kense & Landman, 2011; Makrai *et al.*, 2011; Boerlin *et al.*, 2012). Vertebral osteomyelitis has 5.1% of frequency in these broilers, considering that all broilers sampled had locomotor disorders.

The disease was diagnosed more frequently in males in the studied flocks, in agreement to previously described findings (Wood *et al.*, 2002; Gingerich *et al.*, 2009; Zavala *et al.*, 2011). The higher frequency of the disease observed in males may result from their higher growth efficiency as compared to females (Longo *et al.*, 2006). Females are precocious and complete the process of ossification of long bones earlier than males and interrupting growth (Naldo *et al.*, 1998). Thus, males will need to support a greater muscle mass on less mature bones, when compared to females of the same age.

The individual identification of broilers allowed for the demonstrating of the progressive nature of the disease, since the impaired mobility was clinically varied in intensity, according to the degree of compression of the spinal cord. Only in the severe cases, with advanced spinal cord compression, the classic clinical signs commonly reported in the cases of the disease were observed (Wood *et al.*, 2002; Aziz & Barnes, 2007; Gingerich *et al.*; 2009; Stalker *et al.*, 2010). However, it was interesting to note that in less advanced cases of the disease, clinical signs presented by affected broilers were non-specific and common to other locomotor disorders.

Gross lesions revealed that, in most cases, VO produced a significant enlargement of the affected vertebral body, different from that of spondylolisthesis, which can produce similar clinical signs (Gingerich *et al.*, 2009). Differential diagnosis of these diseases should be performed by longitudinal sectioning of the vertebral column. Although in both diseases there is spinal cord compression of the affected vertebra, only in vertebral osteomyelitis necrotic and/or caseous material in vertebral body are observed, while in spondylolisthesis there is a subluxation in a transverse plane of the adjacent vertebral bodies (Thorp, 1994; Wood *et al.*, 2002; Gingerich *et al.*, 2009).

Histopathology confirmed the chronic nature of the disease, as demonstrated mainly by myelofibrosis and neocartilage formation observed in several cases of this study. In addition, visualization of bacterial colonies attached to the lesion using HE and Goodpasture staining was associated with bacterial isolation and PCR results, providing a more reliable diagnosis. The histopathological changes observed in our cases of vertebral osteomyelitis were similar with other reports of this disease (Stalker *et al.*, 2010; Martin *et al.*, 2011).

The determination of the etiologic agents involved in VO was enabled by bacterial isolation and identification, and detection of DNA by PCR. Invariably, the techniques demonstrated that the bacteria present in vertebral lesions were *Enterococcus* spp., particularly *E. faecalis*, *E. hirae*, *Escherichia coli* and *Staphylococcus aureus*. This result is interesting, considering that the latest reported cases of vertebral osteomyelitis have been mainly associated with the bacterium *E. cecorum* (Wood *et al.*, 2002; Aziz & Barnes, 2007; Gingerich *et al.*; 2009; Stalker *et al.*, 2010), which was not detected in vertebral lesions of this study, despite the specie have been extensively researched.

Recently, Borst *et al.* (2012) suggested that the new cases of VO could be initiated by pathogenic clones of *E. cecorum*, that are spread to different locations of broiler production possibly by horizontal transmission. This is supported by reports of the disease in successive flocks, suggesting persistence of the bacterium in the environment (de Herdt *et al.*, 2008). If this hypothesis is true, and considering the results of our study, it may be inferred that such pathogenic clones of *E. cecorum* are not present in Brazilian flocks.

Enterococcus faecalis and *E. coli* are present in the intestinal tract and can be recovered from the small and large intestine in variable counts, depending on the broiler age (Salanitro & Blake, 1978; Asrore *et al.*, 2015). According to Devriese *et al.* (1991), *E. faecalis* is rarely found in the digestive tract of broilers of three to five weeks of age. However, Gomes (2008) stated that there are controversial reports on the composition of the intestinal microbiota of different segments of the digestive tract of birds and that it would not be possible to determine the existence of a typical microbiota, since the composition of food, climate, stress and pathogens affect the species of bacteria in different ways.

Besides the above factors, the use of growth promoters may influence the composition of the intestinal microbiota, including antibiotics, which can exacerbate some enterobacteria populations due to an imbalance in the normal gut microbiota (Reynolds *et al.*, 1997). Probiotic growth promoting, designated as a food additive, may be composed of pure or mixed cultures of live microorganisms, including *E. faecalis*, *E. faecium* and *E. coli* (Cantarelli *et al.*, 2005). These have the ability to colonize and proliferate in the gastrointestinal tract, promoting changes in the balance of the intestinal microbiota (Silva, 2000; Cantarelli *et al.*, 2005; Bittencourt *et al.*, 2006). However, probiotics may disrupt the intestinal microbiota, and become pathogenic to broilers (Loddi *et al.*, 2000).

We observed that some broilers had only a degenerative process in the vertebral body and, it was associated with bacterial colonies in some cases, but with minimal inflammatory infiltrate and without macroscopic characteristics of osteomyelitis. These results are particularly interesting when the pathogenesis of bacterial condronecrosis with osteomyelitis is considered. In this condition, the high biomechanical stress in long bones and free thoracic vertebrae can trigger a degenerative process of the cartilage with subsequent bacterial colonization without involvement of specific etiologic agents (Wiseman & Prisby, 2013), similar to that observed in the cases of osteomyelitis of the present study.

Interestingly, in 53.6% of cases of the disease, the DNA of *Enterococcus* spp. was confirmed in the vertebral lesions, although it was not possible to identify the species by PCR in three of these cases. However, the conventional (data not shown) and automated techniques enabled the identification of the species as *Enterococcus faecalis*. In a study carried out by Fang *et al.* (2012), the VITEK 2 system correctly identified 99.2% and 91.7% of the samples at the genus and species levels for different *Enterococcus* isolates, respectively. However, in our study the VITEK 2 was found less efficient in the identification of other *Enterococcus* species, i. e. *Enterococcus* not *E. faecalis* and *E. faecium*. Thus, employing PCR for identification of other species of Enterococci is recommended.

Our study showed that vertebral osteomyelitis in broilers has a lower frequency compared to recent reports in other countries, and is not caused by a single infectious agent in Brazil. The bacteria involved in natural cases of the disease were *Enterococcus faecalis*, *E. hirae*, *Enterococcus* spp., *Escherichia coli* and *Staphylococcus aureus*, which can act alone or in conjunction. It is important to emphasize that, given the role of these bacteria in public health, studies that provide the characterization of virulence and detection of antimicrobial resistance genes in microorganisms involved in the disease are essential. In addition, a relation among this disease and rapid growth and weight of broilers should also to be considered.

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References

Asrore, S.M.M., Sieo, C.C., Chong, C.W., Gan, H.M. & Ho, Y.W. (2015). Deciphering chicken gut microbial dynamics based on high-throughput 16S rRNA metagenomics analyses. *Gut Pathogens*, 7, 1-26.

Aziz, T. & Barnes, H.J. (2007). Is spondylitis an emerging disease of broilers? *World Poult*, 23, 44–45.

Aziz, T. & Barnes, H.J. (2009). Spondylitis is emerging in broilers. *World Poultry*, 25, 14.

Baumel, J.J. (1979). Osteología. In: J.J. Baumel, A.S. King, A.M. Lucas, J.E. Breazile & H.E. Evans (Eds.). *Nomina Anatomica Avium* (pp. 53-121). London: Academic Press.

Bittencourt, L.C., Albuquerque, R., Hueza, I., Raspantini, L.E., Cardoso, A.L.S.P. & Tessari, E. (2006). Influência da resposta imune humoral de frangos de corte. Proceedings of the *Conferência Apinco de Ciência e Tecnologia Avícolas* (p. 36). Santos, Brasil.

Boerlin, P., Nicholson, V., Brash, M., Slavic, D., Boyen, F., Sanei, B. & Butaye, P. (2012). Diversity of *Enterococcus cecorum* from chickens. *Veterinary Microbiology*, 157, 405–411.

Boom, R., Sol, C., Beld, M., Weel, J., Goudsmit, J. & Wertheim-van Dillen, P. (1999). Improved silica–guanidiniumthiocyanate DNA isolation procedure based on selective binding of bovine alpha–casein to silica particles. *Journal of Clinical Microbiology*, 37, 615–619.

Borst, L.B., Suyemoto, M.M., Robbins, K.M., Lyman, R.L., Martin, M.P. & Barnes, H.J. (2012). Molecular epidemiology of *Enterococcus cecorum* isolates recovered from enterococcal spondylitis outbreaks in the southeastern United States. *Avian Pathology*, 41, 479-485.

Cantarelli, V.S., Fialho, E.T., Zangeronimo, M., Almeida, E.C. & Gomes Neto, J.C. (2005). *Aditivos e coadjuvantes biológicos na alimentação de suínos* 1st edn. (pp.5-87). Lavras: UFLA/FAEPE.

Centro Pan-Americano de Zoonoses. (1979). Procedimientos para Estudios de Prevalencia por Muestreo. Nota Técnica 18, Rev.1, Ramos Mejia, Buenos Aires. 35p.

Creti, R., Imperi, M., Bertuccini, L., Fabretti, F., Orefici, G., Di Rosa, R. & Baldassarri, L. (2004). Survey for virulence determinants among *Enterococcus faecalis* isolated from different sources. *Journal of Medical Microbiology,* 53, 13–20.

De Herdt, P., Defoort, P., Van Steelant, J., Swam, H., Tanghe, L., Van Goethem, S. & Vanrobaeys, M. (2008). *Enterococcus cecorum* osteomyelitis and arthritis in broiler chickens. *Vlaams Diergeneeskundig Tijdschrift*, 78, 44–48.

Devriese, L.A., Cauwerts, K., Hermans, K. & Wood, A.M. (2002). *Enterococcus cecorum* septicemia as a cause of bone and joint lesions resulting in lameness in broiler chickens. *Vlaams Diergeneeskundig Tijdschrift*, 71, 219–221.

Devriese, L.A., Hommez, J., Wijfels, R. & Haesebrouck, F. (1991). Composition of the enterococcal and streptococcal intestinal flora of poultry. *Journal of Applied Bacteriology*, 71, 46–50.

Fang, H., Ohlsson, A.K., Ullberg, M. & Ozenci, V. (2012). Evaluation of species-specific PCR, Bruker MS, VITEK MS and the VITEK 2 system for the identification of clinical Enterococcus isolates. *European Journal of Clinical Microbiology and Infectious Diseases*, 31, 3073-3077.

Foulquié, M.M.R., Sarantinopoulos, P., Tsakalidou, E. & De Vuyst, L. (2006). The role and application of Enterococci in food and health. *International Journal of Food Microbiology*, 106, 1-24.

Gingerich, E.N., Rankin, S., Barnes, J.H., Owen, R.L. & Davison, S. (2009). Vertebral abscesses due to *Enterococcus cecorum* in broiler chickens: an emerging disease? Proceedings of the *National Meeting on Poultry Health and Processing*. Ocean City, Maryland: USA.

Gomes, A.M. (2008). *Uso de probióticos em substituição aos promotores de crescimento em dietas para frangos de corte* (Master´s degree). Belo Horizonte: Universidade Federal de Minas Gerais.

Gould, F.K. & Freeman, R. (1993). Nosocomial infection with microsphere beds. *Lancet*, 342, 241–242.

Greenacre, B. & Morishita, T.Y. (2014). *Backyard Poultry Medicine and Surgery: A Guide for Veterinary Practitioners.* 1st edn. New York: John Willey & Sons Inc. 368 p.

Hogg, D.A. (1984). The distribution of pneumatisation in the skeleton of the adult domestic fowl. *Journal of Anatomy*, 138, 617-629.

Jackson, C.R., Fedorka-Cray, P.J. & Barrett, J.B. (2004). Use of a Genus- and Species-Specific Multiplex PCR for Identification of Enterococci. *Journal of Clinical Microbiology*, 42, 3558–3565.

Ke, D., Picard, F.J., Martineau, F., Ménard, C., Roy, P.H., Ouellette, M. & Bergeron, M.G. (1991). Development of a PCR Assay for Rapid Detection of Enterococci. *Journal of Clinical Microbiology*, 37, 3497–3503.

Kense, M.J. & Landman, W.J.M. (2011). *Enterococcus cecorum* infections in broiler breeders and their offspring: molecular epidemiology. *Avian Patholoy*, 40, 603–612.

Kola, A., Schwab, F., Barwolff, S., Eckmanns, T., Weist, K., Dinger, E., Klare, I., Witte, W., Ruden, H. & Gastmeier, P. (2010). Is there an association between nosocomial infection rates and bacterial cross transmissions? *Critical Care Medicine*, 38, 46–50.

Loddi, M.M., Gonzales, E., Takita, T.S., Mendes, A.A. & Roça, R.O. (2000). Uso de Probiótico e Antibiótico sobre o Desempenho, o Rendimento e a Qualidade de Carcaça de Frangos de Corte. *Revista Brasileira de Zootecnia*, 29, 1124-1131.

Longo, F.A., Sakomura, N.K., Rabello, C.B.V., Figueiredo, A.N. & Fernandes, J.B.K. (2006). Exigências energéticas para mantença e para o crescimento de frangos de corte. *Revista Brasileira de Zootecnia*, 35, 119-125.

Luna, L.G. (1968). *Manual of histologic staining methods of the Armed Forces Institute of Pathology.* 3rd edn. New York: McGraw-Hill. 258p.

Makrai, L., Nemes, C., Simon, A., Ivanics, E., Dudás, Z., Fodor, L. & Glávits, R. (2011). Association of *Enterococcus cecorum* with vertebral osteomyelitis and spondylolisthesis in broiler parent chicks. *Acta Veterinaria Hungarica*, 59, 11–21.

Marques, E.B. & Suzart, S. (2004). Occurrence of virulence-associated genes in clinical *Enterococcus faecalis* strains isolated in Londrina, Brazil. *Journal of Medical Microbiology*, 53, 1069–1073.

Martin, L.T., Martin, M.P. & Barnes, H.J. (2011). Experimental Reproduction of Enterococcal Spondylitis in Male Broiler Breeder Chickens. *Avian Diseases*, 55, 273–278.

McGaw, L. (2013). Use of Plant-Derived Extracts and Essential Oils against Multidrug-Resistant Bacteria Affecting Animal Health and Production. In.: Rai, M.K. & Kon, K.V. (Eds). Fighting Multidrug Resistance with Herbal Extracts, Essential Oils and their Components. 1st edn. London: Academic Press. pp. 191–203.

Naldo, J.L., Samour, J.H. & Bailey, T.A. (1998). Radiographic monitoring of the ossification of long bones in kori (Ardeotis kori) and white-bellied (Eupodotis senegalensis) bustards. *Research in Veterinary Science*, 65, 161-163.

Oliveira, H.P., Alves, G.E.S. & Rezende, C.M.F. (2002). *Eutanásia em medicina veterinária*. Retrieved from http://www.ufmg.br/coep/eutanasia.pdf

Reynolds, D.J., Davies, R.H., Richards, M.E. & Wray, C. (1997). Evaluation of combined antibiotic and competitive exclusion treatment in broiler breeder flocks infected whith Salmonella enterica serovar enteritidis. *Avian Pathology*, 26, 83-95.

Salanitro, J.P. & Blake, I.G. (1978). Bacteria isolated from the duodenum, ileum and cecum of young chicks. *Applied and Environmental Microbiology*, 35, 782-790.

Sauveur, B. & Mongin, P. (1978). Tibial dyschondroplasia, a cartilage abnormality in poultry. *Annales de biologie animale, biochimie, biophysique*, 18, 87-98.

Silva, E.N. (2000). Antibióticos intestinais naturais: bacteriocinas. In Proceedings of the *Simpósio sobre aditivos alternativos na nutrição animal* (p.15-24). Campinas, Brasil.

Stalker, M.J., Brash, M.L., Weisz, A., Ouckama, R.M. & Slavic, D. (2010). Arthritis and osteomyelitis associated with *Enterococcus cecorum* infection in broiler and broiler breeder chickens in Ontario, Canada. *Journal of Veterinary Diagnostic Investigation*, 22, 643–645.

Thorp, B.H. (1994). Skeletal disorders in the fowl: a review. *Avian Pathology*, 23, 203-236.

Vogelstein, B. & Gillespie, D. (1979). Preparative and analytical purification of DNA from agarose. *Proceedings of the National Academy of Sciences*, 76, 615–619.

Wang, R., Cao, W. & Cerniglia, C.E. (1996). PCR detection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. *Applied and Environmental Microbiology*, 62, 1242–1247.

Wiseman, R.F. & Prisby, R.D. (2013). Bone circulatory disturbances in the development of spontaneous bacterial chondronecrosis with osteomyelitis: a translational model for the pathogenesis of femoral head necrosis. *Frontiers in Endocrinology (Lausanne)*, 3, 1-14.

Wood, A.M., MacKenzie, G., McGiliveray, N.C., Brown, L., Devriese, L.A. & Baele, M. (2002). Isolation of *Enterococcus cecorum* from bone lesions in broiler chickens. *Veterinary Record*, 150, 27.

Zavala, G., Barnes, H.J. & Powell, K.C. (2011). Broiler breeder diseases: a review. In Proceedings *of the XVII World Veterinary Poultry Association Congress*. Cancun, Mexico.

CHAPTER III

Diversity of *Escherichia coli* strains involved in vertebral osteomyelitis and arthritis in broilers in Brazil

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Abstract: ***Background.*** Locomotor disorders and infections by *Escherichia coli* represent major concerns to the poultry industry worldwide. Avian pathogenic *E. coli* (APEC) is associated with extraintestinal infections leading to respiratory or systemic disease known as colibacillosis. The most common lesions seen in cases of colibacillosis are perihepatitis, airsacculitis, pericarditis, peritonitis/salpingitis and arthritis. These diseases are responsible for significant economic losses in the poultry industry worldwide. *E. coli* has been recently isolated from vertebral osteomyelitis cases in Brazil and there are no data on molecular and phenotypic characteristics of *E. coli* strains isolated from lesions in the locomotor system of broilers. This raised the question whether specific *E. coli* strains could be responsible for bone lesions in broilers. The aim of this study was to assess these characteristics of *E. coli* strains isolated from broilers presenting vertebral osteomyelitis and arthritis in Brazil. ***Results.*** Fifteen *E. coli* strains from bone lesions were submitted to APEC diagnosis and setting of ECOR phylogenic group, O serogroup, flagella type, virulence genes content, genetic patterns by Pulsed Field Gel Electrophoresis (PFGE) and Multilocus Sequence Typing (MLST). In addition, bacterial isolates were further characterized through a lethality test, serum resistance test and antibiotic resistance profile. *E. coli* strains harbored different genetic pattern as assessed by PFGE,

regardless of flock origin and lesion site. The strains belonged to seven sequence types (STs) previously described (ST117, ST101, ST131, ST371 and ST3107) or newly described in this study (ST5766 and ST5856). ECOR group D (66.7%) was the most frequently detected. The strains belonged to diverse serogroups (O88, O25, O12, and O45), some of worldwide importance. The antibiotic resistance profile confirmed strains' diversity and revealed a high proportion of multidrug-resistant strains (73%), mainly to quinolones and beta-lactams, including third generation cephalosporin. The percentage of resistance to tetracycline was moderate (33%) but always associated with multidrug resistance. ***Conclusions.*** Our results demonstrated that vertebral osteomyelitis and arthritis in broilers can be associated with highly diverse *E. coli* based on molecular and phenotypic characteristics. There was no specific virulence patterns of the *E. coli* strains associated with vertebral osteomyelitis or arthritis. Also, *E. coli* strains were frequently multidrug resistant and belonged to STs commonly shared by APEC and human ExPEC strains.

Keywords: Broilers - bacterial infections – APEC - virulence genes – pathology - multidrug-resistant *E. coli*.

Background

Escherichia coli is a genetically diverse bacteria comprising non-pathogenic intestinal strains and pathogenic strains responsible for intestinal and extra-intestinal disease [1]. The strains able to cause disease in chickens are known as Avian pathogenic *E. coli* (APEC). APEC is associated with extraintestinal infections leading to respiratory or systemic disease known as colibacillosis. These diseases are responsible for significant economic losses in the poultry industry worldwide, which may occur by decreased hatching rates, mortality, lowered production, carcass condemnation at processing and treatment costs [2]. The most common lesions associated with colibacillosis are perihepatitis, airsacculitis and pericarditis, although other syndromes such as osteomyelitis, arthritis, yolk peritonitis, peritonitis/salpingitis (SPS syndrome), coligranuloma, omphalitis and cellulitis can also be found [3].

Another challenge to modern poultry industry is locomotor disorders, which represent a major economic and welfare problem. Although these disorders may be classified according to underlying pathology as infectious, developmental and degenerative, this classification is difficult since these categories are not mutually exclusive [4]. Infectious conditions such as osteomyelitis, arthritis (or osteoarthritis) and synovitis can be associated with different etiologic agents [3]. Among bacteria, *Staphylococcus* sp. (mainly, *S. aureus*) was isolated from these diseases, although an increase in the incidence of musculoskeletal infection associated with *E. coli* has been reported [5].

