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Full Length Research Paper

Characterization of antimicrobial resistance in *Staphylococcus aureus* isolated from bovine mastitis in Central Ethiopia

Bezina Arega Emeru*, Yohannes Equar Messele, Desiye Tesfaye Tegegne, Shimels Tikuye Yalew, Shelema Kelbessa Bora, Mossisa Dire Babura, MergieTaye Beyene and Gebremeskel Mamu Werid

Ethiopian Institute of Agricultural Research, National Agricultural Biotechnology Research Center, P. O. Box 249, Holeta, Ethiopia.

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***Staphylococcus aureus* is commonly associated with mastitis in dairy herds with potential public health implications. Overall, 303 samples were collected from September 2015 to July 2016 to characterize the phenotypic and genotypic pattern of drug resistance in *S. aureus* isolated from cases of clinical and sub-clinical bovine mastitis in Central Ethiopia. Milk samples were tested by using California Mastitis Test and positive samples were subjected for bacterial culture, disc diffusion test and polymerase chain reaction (PCR) to detect the presence of antimicrobial resistance. Based on California mastitis test (CMT) result and clinical examination, the prevalence of mastitis was 70.6%. *S. aureus* was isolated from 36.9% of CMT positive samples. The phenotypic determination of antimicrobial resistance showed that the isolates were most resistant to ampicillin (80%) followed by trimethoprim-sulfamethoxazole (23.3%), tetracycline (15%), streptomycin (10%) and gentamycin (3.3%) and equally to both erythromycin and chloramphenicol (1.6%). Characterization of the antimicrobial resistance gene was done by using PCR. Most of the isolates (56%) contained *blaZ* gene followed by *ermB* (33%), *ermC* (13.3%) and each *ermA* and *msrA* appeared only in 2% of the isolates. There was no isolate harboring the methicillin resistance *mecA* gene. Thirty six percent of the isolates contained more than one antibiotic resistance genes. The highest multidrug resistance (MDR) gene combination was observed by *blaZ*ermB* (31.25%) genes and the least frequently occurred were *blaZ *ermA* and *msrA*ermB* (3.12%) each. This study showed that consumption of raw milk could be considered as a critical source of antibiotic resistant *S. aureus*.**

Key words: Bovine mastitis, *Staphylococcus aureus*, antimicrobial resistance, Ethiopia.

INTRODUCTION

Mastitis is one of the most important diseases in dairy significant economic losses to the dairy industry. *Staphylococcus aureus* causes one of the most common types of chronic and cows throughout the world and is responsible for subclinical mastitis in dairy animals.

The inappropriate use of antibiotics for medication and growth enhancers in farm animals contributes for emergence of antibiotic resistant organisms. It is more than two decades since the emergence of antimicrobial resistant in staphylococci (Lowy, 2003). Staphylococci are

the main etiological agents of mastitis in dairy cattle and cows are the second largest reservoir of *S. aureus* next to human nares and up to 75 million of cows can be infected by this bacterium from the world cattle population (Sakwinska et al., 2011; Somayyeh and Habib, 2014; Raney, 2009). The overall loss due to mastitis ranges from 31 to 749 kg in first lactation to losses between 117 and 860 kg in subsequent lactation (Hultgren and Svensson, 2009; Ostergaard and Grohn, 1999).

Although there is host range barrier among *S. aureus* lineage, some illustrates the potential hazard of animal origin *S. aureus* on human health which implies possible transmission of genotypes from one species to the other (Smith, 2015; Lowder et al., 2009). Despite the substantial economic impact and potential public health concern, the prevalence as well as the phenotypic and genotypic antibiotic resistance nature of *S. aureus* isolates are less studied in developing countries like Ethiopia. In these countries, scarce in veterinary services, shortage of variety of drugs and poor drug regulatory frameworks could lead to under dosage medication which may end up with development of antibiotic resistant organisms. On the other hand, low hygienic standards of housing and milking can disseminate mastitis causing pathogens including *S. aureus* among individual animals or farms (Marama et al., 2016).

In Ethiopia, prevalence rate ranging from 15.3 to 53.4% has been recorded from different parts of the country (Marama et al., 2016; Sori et al., 2011). However, there are few trends to detect antibiotic resistance genes and to correlate their association with the phenotypic resistance. Hence, this research intended to characterize phenotypic as well as genotypic antibiotic resistance of *S. aureus* isolated from bovine mastitis.

MATERIALS AND METHODS

Sample collection and preparation

A total of 303 lactating cows were screened for subclinical mastitis from September 2015 to July 2016 using California mastitis test (CMT). All the lactating cows were examined carefully and CMT screening procedures were done. Approximately, 2 ml of milk was taken from each teat into the four CMT paddle indentations. Then, equal amount of CMT reagent (COX, USA) was added and swirled gently for 15 s. The screening was done according to the procedure stated in Quinn et al. (1994). The CMT positive samples were kept in cold box and transported immediately to the National Agricultural Biotechnology Research Center Laboratory, Holetta, for further analysis.

Bacterial isolation and identification

Bacteria were cultured and identified from CMT positive milk

samples. The collected samples from each quarter were streaked on blood agar base plates enriched with 7% ovine blood. The inoculums were then incubated aerobically at 37°C for 24 h. After primary culture, identification of *S. aureus* was done by using microscopic and biochemical methods (Quinn et al., 2011; OIE, 2012).

Antimicrobial sensitivity test

Antimicrobial resistance patterns were determined by Kirby-Bauer disc diffusion method on Mueller-Hinton agar (Sigma-Aldrich, USA) (Kirby et al., 1966). The bacteria were inoculated on the plate at a rate of 5×10^5 Bacteria/ml after serial dilution determined by OD measurements according to CLSI recommendation. Antibiotic discs were placed and gently pressed by forceps on the bacterial culture spread on Mueller-Hinton agar (Sigma Aldrich, USA). The inhibition zone was measured after incubation of the plates at 37°C for 18 h under aerobic environment. The response of the isolates to each antimicrobial agent was evaluated by measuring the zone of inhibition categorized as sensitive, intermediate and resistant according to the standards recommended by CLSI (2007). The antimicrobials used in the experiment were ampicillin (10 µg), chloramphenicol (30 µg), gentamycin (10 µg), erythromycin (15 µg), tetracycline (30 µg), streptomycin (10 µg) and trimethoprim-sulfamethoxazole (1.25/23.75 µg).

Isolation of plasmid DNA

Plasmid DNA of the *Staphylococcus* isolates was performed by using Plasmid Midi Kit (QIAGEN). Single colony was taken from each isolate and inoculated into a separate 5 ml LB Broth and incubated over night at 37°C in an orbital shaker. Cells were harvested by centrifugation at speed of 6000 g for 15 min. The isolation procedures were performed according to the manufacturers' protocol. The concentration and the purity of the extract were measured by using a Nano-drop ND-1000 spectrophotometer (Thermoscientific, Wilmington, DE). The integrity of the plasmid DNA was assessed after electrophoresis in 1% agarose gel after mixing with gel loading dye (Thermoscientific, USA).

PCR

The genes involved in antimicrobial resistance (*mecA*, *bla_Z*, *ermA*, *ermB*, *ermC* and *msrA*) were detected by PCR using the primers and cycle conditions described by Murakami et al. (1991) and Sawant et al. (2009) (Table 1). A single colony was picked and inoculated in to 10 ml of nutrient broth (Sigma Aldrich, USA) and incubated overnight at 37°C in shaker incubator at speed of 100 rpm/min. Bacterial plasmid DNA was extracted using kit (Biosbasic, USA) from well grown broth cultures. The PCR reaction was prepared by mixing, reaction buffer (500 Mm KCl, 17.5 mM MgCl₂, 100 mM Tris-HCl, 0.1% TritonX-100) (Himedia, India), 10m MdNTPs, 10 pmol of each primer, 1 U Taq DNA polymerase (Himedia, India) and Nuclease free water which was added up to 25 µl. PCR products were electrophoresed in 1.5% agarose gel after mixing with gel loading dye (Thermoscientific, USA) (0.5 µg/mL) and observed under UV illumination.

*Corresponding author. E-mail: bezinaemeru@gmail.com.

Table 1. Genes involved and their oligonucleotide primers for the polymerase chain reactions.

Gene	Oligonucleotide sequences	Reference
<i>mecA</i>	F- CTTTGGAACGATGCCTAATCTCAT R- AAGAGATTTGCCTATGCTTC	Murakami et al. (1991)
<i>blaZ</i>	F- GCTTGACCACTTTTATCAGC R- ATCGGATCAGGAAAAGGACA	
<i>ermA</i>	R- CACGATATTCACGTTTTACCC F- AAGGGCATTTAACGACGAAA	
<i>ermB</i>	R- CTGTGGTATGGCGGGTAAGT F- TGAAATCGGCTCAGGAAAAG	Sawant et al. (2009)
<i>ermC</i>	F- TGAAATCGGCTCAGGAAAAG R- CAAACCCGTATTCCACGATT	
<i>msrA</i>	F- TGGTACTGGCAAACACAT R-AAACGTCACGCATGTCTTCA	

Table 2. Antimicrobial resistance rate (%) of *S. aureus* isolated from bovine mastitis.

Antimicrobial disc	Sensitivity			Total (%)
	Sensitive (%)	Intermediate (%)	Resistant (%)	
Erythromycin	30 (54.5)	29 (43.9)	1 (1.6)	60 (100)
Chloramphenicol	55 (91.6)	4 (6.6)	1 (1.6)	60 (100)
Gentamycin	56 (93.3)	2 (3.3)	2 (3.3)	60 (100)
Ampicillin	10 (16.6)	2 (3.3)	48 (80)	60 (100)
Tetracycline	40 (66.6)	11 (18.3)	9 (15)	60 (100)
Streptomycin	45 (75)	9 (15)	6 (10)	60 (100)
Trimethoprim sulfamethoxazole	37 (61.6)	9 (15)	14 (23.3)	60 (100)

RESULTS

Prevalence of *S. aureus* and its antimicrobial resistance pattern by using disk diffusion method

Out of the 303 lactating cows, 214 (70.6%) of them were found positive by CMT for either of the four quarters. Among these 214 samples, 187 (87.4%) were bacterial culture positive in which 79 (36.9%) of the culture was identified as *S. aureus*. Antimicrobial resistance test was conducted for 60 of the 79 isolates. Antimicrobial resistance pattern of the *S. aureus* isolates is shown in Table 2. The isolates were resistant to ampicillin (80%) followed by trimethoprim-sulfamethoxazole (23.3%), tetracycline (15%), streptomycin (10%), gentamycin (3.3%) and equally to both erythromycin and chloramphenicol with the least resistance (1.6%).

