Journal of Veterinary Medicine and Animal Health

Volume 8 Number 12 December, 2016
ISSN 2141-2529

Academic Journals

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Journal of Veterinary Medicine and Animal Health

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Vol. 8(12), pp. 207-213, December 2016
DOI: 10.5897/JVMAH2016.0512
Article Number: 62D6B3B61551
ISSN 2141-2529

http://www.academicjournals.org/JVMAH

Journal of Veterinary Medicine and Animal Health

Full Length Research Paper

Evaluation of pacing as an indicator of musculoskeletal pathology in dogs

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Received 4 August, 2016: Accepted 13 October, 2016

Little is currently known about the pacing gait in dogs and it has been speculated that pacing may be utilized by dogs with musculoskeletal pathology. The goals of the present study were to determine if pacing in dogs is associated with musculoskeletal disease and to establish if controlled speed impacts pacing. Dogs underwent orthopedic and lameness assessments. Musculoskeletal pathology, when identified, was further defined with radiography of the affected area. Dogs were considered musculoskeletally normal (MSN) if no pathology was detected and they had no history of musculoskeletal disease. All others were considered musculoskeletally abnormal (MSA). Animals were then evaluated for pacing using digital-video-imaging under three conditions: Off-lead, lead-controlled, and on a treadmill. Thirty-nine dogs were enrolled (MSN: *n* = 20; MSA: *n* = 19). Overall, pacing was observed more frequently in dogs under lead-controlled than off-lead conditions (*P* < 0.001). Lead-controlled MSN dogs were observed to pace significantly more frequently (*n* = 17/20) than lead-controlled MSA dogs (*n* = 10/19; *P* = 0.029). There was no significant difference within each group for the frequency of pacing under treadmill or off-lead conditions. Pacing always occurred at speeds between a walk and a trot, speeds ranged from 0.98 to 2.84 m/s, (mean 1.8 m/s). Pacing was demonstrated in MSN and MSA dogs under all study conditions. Pacing should be considered a gait variation that can be observed in clinically normal dogs. Relative speed and leash walking was determined to be a factor in the use of the pacing gait.

Key words: Pacing, gait analysis, lameness, canine locomotion, symmetrical gait, lateral gait, amble.

INTRODUCTION

Gait and lameness assessments are major aspects of the orthopedic examination; therefore, knowledge of gait patterns is essential for recognition of pathologic conditions (Zink and Van Dyke, 2013). In dogs, the walk, trot, and gallop are considered normal gait patterns; however, it is unclear whether pacing is normal or pathologic in dogs (Hildebrand, 1968; Blaszczyk and Dobrzecka, 1989; Zink and Van Dyke, 2013). The pace (Figure 1) is a 'lateral-couplet' symmetrical gait in which ipsilateral limb pairs move in synchrony (Hildebrand, 1968; Leach et al., 1977; Biknevicius and Reilly, 2006). It has been suggested that dogs which consistently pace in

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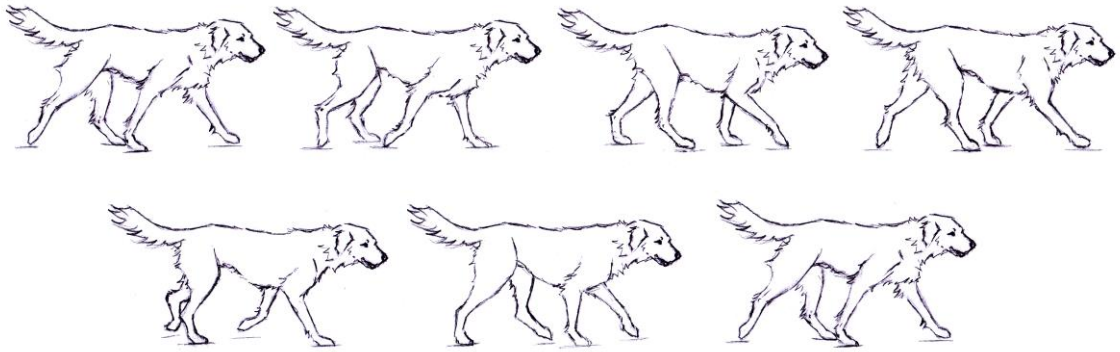

Figure 1. Pace: Two beat 'lateral-couplet' symmetrical gait in which ipsilateral limb pairs move synchronously; image illustrates the stride sequence of the pace; Illustrated by TM Wendland.

Table 1. Lameness grading scale used in this study.

Grade	Description of lameness
0	Clinically sound (no identifiable lameness; animal evenly weight-bears when standing)
1	Barely detectable lameness (possible lameness at walk or trot; animal evenly weight-bears when standing)
2	Mild lameness (subtle but definite lameness at walk and/or trot; animal mildly off-weights affected limb when standing)
3	Moderate lameness (obvious lameness at walk and/or trot; animal definitely off-weights affected limb when standing)
4	Severe lameness (carries limb when trotting but at least occasionally weight bearing walking or standing)

preference to trotting should be examined to rule out injuries that may make trotting difficult (Zink and Van Dyke, 2013). Additionally, it has been implied that dogs which are tired, out of condition, or have a diagnosable orthopedic abnormality may pace (Nunamaker and Blauner, 1985). In contrast, the pace has also been described as normal and naturally occurring in some dogs: For example, several researchers recorded pacing in healthy, athletic dogs in two separate studies and regarded this as a normal gait (Maes et al., 2008; Maes and Abourachid, 2013). Others attribute pacing to dogs with proportionally long legs and/or at speeds between those of a walk and a trot (Hildebrand, 1968; Brown and Dalzell,1986). One final proposition suggests pacing is neither a pathology nor a normality, but that dogs may be trained to pace by handlers consistently leash-walking them at speeds between walk and trot speeds (Zink and Van Dyke, 2013).

Despite the controversy regarding the clinical significance of pacing, there are currently no peer-reviewed publications describing the incidence of pacing in musculoskeletally abnormal dogs to the authors' knowledge. Moreover, little data exists describing speeds at which canids pace and the impact that controlled speed has on gait pattern. Since pacing is easily recognized during visual examination, information on the significance of this gait as a potential indicator of pathology would be useful for veterinarians, trainers, and owners. The goal of the present study was to evaluate dogs with and without musculoskeletal abnormalities for evidence of pacing. We hypothesized that dogs with musculoskeletal pathology would pace more frequently than normal dogs. Another goal of the study was to characterize the relationship between a dog's relative speed and pacing. We hypothesized that dogs would pace at speeds between walk and trot speeds and that they would pace more frequently when speeds were controlled than when dogs were released off-leash.

MATERIALS AND METHODS

Animals

A convenience sample of 39 healthy, privately owned pet dogs were enrolled. The study protocol was reviewed and approved by the Colorado State University Institutional Animal Care and Use Committee (#14-4908A) and written owner consent was obtained. Stress monitoring of the dogs was performed by behavioral assessment and by utilizing a heart rate monitor (Polar Ft1 Heart Rate Monitor, Polar Electro Inc) (Cohen and Obrist, 1975; Vincent and Leahy, 1997; Beerda et al., 1998; Essner et al., 2013). Dogs showing an obvious, strong stress-response during the study were to be excluded.

Dogs were required to be capable of trotting continuously for a minimum of 10 min. Medium to large breed dogs of any weight, breed, and body proportion were included. Dogs were excluded if their body condition scores were less than 4/9 or greater than 6/9 (Brady et al., 2013). A detailed medical history was obtained from each owner. Dogs underwent complete physical examination and orthopedic evaluation by a board certified veterinary surgeon and were assigned a subjective lameness score (scale 0 to 5; 0 = no lameness, and 5 = severe lameness, Table 1). Evaluation was performed prior to application of test conditions so an individual

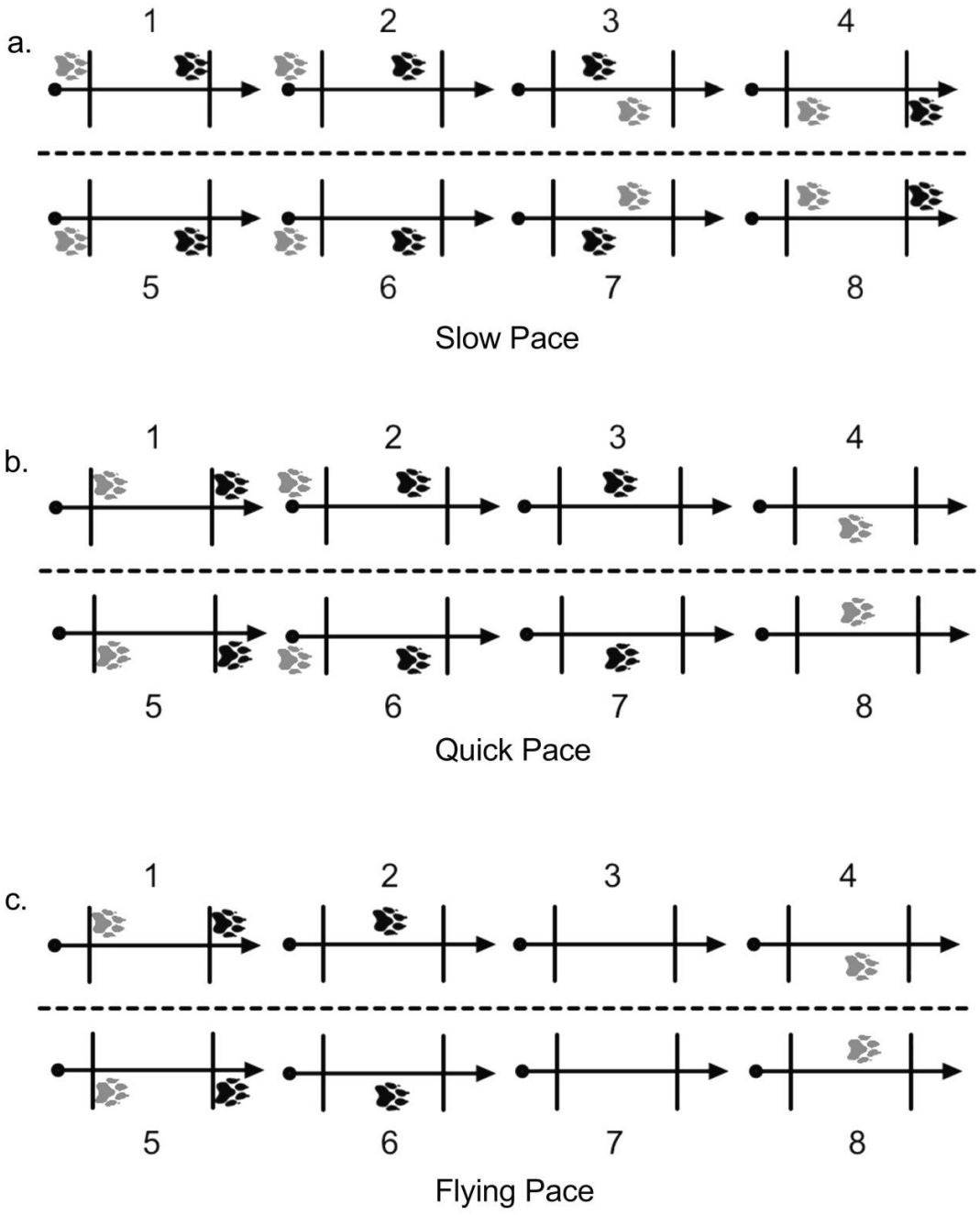

Figure 2. Footfall patterns of the pace at various speeds with black paw prints representing thoracic limb feet and grey paw prints representing pelvic limb feet. Each numbered unit represents foot placement during a moment in time with 8 moments represented for each speed. (a) During the slow pace two feet are in contact with the ground at all times. The left ipsilateral pair supports the dog for half a stride. The rear left foot then lifts as the hind right foot strikes the ground and the dog is supported by diagonal limbs. The left fore foot then lifts at the same time the right fore foot strikes the ground so that the dog is again supported by ipsilateral limbs. (b) The quick pace is similar to the slow pace except that diagonal pairs of feet do not touch the ground at the same time, instead, the contralateral forelimb is lifted as the hind foot strikes the ground. (c) The flying pace uses the same sequence of limb movement but is characterized by a moment of suspension;

dog's pacing status was unknown by the evaluator other than the gait which was observed during orthopedic examination. Dogs with a lameness score of three or greater were excluded from the study. Dogs with no history of lameness, no abnormal findings on physical

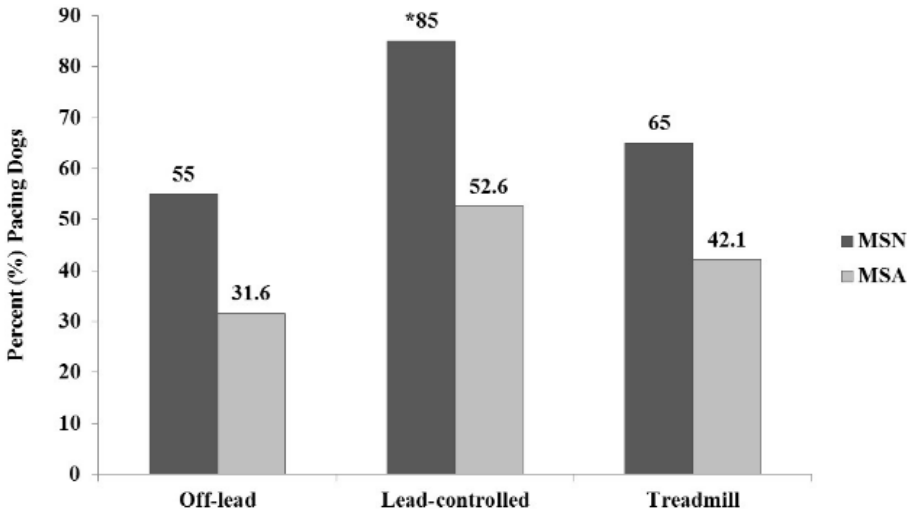

Figure 3. Comparison of pacing frequency between musculoskeletally normal (MSN) and musculoskeletally abnormal (MSA) groups under off-lead, lead-controlled, and treadmill conditions. Dogs in the MSN group (n = 17/20; 85%) were observed to pace significantly more frequently than dogs in the MSA group (n = 10/19; 52.6%) under lead-controlled conditions (P = 0.029).

examination, and a lameness score of zero were assigned to the musculoskeletally normal (MSN) group. All other dogs were assigned to the musculoskeletally abnormal (MSA) group. Any orthopedic abnormality or lameness was further investigated with radiography. Radiographs were evaluated by a board certified veterinary radiologist and findings were recorded. Consistently repeatable abnormal examination findings, even if not supported by radiographic changes were considered 'abnormal' for the sake of this study since no soft tissue imaging (such as MRI, PET-CT, or ultrasound) was performed.

Breed or detailed physical description of mixed-breeds was recorded. Height from the ground to the highest point of the withers and length from the point of the greater tubercle of the humerus to the tuber ischium were recorded. Leg-to-body ratios were calculated as previously described (Brown and Dalzell,1986). Briefly, leg length was measured from the ground to the deepest part of the chest. Chest depth was measured from the bottom of the chest dorsally to the point of the withers. The measured leg length was then divided by the measured chest depth. These measurements were taken with an adjustable 48-inch drywall square (Swanson Tool Co, Inc).

Procedures

Following physical examination and group assignment, dogs were evaluated under three conditions: Off-lead unrestricted movement, lead-controlled movement, and ambulation on a treadmill (Large DogTread, PetZen Products). Animals were recorded with video cameras from the front, back, and sides. The animals' gaits were later evaluated for pacing by reviewing digital videos recorded from all test conditions. Dogs were evaluated on two days to accomplish treadmill habituation: On day one, dogs were habituated to the treadmill by walking and trotting for eight to 10 minutes for at least two discrete sessions separated by a 30-min rest period to increase compliance and to help reduce artifacts in movement induced by the use of the treadmill (Fanchon and Grandjean, 2009; Gustas et al., 2013). The dogs were also introduced to the enclosed outdoor area used for recording unrestricted movement. On day two, video recordings were collected off-lead, followed by lead-controlled, and the treadmill last as outlined in the following.

Off-lead

The dogs were individually released off-lead into a fenced enclosure of approximately 5 × 12 m. They were recorded while free-roaming using a digital video camera held at the dogs' shoulder heights. Continuous recording was performed during a minimum of three walk-to-trot and three trot-to-walk transitions. When a dog stopped ambulating for 10 consecutive seconds, a handler walked within the enclosure and verbally called to the dog to encourage movement.

Lead-controlled

The dogs were led on a loose lead over a flat paved area between two traffic cones marking a 20 m path at incremental speeds estimated by the person handling the dog via a GPS (global positioning system) tracking device (Bad Elf 2200 GPS Pro, Bad Elf LLC). The first pass along the path was made with the dog ambulating at a subjectively determined consistent four-beat walk. Speed was increased during subsequent passes between cones until the dog was subjectively determined to consistently trot. Animals were observed and recorded with digital video imaging from the side, front, and back simultaneously.

Treadmill

Dogs were reintroduced to the treadmill to ensure gait normalization. Reintroduction consisted of a treadmill session starting with the dog walking, increasing the speed until the dog trotted, and subjectively assessing both gaits to be normal. The reintroduction session was limited to five minutes for any test subject. During the reintroduction phase, the speed for a consistent walk was determined and recorded. This walking speed was used as the starting treadmill belt speed for the subsequent treadmill trial. Once dogs were habituated

to the treadmill, they were given a 2 to 5 min break, then underwent a single treadmill session for gait evaluation. Video recording was initiated and dogs were started on the treadmill at the previously determined walking speed. The treadmill belt speed was then increased by 0.3 km/h increments and each increment was maintained for 15 s. Belt speed was increased until the dog reached a consistent working trot, then the session was ended. The treadmill speeds were verbally dictated into the video camera microphone at each incremental change to be noted during video evaluation.

Video evaluation

Each dog was evaluated for pacing by a single observer. Footfall pattern recognition was facilitated by post-processing film to one-quarter speed (Apple i-Movie). A symmetrical lateral-couplet gait in which ipsilateral limbs move in synchrony was considered 'pacing'. Slow, quick, and flying pace (Figure 2) were defined according to previous publications (Hildebrand, 1968; Hollenbeck, 1981; Nunamaker and Blauner, 1985; Brown and Dalzell, 1986; Maes et al., 2008; Zink and Van Dyke, 2013) and were grouped into a single category, 'pacing.' The minimum average speed and maximum average speed at which each dog paced relative to the treadmill belt were also recorded at this time.

Statistical analysis

Statistical analysis was performed utilizing commercially available software (SPSS, IBM). The frequency of pacing in different groups and under different conditions was compared using chi-square tests. The leg-to-body ratios of pacers versus non-pacers were compared using t-test analysis. Statistical significance was set at $P < 0.05$.

RESULTS

Dogs

Dog breeds included Labrador Retriever (*n* = 13), Golden Retriever (9), Border Collie (3), Heeler (2), Pit Bull (2), Standard Poodle Mixes (2), mixed breed (2), Portuguese Water Dog (1), German Shepherd (1), Bernese Mountain Dog (1), Husky mix (1), Shepherd mix (1), and Golden Retriever mix (1). Dogs ranged in age from 1-13 years (mean 4.87 ± 3.2 years). Weight ranged from 13.2 to 49.2 kg (mean 26.85 ± 7.7 kg). Mean height from floor to withers was 59.51 ± 5.33 cm (range 50.80-78.74 cm). Mean body length measured from the greater tubercle of the humerus to the tuber ischium ranged was 63.58 ± 6.93 cm (range 50.80-81.28 cm). No dog required exclusion due to stress.

Sixteen of nineteen MSA dogs had a single abnormality detected and 13/19 MSA dogs had multiple abnormal examination and radiographic findings. Orthopedic examination abnormalities included: Decreased elbow range of motion and/or reactivity to manipulation and/or effusion (*n* = 9), decreased carpal range of motion and/or reactivity to manipulation and/or effusion (9), decreased hip range of motion and/or reactivity to extension and abduction (8), decreased stifle range of motion and/or reactivity to manipulation and/or effusion/periarticular thickening (6), reactivity to palpation of lumbar spine (3), reactivity to shoulder flexion (2), decreased tarsal range of motion, effusion, and crepitus (1). Radiographic abnormalities in the MSA group included: Elbow osteoarthritis (*n* = 7), carpal osteoarthritis (5), coxofemoral osteoarthritis (5), accessory carpal enthesopathy (4), lumbar spondylosis and/or osteoarthritis (3), healed femoral fractures with internal fixation (2), glenohumoral osteoarthristis (1), tarsal osteoarthritis (1), stifle osteoarthritis (1), bilateral stifle osteoarthritis with healed tibial plateau leveling osteotomies (1), irregularly marginated lateral fabella (1).