Brazil, which is currently the largest exporter and the second largest producer of poultry meat in the world, faces challenges with colibacillosis and locomotor disorders the same form as other countries with relevant poultry industry. There are no data on molecular and phenotypic characteristics of *E. coli* strains isolated from lesions in the locomotor system of broilers, although *E. coli* has been recently isolated from vertebral osteomyelitis cases in Brazil [6]. This raises the question whether specific *E. coli* strain could be responsible for bone lesions in broilers. The aim of our work is to provide data on the phenotypic and molecular characteristics of *E. coli* strains isolated from vertebral osteomyelitis and arthritis cases in broilers from Brazil.

Methods

Samples. Fifteen *E. coli* strains isolated between 2012 and 2014 from broilers presenting vertebral osteomyelitis or arthritis at commercial poultry farms in the state of Minas Gerais, Southeast of Brazil, were studied. The broilers were from eight different flocks, which represent seven different farms. They had variable ages and gender. All experimental procedures were approved by the Universidade Federal de Minas Gerais (UFMG), Committee for Ethics in Animal Experimentation (CETEA) under protocol 205/2011.

Clinical signs and pathology. For clinical examination, broilers presenting locomotor disorders were placed in station and encouraged to move for change in gait and posture assessment. Then, broilers were euthanized by cervical dislocation for necropsy and gross evaluation. The locomotor system was analyzed for size, shape, color, flexibility and breaking strength. The vertebral column of all broilers was sectioned along the longitudinal midline for vertebral body and spinal cord analysis. The free thoracic vertebra was considered as T4. Articulations were analyzed for size and aspects of the synovial fluid in the articular space. Samples for bacterial isolation were collected aseptically from broilers presenting osteomyelitis or arthritis. Tissue sections were fixed in 10% neutral buffered formalin for 48 to 56 hours. Then, formalin-fixed-vertebral column, intertarsal and femorotibial articulations with lesions were decalcified in 24% formic acid. For slide preparation, tissues were dehydrated in increasing ethanol concentrations, diaphoanised in xylene, embedded in paraffin to obtain 4-µm thick serial sections and then stained with hematoxylin-eosin (HE) and Goodpasture for further analysis under a light microscope.

Bacterial isolation and identification. Swabs of the lesions were inoculated onto two blood agar (BA) plates and one MacConkey agar (MCK) plate. One BA plate was incubated in microaerophilic conditions at 37 °C for 24 to 72 hours, while the others were incubated at the same temperature and time under aerobic conditions. After the initial growth, morphology of isolated colonies was characterized and these same colonies were subcultured, Gram stained and submitted to catalase and oxidase tests. Bacterial isolates were subjected to automatic bacterial identification through VITEK 2 system (bioMérieux, Inc. Hazelwood, MO, USA) using commercially available identification cards for Gram-negative bacteria in accordance to the manufacturer's recommendations. After bacterial identification, the colonies were inoculated into microtubes containing Brain-Heart Infusion (BHI) broth with 30% glycerol and stored at -80 °C until subsequent molecular and phenotypic tests described below.

APEC diagnosis tests. The diagnosis of APEC strains was performed by different methods previously described. The ability of *E. coli* strains to induce lethality in 1-day-old specific-pathogen-free (SPF) chicks (detailed on section **Lethality test**) was considered gold standard test to assess strain pathogenicity. In addition, two molecular methods based on genetic profiles were used: 1) detection of minimal predictors described by Johnson et al. [7], which classify an *E. coli* strain as pathogenic based on the minimum detection of four out of five virulence genes (*iroN*, *ompT*, *hlyF*, *iss* and *iutA*); and 2) genotyping method developed by Schouler et al. [8], which is based on the identification of different associations of virulence genes (*iutA*, *sitA*, *aec26*, P (F11) fimbriae, O78, frz_{orf4}) that allow the APEC strains classification in four genetic patterns of virulence (A, B, C and D).

Serogrouping. Determination of O antigens was carried out by agglutination using antisera O1, O2, O5, O8, O15, O18, O25, O45, O78, O88, O111 and O120, according to the method described by Blanco et al. [9]. The O antisera were produced in the Laboratorio de Referencia de *Escherichia coli* (Lugo, Spain). Furthermore, PCR was performed to detect O1, O2, O4, O6, O7, O8, O12, O16, O18, O25a, O45a, O45b, O75, O78, O88 and O104 antigens, as previously described (Table 1, supplementary data).

Flagellar type. The strains were submitted to PCR to determine flagella type H4, H7, H8, H21 and H25 (Table 1, supplementary data). Those strains negative for all flagellar types tested by PCR were submitted to motility test. Briefly, bacteria were grown on LB broth overnight. Then, the strains were deeply inoculated in LB plates 0.3% agar using a Pasteur pipette and then incubated at 37 °C overnight for motility evaluation the following day [10].

ECOR phylogenetic grouping. *E. coli* strains were classified into the four main ECOR phylogenetic groups by triplex PCR as described by Clermont et al. [11]. Strains were assigned to phylogenetic groups A, B1, B2, or D according to the amplification of the *chuA* and *yjaA* genes and the TspE4C2 fragment. Strains MG1655, ECOR26, ECOR62, and ECOR50 were used as controls for phylogenetic groups A, B1, B2, and D, respectively.

Virulence genotyping. Total DNA extracts were prepared by a rapid boiling method [12]. The presence of genes encoding virulence factors were determined using primers and PCR amplification programs previously described, together with positive control strains (Table 2, supplementary data). Single PCR assays were used to detect *sfaS*, *focG*, *tsh*, *ibeA*, *aatA*, *neuC*, *irp2*, *ireA*, *sat*, *vat*, *astA*, *fyuA*, *hlyA*, *traT*, *cva/cvi*, *iucD*, *hra*, *iha*, *pic*, *csgA*, *tia*, *malX* (=rpai), *KpsMTII*, *cnf 1* and *cnf 2*. Furthermore, some multiplex assays were performed to detect simultaneously *clbB* and *clbN*, and *fimA*, fim_{avMT78} and *fimH*. DNA fragments were amplified in a 25-µL PCR mix containing 1 U of GoTaq®G2 Flexi DNA polymerase (Promega), 12.5 pmol of the forward and reverse primers, and 5 nmol of deoxynucleotide triphosphate mix (Eurogentec) in 1x GoTaq®G2 Flexi buffer. The PCR conditions were as follows: initial denaturation at 94 °C for 4 to 5 min, followed by 30 cycles of 94 °C for 30 s, annealing temperature according to GC-content of primers for at least 30 s, 72 ºC for 30 s to 45 s according to the size of the amplified fragment (1 min/kbp), and then a final extension at 72 °C for 7 min.

Pulsed-field gel eletrophoresis (PFGE). Pulsed-field gel electrophoresis was conducted as previously described [13]. Bacterial cells (equivalent to an OD_{600} of 1.0) grown in BHI broth were harvested by centrifugation. The cellular pellet was resuspended in 500 µL of buffer TE 100 (10 mM Tris/HCl, pH 9, 100 mM EDTA) and incubated for 30 min at 37 ºC. The bacterial suspension was mixed with an equal volume of 2.0% low-melting-point agarose and dispensed into plug molds (Biorad). Agarose plugs were incubated in a lysozyme solution (10 mM Tris/HCl, pH 9, 100 mM EDTA, 5 mg lysozyme ml^{-1}, 0.05 % sarkosyl) for 2 h at 37 ºC, and then incubated overnight at 55 ºC in a lysis solution (10 mM Tris/HCl, pH 9, 100 mM EDTA, 1 mg proteinase K ml^{-1}, 1 % SDS). After lysis, agarose plugs were washed three times in a TE buffer (10 mM Tris/HCl, pH8, 1 mM EDTA) for 1 h at room temperature, where the first washing buffer was supplemented with 100 mM PMSF (Phenylmethylsulfonyl fluoride). For digestion, plugs were equilibrated in incubation buffer containing *XbaI* restriction enzyme (Takara) overnight. Pulsed-field gel electrophoresis was conducted in a CHEF-DRIII apparatus (Bio-Rad). Gels (1% agarose) were run at 14 ºC for 24 h in TBE buffer (4 mM Tris, 4 mM

borate, 1 mM EDTA, pH 8.3) at 6 V cm^{-1}. Pulse times were increased from 10 to 30 s. *XbaI* restriction fragments of *Salmonella enterica* serovar Braenderup H9812 were used as size markers. Cluster analysis using Dice similarity indices was done in BioNumerics 6.6 software (at 0.5% tolerance and 0.5% optimization) (Applied Maths, Ghent, Belgium) to generate a dendrogram describing the relationships among PFGE profiles.

Multilocus Sequence Typing (MLST). The phylogenetic relationships between strains were studied using MLST method initially described by Maiden et al. [14] and *E. coli* Achtman's scheme (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/). *E. coli* MLST scheme used internal fragments of seven house-keeping genes: *adk* (adenylate kinase), *fumC* (fumarate hydratase), *gyrB* (DNA gyrase), *icd* (isocitrate/isopropylmalate dehydrogenase), *mdh* (malate dehydrogenase), *purA* (adenylosuccinate dehydrogenase) and *recA* (ATP/GTP binding motif). They were amplified in a total volume of 20 µL containing 4 µL of DNA crude extract as a template, 2.5 U of GoTaq®G2 Flexi DNA polymerase (Promega), 10 pmol of each primer, 5 nmol of deoxynucleoside triphosphate 30 mM $MgCl_2$ in 1x buffer. PCR conditions were as follows: 94°C for 5 min; 30 cycles of 94°C for 40 s, variable annealing temperature (54 °C, 60 °C, 64 °C, 58 °C, 62 °C, 62 °C or 58 °C, respectively) for 45 s, and 72°C for 45 s; and a final extension at 72°C for 5 min. The amplicons were sequenced on both strands and sequence type (ST) of each allele was attributed according to Achtman's scheme. Novel STs described in this work were submitted to the *E. coli* MLST database and identified as ST5856 and ST5766.

Lethality test. Strain virulence was evaluated by a lethality test using 1-day-old chicks as previously described [15]. Lethality test was carried out in the experimental infection unit PFIE (Plateforme d'Infectiologie Expérimentale, INRA Val de Loire). For each strain, groups of five 1-day-old SPF chicks were inoculated subcutaneously with 0.5 mL of an overnight culture in LB-Miller broth without agitation (inoculum in stationary phase was ~10^8 CFU). Mortality was recorded at 4 days post inoculation and the strains were classified as pathogenic when at least one chick died [16]. Avian *E. coli* strains BEN2908 and BEN2269 (a non-pathogenic avian *E. coli* isolate of serogroup O2) were used as positive (5 chicks died) and negative control (no chicks died), respectively. The housing, husbandry and slaughtering conditions conformed to European Guidelines for care and use of laboratory animals. French regional ethics committee number 19 (Comité d'Ethique en Expérimentation Animale Val de Loire) approved this protocol under the reference 2012-11-5.

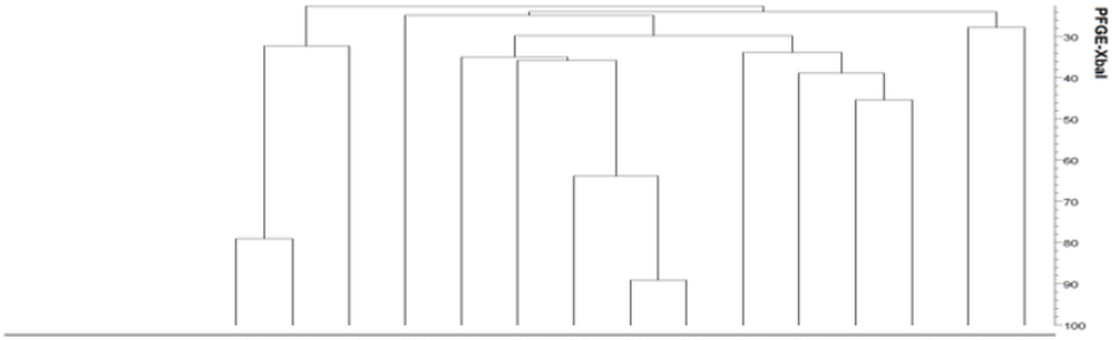
Serum bactericidal test. The serum bactericidal assay was performed as previously described by Dozois et al. [17] with some modifications. Briefly, bacteria were grown overnight in LB broth at 41°C with agitation (180 rpm). Then, bacterial cultures were resuspended in fresh medium (OD_{600} = 0.02), incubated at 41°C with agitation (180 rpm), and harvested during the logarithmic growth phase (DO_{600} = 0.35). Bacteria were washed at room temperature with dulbeco's phosphate-buffered saline (pH 7.0 -7.3) and then resuspended to a concentration of 2x10^6 CFU/mL. A volume of 500 µL of bacterial suspension was added to 500 µL of complement or inactivated (56 ºC, 30 min) SPF chicken serum, which were incubated at 41°C without agitation. Viable cell counts were counted at 0 h and 3 h by plating 10-fold dilutions in sterile saline solution on LB agar plates. Compared to the bacterial count in inactivated serum, a strain was considered resistant when the bacterial count increased or did not change, intermediate when the bacterial count decreased up to one order of magnitude, and sensitive when bacterial count decreased more than one order of magnitude. Serum resistant (BEN2908) and serum sensitive (BEN4134) *E. coli* strains were used as positive and negative controls.

Antibiotic susceptibility testing. Susceptibility testing was performed by the disk diffusion method according to the guidelines of the Antibiogram Committee of the French Society of Microbiology (http://www.sfm-microbiologie.org). The antibiotics tested belong to seven different classes: aminoglycosides (gentamicin, Gen; neomycin, Neo; apramycin, Apr), beta-lactams (amoxicillin, Amx; amoxicillin + clavulanic acid, Amc), cephalosporins (cephalotin, Cef; cefoxitin, Fox; ceftiofur, Xnl), phenicols (florfenicol, Ffc), polypeptides (colistin, Cst), quinolones (nalidixic acid, Nal; flumequine, UB; enrofloxacin, Enr), sulfonamides (trimethoprim, Tmp; Tmp + sulfamethoxazole, TmpStx), and tetracyclines (tetracycline, Tet). The presence of extended spectrum β-lactamases (ESBL) was detected by double-disk synergy method [18]. *E. coli* ATCC 25922 strain was used as quality control.

Results

Epidemiological features of *E. coli* strains and PFGE. *E. coli* strains were isolated from eight flocks in the municipalities of Belo Horizonte, Bom Jesus de Amparo, Igarapé, Igaratinga, Itabira and São Sebastião do Oeste, all located in the state of Minas Gerais, Brazil. Management and biosecurity practices varied among the farms, with broilers number per flock ranging from 20,000 to 40,000. Broiler farms usually raise broilers up to approximately 42 to 45 days before processing them. Broilers studied were 40 to 56 days-old (average of 46 days-old). Antibiotics usage in sampled farms included enrofloxacin, fosfomycin, amoxicillin, and trimethoprim sulfa, which were most commonly used to treat respiratory or enteric diseases. Antibiotics such as zinc bacitracin and colistin were confirmed to be frequently used as growth promoters, although information on its use was not available for all farms.

The fifteen *E. coli* strains presented different genetic profiles and revealed to be highly diverse, even for same flock isolates (Fig. 1).



		EC19	EC02	EC18	EC11	EC03	EC10	EC15	EC13	EC12	EC20	EC09	EC08	EC01	EC17	EC06	
Flock ID		7	1	6	4	2	3	5	5	5	8	3	3	1	6	2	
Lesion		VO	VO	Art	VO	Art	VO	VO	Art	Art	Art	VO	VO	Art	VO	VO	
Serotype		O88	O88	ns	ns	O25	ns	ns	O12	O12	ns	ns	ns	ns	O45	ns	
Flagella		H8	H8	nm	nc	H4	H4	H4	H4	H4	H4	H4	H4	H4	nm	H4	
ST		101	101	5766	3107	131	117	117	117	117	117	117	117	117	371	5856	
ECOR		B1	B1	A	A	B2	D	D	D	D	D	D	D	D	D	D	
APEC (Johnson et al.)		Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	
APEC (Schouler et al.)		No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	
pVAGs		nc	nc	nc	nc	B	B	B	B	B	B	B	B	B	B	B	
Iron aquisition	iutA	■	■			■	■	■	■	■	■	■	■	■	■	■	*86.7%*
	iucD	■	■			■	■	■	■	■	■	■	■	■	■	■	*86.7%*
	iroN	■	■			■	■	■	■	■	■	■	■	■	■		*80.0%*
	chuA					■	■	■	■	■	■	■	■	■	■	■	*73.3%*
	ireA	■	■				■	■	■	■	■	■	■	■	■		*73.3%*
	fyuA					■	■	■	■	■	■	■	■				*53.3%*
	irp2					■	■	■	■	■	■	■					*46.7%*
	sitA	■	■		■	■										■	*33.3%*
Adhesin	fimA	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	*100.0%*
	fimH	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	*100.0%*
	csgA	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	*100.0%*
	aatA	■	■		■		■	■	■	■	■	■	■	■	■	■	*86.7%*
	tsh	■	■				■	■	■	■	■	■		■	■		*67.0%*
	hra													■			*6.7%*
	P(F11)																*0.0%*
	fimAvmt78																*0.0%*
	iha																*0.0%*
	sfaA																*0.0%*
	focG																*0.0%*
Toxin	hlyF	■	■			■	■	■	■	■	■	■	■	■	■		*80.0%*
	vat						■	■	■	■	■	■	■	■			*53.3%*
	astA	■	■						■	■		■					*33.3%*
	clbB																*0.0%*
	clbN																*0.0%*
	sat																*0.0%*
	hlyA																*0.0%*
	cnf1																*0.0%*
	cnf2																*0.0%*
Protectin	traT	■	■			■	■	■	■	■	■	■	■	■	■	■	*86.7%*
	iss	■	■			■	■	■	■	■	■		■	■	■		*73.3%*
	neuC					■											*66.7%*
	cva/cvi	■	■								■	■	■	■		■	*40.0%*
	pic						■				■	■		■			*26.7%*
	kpsMT II					■										■	*13.3%*
Invasin	ibeA					■											*6.7%*
	tia			■													*6.7%*
Miscellaneous	ompT	■	■			■	■	■	■	■	■	■	■	■	■		*80.0%*
	frz orf4					■	■	■	■	■	■	■	■	■	■	■	*73.3%*
	malX					■										■	*13.3%*
	aec26																*0.0%*
VAGs (%)		*40.0% (16/40)*	*40.0% (16/40)*	*10.0% (4/40)*	*12.5% (5/40)*	*47.5% (19/40)*	*47.5% (19/40)*	*45.0% (18/40)*	*47.5% (19/40)*	*47.5% (19/40)*	*50.0% (20/40)*	*47.5% (19/40)*	*42.5% (17/40)*	*47.5% (19/40)*	*37.5% (15/40)*	*32.5% (13/40)*	
Lethality score		3	0	0	0	5	4	5	2	4	2	5	3	1	5	1	
Serum resistance		R	S	I	I	R	R	R	I	S	R	R	S	S	R	R	
N° resistant AB		2	9	7	0	4	6	8	6	6	8	2	4	9	4	1	
Antibiotic resistance profile		NalUB	Neo Amx CefXnl NalUB TmpSxt Tet	Amx CefXnl NalUB Enr Tet	PanSus	Gen Amx NalUB Tet	Amx CefXnl NalUB Enr	Amx Ffc NalUB Enr TmpSxt Tet	Amx NalUB Enr TmpSxt	Amx CefXnl NalUB Enr	Gen Amx CefXnl NalUB TmpSxt	NalUB	AmxAmc CefFox	AmcAmc Cef NalUB Enr TmpSxt Tet	Amx CefXnl NalUB	Gen	

Figure 1. Molecular and phenotypic characterization of 15 *Escherichia coli* strains isolated from broilers with osteomyelitis and arthritis. Black and white boxes represent positive and negative results, respectively. *Flock ID*, number of the flock of origin; *Lesion*, *VO*: vertebral osteomyelitis, *Art*: arthritis; *Serotype*, *ns*: non-serotyped; *Flagella*, *nm*: non-motile, *nc*: non-correspondent to any flagellar type tested; *ST*, Sequence type; *ECOR*: ECOR phylogenetic group; *APEC (Johnson et al.)*: APEC diagnosis according to Johnson et al. (2008); *APEC (Schouler et al.)*; APEC diagnosis according to Schouler et al. (2012); *Yes*: APEC strain, *No*: non-APEC strain; *pVAGs*, pattern of virulence genes described by Schouler et al. (2012), *nc*: non-correspondent to the described patterns; *Iron acquisition*, genes encoding iron acquisition system; *Adhesin*, genes encoding adhesins; *Toxin*, genes encoding toxins; *Protectin*, genes encoding protectins; *Invasin*, genes encoding invasins; *Miscellaneous*, genes encoding different kinds of virulence; *VAGs (%)*, percentage of APEC-associated virulence genes; *Lethality score*, number of chicks that died at the fourth day post-infection with *E. coli*; *Serum resistance*, *R*: serum resistant strain, *I*: intermediate resistant strain, *S*: serum sensitive strain; *Nº resistant AB*: number of antibiotics to which the strain was resistant; *Antibiotic resistance profile*: gentamicin, Gen; neomycin, Neo; apramycin, Apr; amoxicillin, Amx; amoxicillin + clavulanic acid, Amc; cephalotin, Cef; cefoxitin, Fox; ceftiofur, Xnl; florfenicol, Ffc; colistin, Cst; nalidixic acid, Nal; flumequine, UB; enrofloxacin, Enr; trimethoprim, Tmp; Tmp + sulfamethoxazole, TmpStx; tetracycline, Tet; pansusceptible, PanSus.