Determination of antimicrobial resistance genes

Among all 45 isolates tested for the presence of antibiotic

resistance gene, only 14 (35%) were found free from any of the anti-microbial resistance gene (Table 3). Isolates containing antibiotic resistance genes were observed after electrophoresis of the PCR product (Figure 1).

Out of the 45 studied isolates, most of them (55%) contain *blaZ* gene followed by *ermB* (33%), *ermC* (13.3%) and each *ermA* and *msrA* were detected in only 2% of the isolates. No isolate was detected harboring either *ermA* or *msrA* solely. The gene *mecA* was not detected at all (Table 3).

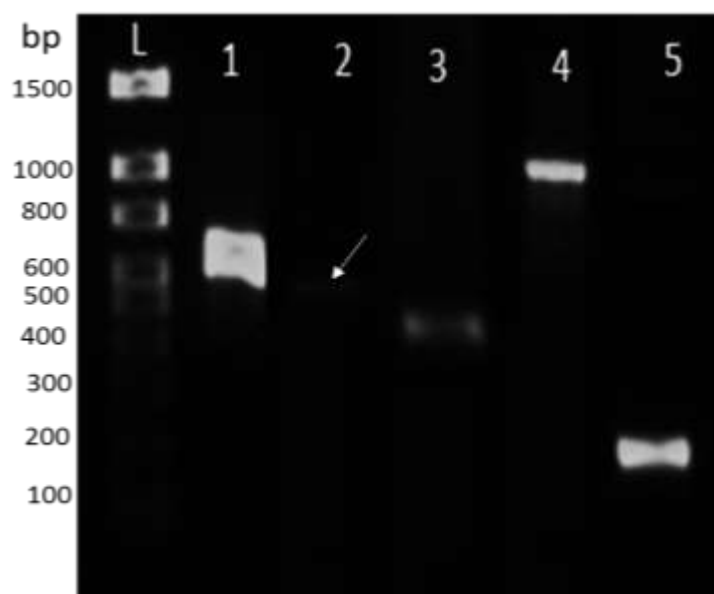
Assessment of multi-drugresistance (MDR) genes

Approximately 36% of the isolates contained more than one antibiotic resistance genes. The highest MDR gene combination was observed by *blaZ*ermB* (22.2%) genes and the least frequently occurred combinations were *blaZ*ermA* and *msrA*ermB* (2.2%) each. Combination of *blaZ*ermC* accounts for 8.8%. The gene *blaZ* occurred in combination with all the *erm* genes (Table 4).

Among the 45 isolates in which the PCR test was

Table 3. Distribution of antimicrobial resistance genes among isolates (out of 45).

Gene	Solely appear (%)	In combination with other (%)	Total (%)
<i>blaZ</i>	10 (22.2)	15 (33.3)	25 (55)
<i>ermA</i>	0 (0.0)	1 (2.2)	1 (2.2)
<i>ermB</i>	4 (4.8)	11 (24.4)	15 (33.3)
<i>ermC</i>	2 (2.4)	4 (8.8)	6 (13.3)
<i>msrA</i>	0 (0.0)	1 (2.2)	1 (2.2)
<i>mecA</i>	0 (0.0)	0 (0.0)	0 (0.0)

**Figure 1.** Agarose gel electrophoresis of representative isolates containing antibiotic resistance marker (1%). L. Ladder (100 bp -1.5 kb DNA ladder, Bio Basic), 1.*blaZ* (517 bp), 2.*ermA* (486 bp), 3.*ermB* (423 bp), 4.*msrA* (1000 bp), 5.*ermC* (272 bp).**Table 4.** Occurrence of multiple drug resistance genes among isolates (out of 45).

MDR	Genes frequency (%)
<i>blaZ</i> * <i>ermB</i>	10 (22.2)
<i>blaZ</i> * <i>ermC</i>	4 (8.8)
<i>blaZ</i> * <i>ermA</i>	1 (2.2)
<i>ermB</i> * <i>msrA</i>	1 (2.2)
subtotal	16 (35.5)

performed, 16 (35.5%) of them showed resistance on the disc diffusion test containing the respective resistance gene. High association between ampicillin resistance and the presence of *blaZ* gene has been observed. Sixty-eight percent of the isolates harboring the *blaZ* gene were found resistant to ampicillin.

DISCUSSION

S. aureus causes one of the most common types of chronic mastitis in dairy animals worldwide. In this study, the prevalence of bovine mastitis was found in 70.6%. This prevalence of mastitis is comparable with previous

reports from Ethiopia who reported a prevalence rate of 71.0 and 75.2% in dairy farms located at Holetta and Jimma towns, respectively (Sori et al., 2011; Mekibib et al., 2009). But, higher result was found in Holetta, Bahirdar and Gondar towns of Ethiopia (Marama et al., 2016; Bitew et al., 2010; Moges et al., 2011). The difference on the prevalence of mastitis among the studies could be due to difference in management system, milking practices, productivity, breed of the animals and location (Marama et al., 2016; Sori et al., 2011).

The prevalence of *S. aureus* was found at 36.9% which is higher than the finding of Marama et al. (2016) and lower than Sori et al. (2011). In Kenya, equivalent prevalence rate (30.6%) of *S. aureus* was reported by Shitandi and Sternesjo (2004). However, prevalence rate of 25.5, 23.3 and 10% was reported from China, Iran and Finland, respectively (Wang et al., 2008; Bahraminia et al., 2017; Pitkalla et al., 2004). The reason for the variability may be due to lactation stage of the cow, age of the cow and milking method (Kivaria et al., 2007; Ergn et al., 2009). In this study, 85% of the *S. aureus* isolates showed resistance to at least one antimicrobial drug. This is similar with the previous finding from Jimma and central Ethiopia with overall antibiotic resistance rates of 92 and 97.5%, respectively (Sori et al., 2011; Mekibib et al., 2009). However, in Brazil relatively higher susceptibility to antimicrobials (49.1%) was reported by Rabello et al. (2005). This variation might occur due to difference in milking practice, purpose of use of the antibiotics and inappropriate therapeutic treatment by nonprofessionals.

Considerable resistance to beta-lactam antibiotic by *S. aureus* appears with serious threat to the world (Lowy, 2003). In the current study, higher resistance to ampicillin (80%) was observed. This is comparable with the previous reports from Gondar (81.5%) and Italy (88%) (Moges et al., 2011; Viridis et al., 2010), but higher than other reports from Hawassa (67.9%) (Teshome et al., 2016). However, Daka et al. (2016) reported lower resistance rate (7.7%) from Hawassa, Ethiopia.

Tetracycline and its derivatives are the most extensively used antibiotics in Ethiopia for treatment of animal diseases. So that, certain level of resistance to this drug was expected. The present finding on resistance against tetracycline was 15% which is nearly half of the figure observed at Gondar and Holetta with the resistance rate of 29.6 and 33.3%, respectively (Moges et al., 2011; Marama et al., 2016). The most effective drugs in this experiment were gentamycin followed by erythromycin and chloramphenicol. These drugs are not first choice for treatment of mastitis in most part of the country hence the chance to develop resistance by *S. aureus* will be minimum. In contrast, there are evidences of erythromycin resistance development in some parts of the country (Moges et al., 2011; Teshome et al. 2016).

Multiple drug resistance is the ability of an organism to resist and grow against two or more antimicrobials. In this study, the isolates had shown inverse relation between

the multi-drug resistance nature and the number of antimicrobials applied. This observation match with the finding of Sori et al. (2011) who demonstrate MDR pattern of 25, 10.45 and 7% for two, three and four types of drugs, respectively. However, it differs from Teshome et al. (2016) who record MDR of 34.8% of the isolates for three and 8.7% for two antimicrobials. The difference observed in the pattern of MDR could be explained by group of drugs with similar chemical structure and mechanism of action may exhibit cross-resistance by the bacteria despite the number of drugs involved (Pechere, 2001).

The two main mechanisms of macrolide resistance are drug-efflux membrane pumps and modification of the drug target site in the ribosome. The third mechanism of resistance is drug inactivation (Bailey et al., 2008; Ojo et al., 2006; Jensen et al., 1999; Lina et al., 1999). It has been known that, isolates harbor *erm* genes (erythromycin resistance rRNA methylase) code for the protein called methyl transferase which induces N6 - dimethylation of an adenine residue of 23S rRNA. This process produces conformational changes in the phosphate site of the rRNA and prevents the macrolide binding at the peptidyl transferase center, hence the protein production will proceed and the bacteria will survive (Kot et al., 2012; Westh et al., 1995). Expression of the three related factors; *ermA*, *ermB*, and *ermC*, are responsible to make the bacteria resistance to macrolides and other related antibiotic groups (Ojo et al., 2006; Westh et al., 1995).

In the present work, among the three erythromycin resistance determinants, *ermB* (33.3%) resistance gene was the most frequently identified followed by *ermC* (13.3%) and *ermA* (2.2%). Bahraminia et al. (2017) reported similar pattern of *erm* genes distribution while studying bovine mastitis caused by *S. aureus*. A work done to determine types of *S. aureus* lineages affecting human showed that *ermA* as the most frequently found resistance gene (Bahraminia et al., 2017; Westh et al., 1995) although there are some findings supporting higher prevalence of *ermC* among the three determinants (Ross et al., 1990). Lina et al. (1999) correlate the distribution of *erm* genes with methicillin resistance and susceptibility in coagulase positive or negative staphylococci species.

The *ermA* gene found more common in methicillin resistant *S. aureus* stains (57.6%) compared to the sensitive ones (5.6%) (Westh et al., 1995).

The contribution of each of the *erm* determinants (*ermA*, *ermB* and *ermC*) towards phenotypic macrolide resistance is essential. Westh et al. (1995) determined that *ermA* and *ermC* are responsible for erythromycin resistance in more than 98% of *S. aureus* strains. In another report *ermC* was the dominant *erm* gene in *S. aureus* and was responsible for erythromycin resistance in 72% of the strains in the years 1983 to 1988 (Duran et al., 2012).

In the current study, despite the high prevalence of *ermB* gene, we have observed only a single erythromycin

resistance isolate (1.6%). This finding agrees with Westh et al. (1995) which reported that *ermB* is less responsible for phenotypic macrolide resistance. However, we have identified a single isolate harboring this gene together with *erm* genes (2.2%). The present finding showed similar results of previous works by Lina et al. (1999) and Ross et al. (1990) which identify prevalence of 3.3 and 3.6%, respectively for similar combination.

