JIVS JOURNAL OF ISTANBUL VETERINARY SCIENCES

Volume 4; Issue 2, August 2020 E-ISSN: 2602-3490

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Contents

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Determination of crude protein and metabolized energy with near infrared reflectance spectroscopy (NIRS) in ruminant mixed feeds

Research Article
Volume: 4, Issue: 2
August 2020
Pages: 31-36

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ABSTRACT

This study aims to determine the crude protein and metabolizable energy values in ruminant mixed feeds based on the measurements obtained from Near Infrared Reflectance Spectroscopy (NIRS) device. The mixed feeds used in the study were produced in Balıkesir. Reference analyses were determined by making chemical analyses of mixed feed samples used in the study. Metabolic energy value of mixed feed samples was calculated by using the determined nutrient values in equations. Portable NIRS device was used in the study. The relationship between estimates obtained from NIRS device and reference values was statistically evaluated. In regression analysis, R2 value was found as 0.0064 for crude protein while it was 0.9397 for metabolizable energy. It has been demonstrated that the NIRS method is a fast, reliable and good estimation method for quantitatively determining the metabolic energy value in ruminant mixed feeds.

Article History
Received: 19.04.2020
Accepted: 24.05.2020
Available online: 26.05.2020

Keywords: mixed feed, metabolizable energy, near infrared reflectance spectroscopy (NIRS)

DOI: https://doi.org/10.30704/http-www-jivs-net.723231

To cite this article: Atalay, H., & Bilal, T. (2020). Determination of crude protein and metabolized energy with near Infrared reflectance spectroscopy (NIRS) in ruminant mixed feeds. *Journal of Istanbul Veterinary Sciences. 4*(2), 31-36, **Abbreviated Title:** *J Ist Vet Sci*

Introduction

Energies are transformed into one form to another in our lives. Green plants store the physical energy of the sun by converting it into chemical energy. Humans and animals transform chemical energy in plants into biochemical energy (ATP) by biochemical reactions (oxidation). Energy is not wasted, instead offered to people to use. Energy which is an abstract concept can be simply defined as the ability of the body to work. There are various forms of energy and these forms can be transformed into heat energy. However, heat energy cannot be transformed into other forms of energy. The chemical energy (gross energy) in the feed can be determined by the heat energy released as a result of the burning of the feed substance in the calorimetry bomb. Metabolizable energy is obtained by removing the energies of feces, urine and fermentation gases (methane) from gross energy (Kirchgessner, 1985).

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Many chemical analyses are used to estimate the quality of mixed feeds and their relationship to animal performance. Neutral Detergent Fiber (NDF) and Acid Detergent Fiber (ADF) analyses recommended by Van Soest are chemical analyses that are obtained using the in vitro technique and used to predict dry matter intake (DMI) and digestible dry matter (DDM). In these analyses, the organic parts of the plants are divided into two as intracellular components (lipids, sugars, organic acids, starch, non-protein nitrogen, soluble protein, pectin and soluble matter) and cell wall components (hemicellulose, cellulose, lignin, lignified N compounds, keratin, silica, heat damaged protein) according to the chemical detergents (Rohweder et al., 1978).

In plants, carbohydrates are divided into two as structural and non-structural carbohydrates. Structural carbohydrates on cell wall are pectin, β -glucan, galactosides, arabinoxylan (pentosan), hemicellulose, cellulose, and lignin, whereas non-structural carbohydrates in the cell are organic acids, starch, and sugars. Carbohydrates are produced by photosynthesis and stored in plants. Carbohydrates in plants consist of cell content (sugars and starch) and cell wall elements (cellulose, hemicellulose, lignin). Starch is highly found in grain feeds while cellulose is found in roughages in high density. Starch and cellulose are glucose molecules linked by different chemical bonds. Starch is digested with enzymes in the digestive system, and cellulose is digested with enzymes produced by microorganisms in the digestive system. Hemicelluloses are also digested by microorganisms in the digestive system and transformed into organic acids used as the main energy source of the organism. The biggest component of the plant cell wall is cellulose. It is a glucose polymer combined with a β-1,4 glycoside bond. Hemicellulose is a heterogeneous polysaccharide (galactose, arabinose) with 5 and 6 carbons such as xylose, glucose and combined with β-1,4 glycoside bond. Lignin is not a carbohydrate, but a phenol alcohol polymer. Microorganisms found in rumen break down cellulose in the feed with the enzymes they synthesize, and thus essential fatty acids, carbon dioxide and methane gas are generated and energy is obtained (Tekce and Gül, 2014).