Clinic and pathological findings of the diseases

Vertebral osteomyelitis. The clinic and pathological findings and the total of broilers examined were previously described in details by Braga et al. [6]. Clinically, affected broilers presented partial or total gait impairment according to the degree of vertebral body enlargement (T4 vertebra) and consequent spinal cord compression, which varied from mild to severe (Fig. 2a). Gross lesions were characterized by caseonecrotic osteomyelitis with protrusion of affected vertebral body and spinal cord compression (Fig. 2b, 2c). Histopathological evaluation of affected vertebral body included caseonecrotic osteomyelitis frequently associated with intralesional Gram-negative bacteria, besides degeneration and necrosis of overlying spinal cord (Fig. 3a).

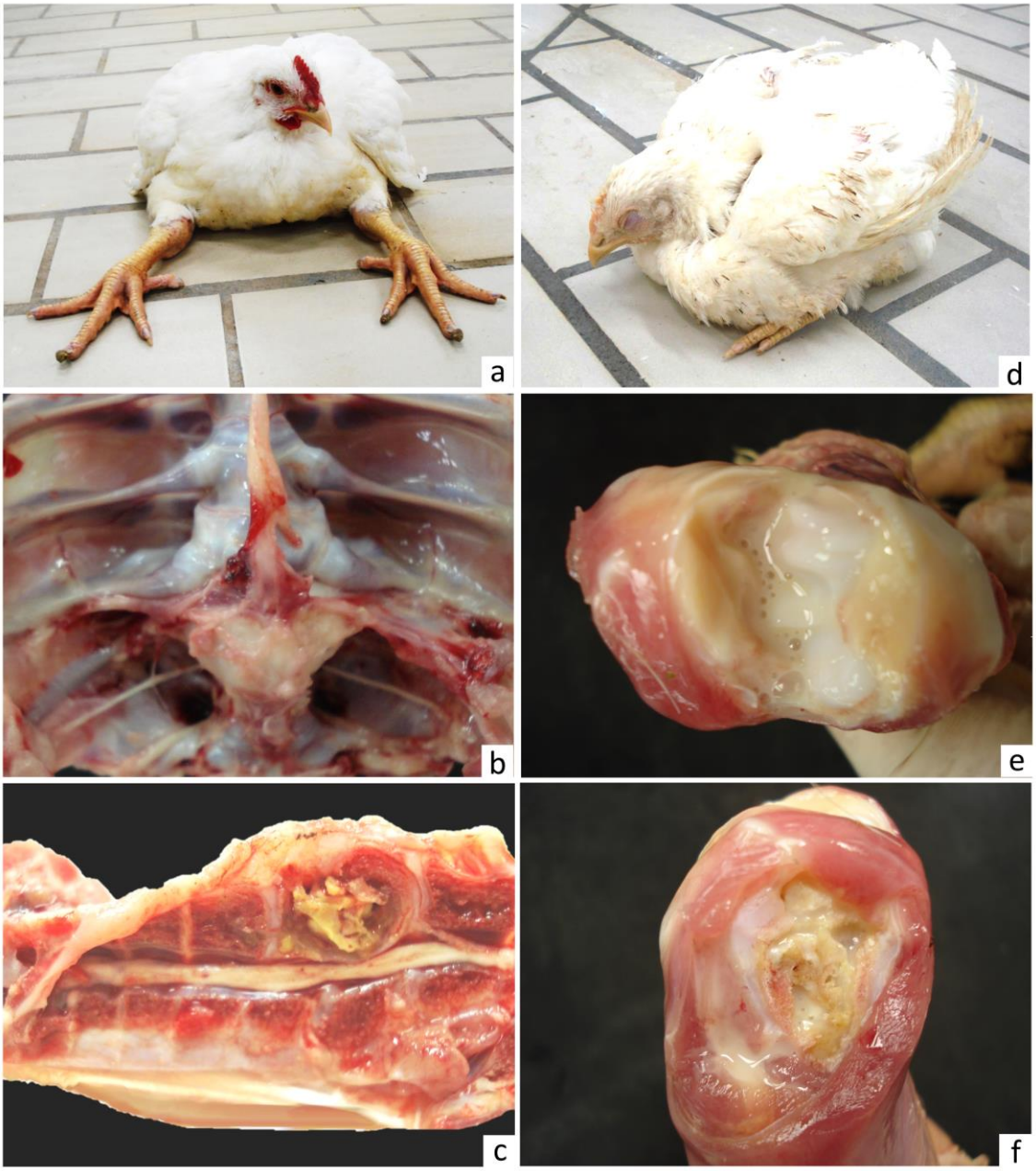


Figure 2. Clinical signs and gross pathology of vertebral osteomyelitis (a, b, c) and arthritis (d, e, f) in broilers. (a) Broiler showing the classical clinical sign of severe cases of vertebral osteomyelitis. (b) Note the enlargement of affected vertebral body (T4), (c) which revels caseonecrotic material and spinal cord compression on longitudinal section. (d) Broiler with bilateral arthritis showing ventral recumbency and retracted legs. (e) Suppurative exudate in articular cavity in acute arthritis, (f) which extended to proximal tibiotarsus causing tibial osteomyelitis.

Arthritis. Broilers presented different degrees of limited mobility depending on the joint lesion site (unilateral or bilateral). When there was involvement of only one leg, broilers could stay in station, although avoiding to support the affected limb on the floor. In bilateral cases, birds often remained in ventral recumbency, with retracted members and supporting their

weight on the pectoral muscles (Fig. 2d). Gross evaluation showed swollen of affected joints and, on cut surface, the aspect of lesions varied according to the course of disease. In acute lesions, there was mild to moderate suppurative exudate within synovial fluid and involving articular capsule, occasionally extending to adjacent tendon sheaths, musculature, and subcutaneous tissue (Fig. 2e). In one case, the inflammatory process extended to adjacent proximal tibiotarsus leading to tibial osteomyelitis characterized by indistinct growth plate and metaphysis, which were replaced by necrosuppurative exudate (Fig. 2f). In chronic cases, there was moderate to severe caseofibrinous arthritis. Occasionally, acute arthritis in an antimere and chronic arthritis in the contralateral antimere were observed in the same broiler. Histopathological analysis of acute lesions revealed moderate to intense fibrinoheterophilic and histiocytic arthritis. In chronic cases, there was caseonecrotic heterophilic and histiocytic arthritis, often associated with myriads of intralesional bacterial colonies (Fig. 3b). Furthermore, necrotic synovitis and synovial hyperplasia were occasionally observed (Fig. 3c). In lesions with greater intensity and extension, involvement of adjacent periarticular structures was characterized by degeneration and necrosis of skeletal muscles or osseous tissue (Fig. 3d) associated with hyperemia, infiltration of heterophils and macrophages and fibrin. In addition, proliferation of fibrous tissue in the articular capsule and adjacent tissue was found in more advanced cases.

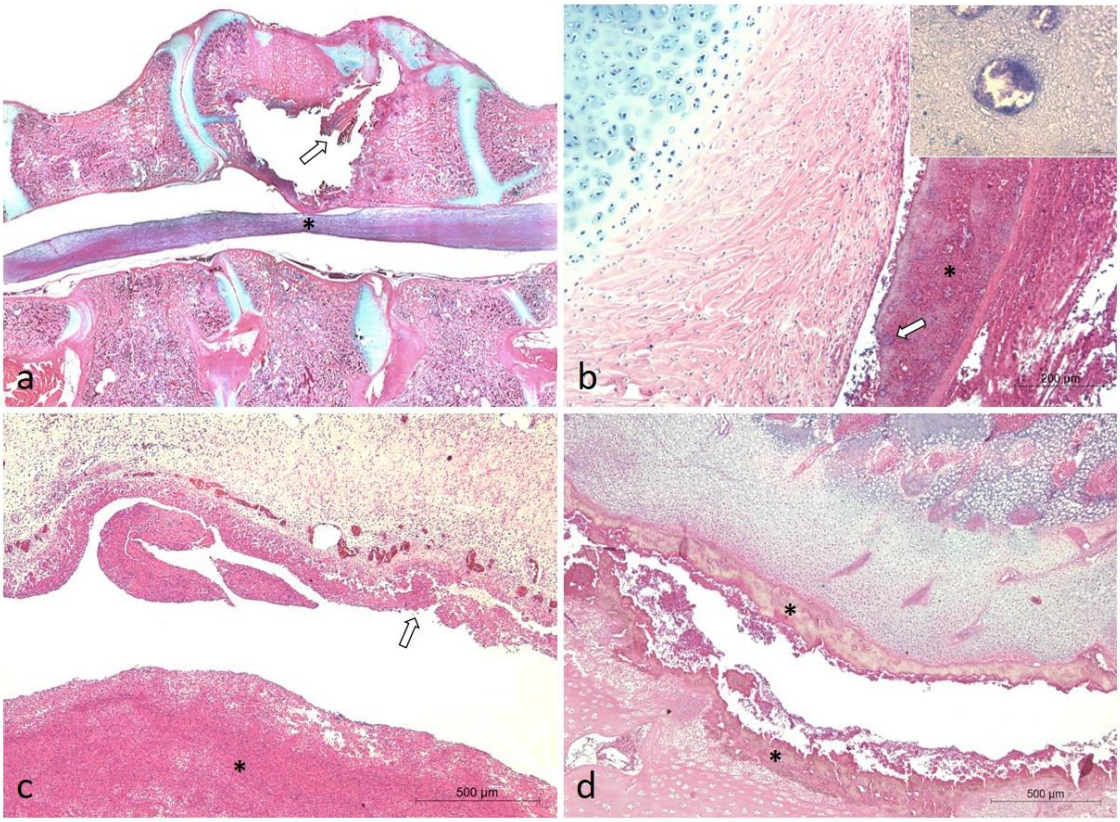


Figure 3. Histopathology of osteomyelitis and arthritis in broilers. (a) Vertebral osteomyelitis showing enlargment of vertebral body (T4) by caseonecrotic material (remanescent, arrow), which compresses spinal cord (*); HE. (b) Caseonecrotic hererophilic and histiocytic exudate (*) in the articular space with intralesional bacterial colonies (arrow); HE. *Inset*: Gram-negative bacteria stained by Goodpasture. (c) Necrotic synovitis (arrow) associated with caseonecrotic exudate within the articular space (*); HE. (d)

Proximal growth plate (physis) of tibiotarsus showing extensive necrosis (*) with heterophilic exudate in a case of tibial osteomyelitis; HE.

APEC diagnosis. According to lethality test, 12 *E. coli* strains were considered as APEC, since four strains killed 5 out 5 chicks, two strains killed 4 out 5 chicks, two killed 3 out 5 chicks, two killed 2 out 5 chicks, and two killed 1 out 5 chicks. One strain (EC02) did not kill any chick, but was considered as APEC according to Johnson et al. [7], which classify an *E. coli* strain as pathogenic based on the presence of minimum four out of five virulence genes carried by plasmids associated with highly pathogenic APEC. The results of molecular tests previously described to diagnose APEC showed an agreement of 80.0% (12/15) (Fig. 1). According to Johnson et al. [7] and Schouler et al. [8], 10 *E. coli* strains were considered APEC and two were considered as non-pathogenic strains. Although there were three discrepancies between both tests, these three APEC strains were diagnosed alternatively by one of the tests and the final criteria for APEC diagnosis was the lethality test.

Group O serotyping and flagella. Different serogroups were detected among the strains, mainly O12 (13.3%), O88 (13.3%), O25 (6.7%), and O45 (6.7%) (Fig. 1). High percentage (60.0%) of strains did not correspond to any of the O somatic antigen surveyed in this study, and were classified as non-serotyped (NS). The most prevalent flagellar types were H4 (66.7%) and H8 (13.3%), and only for one motile strain it was not possible to identify the corresponding flagella (Fig. 1). H4 was detected in O12, O25 and non-serotyped strains, while O88 strains were H8.

MLST and ECOR phylogroups. The strains were assigned to seven different sequence types (STs) (Fig. 1). Most strains (86.7%, 13/15) were grouped in known STs, while 13.3% (2/15) were new STs described in this work. The most frequent ST was ST117 and represented 53.3% (8/15) of *E. coli* strains, followed by ST101 (13.3%, 2/15). ST131, ST371 and ST3107 were identified once each. When classified into ECOR phylogenic groups, most strains were D (66.7%, 10/15), followed by A (13.3% 2/15), B1 (13.3%, 2/15) and B2 (6.7%, 1/15) (Fig. 1).

Virulence genes profile. APEC strains showed highly variable content of virulence genes, although those responsible for iron acquisition and adhesion were detected more frequently (Fig. 1). The non-pathogenic strains showed marked lack of virulence genes when compared to APEC strains, with higher content of adhesin encoding genes.

Bactericidal effect of serum. High percentage of *E. coli* strains, 53.3% (8/15), was serum resistant, while 33.4% (5/15) was characterized as serum sensitive and 13.3% (2/15) as intermediate strains (Fig. 1, supplementary data).

Antibiotic resistance profile. The *E. coli* strains studied presented a large diversity of antibiotic resistance profiles (Fig. 1). One *E. coli* strain was pansusceptible, but high percentage (73.0%) of strains were resistant to more than three classes of antibiotics and defined as multidrug-resistant *E. coli*. These eleven multidrug-resistance strains were mainly characterized by resistance to amoxicillin (100.0%), enrofloxacin (54.5%), ceftiofur (54.5%), and tetracycline (45.4%). The *E. coli* strains were more resistant to nalidixic acid (quinolone class) and amoxicillin (beta-lactam class), 80.0% and 73.3% respectively (Fig. 4). Susceptibility or low resistance to polypeptides (0.0%,) and phenicols (6.7%,) were observed. One non-pathogenic *E.*

coli strain (EC18) was suspected to produce an ESBL by the synergy observed between amoxicillin/clavulanic acid and ceftiofur using the disk diffusion method.

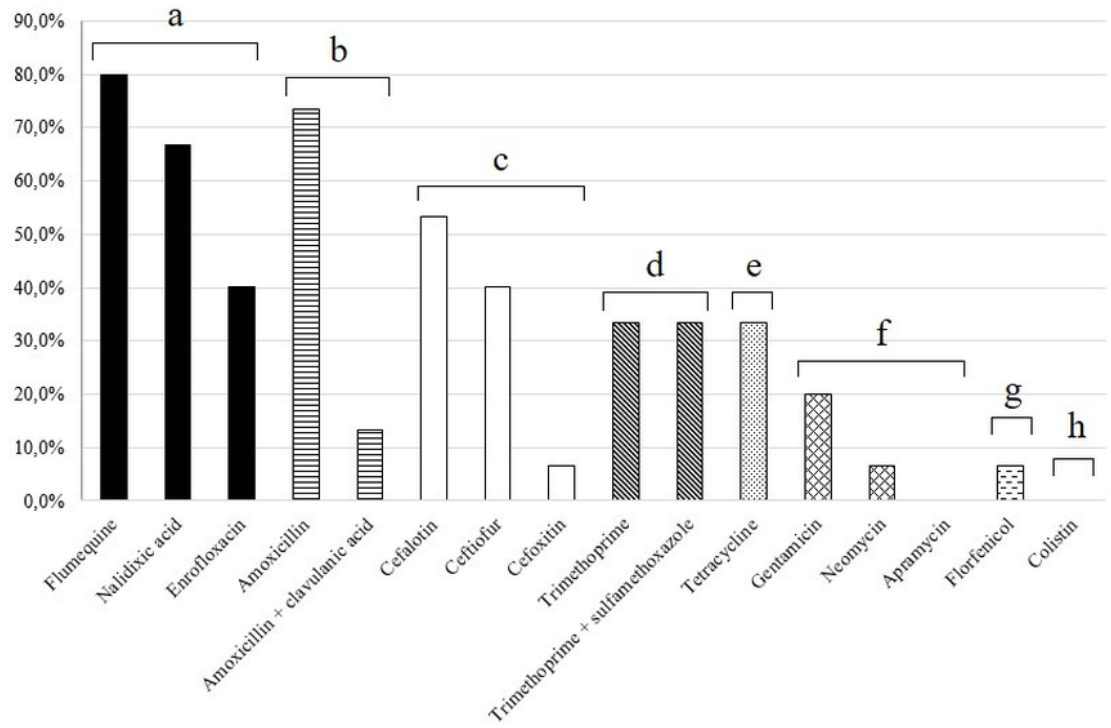


Figure 4. Percentages of antibiotic resistance of *E. coli* strains isolated from vertebral osteomyelitis and arthritis in broilers by antibiotic class: (a) quinolones; (b) beta-lactams; (c) cephalosporins; (d) sulfonamides; (e) tetracyclines; (f) aminoglycosides; (g) phenicols; and (h) polypeptides.

Discussion

Our results showed that *E. coli* strains involved in vertebral osteomyelitis and arthritis cases in broilers in Brazil are highly diverse. We observed that the same disease (i.e., vertebral osteomyelitis) was caused by genetically diverse *E. coli* strains with different pathogenicity traits in the same flock (flock 3). Furthermore, genetically diverse strains were recovered from different diseases (i.e., vertebral osteomyelitis or arthritis) in the same flock (flocks 2, 5 and 6). These findings show that both diseases are not caused by a unique *E. coli* strain. Other authors also report genetic diverse populations of *E. coli* in field cases of colibacillosis in a single flock [19] or in different flocks [20].

E. coli is one of the bacteria described as etiological agent of vertebral osteomyelitis [5]. Recent data on etiological agents of this disease in broilers described involvement of single or mixed bacteria including *Enterococcus* spp., *E. faecalis*, *E. hirae* and *Staphylococcus aureus*, besides *E. coli* [6]. This feature can be similar to what have been previously proposed on turkey osteomyelitis complex (TOC), in which bacterial arthritis and osteomyelitis are associated to involvement of many different opportunistic microorganisms, suggesting that is likely to be influenced by factors such as immunosuppression rather than by the pathogenicity intensity of these bacteria [21].

Diversity in serogroups among *E. coli* strains was also remarkable, as exemplified by the detection of serogroup O12, which up to now was not described in Brazilian *E. coli* strains from human or animal origin. Strains belonging to this serogroup exhibited profile O12:H4-ST117 and were isolated from two broilers from the same flock presenting only arthritis. Previous studies on serogroup determination of *E. coli* isolated from septicemic and healthy broilers revealed that O12 was involved in only 1% of colisepticemia cases, but was one of the serogroups predominantly identified among septicemic *E. coli* [22]. O12 *E. coli* strain was also reported in human, isolated from an immunocompetent woman with a history of repeated amnion infections and spontaneous abortion [23].

An *E. coli* strain O45:HNM-D-ST371 was also detected in this study. This type of strain has been described previously in 16.4% (9/55) of O45 *E. coli* strains isolated from avian colibacillosis cases in Europe and it was identified only in O45 *E. coli* strains of avian origin, different from O45:K1:H7-B2-ST95 identified in avian and human *E. coli* isolates [24]. However, this last one was not detected in Brazilian APEC strains described here and in previous studies [25].

ST117, which represents more than half of our *E. coli* strains, and ST131 were involved in osteomyelitis and arthritis cases. These STs are commonly shared by APEC and human ExPEC strains [25]. Close genetic relations have been detected in ST117 *E. coli* strains of animal and human origin, which have been identified in large poultry producers such as Brazil [25], USA [26], and also Egypt [27], Denmark [28], Sri Lanka [29], and South Korea [30].

We also identified two ST101 APEC strains, which belonged to phylogroup B1, serotype O88:H8 and were non-ESBL as evaluated by the disk diffusion method. This ST was not related to infections caused by APEC until recently, when one O15:H10-B1-ST101 APEC strain was isolated from colibacillosis associated lesions in Spain [20].

ST131 is a globally disseminated multidrug resistance clone, responsible for urinary tract and bloodstream infections in humans. Its rapid emergence and successful spread is strongly associated with antibiotic resistance [31,32,33]. One O25:H4-B2-ST131 *E. coli* strain was detected in a broiler joint with arthritis in this study. In Brazil, this clone was previously detected in APEC strains recovered from broilers with different visceral lesions [34] and from APEC and human ExPEC collections [25]. Although few data are available on this clonal group from poultry, Mora et al. [35] reported an increasing presence of clonal group O25b:H4-ST131 in retail chickens. Interestingly, a retail chicken sample revealed macrorestriction profile indistinguishable from an *E. coli* strain isolated from a human with urinary tract infection [36].

High percentage of multidrug resistant *E. coli* was detected in this study. It is known that *E. coli* strains isolated from poultry frequently show resistance to more than one antimicrobial drug [37], which represents a global concern. It has been shown that poultry workers may have increased risk of carrying multidrug-resistant *E. coli*, which demonstrates that occupational exposure to antimicrobial-resistant *E. coli* from live-animal contact in the broiler industry may be an important route of entry for antimicrobial-resistant *E. coli* into the community [38].

Most *E. coli* strains analyzed in this study exhibited resistance to at least one antibiotic from different main classes: beta-lactams, cephalosporins and quinolones. Resistance to these antibiotic classes is a chronic problem described for avian *E. coli* strains isolated in Brazil [34,39]. A concern is the increasing resistance to ceftiofur, which was evident when we compared our *E. coli* strains to those isolated from broilers in previous years in Brazil [34]. This finding is probably the result of increasing usage of this drug in poultry and highlights the need for responsible and controlled use of antibiotics in animals. A major public health concern is that the use of third-generation cephalosporins, such as ceftiofur, in food animals is leading to

resistance to other extended-spectrum cephalosporin molecules, which are used in the treatment of many different human infections [40].

