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Full Length Research Paper

Performance, immunology and biochemical parameters of *Moringa oleifera* and/or *Cichorium intybus* addition to broiler chicken ration

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Received 21 June, 2017; Accepted 17 August, 2017

This study was aimed to evaluate the influence of *Moringa Oleifera* and/or *Cichorium Intybus* powder supplementation on performance, biochemical parameters, immunology and carcass quality of broiler chicks. Two hundred one-day-old chicks (Ross, 308 hybrid) were randomly allotted into four groups. Each group contained 50 chicks with five replicates. Feed was offered *ad libitum* to all groups. Group C were fed basal control diet. Chicks in the group M were fed basal diet supplemented with 1.5% *M. oleifera* and chicks in group CI were fed basal diet supplemented with 1.5% *C. Intybus*, while the chicks of group MC were fed basal diet supplemented with 0.75% *M. oleifera* plus 0.75% *C. Intybus* during experiment time. Body weight and feed amount were recorded every 15 days. Carcass yields were evaluated at the end of the experiment. The results revealed that supplements improved significantly bird weights, whereas the group C has the least mean value among the treatments. Group MC had better weight (239 ± 80 g) than other groups (2180 ± 48, 2020.5 ± 97 and 1893 ± 54 g, respectively for groups CI, M and C). Feed conversion ratio (FCR) was estimated 1.45, 1.48, 1.54 and 1.58 for MC, CI, M and C groups, respectively. Supplements group have lower total cholesterol than control. Finally, the use of a combination of *C. Intybus* and *M. oleifera* was recommended as good feed additives to improve productivity and enhance immunity.

Key words: Broiler, performance, *Moringa Oleifera*, *Cichorium Intybus*, biochemical parameters, immunology, carcass yields.

INTRODUCTION

Poultry health is affected by the surrounding environment. Infectious pathogens such as bacteria, viruses, parasites and fungi can easily infect poultry when its immune system is suppressed, which lead to different complicated infections (Paliwal et al., 2011a; Sandhu et al., 2009). In European Union, from January 2006, antibiotics use is prohibited to avoid antimicrobial- resistance in bacterial strains and antibiotics residues in human food (Catala-
Gregori et al., 2008); nowadays the herbal substitutes to enhance health status and performance is urgently needed (Panagasa et al., 2007; Singla and Gupta 2012). The immunity is challenged by environment and feed habits, with the concept that feed with natural antioxidants and micronutrients can boost the immune response (Paliwal et al., 2011a, b).

Herbs or extracted oils are safe to be fed to livestock with less risk than antibiotics which has harmful side effects and consider the most effective choice (Barrow, 1992), that is why, many types of plants are widely used in alternative medicine (Endo et al., 1999). The benefits of herbs raised the hope of using them instead of antibiotics (Panagasa et al., 2007). Herbs were recommended to enhance metabolic processes and the health condition of livestock (Panagasa et al., 2012). Some herbs can support the digestive enzymes action, improve feed intake, feed conversion ratio (FCR), carcass yields (Pietrzak et al., 2005), whereas, Halle et al. (2004) recorded no positive impact on broilers.

*M. oleifera* has beneficial anti-inflammatory and antioxidants properties (Yang et al., 2006). Dahot (1988) reported that *Moringa* contains vitamins (A, E, B2, B5, B6, folic acid) and minerals (Ca, Fe). *Moringa* has strong fungicidal and antimicrobial activity (Das et al., 1957). It also has an anti-blood cholesterol effect (Ghasi et al., 2000). Yang et al. (2006) mentioned that *M. oleifera* significantly enhanced immunity and decreased *Escherichia coli* and improved *Lactobacillus* counts in gastrointestinal tract (GIT) of broilers. So *Moringa* improves FCR and enhances immune response of birds. Also, its leaves has natural antioxidant compounds and soluble proteins (Sreelatha and Padma, 2009; Kakengi et al., 2007).

*Cichorium intybus* (chicory) is considered a good source of fiber that can be utilized by simple stomach livestock; also it is palatable for ruminants (Li and Kemp, 2005). Both inulin and oligofructose are the main constituents of chicory. It was documented that oligofructose improved broilers carcass and breast weights, and decrease abdominal fat percent (Ammermal et al., 1989); these findings is supported by those of Yusrizal and Chen (2003) results in which birds' abdominal fat content decreased. Chicory has essential mineral (Foster, 1988) and uronic acids (15%, DM), considered the main constituent of pectin (Voragen et al., 2001). High growth and improved digestibility of non-starch polysaccharide were observed in swine, besides little adverse influence on organic matter and digestibility with high level (16%) of *C. intybus* (Ivarsson et al., 2011). Prebiotic action of inulin was reported by Castellini et al. (2007), in livestock; also Gibson et al. (2004), observed the selective stimulation of lactobacilli and bifidobacteria, in the large intestine in rodents.

Therefore, the purpose of the study was to declare the benefits of *M. oleifera* and/or *C. intybus* as feed additive on broiler chickens in terms of feed intake (FI), growth performance, immunology, biochemical parameters, and carcass yields.

**MATERIALS AND METHODS**

**Experimental chicks, housing and management**

This study was carried out in accordance with the regulations of the Department of Nutrition and Clinical Nutrition, Faculty of Veterinary Medicine, Sohag University, Egypt. Two-hundred (Ross 308 hybrid) chicks (n = 200) raised with traditional litter system with chopped straw were used as bedding material. Room humidity and temperature were controlled and 24 h of lightening was observed throughout the experimental period. Chicks were fed *ad libitum*, and health status was observed daily. All birds were vaccinated for infectious bronchitis at 7 days old followed by Newcastle (Zoetis, Fort Dodge) and infectious bursal disease (Zoetis, Fort Dodge) at 20 days old.

**Experimental design and feeding**

Broilers chicks were divided randomly into four groups (50 chicks per each). Each group contained 50 chicks with 5 replicates of 10 birds per pen. The trial lasted 42 days. Chicks in control group (C) were fed *ad libitum* on the basal control diets (starter for first three weeks of age, and grower-finisher for next three weeks); birds of group M received basal diet enriched with *M. oleifera* (1.5%); chicks of group CI were fed basal diet enriched with *C. intybus* (1.5%); birds of group MC were fed basal diet enriched with *M. oleifera* and *C. intybus* (0.75%:0.75%) during experimental period (Table 1). A standard basal diet was formulated to meet the nutrient requirements of broiler (NRC, 1994) as shown in Table 2.

**Tested parameters**

**Performance measurements**

Chicks were weighed at the start of the experiment and records were taken every 15 days until the end of the experiment. The feed intake was daily recorded for each of the different experiment groups. The average amount of feed intake of each bird was estimated by dividing the consumed amount by the respective number of birds in each group. Mortality was recorded.

Body weight gain (BWG), feed conversion ratio (FCR) and production efficiency factor (PEF) also known as European Production Efficiency Factor (EPEF) were estimated thus: BWG = average final live body weight (LBW) - average initial LBW at a certain period; FCR = total feed intake / total BW gain, and EPEF = ((LBW × FCR)/age in days) × (EPEF - Mousa et al., 2016).

**Carcass yields**

At the end of the trial and before slaughter, chicks were given a feed withdrawal period of 12 h. From each group, ten birds were randomly chosen, weighed and slaughtered. Feathers were removed, carcass was eviscerated and carcass yield was calculated. Selected chicks were deboned and breast, thigh, and abdominal fat were weighed.