The cell wall components are digested by fermenting the feed cell wall with the enzymes produced and secreted by the cellulotic bacteria in the rumen. Cellulotic bacteria rapidly break down the water-soluble sugar, starch and pectin, that is, the cell content of the feed. However, hemicellulose breaks down the cell wall components of the water-insoluble slowly and the cellulose breaks down them even more slowly. Lignin is not a carbohydrate, and it cannot be broken down by cellulotic bacteria (Van soest, 1994; Yavuz, 2005).

Carbohydrates constitute the main component of Ruminant rations and are divided into two as structural (cellulose, hemicellulose) and non-structural (starch, sugar) carbohydrates. It is effective on milk fat of structural carbohydrates, acetic acid / propionic acid ratio in rumen, dry matter consumption, rumen pH, saliva amount and chewing activity (Özen et all, 2005; Saçaklı et all 2007).

The higher roughage is in the ration, the higher acetic acid is produced. The higher protein is, the higher butyric acid is created. And the higher mixed feed is, the higher propionic acid occurs. Acetic acid and butyric acid form milk synthesis and milk fat. Propionic acid provides energy. Cellulotic bacteria are active between pH 6.2-6.8 but are inactive below pH 6. Amylotic bacteria that provide starch digestion are active between Ph 5.2-6.08 (Li et al., 2012).

Near Infrared Analysis is a method used by the feed industry to evaluate the nutrient content of feeds. Near-infrared reflection spectrophotometer is a fast, quick and chemical-free computerized analysis method used to analyze the nutrient content of forage crops and other feedstuffs. Traditional analysis methods are expensive and time-consuming as wet chemicals are used. Since this method uses infrared light instead of chemical, it has low cost and is more environmentally friendly. In the near infrared analysis, the feed sample is exposed to the infrared light source in the spectrophotometer. The reflected infrared light is transformed into

electrical energy and transferred to the computer. The nutrient content of the feed absorbs and reflects near infrared light. Different organic components in feeds can be measured with these different lights reflected in spectrophotometry and each different organic component in a feed can be calculated. The calculation of the reflection of nutrients in the feeds analyzed by chemical methods in spectrophotometry with a computer program constitutes the NIRS analysis. After a feed is placed in the NIRS device, the device compares the wavelength reflections from the feed and matches them from the sample library containing the pre-analyzed feed items and determines the nutrient content (Richard and Church, 2010).

The ability to measure nutrient components (protein, fat) in the feedstuff in NIRS analysis is related to the energy movement of the hydrogen bond. The chemical bonds formed by the hydrogen in the nutrient with other atoms (-CH, -OH, -NH, -SH) absorb energy at specific wavelengths and change the wavelengths (radiation intensity) reflected from the nutrient. The principle of the NIRS analysis is based on the absorption of electromagnetic radiation of near infrared light in the feed material. The radiation reflected from the feed is measured in the energy detector. The electromagnetic wavelength of NIRS is between 700-3000 nm. The analysis is usually performed between 1100-2500 nm wavelength (Ünal, 2005).

This study aims to investigate whether it is possible to determine the crude protein and metabolizable energy values of ruminant mixed feeds by using NIRS analysis. Although analysis with NIRS has advantages such as being very fast and not using chemicals, calibration to the device is an important disadvantage that requires advanced knowledge and experience to overcome.

Materials and methods

Reference Analysis (Chemical Analysis): Examples of ruminant mixed feed used in the study are produced in Balıkesir. 0.5 kg fresh mixed feed samples (n: 10) were taken from the feed factories and brought to the laboratory in air-tight bags and kept at -20 ° C until analysis. Feed samples were milled in the Retsch ZM 200 ultra centrifugal mill in a 1mm screen for analysis. The reference analyses (chemical analyses) of the feeds used in the study were carried out according to the methods reported in Balıkesir University, Faculty of Veterinary Medicine, the laboratory of Animal Nutrition and Nutritional Diseases as the crude protein method 990.03, crude cellulose method 962.09, crude oil method 920.39 (AOAC, 1997). Metabolizable energy values were calculated according to the metabolic energy formula in the ruminant mixed feeds reported the Turkish Standards Institute (Türk Standartlar Enstitüsü, 1991) and given below.