Tetracycline resistance level of the *E. coli* strains studied was lower than that described in other regions of Brazil [34,39] and in other countries, such as China, where resistance to tetracycline can reach about 90% [41]. For many years, tetracycline was used as prevention and as growth promoter in poultry, but the use of antibiotics with these purposes was banned since 2009 in Brazil. In the state of Minas Gerais, where samples were collected, tetracycline use has no longer being recommended by poultry veterinarians due to its prohibition and bacterial resistance (personal information). These data suggest that the discontinued use of tetracycline in poultry in the region may have provided an increase in the number of *E. coli* strains susceptible to this drug, as described for *Salmonella* strains in USA, where it was observed significant reduction of humans and swine strains resistant to tetracycline after its prohibition as prophylactic drug in animal feed [42].

All 15 *E. coli* strains studied were isolated from the exudate of osteomyelitis or arthritis lesions. The strains EC11 and EC18 classified as non-pathogenic were also isolated from broilers with vertebral osteomyelitis and arthritis, respectively. In these cases, necrotic and inflammatory lesions were associated with bacterial colonies constituted by Gram negative rods, including strains classified as non-APEC. The single or double colonies picked up from the pure culture of *E. coli* probably resulted in the selection of a non-APEC clone, once it is known that in the same lesion it is possible to find distinct *E. coli* clone populations. In order to avoid the selection of a non-representative bacterial clone, it is recommended to select and mix several colonies from the pure culture isolated from the lesion for further evaluation [43]. This procedure provides more efficient results, especially regarding to antimicrobial susceptibility, since it can reduce a possible variation in the susceptibility of isolated clones and improve the selection of antibiotics for treatment.

The broilers had no additional gross lesions in other sites at necropsy, except in two: one exhibited vertebral osteomyelitis and intertarsal arthritis and another broiler had intertarsal arthritis with osteomyelitis in proximal tibiotarsus of the same antimere. Although the information on previous respiratory disease was not available for all flocks studied, it is known that colibacillosis is frequent in broilers of the region (laboratory and field observations). Localization of *E. coli* in bones and synovial tissues is a common sequel of colisepticemia [3]. Some studies with turkeys demonstrated that often multiple sites are involved and the bones most often affected are tibiotarsus, femur, thoracolumbar vertebra, and humerus [44]. Bacteria colonizing the vascular sprouts that invade the physis of a growing bone, provoke an inflammatory response that results in osteomyelitis. Transphyseal blood vessels in birds serve as conduits for the process to spread bacteria into the joint and surrounding soft tissues [45].

Conclusion

Our results showed that highly diverse *E. coli* strains can be recovered from osteomyelitis and arthritis in broilers, even in the same flock. Based on molecular and phenotypic characteristics, there is no specific virulence pattern of the *E. coli* associated with vertebral osteomyelitis or arthritis. Some of the strains involved in these diseases are belonged to STs commonly shared by animals and humans, similar to others previously isolated from different lesions in broilers. Most of these strains are multidrug resistant, with increasing rates of ceftiofur resistance, which is a public and animal health concern. These findings highlight the importance of appropriate management practices, which are valuable in preventing and controlling colibacillosis, thus reducing the need for antibiotics use in animals.

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Competing interests. The authors declare that they have no competing interests.

Authors' contributions. RE, JFVB and CS designed of the study. JFVB and RE performed sampling and pathological analysis. JFVB, NKC, AT, RG and CS performed the tests for molecular and phenotypic characteristics of *E. coli*, except antibiotic resistance test, which was performed by JFVB and SB. All the authors reviewed the literature, read and approved the final version of the manuscript.

References

1. Dale AP, Woodford N. Extra-intestinal pathogenic *Escherichia coli* (ExPEC): Disease, carriage and clones. J Infect. 2015;71: 615-626. DOI http://dx.doi.org/10.1016/j.jinf.2015.09.009
2. La Ragione RM, Woodward, MJ. Virulence factors of *Escherichia coli* serotypes associated with avian colisepticaemia. Res Vet Sci. 2002; 73:27–35. DOI http://dx.doi.org/10.1016/S0034-5288(02)00075-9
3. Barnes HJ, Nolan LK, Vaillancourt JP. Colibacillosis. In: Saif YM, Fadly AM, Glisson JR, McDougald LR, Nolan LK, Swayne DE (Ed.), Diseases of Poultry, Blackwell Publishing, Ames (Iowa); 2008. p. 691-732.
4. Bradshaw RH, Kirkden RD, Broom DM. A review of the aetiology and pathology of leg weakness in broilers in relation to welfare. Avian Poult Biol Rev. 2002; 13:45–103. DOI http://dx.doi.org/10.3184/147020602783698421.
5. Riddell C. Leg problems still important. Poult. Diag. 1997;56:28–31.
6. Braga JFV, Silva CC, Teixeira MPF, Martins NRS, Ecco R. Vertebral osteomyelitis associated with single and mixed bacterial infection in broilers. Avian Path. In press.
7. Johnson TJ, Wannemuehler Y, Doetkott C, Johnson SJ, Rosenberger SC, Nolan LK. Identification of minimal predictors of Avian Pathogenic *Escherichia coli* virulence for use as a rapid diagnostic tool. J Clin Microbiol. 2008:46:3987–3996. DOI http://dx.doi.org/10.1128/JCM.00816-08.
8. Schouler C, Schaeffer B, Brée A, Mora A, Dahbi G, Biet F, Oswald E, Mainil J, Blanco J, Moulin-Schouleur M. Diagnostic strategy for identifying avian pathogenic *Escherichia coli* based on four patterns of virulence genes. J Clin Microbiol. 2012;50:1673–78. DOI http://dx.doi.org/10.1128/JCM.05057-11.
9. Blanco M, Blanco JE, Blanco J, González EA, Mora A, Prado C, Fernández L, Rio M, Ramos J, Alonso MP. Prevalence and characteristics of *Escherichia coli* serotype O157:H7 and other verotoxin-producing *E. coli* in healthy cattle. Epidemiol Infect. 1996;117**:** 251-7. DOI http://dx.doi.org/10.1017/S0950268800001424.

10. Clarke MB, Sperandio V. Transcriptional regulation of *flhDC* by QseBC and σ^{28} (FliA) in enterohaemorrhagic *Escherichia coli*. Mol Microbiol. 2005;57:1734-49. DOI http://dx.doi.org/10.1111/j.1365-2958.2005.04792.
11. Clermont O, Bonacorsi S, Bingen E. Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl Environ Microbiol. 2000; 66:4555–8. DOI http://dx.doi.org/10.1128/AEM.66.10.4555-4558.2000
12. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York. 1989.
13. Moulin-Schouleur M, Schouler C, Tailliez P, Kao MR, Brée A, Germon P, Oswald E, Mainil J, Blanco M, Blanco J. Common virulence factors and genetic relationships between O18:K1:H7 *Escherichia coli* isolates of human and avian origin. J Clin Microbiol. 2006;44: 3484–2. DOI http://dx.doi.org/10.1128/JCM.00548-06
14. Maiden MC, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, Zhang Q, Zhou J, Zurth K, Caugant DA, Feayers IM, Achtman M, Spratt BG. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci. 1998;95:3140–5. DOI http://dx.doi.org/10.1073/pnas.95.6.3140
15. Dho M, Lafont JP. Adhesive properties and iron uptake ability in *Escherichia coli* lethal and nonlethal for chicks. Avian Dis. 1984;28: 1016–25. DOI http://dx.doi.org/10.2307/1590278.
16. Dozois CM, Dho-Moulin M, Brée A, Fairbrother JM, Desautels C, Curtiss R. Relationship between the Tsh autotransporter and pathogenicity of avian *Escherichia coli* and localization and analysis of the *tsh* genetic region. Infect Immun. 2000; 68:4145–54. DOI http://dx.doi.org/10.1128/IAI.68.7.4145-4154.2000.
17. Dozois CM, Fairbrother JM, Harel J, Bossé M. Pap-and pil-related DNA sequences and other virulence determinants associated with *Escherichia coli* isolated from septicemic chickens and turkeys. Infect Immun. 1992; 60:2648–2656. DOI http://dx.doi.org/ 0019-9567/92/072648-09$02.00/0.
18. Jarlier V, Nicolas M, Fournier G, Philippon A, Jarlier V, Nicolas M, Fournier G, Philippon A. Extended broad-spectrum, 3-lactamases conferring transferable in resistance to newer 13-lactam hospital agents Enterobacteriaceae: prevalence and susceptibility patterns P-lactamase of nosocomial infections. Rev Infect Dis. 1998;10:867–878. DOI http://dx.doi.org/10.1093/clinids/10.4.867.
19. Ozaki H, Murase T. Multiple routes of entry for *Escherichia coli* causing colibacillosis in commercial layer chickens. J. Vet. Med. Sci. 2009;71:1685–9. DOI http://dx.doi.org/10.1292/jvms.001685
20. Solà-Ginés M, Cameron-Veas K, Badiola I, Dolz R, Majó N, Dahbi G, Viso S, Mora A, Blanco J, Piedra-Carrasco N, González-López JJ, Migura-Garcia L.Diversity of multi-drug resistant Avian Pathogenic *Escherichia coli* (APEC) causing outbreaks of colibacillosis in broilers during 2012 in Spain. PLoS One 10, e0143191. 2015. DOI http://dx.doi.org/10.1371/journal.pone.0143191
21. Huff GR, Huff, WE, Rath NC, Balog JM. Turkey osteomyelitis complex. Poult. Sci. 2000; 79:1050–1056. DOI http://dx.doi.org/10.1093/ps/79.7.1050.
22. Blanco JE, Blanco M, Mora A, Jansen WH, García V, Vázquez ML, Blanco J. Serotypes of *Escherichia coli* isolated from septicaemic chickens in Galicia (Northwest Spain). Vet Microbiol. 1998;61:229–35. DOI http://dx.doi.org/10.1016/S0378-1135(98)00182-5.

23. Blum-Oehler G, Heesemann J, Kranzfelder D, Scheutz F, Hacker J. Characterization of *Escherichia coli* serotype O12:K1:H7 isolates from an immunocompetent carrier with a history of spontaneous abortion and septicemia. Eur J Clin Microbiol Infect Dis. 1997;16: 153–5. DOI http://dx.doi.org/10.1007/BF01709475.
24. Mora A, Viso S, López C, Alonso MP, García-Garrote F, Dabhi G, Maman R, Herrera A, Marzoa J, Blanco M, Blanco JE, Moulin-Schouleur M, Schouler C, Blanco J. Poultry as reservoir for extraintestinal pathogenic *Escherichia coli* O45:K1:H7-B2-ST95 in humans. Vet Microbiol. 2013;167:506–2. DOI http://dx.doi.org/10.1016/j.vetmic.2013.08.007
25. Maluta RP, Logue CM, Casas MRT, Meng T, Guastalli EAL, Rojas TCG, Montelli AC, Sadatsune T, Ramos MDC, Nolan LK, Silveira WD. Overlapped sequence types (STs) and serogroups of avian pathogenic (APEC) and human extra-intestinal pathogenic (ExPEC) *Escherichia coli* isolated in Brazil. PLoS One. 2014;9:1–9. DOI http://dx.doi.org/10.1371/journal.pone.0105016
26. Danzeisen JL[1], Wannemuehler Y, Nolan LK, Johnson TJ. Comparison of Multilocus Sequence Analysis and Virulence Genotyping of *Escherichia coli* from Live Birds, Retail Poultry Meat, and Human Extraintestinal Infection. Avian Dis. 2013;57:104-108.
27. Hussein AH, Ghanem IA, Eid AA, Ali MA, Sherwood JS, Li G, Nolan LK, Logue CM. Molecular and phenotypic characterization of *Escherichia coli* isolated from broiler chicken flocks in Egypt. Avian Dis. 2013;57:602–611.
28. Pires-dos-Santos T, Bisgaard M, Christensen H. Genetic diversity and virulence profiles of *Escherichia coli* causing salpingitis and peritonitis in broiler breeders. Vet Microbiol. 2013; 162: 873–880.
29. Dissanayake DRA, Octavia S, Lan R. Population structure and virulence content of avian pathogenic *Escherichia coli* isolated from outbreaks in Sri Lanka. Vet Microbiol. 2014;168: 403–412.
30. Lim JS, Choi DS, Kim YJ, Chon JW, Kim HS, Park HJ, Moon JS, Wee SH, Seo KH. Foodborne Pathogens and Dis. 2015;12:741-8. DOI 10.1089/fpd.2014.1921.
31. Nicolas-Chanoine MH, Blanco J, Leflon-Guibout V, Demarty R, Alonso MP, Caniça MM, Park YJ, Lavigne JP, Pitout J, Johnson JR. Intercontinental emergence of *Escherichia coli* clone O25:H4-ST131 producing CTX-M-15. J Antimicrob Chemother. 2008;61:273-81.
32. Peirano G, Pitout JDD. Molecular epidemiology of Escherichia coli producing CTX-M β–lactamases: the worldwide emergence of clone ST131 O25:H4. Int J Antimicrob Agents. 2010;35:316-21.
33. Rogers BA, Sidjabat HE, Paterson DL. *Escherichia coli* O25b-ST131:pandemic, multiresistant, community-associated strain. J Antimicrob Chemother. 2011;66:1–14. DOI http://dx.doi.org/10.1093/jac/dkq415
34. Barbieri NL, Oliveira AL, de Tejkowski TM, Pavanelo DB, Matter LB, Pinheiro SRS, Vaz ooTMI, Nolan LK, Logue, CM, Brito BG, de Horn F. Molecular characterization and clonal relationships among *Escherichia coli* strains isolated from broiler chickens with colisepticemia. Foodborne Pathog Dis. 2015;12:74–83. DOI http://dx.doi.org/10.1089/fpd.2014.1815
35. Mora A, Herrera A, Mamani R, López C, Alonso MP, Blanco JE, Blanco M, Dahbi G, García-Garrote F, Pita JM, Coira A, Bernárdez MI, Blanco J. Recent emergence of clonal group O25b:K1:H4-B2-ST131 ibeA strains among *Escherichia coli* poultry isolates, including CTX-M-9-producing strains, and comparison with clinical human isolates. Appl Environ Microbiol. 2010;76:6991–7. DOI http://dx.doi.org/10.1128/AEM.01112-10

36. Vincent C, Boerlin P, Daignault D, Dozois C M, Dutil L, Galanakis C, Reid-Smith RJ, Tellier PP, Tellis PA, Ziebell K, Manges A R. Food reservoir for *Escherichia coli* causing urinary tract infections. Emerg Infect Dis. 2010;16:88–95.
37. Zanatta GF, Kanashiro AMI, Castro AGM, Cardoso ALSP, Tessari ENC, Pulici SCP. Suscetibilidade de amostras de *Escherichia coli* de origem aviária a antimicrobianos. Arq Inst Biol. 2004;71:283–286.
38. Price LB, Graham JP, Lackey LG, Roess A, Vailes R, Silbergeld E. Elevated risk of carrying gentamicin-resistant *Escherichia coli* among U.S. poultry workers. Environ. Health Perspect. 2007;115:1738–42. DOI http://dx.doi.org/10.1289/ehp.10191
39. Korb A, Nazareno ER, Costa LD, Nogueira KS, Dalsenter PR, Tuon FFB, Pomba MC. Tipagem molecular e resistência aos antimicrobianos em isolados de *Escherichia coli* de frangos de corte e de tratadores na Região Metropolitana de Curitiba, Paraná. Pesq Vet Bras. 2015; 35:258-264. DOI http://dx.doi.org/10.1590/S0100-736X2015000300008
40. Zhao S, White DG, Mcdermott PF, Friedman S, English L, Ayers S, Maurer JJ, Holland R, Walker RD, Meng J. Identification and expression of cephamycinase bla_{CMY} genes in *Escherichia coli* and *Salmonella* isolates from food animals and ground meat. Antimicrob Agents Ch. 2001;45:3647–50. DOI http://dx.doi.org/10.1128/AAC.45.12.3647.
41. Zhang T, Wang CG, Lv JC, Wang RS, Zhong XH. Survey on tetracycline resistance and antibiotic-resistant genotype of avian *Escherichia coli* in North China. Poult Sci. 2012; 91:2774–7. DOI http://dx.doi.org/10.3382/ps.2012-02453.
42. Manie T, Khan S, Brözel VS, Veith WJ, Gouws PA. Antimicrobial resistance of bacteria isolated from slaughtered and retail chickens in South Africa. Lett. Appl. Microbiol. 1998;26:253–8. DOI http://dx.doi.org/10.1046/j.1472-765X.1998.00312.x
43. Clermont O, Glodt J, Burdet C, Pognard D, Lefort A, Branger C, Denamur E. Complexity of *Escherichia coli* bacteremia pathophysiology evidenced by comparison of isolates from blood and portal of entry within single patients. Int. J. Med. Microbiol. 2013;303:529–532. DOI http://dx.doi.org/10.1016/j.ijmm.2013.07.002
44. Mutalib A, Miguel, B, Brown T, Maslin W. Distribution of Arthritis and Osteomyelitis in Turkeys with Green Liver Discoloration. Avian Dis. 1996;40:661-4. DOI: 10.2307/1592278
45. Bayyari, GR, Huff, WE, Rath, NC, Balog JM, Newberry LA, Villine JD, Skeeles JK. Immune and physiological responses of turkeys with green-liver osteomyelitis complex. Poult Sci. 1997; 76:280-288.

CHAPTER IV

Genetic diversity and antibiotic susceptibility of *Enterococcus faecalis* isolated from vertebral osteomyelitis in broilers

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Abstract: *Enterococcus faecalis* is intestinal commensal bacterium associated to different diseases in poultry and increasing concern in nosocomial infections in human beings. Recently, the bacterium was associated with vertebral osteomyelitis in broilers. The aim of this study was to determine the molecular characteristics and antibiotic susceptibility profile of *E. faecalis* isolated from this disease in broilers in Brazil. We analyzed 12 *E. faecalis* strains isolated from nine flocks of six farms. The genetic relationship among these strains and others isolated elsewhere were studied by MLST and phylogenic tree analysis. The strains were also submitted to antibiotic susceptibility tests to aminoglycosides, penicillin, polypeptides, beta-lactams, glycopeptides, cephalosporins, and penicillin/novobiocin. *E. faecalis* belonged to eight different sequence types (ST). Six (ST49, ST100, ST116, ST202, ST249, and ST300) were previously described and ST708 and ST709 were first identified in this study. ST49 was the most frequently isolated from vertebral osteomyelitis lesions. The strains showed the highest antibiotic resistance to aminoglycoside, mainly resistant to gentamicin (40.0%), and high susceptibility to vancomycin (10.0%). These are the first data of molecular and phenotypic characteristics of *E. faecalis* isolated from vertebral osteomyelitis in broilers. The evident genetic and antibiotic resistance diversity of the strains highlighted the need for studies that contribute to elucidate what remain unclear about both vertebral osteomyelitis in broilers and the role of antibiotic use animal and its implication in animal and human health.

Keywords: poultry; locomotor diseases; *E. faecalis* infection; MLST; antibiotic susceptibility.

Introduction

Enterococcus spp. are gram-positive latic acid bacteria (Wages, 1998) that are ubiquitous in nature with worldwide distribution in avian species, as also in human and other mammalian. Enterococci are common bacteria in the gastrointestinal tract of animals and humans often seen as beneficial commensal organisms (Tannock, 1995). However, they may also be pathogens responsible for serious systemic infections because their antimicrobial resistance and virulence determinants (Wisplinghoff et al., 2004; Heuer et al., 2006).

In poultry, Enterococci are often associated with different diseases. *Enterococcus* sp. are frequently responsible for infection in the yolk sac in one day-old chicks (Deeming, 2005). *E. cecorum* have been associated with vertebral osteomyelitis and/or arthritis in broilers worldwide (Devriese et al., 2002; Wood et al., 2002; Thayer et al., 2008; Herdt et al., 2009; Stalker et al., 2010; Makrai et al., 2011; Robbins et al., 2012; Aitchison et al., 2014). *E. durans* was isolated from bacteremia and encephalomalacia cases in young chickens (Cardona et al., 1993; Abe et al., 2006). *E. hirae* was reported causing osteomyelitis in broilers (Kolbjørnsen et al., 2011), focal cerebral necrosis in chicks (Devriese et al., 1991; Randall et al., 1993), diarrhea in chicks (Kondo et al., 1997) and endocarditis in broilers (Velkers et al., 2011).

E. faecalis has been associated with systemic AA amyloidosis in laying hens (Landman et al., 1994) and broiler breeders (Steentjes et al., 2002), and arthritis in domestic ducks (Bisgaard, 1981). In broilers, Gregersen et al. (2010) compared *E. faecalis* isolated from different lesions in eight broiler breeders flocks with *E. faecalis* isolated from healthy birds. This analysis results in 12 different STs and lack of correlation between ST and lesion type, although ST82, ST174 and ST177 represented 81% of the strains associated with lesions.