Staphylococcal resistance to penicillin is attained by the gene *blaZ* (β -lactamase). It encodes for extracellular enzyme β -lactamase which hydrolyze the β -lactam ring rendering the β -lactam inactive (Lowy, 2003). In the present study, the gene was widely spread among the isolates (80%) which are in line with the report by Duran et al. (2012). According to Lowy (2003), more than 90% of staphylococcal isolates produce penicillinase (β -lactamase enzyme). This indicates the wide distribution of the resistance gene globally through spread of resistance strains. Although *blaZ* is the primary key player for penicillin resistance among staphylococcus isolates, it is not the sole factor. In this study, not all isolates which show penicillin resistance by disk diffusion test harbor *blaZ* gene. This finding agrees with previous studies by Yang et al. (2015) and Gao et al. (2012) who identified staphylococcus and streptococcus isolates that showed resistance to penicillin but not carrying *blaZ* gene. Point mutation rather than gene acquisition could be another factor for only phenotypic resistance. Biofilm production and multi-drug efflux development are also other possible protection mechanism of the bacteria (Katayama et al., 2005; Wienders et al., 2002).

In this study, no isolate containing *mecA* gene was found. Similarly, another study mentioned that *mecA* is rarely found in many *Staphylococcus* isolates originated from animal infections (Ross et al., 1990). The limited distribution of *Staphylococcus* chromosomal cassette *mec* (SCC*mec*) which carries *mecA* gene by nature may be considered for its rare occurrence.

The occurrence of *S. aureus* resistance to antimicrobial agents is growing in alarming rate. So, the community should be aware by responsible bodies about the risk of consuming raw dairy products. The veterinary service delivery should be improved in order to avoid subjective treatment of animals by non-professionals. Besides, further studies should be conducted to obtain full figure of phenotypic as well as genotypic antimicrobial resistance pattern.

ABBREVIATIONS

CMT, California mastitis test; **OD**, optical density; **PCR**, polymerase chain reaction; **MDR**, multiple drug resistance.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

- Bahraminia F, Emadi SR, Emameini M, Farzaneh N, Rad M, Khoramian B (2017). A high prevalence of tylosin resistance among *Staphylococcus aureus* strains isolated from bovine mastitis. *Veterinary Research Forum* 8(2):121-125.
- Bailey M, Chettiath T, Mankin AS (2008). Induction of *erm(C)* Expression by non-inducing antibiotics. *Antimicrobial Agents and Chemotherapy* 52(3):866-874.
- Bitew M, Tefera A, Tolesa T (2010). Bovine mastitis in dairy farms of Bahir Dar and environs. *Journal of Animal Veterinary Advances* 9:2912-2917.
- Clinical and Laboratory Standards Institute (CLSI) (2007). Performance Standards for Antimicrobial Susceptibility Testing; Seventeenth Informational Supplement 31:1.
- Daka D, G/Silassie S, Yihdego D (2016). Antibiotic-resistance *Staphylococcus aureus* isolated from cow's milk in the Hawassa area, South Ethiopia. *Annals of Clinical Microbiology and Antimicrobials* 11:26.
- Duran N, Ozer B, Duran GG, Onlen Y, Demir C (2012). Antibiotic resistance genes & susceptibility patterns in staphylococci. *The Indian Journal of Medical Research* 135(3):389-396.
- Ergün Y, Aslantaş Ö, Doğruer G, Kireççi E, Sarıbay M, Ateş C, Ülkü A, Demir C (2009). Prevalence and etiology of subclinical mastitis in Awassi dairy ewes in southern Turkey. *Turkish Journal of Veterinary and Animal Sciences* 33(6):477-483.
- França CA, Peixoto RM, Cavalcante MB, Nataniel FM, Oliveira CJB, Veschi JLA, Mota RA, Costa MM (2012). Antimicrobial resistance of *Staphylococcus* spp. from small ruminant mastitis in Pesquisa. *Veterinária Brasileira* 32(8):747-753.
- Gao J, Yu F, Luo L, He JZ, Hou RG, Zhang HQ, Li SM, Su JL, Han B (2012). Antibiotic resistance of *Streptococcus agalactiae* from cows with mastitis. *The Veterinary Journal* 194:423-424.
- Hultgren J, Svensson C (2009). Lifetime risk and cost of clinical mastitis in dairy cows in relation to heifer rearing conditions in southwest Sweden. *Journal of Dairy Science* 92(7):3274-3280.
- Jensen LB, Frimodt-Møller N, Aarestrup FM (1999). Presence of *erm* gene classes in Gram-positive bacteria of animal and human origin in Denmark. *FEMS Microbiology Letters* 170:51-158.
- Katayama Y, Robinson DA, Enright MC, Chambers HF (2005). Genetic background affects stability of *mecA* in *Staphylococcus aureus*. *Journal of Clinical Microbiology* 43(5):2380-2383.
- Kirby W, Bauer A, Sherris J, Turck M (1966). Antibiotic susceptibility testing by single disc method. *American Journal of Clinical Pathology* 45:4.
- Kivaria FM, Noordhuizen JPTM, Nielen M (2007). Interpretation of California mastitis test scores using *Staphylococcus aureus* culture results for screening of subclinical mastitis in low yielding smallholder dairy cows in the Dar es Salaam region of Tanzania. *Preventive Veterinary Medicine* 78:274-285.
- Kot B, Piechota M, Wolska KM, Frankowska A, Zdunek E, Binek T, Kłopotowska E, Antosiewicz M (2012). Phenotypic and genotypic antimicrobial resistance of staphylococci from bovine milk. *Polish Journal of Veterinary Sciences* 15(4):677-683.
- Lina G, Quaglia A, Reverdy M, Leclercq R, Vandenesch F, Etienne J (1999). Distribution of genes encoding resistance to macrolides, lincosamides, and streptogramins among staphylococci. *Antimicrobial Agents and Chemotherapy* 43(5):1062-1066.
- Lowder BV, Guinane CM, Ben Zakour NL, Weinert LA, Conway-Morris A, Cartwright RA, Simpson AJ, Rambaut A, Nübel U, Fitzgerald JR (2009). Recent human-to-poultry host jump, adaptation, and pandemic spread of *Staphylococcus aureus*. *Proceedings of the National Academy of Sciences of the United States of America* 106:19545-19550.
- Lowy FD (2003). Antimicrobial resistance: the example of

- Staphylococcus aureus*. Journal of Clinical Investigation 111(9):1265-1273.
- Marama A, Mamu G, Birhanu T (2016). Prevalence and antibiotic resistance of *Staphylococcus aureus* Mastitis in Holetta Area, Western Ethiopia. Global Veterinaria 16(4):365-370.
- Mekibib B, Furgasa M, Abunna F, Megersa B, Regassa A (2009). Bovine mastitis: prevalence, risk factors and major pathogens in dairy farms of Holetta Town, Central Ethiopia. Veterinary World 13(9):397-403.
- Moges N, Asfaw Y, Belihu K, Tadesse A (2011). Antimicrobial susceptibility of mastitis pathogens from smallholder dairy herds in and around Gondar, Ethiopia. Journal of Animal Veterinary advances 10(12):1616-1622.
- Murakami KW, Minamide W, Wada K, Nakamura E, Teraoka H, Watanabe S (1991). Identification of methicillin resistant strains of *Staphylococci* by polymerase chain reaction. Journal of Clinical Microbiology 29:2240-2244.
- OIE (World Organization for Animal Health) (2012). Manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees). 7th ed., Vol. 2.
- Ojo KK, Striplin MJ, Ulep CC, Close NS, Zittle J, Luis H, Bernardo M, Leitao J, Roberts MC (2006). *Staphylococcus* Efflux *msr(A)* Gene Characterized in *Streptococcus*, *Enterococcus*, *Corynebacterium*, and *Pseudomonas* Isolates. Antimicrobial Agents and Chemotherapy, pp. 1089-1091.
- Ostergaard S, Grohn Y (1999). Effects of diseases on test day milk yield and body weight of dairy cows from Danish Research Herds. Journal of Dairy Science 82(6):1188-1201.
- Pechere JC (2001). Macrolide resistance mechanisms in Gram-positive cocci. International Journal of Antimicrobial Agents 18:25-28.
- Pitkalla A, Haveri M, Pyörälä S, Myllys V, Honkanen-Buzalski T (2004). Bovine mastitis in Finland 2001- Prevalence, distribution of bacteria, and antimicrobial resistance. Journal of Dairy Science (8):2433-41.
- Quinn P, Markey B, Leonard F, Fitz Patrick S, Fanning S, Fitz Patrick E (2011). Veterinary microbiology and microbial disease. 2nd ed, Wiley-Blackwell.
- Quinn PJ, Carter ME, Markey B, Carter GR (1994). Clinical Veterinary Microbiology. Wolfe, London. p. 648.
- Rabello R, Souza C, Duarte R, Lopes R, Teixeira L, Castro A (2005). Characterization of *Staphylococcus aureus* isolates recovered from bovine mastitis in Rio de Janeiro, Brazil. Journal of Dairy Science 88:3211-3219.
- Raney T (2009). The state of food and agriculture: livestock in the balance. Food and Agriculture Organization of the United Nations, Rome, Italy.
- Ross JI, Eady EA, Cove JH, Cunliffe WJ, Baumberg S, Wootton JC (1990). Inducible erythromycin resistance in *Staphylococci* is encoded by a member of the ATP-binding transport super-gene family. Molecular Microbiology 4(7):1207-1214.
- Sakwinska O, Giddey M, Moreillon M, Morisset D, Waldvoege A, Moreillon P (2011). *Staphylococcus aureus* host range and human-bovine host shift. Applied and Environmental Microbiology 77(17):5908-15.
- Sawant A, Gillespie B, Oliver S (2009). Antimicrobial susceptibility of coagulase negative *Staphylococcus* species isolated from bovine milk. Veterinary Microbiology 134:73-81.
- Shitandi A, Sternesjo A (2004). Factors contributing to the occurrence of antimicrobial drug residues in Kenyan milk. Journal of Food Protection 67(2):399-402.
- Smith TC (2015). Livestock-Associated *Staphylococcus aureus*: The United States Experience. PLOS Pathogens 11(2):e1004564. <https://doi.org/10.1371/journal.ppat.1004564>.
- Somayyeh H, Habib D (2014). Staphylococcal species associated with bovine mastitis in the North West of Iran: Emerging of coagulase-negative staphylococci. International Journal of Veterinary Science and Medicine 2:27-34.
- Sori T, Hussien J, Bitew M (2011). Prevalence and susceptibility assay of *staphylococcus aureus* isolated from bovine mastitis in dairy farms of Jimma Town, South west Ethiopia. Journal of Animal Veterinary advances 10(6):745-749.
- Teshome B, Tefera G, Belete B, Mekuria A (2016). Prevalence and antimicrobial susceptibility pattern of *Staphylococcus aureus* from raw camel and goat milk from Somali region of Ethiopia. African Journal of Microbiology Research 10(28):1066-1071.
- Virdis S, Scarano C, Cossu F, Spanu V, Spanu C, De Santis E (2010). Antibiotic resistance in *Staphylococcus aureus* and coagulase negative staphylococci isolated from goats with subclinical mastitis. Veterinary Medicine International 2010: 517060.doi: 10.4061/2010/517060.
- Wang Y, Wu C, Lu L, Ren GN, Cao X, Shen J (2008). Macrolide-lincosamide-resistant phenotypes and genotypes of *Staphylococcus aureus* isolated from bovine clinical mastitis. Veterinary Microbiology 130(1-2):118-25.
- Westh H, Hougaard DM, Vuust J, Rosdahl VT (1995). Prevalence of *erm* gene classes in erythromycin-resistant *Staphylococcus aureus* strains isolated between 1959 and 1988. Antimicrobial Agents and chemotherapy 39(2):369-373.
- Wielders CL, Fluit AC, Brisse S, Verhoef J, Schmitz JF (2002). *mecA* gene is widely disseminated in *Staphylococcus aureus* population. Journal of Clinical Microbiology 40(11):3970-3975.
- Yang F, Wang Q, Wang X, Wang L, Xiao M, Li X, Luo J, Zhang S, Li H (2015). Prevalence of *blaZ* gene and other virulence genes in penicillin-resistant *Staphylococcus aureus* isolated from bovine mastitis cases in Gansu, China. Turkish Journal of Veterinary and Animal Sciences 39:634-636.