Pacing

Pacing was observed in both MSN and MSA groups under all conditions (Figure 3). Pacing was seen significantly more often than expected under lead-controlled conditions (27/39, 69.2%, $P = 0.016$) but not off-lead (17/39, 43.6%, $P = 0.423$) or on the treadmill (21/39, 53.8 %, $P = 0.631$). The overall frequency of dogs pacing under lead-controlled conditions was significantly greater than those pacing during off-lead conditions (27/39, 69.2% compared to 17/39, 43.6%; $P < 0.001$). All dogs that paced under off-lead conditions and/or treadmill conditions also paced during lead-controlled conditions.

Under lead-controlled conditions, MSN dogs (*n* = 17/20, 85.0%) were observed to pace significantly more frequently than MSA dogs (*n* = 10/19, 52.6%, $P = 0.029$). There was no significant difference between the frequency of pacing for MSN dogs (*n* = 11/20, 55.0%) and MSA dogs (*n* = 6/19, 31.6%) under off-lead conditions ($P = 0.140$) or under treadmill conditions (MSN: *n* = 13/20, 65.0%; MSA: 8/19, 42.1%; $P = 0.152$). When MSN/MSA groups were evaluated separately, MSN dogs were more likely to pace under lead-controlled conditions (n = 17/20, 85.0%, $P = 0.002$) but not under off-lead (n = 11/20, 55.0% $P = 0.655$) or treadmill (n = 13/20, 65.0%, $P = 0.180$) conditions. There was no statistically significant difference in the frequency of pacing for any of the conditions for the MSA group (off-lead: $P = 0.108$; lead-controlled: $P = 0.819$; treadmill: $P = 0.491$).

All dogs that paced did so at a speed between a four-beat walk and a two-beat trot with land treadmill speeds ranging from 0.98 m/s to 2.84 m/s (mean minimum speed 1.42 ± 0.21 m/s; mean maximum speed 2.14 ± 0.38 m/s). Leg to body ratios ranged from 0.77 to 1.5 (mean 1.22 ± 0.15) with a mean ratio for pacers of 1.22 ± 0.16 and 1.24 ± 0.13 for non-pacers. There was no significant difference between the leg-to-body ratios of pacers and non-pacers ($P = 0.726$).

DISCUSSION

Many reasons for pacing have been proposed including

body proportion (Hildebrand, 1968; Brown and Dalzell, 1986). Pacing has been correlated with a short-coupled body and long legs since pacing eliminates interference of diagonal pair feet that may occur during trotting (Brown and Dalzell,1986). Hildebrand observed pre-recorded archived film and dog show footage that showed 37 different dog breeds ambulating. The author assigned dogs into three groups based on subjective visual impressions of relative leg length, however, no measurements were taken (Hildebrand, 1968). The dogs that were observed to pace were all long legged breeds and none of the short-legged breeds were observed to pace (Hildebrand, 1968). Of the 37 represented dog breeds, only eight were observed to pace or use a similar lateral-couplet gait (Hildebrand, 1968). Unfortunately, Hildebrand only reported pacing by breed but did not report how many individuals paced. It was also unknown whether these dogs were musculoskeletally normal. Additionally, speed was qualified as slow, moderate, and fast, but never quantified. Further confounding this study, pacing has long been disallowed for most breeds by the dog show community and therefore, dog handlers will make an effort to keep dogs from pacing in the show ring (Brown and Dalzell,1986).

Brown et al. followed up Hildebrand's observations and quantified leg-length to body-length ratios as a calculation obtained from the measured leg length below the chest divided by the measured chest depth, as performed in the present study (Brown and Dalzell,1986). The authors reported that Great Danes and Salukis, breeds that have been reported to pace, have average leg-to-body ratios of 1.22 and 1.32, respectively (Brown and Dalzell,1986), meaning that these dogs have long legs relative to their body proportions. Brown notes that he observed a dog show class of 30 Great Danes in which 10 dogs appeared to pace (Brown and Dalzell,1986), but these observations were never followed by an objective study. The present study has shown much overlap between leg-to-body ratios of pacers and non-pacers with no significant difference between these groups. It should also be noted that the individual with the smallest leg-to-body ratio (0.77) was observed to pace under all three conditions. Of the two individuals with the largest leg-to-body ratio (1.50), one was observed to pace only on lead and the other was not observed to pace. Our results are inconsistent with Hildebrand's and Brown's findings.

Genetics may affect pacing in dogs, but this has not been investigated beyond anecdotal mention (Blaszczyk and Dobrzecka, 1989). A genetic mutation has, however, been linked to pacing ability in horses (Thiruvenkadan et al., 2009; Andersson et al., 2012). Walk, trot, and gallop are naturally occurring gaits in all equids, and some horses are able to use additional gaits (Andersson et al., 2012). This 'gaitedness' is a trait that has been selected for in many specialized breeds (Andersson et al., 2012). Many of these 'gaited' horses pace as a part of their normal gait repertoire while non-gaited horses only walk, trot, or gallop. It would be considered abnormal for these horses to pace (Andersson et al., 2012). It is possible that a similar mutation may be involved in the pacing ability of dogs and this should be investigated once commonly pacing breeds have been identified. With ongoing genetic mapping of the dog and linkage of mutations to phenotypic traits (Meyers-Wallen, 2003), comparison of genetically related pacers versus non-pacers may allow identification and analysis of repeatable gene sequences between groups to determine if certain mutations affect gaitedness.

It has been suggested that dogs are inadvertently trained to pace by leash-walking at speeds too quick for a comfortable walk and too slow for a comfortable trot (Zink and Van Dyke, 2013). Such conditions could potentially force 'non-pacing dogs' to pace. This suggestion is supported by our finding that a greater number of dogs paced under lead-controlled conditions than during off-lead conditions. One study reported self-selected comfortable human walking speeds with an average of approximately 1.43 m/s (Riley et al., 2007), a speed which falls within the minimum and maximum recorded pacing speeds of the medium to large breed dogs in the present study. Dogs paced at widely varying speeds between 0.98 to 2.84 m/s, which warrants further investigation. Pacing speeds vary depending on dog size and particularly the different types of pacing.

Limitations to this study include the lack of objective gait analysis, small sample size and wide inclusion criteria of the study population. The wide inclusion criteria of the present study make it difficult to classify specific characteristics of a pacer, however, it does increase the external validity of the study. It is possible that individual dogs in the MSN group may have had a degree of subtle musculoskeletal disease that was not detected with our screening procedures. Alternatively, it is possible that animals with non-clinical orthopedic disease should have been assigned to the MSN group. Finally, different footing and environments were present in each of the three study conditions making it difficult to exclude these as confounding factors, however, there has been no suggestion in the literature that footing affects pacing.

Despite the small sample size, our results indicate that pacing as a sole clinical sign should not be directly linked with musculoskeletal pathology in dogs. As suggested by the high proportion of sound pacing dogs in this study, it may not represent a sign of pathology at all. Rather, it may be a normal condition or associated with factors such as controlled speed, environment, genetics, or conditioning. In this study population, for instance, leash walking was associated with an increased incidence of pacing. Future research should further clarify the impact of environmental factors and musculoskeletal pathology on gait patterns in dogs. Such research could require serial, life-long evaluation of a larger number of dogs of various breeds. Fortunately, pacing as a gait variation is easily recognized without the use of specialized gait

analysis equipment for this purpose. Further studies should include kinetic and kinematic analysis of pacing and digital musculoskeletal modeling to better understand the biomechanics of pacing and implications for dogs who utilize this gait (Dries et al., 2016; Holler et al, 2010).

In summary, the objectives of the present study were to evaluate dogs with and without musculoskeletal abnormalities for evidence of pacing and to characterize the relationship between a dog's relative speed and pacing under different study conditions. Pacing was demonstrated in MSN and MSA dogs under all study conditions and more frequently when dogs were walked on leash. Pacing should be considered a gait variation that can be observed in clinically normal dogs at speeds between walk and trot.

Conflict of interest statement

The authors have not declared any conflict of interest.

Abbreviations:

MSN, Musculoskeletally normal; **MSA,** Musculoskeletally abnormal.

ACKNOWLEDGEMENTS

This study was funded in part by the Young Investigator Grant program in the Center for Companion Animal Studies, Colorado State University and by the American Humane Association. We would like to thank Molly Vitt for her assistance with this project.

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Vol. 8(12), pp. 214-222, December 2016
DOI: 10.5897/JVMAH2015.0506
Article Number: 04B5D0D61555
ISSN 2141-2529

http://www.academicjournals.org/JVMAH

Journal of Veterinary Medicine and Animal Health

Full Length Research Paper

Evaluation of mucin and cytokines expression with intraepithelial lymphocytes determination in the caecum of broilers administered with *Enterococcus faecium* EF55 and challenged with *Salmonella* Enteritidis SE147

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Received 12 July, 2016: Accepted 13 October, 2016

The protective effect of probiotic strain *Enterococcus faecium* EF55 in chicks challenged with *Salmonella enterica* serovar Enteritidis 147 (SE147) was assessed in caecum and blood. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) detection of mRNAs of chicken intestinal mucin gene (MUC2), Interleukin (IL-15) and Interleukin (IL-17) cytokines, together with flow cytometry determination of intraepithelial lymphocytes (IEL) were investigated in caecum. White blood cell counts were measured haematologically. One-day-old chicks were divided into 4 groups. The probiotic (EF) and probiotic+salmonella groups (EFSE) received perorally 10^9 CFU EF55 7 days. The challenged SE and EFSE groups were single perorally infected with 10^8 CFU SE147 on day 4. No bacteria were given to controls (C). Administration of EF55 to chicks challenged with SE147 (EFSE group) ultimately resulted in increased number of blood heterophils, caecal IgA+ IEL, and decreased caecal expression of MUC compared to SE group on 7 days post infection (d.p.i.). Detection of IL-15 and IL-17 mRNAs cytokine level in caecum showed a tendency to their increase on 1 d.p.i. in EFSE group. The results demonstrated beneficial effect of EF55 on the expression of MUC and production of IgA+ IEL in caecum after SE147 infection. Furthermore, the results suggest certain relation between expression of cytokines, expression of MUC and IgA+ cells.

Key words: blood, chicks, immunity, infection, intestine, probiotics.

INTRODUCTION

Enteric salmonellosis is a major foodborne disease caused by *Salmonella* and its serovars, the most common being Enteritidis and Typhimurium. Chicken is recognized as one of the most important reservoir of *Salmonella*. The colonization of the chicken gut by *S. enterica* could result in the contamination of the environment and food chain. Broiler chicks are most susceptible to colonization by pathogens during the early period of their life, when a

stable gut-microflora is not established, and an introduced stabilized gut-microflora can provide them with vital protection against these undesirable organisms (Chambers and Lu, 2002).

Potential probiotic strains have been found among the intestinal microbiota of food producing animals. Among them, some bacteria have been shown to exert antimicrobial activity against enteric pathogens due to the production of bacteriocins and bacteriocin-like molecules. The *E. faecium* EF55 was shown to produce a bacteriocin-like substance, and the inhibitory activity of this probiotic strain of bacteria was reported against number of enteropathogenic bacteria, including *S. enterica* (Levkut et al., 2009).

Mucosal tissues represent the route of access and site of infection for the majority of pathogens. Mucosal glycoproteins are secreted in large quantities by mucosal epithelia and cell surface mucins are a prominent feature of the apical glycocalyx of all mucosal epithelia. Expression of cell-surface mucins can be upregulated by inflammatory cytokines and other inflammatory factors (Linden et al., 2008). Similarly, some inflammatory cytokines have been demonstrated to play a critical role in induction and expression of SIgA responses at mucosal surfaces (Husband, 2002). Interleukin-15 was shown to be proinflammatory cytokine produced by mononuclear phagocytes (Lillehoj et al., 2001), activated T cells, and stimulates growth and proliferation of intestinal epithelial cells, natural killer cells and activated B cells (Kaiser et al., 2002). Interleukin-17 is a proinflammatory cytokine produced by activated and memory T cells (Hong et al., 2006). Functional studies in mice indicated that IL-17 is involved into stimulation of granulopoiesis, proliferation and differentiation of cells into neutrophils (Fossiez et al., 1996). In chickens IL-17 showed protective role in local immunity against salmonellae (Crhanova et al., 2011). However, the local immune response in the gastrointestinal tract has been scarcely studied in relation to competitive exclusion. It is likely to involve a variety of factors such as the role of native microflora, secretory IgA, mucin barrier, and products of inflammatory cells.

In order to find a certain relation to the quantity of cells and level of cytokines as well as mucin gene expression involved in natural and acquired intestinal immunity in chicks after protection of a probiotic strain *E. faecium* EF55 and challenged with *S. enterica* serovar Enteritidis 147, we chose to study in caecum, the intraepithelial lymphocytes, chicken intestinal mucin gene mRNA expression like human MUC2, as well as proinflammatory cytokines IL-15 and IL-17 mRNA expression together with white blood cell counts in the peripheral blood.

MATERIALS AND METHODS

Animals

One-day-old chicks (220) of Cobb-500 hybrids were randomly divided into 4 groups (n=55) and placed in large pens with a floor-covering of wood shaving for 11 days. The bedding was changed daily during the course of experiment. The temperature was based on the requirement for chicks of this age group: 32°C in the first week and reduction of 2°C each successive day. Water and feed (commercial diet BR1, Barbara Comp., Čaňa, Slovak Republic) were available *ad libitum*. The chicks were kept in the menagerie of the Department of Pathological Anatomy, University of Veterinary Medicine and Pharmacy, Košice, Slovakia (SK P 52004), in accordance with the rules and approval of the Ethics Committee, and the experiment was authorized by the State Veterinary and Food Administration of the Slovak Republic (Č.k.Ro-270710-221).

Experimental design and samplings

The chicks of group 1 (Control) were fed only with commercial diet without application of any bacteria (negative control). *Enterococcus faecium* EF 55 in the dose of 10^9 CFU/ml was administered in 0.2 ml phosphate buffer saline (PBS) to chicks of group 2 (EF) *per os* daily from 1 to 7 days. The birds of group 3 (SE) were individually inoculated *per os* on day 4 of the experiment with *Salmonella enteritidis* SE 147 (provided by Dr. Rychlik from Veterinary Research Institute, Brno, Czech Republic) with a single dose $1x10^8$ CFU/ml in 0.2 ml PBS. The chicks of group 4 (EFSE) were administered with *E. faecium* EF 55 during the first 7 days in the same manner as those of group 2, and were inoculated with *S.* Enteritidis on day 4 of the experiment in the same way as the chicks of group 3. Ten chicks from each group were killed and samples were taken on days 1, 2, 3, 4, and 7 post infection with salmonellae (d.p.i.) for cytokines detection, as well as 4 and 7 d.p.i. for other examinations. The time from 1-4 d.p.i. was termed as early phase, and 7 d.p.i. the late phase of infection. The chicks were anaesthetized with intraperitoneal (i.p.) injection of xylazine (Rometar 2%, SPOFA, Czech Republic) and ketamine (Narkoman 5%, SPOFA, Czech Republic) at the doses of 0.6 and 0.7 $ml.kg^{-1}$ body weight, respectively. After laparotomy, blood was collected into heparinised tubes by intracardial punction and was immediately used for determination of leukocyte numbers. The samples from jejunum and caecum for qRT-PCR assays and caecum for flow cytometry were taken during the necropsy from 5 randomly chosen chicks.

Microbial strains preparation and analysis

Probiotic and bacteriocin-producing strain *Enterococcus faecium* EF55 (by rifampicin marked to differ it from other enterococci) was cultivated in MRS broth (pH 7.0, Merck, Germany) for 18 h at 37°C, the broth culture was centrifuged at 10,000 × g for 30 min. Cells pellet was resuspended in Ringer solution (pH 7.0) to have cell count 10^9 colony forming unit (CFU/ml). Bacterial cell count was confirmed by the standard microbiological dilution method. Appropriate dilutions were spread onto M-Enterococcus agar (Difco, USA) enriched with rifampicin as previously described by

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Strompfová et al. (2003) and cultivated at 37°C for 24 h. Moreover, PCR confirmation (Woodford et al., 1997) was performed. *Salmonella* Enteritidis SE147 strain was cultivated in Trypticase soy broth (TSY, Becton, Dickinson and Company [BD], Cockeysville, USA) overnight at 37°C; dilutions were spread onto Brilliant green agar (Becton & Dickinson, USA) and grown colonies were confirmed by PCR (Chiu and Ou, 1996).

For microbial analysis, caeca and faeces were sampled. Fecal samples were taken at day 1 before the experiment (from each group including 55 chicks, 10 mixture samples was examinated) to exclude salmonella infection and to know microbial backround, then at day 7 and at the end of experiment. As mentioned previously, samples were treated by he standard microbiological dilution method (ISO, ratio 1:9), diluted in Ringer solution and the appropriate dilutions were spread onto the media as follows: M-Enterococcus agar (Difco, USA) to count enterococci, M-Enterococcus enriched with rifampicin to count *E. faecium* EF55, MacConkey agar (BD, USA) for coliforms, CLED agar (Oxoid, Germany) to check enterobacteriae. To check salmonellae including SE147 strain, faecal and caecal samples were pre-cultivated in Rappaport-Vassiliadis medium (Merck, Germany) at 37°C for 48 h; appropriate dilutions (100 µl) were spread onto Brilliant green agar (BD, USA). The colonies grown from the highest dilution of each sample were counted and confirmed by PCR (Chiu and Ou, 1996). The counts of microbiota are expressed in CFU g^{-1} as mean ± SD.

White blood cell count

Leukocytes were counted in a haemocytometer using Fried-Lukačová solution (475 µl of solution plus 25 µl of blood). Differential cell counts of 200 cells per slide were done by light microscopy at 1000 magnification using blood smears stained with Hemacolor (Merck, Germany). The total numbers of different subtypes of white blood cells was then calculated: total leukocytes count x proportion of differential cells counted (%)/100.

Caecal intraepithelial lymphocyte isolation and detection

The ceca were placed into cold PBS and processed immediately after sampling. Modified procedure by Swinkels et al. (2007) was used. Whole caeca (3 cm) were opened longitudinally, washed 3 times with cold PBS and cut into pieces of 0.5 cm. Incubation of pieces was done for 45 min at 37°C in PBS containing 1 mM EDTA and 5 mM DTT. The suspension of IEL was centrifuged for 10 min at 250 g and washed twice in cold PBS. The cells were resuspended in 5.10^4 per 50 µl for flow cytometry imuno-phenotyping. Labelled primary mouse anti-chicken monoclonal antibodies were used in the protocol recommended dilution (Southern Biotechnology Associates, USA). Cells were stained by the direct immunofluorescent method at one of the monoclonal combinations CD3-PE/MHC II-FITC, CD4-PE/CD8αα-FITC, TCRγδ-PE/CD45-FITC, IgM-FITC/IgA-PE. Fifty µl of cell suspension and working solution of specific monoclonal antibodies (MoAbs) were mixed and incubated for 15 min at 37°C. Then, the cells were washed once in PBS (0.5 ml), centrifuged (5 min at 250 g) and resuspended in 0.2 ml PBS with 0.1 % paraformaldehyde. After staining the cells were measured with FACScan flow cytometer (Becton Dickinson, Germany) when a live gate was set for the lymphocyte population in the forward and side scatter plot. The fluorescence data were collected on at least 10,000 lymphocytes and analysed using the Cell Quest programme (BD, Germany). The results were expressed as the relative percentage of lymphocyte subpopulation, which was positive for a specific MoAb.