**Blood biochemical parameters**

Vein blood samples from ten chicks of each group were collected

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null
```
from the wing. Samples were left to stand for 1 h and centrifuged at 4000 rpm for 15 min. The clear serum was kept in sterilized tubes and stored at -20°C for biochemical analysis. Levels of total proteins, albumin, triglycerides and cholesterol were measured in these samples according to the manufacturer’s instruction (Chema Diagnostica, Italy).

Measurement of antibodies (Abs) titers against newcastle disease (ND) and infectious bursal disease (IBD) vaccines

In the collected sera, Abs titer against ND vaccine was measured by haemagglutination inhibition (HI) test according to OIE (2012) and Abs titer against IBD vaccine was measured by enzyme-linked immunosorbent assay (ELISA) test via IBD ELISA kits (Symbiotics Laboratories, USA) according to the manufacturer’s instruction.

Statistical analysis

This data obtained with the standard procedures of analysis of variance (ANOVA), using SPSS Statistics 17.0 (Released 23 August 2008). Differences among means were separated using Duncan’s multiple range test (Duncan, 1955). Significant difference was identified at a level of P < 0.05.

RESULTS AND DISCUSSION

Production performance

The growth data variables are shown in Table 3. The combination of M. oleifera (0.75%) and C. intybus (0.75%) improved significantly (P≤0.05) body weight gain along the experimental period, the best body weight gain was obtained by MC group (257±25 g/bird) in comparison with CI group (246±27 g/bird), C group (235±53 g/bird) and M group (216±16 g/bird). When the experiment was ended (after 42 days), the best cumulative LBW and FCR were recorded in the birds in MC group (2393±30 g/bird and 1.45, respectively) followed by broilers in CI group (2180±48 g/bird and 1.48, respectively) and the birds of C group (2020±97 g/bird and 1.54, respectively), but the M group had the lowest values (1893±54 g/bird and 1.58, respectively). Feed intake had the same order of LBW (MC>C>CI>M groups) of 3458, 3216, 3113 and 2996 g/bird, respectively.

European performance efficiency factor index

The EPEF indexes were significantly different between all the treatments of study, whereas, MC group had better value (385), followed by CI group (344) and C group (293) then M group (279.5). When the EPEF index value is higher, the productive performance is better (Table 3). Health was observed and mortality was recorded throughout the experimental period (Table 3).

The result obtain for chicory as feed additive are in line with that of Yusrizal and Chen (2003) who found that the addition of it significantly improve feed intake, body weight and FCR. These results are supported by those of Ammerman et al. (1989) result, whereas, Waldroup et al. (1995) found no effect. Also, Castellini et al. (2007) found that, green Cichorium feeding decreased amount of feed and rabbit weight gain during suckling period. Result shows improvement along the experiment period in contrast with the report of Aghazadeh et al. (2011) who reported no effects during finishing period and explained this with the fact that fructans effects are age dependent, so stimulate microbial population and as a result enhancing performance during starter period.

Indeed, chicory (inulin) feeding to poultry have a good impact on both health status and production (Roberfroid et al., 2010); also absorption was improved via positive changes of the GIT mucosal membrane (Rehman et al., 2007); besides beneficial microflora growth was enhanced while, pathogenic bacteria growth decreased (Sevane et al., 2014). Also, the fat deposition decreased and fat profile improved (Velasco et al., 2010). 

Yusrizal and Chen (2003) observed that chicory addition increase length of broilers GIT. When GIT is longer, the digestion and metabolism will be better, and so improve performance. Yeung et al. (2005) suggested that inclusion of chicory improve GIT absorption of Ca, Mg and Fe. Izadi et al. (2013) reported improved productive performance via increase surface of absorption through increase villi length, villi length/crypt depth, and villi number; also the performance improvement of broiler allotted on chicory could be related to insoluble non-starch polysaccharides content, which improve rate of digesta removal, and so enhance feed intake (Kalmendal et al., 2011). Chicory has galacturonic acids, which is the main constituent of pectin, the source of uronic acids that has high digestible values (Voragen et al., 2001).

Cichorium has fructo-oligosaccharides and inulin, which could manipulate intestinal microflora and enhance mucosal integrity (Flickinger et al., 2003); it also contains sucrose, cellulose, protein, esculin, coumarins, flavonoids,

### Table 1. Herbal plants plan.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group (C)</td>
<td>Allotted to basal diets without any supplementation</td>
</tr>
<tr>
<td>Moringa oleifera group (M)</td>
<td>Allotted to basal diets supplemented with 1.5% M. oleifera powder</td>
</tr>
<tr>
<td>Cichorium intybus group (CI)</td>
<td>Allotted to basal diets supplemented with 1.5% C. intybus powder</td>
</tr>
<tr>
<td>M. oleifera plus C. intybus group (MC)</td>
<td>Allotted to basal diets supplemented with 0.75% M. oleifera plus 0.75% C. intybus powder</td>
</tr>
</tbody>
</table>
Table 2. Ingredients (kg) and chemical composition of basal and experimental diets for broiler chicks.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Basal diets</th>
<th>Diets supplemented with Moringa</th>
<th>Diets supplemented with chicory</th>
<th>Diets supplemented with Moringa + chicory</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Starters</td>
<td>Grower- finisher</td>
<td>Starter</td>
<td>Grower- finisher</td>
</tr>
<tr>
<td>Corn grain</td>
<td>54.5</td>
<td>62.45</td>
<td>54.5</td>
<td>62.45</td>
</tr>
<tr>
<td>Soy bean meal</td>
<td>28</td>
<td>21</td>
<td>28</td>
<td>21</td>
</tr>
<tr>
<td>Conc. mixture</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Sunflower oil</td>
<td>2.2</td>
<td>1.6</td>
<td>2.2</td>
<td>1.6</td>
</tr>
<tr>
<td>Di-calcium phosphate</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Common salt</td>
<td>0.25</td>
<td>0.1</td>
<td>0.25</td>
<td>0.1</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Minerals-vitamins premix</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Additives tested</td>
<td>1.5 distiller grains</td>
<td>1.5 distiller grains</td>
<td>1.5 Moringa</td>
<td>1.5 Moringa</td>
</tr>
<tr>
<td>Sum (kg)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Estimated analysis

<table>
<thead>
<tr>
<th></th>
<th>ME(kcal/kg)</th>
<th>T. P (%)</th>
<th>Calcium (%)</th>
<th>phosphorus</th>
<th>Lysine (%)</th>
<th>Methionine (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2890</td>
<td>3130</td>
<td>2980</td>
<td>3120</td>
<td>1.5 Moringa</td>
<td>1.5 Moringa + chicory</td>
</tr>
<tr>
<td></td>
<td>21.81</td>
<td>19.43</td>
<td>21.8</td>
<td>19.42</td>
<td>1.5 chicory</td>
<td>1.5 Moringa + chicory</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.90</td>
<td>1</td>
<td>0.90</td>
<td>1.5</td>
<td>1.5 Moringa + chicory</td>
</tr>
<tr>
<td></td>
<td>0.9</td>
<td>0.8</td>
<td>0.9</td>
<td>0.8</td>
<td>1.5</td>
<td>1.5 Moringa + chicory</td>
</tr>
<tr>
<td></td>
<td>1.4</td>
<td>1.2</td>
<td>1.4</td>
<td>1.2</td>
<td>1.5</td>
<td>1.5 Moringa + chicory</td>
</tr>
<tr>
<td></td>
<td>0.58</td>
<td>0.57</td>
<td>0.58</td>
<td>0.57</td>
<td>1.5</td>
<td>1.5 Moringa + chicory</td>
</tr>
</tbody>
</table>

Premix provided the recommended amount of both vitamins and mineral according to NRC (1994).

and vitamins (Meehye and Shin, 1996; Van Loo, 2007). Inulin improves lipid-to-glucose metabolism with potential effects on weight gain, fat deposition and appetite (Urias-Silvas et al., 2007). A beneficial action of chicory (inulin and oligofructose) feeding is decreasing the pH, which could explain thickening of the small intestine wall (Remesy et al., 1992).

Sevane et al. (2014) identified 33 genes associated with protein regulation activity, vital cellular processes, localization and peptidase activity, and so influence productive improvement; 43 genes were also identified that regulate cell division and growth, DNA and RNA synthesis, finally resulting in increasing cellular activity.

Besides, chicory stimulates PPARA, which is a member of peroxisome proliferator-activated receptors, related to energy metabolism regulation, cell growth, dividing and maturation, and in inflammation and immune status (Gervois and Mansouri, 2012).

The result of Moringa is supported by observation of Akhouri et al. (2013), who recorded improved body weight and enhanced FCR of broilers with M. oleifera; it is also in line with the results of Banjo (2012) finding, who reported that supplementation of M. oleifera to diet of broiler...
The titer of IBD and NDV vaccine improved (Wu et al., 1999; Catala et al., 1999) in the presence of flavonoids, inulin and polyphenolic compounds. Result obtained pointed an effect of chicory and *M. oleifera* addition on the improvement of immunity by improving the activity of immunological parameters.

An elevation of antibodies titers of both ND and IBD vaccines allotted on *M. oleifera* and/or *C. intybus* supplemented ration at 30 days old improved in young rabbits that received diet with *M. oleifera*, improvement in protein digestibility and weight gain, while feed intake, FCR, and carcass yields were not affected. Grubben and Denton (2004) reported higher growth of rabbit as a result of vitamin A and essential elements of *M. oleifera*, that promote health. *M. oleifera* also has antimicrobial ability (Caceres et al., 1990). *M. oleifera* leaves contain 0.1 to 0.23% of tannin (Kakengi et al., 2003, 2007), that decrease protein digestion and absorption while lipids and carbohydrate utilization are less affected (Esonu et al., 2001).

**Immunological parameters**

An elevation of antibodies titers of both ND and IBD vaccine was found in groups MC, CI, M than group C (Table 4). The titer of IBD and NDV vaccine improved due to the presence of flavonoids, inulin and polyphenolic compound. Result obtained pointed an effect of chicory and *M. oleifera* addition on the improvement of immunity by improving the activity of genes and fastening pathways related to body defense processes, where the addition of chicory (inulin) stimulated various immune pathways. Sevane et al. (2014) identified 20 genes implicated in immune response pathways, antibodies and immune action. Also, chicory has anti-apoptotic activity, via antioxidant activation, which boosts T-helper activity (Wammes et al., 2013) besides activating enzymes which enhance formation of acyl-CoA, ATP and CoA, and so promote mitochondria action (Wammes et al., 2013). Chicory also regulates glutathione metabolism that also enhances antioxidant defense and regulation of cellular metabolism, where its deficiency leads to oxidative stress (Wu et al., 2004).

Oligofructose suppresses challenged infections of broilers (Van Leeuwen et al., 2005). In quails, inclusion of inulin prevents the pathogenic bacteria growth and enhances the activities of microflora, which have a protective role (Catala et al., 1999). Nodular lymphoid tonsils improvement and infiltration of lymphoid cells were recorded with chicory feeding (Spaeth et al., 1990), besides Kelly-Quagliana et al. (2003) described that both inulin and oligofructose triggers immune defense.

**Table 3. Impact of *M. oleifera* and/or *C. intybus* addition to broiler chicken ration on weight gain, feed intake, FCR and EPEF.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control group (C)</th>
<th><em>Moringa oleifera</em> group (M)</th>
<th><em>Chicory intybus</em> group (CI)</th>
<th><em>M. oleifera</em> plus <em>C. intybus</em> group (MC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of birds</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Initial body weight</td>
<td>45.3</td>
<td>45.2</td>
<td>45.1</td>
<td>45.2</td>
</tr>
<tr>
<td>Body weight (g/bird)</td>
<td>15 days old</td>
<td>216±53&lt;sup&gt;d&lt;/sup&gt;</td>
<td>235±16&lt;sup&gt;c&lt;/sup&gt;</td>
<td>246±27&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>30 days old</td>
<td>1218±145&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1339±95&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1397±135&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>42 days old</td>
<td>1893±97&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2020±54&lt;sup&gt;4&lt;/sup&gt;</td>
<td>2180±48&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Feed intake (g/bird)</td>
<td>15 days old</td>
<td>250.2±23&lt;sup&gt;d&lt;/sup&gt;</td>
<td>266.3±33&lt;sup&gt;c&lt;/sup&gt;</td>
<td>280.5±24&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>30 days old</td>
<td>1503.7±46&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1537.5±27&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1585.8±52&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>42 days old</td>
<td>1242.3±65&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1310.1±39&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1347.4±61&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cumulative</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FCR</td>
<td>1.58</td>
<td>1.54</td>
<td>1.48</td>
<td>1.45</td>
</tr>
<tr>
<td>EPEF</td>
<td>279.5</td>
<td>293.5</td>
<td>344</td>
<td>385</td>
</tr>
<tr>
<td>Mortality</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

<sup>a,b,c,d</sup>Means on the same row with different superscripts are significantly different (P<0.05).

**Table 4. Antibody titer of broilers chicks against ND and IBD vaccines allotted on *M. oleifera* and/or *C. intybus* supplemented ration at 30 days old.**

<table>
<thead>
<tr>
<th>Group</th>
<th>HI of NDV (log2)</th>
<th>Elisa of IBD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group (C)</td>
<td>254.8±47&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1241±23&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group (M)</td>
<td>584.5±59&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2130±70&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group (CI)</td>
<td>296.89±67&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1802±42&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group (MC)</td>
<td>612.23±42&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2209±34&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b,c,d</sup>Means on the same column with different superscripts are significantly different (P<0.05).
mechanism. Without doubt, *M. oleifera* is medicinally used as antioxidants and antifungal (Mohammed and Barhate, 2012; Palival et al., 2011a, b), so high-fat foods could be preserved for long time due to potent antioxidants presence such as flavonoids and total phenolic compounds (Doughari et al., 2008; Jain et al., 2010). The leaves are also free of deleterious substances such as tannins and saponis (Celikel and Kavas, 2008).