Full Length Research Paper

Histological of the midbrain of the grey breasted helmeted guinea fowl (*Numida meleagris galeata*) at post-hatch

Wanmi Nathaniel^{1*}, Usendi Ifukibot Levi², Gosomji Innocent Jonah³ and Pindar David Bukar⁴

¹Department of Veterinary Anatomy, College of Veterinary Medicine, UAM, Makurdi, Benue State, Nigeria.

²Department of Veterinary Anatomy, Faculty of Veterinary Medicine, University of Jos, Plateau State, Nigeria.

³Department of Veterinary Anatomy, Faculty of Veterinary Medicine, UNIABUJA, Nigeria.

⁴Department of Veterinary Anatomy, Faculty of Veterinary Medicine, Ahmadu Bello University Zaria, Kaduna State, Nigeria.

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The study was carried out to investigate the development of the midbrain in the grey breasted helmeted guinea fowl, *Numida meleagris galeata*, at post-hatch. Domestication of this species of bird is in an increase, but scanty documentation on the development of midbrain (mesencephalon) and how it relates to the neurobiology of this bird is lacking. In our findings, there was a steady increase in the midbrain mean weights found to be 0.0418 ± 0.0081 and 0.2236 ± 0.0224 g on the first day and day 61 post-hatch, respectively. Morphological and cellular development shows the midbrain to develop immediately after hatch at day one. This indicates that auditory-visual activities are fully functional in the wild grey breasted helmeted guinea fowl and capable of independent survival immediately after hatching.

Key words: Morpho-histogenesis, midbrain, guinea fowl.

INTRODUCTION

The helmeted guinea fowl (*Numida meleagris galeata*) is a native to Africa and belongs to the Phylum, Chordata; Subphylum, Vertebrata; Class, Aves; Order, Galliformes; and Family, Numidae. It is widely distributed in the Guinea Savannah vegetation zone of Nigeria and estimated at 44 million in captivity (Ayeni, 1980).

Developmentally, in avian, the central nervous system develops from the neural plate, an epithelial sheet that

arises from the dorsal ectoderm of the developing embryo (Hallonet et al., 1990). After neural tube closure, series of vesicles can be clearly distinguished morphologically at the anterior end indicating an anterior-posterior axis development (Joyner, 2002). The most anterior end of the neural tube gives rise to the procerebrum (forebrain) consisting of the telencebrum and diencephalon. The middle part of

*Corresponding author. E-mail: nathanielwanmi2014@gmail.com.

the brain is the mesencephalon, often referred to as the midbrain, while the most posterior region from the brain is the rhombencephalon, which further differentiates into metencephalon (cerebellum and pons) and myelencephalon (medulla oblongata and spinal cord) (Hallonet et al., 1993).

The mesencephalon (midbrain) also comprises of the optic lobe, which is the visual roof, often named optic tectum, in non-mammalian species and superior colliculus in mammalian species (Northcutt, 2002).

Audition is of critical importance to a wide range of behaviours in birds, such as prey capture, individual and species recognition, vocal learning and mate selection. As a result of the range of behaviours that rely upon acoustic cues, studies of avian audition have revealed marked species differences in hearing range and the ability to localize sounds and discriminate pitch, intensity and temporal differences (Agawala et al., 2001). From this and other evidence, it is clear that several groups have evolved unique auditory specializations: localization of prey using only acoustic cues, vocal learning and echolocation in birds. Given that birds share many behavioural and neural similarities (Mogensen and Divac, 1993) with mammals, it is certain that such correlations also exist in bird midbrain.

Several research has been done to study the development of the avian brain, which includes; a pre-hatch study of the structural organization of grey breasted helmeted guinea fowl (Wanmi et al., 2016), the immunoreactivity of glial cells (Maximina et al., 1998), glial cells in the central nervous system (CNS) of healthy Passeriformes birds (Peer, 2012) and the development of chicken cerebellar cortex (Serdar and Emrah, 2010). Despite these studies, there exists a dearth of information on the histomorphogenesis of the grey breasted helmeted guinea fowl in Nigeria.

In this investigation, an attempt will be made to find out the variations in the developmental anatomy of the midbrain in the grey breasted helmeted guinea fowl with regard to their developmental gross structure histogenesis, which may be helpful in the understanding of the neurobiology of this species of bird despite increase awareness on its domestication.

MATERIALS AND METHODS

Experimental design

A total of eighty four fertilized guinea fowl eggs purchased from the National Veterinary Research Institute (NVRI) Vom, Jos, Plateau State, Nigeria and other local breeders within Jos and its environs were used for this study. The eggs were transported to a hatchery, still in Jos and incubated using their standard incubation guide. During incubation, the eggs were turned regularly (minimum of three times) each day for the first 24 days according to method modified by Moreki et al. (2012).

These eggs were allowed to hatch into young ones referred to as keets and were removed from the incubator and kept in a standard laboratory cage.

Extraction of embryo

This was done at pre-hatch using a scalpel blade and clean transparent dish. The blunt side of the scalpel blade was used, with the egg held on the palm, and a gentle tap was made on the egg until a crack was formed. Then, the crack was gently widened manually and the embryo collected in a white dish as modified by Salami (2009).

Extraction of brain

At pre-hatch, because the entire skull is soft and pliable, scalpel blade and rat tooth forceps were used for extraction of the brain. At post-hatch, the keets were euthanized using Nembutal at 40 mg/body weight. Thereafter, decapitation was made and the heads fixed in 10% neutral buffered formalin for 3 to 5 days. After proper fixation, a dissection was made at the angle of the beak up to the level of the occipital bone. The upper portion of the dissected area is pulled off gradually using the rat tooth forceps until the entire brain was exposed. The cranial nerves were severed to ease the lifting of the brain from the cranium. Extracted brain samples were fixed in Bouin's solution for routine staining.

Separation of the midbrain

The cerebellum is located on the dorsal portion of the brain stem with three peduncles: the restiform body connected to the medulla, the brachium pontis that connects cerebellum to the Pons and the brachium, lastly the conjunctivum that connects cerebellum to the midbrain. These peduncles were severed using a scalpel blade to expose the entire brainstem.

The midbrain or mesencephalon is located just caudal to the level of chiasma opticum rostrally and at the level of ponto-mesencephalic region caudally. After the midbrain removal, the anterior portions left are the diencephalon and the cerebrum. At the boundary between the diencephalon and cerebrum, an incision was made revealing the cerebral hemispheres held together by the corpus callosum. Each hemisphere was freed by a transverse incision into the longitudinal fissure. These landmarks were cut across using the scalpel blade to extract the midbrain.

Gross anatomy and morphometry

Seventy seven brain samples were used for morphometric study in which seven brain samples were used per day. The following days were used for sacrificing the keets: day 1, 7, 13, 19, 25, 31, 37, 43, 49, 55, and 61. Two brain samples were used for gross study. The weights of the midbrain were taken using a digital electronic balance; (Model JJ1000, Maximum 1000 g, d=0.01 g, e=10d, No. 211011011098, Made in China and analytical weighing balance, Adventure QHAUS Corporation Item No; AR3130, Maximum Capacity= 310 g Readability= 0.001 g). Photographs of the dorsal and ventral aspects were taken using a cannon digital camera (4x optical zoom lens 5.0 to 20.0 mm, 15.1 mega pixels Apple, Cannon) and a digital handheld microscope, (Magnification 1000x, 5x zoom, 3D stand high speed DSP).

Histological techniques

Six brain samples were used for Hematoxylin and Eosin (H and

Table 1. Mean weights of the guinea fowl midbrain at days 1 to 61 post-hatch period (in gram), (n=7).

Days	Min. wt/g	Max. wt/g	Mean \pm SEM
1	0.0144	0.0703	0.0418 \pm 0.0081
7	0.0248	0.0768	0.0480 \pm 0.0064
13	0.0290	0.0862	0.0576 \pm 0.0019
19	0.0410	0.0914	0.0649 \pm 0.0072
25	0.0519	0.1081	0.0802 \pm 0.0067
31	0.0676	0.1037	0.0882 \pm 0.0014
37	0.0687	0.1301	0.0995 \pm 0.0023
43	0.0791	0.2324	0.1448 \pm 0.0556
49	0.0918	0.2368	0.1531 \pm 0.0499
55	0.1081	0.2512	0.1932 \pm 0.0480
61	0.1989	0.2602	0.2236 \pm 0.0224

n, number of birds used per day; Min, minimum; Max, Maximum; Wt, Weight, g, Gram; SEM, standard error of Mean.

E) technique; fixed brain samples were washed using tap water and dehydrated through ascending grades of alcohol (70, 80, 90, 95, 100%), within intervals of 3 h each, cleared in xylene for 2 h and embedded in liquid paraffin at 50°C according to standard

Procedures as described by Kiernan (1990) were used. Serial transverse sections of 5 μ l were made using Jung rotary microtome (Model 42339, Berlin, Germany). Sections were mounted on glass slides and allowed to dry, deparaffinized, stained, hydrated and cover-slipped using diphynylphthalate propylene xylene (DPX) as mountant (Humanson, 1972). Sections were stained with H and E stains for routine general study, and cresyl violet fast (CVF) stain for nuclei identification. Photomicrographs of sections were taken using a USB digital eyepiece (Amscope DCM 500, Resolution: 14 Mega pixels, made in China) attached to a light microscope (OLYMPUS XSZ107BN, Hamburg, Germany), viewed and captured on the laptop screen at different magnifications (x40, x100, x250 x400 and x560).

Data analysis

Morphometric data on the midbrain was analyzed using Statistical Package for Social Science (SPSS) version 17.0. In the analysis, the descriptive statistics was expressed as mean \pm standard error of the Mean.