ME, (kcal/kg OM) = 3260 + (0,455*HP) – (4,037*HS) + (3,517*HY)

OM = Organic matter

CP = Crude Protein, g/kg OM

CS = Crude Cellulose, g / kg OM

CF = Crude Fat, g / kg OM

NIRS Spectroscopy Measurements: Spectral measurements of ruminant mixed feed samples were conducted on a portable NIRS device (Dinamica Generale Agri NIR Analyzer, Italy). Using the ready and defined calibration set in the Portable NIRS Device, crude protein, crude oil and crude cellulose data were taken, and these data were calculated in the metabolizable energy formula Turkish Standards Institute, 1991 (Türk Standartlar Enstitüsü, 1991) and metabolizable energy values of mixed feeds were calculated.

Statistical Analysis: The data obtained from the study were analyzed with the SAS (SAS Institute, 1999). statistical software package.

Results and Discussion

Descriptive statistics for reference analysis for crude protein and metabolizable energy of ruminant mixed feed are given in Table 1. In Table 2, Wilcoxon test results for reference and NIRS analysis are given for crude protein and metabolizable energy. The differences between reference analyses for crude protein and ME and

Table 1. Descriptive statistics regarding reference analysis of crude protein and metabolizable energy

	Crude protein	Metabolizable energy
n	10	10
Mean	17.94	3237.7
Min	15.62	3224.0
Max	23.73	3264.5
Standard Deviation	2.38	12.4

Table 2. Wilcoxon test results for crude protein and ME

Trait	Group-Comp	Diff	*P* value	Significance
Crude Protein	Ref-NIR	-2.42	0.00617	**
ME	Ref-NIR	-7.18	0.161	ns

NIRS estimates are shown in Figure 1. It was observed that the mean of NIRS estimates for crude protein ratio was significantly higher than the reference analysis results. Although the mean of the NIRS estimates for the ME value was higher than the reference analyses, the difference between the two groups was not statistically significant. Figure 2 shows the results of the regression analysis between the reference analysis of the crude protein and metabolizable energy values and the NIRS results.

In their study, Çelik et al. determined metabolized energy values of dairy cattle and beef cattle as 2549 and 2541 kcal / kg OM, respectively (Çelik et al., 2003). Baran et al. found metabolized energy values of dairy cattle and beef cattle as 2667 and 2673 kcal / kg OM, respectively (Baran et al., 2008a).