Homogenization of tissue and isolation of mRNA

The samples of the caecum taken during necropsies were cut into 20 mg pieces, immediately placed into RNALater (Qiagen, UK) and stored at −70°C prior to RNA purification. Single tissue fragment was transferred into 1 ml of TRI reagent (Molecular Research Centre, USA) and homogenized using zirconia silica beads (BioSpec Products, USA) in vortex mixer (Labnet, USA). To separate the phases, 50 µl of 4-bromanisole (Molecular Research Centre, USA) was added and centrifuged at 12000 rpm for 15 min at 4°C. The upper aqueous phase was collected for RNA purification using the RNeasy mini kit (Qiagen, UK) according to the manufacturer's instructions. Turbo DNA-free kit (Ambion, USA) was used for treatment of RNA samples to remove genomic DNA. Purity and concentration of RNA samples was determined specro-photometrically on NanoDrop 200c (Thermo Fischer Scientific, USA) and 1 µg of total RNA was immediately reverse transcribed using iScript cDNA synthesis kit (Bio-Rad, USA). The resulting cDNA was 10× diluted in UltraPure™ DNase/RNase-Free distilled water (Invitrogen, USA) and used as a template in real-time PCR or stored at −20°C until used.

Quantitative RT-PCR

The mRNA levels of mucin, IL-15, and IL-17 were determined separately. The primers used for the quantitative RT-PCR (qRT-PCR) in this study are listed in Table 1. The expression levels of two housekeeping genes, glyceraldehyde-3-phosphate dehydro-genase (GAPDH) and ubiquitin (UB) were used for data normalisation. Amplification and detection of specific products were performed using Maxima SYBR GREEN qPCR mix (Thermo Fisher Scientific, USA) and the CFX 96 RT thermocycler (Bio-Rad, USA) with the temperature-time profile for 45 cycles: initial denaturation 15 min 95°C then denaturation 95°C for 20 s, annealing 60°C for 30 s and elongation 72°C for 30 s. A melting curve from 50 to 95°C with reading at every 0.5°C was performed for each individual RT-PCR plate. The Ct (cycle threshold) values of genes of interest were normalised to an average Ct value of the house-keeping genes (ΔCt) and the relative expression of each representative was calculated as 2–ΔCt.

Statistical analysis

All data were tested by one-way analysis of variance (ANOVA) and Tukey test in Minitab 16 (SC&C Partner, Brno, Czech Republic). The results are given as means ± SD. Differences between mean values for the groups of chickens were consider significant with $P < 0.05$.

RESULTS

White blood cell counts

Values of peripheral blood leukocytes increased in the birds of SE group compared to the control group ($P < 0.01$) with higher decrease in EF and EFSE groups ($P < 0.001$) on day 4 p.i. (Table 2). On day 7 p.i. an increase of peripheral blood leukocytes in EF, SE and EFSE groups ($P < 0.001$) was observed compared to controls. Moreover, lower significance ($P < 0.01$) of those cells was found between EF and SE groups. The numbers of lymphocytes in SE group were higher than those in

Table 1. List of primers used for chicks' cytokine mRNA quantification.

Primer	Sequence 5'–3'	Reference
GAPDH For	CCTGCATCTGCCCATTT	De Boever et al. (2008)
GAPDH Rev	GGCACGCCATCACTATC	
UB For	GGGATGCAGATCTTCGTGAAA	De Boever et al. (2008)
UB Rev	CTTGCCAGCAAAGATCAACCTT	
IL-15	GCCGCACATCAAACACATATCT	Kolesarova et al. (2011)
IL-15	TGAGACTGGCTCCTTTTCCTT	
MUC For	TCTTCCGCTACCCTGGGCTCT	Smirnov et al. (2004)
MUC Rev	CTCATGCAGTTCTAGCAAGAT	
IL-17 For	TATCAGCAAACGCTCACTGG	Crhanova et al. (2011)
IL-17 Rev	AGTTCACGCACCTGGAATG	

Table 2. Peripheral white blood cell counts ($G.L^{-1} = 10^9.L^{-1}$) in chicks after administration of *E. faecium* EF55 and infection with *S.* Enteritidis SE147 (mean ± SD)

WBC	Days p.i.	Control	EF	SE	EFSE
Leukocytes	4	9.47 ± 1.30^{c}	8.87 ± 0.84^{d}	11.70 ± 0.70^{a}	8.28 ± 0.80^{d}
	7	7.24 ± 0.72^{a}	10.64 ± 0.59^{da}	13.56 ± 1.47^{dc}	12.26 ± 1.20^{d}
Lymphocytes	4	7.70 ± 1.26^{b}	7.32 ± 0.61^{c}	9.30 ± 1.03^{a}	6.18 ± 0.7^{d}
	7	5.59 ± 1.14^{a}	8.20 ± 0.59^{b}	10.05 ± 1.43^{d}	8.04 ± 1.31^{b}
Heterophiles	4	1.75 ± 1.09	1.42 ± 0.50	2.30 ± 1.55	1.95 ± 0.61
	7	1.58 ± 0.69^{a}	2.38 ± 0.23^{a}	3.45 ± 1.34	4.10 ± 1.60^{b}
Monocytes	4	0.10 ± 0.02	0.16 ± 0.04	0.15 ± 0.06	0.15 ± 0.07
	7	0.09 ± 0.02	0.11 ± 0.01	0.15 ± 0.06	0.20 ± 0.11

p.i. – post infection specific superscripts in row indicate significant differences – $^{ab}P < 0.05$; $^{ac}P < 0.01$; $^{ad}P < 0.001$

controls ($P < 0.05$), EF group ($P < 0.01$), and EFSE group ($P < 0.001$) on day 4 p.i. On the other hand, the number of lymphocytes increased in SE group ($P < 0.001$), as well as in EF and EFSE ($P < 0.05$) groups compared to the control chicks on day 7 p.i. There was an increase in the number of heterophils in chicks of EFSE group ($P < 0.05$) compared with the birds in EF group and controls on day 7 p.i.

Phenotyping of intraepithelial lymphocytes in caecum

Only MHC II+, IgM+ and IgA+ subpopulations showed statistical significance. The numbers of intraepithelial MHC II+ cells were higher in birds of SE group ($P < 0.05$) than those in controls and chicks of EF group ($P < 0.01$) on 7 day p.i (Table 3). Intraepithelial IgM+ lymphocytes increased in SE group ($P < 0.05$) compared to control chicks on 4 day p.i. There was an increase of IgA+ cells in the group EFSE ($P < 0.01$) compared to the chicks of the SE group on day 7 p.i.

Intestinal mucin gene mRNA expression

The mRNA **e**xpression of mucin gene was increased in the jejunum of EFSE group ($P < 0.05$) compared to EF group and controls on day 7 p.i. However, mucin gene mRNA expression was the lowest in the caecum in EFSE group in comparison with other groups of chickens, with significance ($P < 0.05$) to EF group on day 7 p.i. (Table 4).

Cytokines mRNA expression in caecum

The infection with *S.* Enteritidis SE147 and administration

Table 3. Counts of caecal intraepithelial lymphocytes (relative percentage) in chicks after administration of *E. faecium* EF55 and infection with *S.* Enteritidis SE147 (mean ± SD).

Subtypes	Days p.i.	Control	EF	SE	EFSE
CD3	4	22.81 ± 9.04	16.87 ± 5.40	25.64 ± 5.67	16.81 ± 5.64
	7	28.32 ± 5.01	23.22 ± 3.39	31.12 ±11.28	26.44 ± 2.00
CD4	4	5.62 ± 4.18	3.22 ± 1.57	4.55 ± 1.85	3.50 ± 1.91
	7	5.03 ± 1.13	3.96 ± 1.80	6.00 ± 2.35	6.60 ± 4.10
CD8	4	16.73 ± 4.78	16.10 ± 3.46	18.87 ± 6.67	9.63 ± 4.70
	7	12.50 ± 3.46	12.00 ± 3.03	16.20 ± 4.14	12.10 ± 1.25
MHC II	4	16.34 ± 4.85	17.87 ± 8.22	12.03 ± 4.13	15.82 ± 4.42
	7	19.61 ± 4.31^{b}	16.58 ±3.50^{c}	27.33 ± 5.35^{a}	21.80 ± 3.52
TCRγ	4	6.68 ± 2.01	5.84 ± 1.77	9.26 ± 3.34	6.96 ± 2.69
	7	12.92 ± 3.35	10.90 ± 1.62	13.70 ± 5.87	11.03 ± 4.08
IgM	4	0.53 ± 0.35^{a}	0.71 ± 0.33	1.93 ± 1.27^{b}	0.63 ± 0.45
	7	3.35 ± 1.80	3.40 ± 0.32	2.48 ± 1.33	4.78 ± 2.51
IgA	4	15.06 ± 7.29	11.15 ± 6.10	12.98 ± 8.17	24.38 ± 9.12
	7	28.45 ± 7.10	28.86 ±13.73	14.61± 2.68^{c}	37.64 ± 11.01^{a}

p.i. – post infection specific superscripts in row indicate significant differences – $^{ab}P < 0.05$; $^{ac}P < 0.01$

Table 4. Relative expression of mucin gene in the jejunum and caecum of chicks after administration of *E. faecium* EF55 and infection with *S.* Enteritidis SE147 (mean ± SD). Results at each time point are the median of 2–ΔCq.

Days p.i.	Control	EF	SE	EFSE
Jejunum				
4	$0.056.10^{-4}$±$0.000.10^{-4}$	$0.062.10^{-4}$±$0.033.10^{-4}$	$0.035.10^{-4}$±$0.003.10^{-4}$	$0.190.10^{-4}$±$0.045.10^{-4}$
7	$0.188.10^{-4}$±$0.033.10^{-4b}$	$0.180.10^{-4}$±$0.032.10^{-4b}$	$0.226.10^{-4}$±$0.057.10^{-4}$	$0.248.10^{-4}$±$0.077.10^{-4a}$
Caecum				
4	$0.329.10^{-4}$±$0.071.10^{-4}$	$0.375.10^{-4}$±$0.113.10^{-4}$	$0.337.10^{-4}$±$0.170.10^{-4}$	$0.470.10^{-4}$±$0.286.10^{-4}$
7	$1.832.10^{-4}$±$0.086.10^{-4}$	$1.734.10^{-4}$±$0.269.10^{-4b}$	$1.634.10^{-4}$±$1.104.10^{-4}$	$0.340.10^{-4}$±$0.248.10^{-4a}$

p.i. – post infection specific superscripts in row indicate significant differences $^{ab}P < 0.05$

of *E. faecium* EF55 did not significantly influence the mRNA expression of IL-15 and IL-17 in the caecum of broilers (Figures 1 and 2). There was only a tendency to increase the mRNA expression of IL-15 and IL-17 on the first day after infection in EFSE group.

Microbial evaluation

Enterococcus faecium EF55 sufficiently colonized the gastrointestinal tract of chicks. Seven days after probiotic strain administration (4 d.p.i.) faeces and caecum had counts of nearly 10^3 CFU/g in EF and EFSE groups. However, during the experiment no significant decrease, but slightly reduction of *S*. Enteritidis SE147 in faeces and caecum in combined EFSE group occurred. No significant differences were found in the counts of enterococci and coliforms bacteria between the experimental and control groups (Table 5).

DISCUSSION

Earlier works demonstrated that infection of chickens with

Table 5. The counts of bacteria in faeces of chicks after administration of *E. faecium* EF55 and infection with *S.* Enteritidis SE147 (mean ± SD).

Bacteria	Days p.i.	Control	EF	SE	EFSE
Faeces					
EF 55	4	–	2.84 ± 0.50	–	2.98 ± 0.80
	7	–	2.83 ± 1.02	–	1.68 ± 0.98
Enterococci	4	7.75 ± 1.03	8.14 ± 1.06	7.51 ± 0.34	7.15 ± 0.80
	7	7.93 ± 0.38	8.04 ± 0.56	7.53 ± 0.80	6.83 ± 1.67
Coliforms	4	6.96 ± 0.45	6.30 ± 0.87	7.13 ± 1.04	7.77 ± 0.64
	7	8.35 ± 0.15	6.19 ± 0.36	7. 63 ± 1.12	5.98 ± 0.62
SE 147	4	–	–	5.16 ± 1.17	5.82 ± 0.51
	7	–	–	5.84 ± 0.37	5.50 ± 0.53
Caecum					
EF 55	4	–	3.28 ± 0.77	–	3.21 ± 0.17
	7	–	1.55 ± 0.63	–	1.25 ± 0.43
Enterococci	4	7.50 ± 0.91	7.63 ± 1.26	6.22 ± 0.42	7.09 ± 0.86
	7	7.59 ± 1.50	7.33 ± 0.80	8.11 ± 1.58	8.37 ± 0.55
Coliforms	4	8.14 ± 0.36	6.98 ± 0.74	7.52 ± 1.17	8.52 ± 0.51
	7	8.20 ± 1.19	8.15 ± 0.90	7.95 ± 1.18	8.07 ± 0.86
SE 147	4	–	–	6.24 ± 0.53	6.10 ± 0.53
	7	–	–	4.77 ± 0.46	5.20 ± 0.94

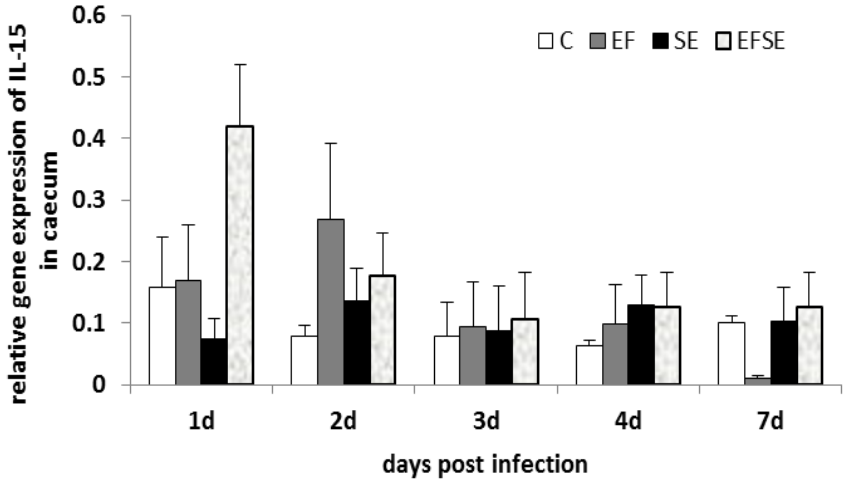

Figure 1. Relative gene expression of IL-15 in the caecum of chicks after administration of *E. faecium* EF55 and infection with *S.* Enteritidis SE147 (mean ± SD). Results at each time point are the median of 2–ΔCq.

S. Enteritidis PT4 increased the total number of peripheral blood leukocytes and lymphocytes (Asheg et al., 2003). Similar quantitative changes in the evaluation of blood leukocytes were found in the current study.

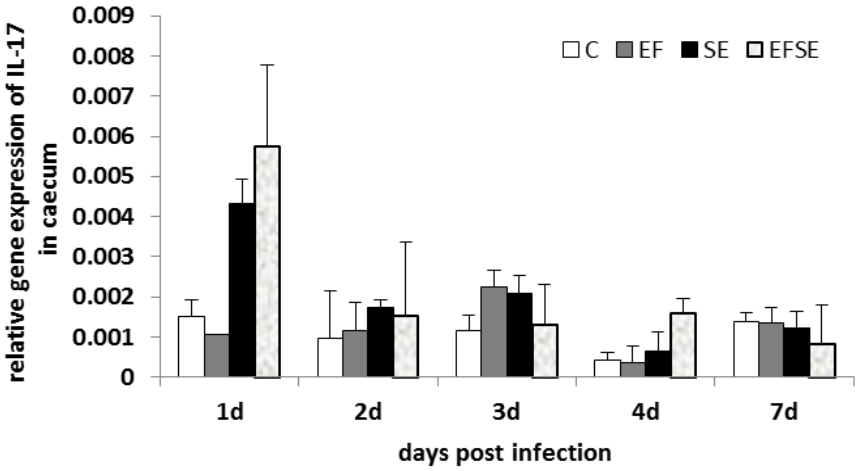

Figure 2. Relative gene expression of IL-17 in the caecum of chicks after administration of *E. faecium* EF55 and infection with *S.* Enteritidis SE147 (mean ± SD). Results at each time point are the median of 2–ΔCq.

Heterophils are critical effector cells in the innate defence of the host against *Salmonellae* infections. The infiltration of heterophils often accompanies the early phase of infection in the gut (Van Immerseel et al., 2002) and these cells can kill invading pathogens and contribute to recruitment of other immune cell types. Our earlier observations indicate that salmonella infection attracts heterophils to the place of infection, cells which have the ability to kill *Salmonella* organisms by releasing antimicrobial peptides (Ševčíková et al., 2003). The results in the current study revealed that administration of *E. faecium* EF55 had immunostimulatory effect on the number of heterophils in the peripheral blood of the chicks infected with *S.* Enteritidis SE147. This suggests the beneficial effect of probiotic against pathogen. Previously, we demonstrated the *Enterococcus faecium* EF55 capability to increase the percentage of phagocytic activity of heterophils in the chickens infected with *Salmonellae* (Levkut et al., 2012). Modulation of heterophils by administration of *E. faecium* EF55 could be one of the important factors in the protection against many intracellular pathogens.

The present results demonstrated milder antimicrobial effect of *E. faecium* EF55 against *S*. Enteritidis SE147 presented by tendency to decrease of followed bacteria counts in caecum and faeces at 7 dpi. On the other hand, previous experiments with administration of EF55 and challenge with *S*. Enteridis PT4 showed significant antimicrobial effect in caecum of chicks 14 dpi (Levkut et al., 2009).

To obtain more information about the numbers and types of lymphocytes that could be recruited for acquired immune responses after infection with *S*. Enteritidis SE147 and administration of *E. faecium* EF55, different intraepithelial lymphocyte subpopulations obtained from caecum were examined. In the current experiment increased frequency of MHC II+ cells, and IgM+ lymphocytes was induced with *Salmonellae* infection, which can be associated with severe host immune response against intracellular bacteria. It is known that MHC II as polymorphic glycoprotein, expressed at the surface of antigen presenting cells, binds exogenously derived antigens and presents them to T lymphocytes. Dendritic cells are able to place class II MHC-antigen complexes on their surface at far higher densities than it can be achieve by macrophages (Kasper et al., 2008). It appears that higher density of MHC II+ cells in caecal epithelium of SE group was associated with alteration of epithelial barrier function and cell damage. On the other hand, administration of *Enterococcus faecium* EF55 has shown in *S*. Enteridis SE147 challenged chicks an increase of the IgA+ subpopulation of caecal intraepithelial lymphocytes in later phase of infection in the current experiment. Other investigators demonstrated that IgA is secreted via mucosal epithelial cells and needs to be retained in the immediate mucosal environment to maximize exclusion of pathogens (Linden et al., 2008). Secretory IgA promotes the clearance of antigens and pathogenic microorganisms from the intestinal lumen. Activity of IgA is directed by blocking access of pathogens to epithelial receptors, entrapping them in mucus, and facilitating their removal by peristaltic and mucociliary activities (Mantis et al., 2011).

Higher jejunal mRNA mucin gene expression in later phase of infection, and on the contrary lower caecal mRNA mucin gene expression in current trial, suggests the modulation in the production of mucin into mucosal environment. The higher jejunal mRNA mucin gene expression (non-specific region for *S*. Enteritidis) can be influenced by the activity of *E. faecium* EF55. Our earlier

work demonstrated that jejunum is very sensitive for mucin secretion after infection (Levkut et al., 2012). Decreased mRNA expression of MUC in the chicks of EFSE group in the late phase of infection can be related to decreased frequency of gramnegative *Enterobacteriaceae* (Strompfová et al., 2003) and *Salmonellae* (Levkut et al., 2012) in the caecum and lack of mucin degrading bacterial species (Macfarlane et al., 2005). The result could be lower mRNA expression of observed mucin gene in the late phase of infection. However, it remains an open question about the correlation of the increased number of IgA+ IEL in current trial and the quantity of SIgA antibodies in biofilm formation. Bollinger et al. (2006) have shown that SIgA and mucin facilitate the formation of biofilm by non-pathogenic *E. coli* on the epithelial cell monolayer grown *in vitro*.