RESULTS

Morphometry

The mean weight of the midbrain of the helmeted guinea fowl at days 1, 43 and 61 post-hatch were; 0.0418 \pm 0.0081, 0.1448 \pm 0.0556 and 0.2236 \pm 0.0224 g, respectively (Table 1).

The chart shows that the midbrain at days less than 37 weighed below 0.10 g, and thereafter, increased progressively (Figure 1).

Gross features

Grossly, the entire brain was fully developed at day one post-hatch. Ventrally, the olfactory bulb, optic tract and optic chiasm were well developed. The cerebellum projected upward and forward. The optic lobes were separated from and located below the cerebrum; attached to the lateral body of the cerebellum and had a flattened dorsal view which broadened ventrally. The midbrain was found to be made up of two ovals like bulge, the optic lobe and midbrain flanked on both sides by optic lobes (Plate 1).

Histological features

At four weeks of post hatch, different forms of nuclei were prominent with centrally located nucleoli in most of the large neurones (Plate 2). Mesencephalic ventricle, with different forms of neurones, possessing nucleoli and devoid of processes was evident when stained with cresyl violet fast (Plate 3). At week eight, nerve processes (dendrites and axon hillock) of large neurones were prominent. Large pyramidal neurones were seen to have dense cytoplasmic inclusions (Plate 4). Throughout with the use of cresyl violet fast stain, most of the neurones pick up dark stained cytoplasmic bodies.

DISCUSSION

From this present study, the mean weight of the midbrain increased with increase in days. This agrees with the statement that brain weights increase as the

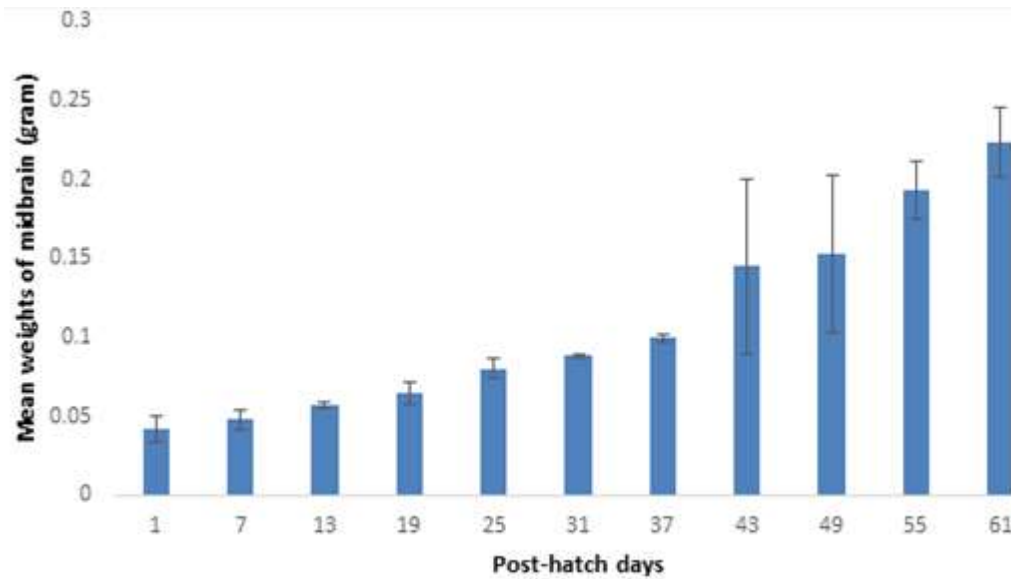


Figure 1. Mean weights of the guinea fowl midbrain at days 1 to 61 post-hatch (in gram).

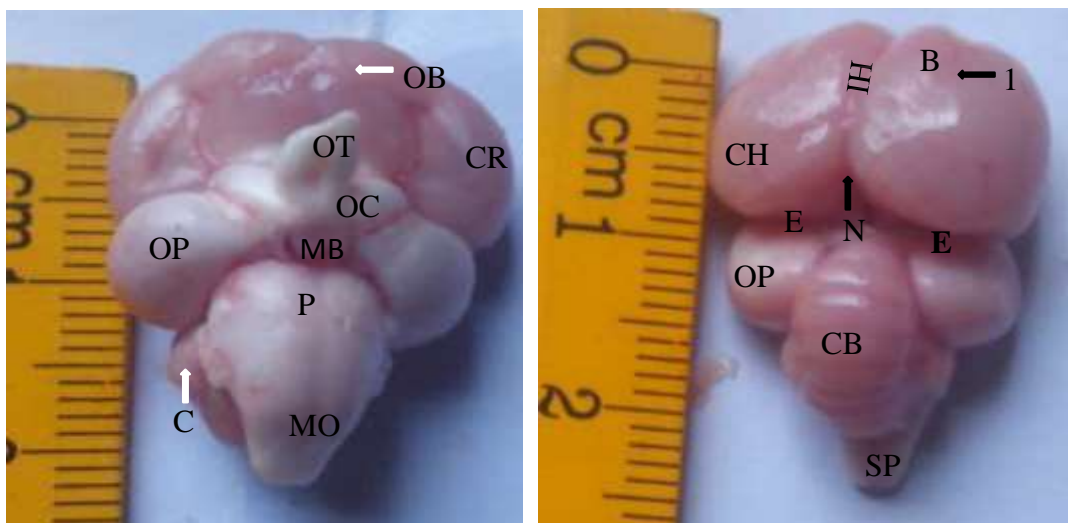


Plate 1. Brain of guinea fowl at day one post-hatch, B, Wulst ;IH, Interhemispheric fissure; CH, cerebral hemisphere; E, transverse fissure; OP, Optic lobe; N, Notch; CB, cerebellum; SP, spinal cord; 1, Vallecula; OB, olfactory bulb; CR, cerebrum; OP, optic lobe; OC, optic chiasm; OT, Optic tract; MB, midbrain; P, Pons; CA, cerebellar auricle; M, medulla oblongata. Magnification X500.

body weights increase, but the mean weight of the brain is lower than that of the body and that Galliformes had the lowest values which were not constant and could vary in birds of the same body weight (Xu et al., 2010). This increase in the weight of the midbrain signifies development is taking place, graphically seen with the chart increasing steadily.

The midbrain appeared like a stalk that is flanked by optic lobe laterally, cranially by optic chiasm. The midbrain in birds and reptiles is one of the regions that

form an integral part of the acoustic-mediated behaviours that receives input from the two parallel auditory pathways and as such play a role in the integration of auditory information and is homologous to both the mammalian inferior colliculus (Carr and Code, 2000).

The optic lobe was large, well developed and located below the cerebrum with the cerebellum projected upward and forward. This indicated that the optic lobe of the helmeted guinea fowl was well developed and this

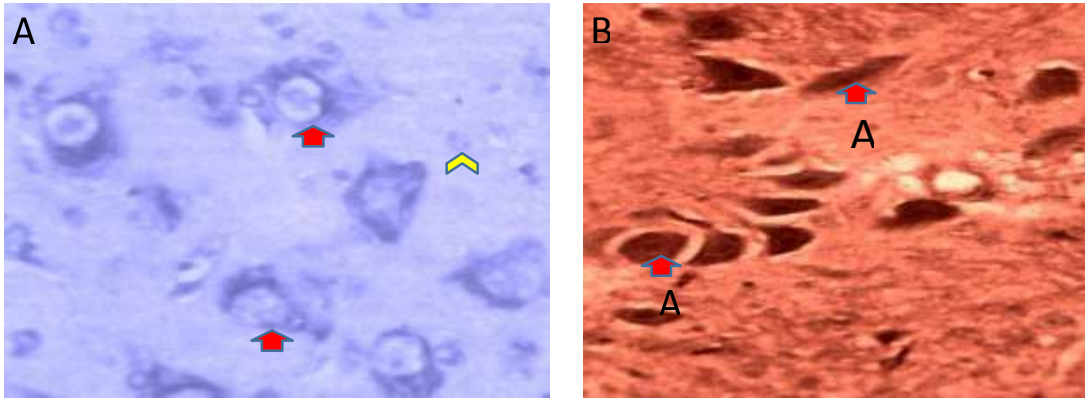


Plate 2. Neurons of midbrain of the helmeted guinea fowl keets at week 4 post-hatch. A, Large neurons; arrowhead, Neuroglia; arrow, pyramidal neurons. A, CFV; B, H&E, magnifications: A, X400; B, X200.

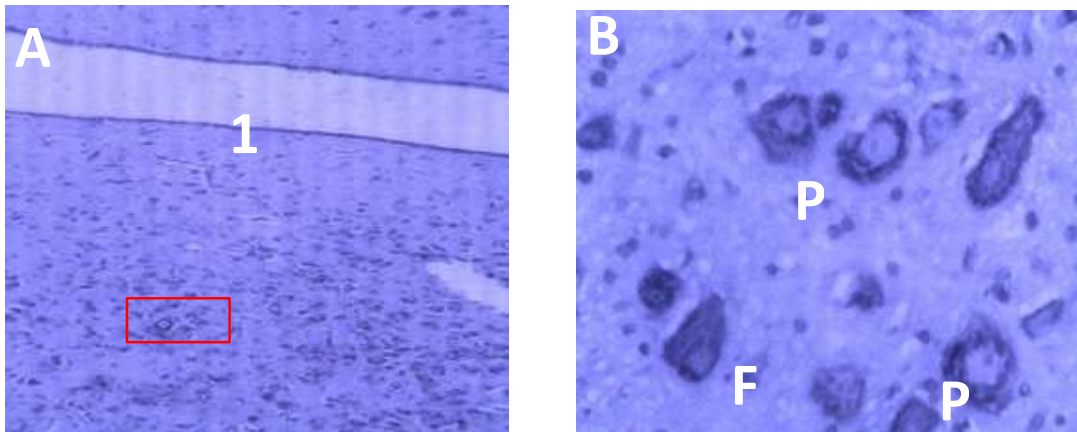


Plate 3. Neuronal components of the midbrain of the helmeted guinea fowl at week 6 post-hatch. 1, Ventricle; P, pyramidal neurons; F, fusiform neurons. Magnifications: A, X140; B, X200.