Antimicrobial activity is the key for the wide use of *M. oleifera* (Suarez et al., 2005). It has immunomodulatory ability in the immune system (Palival et al., 2011a). This herb also serves as promoter to immune system and is used to overcome malnutrition (Palival et al., 2011b). It was chosen due to its phytochemical compound content, which include saponis, carotenoids, phenolic compounds and flavonoids. Saponin and flavonoid are considered natural immunomodulator because they enhance lymphocyte cells development (Anwar et al., 2007). Lipophilic constituents of *Moringa* explained the antimicrobial activity (Jabeen et al., 2006); *Moringa* also contain antibiotic metabolites and cell wall degrading enzymes (Rachmawati and Rifa’I, 2014).

According to Hefni (2013), aqueous extract of *M. oleifera* increases the number of hematopoietic stem cells, B lymphocytes, naive T cells expression and pro-inflammatory cytokines.

**Biochemical parameters**

Table 5 show that blood serum total cholesterol values were different (P ≤ 0.05) among the groups, where lower value as well as triglycerides values was recorded in the chicory-*Moringa* (MC) group, followed by CI and M groups, where C group comes last, while there was a significant improvement in total protein and globulin values in both MC and M groups than CI and C groups. The result was similar to those of Yusrizal and Chen (2003) findings that revealed on addition of chicory to broilers diet a decrease in serum cholesterol level and abdominal fat deposition and an increase in cecum weight and GIT length. Also, result of Yusrizal and Chen (2003) showed that inulin decreased cholesterol content in serum. Jeusette et al. (2004) and Diez (1997) observed a decrease in cholesterol and triglyceride values in the presence of inulin or oligofructose. Delzenne et al. (1995) observe an increase in calcium bioavailability which modifies the bone structure.

The result of the study is similar to the findings of Elson (1995), which recorded that enzyme of synthetic pathway of cholesterol was suppressed by isoprenoids. Moreover, Kim (2000) reported that chicory presence decreased 30% of cholesterol absorption, meanwhile Fremont et al. (2000) recorded that phenolic compounds decrease cholesterol concentration in blood and meat. Similarly, *M. oleifera* extract had hypo-cholesterolemic properties that were explained with low density lipoprotein (LDL) plasma levels due to the presence of B-sitosterol of *Moringa* (Ghasi et al., 2000, Kane and Malloy, 1982). Also, Luqman et al. (2012) confirmed the antioxidant activities as a result of polyphenols and flavonoids found in extract of *M. oleifera*.

The LDL of birds reduced with inclusion of *Moringa* explained by the presence of myriad phytochemicals in *M. Oleifera*. Some compounds present in *Moringa* were reported to have antibacterial and anticancer activity (Fahey, 2005; Mekonnen and Dräger, 2003), as well as antioxidant activity (Win and Jongen, 1996).

**Carcass yield**

As shown in Table 6 it could be observed that there was a significance difference (P≤0.05) among the groups in dressed carcass % of live body weight, where M (70.3) and MC (86.3) groups are better than CI groups (66.1) and C groups (65.3). Treatments had significant effect on breast, thigh and abdominal fat. Broilers which received diets with M had better carcass weight (%), but not the body weight (MC group). The examined groups had higher breast weight (%) than the control group C (P<0.05).

The study result is in line with the finding of Brunsgaard and Eggum (1995), who reported improving carcass dressing and BW percentage.

The chicory inclusion to broilers improves carcass dressing and BW percentage that could be explained as fiber effect, which is obviously observed through the lower GIT length more than in the upper part (Brunsgaard and Eggum, 1995).

---

**Table 5.** Some blood parameters of broilers chicks received ration supplemented with *M. oleifera* and/or *C. intybus* at 42-days old.

<table>
<thead>
<tr>
<th>Group</th>
<th>Protein (g/100 ml)</th>
<th>Albumin (g/100 ml)</th>
<th>Globulin (g/100 ml)</th>
<th>Cholesterol (mg/100 ml)</th>
<th>Triglycerides (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>3.84±0.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.57±0.13&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.27±0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>149.8±8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>53.73±2.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>M</td>
<td>5.67±0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.68±0.06&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.99±0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>122.2±9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>37.62±1.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cl</td>
<td>4.25±0.34&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.96 ±0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.29±0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>117.6±7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>42.71±1.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MC</td>
<td>5.82±0.52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.76±0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.06±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>106.4±4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>41.30±2.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b,c,d</sup>Means on the same column with different superscripts are significantly different (P<0.05).
These results were confirmed by Yusrizal and Chen (2003) findings, who reported that inclusion of chicory lower the blood cholesterol concentration in broilers chicks, besides increase cecum weight and GIT length, but lowered the abdominal fat. A suggested explanation is diminishing stress condition through the action on immune system.

In rats Jaiswal et al. (2009) recorded that glucose concentration lowered with *M. oleifera* extract addition, that confirm the insulin like action of *Moringa* on body tissues, that might be due to cellular glucose utilization or cease gluconeogenesis. *M. oleifera* has abilities to increase glucose utilization by body tissues (Luqman et al., 2012) via suppress hepatic gluconeogenesis or improve glucose utilization by the body tissues (Desta et al., 2011; Kamanyi et al., 1994). Consequently, this could explain the higher dressed carcass of *Moringa* than other treatments.

Generally, abdominal fat deposition is determined by the amount of fat intake and the amount of fat metabolized and excreted. So, if the fat intake and excreted is equal, decreased body fat accumulation could be due to lipolysis or decreased fatty acid production or to the both mechanisms. In contrary to this, the findings of Sizemore and Siegel (1993), who found no effects of dietary fat amount when the calorie protein ratio remained constant, in broiler diets supplemented with chicory. Similar to the result, Ologhobo et al. (2014) reported better values of carcass weights for birds received diets with *M. oleifera* more than birds received the basal one.

Preston and William (1973) result mentioned that birds with heavier weight have higher dressing percentage and eviscerated yield.

Safa and Tazi (2014) found that feeding *M. oleifera* had fair effect on quality of chicks carcass and increased breast weight of chicks, while Zanu et al. (2012) found that carcass characteristics parameters may not be affected by *Moringa* addition.

Finally, it can be concluded that the use of combination of both *M. oleifera* and *C. intybus* is better than each one alone, due to the beneficial synergistic effect on performance, biochemical parameters and immunology, besides improving carcass quality.

Hence, combination of *M. oleifera* and *C. intybus* was recommended as good feed additives with potential antioxidant ability for broiler chicks.

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### CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.


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Rachmwalli I, Rifa’I M (2014). In vitro Immunomodulatory Activity of


Full Length Research Paper

Newcastle disease virus antibody in serum and feather pulp of chickens vaccinated with thermostable vaccine coated on grains and brans in Zaria, Northern Nigeria

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Received 26 June, 2017; Accepted 18 August, 2017

Thermostable Newcastle disease vaccine virus strain I2 (NDV12) was investigated for its efficacy as foodborne vaccine using maize, sorghum and their brans as carriers. Immune response to vaccination and resistance to challenge were assessed by haemagglutination inhibition (HI) test. After primary and secondary vaccination at three and six weeks of age, sera and feather pulp samples were analyzed to determine the antibody titre in the different groups. The highest mean antibody titre of 7.39 ± 0.42 log₂ was recorded for serum when the vaccine was administered through treated sorghum coated with gum Arabic (TSGG) and 7.28 ± 0.37 log₂ for feather pulp in the group given maize bran (MZB) at eight weeks of age. There was no significant difference (p > 0.05) between the HI antibody titre in the feed groups from feather pulp samples at three weeks of age while a significant difference (p < 0.05) in the serum antibody titre was observed between all the feed groups at five weeks of age. There was correlation in antibody titre between serum and feather pulps only at two weeks after second vaccination. The protection rate after challenge in all the groups was low with the highest rate (14%) recorded when the vaccine was administered in treated maize (TMZ) and TSGG. The study concluded that the vaccine could be effective for the protection of village chickens as food-borne vaccine provided the carrier foods are adequately treated to remove antiviral agents. The use of feather samples as suitable alternative to serum for ND serology was discussed.

Key words: Chickens, maize, sorghum, Newcastle disease, thermostable vaccine.

INTRODUCTION

Newcastle disease (ND) is one of the major important viral disease of poultry which had caused huge economic loses to farmers in recent past (Aamir, 2014). Newcastle disease virus (NDV) has a wide range of hosts, as more than 250 bird species have been found to be susceptible by natural or experimental infections, although wild

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waterfowl and shorebirds are regarded to be the reservoir of the virus in nature (Kaleta and Baldouf, 1988). Among avian species, the poultry flocks are commonly affected with this disease, chickens are most susceptible while ducks and geese are least susceptible to ND (Khan et al., 2000). ND is presently one of the most important endemic disease of poultry in Nigeria, causing high morbidity, mortality, decrease in eggs production and it constitutes a major constraint to the development of rural poultry production (Abdu et al., 1992).

In Nigeria, ND is controlled by vaccination of commercial birds with live thermolabile lentogenic or mesogenic NDV vaccines containing between 100 and 1000 bird dose per vial. The vaccines are administered intramuscularly, intracocularly or orally in water (Abdu et al., 2012). This is impracticable for a village farmer because the method of administration requires the catching and handling of birds and there is no guarantee that local birds will drink vaccine treated water (Abdu et al., 2012). The vaccine dose format is also not meant for village flocks containing between 7 and 29 birds (Oltchere, 1990). Thermostable Newcastle disease virus (NDV) vaccines have been used widely to control ND for village poultry flocks, due to their independence of cold chains for delivery and storage (Guoyuan et al., 2015).

The NDV vaccine strain I2 has undergone laboratory test in several countries and has proved to be protective against local virulent strains. In Vietnam, it has been officially recognized as the NDV vaccine for village chicken after extensive laboratory and village trials (Tu et al., 1998). In Tanzania, it has given protection for at least two months after vaccination (Wambura et al., 2000). Field records in Mozambique indicated that NDVII vaccine provides approximately 80% protection in the field of an outbreak when given every month via eye drop (Pangani, 1999). The NDVII vaccine is being tested in several African countries (Alders and Spradbrow, 2001).

The vaccine has been used successfully in village chickens populations in many countries in Asia and Africa including Nigeria (Jayawardane et al., 1990; Jagne et al., 1991; Ibrahim et al., 1992; Echeonwu et al., 2008a). Besides, the successes recorded by many researchers using the V4 and I2 thermostable ND vaccine as feed based vaccine (Nasser et al., 1998; Wambura et al., 2000), there are some basic problems reported to be associated with feed-based vaccination (Cumming, 1992). Firstly, not all types of feed are suitable for the delivery of NDV vaccine in terms of suitability to the chicken and delivery of the virus for protection. Secondly, the type of food vehicle to be used is determined by the availability of that particular feed in a locality (Philemon et al., 2007).

The use of feather shafts of chickens for the diagnosis of viral infections and for monitoring vaccine viruses has been reported (Davidson, 2009). Other researchers (Dong-Hun et al., 2016) also detected viral antigens in feathers of chickens infected with viscerotropic velogenic NDV suggesting that feathers could act as source of viral transmission. The threat of ND to the poultry industry requires routine seromonitoring of vaccinated chickens to show that they have been adequately immunized against the disease (Ameh et al., 2016). To do this effectively, serum samples need to be collected at regular intervals. However, farmers are generally reluctant to allow for collection of serum samples from their birds after vaccination. This study was therefore conducted to determine the suitability of maize and sorghum and their respective brans as delivery systems for NDVII vaccines and also to study the suitability of using feather pulps as an alternative source of sample for seromonitoring of vaccinated chickens against ND in the study area.

**MATERIALS AND METHODS**

**Study area**

The study was conducted at the Nutrition Laboratory of the Veterinary Medicine Department of Ahmadu Bello University Zaria, Nigeria. Zaria is located in Kaduna State, Nigeria: it is a part of the central high plains of Northern Nigeria and about 670 m above sea level. Zaria is located at latitude 11.11°N and longitude 7.73°E. It has two distinct seasons: the dry or harmattan season (October to March) and wet season (April to September) with a population of 975,153 (Oladipo, 1985).

**Experimental birds**

Two hundred day-old unvaccinated cockerels were obtained from the Poultry Research Farm of the National Veterinary Research Institute Vom, Nigeria. The chicks were housed in a brooding room that was cleaned, washed, disinfected and fumigated. All chicks were placed under brooders with chicks mash and water provided ad libitum. At three weeks of age, 18 chicks each were randomly selected and placed in cages with wire meshed floors measuring 56.5 x 56.5 cm until the termination of the experiment.

**Experimental design**

The chicks were divided into 4 groups (A, B, C and D) at three weeks of age. Each group was subdivided into 2 subgroups each consisting of 18 birds. Groups: A1 (treated maize), A2 (treated sorghum), B1 (treated maize plus treated gum Arabic), B2 (treated sorghum plus treated gum Arabic), C1 (maize bran), and C2 (sorghum bran). All birds in subgroups A to C were vaccinated and challenged. Birds in subgroup D1 were not vaccinated but challenged and D2 were unvaccinated and unchallenged and served as positive and negative controls, respectively.