A wide range of cell types respond strongly to exposure of live *Salmonellae* by production of proinflammatory cytokines. Administration of *E. faecium* EF55 revealed a tendency to increase mRNA of IL-15 in the early phase of salmonella infection in both groups with applied probiotic bacteria. Similarly, a tendency to modulate mRNA expression of IL-17 after administration of *E. faecium* EF55 was observed in the current trial in the early phase of infection. Our results suggest that the increased tendency of mRNA expression IL-15 and IL-17 could participate in attraction of mucosal immunocompetent cells including heterophils and lymphocytes into intestinal mucosa. Interleukin-17 has the ability to upregulate synthesis and secretion of numerous cytokines such as IL-6 which is needed for the differentiation of IgM+ cells into plasma cells (Lillehoj and Okamura, 2003). Finally, the tendency of increased density of caecal IgM+ IEL was found in the groups with administration of *E. faecium* EF55 in late phase of salmonella infection. Higher frequency of caecal IgA+ IEL could suppress the mRNA expression of IL-15 and IL-17 in that late phase of infection with *S*. Enteritidis. Previous investigators demonstrated the influence of SIgA on the composition of the intestinal microbiota, downregulation of proinflammatory responses which are normally associated with the uptake of highly pathogenic bacteria (Mantis et al., 2011). Finally, the changes in the composition of intestinal bacteria after application of EF55 were observed in our other experiment (Strompfová et al., 2003).

Conclusion

The results of the current study suggest that there was influence of *E. faecium* EF55 on mRNA expression of MUC2 gene and production of IgA+ IEL with the anti-inflammatory effect in birds presented by decline salmonellae counts in caecum and faeces of challenged (EFSE) group. This suggestion is supported by the beneficial effect of *E. faecium* EF55 on the chicks challenged with *S*. Enteritidis SE147 having higher number of lymphocytes and heterophils in the early or late phase of infection. *E. faecium* EF55 also increased the number of IgA+ IEL in *S*. Enteritidis challenged group, which suggests higher immune exclusion. Finally, the administration of *E. faecium* EF55 had a tendency to increase mRNA expression of IL-15 and IL-17 in the chicks infected with salmonellae in the early phase of infection. The results require further characterization of mucin formation in relation to immunocompetent cells, expressed cell molecules and biofilm formation after application of *E. faecium* 55 to the chickens infected with pathogen.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This project was supported by the following grants: the Slovak Research and Development Agency under the contract APVV-0302-11 and APVV-15-0165; VEGA Slovakia, Grant No. 1/0562/16; INFEKTZOON – the Centre of Excellence for Infections of Animals and Zoonoses, ITMS code 26220120002, supported by the Research and Development operational program financed by the European Regional Development Fund.

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academicJournals

Vol. 8(12), pp. 223-227, December 2016
DOI: 10.5897/JVMAH2015.0425
Article Number: 0C04A0D61559
ISSN 2141-2529

http://www.academicjournals.org/JVMAH

Journal of Veterinary Medicine and Animal Health

Full Length Research Paper

Poultry coccidiosis: Prevalence and associated risk factors in extensive and intensive farming systems in Jimma Town, Jimma, Ethiopia

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Received 14 October, 2015: Accepted 16 March, 2016

Despite the presence of large number of chicken in Ethiopia, contribution to national economy or benefit from this sector of activity is very limited due to diseases and management, out of which poultry coccidiosis is a leading problem. A cross sectional study was undertaken in Jimma town and College of Agriculture and Veterinary Medicine's (JUCAVM) poultry farm from November 2014 to April 2015 with the objective of determining the prevalence of poultry coccidiosis and associated risk factors. The fecal samples collected from live poultry and some after postmortem were subjected to flotation technique to increase sensitivity of the coprological examination of coccidian oocysts using light microscopy (10x). Postmortem examination was also performed to observe the pathological lesion and harvest coccidian oocysts. Out of the total 384 chicken examined, 152 (39.6%; 95% CI 34.7, 44.5) were positive for coccidian oocysts. Statistically significant difference (*P*=0.003) in the prevalence of coccidiosis was observed between young and adult as well as between exotic and local breeds of chickens. Higher infection proportion was detected in birds under intensive management system as compared to birds in back yard. The difference was statistically significant (*P*=0.000). The prevalence was also statistically significantly different (*P*=0.000) between exotic and local breed chickens. Higher prevalence was observed in chickens that were clinically diseased than subclinically harboring the oocysts which was statistically significant (*P*=0.03). However, no statistical significant association was observed between the prevalence and risk factors like sexes, feeding and watering sources. This study demonstrated that coccidiosis is an important problem of poultry for owners in Jimma town and JUCAVM poultry farm. Therefore, appropriate preventive strategies have to be designed to reduce the burden of this disease.

Key words: Coccidiosis, *Eimeria,* poultry, prevalence, risk factors, Jimma.

INTRODUCTION

The poultry industry plays an important role in the provision of animal protein (meat and egg) to human being, and is in general vital in the national economy by generating revenues (Nnadi and George, 2010).

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Approximately, 20 billion poultry exist worldwide among which about 75% are in developing countries Approximately, 20 billion (Gebremariam et al., 2011). Poultry is one of the most intensively reared of the domesticated animal species. Its importance in national economies of developing countries and its role in improving the nutritional status and income of farmers have been recognized (Nnadi and George, 2010).

The total poultry population in Ethiopia is estimated at 56.5 million, of which about 99% are bred under traditional backyard system of management, while 1% is exotic breeds maintained under intensive management system. There are 5-20 birds per households, with simple rearing system in backyard, with inadequate housing, feeding and health care (Tadelle and Ogle, 2001).

Despite the presence of large number of chicken in Ethiopia, contribution to national economy or benefit from this sector of activity is very limited due to nutritional and management factors, as well as diseases, notably: coccidiosis, newcastle disease, salmonellosis and chronic respiratory disease (Abel, 2008).

Coccidiosis is one of the serious poultry diseases that infect the lining of the intestines. It is a complex disease of poultry caused by different species of *Emiria* parasite. The damaged tissue caused by coccidia results in lower feed intake, interference with normal digestion and nutrient absorption, dehydration and blood loss (Pangasa et al., 2007). Chickens suffering from coccidiosis quickly become less productive and poor performers. Laying hens will experience a reduction in rate of egg production (Nematollahi et al., 2009). Coccidiosis affects the chickens in both clinical and sub-clinical forms (Sandhu et al., 2009). Factors contributing to outbreaks of clinical coccidiosis

include litter moisture exceeding 30%, immune suppression, suboptimal inclusion of anticoccidials in feed and environmental and managemental stress such as overstocking, poor feeding systems, and inadequate ventilation (Baba et al., 1982; Singla et al., 2007). Sub-clinical coccidiosis manifests mainly by poor weight gain and reduced efficiency of feed conversion contributing to big economic losses (Razmi and Kalideri, 2000).

The losses caused by coccidiosis without including the sub clinical coccidiosis are estimated to be 2 billion USD throughout the world. Quantitative losses due to coccidiosis in Ethiopia is not well documented, but it has been reported that it contributes to 8.4% loss in profit in large scale farms and 11.9% loss in profit in small scale farms (Gari et al., 2008). Losses due to mortality following a severe outbreak may be devastating and incidence rates as high as 80% were observed to occur in the form of an outbreak in Ethiopia (Kinung'hi et al., 2004). In all parts of the world where confinement rearing is practiced, coccidiosis represents a major disease problem demanding the attention of poultry producers, feed manufactures, and poultry disease experts (Nematollahi et al., 2009). To the authors' knowledge, the prevalence and associated risk factors of poultry coccidiosis in Jimma town backyard and intensive poultry farms has not been well addressed. Therefore, the objectives of this study were to determine the current prevalence and associated risk factors of poultry coccidiosis in Jimma town in Ethiopia.

MATERIALS AND METHODS

Study area

The study was conducted in Jimma town and Jimma University College of Agriculture and Veterinary Medicines's (JUCAVM) poultry farm, Oromia Regional State, Southwestern Ethiopia. Jimma is located at 352 km southwest of Addis Ababa, the Ethiopian capital city. Geographically, Jimma is located at 7°13' and 8°56' N latitude and 35°52' and 37°37'E longitude. The area has an altitude ranging between 1720 and 2110 m above sea level with an annual rain fall ranging between 1200 and 2000 mm. The annual mean temperature ranges from 12 to 28°C (NMA, 2013). In Kersa district, there are 70133 local and 1831 improved chickens to which Jimma town contributes a large share of poultry farms. The study wards of the town were *Bosaketo*, *Hirmata*, *Saxosamaro*, *Manderakochi* and *Mantina* and the JUCAVM poultry farm.

Study design and population

A cross-sectional study was undertaken from November 2014 to April 2015 on randomly selected exotic and local chickens, either from JUCAVM poultry farms or owned by local individuals reared in backyard. The study areas are selected based on convenience method because of their potential poultry bred. Chickens were selected by simple random sampling methods for inclusion into this study. The study included both sexes, different breeds and chickens under different managements.

Risk factors considered and questionnaires

The study was conducted on intensively and extensively reared chickens with consideration of age, breed, clinical status, sex, feed, water and management system of the chickens as risk factors. The information regarding the feed types, the source of drinking water, and hygiene status of the house where the chickens were bred was gathered directly from the owners and visual observation.

Sample size determination

The sample size was determined by assuming the required 5% precision and 50% expected prevalence when normal approximation is used for inference with 95% confidence interval as described in Thrusfield (2005) and these values had given the minimum required sample size equal to 384.

Study methodology

Coprological examination

Each of the birds' faecal samples was collected with a spatula from

Table 1. Final multivariable logistic regression model output of factors associated with fecal coccidian oocyst shedding of chickens from Jimma, Ethiopia.

Variables	Category	No. examined	No. positive	Prevalence (%)	95% CI	-2log LH p-value	OR	95% CI
Age	Young	112	66	58.9	49.8-68	0.003	0.26	0.08-0.84
	Adult (Ref.)	272	86	31.6	26.1-37.1			
Breed	Local(Ref.)	203	49	24.1	18.3-30	0.000	1.61	0.73-3.52
	Exotic	181	103	56.9	49.7-64.1			
Magt system	Backyard (Ref.)	191	44	23	17.1-29	0.000	2.63	1.19-5.80
	Intensive	193	108	56	49-63			
Feed	Industrial products	188	85	45.2	38.1-52.3	0.411	2.99	1.57-5.60
	Local grains (Ref.)	196	67	34.2	27.5-40.8			
Drinking Water	Tap water	160	45	28.1	21.2-35.1	0.128	0.59	0.35-1.01
	River water(Ref.)	224	107	47.8	41.2-54.3			
Clinical status	Clinical	181	82	45.3	38.1-52.6	0.03	1.57	1.04-2.38
	Subclinical(Ref.)	203	70	34.5	27.9-41			
Sex	Female	214	65	30.4	24.2-36.5	0.23	1.09	0.58-2.05
	Male(Ref.)	97	23	23.7	15.2-32.2			
Total		384	152	39.6	34.7-44.5			

No.: number of; OR: odds ratio; Ref: reference cell; Magt: management; LH: likelihood.

freshly voided faeces. The faecal samples were placed into sampling bottles, identified appropriately, and transported to College of Agriculture and Veterinary Medicine's parasitological Laboratory for processing. Before microscopic observation, floatation technique was used to concentrate the oocysts in order to increase the sensitivity of the examination. Sodium chloride solution was used as floating medium. The diagnosis of the oocysts in the faeces was made using 10x optical lens of the microscope.

Post mortem examinations of birds

Post mortem examination was conducted on dead birds to observe gross lesions associated with coccidiosis. It was carried out following the procedures described by Conway and McKenzie (2007). The gastro-intestinal tract was thoroughly examined for gross pathological changes as previously described (Lobago et al., 2005; Gari et al., 2008).Then, the mucosal scrapings were taken to demonstrate the protozoan developmental stage along with lesions by microscopic examination according to Lobago et al. (2005).

Data management and analysis

The raw data were entered, edited and cleaned in office Excel spread sheet. Computation of descriptive statistics was conducted using statistical package for social science (SPSS) version 20.0. The point prevalence was calculated as the percentage of infected chickens among the total number of samples examined. The strength and orientation of association between the prevalence of the disease and each risk factor was assessed by the Miltivariable logistic regression statistical model was used for association analysis The association between explanatory variables (factors evaluated) and the response variable (infection by coccidiosis) was considered statistically significant if the computed p-value was less than 0.05 (P<0.05).

RESULTS

Coprological result and risk factors

Out of 384 chickens examined for the presence of oocysts of *Emeria* spp., 152 (39.6%; 95% CI 34.8 and 44.5%) were found positive on coprological examination. Amongst the 25 chickens examined by post-mortem, 15 (60%) were positive to coccidiosis. The prevalence of coccidiosis was significantly higher (P-value=0.003) in chickens aged 3 to 18 weeks (young) as compared to their adult counterparts older than18 weeks, the odds of getting coccidiosis for the adults being 0.26 times less than the young (Table 1). The prevalence of coccidiosis was significantly higher (P=0.000) in exotic (56.9%) than local breeds (24.1%), exotic breeds having1.61 times more odds of harboring coccidiosis than the local (Table 1). The prevalence of the infection was a little bit higher in female (30.4%) than in male (23.7%) (Table 1). The prevalence was higher in clinical (45.3%) than subclinical (34.5%) diseased birds, clinical birds having 1.57 times more odds of harboring coccidiosis than the subclinical cases. This association was statistically significant (p = 0.03) (Table 1). Higher infection rate was detected in birds under intensive management system (56%) as compared to birds in backyard (23%) (p< 0.0001) (Table 1). The odds of oocyst harboring for the intensively

Table 2. Results of postmortem lesion examinations for poultry coccidiosis, Jimma town, Ethiopia

Site affected	Nature of gross lesion	Number of cases with lesion
Duodenum	White lesions and the mucosa sometimes show hemorrhagic appearance.	5
Jejunum	Petechiae, the jejunum thickened and ballooned with red pinpoint.	1
Ileum	Thin intestinal wall and hemorrhages	3
Caecum	Thickened and ballooned, its content mixed with blood	6

managed was 2.63 times than that of the backyards indicating positive association. In this study, the prevalence of coccidiosis was higher in chickens that were drinking river water (47.8%) than chickens that were drinking tap water (28.1%). The odds of getting coccidiosis for the chickens that were drinking tap water is 0.59 times less than the chickens that were drinking river water (Table 1).

Results of postmortem examination

Postmortem examination was carried out on 25 dead birds, out of which 15 (60%) were positive to coccidiosis by coprological examination, mucosal scraping examination and observation of pathological changes on mucosa of intestine and caecum. Observations from postmortem examinations are summarized in Table 2.

DISCUSSION

The cross-sectional study was primarily conducted to assess the prevalence of poultry coccidiosis and investigate potential risk factors related to the oocyst distribution. This is an important study because quantitative assessment of this disease will provide proxy of the economic burden of this important disease.

Coccidiosis is classified as an intestinal disease affecting small intestine and caecal portion of the large intestine. The present study showed that coccidiosis is an important health problem of chickens in the study area. In this study, the overall prevalence was 39.6%. This result was in line with some previous reports (Netsanet, 2003; Lobago et al., 2005; Mwale and Masika, 2011), and seemed to be higher than other reports (Oljira et al., 2012) (20.57%), (Garbi et al., 2015) (19.5%), (Gari et al., 2008) (22.58%) in poultry farms in Ethiopia and abroad. The wet climate and convenient temperatures of the current study area may be more favorable for the occurrence of coccidiosis. Observation showed that the poor poultry management and the environment such as overcrowding, leaking water troughs and accumulation of feaces support the development of oocysts. Most of these factors are the common problem in the current study area. The result obtained from this study also corresponds with the statement and findings of Slayer and Mallison (1995) who stated that overcrowding, accumulation of faeces and contamination of feed and water by faecal materials increases the number of *Eimeria* oocyst.

In this study, the prevalence of the disease was significantly higher in exotic breeds than local breeds. Higher prevalence in exotic breeds was also reported by Gari and colleagues (2008) who stated that the frequency of occurrence of coccidial infection in Rhode Island Red (RIR) breed was significantly higher than the local breed and this could be due to management system and breed factor (Williams, 2001).

Age difference plays a significant role in prevalence distribution of coccidia oocyst shedding. Indeed, a strong statistical association (P=0.003) was observed between the prevalence of coccidian oocyst shedding of age groups (Table 1). This agreed with the report of McDougald and Reid (1997) who also found that most *Eimeria* species affect birds between 3 and 18 weeks of age. Resistance to the disease usually increases with age of birds. This perhaps explains the decrease in prevalence with increasing age of birds (Uza et al., 2001).

Significantly higher infection rate was detected in birds under intensive management system (56%) as compared to birds in backyard (23%). The odds of oocyst harboring for the intensively management was 2.63 times higher than that of the backyards. The high prevalence in birds under intensive management system in the current study may be caused by improper cleaning and disinfection of the house (observation during data collection), overcrowding and contamination of feed and water by feaces (Slayer and Mallison, 1995).

Also, the prevalence of coccidiosis was higher in chickens that were drinking river water (47.8%) than the chickens that were drinking tap water (28.1%). Accumulation of faeces and contamination of feed and water as in the case of river water by faecal materials increases the number of *Eimeria* spp. oocysts (Slayer and Mallison, 1995).

Conclusion

In general, this study showed that poultry coccidiosis is an important chicken health problem for poultry owners in

Jimma which needs careful attention demanding interventions that will reduce the burden of coccidiosis. Age, management factors and breed difference are important risk factors that need to be worked on to minimize the impact of coccidiosis. Clinically diseased birds are more prone in harboring the oocyst. It is better if further research utilizing molecular techniques is conducted to identify the prevalent *Eimeria* species, as this will contribute to designing the appropriate preventive techniques.

Conflicts of interest

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENT

The authors are grateful to Jimma University College of Agriculture and Veterinary Medicine for providing financial and logistics aid to conplete this study.

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academicJournals

Vol. 8(12), pp. 228-236, December 2016
DOI: 10.5897/JVMAH2016.0522
Article Number: 483E27561563
ISSN 2141-2529

http://www.academicjournals.org/JVMAH

Journal of Veterinary Medicine and Animal Health

Full Length Research Paper

Coliform organisms associated with milk of cows with mastitis and their sensitivity to commonly available antibiotics in Kaduna State, Nigeria

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Received 16 September, 2016; Accepted 20 October, 2016

An examination of raw milk for the isolation of coliforms as members of mastitis-causing organisms was conducted on 300 cows from four Local Government Areas in Kaduna State, northern Nigeria. A 10.3% prevalence was recorded for coliform organisms using the Microgen GN-ID A+B Kit (Medica-Tec™), these were; *Enterobacter* spp., *Citrobacter* spp., *Klebsiella* spp., *Serratia marcescens*, *Proteus* spp. and *Pantoea agglomerans* (similar to *Enterobacter aerogenes*). Antibiotic sensitivity test using commonly available antibiotics showed that all isolates were sensitive to amoxicillin and ciprofloxacin, but resistant to tetracycline and erythromycin. Age, parity number, stage of lactation, management system, hygiene of milking process, and presence of lesion on udder/teat were found to be significantly associated ($p<0.05$) with the prevalence of mastitis in cows. The lowest prevalence (24%; 48 of 200) was recorded in cows within 3 to 4 years of age while, the highest (60.6%; 20 of 33) was in cows aged above 5 years. Stage of lactation was significant with the prevalence of mastitis being the highest (45.5%; 30 of 66) during the initial stage of lactation (0 to 5 month). It was concluded that, the relatively high prevalence of coliforms in bovine mastitis in dairy herds could significantly reduce milk production and cause economic losses. Good hygiene in milking process, milking clinically infected cows last, culling chronic mastitis cases, treating clinically infected cows and dry period therapy could reduce the prevalence of coliform mastitis in Kaduna State, Nigeria.

Key words: Cow, milk, mastitis, coliforms, antibiotic sensitivity.