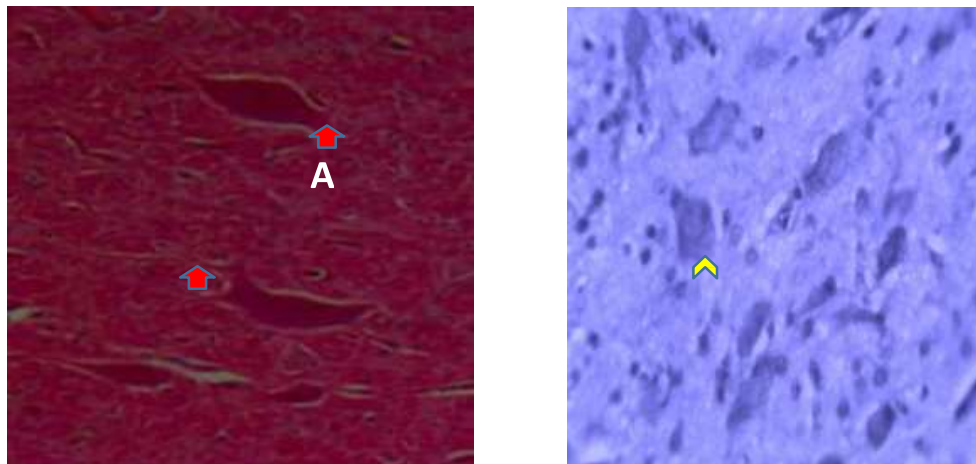


Plate 4. Midbrain of the helmeted guinea fowl at week eight post-hatch indicating. A, Axon hilux; Arrow, Dendrite of fusiform neurons, arrowhead, pyramidal neurons. Magnifications: Week 8 post-hatch, A, X140; B, X200.

was similar to the reports of Martin et al. (2007) in the emu and pigeon and Iwaniuk (2003) in parrots.

The kiwi and barn owl have relatively small optic lobes (Wylie et al., 2006). The position of the optic lobe depends on the extension of the caudotemporal part of the cerebral hemisphere and the position in the parrot, kiwi and corvids were ventral (Iwaniuk, 2003), lateral and below the cerebral hemispheres in the diurnal raptor species, respectively (Shermean, 2013). The midbrain contains neuropil darker stained granule cells (small and large) similar to the observation of Ghatar (1992) in the developing brain of young rats.

These dark stained granules in developing brain may be due to the large proliferation of chromatin in the brain (Karten and Hodes, 1967) in which different forms of neurones with prominent and centrally located nucleoli occurred at a later stage.

The midbrain act as a relay station for part of the extrapyramidal system, in which lesion of the neurones in the chicken has led to transient tremor, ataxia and ipsilateral oculomotor disturbances (Mackenna and Callander, 1997).

Conclusion

Grossly and histologically, major features were fully formed on day one post-hatch. This signifies that, the keet of wild helmeted guinea fowl has a better developed sense of hearing immediately after hatching. Large neurones of the midbrain are capable of controlling gaits in this bird.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

- Agawala STA, Sanders CW, Ragsdale R (2001). Sonic hedgehog control of size and shape in midbrain pattern formation. *Science* 291:2147-2150.
- Ayeni JSO (1980). The biology and utilization of the helmeted guinea fowl (*Numida meleagris galeata*, Pallas). Ph.D. Thesis unpublished. University of Ibadan, Ibadan, Nigeria.
- Carr CE, Code RA (2000). The central auditory system of reptiles and birds. In: Dooling RJ, Fay RR, Popper AN, editors. *Comparative Hearing in Birds and Reptiles*. New York: Springer-Verlag, pp. 197-248.
- Ghatak NR (1992). Occurrence of Oligodendrocytes within Astrocytes in Demyelinating Lesions. *Journal of Neurology and Experimental Neuroscience* 5(1):40-46.
- Hallonnet ME, Dehl LE, Douarin MN (1993). Tracing neuroepithelial cells of mesencephalic and metencephalic alar plates during cerebellar ontogeny in quail-chick chimeras. *European Journal of Neuroscience* 5:1145-1155.
- Hallonnet ME, Le Douarin M, Teillet MA (1990). A new approach to development of the cerebellum provided by the quail-chick marker system. *Development* 108:19-31.
- Humanson GL (1972). *Animal Tissue Technique*. New York: Blakiston. pp. 403-409. Available at: <https://archive.org/details/animaltissue00huma>
- Iwaniuk AN (2003). The Evolution of Brain Size Structure in Birds unpublished. *PhD Thesis*. Monash University Clayton, Australian.
- Joyner AL (2002). Establishment of anterior-posterior and dorsal-ventral pattern in the early central nervous system, *Journal of Rossant, P.P.L. Tam Editions, mouse development patterning, morphogenesis and organogenesis*, Academic Press, San Diego. pp. 107-126.
- Karten HJ, Hodes W (1967). *A stereotaxic atlas of the brain of the pigeon (Columba livia)*. Baltimore: Johns Hopkins Press.
- Kiernan JA (1990). *Histological and Histochemical Methods: Theory and Practice*. Oxford Pergamon Press, London. pp. 320-344.
- Martin GR, Wilson KJ, Wild JM, Parsons S, Kubke MF, Corfield J (2007). Kiwi forego vision in the guidance of their nocturnal activities. *Plos One* 2:198-209.
- Mackenna BR, Callander R (1997). *Illustrated Physiology 8th Edition*, New York, San, Francisco. Toronto, Churchill, Livingston Press. pp. 288-305.
- Maximina MM, Carmen Y, Jean DB, Carmwn CC, Jaime RP, Georges T, Giorgio G (1998). Heterogeneous immunoreactivity of glial cells in the mesencephalon of a lizard; a double labelling immunohistochemical study. *Journal of Morphology* 235:109-119.
- Mogensen J, Divac I (1993). Behavioural effects of ablation of the pigeon-equivalent of the mammalian prefrontal cortex. *Behavioural Brain Research* 55:101-107.
- Moreki JC, Nobo G, Nsoso SJ (2012). Feed intake, body weight, average daily gain, feed conversion, ratio and carcass characteristic of Helmeted guinea fowl fed varying levels of phane meal (*Imbrasia belina*) as replacement of fishmeal under intensive system *International Journal of Poultry Science* 11(6):278-284.
- Northcutt RG (2001). Changing views of brain evolution. *Brain Research Bulletin* 55:663-674.
- Northcutt RG (2002). Understanding vertebrates brain evolution. *Integrative and Comparative Biology* 42(4):743-756.
- Peer J (2012). Perineural glial clusters in the avian telencephalon. *Animal Behaviour*. 83:168-179.
- Salami SO (2009). Studies on the onset of osteogenesis in grey-breasted guinea fowl (*Numida meleagris galeata*). PhD Thesis, Ahmadu Bello University, Zaria, Nigeria.
- Shermean AA (2013). Anatomical and Histological Study of the Optic Tectum in Diurnal Raptor Species Buteo (Buzzard). *Journal of University of Babylon for Pure and Applied Sciences* 21(1).
- Xu G, Kemp PS, Hwu JA, Beagley AM, Bayly PV, Taber LA, (2010). Opening angles and material properties of the early embryonic chick brain. *Journal of Biomechanical Engineering* 132:011005.
- Wanmi N, Onyeanusi BI, Nzalak JO, Aluwong T (2016). Histomorphogenesis of the cerebellum of the grey breasted helmeted guinea fowl (*Numida meleagris galeata*) at pre-hatch and post-hatch study. *Animal Molecular Breeding* 6(4):1-10.
- Wylie DR, Hurd P, Iwaniuk AN (2006). The pretectal nucleus lentiformis mesencephalon is hypertrophied in homing birds. *Brain, Behavior and Evolution* 32:210-223.

Full Length Research Paper

Comparison of fluorescence polarization assay and enzyme-linked immunosorbent assay for the diagnosis of bovine paratuberculosis

Raquel Torres-Velez^{1,2}, Marco Antonio Santillán-Flores^{1*}, Dionisio Cordova-López¹, Olga Lidia Martínez-Martínez² and Claudia Celic Guzmán-Ruiz³

¹CENID Microbiología INIFAP: Carretera México Toluca Km 15.5, Colonia Palo Alto, Cuajimalpa, C.P. 05110, CDMX, México.

²Universidad del Valle de México CAMPUS Coyoacán: Calzada de Tlalpan 3016, Ex hacienda de Coapa, C. P. 04910, Delegación Coyoacán CDMX, México.

³Universidad de Guanajuato, División Ciencias de la Vida, Campus Irapuato –Salamanca. Ex hacienda, El Copal Km 9, Carretera Irapuato-Silao, CP 36820. Irapuato, Guanajuato, México.

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***Mycobacterium avium* subspecies *paratuberculosis* (Map), is the etiological agent of paratuberculosis, a disease that affects cattle and causes economic losses to the animal husbandry industry. Its opportune diagnosis, in herds, is part of the control measures of the disease; therefore, the objective of this work was to compare paratuberculosis detection of infected bovines with the Fluorescence Polarization Assay (FPA) and Enzyme-linked Immunosorbent Assay (ELISA). Six-hundred and three sera and feces samples, from bovines older than 2 years old were studied. The sera were assessed with the FPA technique using as antigen a protein fraction of 35 kDa obtained from the raw extract of the Map strain 3065, and for ELISA the protoplasmic antigen of the same strain was used. DNA was obtained from the feces and assessed by nested PCR. The correlation of results was established by Kappa Test. The FPA test had sensitivity (Se) of 88.50% and specificity (Sp) of 91.42% ($p \leq 0.000$); for ELISA Se 83.86% and Sp 89.87% ($p \leq 0.000$) were obtained. Concordance (K) between tests was 0.6742%, and when compared with nested polymerase chain reaction (PCR), the FPA test had $K = 0.7314\%$, while for ELISA it was 0.5771%. The FPA technique using as antigen the protein fraction of 35 kDa showed a higher sensitivity and specificity, moreover it was a simple technique for the determination of the antigen-antibody interaction, and therefore it becomes an alternative diagnostic tool to detect paratuberculosis infected bovines.**

Key words: Paratuberculosis, diagnosis, fluorescence polarization assay (FPA), enzyme-linked immunoabsorbent assay (ELISA).

INTRODUCTION

Paratuberculosis or Johne's disease is an infectious contagious disease that is characterized by the production of chronic granulomatous enteritis. The etiological agent is *Mycobacterium avium* subsp.

paratuberculosis (Map) (Arsenault et al., 2014). This serious economically important disease has a worldwide distribution with prevalence rate variation from five to 30% (Mortier et al., 2015).

Animals younger than 6 months of age are infected by ingesting bacilli through feed and water, as well as, sucking on teats contaminated with infected feces (Castellanos, 2010).

Affected bovines suffer chronic enteritis, the signs of which are diarrhea, submandibular edema, weight loss, and low body condition that leads eventually to death (Cirone et al., 2007; Speer et al., 2006). Clinical signs are only observed in adults between 18 and 24 months of age. Appetite and general behavior of the animal remain normal during the early stages, but milk production and body condition worsen due to nutrient malabsorption. As the disease advances, there is lethargy, depression, bristly hair, hypoproteinemia and submandibular edema (Mortier et al., 2015).

Tests based on the humoral immune response are the most frequently used for the diagnosis of the infection. One of the more evaluated tests has been the ELISA which is considered a good option for diagnosis, since there are several brands of commercial kits; it has a sensitivity of 50 to 83% and a specificity of 70 to 89% (Martinez et al., 2012).