**Source of NDVII vaccine and challenge virus**

The NDVII vaccine was obtained from the Viral Research Department, National Veterinary Research Institute (NVRI) Vom, Plateau State, Nigeria. The vials of the vaccines were 50 dose vials meant to be reconstituted in 50 ml of chlorine free water and to be given orally at 1 ml/bird. The virus strain used for the challenge study was the NDV (Kudu 113 strain) isolated and characterized in a previous study (Echeonwu et al., 1993) with EI50 titre of 10^2.5. The virus was obtained from the Virology Division of the NVRI, Vom.
Preparation and coating of food carrier with vaccine virus

Five kilograms each of maize, sorghum and their bran and 4 kg of gum Arabic were used. The maize and sorghum were milled once to remove the husk and then crushed into a gritty mash. These were soaked in chlorine free water for 72 h, while changing the water daily. The soaked grains were then washed with clean water, sieved and placed to dry in the sun. They were then weighed and packaged into polythene bags of 1 kg/package and stored at room temperature until used. The maize and sorghum brans were not subjected to any treatment; they were dried, packaged and kept at room temperature until used. About 2 kg of gum Arabic (used as additive) was soaked to dissolve overnight in 1,000 ml of distilled water. The gum Arabic was then boiled for an hour, allowed to cool and then autoclaved at 121°C for 15 min.

The method described by Alders and Spradbrow (2001) was used for coating the feed grain and brans with the vaccine virus. The quantity of grains or brans consumed by 18 birds (10 g per bird) was measured and the time taken to consume the vaccine feed was noted. Three vials of the 50 doses of NDVJ2 vaccines were reconstituted in 100 ml of PBS (pH 7.4). Then 50 ml of the treated diluted gum Arabic was thoroughly mixed with the reconstituted vaccine (total 150 ml) and then mixed with the feed in a bowl and then spread on trays and kept at room temperature for 30 min before administrating to the birds.

Vaccinations

First and second dose of NDVJ2 coated on the treated grains and bran were given to the birds at 3 and 6 weeks of age, respectively.

Serum samples

About 1 to 2 ml of blood was collected through the wing vein of each bird with a 2 ml syringe and 21 G needles on days 7, 14 and 21 before primary vaccination and at 2 and 3 weeks post vaccination. The blood samples were deposited into sterile test tubes and sera were separated by allowing the blood to clot in the test tubes slanted in racks at room temperature for 1 to 2 h. Sera collected were stored in a freezer at -20°C until tested.

Preparation of feather pulp for serology

The method described by Roy et al. (1998) with slight modification was used for preparing the feather pulp samples. Four down feathers, two from each wing were plucked from each bird, weighed and cut at the base to remove the pulp using a scissor. Laboratory pestle and mortar was used to grind the feather pulp which was then mixed with 2 ml of PBS, centrifuged at 2000 rpm for 5 min and the supernatant tested for NDV HI antibodies.

Haemagglutination (HA) and haemagglutination inhibition (HI) tests

Five millilitres of chicken blood was collected from newly hatched commercial chicks and transferred into 10 ml of Alsever’s solution and gently mixed. The red blood cells (RBCs) were washed three times with PBS pH 7.2 by centrifugation at 2000 rpm for 5 min each. The concentration of the RBCs used was 1% in 99 ml of PBS. The titre of a live La Sota NDV strain antigen obtained from NVRI was determined by the HA test. Four HA units were used in the HI test. All sera collected were tested for NDV specific antibody by the haemagglutination inhibition (HI) test using methods described by OIE (2004). The antibody level for each serum and feather pulp sample was expressed as a log to the base two and recorded. The geometric mean titers (GM) were calculated. In this study, the published cut off value was used for the protective HI antibody titer (HI titer≥log2 3, that is, GM≥3) for ND vaccination in chickens (Alexander et al., 2004; OIE, 2004).

Challenge studies

At nine weeks of age, three weeks after the second vaccination, all the birds except the negative controls were challenged with NDV Kudu 113 strain. Each bird received a dose of 0.2 ml through the ocular nasal route. After challenge, the birds were observed for two weeks for clinical signs, gross lesions and death.

Data analysis

The mean HI antibody titre and percentage of birds with detectable ND antibody were calculated. Data collected were analyzed using Statistical Package for Social Sciences (SPSS) version 17 program. One way analysis of variance (ANOVA) was performed with Tukey post hoc multiple comparison, which determined statistical significant difference between subgroups at 95% confidence interval with p<0.05 considered as significant. The correlation coefficients were calculated to compare the mean HI ND antibody titres between serum and feather samples in the different groups. Mortality and protection rates were also calculated.

**RESULTS**

**Antibody titre using serum**

At three weeks of age the mean HI antibody titre was ≥ 3 log2 in all birds except those in group C2 (1.17 ± 0.38 log2) and D2 (1.67± 0.56 log2). Two weeks after primary vaccination (at five weeks of age), the HI ND antibody titre dropped in all groups with group A1 and C2 having the lowest mean HI ND antibody titre of 0.38 ± 0.23 log2. At six weeks of age, the lowest mean HI ND antibody titre was recorded in group A2 (0.33 ± 0.33 log2), while at eight weeks of age after secondary vaccination the highest mean HI ND antibody titre of 7.39± 0.42 log2 was recorded in groups B2 (Table 1).

**Antibody titre using feather pulp**

All the birds had low HI mean antibody titre before vaccination at three weeks of age with no detectable antibody titre in groups A1, A2 and C2 (Table 2). The response of birds to primary vaccination was high at five weeks of age with the highest mean HI antibody titre of 8.67 ± 0.58 log2 recorded in group A2; all other groups had mean HI antibody titre ≥ 3 log2. At six weeks of age the mean HI antibody titre in all the groups were ≥ 3 log2 except in group D2 which had the lowest HI antibody titre of 0.61 ± 0.39 log2. Antibody titre of birds in all the groups increased two weeks after booster vaccination (eight weeks of age) with the highest mean HI ND antibody titre recorded in group B2 (7.22 ± 0.58 log2) and 7.28 ± 0.37
log₂ in group C, while the control groups had the lowest HI antibody titre of 0.26 ± 0.15 log₂ in group D1 and 0.33 ± 0.14 log₂ in group D2. At nine weeks of age, the mean HI antibody titre dropped again in all the groups except in groups B2, C1, C2 and D1 which had mean HI antibody titre ≥ 3 log₂ (Table 2).

Percentage of birds with ND antibody HI titres of ≥ 3 log₂ (serum)

At three weeks of age prior to primary vaccination, 88% of birds in group A1 and 94% of birds in group D1 had the highest HI antibody titre of ≥ 3 log₂ (Table 3). At five weeks of age, 33% of birds in group A2 and 27% in group B2 had HI antibody titre of ≥ 3 log₂. At six weeks of age, groups A1 and C1 had 77% of birds with HI antibody titre ≥ 3 log₂. At eight weeks of age, 100% of birds in group B1 had HI antibody titre ≥ 3 log₂. Prior to challenge at nine weeks of age, 44% of the birds in groups A1 and 50% in group A2 had HI antibody titres ≥ 3 log₂ (Table 3).

Table 2. Mean haemagglutination inhibition antibody (log₂) titres from feather pulp samples of birds vaccinated with Newcastle disease vaccine strain I₂.

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccine carriers</th>
<th>No. of birds</th>
<th>Age (weeks) Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>A1</td>
<td>TMZ</td>
<td>18</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>A2</td>
<td>TSG</td>
<td>18</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>B1</td>
<td>TMZG</td>
<td>18</td>
<td>0.17±0.12</td>
</tr>
<tr>
<td>B2</td>
<td>TSGG</td>
<td>18</td>
<td>0.17±0.17</td>
</tr>
<tr>
<td>C1</td>
<td>MZA</td>
<td>18</td>
<td>0.56±0.33</td>
</tr>
<tr>
<td>C2</td>
<td>SGB</td>
<td>18</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>D1</td>
<td>Control 1</td>
<td>18</td>
<td>0.26±0.17</td>
</tr>
<tr>
<td>D2</td>
<td>Control 2</td>
<td>18</td>
<td>0.06±0.06</td>
</tr>
</tbody>
</table>

A1: Treated maize; A2: treated sorghum; B1: treated maize plus gum Arabic; B2: treated sorghum plus gum Arabic; C1: maize bran; C2: sorghum bran; D1 and D2: positive and negative controls not vaccinated.

Percentage of birds with ND antibody HI titres of ≥ 3 log₂ (feather pulp)

At five weeks of age, 94% of the birds in groups A1, A2, and C1 had HI antibody titres of ≥ 3 log₂, while group C2 had 83% of birds with ≥ 3 log₂ at five weeks of age. The highest percentage of birds (83%) with titres ≥ 3 log₂ at six weeks of age was recorded in group B2. All the birds (100%) in group C1 had HI antibody titres of ≥ 3 log₂ followed by 94% of the birds in group B2 and 83% of the birds in group A1 at eight weeks of age. At nine weeks of age, 77% of birds in group C2, 66% in group D1, and 11% in group B1 had HI antibody titres of ≥ 3 log₂ (Table 4).

Correlation analysis

The results of Pearson’s and Spearman’s rho correlation to compare the mean ND HI antibody titre between feather and serum are shown in Table 5. There was a
Table 3. Percentage of birds with NDV2 antibody titers of ≥ 3 log₂ in serum samples following vaccination at 3 and 6 weeks of age.

<table>
<thead>
<tr>
<th>Group</th>
<th>Age in weeks</th>
<th>Percent positive with titres of ≥ 3 log₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>A1</td>
<td>88.9</td>
<td>11.1</td>
</tr>
<tr>
<td>A2</td>
<td>72.2</td>
<td>33.3</td>
</tr>
<tr>
<td>B1</td>
<td>61.1</td>
<td>11.1</td>
</tr>
<tr>
<td>B2</td>
<td>77.8</td>
<td>27.8</td>
</tr>
<tr>
<td>C1</td>
<td>66.7</td>
<td>11.1</td>
</tr>
<tr>
<td>C2</td>
<td>22.2</td>
<td>0.0</td>
</tr>
<tr>
<td>D1</td>
<td>94.4</td>
<td>22.2</td>
</tr>
<tr>
<td>D2</td>
<td>11.1</td>
<td>0.0</td>
</tr>
</tbody>
</table>

A1: Treated maize; A2: treated sorghum; B1: treated maize plus gum Arabic; B2: treated sorghum plus gum Arabic; C1: maize bran; C2: sorghum bran; D1 and D2: positive and negative controls not vaccinated.

Table 4. Percentage of birds with NDV2 antibody titres of ≥ 3 log₂ in feather pulp samples.

<table>
<thead>
<tr>
<th>Group</th>
<th>Age in weeks</th>
<th>Percent positive with titre of ≥3 log₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>A1</td>
<td>0</td>
<td>94.4</td>
</tr>
<tr>
<td>A2</td>
<td>0</td>
<td>94.4</td>
</tr>
<tr>
<td>B1</td>
<td>0</td>
<td>44.4</td>
</tr>
<tr>
<td>B2</td>
<td>11.1</td>
<td>77.8</td>
</tr>
<tr>
<td>C1</td>
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<td>94.4</td>
</tr>
<tr>
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</tr>
<tr>
<td>D1</td>
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<td>44.4</td>
</tr>
<tr>
<td>D2</td>
<td>0.0</td>
<td>44.4</td>
</tr>
</tbody>
</table>

A1: Treated maize; A2: treated sorghum; B1: treated maize plus gum Arabic; B2: treated sorghum plus gum Arabic; C1: maize bran; C2: sorghum bran; D1 and D2: positive and negative controls not vaccinated.

Table 5. Results of correlation analysis for antibody titres in serum and feather pulp after vaccination of chickens with NDV2 at various ages.

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>Correlation coefficients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pearson</td>
</tr>
<tr>
<td>3</td>
<td>0.041</td>
</tr>
<tr>
<td>5</td>
<td>0.098</td>
</tr>
<tr>
<td>6</td>
<td>0.193*</td>
</tr>
<tr>
<td>8</td>
<td>0.318**</td>
</tr>
<tr>
<td>9</td>
<td>-0.078</td>
</tr>
</tbody>
</table>

*Correlation is significant at 0.05 level. **Correlation is significant at 0.01 level.

Mortality rate and protection rate

The mortality and protection rates of birds challenged with NDV Kudu 113 are presented in Figure 1. The highest mortality rate (100%) was recorded in groups B1, C2 and the control group, while the lowest (78%) was recorded in groups A1 and B2. Groups A2 and C1 had 89% mortality rate. Protection rate after challenge was low for all the groups; A1 had (14%), A2 (1.2%), B2...
Figure 1. Mortality and protection rate in experimental birds after challenge with Newcastle disease virus Kudu 113 at three weeks after second vaccination. A1: Treated maize; A2: treated sorghum; B1: treated maize plus gum Arabic; B2: treated sorghum plus gum Arabic; C1: maize bran; C2: sorghum bran; D1 and D2: positive and negative controls not vaccinated.

(14%), C1 (1.2%), while B1, C2 and D1 were not protected (Figure 1).

DISCUSSION

The reported protective antibody titre for ND vaccines are HI ≥ 4 log₂ (OIE, 2000) with reference to conventional ND vaccine designed for intensively reared commercial chickens. However, HI ND antibody titre of ≥ 3log₂ was considered to be adequate for food-based vaccines orally administered to scavenging chickens (Echeonwu et al., 2007). The mean HI antibody titre at three weeks of age was low in all the groups with feather pulp samples in contrast to the high antibody titre recorded at same age in serum. The observed low antibody titre in the feather pulp might be due to movement of antibodies which was more in the central circulation than peripheral at three weeks of age and more in the peripheral circulation at five weeks of age. However, since the birds had no previous vaccination before the primary vaccination, the high antibody titre detected in serum at three weeks of age could be due to the presence of maternal antibody which may also be responsible for the low antibody titre recorded in serum two weeks after primary vaccination at five weeks of age. It has been established that chicks from immunized parents possess high level of maternal antibody which protect the chicks against virulent virus and interferes with vaccine antigens (Saeed et al., 1988; Rahman et al., 2002). The percentage of vaccinated birds with HI antibody titres ≥ 3log₂ showed a marked increase at six and eight weeks of age in both serum and feather pulp samples. Flock immunity reported by Boven et al. (2008) as the only means to prevent the transmission of NDV can only be achieved when ≥ 85% of vaccinated birds have antibody titres of ≥ 3log₂. In the present study, this was achieved in groups B2, C1 and C2 for serum and groups B2 and C1 for feathers at eight weeks of age. However, prior to challenge at nine week of age, the percentage dropped in both serum and feather with none of the groups having percentage mean HI antibody titre sufficient to protect the birds from challenge. However, it was observed that some birds with low or undetectable ND HI antibody titres survived after challenge. This
observation has been reported by Ibrahim et al. (1981) who concluded that low HI antibody titre following NDV4 vaccination were not indicative of susceptibility to challenge, an observation also confirmed by other researchers (Bell et al., 1995; Wambura et al., 2000; Tu et al., 1998). In addition to serum antibody, secretory antibody (IgA) at mucosal surfaces and cell mediated immunity are thought to play a role in resistance to challenge (Alexander, 2003).