INTRODUCTION

Almost any microbe that can opportunistically invade tissues and cause infection can cause mastitis. However, most infections are caused by various species of streptococci, staphylococci, and gram-negative rods, especially lactose-fermenting organisms of enteric origin, commonly termed coliforms. Escherichia coli,

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Enterobacter aerogenes, *Klebsiella pneumoniae* and *Serratia marcescens* are four common coliform bacteria that cause mastitis (Radostits et al., 2007; Junaidu et al., 2011). Coliform bacteria are normal inhabitants of soil, digestive tract and manure. They accumulate and multiply in contaminated bedding. Coliform numbers of 1,000,000 or more per gram of bedding increase the likelihood of an udder infection and clinical mastitis (Podder et al., 2014). *Klebsiella pneumoniae* is common in sawdust bedding,
especially rough-cut sawdust that contains bark or soil (Kagkli et al., 2006). Coliforms invade the udder through the teat sphincter when teat ends come in contact with coliform bacteria. Once coliform bacteria enter the mammary gland, they either multiply rapidly or remain dormant. However, the immune response of the cow is highly successful in destroying these bacteria. Many inflammatory and systemic changes seen in severe coliform mastitis result from the effects of release of lipopolysaccharide (LPS) endotoxin (a component of the bacteria cell wall) and subsequent activation of cytokine and arachidonic acid–derived mediators of inflammation and the acute phase response. By the time therapy is initiated, maximal release of LPS has likely occurred (Burns et al., 2000; Smith and Hogan, 2001). Coliform bacteria are responsible for a great number of acute clinical mastitis cases in dairy cows. Severely affected cows may show signs of high fever, udder inflammation (swelling), depressed appetite, dehydration (sunken eyes), diarrhea, decreased production and abnormal milk. Coliform bacteria are also capable of producing subclinical infections that persist for longer periods of time (Radostits et al., 2007). Studies have shown that coliform mastitis apart from resulting to agalactiae can cause substantial losses to producers (NMC, 2000). This disease costs the US dairy industry about 1.7 to 2 billion USD each year (Jones and Bailey, 2010). Consequently, due to economic losses resulting from infection, the relatively high incidence of infection as compared to other clinical mastitis pathogens, and the occasional severe nature of infection, coliform mastitis continue to be a major problem confronting dairy producers (FAO, 2008; Junaidu et al., 2011).

Mastitis caused by coliforms results in a higher incidence of cow death or agalactia-related culling (30 to 40%) than mastitis caused by other pathogens (2%). Prognosis for cases of *Klebsiella* infection should be particularly guarded, because these cows are twice more likely to be culled or die than those infected by other coliforms (Jones and Bailey, 2010). *Serratia* mastitis may arise from contamination of milk hoses, teat dips, water supply, or other equipment used in the milking process. The organism is resistant to disinfectants (Kagkli et al., 2006). The bedding used to house cattle is the primary source of environmental pathogens, but contaminated teat dips, intramammary infusions, water used for udder preparation before milking, water ponds or mud holes, skin lesions, teat trauma, and flies have all been incriminated as sources of infection (Matofari et al., 2003; Kivaria and Noordhuizen, 2007). A study carried out in Sokoto State, Nigeria reported *E. coli* (9.78%), *Klebsiella* spp. (4.35%), *Proteus* spp. (8.69%) and *Enterobacter* spp. (1.09%) (Junaidu et al., 2011). A cross-sectional study carried out in Gondar town, Ethiopia had 54 different bacterial species identified but, *E. coli* (29.6%), *Pseudomonas aeruginosa* (18.5%), and *Klebsiella pneumoniae* (16.7%), were the most commonly identified gram-negative staining bacterial pathogens (Garedew et al., 2012). Majority of coliform isolates from a study conducted using the raw milk consumed in Khartoum, Sudan were *E. coli* 32%, *Enterobacter* spp. 29.2%, *Klebsiella* spp. 19.4%, *Serratia* spp. 11.1% and Citrobacter 1.0% (Salman and Hamad, 2011).

MATERIALS AND METHODS

Study design

A cross-sectional study was carried out on 26 farms in Kaduna State targeting peri-urban farms that provide milk to the community. A total of 300 cows were sampled. Selection of farms was based on; different management conditions, willingness of the farmers/pastoralists to participate in the study, and accessibility of the location, so that samples collected could be immediately transferred to the laboratory for further analysis. All cows in-milking were sampled in every farm visited and it was ascertained that the cows did not receive any treatment before sampling.

Clinical examination of udder

Individual cows were properly restrained, identified and clinically examined. Clinical findings like abnormalities of udder secretions, abnormalities of udder size, consistency, and temperature of mammary gland were examined by visual inspection and palpation. Pain reaction upon palpation, changes in the milk (blood tinged milk, watery secretions, clots, pus), and change in consistency of udder were considered as indications of the presence of clinical mastitis.

Sample collection

The teats were disinfected using a disposable paper towel immersed in 70% ethyl alcohol. California Mastitis Test (CMT) was performed on the first stream of milk before volumes of about 10ml (composite) were aseptically collected into labelled sterile universal bottles. The samples were kept in a cool box containing ice packs and transported to the laboratory immediately.

Media cultivation and identification

A 10 fold serial dilution was done using 3 tubes containing 9mls of sterile physiological saline (PSS). The 10^{-2} and 10^{-3}dilutions were used to inoculate MacConkey and Nutrient Agar respectively, by surface plating. One tenth of a milliliter of the diluted sample was added to the culture media and evenly spread out with an 'L' shaped sterile glass rod. Plated culture media were aerobically

Table 1. Identification of coliform bacteria isolated from 300 milk samples using Microgen GN-ID A+B Kit (Medica-Tec™).

S/N	Bacteria isolated	No. of isolates	Frequency (%)
1	*Enterobacter cloacae*	1	3.2
2	*Enterobacter aerogenes*	1	3.2
3	*Enterobacter gergoviae*	2	6.5
4	*Citrobacter freundii*	2	6.5
5	*Citrobacter koseri*	1	3.2
6	*Klebsiella pneumoniae*	11	35.5
7	*Klebsiella oxytoca*	5	16.1
8	*Serratia marcescens*	4	13.0
9	*Proteus mirabilis*	3	9.7
11	*Pantoea agglomerans*	1	3.2
Total		31	100

incubated at 37°C for 24 h. Individual pinkish colonies on MacConkey agar were picked and sub cultured onto freshly prepared MacConkey agar plate and incubated at 37°C for 24 h to purify the coliform isolate. Representative colonies were stored in slant bottles containing freshly prepared nutrient agar and kept in the refrigerator (4°C) until required for further work (David, 2011).

Biochemical tests for coliforms

Conventional biochemical tests (TSI, SIM, Citrate, Urea and MRVP) were done to preliminarily identify the bacteria isolates before using a commercial kit for *Enterobacteriaceae* (Medica-Tec™ Microgen GN-ID A+B Kit) to confirm the identities of the isolates according to the manufacturers' instructions.

Antibiotic sensitivity testing

Antibiotic sensitivity was carried out on the coliform isolates using commercially prepared antibiotics (Oxoid). The antibiotics employed were: gentamicin, amoxicillin, chloramphenicol, streptomycin, ciprofloxacin, tetracycline and erythromycin. Colonies of the isolate of interest was inoculated into peptone water, the inoculum was standardized to 0.5 McFarland and incubated for 4 hours. A sterile cotton swab was soaked into the peptone water culture and excess fluid expressed from the swab by rolling it on the side of the culture bottle. The swab was used to streak the entire surface of the Mueller-Hinton agar plate. Then antibiotic discs were placed on the surface of the plate 15 mm apart. The plates were then incubated at 37°C for 24 h. The zone of inhibition was measured in millimeters (mm) and compared against a reference standard which contains measurement ranges and their equivalent qualitative categories of susceptible/sensitive (CLSI, 2013).

Administration of questionnaires

Two sets of structured questionnaire were developed and administered through discussion with the Zonal Veterinary Assistants and Livestock Extension Specialists, in order to capture information on herd/farm and individual cow. A total of 26 questionnaires on herd information and 300 questionnaires on individual cow information were distributed. The questionnaire was composed in English and translated to the Fulani herdsmen in Hausa language. Each questionnaire took about 10 to 15 minutes to be administered.

Data analyses

The Statistical Package for Social Science (SPSS) version 20 was used to analyze the data. Each cow-in-milking was a statistical unit. Outcome of coliform examination and sensitivity to antibiotics were compiled in tabular forms. Data obtained from questionnaires were analyzed to determine the prevalence and distribution of mastitis in cows of the study area. Significance of risk factors on the prevalence of mastitis in cows was calculated using chi-square ($\chi 2$) technique to test the association between affected cows and risk factors like age, parity and stage of lactation. In addition, logistic regression analysis was used to calculate odds ratio (with 95% confidence interval and $P < 0.05$ regarded as significant) to measure the degree of association between risk factors and the disease in cows. Results of different isolates were compared.

RESULTS AND DISCUSSION

Thirty-one (10.3%) milk samples were positive for coliforms in this study (Table 1) and are in close agreement with that of Abdurahman (2006) in eastern Ethiopia and Kalla et al. (2008) in Kano State, Nigeria. However, higher prevalence of coliform mastitis were reported by Sena et al. (2001) in India, Woubit et al. (2001) in Southwestern Ethiopia and in Jordan by Hawari and Hassawi (2008). The highest prevalence of coliform mastitis so far were documented by Junaidu et al. (2011) who reported a 52% for Sokoto State, Nigeria and Karimurbo et al. (2005) who reported 66% in Tanzania. The differences in sample size, environmental condition, breed and feeding regimen are possibly the major causes for this discrepancy. The isolated genera of coliform bacteria in this study were *Klebsiella*, *Pantoea*, *Enterobacter*, *Serratia*, *Citrobacter* and *Proteus* (Table 1). This is in close agreement with Matofari et al. (2003), Abdurrahman (2006), Kalla et al. (2008), Giannino et al. (2009), Abera et al. (2010) and Garedew et al. (2012)

Table 2. *In-vitro* Sensitivity testing of bacterial isolates to seven antibiotics.

Bacterial species	No. of isolates	No (%) sensitive to each antibiotic						
		C	A	CH	S	T	G	E
Enterobacter spp.	4	4(100)	4(100)	2(50)	2(50)	1(25)	2(50)	0
Citrobacter spp.	3	3(100)	3(100)	1(33.3)	1(33.3)	1(33.3)	2(66.7)	0
Klebsiella spp.	16	10(62.5)	16(100)	10(62.5)	10(62.5)	5(31.3)	10(62.5)	0
Serratia spp.	4	4(100)	4(100)	2(50)	0	0	4(100)	2(50)
Proteus spp.	3	3(100)	3(100)	2(66.7)	0	0	3(100)	0
Pantoea spp.	1	1(100)	1(100)	1(100)	0	0	1(100)	0

C = Ciprofloxacin, A= Amoxicillin, CH= Chloramphenicol, S = Streptomycin, T = Tetracycline, G= Gentamicin and E= Erythromycin.

Who all found *Escherichia coli, Klebsiella* spp., *Enterobacter* spp., *Citrobacter*, *Serratia* and *Proteus* as major mastitogens. Although *E. coli* was not isolated from this study, the other coliform bacteria are generally found in high concentrations in organic matter, such as bedding and manure (environment). Therefore from an epidemiologic standpoint, the primary source of infection for most pathogens in this study is environmental. *Klebsiella* spp. accounted for 51.6% of the isolates in this study; Podder et al. (2014) asserted that *Klebsiella pneumoniae* is well adapted to survive in the udder and usually establishes a mild subclinical infection of long duration, from which it is shed in milk, facilitating transmission to healthy animals, mainly during milking procedures. This might be due to the fact that there are no established mastitis control practices that are employed by the farmers, but instead mastitis control relies heavily on drug use.

Moreover, the unhygienic housing and milking practices observed among the dairy herds increase both exposure and infection pressure to cows, with the subsequent high infection levels. The present study showed that there was high prevalence of coliform mastitis and, *Klebsiella* spp. were the dominant coliform isolates in the sampled area. Studies have shown *Klebsiella* spp, *Enterobacter* spp, *Citrobacter* spp. and *Serratia* spp. to be the most frequently isolated microrganisms in connection with nosocomial infections (Radostits et al., 2007; Podder et al., 2014). Recently, all species and all serotypes of *Klebsiella*, all species of *Proteus*, *Citrobacter*, *Enterobacter*, *Pantoea* and *Serratia* are listed by the United States Public Health Service, Department of Health and Human Services bioterrorism list of dangerous biological agents that have the potential to pose a severe threat to public health and safety, to animal health, to plant health, or to animal and plant products (Bynum, 2011). All of these organisms were isolated from cow milk samples and are therefore of public health importance.

Antibacterial susceptibility testing of coliform isolates showed that, all coliform isolates were sensitive to amoxicillin followed by decreasing susceptibilities to ciprofloxacin, gentamicin, streptomycin, tetracycline and erythromycin (Table 2). The pattern of susceptibility and resistance exhibited in the present study may be due to prolonged and indiscriminate usage and prescription of particular antibiotics which often leads to possible resistance development in animals (Kwaga, 2012; Sharma, 2014). On table 3, questionnaires data analysis showed that 89(29.7%) milk samples were CMT positive of these; 13(4.3%) were contaminated with only yeasts, 7(2.3%) with only coliforms while, 24(8%) with yeasts and coliforms. More than 60% of cows aged above 5 years were affected with mastitis (Table 3) this is in agreement with Sena et al. (2001) who reported that animals aged more than 5 years were more susceptible to mastitis. After the age of 7 years, immunity of the animals is affected making them vulnerable to diseases.

The study determined the increasing prevalence of mastitis with an increase in parity number which agrees with findings by Abdurahman (2006). Radostits et al. (2005) stated that high yielding animals are more susceptible to mastitis than low-yielding ones. Also observed were cows with lesions on their teats and/or udder had a 10.7 time chances of developing mastitis than cows that had no teats lesions (Table 4). This is similar to the findings of Mulei (1999), who found in the Kiambu district of Kenya that mammary gland quarters with teat lesions were 7.2 times more likely to have microorganisms isolated from them than those without any teat lesions. Also, cows managed by nomadic system were 4 times likely to develop mastitis than cows in the sedentary system. In addition, animals with poor hygiene of milking process had a 3.4 times chance of developing mastitis. The nomadic system of cattle rearing and poor hygiene of milking process were also identified as risk factors for occurrence of coliform mastitis in another study in Ethiopia (Abdurahman, 2006). This might be due to absence of udder washing, milking cows that had been suckled by calves without washing the udder/teats, milking of cows with milkers who had cuts and chaps on their hands, and using of common udder cloths, which could be fomites for spread especially for contagious mastitis (Tables 5 and 6). On Table 7, twenty-

Table 3. Distribution of CMT positive milk samples showing number of single infection and mixed infection.

S/N	Farms in LGA	No. of samples examined	No of sample +ve for CMT	No of sample +ve for yeast	No of sample +ve for coliform
Kaduna north					
1	A1	9	5	1	0
2	A2	11	3	1	0
3	A3	10	3	2	2
4	A4	11	1	1	0
5	A5	12	4	1	1
6	A6	10	0	0	0
7	A7	14	10	3	1
8	A8	13	1	2	0
Igabi					
9	B1	12	5	3	3
10	B2	11	4	0	2
11	B3	5	0	0	0
12	B4	16	2	2	0
13	B5	15	3	3	1
14	B6	12	12	6	5
15	B7	13	1	1	1
16	B8	10	7	1	1
Chikun					
17	C1	8	2	2	3
18	C2	14	0	0	0
19	C3	11	2	0	1
20	C4	18	3	2	2
21	C5	14	5	2	2
Kaduna south					
22	D1	9	1	0	1
23	D2	14	7	3	3
24	D3	9	0	0	0
25	D4	11	2	0	2
26	D5	8	6	1	0
	Total	300	89(29.7)	37(12.3)	31(10.3)

Table 4. Associations of risk factors of mastitis with occurrence of mastitis in cows in Kaduna State, Nigeria using logistic regression analysis.

Risk factor	P value	Crude odds ratio (95%CI)	Adjusted odds ratio (95%CI)
Age (years)	0.03	4.7(2.4-10.6)	3.3(1.6-7.2)
Parity	0.00	3.3(1.7-7.8)	2.1(1.8-5.8)
Stage of lactation	0.03	9.1(4.2-19.8)	4.0(1.7-9.5)
Management system	0.04	10.1(3.9-21.2)	4.0(1.9-8.4)
Hygiene of milking Process	0.00	8.7(4.4-17.3)	3.4(1.6-10.2)
Lesion on udder/teat	0.00	21.8(7.9-68.5)	10.7(3.8-36.5)

one (80.8%) farmers consumed boiled or pasteurized milk while 5(19.2%) of the farmers consumed raw milk.

Although, milk consumption in Africa is fairly low compared to the rest of the world, in tribes where milk

Table 5. Different determinants influencing the prevalence of mastitis.

Parameter	No. of cows examined	No. of affected cows(CMT+ve)	Prevalence (%)
Age (years)			
<2	67	21	31.3
3–4	200	48	24.0
>5	33	20	60.6
Parity			
1	83	21	25.3
2	123	33	26.8
>3	94	35	37.2
Stage of lactation (months)			
0–5	66	30	45.5
6–10	144	16	11.1
>10	90	39	43.3
Management system			
Sedentary system	54	17	31.5
Nomadic system	246	85	34.6
Hygiene of milking process			
Good	157	22	14.0
Poor	143	88	61.5
Lesion on udder/teat			
Yes	62	24	38.7
No	238	71	29.8

consumption is popular, such as the Fulani and Maasai, milk is typically consumed unpasteurized (Mulei, 1999; Ocholi et al., 2004). Those favoring the consumption of raw milk believe that raw milk and associated products are healthier and taste better but, under such conditions, public health hazards definitely exist as seen from the type of diseases farmers suffered from such as; sore throat (42.5%), campylobacteriosis (23%), thrush (11.5%), tuberculosis (11.5%) and typhoid (11.5%). Agencies such as the Centers for Disease Control and Prevention (CDC), Food and Drug Administration (FDA) and other regulatory agencies around the world note that pathogens from raw milk, including potentially agents of tuberculosis, diphtheria, typhoid, and streptococcal infections, make it unsafe to consume (FDA, 2009). Similarly, a recent review authored by the Belgian Federal Agency for the Safety of the Food Chain and experts from Belgian universities and institutions concluded that "raw milk poses a realistic health threat due to a possible contamination with human pathogens. It is therefore strongly recommended that milk should be heated before consumption. With the exception of an altered organoleptic (flavor) profile, heating (particularly ultra-high temperature and similar treatments) will not substantially change the nutritional value of raw milk or other benefits associated with raw milk consumption" (Claeys et al., 2013).

CONCLUSION AND RECOMMENDATIONS

The diversity of organisms isolated from milk of cows in this study might have been influenced by the type of management system employed or, the milk samples may have been contaminated by infected persons during milking process or the environment. Good hygiene in milking process, milking clinically infected cows last, culling chronic mastitis carriers, treating clinically infected cows and dry period therapy could reduce the prevalence of coliform mastitis in Kaduna State, Nigeria.

Veterinarians, livestock extension workers and farmers should implement *in-vitro* susceptibility testing prior to the use of antibiotics for treatment of intra-mammary infections in cows. Also, efforts must be made to encourage dairy farmers to apply safe substitutes such as probiotics and bioactive natural compounds for

Table 6. Milking practices on farms.

Variable	No	Frequency
Milking methods		
Hand	25	96.2
Machine	1	3.8
No of milking times/day		
Once	25	96.2
Twice	1	3.8
Udder preparation before milking		
Calves suck before milking	21	80.8
Wash with soap and water	1	3.8
Wash with water only	4	15.4
Type of milking container		
Plastic	20	70.0
Calabash	5	19.2
Stainless steel	1	3.8
Cleaning of milking container		
Wash with water only	25	96.2
Wash with water and soap	1	3.8
Is there decrease in milk yield?		
Yes	24	92.3
No	2	7.7

Table 7. Common diseases affecting farmers.

Variable	No	Frequency (%)
Have you heard of mastitis?		
Yes	26	100
No	0	0
Have your cows had mastitis before?		
Yes	20	77
No	6	33
What do you do when your cows are sick?		
Report to Veterinarian	21	80.8
Use veterinarian and traditional methods	5	19.2
Do you consume milk from your cows?		
Yes	26	100
No	0	0
What do you do before you drink milk?		
Boil/Pasteurize	21	80.8
Don’t boil/pasteurize	5	19.2

Table 7. Cont`d

Have you been sick after consuming milk?		
Yes	26	100
No	0	0
If yes, what was it?		
Sore throat	11	42.5
Campylobacteriosis	6	23.0
Thrush	3	11.5
Tuberculosis	3	11.5
Typhoid	3	11.5

prophylactic and therapeutic use.