In recent years, Enterococci have emerged as major cause of nosocomial infections, particularly *E. faecalis* (Kola et al., 2010), causing extraintestinal infections in humans (Creti et al., 2004). These bacteria have intrinsic resistance to many antibiotics and they acquired new resistance phenotypes, with special concern about vancomycin-resistant enterococci (VRE) (Cetinkaya et al., 2000; Willems and Bonten, 2007), which became a major problem in nosocomial infections. A retrospective study of 10 human patients with non-vertebral osteomyelitis showed that eight of these cases were due to infection by vancomycin-resistant *Enterococcus faecalis* with one death reported due to bacteremia (Holtom et al., 2002).

Specific genetic lineages of hospital-adapted strains have emerged and some *E. faecalis* are considered high-risk enterococci, such as clonal complex CC2, CC9, CC28 and CC40. These strains are characterized by the presence of antibiotic resistance determinants and/or virulence factors usually located on pathogenicity islands or plasmids, which highlights the major role of mobile genetic elements in establishing of problematic strains (Franz et al., 2011). In general, most of *E. faecalis* genetic diversity is attributed to the inclusion of mobile genetic elements (i.e., plasmids and transposons) into a widely conserved genome (Palmer et al., 2012).

Recently, Getachew et al. (2013) studied genetic relationship among *E. faecalis* isolated from human, chicken and pigs, and showed that only one strain isolated from chicken was clonal to that of human. Furthermore, there was evident that VRE were predominantly host specific with clinically important strains found mainly in humans. From these findings, the authors suggested that the infrequent detection of a human VRE clone in a chicken may in fact suggest reverse transmission of VRE from humans to animals.

Resistant bacteria in animals and their by-products and the possible transmission to humans through contamination of carcasses represent a concern in animal and public health (Moreno et al., 2006). These highlight the need for molecular characterization which allows comparison between human beings and animals bacterial strains. Although most reports of vertebral osteomyelitis in broilers have been associated to *E. cecorum*, *E. faecalis* was recently isolated from cases of the disease in Brazil (Braga et al., 2016). This study aimed to provide information based on molecular characteristics and antibiotic susceptibility profile of *E. faecalis* isolated from vertebral osteomyelitis in broilers.

Material and methods

Samples. We analyzed 12 *E. faecalis* isolated from natural cases of vertebral osteomyelitis in broilers previously reported by Braga et al. (2016). These broilers belonged to nine different flocks and six municipalities from the largest poultry production area in the state of Minas Gerais, southeast Brazil. *E. faecalis* was recovered from a total of 31 cases of vertebral osteomyelitis with 608 broilers necropsied. After clinical evaluation, the broilers were submitted to necropsy to search for macroscopic evidence of vertebral osteomyelitis by sagittal section of vertebral column. In such cases, the caseonecrotic material within the affected vertebral body (fourth or free thoracic vertebra and adjacent vertebrae) was used to bacterial isolation and DNA detection. Samples for bacterial isolation were collected aseptically using swab from lesions and vertebral samples were collected in sterile microtubes and frozen at -20 °C for DNA extraction and Polymerase Chain Reaction (PCR) specific for *Enterococcus faecalis*. Histopathology was performed for all cases including special staining to identify bacterial colonies associated with vertebral osteomyelitis (Braga et al., 2016). The procedures in this study were performed in accordance with the recommendations by the Animal Experimentation Ethics Committee of Universidade Federal de Minas Gerais (Protocol 205/2011).

Bacterial isolation and identification. The swabs of the vertebral lesions were inoculated onto two blood agar (BA) and one MacConkey agar (MCK) plates. One BA and the MCK plates were incubated under aerobic conditions, at 37 °C for 24 to 72 hours. The other BA plate was incubated in microaerophilic conditions at the same temperature and time. The morphology of isolated colonies was characterized and they were Gram stained and submitted to catalase and oxidase tests (Teixeira et al., 2007). Bacterial isolates were subjected to automatic bacterial identification by VITEK 2 system (bioMérieux, Inc. Hazelwood, MO, USA), in accordance to the manufacturer's recommendations. After bacterial identification, the colonies were plated on Mueller-Hinton agar (MH) for growth and then inoculated into microtubes containing Brain-Heart Infusion (BHI) broth and 30% glycerol and stored at - 80 °C.

Antibiotic susceptibility profile. *E. faecalis* isolated from vertebral osteomyelitis lesions were submitted to antibiotic susceptibility test as described by CLSI/NCCLS (2008). Briefly, the colonies were inoculated onto MH at 37 °C for 24 hours, and then diluted into Mueller-Hinton broth at the concentration of 1-2 x 10^8 CFU/mL, corresponding to 0.5 McFarland standard. Then, the inoculum was spread on MH plate using a drigalski loop in different directions to ensure a uniform distribution. After that, the discs containing the antibiotics were distributed and the plates incubated at 37 °C for 18 hours. The antibiotic classes tested and the antibiotic concentrations by disk were as follow: aminoglycosides (neomycin, 30 mcg; gentamicin, 10 mcg; gentamicin high-level aminoglycoside resistance - HLAR, 120 mcg; streptomycin HLAR, 300 mcg), penicillin (ampicillin, 10 mcg), polypeptides (bacitracin, 10 IU), beta-lactams (amoxicillin, 10 mcg), glycopeptides (vancomycin, 30 mcg), cephalosporins (ceftiofur, 30 mcg), and penicillin/novobiocin (40 mcg).

DNA extraction. To detect *E. faecalis* DNA in vertebral lesions, **t**otal DNA was extracted directly from caseonecrotic material using the method previously described by Vogelstein and Gillespie (1979) and Boom et al. (1999). Briefly, tissue samples were ground in a mortar and pestle combined with three volumes of 6M sodium iodide and then the total DNA was recovered on silicon dioxide microspheres. Also, *E. faecalis* DNA was extracted directly of the reference colonies (CCCD-E006, Cefar Diagnostics), used as control for PCR, and of the

bacterial colonies isolated from vertebral osteomyelitis lesions to perform MLST. In these cases, the DNA extraction was performed by boiling (Marques and Suzart, 2004) with modifications. Briefly, *E. faecalis* colonies was taken directly from MHA and transferred with a 10 µL calibrated loop into a microtube containing 300 µL of ultrapure water and homogenized for 10 seconds by vortexing. Then, the microtube was placed in a dry bath at 100 °C for 30 minutes and centrifuged at 14,000 x g for two minutes. The supernatant was placed in a new microtube and stored at -80 °C. The quantity and purity of DNA extracted from vertebral samples and bacterial colonies were assessed by spectrophotometry.

Polymerase Chain Reaction (PCR). The DNA extracted from vertebral lesions was subjected to *E. faecalis* specific PCR using specific primers (*FL-1* 5´-ACTTATGTGACTAACTTAACC-3´ and *FL-2* 5´-TAATGGTGAATCTTGGTTTGG-3´) to amplify a region of *sodA* gene (manganese dependent superoxide dismutase) and amplification protocols previously described by Jackson et al. (2004), which resulted in a 360 base pairs product. PCR reactions were performed using 200 to 300 ng of DNA template on a final volume of 25 µL (PCR Master Mix Promega) in accordance to the manufacturer's recommendations. A reference *E. faecalis* strain (CCCD-E006, Cefar Diagnostics) was used as positive control. As negative control, reactions were performed with all reagents except for template DNA. The final product of each reaction was subjected to electrophoresis in 1.5% agarose gel containing ethidium bromide along with molecular weight marker of 100 bp (LowRanger100bp DNA Ladder Norgen®).

Multilocus Sequence Typing (MLST). The genetic relationships among *E. faecalis* strains were determined by MLST as previously described by Ruiz-Garbajosa et al. (2006). The oligonucleotide sequences and polymerase chain reaction (PCR) conditions were available at *E. faecalis* MLST homepage (http://pubmlst.org/efaecalis/). These PCRs amplified seven housekeeping genes: glucose-6-phosphate dehydrogenase (*gdh*), glyceraldehydes-3-phosphate dehydrogenase (*gyd*), phosphate ATP binding cassette transporter (*pstS*), glucokinase (*gki*), shikimate-5-dehydrogenase (*aroE*), xanthine phosphoribosyltransferase (*xpt*) and acetyl-CoA acetyltransferase (*yiqL*) published at *E. faecalis* MLST homepage. All amplification reactions were performed under the following conditions: initial denaturation at 94 ºC for 5 min; 30 cycles at 94 ºC for 30s, 52 ºC for 30s and 72 ºC for 1min; and final extension at 72 ºC for 7 min. Reactions were performed in 25 µL final volume using PCR Master Mix (Promega) in accordance to the manufacturer's recommendations. The sequences of the Brazilian *E. faecalis* isolates were aligned with sequences of reference strains in BioEdit using CLUSTALW (Thompson et al., 1994). The genetic distance matrix was obtained using Kimura's two-parameter model (Kimura, 1980), and an evolutionary tree was created using the neighbour-joining method (Saitou and Nei, 1987) with Mega6 (Tamura et al., 2013). Bootstrap values from 1000 replicates were displayed as percentages. The allele based evolutionary relatedness of *E. faecalis* was illustrated by construction of a population snapshot with all published STs using the eBURST program available online (http://eburst.mlst.net/v3/mlst_datasets/). New allele was deposited in the *E. faecalis* MLST database at the same website.

Results

The *E. faecalis* strains were isolated from T4 vertebra with osteomyelitis in broilers. Clinicopathological findings of these cases were detailed by Braga et al. (2016). Briefly, affected broilers presented different impaired mobility degrees that correlated to the degree of

spinal cord compression caused by swelling of the vertebral body due to the infectious osteomyelitis. Sagittal section of this lesion revealed whitish to yellowish and friable caseonecrotic material replacing the normal vertebral body and confirmed compression of spinal cord. On the histopathology there were necrosis and inflammation of vertebral body often associated with intralesional bacterial colonies. In some cases, inflammatory cells were represented mainly by neutrophils and fibrin exudate, while others had predominance of macrophages, fibroplasia and neocartilage formation.

MLST analysis revealed high genetic diversity of *E. faecalis* and the analysis of concatenated sequences is represented in Fig. 1. Eight different STs were detected among the strains isolated from vertebral osteomyelitis in broilers. ST49 was the most frequently detected, corresponding to 41.7% (5/12) of the isolates, which belonged to three flocks (1, 4 and 8) in different municipalities (Fig. 2). This ST is the founder of a clonal complex that also includes ST203 and ST309. The other seven *E. faecalis* strains analyzed belonged to seven distinct STs, corresponding to each one to 8.3% (1/12) of the strains. Five of these STs were previously described on *E. faecalis* MLST database, they are: ST100, ST116, ST202, ST249, and ST300. The other two STs were not detected in previous studies and were described here for the first time. Their sequences were deposited on *E. faecalis* online database under the numbers ST708 and ST709. These *E. faecalis* isolates were the only singletons among the strains analyzed (Fig. 3).

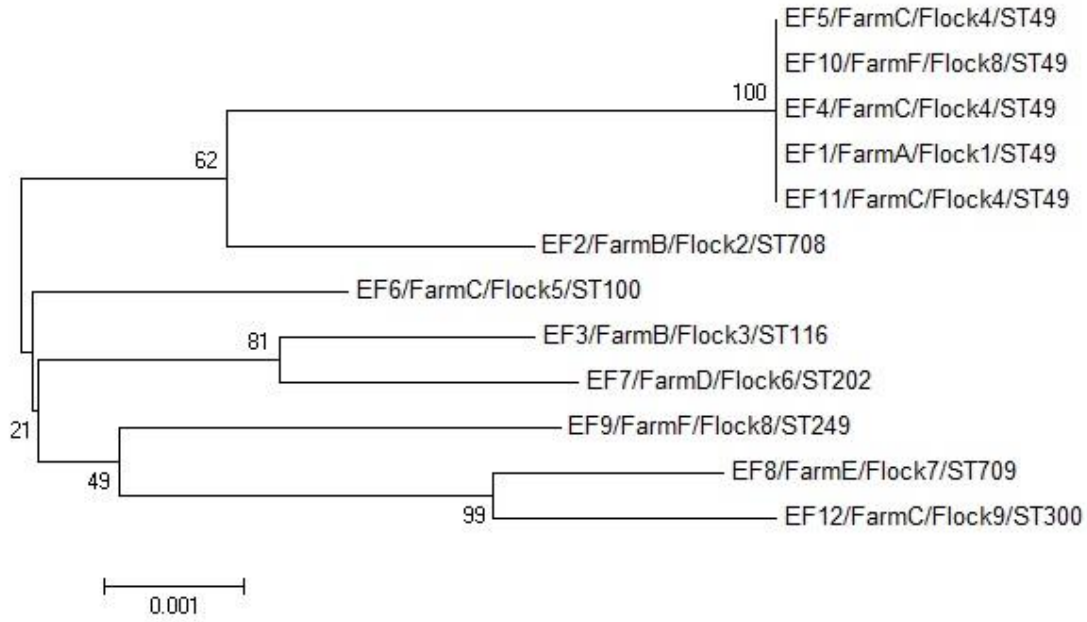

Figure 1. Evolutionary relationships among the concatenated sequences of the identified sequence types of *E. faecalis* isolated from vertebral osteomyelitis in broilers in Minas Gerais state, southeast Brazil, in 2012. The strain identification and its farm of origin, flock number and ST number are shown. Construction of Neighbour-joining tree was performed using Kimura 2-parameter with bootstrap values of 1000 replicates.

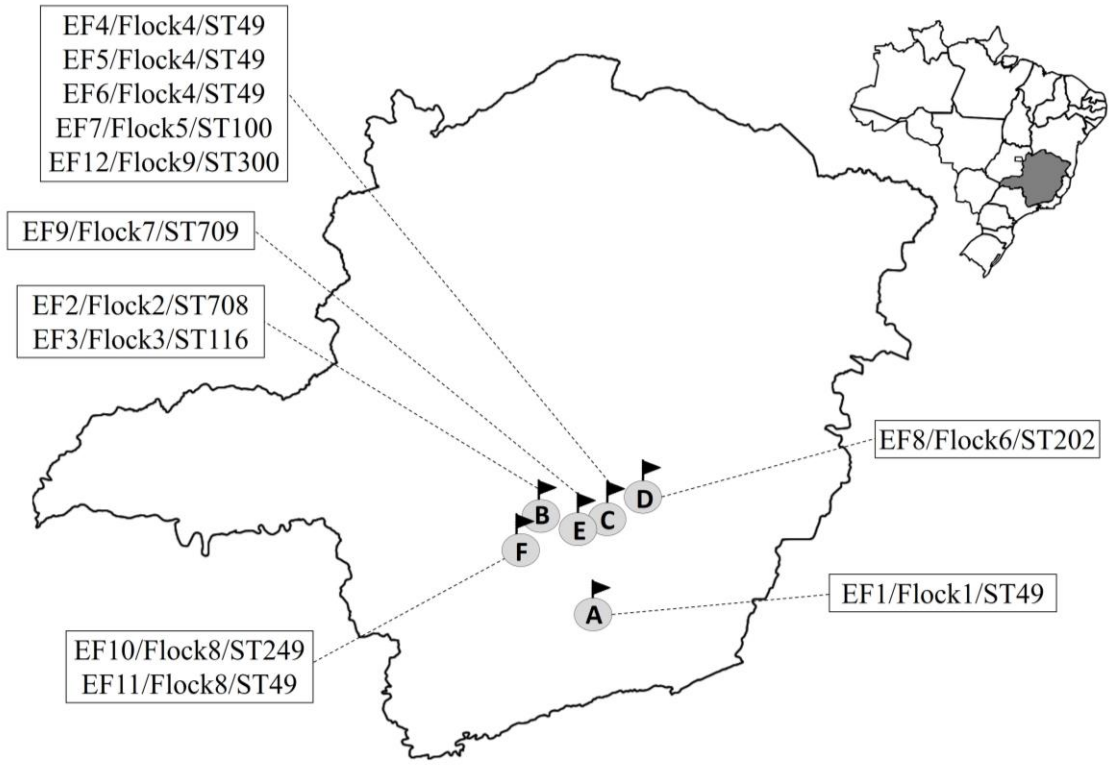

Figure 2. Geographical distribution of *E. faecalis* isolated from vertebral osteomyelitis in broilers in Minas Gerais state, southeast Brazil, in 2012. The letters (A, B, C, D, E and F) represent the different municipalities included in the study, which are linked to its respective boxes with details of the strains isolated in the place ("Strain ID/Number of the flock/Sequence Type number"). Distance among farms: A to F (130 km); F to B (47 km); B to E (45 km); E to C (42 km); C to D (54 km); and D to A (161 km), comprising a total area of 10,434 km^2.

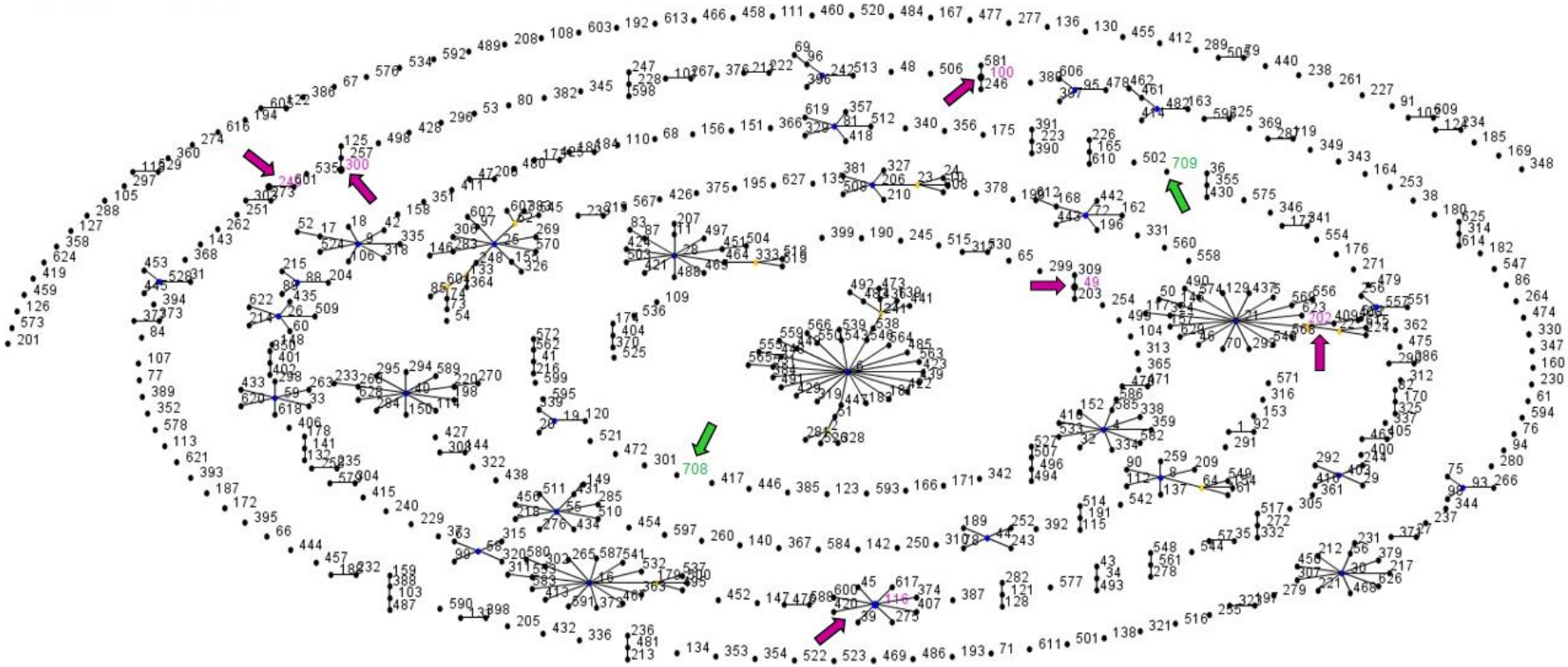

Figure 3. Population snapshot of STs included in the MLST database for *E. faecalis* isolated from vertebral osteomyelitis in broilers in Minas Gerais state, southeast Brazil, in 2012. Each ST is represented as a node with the ST number. Clusters of linked STs correspond to clonal complexes. Black lines connect single locus variants. Primary founders are represented in blue in the cluster, and subgroup founders in yellow. Pink arrows indicates STs available in *E. faecalis* database that were also identified among the isolates described in this study. STs pointed by green arrows are firstly described in this study.

Antimicrobial susceptibility profile of *E. faecalis* strains is shown in Fig. 4. The antimicrobial susceptibility test revealed that 70.0% (7/10) of *E. faecalis* isolates were mainly resistant to aminoglycosides. The highest resistance levels were to gentamicin (40.0%, 4/10) and neomycin (30.0%, 3/10), and 50.0% (5/10) of the isolates showed high-level aminoglycoside-resistance. This was characterized by streptomycin aminoglycoside-resistant in 30.0% (3/10) and gentamicin aminoglycoside-resistant in 20.0% (2/10) of the *E. faecalis* strains analyzed. Resistance to vancomycin was observed in only one *E. faecalis* strain (10.0%, 1/10). All *E. faecalis* isolates (100.0%, 10/10) of vertebral lesions were sensitive to ampicillin, amoxicillin and penicillin plus novobiocin. Most strains (40.0%, 4/10) were simultaneously resistant to two antibiotics represented by the combination of gentamicin/gentamicin aminoglycoside-resistant (20.0%, 2/10) or neomycin/streptomycin (20.0%, 2/10). Resistance to only one antibiotic and three antibiotics simultaneously was observed in 30.0% (3/10) and 10.0% (1/10) of the *E. faecalis* isolates, respectively. Susceptibility to all antibiotics tested was observed in 20.0% (2/10) of the analyzed strains.