There was a general increase in the HI ND antibody titre two weeks after secondary vaccination in serum and feather. Similar results were reported by other workers (Ideris et al., 1990; Spradbrow 1993) who stated that protective immunity is often not apparent until after the second oral vaccination. Similar findings were reported by Baba et al. (2006) and Nasser et al. (2000) that titres among vaccinated birds generally peaked by day 21 post vaccination and declined subsequently. The results of the current study show that administration of 2nd booster vaccination significantly and progressively increased HI antibody titer in all the treatment groups.

The duration of active protective immunity varies with the immune status of the bird and nature of the immune stimulus, which depends on the dose and strain of NDV vaccine and route of administration of the vaccine (Ibrahim et al., 1981; Westbury, 1984). Previous studies found that drinking water induced acceptable immune response and protection, but it is inappropriate particularly in cold weather because vaccination had to be conducted in the mornings, and not all chicken drink water in the mornings even after feeding (Mogoje, 2006). In another study, the administration of a partially thermostable ND vaccine via eye drop application gave the best response, while the vaccine administered via cooked maize meal gave the lowest response. Eye drop vaccination is impractical to implement in village environment (Mogoje, 2006).

There was positive correlation between the mean HI ND antibody titre of serum and feather pulp only at eight weeks of age. This is in contrast to the reports of Roy et al. (1998), who recorded consistent high ND HI antibody titre in serum than in feather three weeks after primary vaccination and three weeks after secondary vaccination in an experiment to compare ND vaccines by serology in tears and feather pulp samples. In the present study, fluctuations were observed in the level of ND HI antibody titre in both serum and feather after primary and secondary vaccination with a marked difference observed at three weeks of age in feather with very low antibody titre than serum and at five weeks of age, feather pulp samples had higher ND antibody titre than serum. These fluctuations could be due to the lack of uniformity of feather pulp samples, since the amount of pulp in each feather will produce some variation in results as reported by Garrido et al. (1992). Results from the present study showed high mortality and low protection rate in all the groups. The difference in the protection rate after challenge with virulent NDV may be due to differences in the vehicles used in the administration of the vaccine. The highest mortality rate (100%) in vaccinated birds was recorded in birds vaccinated with TMZG and SGB. The lowest protection rate was also recorded in birds vaccinated via TMZG and SGB. However, birds vaccinated with TMZ and TSGG had the lowest mortality. These findings is similar to what was reported by Nasser et al. (2000) in vaccination trials in Ethiopia, where untreated and parboiled sorghum used as vaccine carriers for NDVI2 gave low protection to vaccinated birds after challenge. Similarly, in Nigeria Musa et al. (2010) reported that untreated sorghum, parboiled sorghum, sorghum coated with gum Arabic and a commercial feed mash used as vaccine feed carriers for NDVI2 gave low antibody titre and low protection following challenge with a velogenic NDV. The results of these investigations are in contrast to the findings of Echeckonwu et al. (2007, 2008b) who tested NDVI2 and V4 vaccines on millet, maize and guinea corn grains and bran in Nigeria and the vaccinated birds were protected after challenge.

Results from their study indicated that the vaccines could be effective for protection of village chickens as food-borne vaccines provided the carriers are adequately processed. Furthermore, different grains induced different level of HI antibody titer. This implies the presence of inherent variation in virus carrying capacity of different grains (Reta et al., 2016). This is an opportunity to screen grains of different species and varieties. Interestingly, treating grains (either cracking or parboiling) increased their efficacy as vaccine carrier. Similar results have been reported in Nigeria by Olabode (2010) as to the efficacy of treated grain particularly maize compared to untreated grain. Grains have been known to contain tannins, anthraquinone, cardiac glycosides and alkaloids. Some of these chemicals have been shown to have antiviral properties (Oakeley, 2000; Musa et al., 2010). The higher HI titer induced by treated grains than untreated ones could be due to the fact that cracking grains increase the surface area of the grains to adsorb the vaccine virus (Oakeley, 2000; Wambura et al., 2007; Olabode, 2010). Cracked maize and treated sorghum were found to be better vaccine carriers in this study, though the protection rate was low.

This is in contrast with the work of Lawal et al. (2016) in Nigeria using maize grit as vaccine carrier for NDVI2. Their study showed that 94.3% of the vaccinated village chickens seroconverted with protective levels of antibodies against ND virus. However, it should be noted that the protection level of the grain based NDVII2 vaccine varies under laboratory conditions, that is, >90% protection (Aini et al., 1990) and under real village conditions, that is, <60% (Aini et al., 1992) and with vaccine delivered by farmers (Aini et al., 1990). Hence, it is necessary to conduct pilot field trial at village level to evaluate the results of the current study under real village conditions.
Conclusions

The study concluded that treated maize, treated sorghum, untreated maize bran and untreated sorghum bran when used as feed carriers for NDV in this study gave low protection to vaccinated birds after challenge with velogenic NDV. The NDV vaccine could be useful for the protection of village chicken against ND provided the carrier feeds are adequately treated to remove antiviral substances. The use of different processing methods for maize and sorghum should be employed to treat these grains and other locally available feeds such as millet to reduce or eliminate possible antiviral substances in them and to test their suitability as ND vaccine carriers. Correlation was found between the NDV HI antibody titre in serum and feather pulp only at eight weeks of age and thus, feather pulp samples cannot be used as an alternative to serum for seromonitoring of vaccinated birds. However, feathers can be easily collected from live or dead birds, and thus can serve as suitable samples for diagnosis of NDV in chickens. The selection of feather for seromonitoring is important; however, since the amount of pulp in each feather will produce variation in results, further research is therefore necessary.

ACKNOWLEDGEMENTS

The authors appreciate the contributions of Prof. S. B. Oladele of the Department of Veterinary Microbiology, Ahmadu Bello University, Zaria, Dr. Musa U. of the Veterinary Research Institute, Vom and thank all staff of the Nutrition Laboratory, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, for their technical assistance.

REFERENCES


Full Length Research Paper

Detection of *Mycobacterium avium* subspecies *paratuberculosis* excretion in bovine feces, using quantitative real time polymerase chain reaction (Q-PCR)

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Received 30 June, 2017; Accepted 22 August, 2017

*Mycobacterium avium* subspecies *paratuberculosis* (Map) causes paratuberculosis, an infectious enteritis that affects domestic ruminants. The main source of infection for herds comes from the elimination of the bacilli through feces. The objective of this study was to determine Map excretion in bovine feces. The study included forty, one-year-old bovine, Holstein breed females, from a herd that had >25% paratuberculosis prevalence. Samples of blood and feces were obtained four times with three-month intervals. Feces samples were subjected to bacteriological culture and DNA extraction for IS900 quantitative real time polymerase chain reaction (Q-PCR). Enzyme-linked immunosorbent assay (ELISA) was done from serum samples to detect the presence of anti-Map antibodies. Correlation between IS900 Q-PCR, culture and ELISA was established by the Kappa (K) test; statistical analysis was carried out by the Pearson Chi² test. On the first sampling, five animals were detected by IS900 Q-PCR, shed 1 x 10² to 2.6 x 10³ Map copies per gram of feces; on the second sampling, 6 animals shed 3.25 x 10³ to 8.5 x 10⁴; on the third sampling two animals shed 2.03 x 10³ to 1.10 x 10⁴ and on the fourth, three animals shed 7.92 x 10⁴ to 1.4 x 10⁵ Map copies. Correlation between tests in samplings first, second and fourth, was 0.53 to 0.73, and for third was 0.22. With the use of IS900 Q-PCR, it was possible to detect animals that were eliminating Map in feces from 12 months of age without clinical manifestations. The IS900 Q-PCR is an alternative method to carry out programs of control of paratuberculosis that allow the detection of animals that shed Map in the early stages of the infection.

**Key words:** Paratuberculosis, *Mycobacterium avium* subspecies *paratuberculosis*, excretion, quantitative polymerase chain reaction (Q-PCR), bacteriological culture, enzyme-linked immunosorbent assay (ELISA).

INTRODUCTION

Paratuberculosis is a chronic infectious disease, caused by *Mycobacterium avium* subspecies *paratuberculosis* (Map) that affects domestic and wild ruminants. Map is classified within the *Mycobacterium avium-intracellulare* complex (*M. avium* subspecies *avium*, *M. avium* subspecies *paratuberculosis*, *M. avium* subspecies...
silvaticum and M. intracellulare) and it differs from other subspecies of the complex by its dependence on mycobactin for in vitro growth (Stevenson et al., 2002). Map is an intracellular bacteria characterized by resistance to the environmental conditions; it can remain viable for 270 days in stagnant waters, 47 months in dry desiccated organic matter, 45 days in milk and 60 days in fresh cheese (Abalos, 2001; Cironi et al., 2007).

The disease has a chronic course and mainly affects adult animals of two to three years of age. It is characterized by progressive loss of body condition due to the lesions produced in the ileum, ileocecal, cecum and proximal colon mucosa and mesenteric lymph nodes; diffuse hypertrophy of the jejenum and ileum mucosa is developed, which acquire a rough appearance, causing low nutrient absorption (Valentin-Weigand, 2002).

Paratuberculosis has an important impact on animal production by generating a reduction in milk and meat production, early discarding of animals and low fertility with predisposition for mastitis (Eda et al., 2006). With this disease, carcasses have a lower commercial value and there is an increase in control program costs (Soto et al., 2002a). Map is excreted intermittently and animals are infected by consuming feed and water contaminated with paratuberculosis microorganisms shed by infected animals. Map concentration in feces may surpass $10^6$ colony forming units (CFU)/g (Valentin-Weigand, 2002). Young animals less than six months of age are the most susceptible to infection; calves that are in subclinical stages of the disease shed the bacilli in feces without showing the characteristic signs of the disease (Park et al., 2006). The disease distribution is worldwide, especially in domestic ruminants confined rearing conditions; prevalence of the disease varies between 5 and 55% (Millan-Suazo et al., 2015; Avila et al., 2011).

To confirm diagnosis, bacteriological culture is carried out, although its disadvantage is that it is a slow process that needs more than six weeks to have a positive Map culture (Soto et al., 2002b). Currently, there are molecular tests developed to diagnose paratuberculosis and the most used is the polymerase chain reaction (PCR). It had been mainly applied as a qualitative method, but there is also the option to do the PCR test in real time or quantitative PCR (Q-PCR) that is a variation of the standard PCR used for quantification of DNA or messenger RNA (mRNA) of a sample. It is possible to determine the number of copies or the relative amount of a specific DNA or RNA sequence by using specific sequencing primers. The amount of amplicon produced in each PCR cycle is used for quantification. Product quantification occurs by the addition of fluorophores that join the amplicon in a directly proportional manner, and therefore, the higher amount of product there is, the higher fluorescence emission. Real time PCR programs detect the amount of fluorescence produced in each PCR cycle and the analysis programs represented the said fluorescence in graphs, in relation to the number of cycles (UCV, 2012; Vinueza-Burgos, 2009). Most of the PCR protocols, for DNA/RNA Map detection, use primers that amplify the 900-insertion sequence (IS900), and therefore it has been accepted as a standard Map marker. IS900 is a 1451 base-pair sequence that codifies for a hypothetical transposase of 399 amino acids, with 14 to 20 copies in the MAP genome inserted in a consensus region within highly conserved loci in the Map genome (Kralik et al., 2011; Bull et al., 2000). It is thought that, for every animal diagnosed with the clinical profile of paratuberculosis, there are approximately 25 other individuals that have a subclinical profile and that these will intermittently shed the bacillus through feces. It is therefore important that the diagnosis of this disease be done using diagnostic techniques, such as q-PCR, that can detect animals that are shedding the bacillus before clinical signs are present, in order to establish control measures within the herd. The purpose of this study was to determine the shedding of Map in bovine feces using Q-PCR.

MATERIALS AND METHODS

The work was carried out using 40 Holstein breed female cows at least 1-year-old that originated from a dairy herd with previous clinical history of paratuberculosis (>25% prevalence) in Hidalgo Mexico, confirmed with microbiological culture IS900 nested PCR and ELISA. Follow up was done by taking four samples of blood and feces, every three months. Blood was obtained from the caudal vein using vacutainer tubes and centrifuged at 180 × g for 10 min; the resulting serum was then divided into aliquots in microtubes. Feces were collected directly from the rectum using palpation gloves. All samples were stored at -20°C until analyzed (Martinez et al., 2012).

DNA extraction

Two grams of feces were decontaminated using 50 ml of 7.6% 1-hexadecylpyridinium chloride (HCP) (Sigma Aldrich) for 18 h. DNA extraction was then carried out following the protocol described by Jaimes et al. (2008). DNA was stored at -20°C until used in q-PCR.

Real time Q-PCR

The Q-PCR reaction was done using the primers for ISIS IS900 PTB FP 5’TATAGGGTACGGAGTGTTGC3’ and PTB RP 5’GGAGTAATGGTGCCTTTACC 3’ (Kim et al., 2002). Each 25-μl q-PCR reaction contained 3 μl DNA (45 ng/μl), 12.5 μl Maxima

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SYBR green / ROX q-PCR Master mix (3 mM MgCl₂, 200 μM each dATP, dGTP, dCTP, 400 μM dUTP, 1.25 IU DNA polymerase and 0.5 IU Uracil-N-Glycosylase (UNG) (Thermo)), 1 μl each PTBF and PTBRP primers (0.05 μM each/one), and 7.5 μl water. DNA from a standard Map strain (ATCC #43545) was used as positive control, while distilled water was used as the negative control.