Conflict of interests

The authors have not declared any conflict of interests.

ACKNOWLEDGMENT

Mbuk E. U. would like to thank the Staff of the veterinary bacterial zoonosis laboratory, Ahmadu Bello University, Zaria. Special thanks are due to the pastoralists of the study area.

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academicJournals

Vol. 8(12), pp. 237-243, December 2016
DOI: 10.5897/JVMAH2016.0521
Article Number: EAD869261647
ISSN 2141-2529

http://www.academicjournals.org/JVMAH

Journal of Veterinary Medicine and Animal Health

Full Length Research Paper

Genetic evolution of infectious bursal disease virus in Senegal

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Received 8 September, 2016: Accepted 20 October, 2016

In recent years very virulent (VV) IBDV strains and classical (CV) IBDV strains re-emerged and caused devastating outbreaks in different parts of the world. In this study, genetic evolution of fifteen IBDVs collected in Senegal in 1979, 1999, 2007, 2012, 2013 and 2014 was characterized to gain information for a better control of IBD. Following RT-PCR, nucleotide sequence of the VP2 hypervariable region was determined and compared with sequences available in GenBank. Phylogenetic analysis showed that the viruses diverged into two genotypes: Very virulent (VV) IBDV and classical virulent (CV) IBDV. The Senegalese field strains of the first genotype (VV) IBDV had 98.9 to 100% identity among themselves, whereas their identity with reported Nigerian (VV) IBDVs ranged between 96.7 and 99%. The close phylogenetic relationship of the Senegalese and Nigerian strains suggests that they likely derived from a common ancestor. In the phylogenetic tree, all the Senegalese (VV) IBDV strains belonged to the African very virulent types (VV2). The genotyping of senegalese field IBDV strains indicated that the majority of viruses circulating in Senegal are (VV) IBDVs and highlights a genetic stability.

Key words: Infectious bursal disease, phylogenetic analysis, Senegal.

INTRODUCTION

Infectious bursal disease (IBD) is a highly contagious acute viral disease of young chickens (3 to 6 weeks old) that causes immunosuppression by damaging the bursa of Fabricius. The disease is either fatal or causes impaired growth of young chickens, resulting in significant economic losses for the poultry industry (Islam et al., 2005). Infectious bursal disease virus (IBDV) is a member of the genus *Avibirnavirus* of the family *Birnaviridae*. There are two distinct serotypes of IBDV, 1 and 2. Serotype 1 viruses are pathogenic to chickens, while serotype 2 viruses are non-pathogenic to chickens (Ismail et al., 1998). IBDV can also be grouped into

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pathotypes based on their pathogenicity in chickens: They are referred to as classical virulent (CV), antigenic variant, very virulent (VV) and attenuated (Műller et al., 2003).

IBDV is a double stranded RNA virus with a bisegmented genome. The larger segment A encodes four viral proteins designated as VP2, VP3, VP4 and VP5. The smaller segment B encodes only VP1 which has polymerase activity (Tamura et al., 2011). The hypervariable region (HVR) within VP2, between amino acid residues 206 and 350, has the highest amino acid sequence variation among serotype 1 strains, and the nucleotide and deduced amino acid sequences of this region are widely used for molecular diagnosis and genotyping of IBDVs. This region includes two major sets of hydrophilic amino acids, termed peak A and B, from amino acid positions 210 to 225 and 312 to 324, respectively, with smaller hydrophilic peaks present in-between (Bayliss et al., 1990). So far, the four amino acid changes in VP2 at positions 222A, 256I, 294I, and 299S have been shown to be present in all European-like (VV) IBDVs and serve to differentiated (VV) from (CV) viruses (Zierenberg et al., 2000).

During IBDV replication, the RNA-dependent polymerase (RdRp)-error prone activity potentially leads to a high rate of mutations resulting in antigenic variation in protein domains involved in virus neutralization. This process is thought to be a contributor to the emergence of variant viruses or highly virulent forms seen in the USA and others countries. In the USA, it was demonstrated that the new isolates were affected by antigenic drift against which classical IBDV vaccines were not protective (Snyder et al., 1992), whereas in Europe, the first cases of acute IBDV were described in the 1990s (Dormitorio et al., 1997). Surprisingly, some of these first acute outbreaks occurred in broilers, at the end of the fattening period, at farms where all the necessary hygiene and prophylactic measures had been taken. These findings indicated a dramatic change in the field situation (Van den Berg, 2000).

In Senegal, the first IBD outbreaks were reported in 1975 involving 21 to 60 day old broilers and pullets (Sagna, 1975). Subsequently, IBD has become a major player among infectious diseases of broilers and pullets, despite routine implementation of vaccination. For improved control of this disease in Senegal, genetic evolution of fifteen Senegalese IBDV strains isolated in 1979, 1999, 2007, 2012, 2013 and 2014 was studied. The hypervariable region of the VP2 gene of these field strains was sequenced and compared to each other and to published sequences obtained from GenBank.

MATERIALS AND METHODS

Bursa sample collection and preparation of viral suspensions

For virus detection, the bursa samples were aseptically collected from IBD suspected dead chickens. The samples were collected from commercial broilers and pullet flocks in 1979, 1999, 2007 and during the period from 2012 to February 2014 (Table 1).

The samples were subjected for postmortem examination, placed into individual sterile universal bottles and transported under cold chain to the National Veterinary Research Laboratory, Dakar, Senegal. A freeze-dried live vaccine (HIPRAGUMBORO® CH/80, Hipra laboratory, Spain) containing the CH/80 strain of IBDV was taken as positive control and phosphate buffered saline (PBS) as negative control.

In order to prepare viral suspensions, ten milliliter of PBS (pH 7.2) was added to approximately 100 mg of bursal tissue from each sample and homogenized using a pestle and mortar. The homogenates were centrifuged at 12000 rpm, 4°C for 5 min and the supernatants collected for molecular analysis.

IBDV detection by reverse transcription-polymerase chain reaction (RT-PCR)

The viral RNA was extracted using the RNeasy Plus Mini Kit (QIAGEN) from bursal suspensions according to the manufacturer's protocol and was subjected to RT-PCR.

A 743-bp segment of the highly variable region of VP2 from nucleotides 737 to 1479 was amplified using primers 743-1 (5′-GCCCAGAGTCTACACCAT-3′) and 743-2 (5′-CCCGGATTATGTCTTTGA- 3′) (Jackwood et al., 2011). These primers used for both RT and PCR are located in a conserved sequence region and amplify this portion of genome segment A from all serotype 1 IBDV strains including vvIBDV and non-vvIBDV. They do not amplify genome segment A from serotype 2 viruses. The RT-PCR reactions were conducted using the QIAGEN One Step RT-PCR kit according to the manufacturer's instructions. For one test, the reaction mixture consisted of 15 µl RNase free water, 5 µl purified RNA, 10 µl PCR Buffer Tampon 5X , 2 µl of 10 mM dNTPs mix, 3 µl of each primer (10 µM), 10 µl of Q 5X Solution and 2 µl of one Step RT-PCR Enzyme mix. PCR tubes were placed in the Bio-Rad MJ Mini Personal Thermal Cycler and subjected to 35 amplification cycles.

The RT incubation was at 50°C for 30 min followed by 95°C for 15 min to activate Taq polymerase enzyme and 35 cycles of 95°C for 30 s, 53°C for 1.5 min and 72°C for 1.0 min. At the end of the 35 PCR cycles, a 7.0 min at 72°C extension period was added.

The PCR products were analysed on 2% agarose gels prepared in Tris-Acetate-EDTA (TAE) buffer and stained with ethidium bromide. Eight micro-liter of the PCR product from each of the tubes were mixed with 2 µl 6X Load Dye and electrophoresed along with a DNA ladder 100 pb (Promega) at a constant 100 V for 60 min in 1 X TAE buffer. Amplified product was viewed under UV light for the expected 743 bp. Before sequencing, the amplified products were purified by "QIAquick PCR Purification Kit" (QIAGEN).

Sequencing and phylogenetic analysis

The RT-PCR products for fifteen selected positive samples were sequenced at the Genomics Platform GeT-Purpan, UDEAR UMR 5165 CNRS/UPS, CHU PURPAN, France on a 3130XL Applied Biosystems capillary sequencer. Samples were first purified using the QIAquick PCR Purification Kit (Qiagen). Sanger sequencing was carried out using the same primers as those used for the PCR. Sequences were aligned with reference strains from the GenBank database using Clustal W and a phylogenetic tree of the nucleotides was constructed using MEGA version 5.10 (Tamura et al., 2011) with up to 1000 bootstrapping replicates. The neighbor joining (NJ) method and Pairwise distance were used.

Accession numbers of sequence data. The nucleotides sequence data reported in this article have been submitted to the GenBank

Table 1. Description of IBDV strains investigated in this study.

Isolate	Isolation year	locality	Flock type	Field mortality (%)	Vaccination	Age (days)	GenBank accession number
IBDV/DK9/SN/2013	2013	Dakar	Broilers	57.6% (day 3)	+	19	KP773414
IBDV/DK3/SN/2012	2012	Dakar	Broilers	> 17% (day 1)	+	28	KP773415
IBDV/DK6/SN/2012	2012	Dakar	Broilers	NI	NI	38	KP773416
IBDV/DK2/SN/2013	2013	Dakar	Pullets	> 16.7% (day 1)	+	22	KP773417
IBDV/DK1/SN/2014	2014	Dakar	Pullets	50-54%	+	31	KP773418
IBDV/DK3/SN/2014	2014	Dakar	Broilers	20.5% (day 1)	NI	32	KP773419
IBDV/KL1/SN/2013	2013	Kaolack	Pullets	16% (day 1)	+	33	KP773420
IBDV/TH1/SN/2014	2014	Thiès	Broilers	>28%	NI	26	KP773421
IBDV/TH2/SN/2014	2014	Thiès	Broilers	41.7%	+	23	KP773422
IBDV/ST1/SN/2012	2012	ST Louis	Pullets	46 -37.6% (day 2)	+	40	KP773423
IBDV/ST1/SN/2013	2013	ST Louis	Broilers	> 63.1%	+	34	KP773424
IBDV/DK1/SN1979	1979	Dakar	NI	NI	NI	NI	KP773425
IBDV/DK2/SN/1979	1979	Dakar	NI	NI	NI	NI	KP773426
IBDV/DK1/SN/1999	1999	Dakar	NI	NI	NI	NI	KP773427
IBDV/DK1/SN/2007	2007	Dakar	NI	NI	NI	NI	KP773428

NI, No Information; +, Vaccination via drinking water with intermediate or intermediate plus IBDV strains.

sequence database and have been assigned the accession numbers.

RESULTS

Nucleotide sequence analysis

The nucleotide sequence of the hypervariable domain of VP2 from nucleotide positions 737 to 1479 was determined for the fifteen samples in this study (Table 1) and sequences were submitted to GenBank. Thus, the analysis revealed that the Senegalese field IBDV strains had 98.9 to 100% identity among themselves, whereas their identity with the reported Nigerian (VV) IBDV strains (IBDV33/Abuja. NG/2011, IBDV61/Kaduna. NG/2009, IBDV43/Kaduna. NG/2011, IBDV79/Kaduna. NG/2011, IBDV2/Kaduna. NG/2009, IBDV58/Kaduna. NG/2010) ranged between 96.7 and 99%. These Senegalese field isolates were more close to the Nigerian (VV) IBDV isolates IBDV33/Abuja. NG/2011, IBDV61/Kaduna. NG/2009, IBDV43/Kaduna. NG/2011, IBDV79/Kaduna. NG/2011, IBDV2/Kaduna. NG/2009, IBDV58/Kaduna. NG/2010 (identity up to 99%) than the reported Asian and European (VV) IBDV strains OKYM and Cro-Ig/02 (identity up to 96.9%). The Senegalese field isolates IBDV/DK3/SN/2014 and IBDV/KL1/SN/2013 shared 94.6 to 96.9% identity within themselves. The identity of these Senegalese field isolates with the American and European reported (CV) IBDVs (STC, Cu-1, Vaccine Cevac-IBD-L and Faragher/52-70) ranged between 94.6 and 100% identity. All the fifteen Senegalese field isolates studied had a minimum identity of 68.9% with serotype 2 strain IBDVP234 and a maximum identity of 71.0%.

Deduced amino acid sequence analysis

Amino acid sequence analysis revealed 100% sequence similarity among the Senegalese field isolates (Figure 1). Their identity with the Nigerian IBDV strains (IBDV33Abuja. NG/2011, IBDV61/Kaduna. NG/2009, IBDV43Kaduna. NG/2011, IBDV79Kaduna. NG/2011, IBDV2Kaduna. NG/2009, and IBDV58Kaduna. NG/2010) was also 100%. Their similarity with other reported (VV) IBDVs (Cro-Ig/02, OKYM, IBDV80/Kaduna. NG/2011, 050 Oyo. NIE/99, 042 Oyo. NIE/00, 034 Oyo. NIE/99, and 082 Ogun. NIE/97) ranged between 96.9 and 99.2%. The amino acid sequence of the Senegalese field isolates IBDV/DK3/SN/2014 was similar to the strain IBDV/KL1/SN/2014 (99.2%). The Senegalese field isolate IBDV/DK9/SN/2013, IBDV/DK3/SN/2012, IBDV/DK6/SN/2012, IBDV/DK1/SN/2014, IBDV/ST1/SN/2012, IBDV/TH1/SN/2014, IBDV/ST1/SN/2013, IBDV/DK1/SN/1999, IBDV/DK2/SN/2013, IBDV/DK1/SN/2007, IBDV/DK1/SN/1979 and IBDV/TH2/SN/2014 harbored amino acids 222(A), 242(I), 256(I), 294(I), 299(S) and 300 (A). The strains IBDV/DK3/SN/2014 and IBDV/KL1/SN/2013 had 222(P) or 222(S), 256(V) and 299(N) which allow for the distinction between (VV) and (CV) IBDVs from (CV) IBDVs. All the Senegalese strains had the same amino acid markers at position 253 (Q), 279 (D) and 284(A) (Figure 1).

Phylogenetic analysis

Phylogenetic analysis based on nucleotide sequence revealed that all fifteen Senegalese field isolates fell

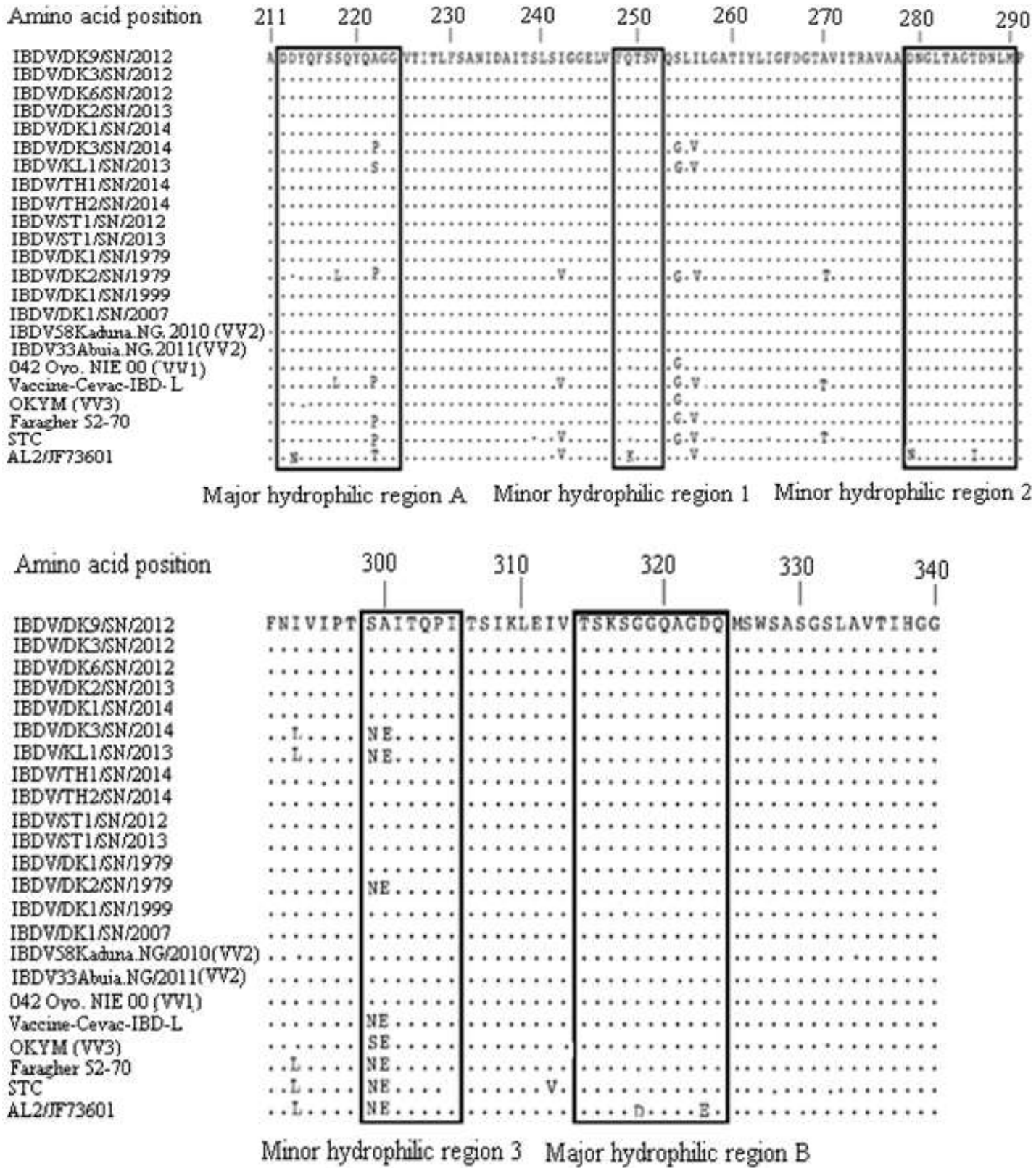

Figure 1. Deduced amino acid sequences of VP2 hypervariable domain from amino acid positions 211 to 340 of serotype 1 strains. Compared amino acid sequences and corresponding accession numbers include those of Senegalese field IBDV strains (accession numbers will be created in GenBank), representative sequences of European, Asian and Nigeria VV (VV3) (OKYM), Nigerian VV1 representative sequence (VV1) (042 Oyo.NIE.00), Nigerian VV2 representative sequence (IBDV58.Kaduna.NG/2010, IBDV33Abuja.NG/2011), Variant strain (AL2, JF73601), classical virulent strains (STC, Faragher/52-70) and the classical Vaccine Cevac-IBD-L).

within the serotype 1 viruses and form two separate genotypes. The (VV) genotype included the Senegalese strains IBDV/DK9/SN/2013, IBDV/DK3/SN/2012, IBDV/DK6/SN/2012, IBDV/DK1/SN/2014, IBDV/ST1/SN/2012, IBDV/TH1/SN/2014, IBDV/ST1/SN/2013, IBDV/DK1/SN/1999, IBDV/DK2/SN/2013, IBDV/DK1/SN/2007, IBDV/DK1/SN/1979 and IBDV/TH2/SN/2014 as well as the reported (VV) IBDVs isolates from Nigeria, Europe and Asia. In the tree, the (VV) genotype was subdivided into three major clusters namely VV1, VV2 and VV3. All these Senegalese (VV) IBDV strains were grouped with Nigerian (VV) IBDV strains (IBDV33/Abuja.NG/2011, IBDV61/Kaduna.NG/2009, IBDV43/Kaduna.NG/2011, IBDV79/Kaduna.NG/2011, IBDV2/Kaduna.NG/2009, and IBDV58/Kaduna.NG/2010) in the cluster VV2. The reported strains 050 Oyo.NIE/99, 042 Oyo.NIE/00, 034 Oyo.NIE/99 and 082 Ogun.NIE/97 formed the cluster VV1 and the reported strains Cro-Ig/02, OKYM, IBDV80/Kaduna.NG/2011 were regrouped in the cluster VV3. In the classical genotype cluster, the Senegalese strains IBDV/DK3/SN/2014 and IBDV/KL1/SN/2013 were closely related to the classical strains STC, Cu-1, Vaccine Cevac-IBD-L and Faragher/52-70 isolated in the U.S.A. and Europe. The (CV) IBDV strain IBDV/DK2/SN/1979 showed 100% nucleotide sequence identity compared with the Cevac-IBD-L vaccine (Figure 2).