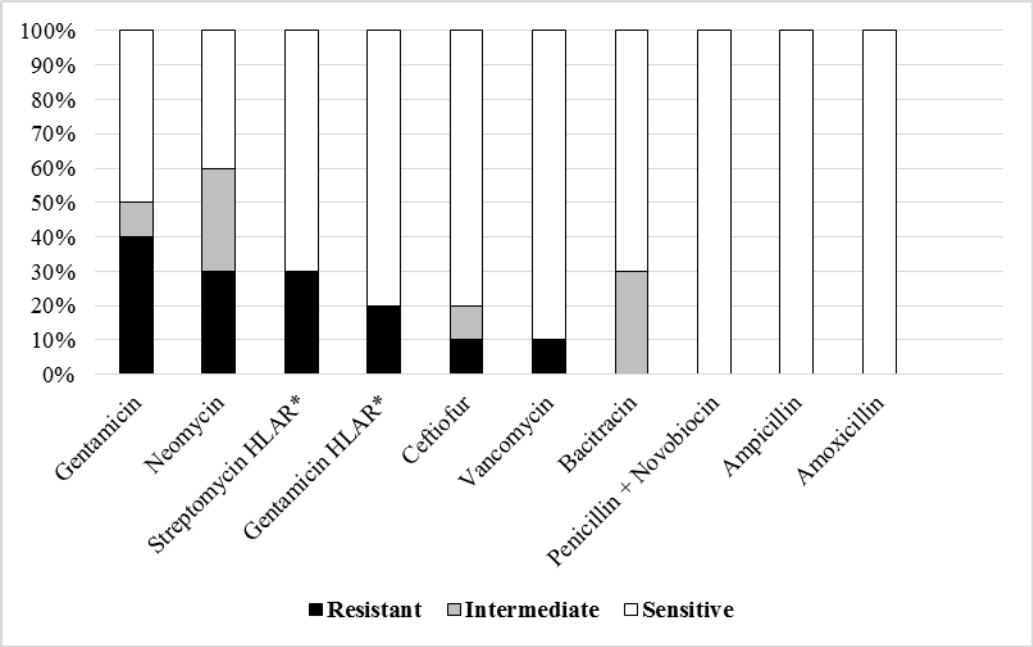

Figure 4. Antibiotic susceptibility profile of *E. faecalis* strains isolated from vertebral osteoymielitis in broilers in Minas Gerais state, southeast Brazil, in 2012. Black column: percentage of antibiotic resistant strains; Gray column: percentage of strains with intermediate antibiotic resistance; White column: percentage of antibiotic sensitive strains. HLAR*: high-level aminoglycoside resistant.

Discussion

These are the first data about molecular typing of *E. faecalis* isolated from vertebral osteomyelitis. Our results showed the diversity of isolates involved in the disease evidenced by the number of different STs (eight) proportionally to the number of samples analyzed (twelve). Two different situations were noted: the detection of the same ST (ST49) in three different vertebral osteomyelitis cases in the same flock (Flock 4, Fig. 2); the detection of different STs (ST49 and ST249) in two different cases of the disease in the same flock (Flock 8, Fig. 2).

Although most of the cases of vertebral osteomyelitis in different flocks were not caused by a unique *E. faecalis* ST, the ST49 was the most frequently detected in the region.

E. faecalis ST49 was previously detected in blood and feces samples from human patients in Spain and Nigeria (data available in http://pubmlst.org/efaecalis/). Recently, Tedim et al. (2015) observed that ST49 was more frequently detected in hospitalized human patients when compared to non-hospitalized human patients. *E. faecalis* ST49 was also detected in broiler breeders flocks by Gregersen et al. (2010). However, these authors obtained these two strains from apparently healthy broiler breeders. It worth mentioning that the *E. faecalis* described in this study were isolated from vertebral osteomyelitis cases that were analyzed by histopathological special stains that confirmed Gram-positive cocci bacteria associated with the lesions (Braga et al., 2016).

Information available about the association between *E. faecalis* STs and lesions types still seems to be conflicting. Gregersen et al. (2010) observed no correlation between ST and lesion type caused by *E. faecalis* in broilers breeders. In contrast to the findings of these authors and the present study, Petersen et al. (2009) reported that 71.4% (15/21) of the *E. faecalis* strains associated with arthritis and amyloid arthropathy in five different countries were ST82. This ST was not detected among the *E. faecalis* strains isolated from vertebral osteomyelitis of this study and from other Brazilian *E. faecalis* isolated strains, to the author´s knowledge. However, ST82 was the second isolated most frequently detected by Gregersen et al. (2010), which was actually associated with different diseases in broiler breeders.

E. faecalis ST249 was identified in one case of vertebral osteomyelitis analyzed. This ST was previously detected in both two apparently healthy and three sick broilers by Gregersen et al. (2010). In these last cases, the isolated were obtained from birds showing septicaemia or bacteremia, valvular endocarditis, and amyloidosis. According to Fertner et al. (2011), *E. faecalis* ST249 was among the three STs most frequently detected in cloacal swabs from chicks 24h after hatching.

The other STs (ST100, ST116, ST202, ST249 and ST300) have already been described in *E. faecalis* isolated elsewhere. ST116, ST202 and ST300 were isolated from retail chicken meat in Korea by Choi and Wood (2013), but there is no report of these STs related to disease in birds. In humans, *E. faecalis* ST116 was isolated from a human hospitalized patient in Cuba (Quiñones et al., 2009). ST100 was identified from swine samples in Denmark (Shankar et al., 2006), however, there is no description of this ST related to birds to the author´s knowledge.

High-level aminoglycoside resistance (HLAR) was detected in 50.0% (5/10) of the *E. faecalis* isolates. This trait was detected in the previously described ST49 (2/5) and ST100 (1/5), as well in both ST708 (1/5) and ST709 (1/5) described in this study. High-level gentamicin-resistance (HLGR) was also observed in 10.9% (11/101) of the food-borne *E. faecalis* isolated from retail chicken meat in Korea (Choi and Wood, 2013). Among these HLGR strains, the authors identified ST116, ST202 and ST300, which were all detected in our study, but it was not characterized by HLGR.

Intrinsically antimicrobial-resistant enterococci have acquired HLAR genes, which has made the treatment of enterococcal infections a challenge for clinicians (Bonten et al., 2001). High-level resistance to gentamicin is associated to bifunctional 6′-aminoglycoside acetyltransferase and 2″-aminoglycoside phosphotransferase (AAC6′-APH2″) of aminoglycoside-modifying enzymes (AMEs), which reduce the effect of aminoglycosides (Udo et al., 2004). Streptomycin is the exception and is modified by the 6-nucleotidyltransferase (ANT6) (Chow, 2000). According to Choi and Woo (2013), the emergence of food-borne HLGR *E. faecalis* suggests that chicken could be a potential source of transmission of antimicrobial resistance and virulence factors.

Resistance to vancomycin in the antibiotic susceptibility test was detected only in EF8 *E. faecalis* strain (ST202). Vancomycin-resistant enterococci (VRE) have emerged as nosocomial pathogens in the past 10 years, causing epidemiological controversy (Bonten et al., 2001). Resistance to vancomycin in enterococci is encoded by different genes. *VanA* is one of these genes, which detection in *E. faecium* isolated from food animals and meat was associated with the use of the glycopeptide avoparcin for growth promotion. In *E. faecalis*, this resistance trait is rarely found and it was mainly detected in hospitalized human patients. However, vanA-positive *E. faecalis* isolated from meat or animals were associated with poultry production in Asia and New Zealand (Agersø et al., 2008). Interestingly, Getachew et al. (2013) analyzed VRE isolates from human beings, chickens and swine in Malaysia and observed that the VRE belonged to six different STs, but no one of them was ST202. Among the conclusions, these authors highlights that the infrequent detection of a human VRE clone in a chicken may in fact suggest a reverse transmission of VRE from humans to animals.

None of the *E. faecalis* strains isolated from vertebral osteomyelitis cases was resistant to bacitracin. However, it worth to note that 30.0% (3/10) of the isolates had intermediate susceptibility to bacitracin, which is frequently used as growth promoter in broilers in Brazil. In contrast to its low frequency of use in humans, bacitracin has an important role in poultry as growth promoter, prophylaxis and therapy, specially by suppressing necrotizing enteritis caused by *Clostridium perfringens* (Phillips, 1999; Manson et al., 2004).

The use of zinc bacitracin in poultry production in New Zealand has selected enterococcal strains harboring bacitracin resistance genes, which are transferable and plasmid borne in *E. faecalis* (Manson et al., 2004). In Canada, where bacitracin is one of the antibiotics used in feed as growth promoters, Diarra et al. (2010) detected resistance to bacitracin in 98.6% of the enterococci isolated from cecal samples of broilers at slaughter and *E. faecalis* was one of the species that demonstrated relatively high resistance levels to this antibiotic. To the knowledge of the authors, there is no information available on *E. faecalis* resistance profile to bacitracin in broilers of Brazil. However, a study performed in the State of Minas Gerais showed that 47.3% of the *C. perfringens* strains isolated from broilers intestines in slaughterhouse were considered resistant to bacitracin (Silva et al., 2009). According to Phillips (1999), there is evidence of bacitracin acquired resistance in enterococci isolates from animals, but there is no evidence that its frequency has increased over the time or that it is related to its use in humans and animals.

Antimicrobial resistance genes in enterococci are considered danger to animal and human health, especially those located on mobile elements, once these bacteria may transfer antimicrobial resistance genes to other possibly pathogenic bacteria in the chicken intestine and also to zoonotic bacteria. Moreover, these enterococci may be transferred to human beings, where they could cause disease or further spread their antimicrobial resistance genes among the gastrointestinal bacteria (Cauwerts et al., 2007). Olsen et al. (2011) recently demonstrated that *E. faecalis* of human beings and poultry origin shared virulence genes supporting the zoonotic potential of *E. faecalis*. Similarly, Poulsen et al. (2012) studied *E. faecalis* isolated from humans with urinary infection and poultry and concluded that the homology of these isolates indicates the zoonotic potential and global spread of *E. faecalis* ST16, which recently was reported in human beings with endocarditis and in swine in Denmark.

E. faecalis acquires antimicrobial resistance through transfer of plasmids and transposons, chromosomal exchange, or mutation (Coque, 2008). Transferable genetic elements in enterococci have a broad host range and are transferrable to both gram-negative and gram-positive bacteria. Therefore, *E. faecalis* could also act as a source of antimicrobial resistance genes for poultry intestinal pathogens (Donelli et al., 2004). This highlights the role of

antimicrobial resistance and use in animals and its implication to animal human health, which actually still raises many questions to be solved.

A study stated evidences that identified bacteria living in humans, animals, and those found in the environment are the main reservoirs of resistance genes. However, information about the conditions and factors that lead to the mobilization, selection and movement of these bacteria into and among animals and human populations is still insufficient (Bush et al., 2011). It is interesting to note that, antibiotic resistance may also be favored by different anthropogenic activities, other than the use of antibiotics in animals. According to Davies and Davies (2010), since a long time antibiotics were designated for clinical applications in human beings, also have been manufactured for prophylaxis in humans, fish and pets; as pest control for plants, biocide in toiletries, household cleaning products and also in water waste irresponsible disposal. Thus, they are widely disseminated, providing constant selection and maintenance pressure for populations of resistant strains in all environments.

Conclusions

These are the first data of molecular typing and antibiotic resistance profile of *E. faecalis* isolated from vertebral osteomyelitis in broilers. Our results showed diversity of STs involved with this disease, with ST708 and ST709 firstly described in this study. ST49 was the most frequently detected. The strains revealed high frequency of aminoglycoside resistance, with high-level gentamicin and streptomycin resistance detected, and low frequency of vancomycin-resistance. Also, most strains were sensitive to ampicillin, amoxicillin and penicillin plus novobiocin, giving alternatives for the application. The evident diversity of molecular and phenotypic characteristics of the *E. faecalis* strains highlights the need for future studies in human and veterinary medicine. More information could help to elucidate the questions that remain unclear about vertebral osteomyelitis in broilers, the evolutionary aspects of *E. faecalis* and the role of antibiotic use in animal and in human beings and their impact in the veterinary poultry production and human health.

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Conflict of interest. The authors declare that they have no conflict of interests.

References

Abe, Y.; Nakamura, K.; Yamada, M.; Yamamoto, Y., 2006. Encephalomalacia with *Enterococcus durans* infection in the brain stem and cerebral hemisphere in chicks in Japan. Avian. Dis. 50, 139-41.

Agersø, Y.; Lester, C.H.; Porsbo, L.J.; Ørsted, I.; Emborg, H.D.; Olsen, K.E.P.; Jensen, L.B.; Heuer, O.E.; Frimodt-Møller, N.; Aarestrup, F-M.; Hammerum, A.M., 2008. Vancomycin-

resistant *Enterococcus faecalis* isolates from a Danish patient and two healthy human volunteers are possibly related to isolates from imported turkey meat. J. Antimicrobiol. Chemoth. 62, 844-845.

Aitchison, H.; Poolman, P.; Coetzer, M.; Griffiths, C.; Jacobs, J.; Meyer, M.; Bisschop, S., 2014. Enterococcal-related vertebral osteoarthritis in South African broiler breeders: A case report. J. S. Afr. Vet. Assoc. 85, 01-05.

Bisgaard, M., 1981. Arthritis in ducks: aetiology and public health aspects. Avian Pathol. 10, 1121.

Bonten, M.J.M.; Willems, R.; Weinstein, R.A., 2001. Vancomycin-resistant enterococci: why are they here, andwhere do they come from? Lancet Infect. Dis. 1, 314–325.

Boom, R.; Sol, C.; Beld, M.; Weel, J.; Goudsmit, J.; Wertheim-van Dillen, P., 1999. Improved silica–guanidiniumthiocyanate DNA isolation procedure based on selective binding of bovine alpha–casein to silica particles. J. Clin. Microbiol. 37, 615–619.

Braga, J.F.V.; Silva, C.C.; Teixeira, M.P.F.; Martins, N.R.S.; Ecco, R., 2016. Vertebral osteomyelitis associated with single and mixed bacterial infection in broilers. Avian Pathol. In press.

Bush, K.; Courvalin, P.; Dantas, G.; Davies, J.; Eisenstein, B.; Huovinen, P.; Jacoby, G.A.; Kishony, R.; Kreiswirth, B.N.; Kutter, E.; Lerner, S.A.; Levy, S.; Lewis, K.; Lomovskaya, O.; Miller, J.H.; Mobashery, S.; Piddock, L.J.; Projan, S.; Thomas, C.M.; Tomasz, A.; Tulkens, P.M.; Walsh, T.R.; Watson, J.D.; Witkowski, J.; Witte, W.; Wright, G.; Yeh, P.; Zgurskaya, H.I., 2011. Tackling antibiotic resistance. Nat. Rev. Microbiol. 9, 894-896.

Cardona, C.J.; Bickford, A.A.; Charlton, B.R.; Cooper, G.L., 1993. *Enterococcus durans* infection in young chickens associated with bacteremia and encephalomalacia. Avian Dis. 37, 234-239.

Cauwerts, K.; Decostere, A.; De Graef, E.M.; Haesebrouck, F.; Pasmans, F., 2007. High prevalence of tetracycline resistance in Enterococcus isolates from broilers carrying the erm(B) gene. Avian Pathol. 36, 395–399.

Cetinkaya, Y.; Falk, P.; Mayhall, C.G., 2000. Vancomycin-resistant enterococci. Clin. Microbiol. Rev. 13, 686-707.

Choi, J-M.; Woo, G-J., 2013. Molecular characterization of high-level gentamicin-resistant *Enterococcus faecalis* from chicken meat in Korea. Int. J. Food Microbiol. 165, 1–6.

Chow, J.W., 2000. Aminoglycoside resistance in enterococci. Clin. Infect. Dis. 31, 586–589.

Coque, T.M. Evolutionary Biology of Pathogenic Enterococci. In *Evolutionary Biology of Bacteria and Fungal Pathogens*; Baquero, F., Nombela, C., Cassell, G.H., Guitierrez, J.A., Eds.; ASM Press: Washington, DC, USA, 2008; pp. 501–521.

Creti, R.; Imperi, M.; Bertuccini, L.; Fabretti, F.; Orefici, G.; Di Rosa, R.; Baldassarri, L., 2004. Survey for virulence determinants among *Enterococcus faecalis* isolated from different sources. J. Med. Microbiol. 53, 13–20.

Davies, J.; Davies, D., 2010. Origins and Evolution of Antibiotic Resistance. Microbiol. Mol. Biol. Rev. 74, 417–433.

Deeming, D.C., 2005. Yolk sac, body dimensions and hatchling quality of ducklings, chicks and poults. Brit. Poult. Sci. 46, 560-564.

Devriese, L.A.; Cauwerts, K.; Hermans, K.; Wood, A.M., 2002. *Enterococcus cecorum* septicemia as a cause of bone and joint lesions resulting in lameness in broiler chickens. Vlaams Diergen. Tijds. 71, 219–221.

Devriese, L.A.; Hommez, J.; Wijfels, R.; Haesebrouck, F., 1991. Composition of the enterococcal and streptococcal intestinal flora of poultry. J. Appl. Bacteriol. 71, 46–50.

Diarra, M.S.; Rempel, H.; Champagne, J.; Masson, L.; Pritchard, J.; Topp, E., 2010. Distribution of Antimicrobial Resistance and Virulence Genes in *Enterococcus* spp. and Characterization of Isolates from Broiler Chickens. Appl. Environ. Microbiol. 76, 8033–8043.

Donelli, G.; Paoletti, C.; Baldassarri, L.; Guaglianone, E.; di Rosa, R.; Magi, G.; Spinaci, C.; Facinelli, B. Sex pheromone response, clumping, and slime production in enterococcal strains isolated from occluded biliary stents. J. Clin. Microbiol. 2004, 42, 3419–3427.

Fertner, M.E.; Olsen, R.H.; Bisgaard, M.; Christensen, H., 2011. Transmission and genetic diversity of *Enterococcus faecalis* among layer chickens during hatch. Acta Vet. Scand. 53:56.

Franz, C.M.A.P.; Huch, M.; Abriouel, H.; Holzapfel, W.; Gálvez, A., 2011. Enterococci as probiotics and their implications in food safety. Int. J. Food Microbiol 151, 125–140.

Getachew, Y.; Hassan, L.; Zakaria, Z.; Aziz, S.A., 2013. Genetic Variability of Vancomycin-Resistant *Enterococcus faecium* and *Enterococcus faecalis* Isolates from Humans, Chickens, and Pigs in Malaysia. Appl. Environ. Microbiol. 79, 4528-4533.

Gregersen, R.H.; Petersen, A.; Christensen, H.; Bisgaard, M., 2010. Multilocus sequence typing of *Enterococcus faecalis* isolates demonstrating different lesion types in broiler breeders, Avian Pathol. 39, 435-440.

Herdt, P.; Defoort, P.; Van Steelant, J.; Swam, H.; Tanghe, L.; Van Goethem, S.; Vanrobaeys, M., 2009. *Enterococcus cecorum* osteomyelitis and arthritis in broiler chickens. Vlaams Diergen. Tijds. 78, 44–48.

Heuer, O.E.; Hammerum, A.M.; Collignon, P.; Wegener, H.C., 2006. Human health hazard from antimicrobial-resistant enterococci in animals and food. Clin. Infect. Dis. 43, 911-916.

Holtom, P.D.; Zamorano, D.; Patzakis, M.J., 2002. Osteomyelitis attributable to vancomycin-resistant enterococci. Clin. Orthop. Relat. Res. 403, 38-44.

Jackson, C.R.; Fedorka-Cray, P.J.; Barrett, J.B., 2004. Use of a Genus- and Species-Specific Multiplex PCR for Identification of Enterococci. J. Clin. Microbiol. 42, 3558–3565.

Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111-20.

Kola, A.; Schwab, F.; Barwolff, S.; Eckmanns, T.; Weist, K.; Dinger, E.; Klare, I.; Witte, W.; Ruden, H.; Gastmeier, P., 2010. Is there an association between nosocomial infection rates and bacterial cross transmissions? Crit. Care Med. 38, 46–50.

Kolbjørnsen, Ø.; David, B.; Gilhuus, M., 2011. Bacterial osteomyelitis in a 3-week-old broiler chicken associated with *Enterococcus hirae*. Vet. Pathol. 48, 1134-1137.

Kondo, H.; Abe, N.; Tsukuda, K.; Wada, Y., 1997. Adherence of *Enterococcus hirae* to the duodenal epithelium of chicks with diarrhoea. Avian Pathol. 26, 189-194.

Landman, W.J.M.; Gruys, E.; Dwars, R.M., 1994. A syndrome associated with growth depression and amyloid arthropathy in layers: a preliminary report. Avian Pathol. 23, 461-470.

Makrai, L.; Nemes, C.; Simon, A.; Ivanics, E.; Dudás, Z.; Fodor, L.; Glávits, R., 2011. Association of *Enterococcus cecorum* with vertebral osteomyelitis and spondylolisthesis in broiler parent chicks. Acta Vet. Hung. 59, 11–21.