The Q-PCR was carried out in a SmartCycler® (Cepheid) with the following cycling conditions: 95°C/10 min, 40 cycles of 95°C/15 s, 60°C/30 s and 72°C/10 s, followed by a final cycle of 72°C/30 s. Samples were worked in duplicate and the results were analyzed following the guidelines in the SmartCycler operating manual. A linear regression was performed on CT versus log IS900 copy number and R² was 0.99. The standard error of y was used to create two equations to estimate the upper and lower concentration, for calculation of *M. avium* subsp. *paratuberculosis* IS900 copy number; this was done following the protocol described previously by Moravkova et al. (2012), Slana et al. (2008) and Kim et al. (2002).

**Bacteriological culture**

Feces from all animals were cultured in duplicate following the technique described by Aly et al. (2010). All samples were decontaminated using 0.5% Zephiran and sown in Herrold’s egg yolk solid media with and without 2 mg/L mycobactin J (Allied Monitor INC) and incubated at 37°C for 6 months. Ziehl-Neelsen acid-fast stain was carried out on cultures that showed positive growth; DNA was also extracted and nested PCR carried out using primers that amplify a 210 bp region of IS900 (Jaimes et al., 2008).

**Enzyme linked immunosorbent assay (ELISA)**

Protolymphatic antigen of the 3065 Map strain was adhered to ELISA microplates following the protocol described by Martinez et al. (2012). Sera were diluted to a working concentration of 1:160 using a solution of 0.02% *Mycobacterium phlei* (Allied Monitor INC). A Map positive serum was used as positive control and a negative serum as negative control (Allied Monitor INC). The bovine anti-IgG conjugate tagged with horseradish peroxidase (Sigma-Aldrich) was used at a 1:2000 dilution, while 2, 2’-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic acid (ABTS, AMPRESCO) was used as substrate, and 1.5 mM sodium nitrate (sigma-Aldrich) in 0.1 M citric acid was used as stop solution. Plate reading was done at 650 nm using a spectrophotometer (ELX800, BioTek). The cut-off was established at 0.22 optical deviations (OD).

**Statistical analysis**

Statistical analysis was carried out using the STATA® 7.0 software package (StataCorp LP, College Station, TX, USA). Cohen’s Kappa test (k) or inter-rater agreement index was used to establish the association between the results obtained with the IS900 Q-PCR, culture and ELISA. Differences between diagnostic techniques were tested using Pearson’s Chi² (χ²). The scale proposed by Prieto et al. (2014), was used to determine the concordance among techniques. Range (%) to <0.00 is poor; to 0.00 and 0.20 is slight; to 0.21 and 0.40 is fair; to 0.41 and 0.60 is moderate; to 0.61 and 0.80 is substantial; to 0.81 and 1.0 is perfect.

**RESULTS**

Follow-up of the 40 female cows was done every three months starting at 12 months of age and continued until they reached 21 months, to determine the age at which the animals begin shedding the bacilli in feces. All cows came from a herd with a history of >25% prevalence of paratuberculosis. The results obtained in the work are shown in Table 1, with the use of IS900 Q-PCR, amplification curves of the Map positive control and samples that were found to be positive were present from cycle 20 up to cycle 30 of the Q-PCR run (Figure 1). Animals that were shedding the bacteria could be identified from the first sampling, which corresponded to 12 months of age. Map shedding in feces is intermittent, since only one animal was detected as positive in all samplings.

The presence of anti-Map antibodies was lower (2.5 to 7.5%); tests based on the detection of humoral immune response are not feasible during early stages of infection (subclinical stage), because the concentration of anti-Map antibodies in early stages of infection are low.

Bacterial growth could only be observed in tubes that had mycobactin added from five weeks on. Ziehl-Neelsen stain revealed the presence of acid-fast bacilli, and the nested PCR amplified the expected 210 bp amplon which is specific to Map. The sensitivity of the bacteriological culture was less than 10%, the animals that were positive to the bacteriological culture in all the samplings were also positive to the IS900 Q-PCR.

Association between the three diagnostic tests used in this study was determined with Cohen’s Kappa test (Tables 2 and 3). When comparing IS900 Q-PCR with bacteriological culture and ELISA, first, second and fourth samplings showed moderate to substantial concordance, while in third sampling the concordance was fair.

Results of the negative predictive values (VP-) indicate that IS900 Q-PCR is a highly specific test since the

<table>
<thead>
<tr>
<th>Sampling</th>
<th>IS 900 Q-PCR (%)</th>
<th>Map culture (%)</th>
<th>ELISA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive (+)</td>
<td>Negative (-)</td>
<td>Positive (+)</td>
</tr>
<tr>
<td>1st</td>
<td>5 (12.5)</td>
<td>35 (87.5)</td>
<td>2 (5.0)</td>
</tr>
<tr>
<td>2nd</td>
<td>6 (15.0)</td>
<td>34 (85.0)</td>
<td>4 (10.0)</td>
</tr>
<tr>
<td>3rd</td>
<td>2 (5.0)</td>
<td>38 (95.0)</td>
<td>2 (5.0)</td>
</tr>
<tr>
<td>4th</td>
<td>3 (7.5)</td>
<td>37 (92.5)</td>
<td>2 (5.0)</td>
</tr>
</tbody>
</table>
Figure 1. IS900 Q-PCR carried out from genomic DNA obtained from bovine feces of the first sampling.

Table 2. Cohen's Kappa (K), positive predictive value (VP+), negative predictive value (VP-) and χ² for each sampling, comparing IS900 q-PCR and Bacteriological culture (n=40).

<table>
<thead>
<tr>
<th>Sampling number</th>
<th>IS900 Q-PCR vs. bacteriological culture</th>
<th>K</th>
<th>VP+ (%)</th>
<th>VP- (%)</th>
<th>χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td></td>
<td>0.538</td>
<td>40</td>
<td>100</td>
<td>0.0001</td>
</tr>
<tr>
<td>2nd</td>
<td></td>
<td>0.771</td>
<td>66</td>
<td>100</td>
<td>0.0000</td>
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<tr>
<td>3rd</td>
<td></td>
<td>0.294</td>
<td>33</td>
<td>92</td>
<td>0.0000</td>
</tr>
<tr>
<td>4th</td>
<td></td>
<td>0.411</td>
<td>33</td>
<td>100</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

average VP-reached 98% when compared with the microbiological culture and 97.5% when compared with ELISA. As such, individuals that had a negative result in the IS900 Q-PCR test had been correctly diagnosed as they were true negatives. The positive predictive value (VP+) reached 43 and 47.5% when comparing IS900 Q-PCR with the microbiological culture and ELISA, respectively (Tables 2 and 3).

Quantification of Map shedding

Map shedding is intermittent and starts in the early stages of the infection. The IS900 Q-PCR detected bovine from 12 months of age, that were already excreting the bacillus in feces, and as the samplings advanced, the elimination increased logarithmically (bovine 1, 10 and 36). On the first sampling, it was detected that five animals shed between \(1 \times 10^2\) and \(2.6 \times 10^3\) copies of the IS900 from Map per gram of feces. In the second sampling, six animals shed between \(3.25 \times 10^4\) and \(8.5 \times 10^6\). On the third sampling, two animals were detected that shed between \(2.03 \times 10^5\) and \(1.10 \times 10^6\), while in the fourth sampling three animals shed between \(7.92 \times 10^4\) and \(1.4 \times 10^7\) (Table 4).

DISCUSSION

Forty dairy cows, which had a mean of 12 months of age at the first sampling, were followed for a year in order to determine the time of shedding of Map using IS900 Q-PCR and bacteriological culture. From the first sampling, animals that shed Map were detected, demonstrating that bovines infected with paratuberculosis can shed the bacillus even in early infection stages without any clinical
Table 3. Cohen’s Kappa (K), positive predictive value (VP+), negative predictive value (VP-) and \( \chi^2 \) for each sampling, comparing IS900 q-PCR and ELISA (n=40).

<table>
<thead>
<tr>
<th>Sampling number</th>
<th>IS900 q-PCR vs. ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K</td>
</tr>
<tr>
<td>1st</td>
<td>0.53</td>
</tr>
<tr>
<td>2nd</td>
<td>0.62</td>
</tr>
<tr>
<td>3rd</td>
<td>0.22</td>
</tr>
<tr>
<td>4th</td>
<td>0.73</td>
</tr>
</tbody>
</table>

Table 4. Quantification of amplicon copies by q-PCR in DNA samples taken from bovine feces positive to IS900.

<table>
<thead>
<tr>
<th>Sampling</th>
<th>Mean age in months</th>
<th>Identification number</th>
<th>Number of IS900 map copies/g of feces</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>12</td>
<td>1</td>
<td>5.30 \times 10^2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>3.50 \times 10^2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>2.60 \times 10^3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>32</td>
<td>1.90 \times 10^2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>41</td>
<td>1.50 \times 10^3</td>
</tr>
<tr>
<td>2nd</td>
<td>15</td>
<td>1</td>
<td>5.11 \times 10^4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>5.00 \times 10^4</td>
</tr>
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<td></td>
<td></td>
<td>25</td>
<td>3.25 \times 10^4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>32</td>
<td>6.50 \times 10^6</td>
</tr>
<tr>
<td></td>
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<td>41</td>
<td>8.50 \times 10^8</td>
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<tr>
<td></td>
<td></td>
<td>36</td>
<td>7.00 \times 10^4</td>
</tr>
<tr>
<td>3rd</td>
<td>18</td>
<td>1</td>
<td>1.10 \times 10^6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>36</td>
<td>2.03 \times 10^5</td>
</tr>
<tr>
<td>4th</td>
<td>21</td>
<td>1</td>
<td>1.40 \times 10^7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>7.92 \times 10^5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>36</td>
<td>4.77 \times 10^6</td>
</tr>
</tbody>
</table>

signs of the disease (Park et al., 2016; Fang et al., 2002). Previously, Khol et al. (2010), had followed for 380 days a 2.5 year-old cow that had become naturally infected with Map, sampling feces on 9 occasions. Their results showed that the number of IS900 copies that were detected increased between 10^5 and 10^7 copies per gram of feces throughout the study, although in their third sample a slight decrease was observed, demonstrating that Map shedding is intermittent and can start from early infection stages (subclinical phase) before the infected animal becomes positive to the serological test. The results agree with those of Khol et al. (2010) in that Map shedding is intermittent and can start from early infection stages (subclinical phase) before the infected animal becomes positive to the serological test. The results obtained agree with those of kralik et al. (2011), because in the first sampling three animals were detected as positive and the quantification was obtained as 1.9 to 5.30 \times 10^2 copies of IS900 of Map per gram of feces.

Several authors have carried out studies on the correlation of q-PCR tests, bacteriological culture and ELISA with the diagnosis of paratuberculosis, with varying results. Pinedo et al. (2008) found that when comparing PCR tests with bacteriological culture and ELISA, Kappa values were 0.39 and 0.01, respectively. In contrast, Fang et al. (2002), when correlating IS900 Q-PCR with bacteriological culture, a Kappa value of 0.94 was found. In this study, Kappa values in the first, second and fourth samplings ranged from 0.53 to 0.73 indicating a moderate correlation, although the Kappa value in third sampling reached 0.22 indicating a fair correlation. It is possible that the latter is due to the fact that in this disease shedding of bacilli is intermittent and at the time of collecting the sample, it could have been absent from feces. Nevertheless, mistakes in DNA extraction or
sample contamination could also explain the results and it cannot be excluded (Khol et al., 2010). Correlation between bacteriological culture and Q-PCR depends on the stage of the infection present in the individual at the time of sample collection which has an effect on the amount of bacilli shed (Kralik et al., 2011; Aly et al., 2010; Pinedo et al., 2008). The ELISA used in the study detected less number of animals as positive, this is because, in early infection stages the predominant immune response is cell type, and the elimination of bacillus in feces begins in an intermediate way. Other reason for which the antibody production may be affected, is the cause of loss of immune response after infection. This suppressor activity is known as anergia. Animals in early infection stages, even if they excrete the bacteria in their feces, may not necessarily be detected by the ELISA test, since it may take several months, or years, before the level of circulating antibodies is sufficient to trigger a positive reaction (Soto et al., 2002a).

Bacteriological culture from tissues, feces, milk, and semen, among others, is considered the gold standard for the diagnosis of paratuberculosis. Nevertheless, such testing has its limitations since, although it has a 100% specificity, it has very low sensitivity mainly due to the intermittent nature of the shedding of bacilli through feces, milk and semen (Khol et al., 2010). Furthermore, the culture media needs to be enriched by mycobactin to improve the development of the bacillus and the incubation times are more than six-weeks long for isolating Map. Another important disadvantage of bacteriological culture is the high risk of contamination with other microorganisms, especially fungi, and the decrease in viable bacilli after the sample decontamination process. By using Q-PCR, results are obtained within at least two days, depending on the amount of samples to be processed, representing a clear advantage in time-to-diagnosis when compared with bacteriological isolation allowing the quantification of bacilli shedding in feces (Kralik et al., 2011; Khol et al., 2010). With the simultaneous use of IS900 Q-PCR, fecal culture, and the ELISA test, it is possible to detect a greater number of infected animals.

In control and diagnostic testing, the probability that an individual that has a positive result is indeed positive is known as the positive predictive value, while the negative predictive value is the percentage of individuals with a negative test that do not have the disease. It is important to note that the predictive value is closely related with the sensitivity and specificity of a test, as well as with the prevalence of the disease in the population where the test is to be carried out (Martínez et al., 2012). The positive predictive values (VP+) obtained in this study, ranging between 43 and 47.5%, are considered low and are probably due to the long sampling interval (90 days) and the intermittent shedding of bacilli. Nevertheless, the negative predictive values (VP-) where high (97.5-98%) which demonstrates that the IS900 q-PCR is highly specific and that negative tests were appropriately classified.

The IS900 marker is considered the standard for endpoint PCR and Q-PCR since there are between 14 and 20 copies of the sequence within the mycobacterium genome improving the sensitivity for detecting Map. Other genetic markers have been evaluated for detecting Map, such as the SF57, ISMav2 and HspX sequences, but they have the limitation that they are only present in 1 to 6 copies within the Map genome, therefore sensitivity is reduced, although specificity is increased. To increase specificity when using primers for IS900, these should be designed so that they amplify regions that are close to the 5’ end of this inserted sequence since it is highly conserved (Cook and Britt, 2007; Tasara and Stephan, 2005; Kim et al., 2002; Fang et al., 2002).