DISCUSSION

Infectious Bursal disease is one of the major problems faced by modern poultry farming in Senegal and is responsible for considerable economic losses particularly in broiler farms. The first cases of IBD diagnosed clinically were recorded in 1975 in Dakar with an important mortality rate (Sagna, 1975). Since then IBD outbreaks have been noted in broilers and layers chickens in modern poultry farming throughout Senegal. The Senegalese VV strains showed intra-group nucleotide sequence identities within themselves of 98.9 to 100% and amino acid sequence identities of 100%. They were all grouped in the same cluster VV2 with six Nigerian (VV) IBDVs (IBDV33/Abuja.NG/2011, IBDV61/Kaduna.NG/2009, IBDV43/Kaduna.NG/2011, IBDV79/Kaduna.NG/2011, IBDV2/Kaduna.NG/2009 and IBDV58Kaduna.NG/2010). These Senegalese strains were closely related to the reference (VV) IBDV strains from Europe (Cro-Ig/02) and from Asia (OKYM) but belonged to a different lineage. The Senegalese strain IBDV/TH2/SN/2014 and the Nigerian strains IBDV33/Abuja.NG/2011 and IBDV61/Kaduna.NG/2009 were found to share 99 and 100% identity at the nucleotide and amino acid levels, respectively. It could be speculated that the same IBDV strains were exchanged between the two countries or that the outbreaks in the two countries might have a common source. However, the epidemiological links between the outbreaks in the two countries are not known. These findings demonstrate the presence of African (VV) IBDV type in Senegal since 1979. Kasanga et al. (2007) reported that the African genetic lineage may have spread worldwide later on. This suggests that similar viruses clustering in VV2 may exist in other countries in West Africa.

Genetic stability over time within the VP2 hypervariable domain has been detected in Italy (Martin et al., 2007). This is in contrast to the sequence heterogeneity detected among (VV) IBDV isolates from other African countries. Indeed, Adamu (2013) reported in Nigeria a high level of genetic heterogeneity and two distinct genetic clusters, specifically VV1 and VV2. The high degree of sequence diversity among Nigerian (VV) IBDV isolates suggests that West-Africa may be the origin of the newly emerged (VV) IBDV variant found across the Old World (Owoade et al., 2004). In Tanzania, Kasanga et al. (2007) reported the circulation of the (VV) IBDV isolates from both Asian/European lineages.

The phylogenetic analysis showed that the Senegalese strains IBDV/DK3/SN/2014, and IBDV/KL1/SN/2013 belonged to the (CV) genotype. These strains were related to the reference (CV) IBDV from the U.S.A. (STC, Cu-1) and from Europe (Faragher/52-70) but they also clustered in a different sub-lineage. According Sagna (1975) the Cevac-IBD-L vaccine containing intermediate plus strains was widely imported and used by the Senegalese poultry farmers since 1975. And this vaccine preserved an important residual pathogenic power which caused IBD.

Antigenic variation of IBDVs is largely due to mutations occurring in two main hydrophilic regions of VP2, located between amino acid residues 212 to 223 and amino acid residues 314 to 324 (Vakharia et al., 1994). It has been reported that the amino acid composition at position 222 can be used to define groups of IBDVs. Classical viruses typically harbor a proline at position 222, variant viruses a threonine and a serine at position 222 can be found in both classical and variant viruses (Dormitorio et al., 1997). The results revealed that all the Senegalese (VV) IBDV strains contained the putative virulence marker amino acids at positions 222(A), 242(I), 256(I), 294(I) and 299(S) (Figure 2), which have been identified in most (VV) IBDVs (Kasanga et al., 2007). The amino acids at positions 222 (A), 256 (I) and 294 (I) were reported to be unique to all known (VV) IBDV strains (Banda et al., 2004). In addition, these strains retained the amino acid substitution at position 300 (E→A) in the minor hydrophilic region of the VP2 variable domain, as was also observed in the African (VV) IBDVs from Tanzania (Owoade et al., 2004), Zambia (Kasanga et *a*l., 2013) and Nigeria (Adamu et al., 2013). All the Senegalese IBDV strains had the same amino acid sequences at positions 253 (Q), 279 (D) and 284 (A). Similarly, Mawgod et al. (2014) reported the same amino acid

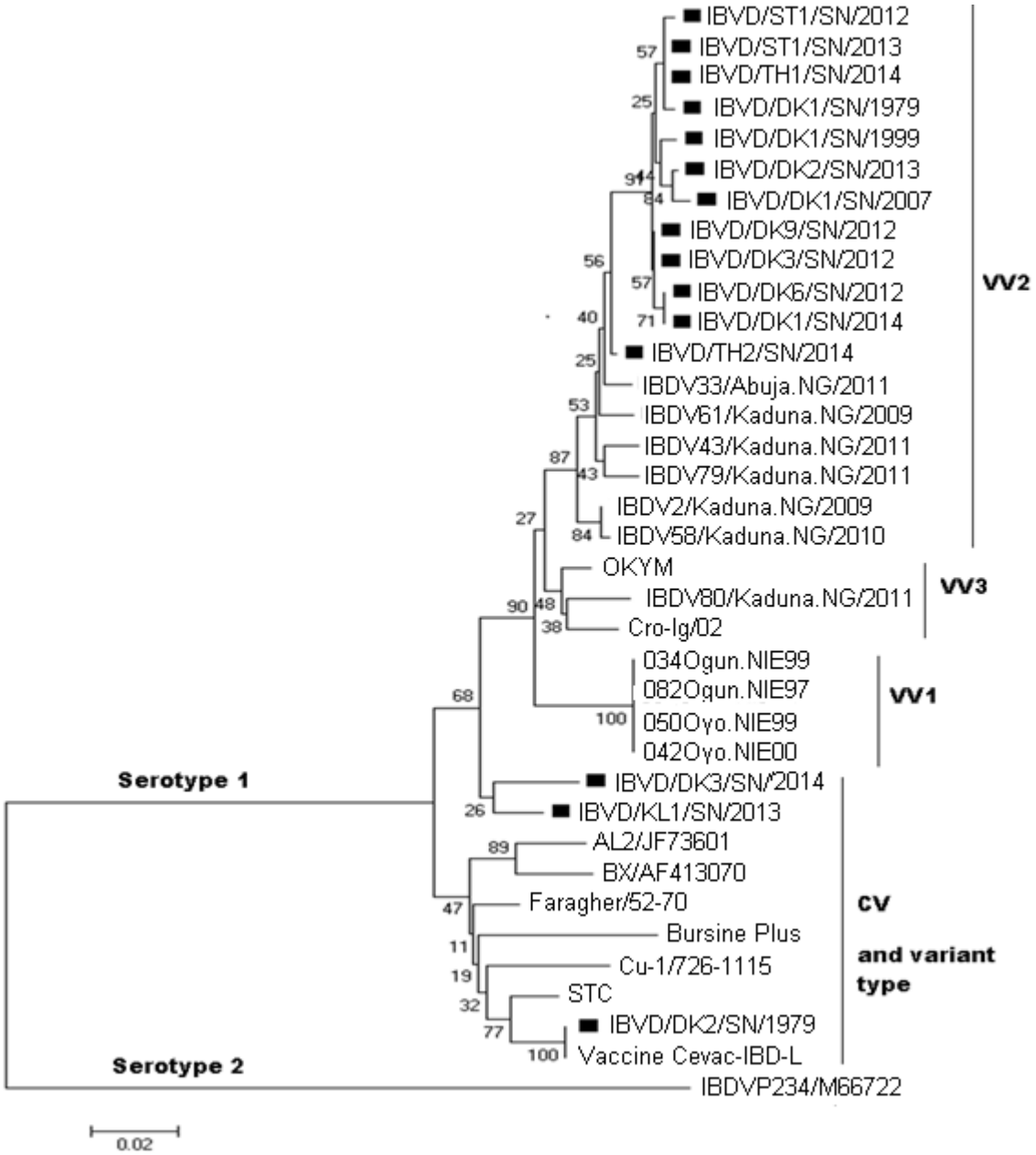

Figure 2. Phylogenetic tree calculated by the neighbor-joining (NJ) method based on the hypervariable domain of the VP2 capsid gene of IBDV serotype 1 strains using nucleotide alignment created in Clustal W, MEGA 5.10. The numbers at nodes indicate the bootstrap values (1000 replicates) in important junctions of the tree. VV, Very virulent genotype subdivided in clusters VV1, VV2 and VV3; and CV, Classic Virus; Isolates from Senegal are marked with ■ sign; Accession numbers: VV1 (050 Oyo.NIE/99, AJ586956; 042 Oyo.NIE/00, AJ586959; 034 Oyo.NIE/99, AJ586953; and 082 Ogun.NIE/97, AJ586934); VV2 (IBDV33/Abuja.NG/2011, JX424059; IBDV61/Kaduna.NG/2009, JX424069; IBDV43/Kaduna.NG/2011, JX424064; IBDV79/Kaduna.NG/2011, JX424078; IBDV2/Kaduna.NG/2009, JX424048; IBDV58/Kaduna.NG/2010, JX424067); VV3 (IBDV80/Kaduna.NG/2011, JX424079; Cro-Ig/02, EU184685; OKYM, D49706; CV (Faragher/52-70, Y14958; Cu-1/726-1115, D00867; STC, D00499; Bursine plus, AF498632; Vaccine Cevac-IBD-L, EU544157; Variant (AL2, JF73601; BX, AF413070); IBDVP234, M66722.

sequences at these positions of the VP2 in field strains in Egypt. The amino acids at positions 253 and 284 were found to be responsible for pathogenicity and are unique to highly virulent IBDVs (Brandt et al., 2001).

These observations confirm the circulating of (VV) IBDVs in Senegal with high mortality. Consequently, a

variety of freeze-dried live vaccines containing intermediate plus IBDV strains are widely used in Senegalese commercial chickens to get better protection against currently circulating (VV) IBDV. Thus, the pathogenicity and protective efficacy of these vaccine strains need to be evaluated for a better control of the IBD in Senegal.

In conclusion, we have studied the genetic evolution of the Senegalese field IBDVs isolated in 1979, 1999, 2007, 2012, 2013 and 2014. Thus, on the basis of nucleotide and deduced amino acid sequences, the study reveals that the majority of viruses circulating in Senegal is (VV) IBDV and indicated (VV) IBDV as a main cause of substantial economic losses in the Senegalese poultry industry. We found that all Senegalese (VV) IBDVs cluster phylogenetically with (VV) IBDVs from Nigeria in an African genetic lineage, independent of the Asian/European genetic lineage. The origins of the African genetic lineage remain unclear, and need to be investigated. The study indicates genetic stability over time within the VP2 variable domain since 1979.

Conflicts of interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

We acknowledge the veterinarians operating in the field for their help in providing clinical samples. The authors thank poultry farmers for their participation and Renaud Berger and Charles Euloge Lamien for his help analyzing and submitting the sequences.

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academicJournals

Vol. 8(12), pp. 244-247, December 2016
DOI: 10.5897/JVMAH2016.0505
Article Number: 1F8B65A61649
ISSN 2141-2529

http://www.academicjournals.org/JVMAH

Journal of Veterinary Medicine and Animal Health

Full Length Research Paper

Prevalence of poultry coccidiosis in and around Yabello, southern Ethiopia

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Received 12 July, 2016: Accepted 4 October, 2016

This study was conducted in and around Yabello, Southern Ethiopia, with the objective of determining the prevalence of poultry coccidiosis and its associated risk factors. Faecal samples were collected from total of 384 chickens. Floatation technique was used to detect coccidian oocyst. The result revealed that out of 384 chickens, 74 were positive to coccidiosis and overall prevalence was 19.3%. The frequency of detection of oocyst in the faecal samples from exotic and local breed chicken were 18 and 20.4%, respectively. The result of the current study disclosed that the prevalence of coccidiosis was almost similar in female (19.6%) and male (18.2 %) chickens. The prevalence rates of 18.4 and 21.9% were recorded in chicken grouped under the age category of 2-8 weeks (young) and greater than 8 weeks (adult), respectively. The prevalence rates of 21.6 and 18% were also recorded in good and poor body condition, respectively. Furthermore, coccidiosis occurrence in intensive and extensive management system were 22.1 and 16.7%, respectively. In conclusion, even though the difference for all risk factors was statistically insignificant (p>0.05), the study showed that coccidiosis is important disease of poultry in Yabello district, Southern Ethiopia and this is an indication for intervention to tackle the disease without any priority within the risk factors.

Key words: Coccidiosis, poultry, prevalence, Yabello district.

INTRODUCTION

Poultry coccidian are strictly host-specific and the different species parasitize specific parts of the intestine. The disease is characterized by droopiness, paleness of the comb, diarrhoea and occasional appearance of blood in droppings (Lillehoj and Trout, 1993). The oocysts exist in the litter, premises and are distributed by clothes, shoes, dust and others (Radiostitis et al., 2007; Singla and Gupta, 2012). Several factors influence the severity of infection like age and the number of ocysts eaten (Vegad, 2008). The most common and pathogenic species that affect the poultry is *Emeria tenella,* resulting in 100% morbidity and a high mortality due to extensive damage of digestive tract (Singla et al., 2007; Awais et al., 2012)

Poultry coccidiosis is responsible for tremendous worldwide economic losses to the poultry industry with an estimated world annual loss of more than 3 billion USD (Dkhil, 2013). In Ethiopia, some reports indicated coccidiosis loss from 8.4 and11.86% profit in large and small scale farms, respectively (Kinunghi et al., 2004).

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Losses due to mortality following a severe outbreak may be devastating and incidence rates as high as 80% were sometimes observed in the country (Alamargot, 1987).

Although, there are some previous works done on poultry coccidiosis in different regions of Ethiopia, there is no literature documentation in this study area. Therefore, this study was designed with the objectives of determining the prevalence of poultry coccidiosis and identifying the associated risk factors.

MATERIALS AND METHODS

Study area

The study was conducted in and around Yabello district, Southern Ethiopia (Figure 1) from November 2015 to April 2016. The area is located in Oromia regional state situated at 565 km south of Addis Ababa, geographically located at 50 23'49 N latitude 390 31'52 E longitudes with elevation 1857 of meters. The area have bimodal rainfall with 60% occurring in the long rainy season extending from mid-March to May and erratic short rain season from mid-September through mid-November. Other seasons such as the cool dry season extend from June to August and the major dry season from December to February (BZPADO, 2009/10). The farming system comprise of mainly pastoral and seldom Agro-pastoral areas. The region has predominantly a semiarid climate. The annual temperature varies between 21 and 38°C and the rainfall ranges from 350 to 900 mm, with considerable spatial and temporal variability in quantities and distribution. The area holds 1,496,652 cattle, 452,177 goats, 193,021 sheep, 467,119 camels, 355,700 chickens, 13,945 mules and 61,699 donkeys. Both local and exotic poultry breeds are available in the study area (CARE-Ethiopia, 2007).

Study population

The study was conducted on poultry found in and around Yabello district by dividing them into sex, breeds and ages as young (2 to 8 weeks) and adult above 8 weeks of age (Oljira et al., 2012). This range of age was selected because the disease is more common in young poultry (Pangasa et al., 2007; Taylor et al., 2007). Systematic random sampling was used to select the study population from the study area. Epidemiological information regarding ages, sex, breed, body condition and housing type, date of sample collection and origin or name of the farm were collected.

Study design and sample size determination

Random sampling method was used to select the chickens from the target population. The minimum sample size required for this study was determined by using the following sample determination formula described by Thrusfield (2005):

$n= 1.96^2 Pexp (1-Pexp)/d^2$

Where: n = sample size; Pexp = minimum expected prevalence = 50%; 1.96 = the value of z at 95% confidence interval; d = desired accuracy level at 95% interval.

By rule of thumb where there is no documented information about the prevalence of poultry coccidiosis in the study area, it is possible to take 50% prevalence as minimum expected prevalence. In this study, the sample size was calculated using 50% prevalence with 5% desired level of precision and 95% of confidence interval. By substituting these values in the formula given above, the size was found to be 384.

Data collection

A cross-sectional study was conducted from November 2015 to April 2016 to estimate the prevalence and risk factors of poultry coccidiosis in and around Yabello. Data was gathered from randomly selected chickens. Age, sex, breed, body condition, and housing type were considered as risk factors to test for occurrence of coccidiosis.

A total of 384 fecal samples were collected during the entire period of the study directly from the rectum of selected animals using spatula and from freshly dropped feces excluding soil contamination after wearing disposable plastic gloves and placed into air tight sample vials and transported to Yabello Regional Laboratory on the same day of collection and preserved at refrigeration temperature until processing, that is, within 48 h. During sampling date of sample collection, age, sex, breed, body condition and management system were recorded for each sampled animal. Fecal sample was qualitatively examined by centrifugation floatation technique (Ryley et al., 1976).

Data management and analysis

Data collected from the study sites were coded and entered in to a Microsoft excel spread sheet program for analysis. Statistical analysis was done on Statistical package for Social sciences (SPSS) software version 16 (Polar engineering and consulting, http://www.winwrap.com/). Descriptive statistics like percentage was used to express prevalence while chi-square (X^2) test was used to compare the association between variables and a statistically significant association between variables was considered at p- value less than 0.05.

RESULTS

Out of 384 fecal samples examined, 74 were found to be positive for *Eimeria* oocysts with the overall prevalence of 19.3 %. The highest prevalence rate (22.1%) was observed in chicken reared in intensive management system and the lowest prevalence rate (16.7%) was observed in extensive management system. The prevalence rates of 18.4 and 21.9% were recorded in chicken grouped under the age category of 2-8 weeks (young) and greater than 8 weeks (adult), respectively. The prevalence rates of 18 and 21.6% were also recorded in good and poor body condition, respectively. As shown in Table 1, the prevalence of coccidiosis was relatively different in different risk factors. However, difference between all risk factors was not statistically significant (p>0.05).

DISCUSSION

Coccidiosis is the most prevalent and important disease of poultry and its prevalence and economic significance has been reviewed by different workers in different

Figure 1. The location of Yabello, Southern Ethiopia. Adapted from http://fieldguids.com/news/fresh-2011-inieraries-ethiopia-phillipines-more/.

Table 1. Prevalence of coccidiosis by age, sex, body condition, breed and management type

Risk factors	Category	No. examined	Positive	Prevalence (%)	Chi-square	P-value
Sex	Male	99	18	18.2	0.102	0.750
	Female	285	56	19.6		
Age	Adult	96	21	21.9	0.558	0.455
	Young	288	53	18.4		
Breed	Local	206	42	20.4	0.357	0.550
	Exotic	178	32	18.4		
Management system	Extensive	203	34	16.7	1.761	0.185
	Intensive	181	40	22.1		
Body condition	Good	250	45	18	0.744	0.388
	Poor	134	29	21.6		

production system (Luu et al., 2013). The result of the present study illustrate that poultry coccidiosis is endemic in and around Yabello, Southern Ethiopia, with an overall prevalence of 19.3% (74/384). The present result support the previous finding in central Ethiopia (Ashenafi et al., 2004) and Addis Ababa (Alemayehua et al., 2012) with prevalence rate of 25.8 and 23.1%, respectively. Moreover, this result is in agreement with the finding in Ambo by Oljira et al. (2012) who reported a prevalence of 20.57%. However, this prevalence of coccidiosis result is much lesser than the findings of Dinka and Tolossa (2012) in DebreZeit, Ethiopia (71.1%) and Alemargot in Addis Ababa (80%). This variation in prevalence of the disease may be due to epidemiology of coccidian infection and differences in management systems of the farms.