Another technique that allows the determination of the antigen-antibody interaction is the FPA. The principle of this test is based on the property that antibodies have a high affinity and specificity to join a particular antigen; this property is used to recognize a specific analyte of interest, and by different mechanisms, generate a light signal that may be measured. In the case of FPA, the signal which is being measured is polarized fluorescent light. The size of the molecule is the main factor that influences the rotation speed. For example, a small molecule rotates at a higher speed than a larger molecule. These molecules move and rotate freely in the solution, and the result that is obtained is going to depend on how much the molecule has been able to rotate during the time the excited fluorescence state lasts. The smaller the molecule, the faster it shall rotate, and its polarization of fluorescence shall be lower (Nielsen and Gall, 2001; Marcelo et al., 2017).

If a molecule is marked with a fluorochrome, the rotation time through a 68.5° angle can be established by measuring the intensity of polarized light in vertical or horizontal planes. A large molecule emits more light in a single plane (more polarized) than a small one that rotates faster and emits more depolarized light (Nielsen and Gall, 2001; Marcelo et al., 2017). Polarized fluorescence has been used for the diagnosis of bovine brucellosis and tuberculosis, demonstrating a sensitivity of 95.5% and specificity of 99.0% for brucellosis and 92.9 and 98.3%, respectively for tuberculosis (Surujballi et al., 2002; Jolley et al., 2007; Marcelo et al., 2017).

Paratuberculosis diagnostic methods must be based on their capacity to detect infected animals; the specificity and reliability of serodiagnosis tests is determined by the type of mycobacterial antigens that are used, hence the identification of the structural antigenic components must be well characterized and specific for each mycobacterial species to be able to provide the means to improve specificity and sensitivity of immunodiagnostic assays. The economic losses caused by paratuberculosis on the animal husbandry industry are a serious problem, and therefore it is necessary to have quick tests for diagnostic of the disease. The objective of this work was to compare FPA and ELISA to detect paratuberculosis infected bovines.

MATERIALS AND METHODS

Samples

The sampling was carried out in dairy herds, with and without prior paratuberculosis diagnosis, which belonged to cooperating cattlemen. The dairy herds were located in the States of Mexico, Hidalgo, Queretaro and Aguascalientes (Table 1). To estimate the sample size, the cattle census of each State of the Mexican Republic included in the study were taken into consideration, as well as a paratuberculosis prevalence of 10% with a confidence level of 95% and 5% error. Six-hundred and three serum and feces samples were taken from bovines, older than two years of age, most of them from the Holstein breed. The blood was taken from the caudal vein in vacutainer tubes and centrifuged at 180 xg, for ten minutes to obtain the serum; the feces samples were directly taken from the rectum with palpation gloves and were kept at 4°C until their use.

Fluorescence polarization assay (FPA)

The protocol described by Torres (2015), was followed to obtain and mark with fluorochrome the protein fraction of 35 kDa from Map 3065 strain.

The sera were diluted 1:100 in borosilicate tubes with Tris buffer (pH 7.2, 0.01 M), then incubated for 30 minutes at room temperature.

The initial reading of the diluted sera was carried out in an FPA Sentry 2000 reader; 10 µl of fluorescein marked antigen was incubated for 5 min at room temperature and a second reading was carried out. The results were expressed in milli-polarization units (mP), which is the difference between both readings. The samples were processed in duplicate with positive and negative serum controls of Map (Allied & Monitor Inc). Results were considered positive when they were equal to or greater than 126 mP (Torres, 2015).

Enzyme-linked immunosorbent assay (ELISA)

The protocol described by Martinez et al. (2012), was followed to fix the protoplasmic antigen of Map from strain 3065 to the microplates. A dilution 1:160 was obtained with the problem sera using a 0.02% M.

*Corresponding author. E-mail: mayco1768@gmail.com, santillan.marco@inifap.gob.mx.

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Table 1. Total samples and number of herds included in the study.

State	Number of Samples	Number of herds
Hidalgo	270	5
State of Mexico	150	3
Queretaro	101	3
Aguascalientes	82	3

Table 2. Samples subjected to n-PCR, ELISA and FPA for paratuberculosis diagnosis.

Test	Positives	Negatives	Total
n-PCR	162	441	603
FPA	142	461	603
ELISA	141	462	603

Table 3. Sensitivity and specificity results of FPA, ELISA and PCR assays for the diagnosis of paratuberculosis.

Test	Sensitivity (%)	Specificity (%)	P
FPA	88.50	91.42	0.000
ELISA	83.86	89.87	0.000
n-PCR	100	100	0.000

phlei, 0.1% gel and 0.05% Tween 80 solution. The diluted sera were incubated for an hour at room temperature. Map positive and negative sera controls were used (Allied Monitor & Inc). Each well received 100 µl of each, suspected serum, negative and positive controls; then incubated at room temperature for 30 min, and then washed four times with 300 µl PBS-Tween 20 (1X). 100 µl of a 1:10 000 dilution of Anti-IgG bovine HRP was added to each well and incubated at room temperature for 30 min. The plate content was discarded and washed with PBS-Tween 20 (1X), four times; 100 µl of the substrate solution 2,2'-azino-bis-(3-ethyl-benzothiazoline-sulfone-6)-(diammonium) (ABTS, AMPRESCO) was added to each well and incubated for 30 min at room temperature and 1.5 mM sodium nitrate (Sigma Aldrich) in 0.1M citric acid (Sigma Aldrich) was used as stop solution. Plate reading was carried out at 650 nm in a spectrophotometer (ELx800 Biotek). Sera with results equal to or greater than 0.22 optic density (OD) were considered positive to the ELISA test.

Nested polymerase chain reaction (n-PCR)

DNA extraction from feces was carried out according to the protocol described by Jaimes et al. (2008).

The primers described by Erume et al. (2001), were used for the first PCR reaction: Paratb1 (5' TGA TCT GGA CAA TGA CGG TTA CGG A 3') and Paratb 4 (5' CGC GGC ACG GCT CTT GTT 3') and the product that was obtained had 563 bp; for the second reaction, Paratb 2 (5' GCC GCG CTG CTG GAG TTA A 3') and Paratb 3 (5' AGC GTC TTT GGC GTC GGT CTT G 3') were used, obtaining a final product of 210 bp.

For the first reaction, 2 µl (15 ng/ µl) of DNA, obtained from bovine feces were used, with the following reagent conditions: for 48 µl of premix solution, 5 µl were used of Reaction Buffer 10X (67 mM/µl), 4 µl MgCl₂ 30 mM 20 X, 1 µl DNTP (200mM), 1 µl Paratb 1 (25 pMol), 1 µl Paratb 4 (25 pMol), 0.25 µl Polimerase 500 U and 35.75 µl double-distilled water. Amplification was carried out in an Axigene thermocycler using the following program: an initial

denaturing at 98°C/3 min, followed by 35 cycles of denaturing at 98°C/30 s, annealing at 65 °C/30 s, and extension at 72 °C/30 s, followed by a final extension at 72°C/3 min and then 4°C /three min. For the second reaction, 3 µl of the first reaction were taken as a DNA template and were transferred into PCR microtubes containing the same amount and concentration of reagents previously described with the exception that the Paratb1 and Paratb4 primers were substituted by primers Paratb2 (25 pMol) and Paratb3 (25 pMol). The same thermocycler program was used. For the visualization of the amplification products, electrophoresis was carried out on 2% agarose gels and stained with ethidium bromide.

Establishing test sensitivity and specificity

Receiver operational characteristics (ROC) analysis was carried out, which is a graphic representation of sensitivity and specificity of a binary system according to the discrimination threshold variation. The Kappa Test or Concordance Index was estimated to measure the association with the results obtained in FPA, ELISA and nested PCR. The statistical analysis was carried out with the Intercooled Stata 7.0 and Epidat 3.1 software packages.

RESULTS

The results obtained with the FPA (142/603) and ELISA (141/603) tests were similar; the FPA test identified only one more serum positive (Table 2). The sensitivity and specificity of the FPA technique were 88.50 and 91.42%, respectively, when compared with ELISA and n-PCR. The FPA assay shows high sensitivity and specificity (Table 3, Figure 1). Concordance between tests showed a good correlation of FPA when compared to ELISA

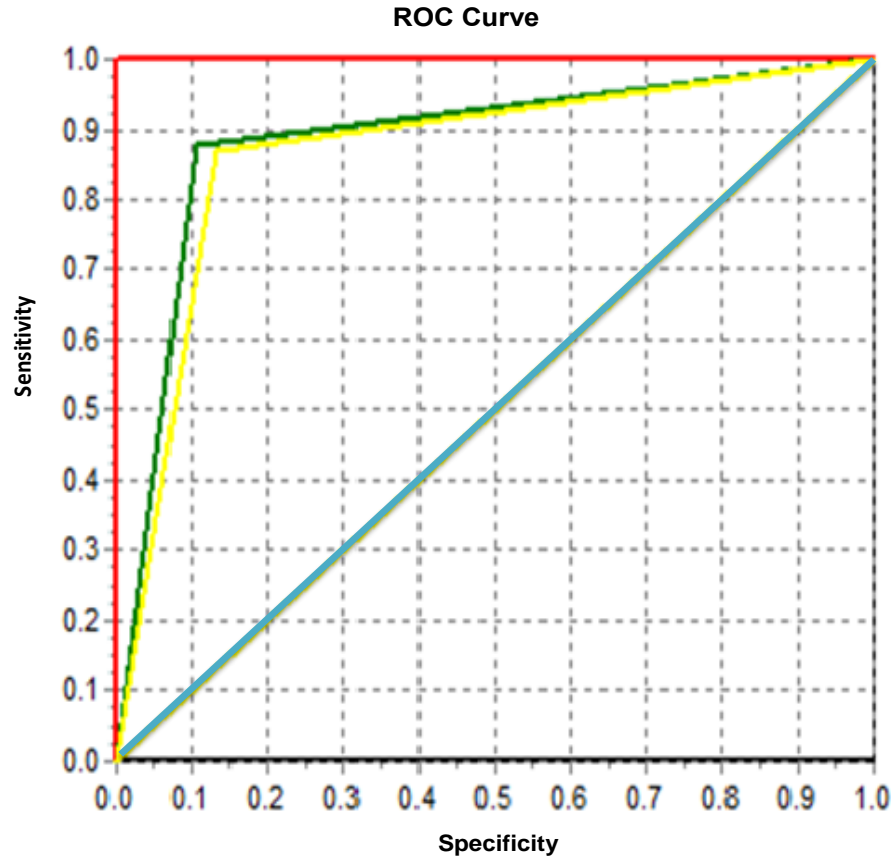


Figure 1. ROC curve, sensitivity and specificity of nested PCR (red), FPA (green) and ELISA (yellow), area under the curve (blue).