In this study, the IS900 Q-PCR detected that 12.5% of animals of the 12 months of age that were included in the first sampling where shedding bacilli through feces. This is important since the majority of the paratuberculosis clinical cases are observed in animals with two to six years of age and young animals, in general, do not show clinical signs but are already shedding the bacillus through their feces. As such, it is important that diagnostic tools are available that can detect the shedding of bacilli in the subclinical stage of the disease. In this sense, the IS900 Q-PCR is a good diagnostic alternative for detecting animals that are shedding the pathogen and prevent the spread of the disease by allowing the implementation of control and eradication measures for paratuberculosis.

Conclusions

The IS900 Q-PCR allows the diagnosis of early-stage paratuberculosis in animals from 12 months of age that do not have clinical signology of paratuberculosis. The technique is an alternative to carry out programs of control of paratuberculosis that allow the detection of animals that eliminate Map in early stages of infection.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

This study received financial support from CONACYT-SAGARPA research grant number 48176.

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Serological evidence of African horse sickness virus infection of donkeys in Karamoja sub-region, North-eastern Uganda

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Received 12 June, 2017; Accepted 17 August, 2017

African horse sickness virus (AHSV) causes a non-contagious, infectious insect-borne disease of equids and it is endemic in many areas of sub-Saharan Africa but extends beyond its endemic zones to the Arabian Peninsula, Asia and Europe. The usual mode of transmission is by biting midge, a biological vector and Culicoides imicola appears to be the principal vector. Serum samples were screened from camels and donkeys for AHSV antibodies using competitive enzyme-linked immunosorbent assay (cELISA). Results revealed that 16/22 (73%) donkeys had been exposed to AHSV. All 85 camels screened in the study tested negative to AHSV. This was the first study of AHSV in Uganda and it was geared at creating awareness for the veterinary service needs of these animal species which is non-existent so far.

Key words: African horse sickness virus (AHSV), Culicoides spp., camels, donkeys, Uganda.

INTRODUCTION

African horse sickness (AHS) is caused by a double stranded RNA virus of the family Reoviridae of the genus Orbivirus. There are nine antigenically distinct serotypes of AHS virus (AHSV) identified by virus neutralization (Howell, 1962; McIntosh, 1958). The hosts for AHSV are equids: horses, mules, donkeys and zebra. Zebra is believed to be the reservoir host (Barnard, 1998). Antibody is found in camels, African elephants, and black and white rhinoceroses, but their role in epidemiology is unlikely to be significant (OIE, 2009). Dogs acquire peracute fatal infection after eating infected horse meat (Bevan, 1911; Piercy, 1951), but are not a preferred host by Culicoides spp., therefore, are unlikely to play a role in transmission (McIntosh, 1955). Clinical manifestation of AHS in horses involve damage to the circulatory and respiratory systems resulting in serous effusion and haemorrhage in various organs and tissues (Awad et al., 1981; Coetzer and Erasmus, 1994; Lubroth, 1988). African horse sickness (AHS) is peracute, acute, subacute or mild but the disease is more severe in horses. Clinical manifestations of AHSV involve four forms: horse sickness fever, in the majority of cases (which usually
affects only mules, donkeys and partially immune horses); the subclinical cardiac form is suddenly followed by marked dyspnea and other signs typical of the pulmonary form. This could manifest as the cardio-pulmonary or mixed form or the peracute or pulmonary form (Maurer and McCully, 1963; Newsholme et al., 1983; Theiler, 1921). A nervous form may occur, though it is rare. All forms of disease can occur in any one outbreak but in susceptible populations of horses the mixed and pulmonary forms tend to predominate so mortality rates in these animals will be very high. Mortality rate ranges from 50 to 95% in horses to rare in African donkeys and zebra. Following recovery to AHVS, animals develop good immunity to the infecting serotype and partial immunity to other serotypes. There is no treatment for AHVS; the disease is managed by supportive treatment. Disease prevention is by vaccination with a polyvalent vaccine since all AHVS serotypes are present in South Africa and in most parts of sub-Saharan Africa. Several methods are employed for the diagnosis of AHVS, including virus inoculation of cell cultures, mice inoculation (Howell, 1962), postmortem, serology and molecular assays (Costa et al., 2016; Fowler et al., 2016; de Waal et al., 2016; Sánchez-Matamoros et al., 2016; Weyer et al., 2015). AHVS is not contagious, but is known to be spread by insect vectors. The biological vector of the virus is the Culicoides (midges) species (Theal, 1900; Wetzel et al., 1970). Culicoides midges, in general, breed in damp soil rich in organic matter, however C. bolitinos breeds in bovine dung, and it therefore not as dependent on annual rainfall and soil-type. Adult midges become infected by taking blood meals from viraemic animals. However, this disease can also be transmitted by species of mosquitoes including Culex, Anopheles, and Aedes, and species of tics such as Hyalomma and Rhipecephalus. Biting flies may also be able to transfer the virus. In Uganda, camels and donkeys are distributed in North-eastern Uganda in Karamoja and Sebei sub-regions. Zebras are found in the various conservation areas throughout the country while horses are sparsely distributed in Uganda. The horse medicine aspect of veterinary service in Uganda is not developed possibly because horses are not common in Uganda and their economic importance is limited. For this reason few people keep horses for prestige and deaths in these horses are common because during an emergency, the Ugandan veterinarians lack the expertise in horse medicine. This is the first report of AHVS in Uganda and it is geared at creating awareness for the need for equine veterinary intervention in these animals.

MATERIALS AND METHODS

Serum samples were collected from Karamoja sub-region in two districts namely: Moroto: N 2° 31' 41.604", E 34° 39' 28.794" and Amudat: N 1° 47' 29.841", E 34° 54' 23.583" districts, Uganda. The camels and donkeys were classified as: infant, juvenile, sub-adult and adult. Both sexes were sampled. Serum was collected from donkeys and camels from Karamoja sub-region in March, 2016.

Serological analysis

The animals were bled by the jugular vein following restraint. 2.5 ml blood was collected into plain vacutainer tubes without anti-coagulant. Serum was separated from the blood cells by centrifugation at 2500 rpm for 15 min and stored at -20°C until use in a competitive enzyme-linked immunosorbent assay (cELISA) (Inmunologia Y Genetica Aplicad, S. A. Madrid, Spain). In total, 110 samples were collected. These included 25 donkeys and 85 camels. Purposive sampling was employed due to the availability of the animals.

RESULTS AND DISCUSSION

16/22 donkeys tested positive to AHVS antibodies. All the 85 camels screened alongside the donkeys tested negative to the viral antibodies. Corrected optical densities (ODs) were calculated from sample ODs and blank ODs. Sample Id represents animal species, age, sex and sample number.

Results revealed that 16/22 (73%) of serum samples from donkeys tested positive to AHVS antibodies (Table 1). All the 85 camels tested negative to AHVS. No previous research has been done on AHVS in Uganda. Literature on AHVS research in Africa and other parts of the world is scanty although reports in South Africa exist (Lieberberg et al., 2016). Not much research interest on biting mides (Culicoides spp.) in Uganda (Mayo et al., 2016; Lieberberg et al., 2016; Probst et al., 2015) and not much interest in equine and cameline species in Uganda and their economic importance hence population structure is limited. Nakayima et al. (2017a, b) reported endo-parasites and equine piroplasmosis in these animals in Karamoja sub-region in the absence of veterinary care and these diseases are also prevalent around the globe (Singh et al., 2012; Sumbria et al., 2016; Singla and Sumbria, 2017).

The distribution of AHVS is determined by several factors including the efficiency of control measures, availability of vertebrate hosts or reservoirs, vector abundance, seasonality and climate. AHVS apparent infection rate rapidly fall to zero at temperatures below 15°C since virus replication does not seem to occur below this temperature (Wellby et al., 1996). However, overwintered midges could harbor “latent” virus in some of these surviving midges that will commence replication and transmission should temperatures rise to permissive levels for example during spring. The major vector of AHVS, Culicoides imicola adults are active at temperatures as much as 3°C lower than the minimum required for AHVS replication (Sellers and Mellor, 1993). The seasonality of AHVS is explained by vector activity; after the rainy season in the tropics, in the summer and autumn in temperate regions. Bluetongue virus shares the same vector species (Culicoides) (Boorman et al., 1975; Mellor, 2000; Mellor et al., 1975; Venter et al., 2000;
Table 1. Sero-prevalence of AHSV in donkeys from Karamoja sub-region, North-eastern Uganda.

<table>
<thead>
<tr>
<th>S/N</th>
<th>Animal species</th>
<th>Sample ID</th>
<th>OD reading</th>
<th>Corrected OD</th>
<th>AHSV result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Donkey</td>
<td>D/A/F/02</td>
<td>0.152</td>
<td>101.7</td>
<td>Positive</td>
</tr>
<tr>
<td>2</td>
<td>Donkey</td>
<td>D/SA/F/03</td>
<td>0.459</td>
<td>83.2</td>
<td>Positive</td>
</tr>
<tr>
<td>3</td>
<td>Donkey</td>
<td>D/A/F/04</td>
<td>0.137</td>
<td>102.6</td>
<td>Positive</td>
</tr>
<tr>
<td>4</td>
<td>Donkey</td>
<td>D/A/F/05</td>
<td>0.11</td>
<td>104.3</td>
<td>Positive</td>
</tr>
<tr>
<td>5</td>
<td>Donkey</td>
<td>D/A/F/06</td>
<td>0.103</td>
<td>104.7</td>
<td>Positive</td>
</tr>
<tr>
<td>6</td>
<td>Donkey</td>
<td>D/A/F/07</td>
<td>1.827</td>
<td>0.4</td>
<td>Negative</td>
</tr>
<tr>
<td>7</td>
<td>Donkey</td>
<td>D/A/F/08</td>
<td>1.974</td>
<td>-5.0</td>
<td>Negative</td>
</tr>
<tr>
<td>8</td>
<td>Donkey</td>
<td>D/A/F/09</td>
<td>1.841</td>
<td>-5.0</td>
<td>Negative</td>
</tr>
<tr>
<td>9</td>
<td>Donkey</td>
<td>D/A/M/10</td>
<td>0.126</td>
<td>103.3</td>
<td>Positive</td>
</tr>
<tr>
<td>10</td>
<td>Donkey</td>
<td>D/A/M/11</td>
<td>1.972</td>
<td>-8.4</td>
<td>Negative</td>
</tr>
<tr>
<td>11</td>
<td>Donkey</td>
<td>D/A/F/12</td>
<td>0.098</td>
<td>105.0</td>
<td>Positive</td>
</tr>
<tr>
<td>12</td>
<td>Donkey</td>
<td>D/A/F/13</td>
<td>0.098</td>
<td>105.0</td>
<td>Positive</td>
</tr>
<tr>
<td>13</td>
<td>Donkey</td>
<td>D/A/F/14</td>
<td>0.121</td>
<td>103.6</td>
<td>Positive</td>
</tr>
<tr>
<td>14</td>
<td>Donkey</td>
<td>D/A/M/15</td>
<td>0.114</td>
<td>104.0</td>
<td>Positive</td>
</tr>
<tr>
<td>15</td>
<td>Donkey</td>
<td>D/A/M/16</td>
<td>0.132</td>
<td>102.9</td>
<td>Positive</td>
</tr>
<tr>
<td>16</td>
<td>Donkey</td>
<td>D/A/F/17</td>
<td>0.689</td>
<td>69.2</td>
<td>Positive</td>
</tr>
<tr>
<td>17</td>
<td>Donkey</td>
<td>D/A/F/18</td>
<td>0.097</td>
<td>105.1</td>
<td>Positive</td>
</tr>
<tr>
<td>18</td>
<td>Donkey</td>
<td>D/A/M/19</td>
<td>0.096</td>
<td>105.1</td>
<td>Positive</td>
</tr>
<tr>
<td>19</td>
<td>Donkey</td>
<td>D/SA/F/20</td>
<td>1.755</td>
<td>4.7</td>
<td>Negative</td>
</tr>
<tr>
<td>20</td>
<td>Donkey</td>
<td>D/A/F/55</td>
<td>0.605</td>
<td>74.3</td>
<td>Negative</td>
</tr>
<tr>
<td>21</td>
<td>Donkey</td>
<td>D/SA/F/56</td>
<td>0.335</td>
<td>90.7</td>
<td>Positive</td>
</tr>
<tr>
<td>22</td>
<td>Donkey</td>
<td>D/C/M/57</td>
<td>0.135</td>
<td>102.8</td>
<td>Positive</td>
</tr>
</tbody>
</table>

With the advent of climate change the midge vector has now significantly extended its range northwards into Europe. Since 1998, bluetongue virus has caused disease outbreaks and has become endemic in Europe. AHSV is widely distributed across sub-Saharan Africa (Mellor and Boorman, 1995; Howell, 1963), from Senegal and Gambia in the west to Ethiopia and Somalia in the east, and extending as far south as northern South Africa, and may extend at times to Egypt in the north (Howell, 1963). The Sahara desert serves as an effective geographical barrier preventing the infection from spreading northwards. Probably AHSV has its first historical reference traced to an epizootic in Yemen which occurred in 1327 (Moule, 1896; Sailleau et al., 2000). However, the virus is believed to have originated from Africa following the introduction of susceptible equine breeds during exploration of central and eastern Africa (M’Fadyean, 1900). The earliest account of the disease in Africa traces back to 1569 (Theal, 1900). The first detection of AHSV in South Africa was in 1719, a major outbreak that killed 1,700 animals in the Cape region. However, before this, the wildlife reservoirs could have been circulating the disease (Mornet and Gilbert, 1968). The disease is endemic in these areas with subsequent outbreaks and massive horse deaths (Mellor and Hamblin, 2004). During outbreaks of AHS in endemic areas, different virus serotypes may be active simultaneously within an area, but one serotype usually dominates during a particular season, followed in the following year by the dominance of another serotype. AHSV is a major challenge to horses in endemic areas in sub-Saharan Africa, but it repeatedly caused large epizootics in the Mediterranean region (North Africa and southern Europe in particular) as a result of trade in infected equids.

Conclusion

AHSV could be endemic in the equine population in Uganda but goes undiagnosed. Zebras in wildlife conservation areas and donkeys could be acting as reservoirs to the infection. No information about the disease is available in Uganda hence no control measures in place. This is a threat to the horse population in Uganda and neighboring countries. There is need to improve knowledge of equine and cameline medicine and welfare in Uganda.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

This study was financially supported by the Government...
of Uganda under National Agricultural Research Organization (NARO).

REFERENCES


Aerobic and anaerobic bacterial isolates from the respiratory tract of sheep slaughtered at Addis Ababa Abattoirs Enterprises, Central Ethiopia

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Received 16 March, 2017: Accepted 25 July, 2017