The result of current study revealed that the prevalence of coccidiosis was almost alike in both female (19.6%) and male (18.2%) chicken. This may be due to equal chance of exposure for parasitic infection. This result is in agreement with report of previous studies done by Oljira et al. (2012) who reported a higher prevalence of poultry coccidiosis in female chickens than male chickens. This variation in prevalence of the disease may be due to climatic condition of coccidian infection and differences in management systems of the farms. However, the present result disagrees with the finding of Alemayehu et al. (2012) in Addis Ababa and Gebretensae et al. (2014) in Gondar who reported that a higher prevalence of poultry coccidiosis in male chickens than female chickens.

This study also indicated that the prevalence of coccidiosis was relatively higher in adult (greater than 8 weeks) 21.9% than young (2 to 8 weeks) 18.4% chickens. It was observed that there was no statistically

significant difference (p>0.05) in the prevalence of coccidiosis among the two different age groups examined. However, slight variation was observed between the age categories.

The prevalence of coccidiosis was relatively higher in local (20.4%) than exotic 18.4%. This result agrees with the finding of Gebretensae et al. (2014) in Gondar who stated that the prevalence rate of coccidiosis was relatively higher in local than exotic breeds, as locals are allowed to scavenge in villages without any restriction and thus more likely get contact with the sporulated oocysts in feaces.

The effect of body condition on the prevalence of the disease was assessed and relatively high prevalence was recorded in those chickens which have poor body (21.6%) than those chickens which have good body condition (18%). Chickens which are managed in intensive housing system were more affected (22.1%) than extensive (16.7%) housing systems. This result in line with report of previous studies carried out by Taylor et al. (2007) who reported that coccidiosis was the most common problem to chickens kept under intensive management system especially those on deep litter management.

In conclusion, the present study showed that coccidiosis is prevalent in the study area. Even if the association between different risk factors are not statistically significant, still the disease has great socio-economic impact. Therefore, it is recommended that biosecurity practices should be a primary concept in the prevention and control of coccidiosis. Awareness should be a created among the local chicken farmers through training on general knowledge of coccidiosis occurrence, medication procedures and prevention and control methods should be undertaken for sustainable control. Moreover, stress conditions such as overcrowding should be minimized by reducing the number of chicken in intensive housing which triggers the disease occurrence.

Conflict of interest

The authors have not declared any conflict of interest

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academicJournals

Vol. 8(12), pp. 248-254, December 2016
DOI: 10.5897/JVMAH2016.0530
Article Number: AE3605461709
ISSN 2141-2529

http://www.academicjournals.org/JVMAH

Journal of Veterinary Medicine and Animal Health

Full Length Research Paper

The epidemiology of major ectoparasites of sheep and the effectiveness of the control campaign employed in Tiyo and Diksis Districts, Oromia Region, Ethiopia

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Received 24 October, 2016: Accepted 17 November, 2016

A cross-sectional study was conducted from November 2013 to July 2014 with the objectives to determine the prevalence of ectoparasite of sheep in the Tiyo and Diksis districts, determine the effectiveness of control program against sheep ectoparasites in the study area and major risk factors associated with effectiveness of control program. A total of 646 sheep (323 from each districts) were examined for the presence of ectoparasites. From the total sheep examined, 371 (57.43%) were infested with one or more ectoparasites. The ectoparasites identified were lice 49.23%, sheep keds 7.4%, tick 9.75% and mixed infestation 8.98%. Favorable climatic conditions, poor husbandry and animal management, lack of awareness by the farmers, and weak animal health extension services are believed to have contributed for widespread distribution and occurrences of ectoparasites. Even if control campaign is practiced in the study areas, higher prevalence of sheep ectoparasite was recorded. The growing threat of ectoparasites to small ruminant production and the tanning industry needs well-coordinated, appropriate and urgent control intervention.

Key words: Control program, ectoparasites, prevalence, sheep, Tiyo/Diksis.

INTRODUCTION

Ethiopia is currently considered among the largest livestock producer and biggest exporter of livestock in Africa (CSA, 2012). Small ruminants represent the most important part of the Ethiopian livestock system; about 24.2 million sheep are estimated to be found in the country (CSA 2012). In Ethiopia, sheep is reared in all agro climatic zones. The highland area comprises 70% of the sheep, while the lowland pastoral and agro pastoral area have 30% of the sheep population (Degume, 2002). Agriculture is the mainstay of the Ethiopian economy. It employs over 80% of the adult population and account for 45% of the GDP and 85% of the export earnings

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Livestock production performs several functions primarily as source of household incomes, food and animal drought power for livestock producers (UNECA, 2012). However, the current levels of contributions of sheep in Ethiopia, either the macro or micro level is below the expected potential. Among major constraints hindering the productivity of sheep in the country are diseases, among which sheep skin diseases caused by ectoparasites accounts a wide range of health problems that confront the productivity. Ectoparasites are very common and widely distributed in all agro-ecological zones in Ethiopia (Berhanu et al., 2007; Kumsa et al., 2012; Yacob 2014).

Parasitic diseases caused by helminths and arthropods are among the major diseases of sheep causing serious economic loss to small holder farmer, the tanning industry throughout the globe (Singla, 1995; Mulugeta et al., 2010; Kumsa et al., 2012). Skin diseases cause mortality, decreased production and reproduction; in addition to these, currently skin diseases affecting the tanning industry very seriously causing enormous down grading and rejection of skins and hides (Bayou, 1998; ESGPIP, 2010; Yacob, 2013). It is reported that 35% of sheep skin rejections in Ethiopia are attributed to ectoparasites (Bayou, 1998; Kassa, 2005). All these established facts imply that ectoparasites pose serious economic losses to the farmer, the tanning industry and the country as a whole (Berhanu et al. 2007; Chanie, et al. 2010). In many part of the Ethiopia, skin diseases due to ectoparasite have prevented many farmers from keeping sheep and becoming serious threat to sheep production (Demissie et al., 2000; Asnake et al., 2013; Yacob, 2014).

The control program against ectoparasites and skin diseases have been designed by the Ministry of Agriculture and Rural Development of Ethiopia (MoARD) in 2005 and launched in Tigray, Amhara and Afar regions. In Oromia regional state, this activity started in 2010 and still ongoing. Even though national and regional efforts and emphasis given to the control programs against ectoparasites; as some reports from north-west Amhara region indicate, the problem seems to be still alarming (Sisay et al., 2013; Yacob, 2014).

Despite the large population of sheep in the region and national and regional efforts and emphasis given to the control programs against ectoparasites, ectoparasites are also among serious problems in Arsi zone of Oromiya region (Hailu, 2010). Even control program against ectoparasites in the study areas started, the effectiveness of control campaign on the status of ectoparasite infestation on sheep was not yet studied. Therefore, the present study was conducted with the objective to determine the prevalence of ectoparasites and to define the effectiveness of control program by studying major risk factors associated with the effectiveness of control program against sheep ectoparasites.

MATERIALS AND METHODS

Study area and population

A cross-sectional study was employed from November 2013 to July 2014 to address the objectives of the study. The study area is found in the central part of the Oromiya Regional State, astronomically lies between 60 45' N to 80 58'N and 380 32' E to 400 50' E. The mean annual temperature of the study area is found between 20 and 25°C in the low land and 10 and 15°C in the central high land with the mean annual rainfall varying from 633.7 to 1059.3 mm. The production system implemented in the study area is mixed crop livestock. According to CSA (2012), the study areas contain a total population of cattle 2,295,138, sheep 1,207,182, goats 653,327, Equines 593,272, poultry 1,449,583 and 94,456 beehives.

Sampling method and sample size determination

The study involves districts, peasant associations (PAs) and sheep as a sampling unit. The study districts were selected purposively based on their history of representation of ectoparasite controlled area; five PAs from each district were also selected randomly with corresponding control status. Sheep found in selected PAs were included in the study randomly from animals grazing in communal pastures.

The required sample size was determined as described by Thrusfield (2007). The expected prevalence of ectoparasite was taken from previous work of Hailu in the area before initiation of control program was 70% and by setting 95% confidence level and 5% desired level of precision. Hence, 646 sheep were sampled from the study districts, 323 sheep were included in the study from each district.

Clinical examinations

A total of 646 sheep randomly selected from the study districts were clinically examined for presences of ectoparasites and/or lesions. Before clinical examination, the sex, age, body condition and management of the selected sheep was recorded. Body condition score (poor and good) and age categorization of the animal was made by modifying the system described by Gatenby (1991). Sheep up to 1 year of age were categorized as young and older than 1 year of age as adult.

The clinical examination was performed by multiple fleeces parting in the direction opposite that in which hair or wool normally rests and visual inspection and palpation of the skin for parasites and/or lesion on all parts of the animals including the ears and the digits. Those sheep found infested by ectoparasites were considered as positive.

Sample collection

Sheep keds, ticks and lice were collected manually from their sites of attachment. The ticks were removed from the host skins whilst retaining their mouth parts for identification using forceps. Coat brushing techniques were used for collection of lice (Soulsby, 1982; Walker et al., 2003). They were placed in labeled bottles containing 70% ethanol. Samples were processed as per the standard

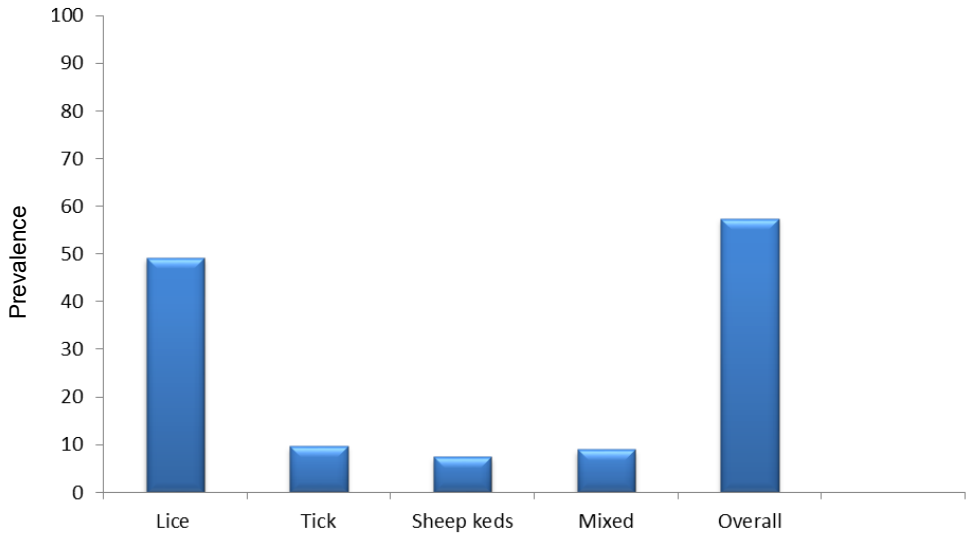

Figure 1. Prevalence of lice, tick, sheep keds in the study area.

Table 1. Frequencies and percentages of ectoparasites identified.

Ectoparasite group	Frequency	Prevalence (%)
Lice	318	49.23
ticks	63	9.75
Sheep ked	48	7.4
Mixed infection	58	8.98
Overall	371	57.43

procedure (Gupta and Singla, 2012) and identified under a stereoscopic microscope (Urquhart et al., 1996; Wall and Sheare, 2001).

Data analysis

Raw data was carefully recorded and stored in Microsoft Excel database system used for data management. Statistical software package SPSS version 20.0 was used for data analysis. Descriptive statistics and percentages were used to summarize the proportion of infested and non-infested animals. The effects of different environmental, managemental and host risk factors were analyzed by regression and $\chi 2$ test. Statistical significance was set at $p \leq 0.05$.

RESULTS

Overall prevalence

Out of 646 sheep examined from the study districts 371 (57.43%) were infested with one or more ectoparasites. The major ectoparasites identified on sheep were lice (49.23%), ticks (9.75%), sheep keds (7.4%) and (8.98%) mixed infestation (Table 1 and Figure 1).

Sex wise prevalence

The overall prevalence of ectoparasite in female and male was 63.11 and 44.4%, respectively (Table 2). Statistically significant variation in the overall prevalence of ectoparasites was recorded between male and female sheep of the study districts (OR= 0.455, p=0.000). Likewise significant difference on the prevalence of lice was also recorded between ram and ewe of the study area (OR= 0.567, p=0.000)

Age wise prevalence

The overall prevalence of ectoparasite in young and adult sheep was 56.9 and 57.8% (Table 3). Difference was

Table 2. Prevalence of ectoparasites in sheep by sex.

Ectoparasite type	Sex		P value
	Ram (n=196)	Ewe (n=450)	
Lice	38.78 (76)	53.8 (242)	0.000
Sheep ked	9.7 (19)	6.4 (29)	0.421
Ticks infestation	8.2 (16)	10.4 (47)	0.127
Overall	44.4 (87)	63.11 (284)	0.000

Table 3. Prevalence of ectoparasite in sheep by age.

Ectoparasite	Age	
	Young (n=267)	Adult (n=379)
Lice	49.1 (131)	49.3 (187)
Ticks	10.1 (27)	9.5 (36)
Sheep keds	7.5 (20)	7.4 (28)
Overall	56.9 (152)	57.8 (219)

Table 4. Prevalence of ectoparasite in sheep by body condition.

Ectoparasite	Body condition	
	Poor (n=363)	Good (n=283)
Lice	48.5 (176)	50.2 (142)
Sheep ked	7.7 (28)	7.1 (20)
Ticks	9.1 (33)	10.6 (30)
Overall	56.7 (206)	58.3 (165)

seen to be statistically insignificant (p>0.05) in the overall prevalence of ectoparasite infestations between young and adult sheep of the study area.

Body condition based prevalence

The overall prevalence of ectoparasites in good and poor body condition sheep were 58.3 and 56.7%, respectively (Table 4). Prevalence of ectoparasite infestations (p>0.05) between sheep with poor and good body condition did not show statistically significant difference.

Management based prevalence

The overall prevalence of ectoparasites in sheep reared separately and sheep reared with other animals were 43.5 and 79.3%, respectively (Table 5). Statistically significant variation in the overall prevalence of ectoparasites was recorded between sheep reared separately and sheep reared together with other species of animals in the study districts (OR= 0.015, p=0.000); higher in the sheep reared together with other species of animals than reared separately. Similarly significant difference on the prevalence of lice and tick were also recorded between sheep reared separately and those reared with other animal species, respectively (OR= 0.493, p=0.002) (OR=0.732, p=0.000). The prevalence of lice and tick infestation was significantly higher in sheep reared together with other animal species than sheep reared separately.

DISCUSSION

The results of the present study revealed an overall prevalence of 57.43% of sheep ectoparasites in the study area, which are in agreement with the previous reports from different parts of the country (Tefera, 2004; Yacob et

Table 5. Prevalence of ectoparasites in sheep reared separately and reared with other species of animals.

Ectoparasite	Management		P value
	Sheep reared separately (n=395)	Sheep reared with other animals (n=251)	
Lice	37.2 (147)	68.12 (171)	0.000
Sheep ked	45.8 (n=22)	54.2 (n=26)	0.346
Tick	33.33 (n=21)	66.67 (n=42)	0.002
Overall	172 (n=43.5)	199 (n=79.3)	0.000

al., 2008; Hailu, 2010; Mulugeta et al., 2010; Rahmeto et al., 2011; Asmare et al., 2012; Dawit et al., 2012; Shibeshi et al., 2013; Tewodros et al., 2012: Taddese et al., 2013). The higher prevalence rate is attributable to several important factors including management problems, conducive environment, malnutrition and poor husbandry systems, poor awareness of farmers and inadequate veterinary services in the study districts (Pegram et al., 1981; Mekonnen et al., 2001; Mekonnen et al., 2007).

Even though control campaign was implemented in the study area, the prevalence of ectoparasite was as high as the areas where control campaign was not under implementation. Among the factors which might be responsible for high prevalence of ectoparasite in the study area were the type of acaricides in use and method of acaricides application (spray).

Development of acaricide resistance in ectoparasites is reported worldwide, wherever acaricides are in use. In order for a chemical to be effective as an ectoparasiticides, it needs to be delivered to the site of infestation or potential infestation in sufficient quantities to be effective for the maximum period of time. The spraying method of ectoparasite control is not as efficient as dipping (Drummond, 1983). Spraying method may not expose ectoparasites found in the inner parts of the ear, under part of the tail, the tail brush and the areas between the teat and the interdigital space to the acaricides and also liquid acaricide might not reach the base of the skin in wooly sheep, hence, ectoparasite may escape treatment and stay alive to induce re-infestation.

Some species of ectoparasite such as tick spend more time off the host and can exist for a very long period of time without feeding. For ectoparasites that are free living in one or more life cycle stage or are present on the host for only short period such as ticks, fleas and flies, acaricides may be directed at the free living stages in the environment (Wall and Shearer, 2001). Also contaminated bedding and farm equipment's with lice and fleas can perpetuate the re-infestation of the flocks (ESGPIP, 2010). However, in the study areas, the control campaign only focus on the application of the acaricides (diazinon) on the sheep only, but no more application of environmental control of the ectoparasites on house and bedding or farm equipment; hence the free living stages and those found in the bedding may be responsible for the occurrence of high prevalence of sheep ectoparasite of the study areas.

Government control measures, such as depopulation of affected animals, quarantines and movement restrictions, may reduce disease transmission and losses due to disease (Daniel et al., 2006). In Ethiopia, there is no policy which restricts free movement of animals from one place to the other. Hence, infested sheep may introduce into the study areas from other areas (neighboring districts or zones) during marketing of sheep, searching of food and water.

Quarantine is the best method of ectoparasites control. It is used to isolate animals when they are introduced from other area to new area; treatment and follow up should be carried out if the animal is infested (Thrusfeild, 2007). However, knowledge of the farmers on quarantine and treating of newly introduced animal is negligible.

As a fact that ectoparasite are most often introduced to herds by bringing in infested animals (Kufman et al., 2012). Therefore, ectoparasite might be introduced to the controlled area. Actions the USDA takes to minimize the risk of disease introduction are driven by the awareness that animal health is a public good (Daniel et al., 2006). The awareness of farmers of the study areas regarding to transmission of ectoparasite, host range and management of animal is minor.

Therefore, the farmer's rear and house different age group and different species of animals together, hence, such condition may favor the transmission of ectoparasites which are characterized by infesting different species of animals.

Among the ectoparasites known to infesting wide range of domestic animals ticks and lice are the principal one; so in the study area, control campaign is implemented only on sheep but the rest species of animal remains untreated. Hence, untreated animals can also serve as a source for the re-infestation of sheep following spraying acaricides by transmitting ectoparasite through direct contact with sheep or by contaminating the environments (pasture, house and bedding or farm equipment).

CONCLUSION AND RECOMMENDATIONS

This study was conducted to identify the major ecto-parasites prevalence, effectiveness of control campaign and associated risk factors of effectiveness of control strategy on sheep of the study districts. Even though control campaign is implemented, ectoparasites infestation is still among the major causes of sheep production constraints and quality deteriorations of exported skin in the study areas. Among the major possible reason of finding high prevalence of ectoparasite infestation, following acaricide spraying are proper formulation and application of acaricides, awareness of the farmer on the transmission of ectoparasite and quarantine of newly introduced animal, unavailability of policy which restricts free animals movement from one place to the other. Control of ectoparasites requires integrated ectoparasite management systems that combine sanitation, application of ectoparasiticides, reduction of breeding sites, environmental sprays, weed and vegetation controls and other biological control. Based on findings, one can clearly conclude that even though control campaignwere implemented, several species of ticks, lice, and sheep keds represent common health and productivity problems of sheep. Therefore, control programs should be designed and implemented with the participation of all stakeholders (farmers, tanners, and government and policy makers) and there should be strong coordination between neighboring regions and/or districts with strict follow up and control, strategic and appropriate application of acaricides (dipping than spraying) during control campaign for effective ectoparasite control is required, effective extension system and programs that could raise public awareness on management of animals, effect of ecto-parasites and control methods should be implemented.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Addis Ababa University, College of Veterinary Medicine, Hide and Skin Thematic Research Committee for funding this research project. They are most deeply grateful to the officials and experts of Assela Regional Veterinary Laboratory for their unforgettable hospitability and supports they gave during our stay in the laboratory session. Our special gratitude shall also goes to official administrators, district and PA leaders and farmers of the study areas for allowing us to perform this research and assisting us to collect the samples and related information.

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Journal of Veterinary Medicine and Animal Health

Volume 8 Number 12 December, 2016
ISSN 2141-2529

Academic Journals