Manson, J.M.; Keis, S.; Smith, J.M.B.; Cook, G.M., 2004. Acquired Bacitracin Resistance in *Enterococcus faecalis* Is Mediated by an ABC Transporter and a Novel Regulatory Protein, BcrR. Antimicrobiol. Agents Ch. 48, 3743–3748.

Marques, E.B.; Suzart, S., 2004. Occurrence of virulence-associated genes in clinical *Enterococcus faecalis* strains isolated in Londrina, Brazil. J. Med. Microbiol. 53, 1069–1073.

Moreno, M.R.F.; Sarantinopoulos, P.; Tsakalidou, E.; De Vuyst, L., 2006. The role and application of enterococci in food and health. Int. J. Food. Microbiol. 106, 1-24.

CLSI/NCCLS (Clinical and Laboratory Standards Institute, former National Committee for Clinical Laboratory Standards), 2008. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. 6 ed. (M7-A6), Wayne, v.23. 81p.

Olsen, R.H.; Schonheyder, H.C.; Christensen, H.; Bisgaard, M. *Enterococcus faecalis* of human and poultry origin share virulence genes supporting the zoonotic potential of *E. faecalis*. *Zoonoses Public Health* 2011, *59*, 256–263.

Palmer, K.L.; Godfrey, P.; Griggs, A.; Kos, V.N.; Zucker, J.; Desjardins, C.; Cerqueira, G.; Gevers, D.; Walker, S.; Wortman, J.; Feldgarden, M.; Haas, B.; Birren, B.; Gilmorea, M.S., 2012. Comparative genomics of enterococci: variation in *Enterococcus faecalis*, clade structure in *E. faecium*, and defining characteristics of *E. gallinarum* and *E. casseliflavus*. MBio. 3, e00318-11.

Petersen, A.; Christensen, H.; Philipp, H-C.; Bisgaard, M., 2009. Clonality of *Enterococcus faecalis* associated with amyloid arthropathy in chickens evaluated by multilocus sequence typing (MLST). Vet. Microbiol. 134, 392–395.

Phillips, I., 1999. The use of bacitracin as a growth promoter in animals produces no risk to human health. Journal of Antimicrobial Chemotherapy. 44, 725-728.

Poulsen, L.L.; Bisgaard, M.; Son, N.T.; Trung, N.V.; An, H.M.; Dalsgaard, A., 2012. *Enterococcus faecalis* clones in poultry and in humans with urinary tract infections, Vietnam. Emerg. Infect. Dis. 18, 1096-1100.

Quiñones, D.; Kobayashi, N.; Nagashima, S., 2009. Molecular epidemiologic analysis of *Enterococcus faecalis* isolates in Cuba by Multilocus Sequence Typing. Microb. Drug Resist. 15, 287-293.

Randall, C.J.; Wood, A.M.; MacKenzie, G., 1993. Encephalomalacia in first-week chicks. Vet. Rec. 132, 419.

Robbins, K.M.; Suyemoto, M.M.; Lyman, R.L.; Martin, M.P.; Barnes, H.J.; Borst, L.B., 2012. An outbreak and source investigation of enterococcal spondylitis in broilers caused by *Enterococcus cecorum*. Avian Dis. 56, 768-773.

Ruiz-Garbajosa, P.; Bonten, M.J.M; Robinson, D.A.; Top, J.; Nallapareddy, S.R.; Torres, C.; Coque, T.M.; Cantón, R.; Baquero, F.; Murray, B.E.; del Campo, R., Willems, R.J.L., 2006. Multilocus Sequence Typing Scheme for *Enterococcus faecalis* Reveals Hospital-Adapted Genetic Complexes in a Background of High Rates of Recombination. J. Clin. Microbiol. 44, 2220–2228.

Saitou, N.; Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406-25.

Shankar, N.; Baghdayan, A.S.; Willems, R.; Hammerum, A.M.; Jensen, L.B., 2006. Presence of pathogenicity island genes in *Enterococcus faecalis* isolates from Pigs in Denmark. J. Clin. Microbiol. 44, 4200–4203.

Silva, R. O. S.; Salvarani, F.M.; Assis, R.A.; Martins, N.R.S.; Pires, P.S.; Lobato, F.C.F. Antimicrobial susceptibility of *Clostridium perfringens* strains isolated from broiler chickens. Brazilian Journal of Microbiology (2009) 40:262-264

Stalker, M.J.; Brash, M.L.; Weisz, A.; Ouckama, R.M.; Slavic, D., 2010. Arthritis and osteomyelitis associated with *Enterococcus cecorum* infection in broiler and broiler breeder chickens in Ontario, Canada. J. Vet. Diagn. Invest. 22, 643–645.

Steentjes, A.; Veldman, K.T.; Mevius, D.J.; Landman, W.J.M., 2002. Molecular epidemiology of unilateral amyloid arthropathy in broiler breeders associated with *Enterococcus faecalis*. Avian Pathol. 31, 3139.

Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A.; Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol. Biol. Evol. 30, 2725–2729.

Tannock, G.W., 1995. Normal microflora: An introduction to microbes inhabiting the human body, 1st ed. London: Chapman and Hall, 116p.

Tedim, A.P.; Ruiz-Garbajosa, P.; Corander, J.; Rodríguez, C.M.; Cantón, R.; Willems, R.J.; Baquero, F.; Coque, T.M., 2015. Population biology of intestinal *Enterococcus* isolates from hospitalized and nonhospitalized individuals in different age groups. Appl. Environ. Microbiol. 81, 1820-1831.

Teixeira, L., Carvalho, M., Facklan, R., 2007. *Enterococcus*. In: Murray, P., Baron, E., Landry, M., Jorgensen, J., Pfaller, M. (eds). Manual of Clinical Microbiology. 9th ed. Washington, DC: ASM Press. 430–442.

Thayer, S.G.; Waltman, W.D.; Wages, D.P., 2008. *Streptococcus* and *Enterococcus*. In: Saif, Y.M.; Fadly, A.M.; Glisson, J.R.; McDougald, L.R.; Nolan, L.K.; Swayne, D.E. (Eds.), Diseases of Poultry. 12th ed. Ames, Iowa: Blackwell Publishing, pp. 900–908.

Thompson, J.D.; Higgins, D.G.; Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673-4680.

Udo, E., Al-Sweih, N., John, P., Jacob, L., Mohanakrishnan, S., 2004. Characterization of high-level aminoglycoside-resistant enterococci in Kuwait hospitals. Microb. Drug Resist. 10, 139–145.

Velkers, F.C.; Graaf-Bloois, L.V.; Wagenaar, J.A.; Westendorp, S.T.; van Bergen, M.A.P.; Dwars, R.M.; Landman, W.J.M, 2011. *Enterococcus hirae*-associated endocarditis outbreaks in broiler flocks: clinical and pathological characteristics and molecular epidemiology. Vet. Quart. 31, 3-17.

Vogelstein, B.; Gillespie, D., 1979. Preparative and analytical purification of DNA from agarose. P. Natl. Acad. Sci. USA. 76, 615–619.

Wages, D.P., 1998. Streptococcosis. In: Swayne, D.E.; Glisson, J.R.; Jackwood, M.W.; Person, J.E.; Reed, W.M. (Eds.), Isolation and identification of Avian Pathogens, 4th ed. American Association of Avian Pathologists: Kennett Square, PA, pp.58-60.

Willems, R.J.L.; Bonten, M.J.M., 2007. Glycopeptide-resistant enterococci: deciphering virulence, resistance and epidemicity. Curr. Opin. Infect. Dis. 20, 384-390.

Wisplinghoff, H.; Bischoff, T.; Tallent, S.M.; Seifert, H.; Wenzel, R.P.; Edmond, M.B., 2004. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clin. Infect. Dis. 39, 309-317.

Wood, A.M.; Mackenzie, G.; Mcgillveray, N.C.; Brown, L.; Devriese, L.A.; Baele, M., 2002. Isolation of *Enterococcus cecorum* from bone lesions in broiler chickens. Vet. Rec. 150, 27.

GENERAL CONCLUSIONS

1. Vertebral osteomyelitis has a frequency of 5.1% in broilers with locomotor disorders in the state of Minas Gerais;

2. The clinical and pathological changes in broilers with vertebral osteomyelitis varied according to the extent of vertebral lesions; Only broilers with severe lesions and high degree of spinal cord compression had the classic clinical signs of disease;

3. Histopathological analysis, specially performed using special histological staining, contributed to determine the etiological agent, since some cases of vertebral osteomyelitis were associated to multiple bacteria;

4. Vertebral osteomyelitis in broilers in the state of Minas Gerais was caused by different etiological agents. The agents and its frequency were as follows: *Enterococcus* spp. (53.6%), *E. faecalis* (35.7%) and *E. hirae* (7.1%); *Escherichia coli* (35.7%), in co-infection with *E. faecalis* in 7.1% of the cases; *Staphylococcus aureus* (14.3%), in 7.1% of the cases in co-infection with *Enterococcus* spp. or *E. hirae*;

5. The *Escherichia coli* strains associated with vertebral osteomyelitis and arthritis in poultry in the state of Minas Gerais had high genetic diversity and the absence of a pattern related to the type of lesion presented by the broiler;

6. The *E. coli* strains had variable content of virulence genes and belonged to different phylogenetic groups and serogroups;

7. High frequency (73%) of multidrug-resistant *E. coli* was observed among the strains isolated from vertebral osteomyelitis and arthritis;

8. Broilers with locomotor disorders associated with *E. coli* infection presented clinical signs that can help in the differential diagnosis of vertebral osteomyelitis and unilateral/bilateral arthritis, depending on the extension of the lesions;

9. *E. faecalis* STs involved with this disease are genetically diverse; ST708 and ST709 were described for the first time in this study; ST49 was the most frequently detected; and

10. *E. faecalis* strains were frequently aminoglycoside-resistant with detection of high-level gentamicin-resistant and high-level streptomycin-resistant strains, and low frequency of vancomycin-resistant strains.

REFERENCES OF INTRODUCTION

ABPA (Associação Brasileira de Proteína Animal), 2015a. Disponível em: <http://abpa-br.com.br/files/publicacoes/c59411a243d6dab1da8e605be58348ac.pdf> Consultado em: 14 fev. 2016.

ABPA (Associação Brasileira de Proteína Animal), 2015b. Disponível em: <http://abpa-br.com.br/noticia/artigos/todas/avicultura-e-suinocultura-do-brasil-producao-e-exportacao-previsoes-para-2015-e-2016-1478>. Consultado em: 05 jan. 2016.

ALMEIDA PAZ, I.C.L. Avaliação de problemas locomotores e bem-estar em frangos de corte criados em diferentes tipos de cama. Conferência FACTA, 2010, Santos. **Anais...** Santos: Mendes Convention Center, 2010.

AMARAL, T.F. 2003. Disponível em: http://www.aviculturaindustrial.com.br/noticia/cenario-da-avicultura-de-minas-gerais-exclusivo/20030715163603_05122; Consultado em: 15 dez. 2015.

ANGEL, R. Metabolic disorders: limitations to growth of and mineral deposition into the broiler skeleton after the hatch and potential implications for leg problems. **J Appl Poult Res**, v.16, p.138-149, 2007.

ARAÚJO, G.M.; VIEITES, F.M.; BARBOSA, A.A. et al. Variação aniônica da dieta sobre características ósseas de frangos de corte: resistência à quebra, composição orgânica e mineral. **Arq Bras Med Vet Zootec**, v.63, p.954-961, 2011.

BESSEI, W. Welfare of broilers: a review. **World Poultry Sci J**, v.62, p.455–466, 2006.

COTO, C.; YAN, F.; CERATTE, S. et al. Effects of dietary levels of calcium and nonphytate phosphorus in broiler starter diets on live performance, bone development and growth plate conditions in male chicks fed a corn-based diet. **Int J Poult Sci**, v.7, p. 638-645, 2008.

EMMANS, G.C.; KYRIAZAKIS, I. Issues arising from genetic selection for growth and body composition characteristics in poultry and pigs. In: HILL, W.G.; BISHOP, S.C.; MCGUIRK, B. et al. **The challenge of genetic change in animal production**. Penicuik, UK: BSAS, p.39-53, 2000.

FAO–PPLPI Research Report (Industrial Livestock Production and Global Health Risks, June 2007). Disponível em: http://www.fao.org/ag/AGAinfo/projects/en/pplpi/docarc/rep-hpai_industrialisationrisks.pdf; Consultado em: 20 mar. 2014.

JULIAN, R.J. Rapid growth problems: ascitis and skeletal deformities in broilers. **Poult Sci**, v.77, p.1773-1780, 1998.

KESTIN, S.C.; KNOWLES, T.G.; TINCH, A.E. et al. The prevalence of leg weakness in broiler chickens assessed by gait scoring and its relationship to genotype. **Vet Rec**, v.131, p.190–194, 1992.

MARTIN, L.T.; MARTIN, M.P.; BARNES, H.J. Experimental Reproduction of Enterococcal Spondylitis in Male Broiler Breeder Chickens. **Avian Dis**, v.55, p.273–278, 2011.

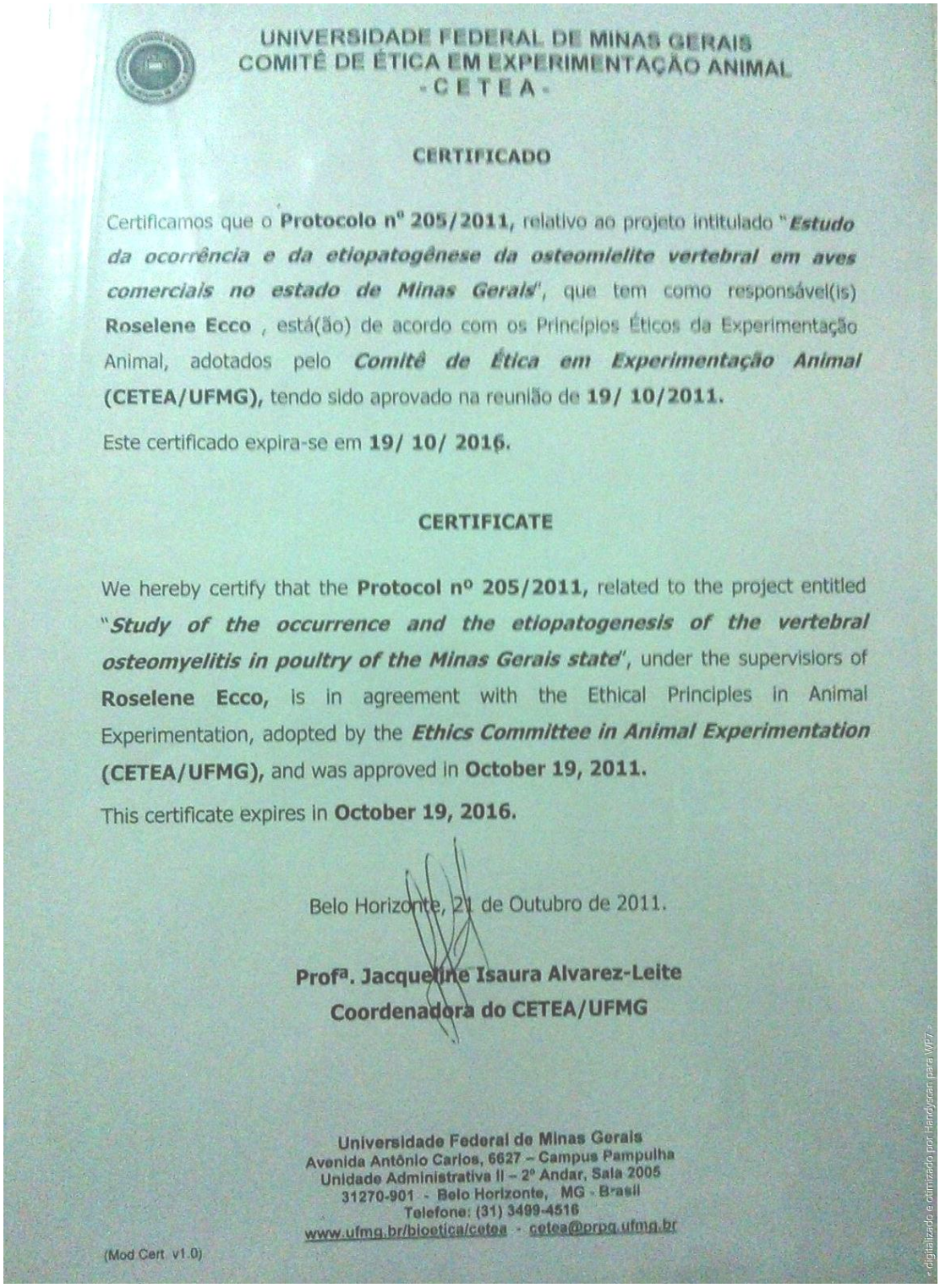
SCAHAW (Scientific Committee on Animal Health and Animal Welfare), 2000. The Welfare of Chickens Kept for Meat Production (Broilers). European Commission. Disponível em: http://ec.europa.eu/food/fs/sc/scah/out39_en.pdf; Consultado em: 20 mar. 2014.

SILVA, F.A.; MORAES, G.H.K., RODRIGUES, A.C.P. et al. Efeitos do ácido L-Glutâmico e da vitamina D3 no desempenho e nas anomalias ósseas de pintos de corte. **Rev Bras Zootecn**, v.30, p.2059-2066, 2001.

APPENDIX

APPENDIX I

Certificate of approval of the procedures performed in this study issued by Animal Experimentation Ethics Committee of the Universidade Federal de Minas Gerais



UNIVERSIDADE FEDERAL DE MINAS GERAIS
COMITÊ DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL
- C E T E A -

CERTIFICADO

Certificamos que o **Protocolo nº 205/2011,** relativo ao projeto intitulado "***Estudo da ocorrência e da etiopatogênese da osteomielite vertebral em aves comerciais no estado de Minas Gerais***", que tem como responsável(is) **Roselene Ecco** , está(ão) de acordo com os Princípios Éticos da Experimentação Animal, adotados pelo ***Comitê de Ética em Experimentação Animal*** **(CETEA/UFMG),** tendo sido aprovado na reunião de **19/ 10/2011.**

Este certificado expira-se em **19/ 10/ 2016.**

CERTIFICATE

We hereby certify that the **Protocol nº 205/2011,** related to the project entitled "***Study of the occurrence and the etiopatogenesis of the vertebral osteomyelitis in poultry of the Minas Gerais state***", under the supervisiors of **Roselene Ecco,** is in agreement with the Ethical Principles in Animal Experimentation, adopted by the ***Ethics Committee in Animal Experimentation*** **(CETEA/UFMG),** and was approved in **October 19, 2011.**

This certificate expires in **October 19, 2016.**

Belo Horizonte, 21 de Outubro de 2011.

Profª. Jacqueline Isaura Alvarez-Leite
Coordenadora do CETEA/UFMG

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(Mod Cert v1.0)

APPENDIX II

CHAPTER 2

Confirmation of article acceptance for publication in *Avian Pathology*

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Fwd: Avian Pathology - Decision on Manuscript ID CAVP-2015-0097.R1

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Dear Dr. Ecco:

I am pleased to inform that your manuscript entitled "Vertebral osteomyelitis associated with single and mixed bacterial infection in broilers" has now been accepted for publication in Avian Pathology.