The present study was an endeavor to isolate and identify the various bacteria localizing pneumonic lungs and the associated tracheas of sheep slaughtered at Addis Ababa Abattoirs Enterprise, Central Ethiopia, in both aerobic and anaerobic conditions. A total of 60 pneumonic lungs and 60 tracheal swabs were examined bacteriologically. From all the samples collected, a total of 440 bacterial isolates (239 from the aerobic culture and 201 from the anaerobic culture) were obtained. The result of aerobic isolates include: Staphylococcus species (31.38%), Pasteurella hemolytica (29.71%), Bacillus species (10.04%), Bibersteinia trehalosi (6.69%), Micrococcus (3.77%), Escherichia coli (3.35%), Streptococcus species (2.51%), Rhodococcus equi (2.93%), Pseudomonas species (2.09%), Klebsiella pneumoniae (0.84%), Actinobacillus species and Bordetella species (1, 29%); whereas Staphylococcus species (26.87%), P. hemolytica (37.81%), Bacillus species (3.98%), B. trehalosi (10.45%), Micrococcus (3.48), E. coli (6.97%), Streptococcus species (0.5%), Rhodococcus equi (0.5%), Klebsiella pneumoniae (2.99%) and Actinobacillus species (1.49%) were among anaerobic isolates. Thus, isolation of multiple bacterial species from the respiratory tracts of pneumonic sheep in this study signifies their possible role in the involvement of respiratory diseases. Appropriate prevention and control methods should be established along with identification of the most pathogenic species by future studies.

Key words: Bacteria, lung, pneumonia, sheep, trachea.

INTRODUCTION

Ethiopia is home to various indigenous sheep breeds. From the total livestock population of the country, sheep owns about 46%. Despite this huge resource, Ethiopian sheep productivity remains far lower than expected. The

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major biological constraints contributing to low productivity include bacterial and parasitic infections (Kaur et al., 2009; Leta and Meles, 2014; CSA, 2014-15; Fikru and Gebeeyehu, 2015; Gebremeskel et al., 2017; Pawar et al., 2017; Singh et al., 2017). The lungs are continuously exposed to air that contains dust, bacteria, fungi, viruses and various noxious agents and defense against these potentially harmful materials is controlled by a complex of protective mechanisms (Mohan et al., 2013). Stress factors such as inclement of weather, cold and stress of weaning, transportation, poorly ventilated housing and nutritional deficiencies have predisposing roles. In addition, concurrent infections with some viruses, bacteria or parasitic infestation degrade the potential of the host to combat infections (Gebremeskel et al., 2017; Gupta et al., 2009; Radostits et al., 2000).

The impact of respiratory disease is extensive and can be measured as the sum of the direct economic losses occurring due to mortality, morbidity, treatment and prevention costs. Loss of production (reduced animal performance and carcass quality) and the indirect costs such as labor, infrastructures and intangibles (Jim, 2009; Monot et al., 2015). A number of bacterial, viral and parasitic agents participate in sheep respiratory diseases, however most important include: mycoplasma species such as Mycoplasma ovipneumoniae, Mycoplasma arginini, Mycoplasma agalactiae (Lin et al., 2008), lung worms particularly (Dictyocaulus filaria) (Borji et al., 2012) and bacteria like Pasteurella multocida, Mannheimia haemolytica, Chlamydia psittaci, Histophilus somni, which can suppress the animal's immune system, allowing opportunistic microorganisms to colonize the lung and cause the disease (Angen et al., 1998; Radostitis et al., 2000; Tesfaye et al., 2013; Fulton, 2009; Alemneh and Tewodros, 2016). Therefore, objectives this study is to identify most bacterial pathogens involved in pneumatic lung of sheep as well to compare and contrast the types of isolated bacterial species in different sites of the respiratory system both in aerobic and anaerobic environments.

MATERIALS AND METHODS

Study area

The study was conducted from October 2010 to April 2011 at Addis Ababa abattoir enterprises, central Ethiopia. Geographically Addis Ababa is located 9°2' N and 38° 42'E having elevation of 2400 above sea level (a.s.l) and mean annual rainfall of 1800 mm. The city has average minimum and maximum annual temperature of 10.7 and 23.6°C, respectively (NMSA, 2005; Jury and Chris, 2013).

Study animals and sampling strategy

The study was conducted on 60 randomly selected sheep lungs with pneumatic lesions and tracheal swabs slaughtered at Addis Ababa Abattoirs Enterprises. Samples were kept separately and transported to the School of Veterinary Medicine, Microbiology Laboratory of Addis Ababa University in a cool box containing ice pack at 4°C.

Sample collection

Tracheal swabs

Samples were taken with sterile cotton swabs moistened with tryptose soya broth from the trachea of sheep. Two swabs were introduced directly into the trachea of slaughtered sheep and rubbed smoothly against the mucosa in a circular motion. The swabs were allowed to remain in contact with the secretions for up to 1 min, and the two swabs collected from each sheep were kept in a tryptose soya broth transport medium and transported to the laboratory (Lees et al., 1990; OIE, 2008).

Pneumonic lung tissue

Samples of pneumatic lung tissue were collected at post mortem for microbial culture. Each piece of tissue was placed in a fully labeled separate sterile screw capped universal bottle. Containers were fully labeled with the date, tissue and sterile instruments (knife, scalpel, forceps and scissors) were used for collecting specimens for microbiological cultures. After collection and transportation to the laboratory, the samples were processed immediately (Gebremeskel et al., 2017).

Bacteriological sample processing

Culturing the tracheal swabs

The broth culture samples were incubated overnight under aerobic and anaerobic conditions, respectively. After 24 h of incubation the samples were thoroughly agitated, mixed and a loop of broth cultures was taken and streaked over labeled Petri plates containing blood agar base supplemented with 7% sheep blood as described by Quinn et al. (1994).

Culturing the lung tissues

The outer surfaces of the lungs were first seared with a heated spatula, followed by cutting and mincing of the inner surface of the lungs using sterile scissors and forceps, and then transferred to sterile Petri dish. The minced interior part of the lungs were further incised with sterile scalpel blade, then printed on the blood agar and streaked with wire loop. All bacteriological procedures were conducted in a level two biological safety cabinet.

Cultural characterization and bacteriological examination

The growths of typical colonies on blood agar were characterized based on the presence or absence of hemolysis, the type of hemolysis and general appearance of the colonies (color, shape, size, consistency etc.). On MacConkey agar, the colonies were examined for the presence or absence of growth, general appearance and ability to ferment lactose (Sharma and Adiakha, 1996; Alemneh and Tewodros, 2016). All cultures were incubated under aerobic and anaerobic conditions at 37°C for 24 to 48 h.

Isolation and Identification

Single colony type from pure cultures on blood agar was transferred
to nutrient agar for a series of primary and secondary biochemical tests. Primary tests such as Grams staining, motility, catalase, oxidase, and oxidative-fermentative (O-F) tests were conducted. In addition, secondary biochemical tests including indole, methyl red, and citrate utilization tests were performed for further confirmation of the isolates. General procedures for isolation and identification of Gram positive and Gram negative bacteria were as described by Carter (1984) and Quinn et al. (1994).

**Data analysis**

Descriptive statistics was performed to analyze the data obtained from the study. The number of each species/genera was expressed as a percentage in comparison to the total number of isolates.

**Ethical approval**

The study considered direct observation of slaughter animals in the abattoirs and took appropriate samples for further microbiological examination. As a result of this study, no animal was subjected to suffer. Nevertheless, ethical approval was conducted by Research Ethical Approval Committee of Addis Ababa University, School of Veterinary Medicine, Ethiopia.

**RESULTS**

The predominant species among the aerobic isolates were *Staphylococcus* species (31.38%), followed by *M. haemolytica* (29.71%), *Bacillus* species (10.04%), *B. trehalosi* (6.69%), *Micrococcus* species (3.77%), *E. coli* (3.35%), *Rhodococcus equi* (2.93%), and *Pseudomonas* species (2.09%). On the other hand *Streptococcus, Bordetella, Klebsiella* and *Actinobacillus* were among the least encountered bacterial genera as indicated by Figure 1.

The majority of the isolates (aerobic and anaerobic) colonize the two anatomical sites investigated with *Streptococcus, Rhodococcus equi, Pseudomonas,* and *Actinobacillus* as exceptions which were not seen in trachea. However, a general increase in the isolation rate was observed as one that goes down the respiratory tract. In a nutshell, Gram positive bacteria were the predominant species inhabiting the respiratory tract in aerobic condition, whereas Gram negative bacteria predominate in anaerobic conditions.

A total of 191 facultative bacteria were isolated in anaerobic condition with Gram negative bacteria as the dominant isolates. The isolated bacteria with their isolation rate include *M. haemolytica* (37.81%), *Staphylococcus* species (26.87%), *B. trehalosi* (10.45%), *E. coli* (6.97%), *Bacillus* species (3.98%), *Micrococcus* species (3.48%), *K. pneumoniae* (2.99%), *Actinobacillus* species (1.49%), *Streptococcus* species (0.5%) and *Rhodococcus equi* (0.5%) as denoted by Figure 2.

**DISCUSSION**

The present study assessed the frequency and type of aerobic and facultative anaerobic bacteria isolated from ovine pneumatic lungs and their associated trachea at Addis Ababa Abattoir Enterprises, Central Ethiopia among which several of them were isolated. A number of workers such as Gebremeskel et al. (2017), Akloul and Mohammed (2016), Sarkar et al. (2016), Porter et al. (1994), Al Sultan (1995), Barbour et al. (1997), Almeida et al. (1986), Okolo (1985), Esra et al. (2009) and Richard et al. (1986) have isolated similar bacteria from pneumatic lungs of domestic animals. The invariable isolation of those organisms from pneumatic lungs of various animal species may indicate their significance in various respiratory syndromes in different animal species (Megra et al., 2006).

Among 239 isolates, *Staphylococcus* species were the dominant bacteria having high proportion accounts (23.25 and 35.95%) of the trachea and lung isolates, respectively. In agreement to this study *Staphylococcus* species were isolated and reported in high proportion by few studies such as Alley (1975) and Queen et al. (1994). Other investigations includes that by Esra et al. (2009) who isolated *Staphylococcus* spp. from unhealthy Holstein cattle nasal cavities with high frequency and percentage, and Asaduzzaman et al. (2013) who reported on *Staphylococcus* species from Black Bengal goat in Bangladesh. These indicate that there is a probability of association between these bacteria and pneumatic syndrome of lung. The bacteria are commensally living in the mucous membrane of the upper respiratory tract of animals and are opportunistic pathogens (Rashid et al., 2014; Quinn et al., 1994).

*Bacillus* species has been described as ubiquitous microbes found in nature as normal micro-flora. Thus, their role in the pathogenesis of respiratory infections is thought to be insignificant (Carter et al., 1995; Garedew et al., 2010). A high proportion of *Bacillus* species was obtained mainly from the lungs in this study. Other studies reported similar data such as Asaduzzaman et al. (2013), Garedew et al. (2010), Rajiv et al. (2000) and Shigidi (1973) from various domestic animals.

*Micrococcus* species is known primarily to be a normal flora of the respiratory tract. However, in most of the infections along with other pathogens, it may flare up and act as a secondary invader (Akloul and Mohammed, 2016; Carter, 1984). Our finding indicated that only 3.77% of *Micrococcus* species were isolated from ovine pneumatic lungs, with similar studies found to have agreed with present results such as Marru et al. (2013) and Esra et al. (2009).

Streptococcal are resident flora of the upper respiratory tract mucous membrane and commonly associated with suppuration and abscess formation (Quinn et al., 1994; Obasi et al., 2001). Although streptococci have not been associated with respiratory problems in ruminants, they are well known pathogens in humans, equine and camels. In this study, isolation of these bacteria might be an indication of its involvements as opportunistic
Bacterial isolates (%)

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unidentified</td>
<td>4.6</td>
<td>18.33</td>
<td>33.33</td>
</tr>
<tr>
<td>Actinobacillus spp.</td>
<td>0.84</td>
<td>3.33</td>
<td>0</td>
</tr>
<tr>
<td>Klebsiella pneumonia</td>
<td>0.84</td>
<td>3.33</td>
<td>0</td>
</tr>
<tr>
<td>E. coli</td>
<td>3.35</td>
<td>6.67</td>
<td>0</td>
</tr>
<tr>
<td>Bordetella spps</td>
<td>1.26</td>
<td>5</td>
<td>1.67</td>
</tr>
<tr>
<td>Pseudomonas spps</td>
<td>2.09</td>
<td>8.33</td>
<td>0</td>
</tr>
<tr>
<td>Rhodococcus equi</td>
<td>2.93</td>
<td>11.6</td>
<td>0</td>
</tr>
<tr>
<td>B. trehalosi</td>
<td>6.69</td>
<td>11.6</td>
<td>0</td>
</tr>
<tr>
<td>M. hemolytica</td>
<td>29.71</td>
<td>41.6</td>
<td>76.67</td>
</tr>
<tr>
<td>Bacillus spps</td>
<td>10.04</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>Micrococcus spps</td>
<td>3.77</td>
<td>13.33</td>
<td>1</td>
</tr>
<tr>
<td>Streptococcus spp</td>
<td>2.51</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Staphylococcus spp</td>
<td>31.38</td>
<td>91.67</td>
<td>3.33</td>
</tr>
</tbody>
</table>

Figure 1. Bacterial isolates (%) under aerobic condition from respiratory tract of sheep slaughtered at Addis Ababa Abattoirs Enterprises. (A) Overall proportion of bacterial isolates; (B) Bacterial Isolates from lung; (C) Bacterial isolates from trachea.

Pathogens in sheep pneumonia. *M. haemolytica*, is a normal flora of the upper respiratory tract and may play a secondary role after the primary initiating agent suppressed the host’s defense mechanisms, and favors the multiplication of *Pasteurella* species leading to bronchopneumonia (Aiello and May, 1998; Buxton and Frazer, 1977). Stress factors with or without viral infections interact to suppress the host defense mechanisms which allow the proliferation of commensal bacteria in the respiratory tract of animals (Baker, 1998).

Conclusion

This study revealed that the isolation of multiple bacterial species from the respiratory tracts of pneumonic sheep signifies their possible role in the involvement of respiratory disease complex. However, viruses and mycoplasma species are expected to reside in the respiratory tract. Therefore, the extent of the impact of respiratory diseases on sheep production and a complete understanding of the respiratory microbial flora, both culturable and unculturable condition give future research warranty.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

The authors are indebted to Addis Ababa University for the support to conduct this study.
Figure 2. Bacterial isolates (%) under anaerobic condition from the respiratory tract of sheep slaughtered at Addis Ababa Abattoirs Enterprises. (A) Overall proportion of bacterial isolates; (B) Bacterial isolates from lung; (C) Bacterial isolates from trachea.

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