(0.6742%) and n-PCR (0.7314%); the positive predictive value of FPA was 75.6% when compared with ELISA and 87.7% compared with polymerase chain reaction (PCR). Likewise, the negative predictive values were 90.8 % and 89.3% respectively, which indicates that the test has a greater specificity (Table 4).

DISCUSSION

Currently, research lines in mycobacteriosis are focusing on the assessment of low molecular weight protein type antigens. These antigens, since they are highly specific, are the most viable candidates to be used in diagnostic tests to detect animals infected with tuberculosis and/or paratuberculosis. The low molecular weight antigens have been previously used in serological diagnostic techniques such as ELISA and FPA, where the sensitivity and specificity has been demonstrated to be higher in both techniques (Beck et al., 2005; Chaubey et al., 2016). In this work, the FPA and ELISA techniques were evaluated using as a confirming test n-PCR. FPA had an 88.5% sensitivity and 91.42% specificity, while ELISA had 83.86% and 89.87% respectively; when the

concordance analysis was carried out, an acceptable result of ($K=0.6742$) was obtained. Therefore, both tests are a good option to determine the presence of anti-Map antibodies in cattle.

With n-PCR, 162 positive samples were detected while with FPA and ELISA 142 and 141 positives were detected, respectively. It is possible to find samples that are positive in n-PCR but negative in the serology tests, because in the subclinical stage of paratuberculosis the humoral immune response decreases and the cellular immune response increases. Another reason for alteration of antibody production can be the loss of immune response to the infection. This suppressing activity is known as anergy and occurs in immunocompromised animals such as old animals or that are in the final stages of the disease. Nevertheless, it is considered that these animals may be eliminating the bacilli that are not detected by serological diagnosis but are detected by n-PCR (Martinez et al., 2012; Chaubey et al., 2016). Nielsen and Gall (2001), mentioned that the FPA test is more specific than sensitive; albeit Jolley et al. (2007), when using the FPA test with the 22 kDa protein MPB70 from an *M. bovis* strain for the diagnosis of bovine tuberculosis, obtained a 99.9% specificity. Also,

Table 4. Concordance index results (K), positive predictive value (VP), negative predictive value (VN) and statistical value (P); when comparing FPA, ELISA and n-PCR.

Test	K	VP + (%)	VP - (%)	P
FPA vs ELISA	0.6742	75.6	90.8	0.000
FPA vs n-PCR	0.7314	87.7	89.3	0.000
ELISA vs n-PCR	0.5771	87.6	86.4	0.000

Surujballi et al. (2009), used FPA for diagnosis of tuberculosis in cervids and obtained a sensitivity and specificity above 81.00%.

FPA has been used in the diagnosis of bovine brucellosis, and the sensitivity and specificity has been reported at 99.00 and 95.50%, respectively (Marcelo et al., 2017). Even though the FPA technique is simple and has several advantages over other serological techniques, up until now there had not been studies on its use and application to the diagnosis of paratuberculosis. The results obtained in this work show that the use of FPA might contribute to and be an alternative for the diagnosis of bovine paratuberculosis.

The Map 3065 strain protoplasmic antigen that was used for ELISA, has the characteristic of being a complex preparation of lipids, carbohydrates, proteins and nucleic acids that contain antigenic determinants in common with most of the Map strains, with which an acceptable sensitivity and specificity has been obtained (Martinez et al., 2012). In comparison, the antigen that is used in the FPA test is a protein fraction of 35 kDa, and as such has more specificity. Proteins below 50 kDa are involved in the humoral and cellular response to intracellular microorganism infections, and therefore they are candidates to be used as antigens in the FPA test since they allow for more specificity of the assay (Nielsen and Gall, 2001; Franco et al., 2005; Mon et al., 2012; Chaubey et al., 2016).

Bauman et al. (2019) evaluated Bulk tank milk (BTM) to determine Map's presence at the herd level in production, using fecal culture, PCR, mELISA paratuberculosis tests in 29 dairy goat herds and 21 dairy sheep flocks; their results showed that mELISA was more sensitive for identifying farms with affected animals (those with detectable circulating antibodies in their serum or milk). Increasing sensitivities to 87.50% (serum ELISA as reference test) and 71.40% (milk ELISA as reference test) in dairy goats and 72.70% (serum ELISA as reference test) and 87.50% (milk ELISA as reference test) in dairy sheep; While the sensitivity was 50% and specificity 83.00%; when used PCR of the BTM and fecal culture. BTM has been evaluated in dairy cattle as a potential herd-level paratuberculosis testing strategy so inclusion of FPA for the detection of anti-map antibodies in milk, is an alternative that would allow to have a diagnostic test with greater sensitivity and specificity, as well as determine the

health status of paratuberculosis of the herd in production.

Control of paratuberculosis in herds is based on the opportune identification and elimination of infected animals. Nevertheless, the low sensitivity that diagnostic tests have and the subclinical presentation of the infection, make the diagnosis of the disease difficult, especially in young animals (Chaubey et al., 2016).

Conclusion

The FPA test using the 35 kDa antigen may be considered as an alternative that allows better sensitivity and specificity to diagnose paratuberculosis.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

- Arsenault RJ, Maattanen P, Daigle J, Potter A, Griebel P, Napper S, (2014). From mouth to macrophage: mechanisms of innate immune subversion by *Mycobacterium avium* subsp. *paratuberculosis*. *Veterinary Research* 45(1):54.
- Bauman CA, Jones-Bitton A, Jansen J, Kelton D, Menzies P (2019). Evaluation of bulk tank milk PCR and bulk tank milk modified ELISA tests for the detection of paratuberculosis at the herd level in goat and sheep dairies in Ontario, Canada. *Journal of Dairy Science* 102(1):511-520.
- Beck ST, Leite OM, Arruda RS, Ferreira AW (2005). Humoral response to low molecular weight antigens of *Mycobacterium tuberculosis* by tuberculosis patients and contacts. *Brazilian Journal of Medical and Biological Research* 38:587-596.
- Castellanos RE (2010). Caracterización molecular de aislados de *Mycobacterium avium* subespecie *paratuberculosis*. Mapa epidemiológico en España (2010). Tesis doctoral. Universidad Complutense de Madrid, Facultad de Veterinaria. España.
- Chaubey KK, Gupta RD, Gupta S, Singh SV, Bhatia AK, Jayaraman S, Kumar N, Goel A, Rathore AS, Sahzad, Sohal JS, Stephen BJ, Singh M, Goyal M, Dhama K, Derakhshandeh A (2016). Trends and advances in the diagnosis and control of paratuberculosis in domestic livestock. *The Veterinary Quarterly* 36(4):203-227.
- Cirone K, Morsella C, Romano M, Paolicchi F (2007). *Mycobacterium avium* subsp. *paratuberculosis*: presencia en los alimentos y su

- relación con la enfermedad de Crohn. *Revista Argentina de Microbiología* 39:57-68.
- Erumé J, Sperser J, Rosengarten R (2001). Rapid detection of *Mycobacterium avium* subsp. *paratuberculosis* from cattle and zoo animals by nested PCR. *African Health Sciences* 1(2):83-89.
- Franco J, Camarena JJ, Noriega JM, Blanuer R, Ruiz MJ, Marin J (2005). Serological response (Western blot) to fractions of *Mycobacterium tuberculosis* sonicate antigen in tuberculosis patients and contacts. *International Journal of Tuberculosis and Lung Disease* 5(10):958-962.
- Jaimes NG, Santillán FMA, Hernández COA, Córdova LD, Guzmán RCC, Arellano RB, Díaz AE, Tenorio GVR, Cuéllar OJA (2008). Detección de *Mycobacterium avium* subespecie *paratuberculosis*, por medio de PCR-anidada a partir de muestras de heces de ovinos. *Vet. Méx.* 39(4):377-386.
- Jolley ME, Nasir MS, Surujballi OP, Romanowska A, Renteria TB, De la Mora A, Lim A, Bolin SR, Michel AL, Kostovic M, Corrigan EC (2007). Fluorescence polarization assay for the detection of antibodies to *Mycobacterium bovis* in bovine sera. *Veterinary Microbiology* 120(1-2):113-121.
- Marcelo I, Hernan B, Ruth S, Milena G, Judith G, Julio P, David H, Jorge M, Martin C, Byron P (2017). Determining a diagnostic cut-off on fluorescence polarization assay (FPA) for bovine brucellosis in Carchi, Ecuador. *Open Journal of Animal Sciences* 7:425-432.
- Martínez CAG, Santillán FMA, Guzmán RCC Favila HLC, Córdova LD, Díaz AE, Hernández AL, Blanco OM (2012). Desarrollo de un Inmuno-Ensayo Enzimático (ELISA) para el diagnóstico de paratuberculosis en bovinos. *Revista Mexicana de Ciencias Pecuarias* 3(1):1-18.
- Mon ML, Viale M, Baschetti G, Alvarado Pinedo F, Gioffre A, Travería, G, Willemsen P, Bakker D, Romano MI (2012). Search for *Mycobacterium avium* subspecies *paratuberculosis* antigens for the diagnosis of paratuberculosis. *Veterinary Medicine International* 2012.doi:10.1155/2012/860362.
- Mortier RA, Barkema HW, De Buck J (2015). Susceptibility to and diagnosis of *Mycobacterium avium* subspecies *paratuberculosis* infection in dairy calves: A review. *Preventive Veterinary Medicine* 121(3-4):189-98.
- Nielsen K, Gall D (2001). Fluorescence polarization assay for the diagnosis of brucellosis. *Journal of Immunoassay and Immunochemistry* 22(3):183-201.
- Speer CA, Scott MC, Bannantine JP, Waters WR, Mori Y, Whitlock RH, Eda S (2006). A novel enzyme-linked immunosorbent assay for diagnosis of *Mycobacterium avium* subsp. *paratuberculosis* infection (Johne's Disease) in cattle. *Clinical and Vaccine Immunology* 13(5):535-540.
- Surujballi O, Romanowska A, Sugden EA, Turcotte C, Jolley ME (2002). A fluorescence polarization assay for the detection of antibodies to *Mycobacterium bovis* in cattle sera. *Veterinary Microbiology* 87(2):149-157.
- Surujballi OM, Lutze-Wallace C, Turcotte C, Savic M, Stevenson D, Romanowska A, Monagle W, Berlie-Surujballi G, Tangorra E (2009). Sensitive diagnosis of bovine tuberculosis in a farmed cervid herd with use of an MPB70 protein fluorescence polarization assay. *Canadian Journal of Veterinary Research* 73(3):171-176.
- Torres VR (2015). Obtención y evaluación de proteínas de bajo peso molecular a partir de un extracto crudo de *Mycobacterium avium* subsp. *paratuberculosis* para el diagnóstico de paratuberculosis por fluorescencia polarizada. Tesis de Maestría. Posgrado-FMVZ UNAM. México.

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