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APPENDIX III

CHAPTER 3

Confirmation of article submission for publication in *BMC Veterinary Research*

Juliana Fortes <jufortes22@gmail.com>

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APPENDIX IV

CHAPTER 3

Supplementary Data

Material and Methods

Serogrouping and Flagellar type

Table 1. Primers used for PCR amplification

Target	*Primer*	*Sequence (5'-3')*	*Fragment size (bp)*	*TM (ºC)*	*(+) Control*	*Reference*
Serogroup O	gndbis.f	ATACCGACGACGCCGATCTG	-	-	-	
Serogroup O1	rfbO1.r	CCAGAAATACACTTGGAGAC	189	56	BEN1438	
Serogroup O6	rfbO6a.r	AAATGAGCGCCCACCATTAC	584	59	BEN2936	
Serogroup O7	rfbO7.r	CGAAGATCATCCACGATCCG	722	59	BEN2845	
Serogroup O12	rfbO12.r	GTGTCAAATGCCTGTCACCG	239	59	BEN355	
Serogroup O16	rfbO16.r	GGATCATTTATGCTGGTACG	450	59	BEN2198	Clermont et al. [1]
Serogroup O18	rfbO18.r	GAAGATGGCTATAATGGTTG	360	59	BEN2744	
Serogroup O25a	rfbO25a.r	GAGATCCAAAAACAGTTTGTG	313	59	ECOR51	
Serogroup O45a	rfbO45a.r	GCGCAATAAATGGCTGACTG	312	58	BEN4190	
Serogroup O45b	rfbO45b.r	TGCGAGTAGACTATCTCAAG	436	58	BEN5054	
Serogroup O75	rfbO75.r	GTAATAATGCTTGCGAAACC	419	59	ECOR64	
Serogroup O88	rfbO88.r	AAGGAAAAACGCTGGGAGAG	494	55	ECOR26	Clermont et al. [2]
Serogroup O104	rfbO104.r	TGGCTTAGGATACTTGCAGC	410	52	BEN4438	Clermont et al. [1]
Serogroup O2	wzyO2-F wzyO2-R	TGCAACTCATTGGTCTGCTTTGCC CGGAAAGCCATAACAGGTAGAGAG	351	56	ECOR62	Fratamico et al. [3]

Serogroup O4	wzxO4-F wzxO4-R	TTGTTGCGATAATGTGCATGTTCC AATAATTTGCTATACCCACACCCTC	664	58	ECOR66	Li et al. [4]
Serogroup O8	O8-F O8-R	CCAGAGGCATAATCAGAAATAACAG GCAGAGTTAGTCAACAAAAGGTCAG	448	55	BEN352	Li et al. [4]
Serogroup O78	AT7 AT8	GGTATCGGTTTGGTGGTA AGAATCACAACTCTCGGCA	992	52	ECOR70	Liu et al. [5]
Flagella H4	fliC-H4-F fliC-H4-R	GGCGAAACTGACGGCTGCTG GCACCAACAGTTACCGCCGC	201	66	BEN4185	Bielaszewska et al. [6]
Flagella H7	fliC-H7f fliC-H7r	CCACGACAGGTCTTTATGATCTGA CAACTGTGACTTTATCGCCATTCC	96	58	BEN4190	
Flagella H8	fliC-H8f fliC-H8r	AAAGGCTCCATTGAATACAAGG TTGACCATCAATATTTGCGGTC	108	62	BEN4198	Bugarel et al. [7]
Flagella H21	fliC-H21f fliC-H21r	TACTAGTGCAACCGTTGCC AGATCAGATAGTGTCGCTGC	102	58	BEN4197	
Flagela H25	fliC univ-F fliC-H25-R	ATGGCACAAGTCATTAATAC TGCGGGATAGATGTGATAGCA	559	57	BEN1424	Iguchi et al. [8]

Virulence genotyping

Table 2. Primers used for PCR amplification

Gene		*Primer*	*Sequence (5´-3´)*	*TM (°C)*	*Product (bp)*	*(+) Control*	*Reference*
aatA	APEC autotransporter gene	aatA-F aatA-R	ATGAATAAGAATATACGAATTTTAC ACCATTATTATTTAGCGTAAAG	52	300	BEN194	Dai et al. [9]
aec26	Avian *E. coli* gene 26 (=A9)	aec26-F aec26-R	ATGAGCGATATGAGTGAAGC TTATCGGAGTAATTTATTGA	53	760	BEN2908	Schouler et al. [10]
astA	Aggregative stable enterotoxin	astA-F astA-R	TGCCATCAACACAGTATATC TCAGGTCGCGAGTGACGG	58	116	BEN194	Yamamoto and Nakazawa [11]
chuA	Heme binding protein	chuA.1 chuA.2	GACGAACCAACGGTCAGGAT TGCCGCCAGTACCAAAGACA	59	279	BEN2908	Clermont et al. [12]
clbB	Colibactin polyketide synthesis system	clbB-F clbB-R	GATTTGGATACTGGCGATAACCG CCATTTCCCGTTTGAGCACAC	62	579	BEN2742	Johnson et al. [13]
clbN	Colibactin polyketide synthesis system	clbN-F clbN-R	GTTTTGCTCGCCAGATAGTCATTC CAGTTCGGGTATGTGTGGAAGG	62	733	BEN2742	Johnson et al. [13]
cnf1	Cytotoxic necrotizing factor type 1	cnf1-A cnf1-B	GAACTTATTAAGGATAGT CATTATTTATAACGCTG	50	543	BEN2987	Blanco et al. [14]
cnf2	Cytotoxic necrotizing factor type 2	cnf2-F cnf2-R	AATCTAATTAAAGAGAAC CATGCTTTGTATATCTA	48	543	BEN2340	Blanco et al. [14]
csgA	Structural subunit of the curli fimbriae	csgA-F csgA-R	AGAGACAGTCGCAAATGGCTA AGTACTGATGAGCGGTCGCGT	55	538	BEN2936	This work
cva/cvi	Strutural genes of colicin V operon	cva/cvi-F cva/cvi-R	TCCAAGCGGACCCCTTATAG CGCAGCATAGTTCCATGCT	60	598	BEN2908	Ewers et al. [15]
fimA	Major type 1 subunit fimbriae (pilin)	fimA1 fimA2	CGGCTCTGTCCCTSAGT GTCGCATCCGCATTAGC	52	500	BEN2908	Moulin-Schouleur et al. [16]
fim_{avMT78}	fimA variant of MT78	fimA201 fimA215	TCTGGCTGATACTACACC ACTTTAGGATGAGTACTG	52	266	BEN2908	Marc and Dho-Moulin [17]
fimH	Minor fimbrial subunit, D-mannose specific adhesin	fimH2 fimH17	GATCTTTCGACGCAAATC CGAGCAGAAACATCGCAG	52	389	BEN2908	Arné et al. [18]

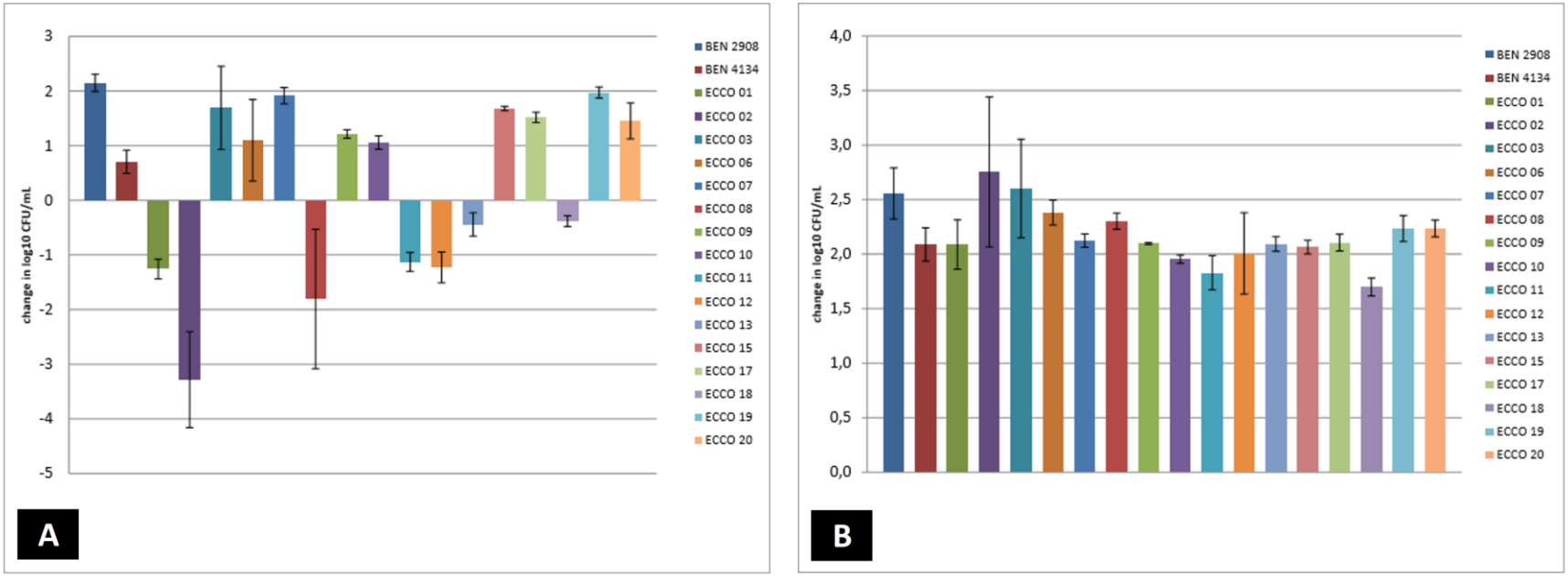
focG	G adhesin of the type F1C fimbriae	focG-F focG-R	CAGCACAGGCAGTGGATACGA GAATGTCGCCTGCCCATTGCT	63	362	BEN2936	Johnson and Stell [19]
frz$_{orf4}$	Sugar metabolism (=D7)	frz-F frz-R	TCAGTAAGAACGAAAGTGTG ACAGGAACAATCCCGTGGAT	53	565	BEN2908	Moulin-Schouler et al. [16]
fyuA	Ferric yersinia uptake	fyuA-F fyuA-R	GCGACGGGAAGCGATGACTTA CGCAGTAGGCACGATGTTGTA	64	774	BEN2908	Schubert et al. [20]
hlyA	Hemolysin A	hlyA-F hlyA-R	GTCCATTGCCGATAAGTTT AAGTAATTTTTGCCGTGTTTT	50	351	J96	Ewers et al. [21]
hlyF	Putative avian hemolysin	*hlyF-F* *hlyF-R*	GGCCACAGTCGTTTAGGGTGCTTACC GGCGGTTTAGGCATTCCGATACTCAG	63	450	BEN2908	Johnson et al. [13]
hra	Heat-resistant agglutinin	hra-F hra-R	GTAACTCACACTGCTGTCACCT CGAATCGTTGTCACGTTCAG	62	139	BEN2908	Ewers et al. [15]
ibeA	Invasion brain endothelium	ibeA-F ibeA-R	TGAACGTTTCGGTTGTTTTG TGTTCAAATCCTGGCTGGAA	55	814	BEN2908	Germon et al. [22]
iha	Bifunctional enterobactin receptor/adhesin protein	iha-F iha-R	TAGTGCGTTGGGTTATCGCTC AAGCCAGAGTGGTTATTCGC	60	609	BEN2936	Ewers et al. [15]
ireA	Iron-responsive element	ireA-F ireA-R	ATTGCCGTGATGTGTTCTGC CACGGATCACTTCAATGCGT	60	385	BEN2936	Ewers et al. [15]
iroN	Salmochelin siderophore receptor gene	*iroN-F* *iroN-R*	AATCCGGCAAAGAGACGAACCGCCT GTTCGGGCAACCCCTGCTTTGACTTT	63	553	BEN2908	Johnson et al. [13]
irp2	Iron-repressible protein	irp2-F irp2-R	AGGATTCGCTGTTACCGGAC TCGTCGGGCAGCGTTTCTTCT	62	286	BEN2908	Schubert et al. [20]
iss	Episomal increased serum survival gene	*iss-F* *iss-R*	CAGCAACCCGAACCACTTGATG AGCATTGCCAGAGCGGCAGAA	63	323	BEN2908	Johnson et al. [13]
iucD	Aerobactin synthesis	iucD-F iucD-R	CCTGATCCAGATGATGCTC CTGGATGAGCAGAAAATGACA	56	193	BEN2908	Frömmel et al. [23]
iutA	Aerobactin siderophore receptor	iutA1 iutA15	ATGAGCATATCTCCGGACG CAGGTCGAAGAACATCTGG	56	587	BEN2908	Moulin-Schouler et al. [16]
kpsMT II	Group II capsule polysaccharide synthesis	kpsMTII-F kpsMTII-R	GCGCATTTGCTGATACTGTTG CATCCAGACGATAAGCATGAGCA	63	272	BEN2936	Johnson and Stell [19]
malX (=rpai)	Pathogenicity-associated island marker CFT 073	malX-F malX-R	GGACATCCTGTTACAGCGCGCA TCGCCACCAATCACAGCCGAAC	68	922	BEN2908	Johnson and Stell [19]

neuC	Capsule K1	neu1 neu2	AGGTGAAAAGCCTGGTAGTGTG GGTGGTACATTCCGGGATGTC	61	676	BEN2908	Moulin-Schouler et al. [16]
ompT	Episomal outer membrane protease	*ompT-F* *ompT-R*	TCATCCCGGAAGCCTCCCTCACTACTAT TAGCGTTTGCTGCACTGGCTTCTGATAC	63	496	BEN2908	Johnson et al. [13]
P(F11)	felA	fel1 fel2	GGTCAASCAGCTAAAAACGGTAAGG CCTTCAGAAACAGTACCGCCATTCG	61	239	BEN2905	Moulin-Schouler et al. [16]
	papC	pap1 pap2	GACGGCTGTACTGCAGGGTGTGGCG ATATCCTTTCTGCAGGGATGCAATA	61	328	BEN2905	Le Bouguénec et al. [24]
pic	Serine protease autotransporter	pic-F pic-R	ACTGGATCTTAAGGCTCAGG TGGAATATCAGGGTGCCACT	60	411	BEN2936	Ewers et al. [15]
sat	Secreted autotransporter toxin	sat-F sat-R	TGCTGGCTCTGGAGGAAC TTGAACATTCAGAGTACCGGG	60	667	BEN2936	Ewers et al. [15]
sfaS	S adhesin of the type S fimbriae	sfaS-F sfaS-R	GTGGATACGACGATTACTGTG CCGCCAGCATTCCCTGTATTC	63	242	BEN2742	Johnson and Stell [19]
sitA	Iron transport protein (=A12)	sitA-F sitA-R	ATGCACTCGATAAAAAAAGT TTAAGAAGGTCGATATACGT	53	860	BEN2908	Schouler et al. [10]
tia	Toxigenic invasion locus	tia-F tia-R	AGCGCTTCCGTCAGGACTT ACCAGCATCCAGATAGCGAT	60	512	ECCO 18	Ewers et al. [15]
traT	Protectin-transfer and serum resistance protein	tratT-F traT-R	GGTGTGGTGCGATGAGCACAG CACGGTTCAGCCATCCCTGAG	68	290	BEN2908	Johnson and Stell [19]
tsh	Thermosensitive haemagglutinin	tsh-F tsh-R	GGTGGTGCACTGGAGTGG AGTCCAGCGTGATAGTGG	55	640	BEN2277	Dozois et al. [25]
TspE4.C2	Anonymous DNA fragment	TspE4C2.1 TspE4C2.2	GAGTAATGTCGGGGCATTCA CGCGCCAACAAAGTATTACG	59	152	BEN2908	Clermont et al. [12]
uidA	*E. coli* beta-glucuronidase	uidA-F uidA-R	ATGGAATTTCGCCGATTTTGC ATTGTTTGCCTCCCTGCTGC	60	187	BEN2908	Heijnen and Medema [26]
vat	Vacuolating autotransporter toxin	vat-F vat-R	GTGTCAGAACGGAATTGTC GGGTATCTGTATCATGGCAAG	60	230	BEN2936	Frömmel et al. [23]
yjaA	Conserved protein with unkown function	yjaA.1 yjaA.2	TGAAGTGTCAGGAGACGCTG ATGGAGAATGCGTTCCTCAAC	59	211	BEN2908	Clermont et al. [12]

Results

Serum bactericidal test

Figure 1. Serum resistance of *E. coli* strains in complet SPF chicken serum (A) and inactivated SPF chicken serum (B).



References

1. Clermont O, Johnson JR, Menard M, Denamur E. Determination of *Escherichia coli* O types by allele-specific polymerase chain reaction: application to the O types involved in human septicemia. Diagn Microbiol Infect Dis. 2007;57:129–13.

2. Clermont O, Olier M, Hoede C, Diancourt L, Brisse S, Keroudean M, Glodt J, Picard B, Oswald E, Denamur E. Animal and human pathogenic *Escherichia coli* strains share common genetic backgrounds. Infect Genet Evol. 2011;11:654–662.

3. Fratamico PM, Yan X, Liu Y, DebRoy C, Byrne B, Monaghan A, Fanning S, Bolton D. *Escherichia coli* serogroup O2 and O28ac O-antigen gene cluster sequences and detection of pathogenic *E. coli* O2 and O28ac by PCR. Can J Microbiol. 2010;56:308–316.

4. Li D, Liu B, Chen M, Guo D, Guo X, Liu F, Feng L, Wang L: A multiplex PCR method to detect 14 *Escherichia coli* serogroups associated with urinary tract infections. J Microbiol Methods. 2010;82:71–7.

5. Liu B, Wu F, Li D, Beutin L, Chen M, Cao B, Wang L: Development of a serogroup-specific DNA microarray for identification of *Escherichia coli* strains associated with bovine septicemia and diarrhea. Vet Microbiol. 2010;142:373–8.

6. Bielaszewska M, Mellmann A, Zhang W, Köck R, Fruth A, Bauwens A, Peters G, Karch H: Characterisation of the *Escherichia coli* strain associated with an outbreak of haemolytic uraemic syndrome in Germany, 2011: A microbiological study. Lancet Infect Dis. 2011;11:671–676.

7. Bugarel M, Beutin L, Martin A, Gill A, Fach P: Micro-array for the identification of Shiga toxin-producing *Escherichia coli* (STEC) seropathotypes associated with Hemorrhagic Colitis and Hemolytic Uremic Syndrome in humans. Int J Food Microbiol. 2010;142:318–329.

8. Iguchi A, Iyoda S, Ohnishi M: Molecular characterization reveals three distinct clonal groups among clinical shiga toxin-producing *Escherichia coli* strains of serogroup O103. J Clin Microbiol. 2012;50:2894–900.

9. Dai J, Wang S, Guerlebeck D, Laturnus C, Guenther S, Shi Z, Lu C, Ewers C: Suppression subtractive hybridization identifies an autotransporter adhesin gene of E. coli IMT5155 specifically associated with avian pathogenic *Escherichia coli* (APEC). BMC Microbiol. 2010;10:236.

10. Schouler C, Koffmann F, Amory C, Leroy-Sétrin S, Moulin-Schouleur M: Genomic subtraction for the identification of putative new virulence factors of an avian pathogenic *Escherichia coli* strain of O2 serogroup. Microbiol. 2004;150:2973–84.

11. Yamamoto T, Nakazawz M. Detection and sequences of the enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 gene in enterotoxigenic *E. coli* strains isolated from piglets and calves with diarrhea. J Clin Microbiol. 1997;35:223-7.

12. Clermont O, Bonacorsi S, Bingen E: Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl Environ Microbiol. 2000;66:4555–8.

13. Johnson TJ, Wannemuehler Y, Doetkott C, Johnson SJ, Rosenberger SC, Nolan LK: Identification of Minimal Predictors of Avian Pathogenic *Escherichia coli* Virulence for Use as a Rapid Diagnostic Tool. J Clin Microbiol. 2008;46:3987–3996.

14. Blanco M, Blanco IE, Blanco J, Alonsob MP, Balsalobre C, Madrid C, Juirez A. Polymerase chain reaction for detection of *Escherichia coli* strains producing cytotoxic necrotizing factor type 1 and type 2 (CNFl and CNF2). J Microbiol Methods. 1996;26:95–101.

15. Ewers C, Li G, Wilking H, Kießling S, Alt K, Antáo EM, Laturnus C, Diehl I, Glodde S, Homeier T, Böhnke U, Steinrück H, Philipp HC, Wieler LH: Avian pathogenic, uropathogenic, and newborn meningitis-causing *Escherichia coli*: How closely related are they? Int J Med Microbiol. 2007;297:163–176.

16. Moulin-Schouleur M, Schouler C, Tailliez P, Kao M-R, Brée A, Germon P, Oswald E, Mainil J, Blanco M, Blanco J: Common virulence factors and genetic relationships between O18:K1:H7 *Escherichia coli* isolates of human and avian origin. J Clin Microbiol. 2006;44:3484–92.

17. Marc D, Dho-Moulin M: Analysis of the fim cluster of an avian O2 strain of *Escherichia coli*: serogroup-specific sites within fimA and nucleotide sequence of fimI. J Med Microbiol. 1996;44:444–52.

18. Arné P, Marc D, Brée A, Schouler C, Dho-Moulin M: Increased tracheal colonization in chickens without impairing pathogenic properties of avian pathogenic *Escherichia coli* MT78 with a fimH deletion. Avian Dis. 2000;44:343–55.

19. Johnson JR, Stell AL: Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. J Infect Dis. 2000;181:261–72.

20. Schubert S, Rakin a., Karch H, Carniel E, Heesemann J: Prevalence of the "high-pathogenicity island" of Yersinia species among *Escherichia coli* strains that are pathogenic to humans. Infect Immun. 1998;66:480–485.

21. Ewers C, Schüffner C, Weiss R, Baljer G, Wieler L: Molecular characteristics of *Escherichia coli* serogroup O78 strains isolated from diarrheal cases in bovines urge further investigations on their zoonotic potential. Mol Nutr Food Res. 2004;48:504–14.

22. Germon P, Chen YH, He L, Blanco JE, Brée A, Schouler C, Huang SH, Moulin-Schouleur M: ibeA, a virulence factor of avian pathogenic *Escherichia coli*. Microbiol. 2005;151:1179–1186.

23. Fr̈mmel U, Lehmann W, R̈diger S, B̈hm A, Nitschke J, Weinreich J, Groß J, Roggenbuck D, Zinke O, Ansorge H, Vogel S, Klemm P, Wex T, Schr̈der C, Wieler LH, Schierack P: Adhesion of human and animal *Escherichia coli* strains in association with their virulence-associated genes and phylogenetic origins. Appl Environ Microbiol. 2013;79:5814–5829.

24. Le Bouguenec C, Archambaud M, Labigne a.: Rapid and specific detection of the pap, afa, and sfa adhesin-encoding operons in uropathogenic *Escherichia coli* strains by polymerase chain reaction. J Clin Microbiol. 1992;30:1189–1193.

25. Dozois CM, Dho-Moulin M, Brée A, Fairbrother JM, Desautels C, Curtiss R: Relationship between the Tsh autotransporter and pathogenicity of avian *Escherichia coli* and localization and analysis of the tsh genetic region. Infect Immun. 2000;68:4145–4154.

26. Heijnen L, Medema G: Quantitative detection of E. coli, E. coli O157 and other shiga toxin producing E. coli in water samples using a culture method combined with real-time PCR. J Water Health. 2006;04(Suppl 2):487–498.