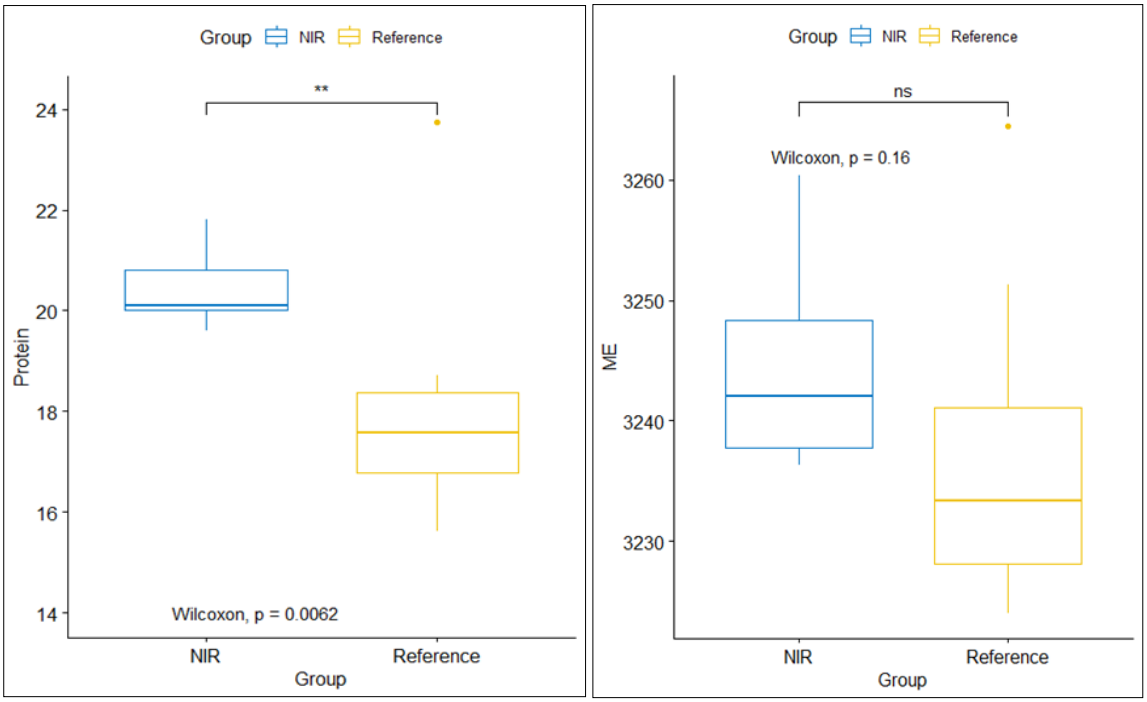

Figure 1. Differences between reference analysis and NIR estimates for crude protein ratio and Metabolizable energy value

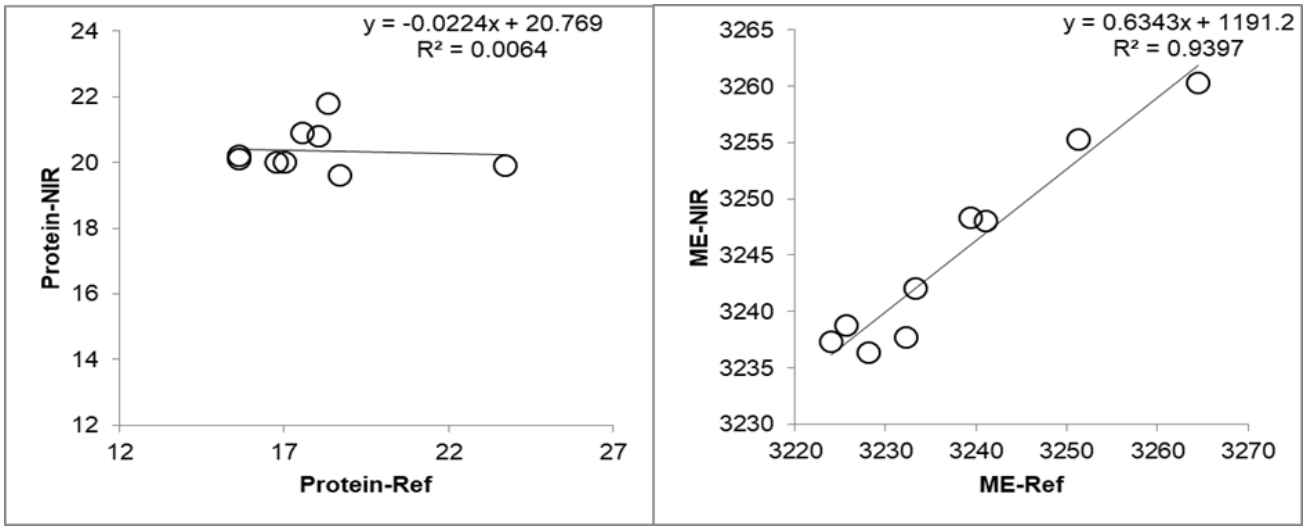

Figure 2. Regression analysis between reference analysis and NIR results of crude protein and metabolizable energy values.

Baran et al., also found crude protein values in dairy cattle and beef cattle as 14.81% and 13.17%, respectively (Baran et al., 2016). In their study, Baran et al., obtained crude protein values in dairy cattle) and beef cattle as 15.13% and 12.60%, respectively (Baran et al., 2008a). In the study conducted by Baran et al., the crude protein values of dairy cattle and beef cattle were 14.39% and 13.10%, respectively (Baran et all., 2008b). In the thesis study conducted by Gündüz, 2013 crude protein value in milk feed was shown to be 21.26 % (Gündüz, 2013).

NIRS calibrations have been reported as accurate enough to estimate moisture, crude protein, crude fat, crude fiber and energy content in ruminant mixed feeds (Boover et al., 1995), which is similar to our results.

It has been stated that accurate NIRS estimates can be obtained for crude protein, ether extract, crude fiber, acid detergent fiber, neutral detergent fiber and gross energy in ostrich total mixed ratios (TMRs). It has been also expressed that less accurate NIRS estimates can be obtained for dry matter, ash, calcium and phosphorus in Ostrich TMRs (Swart et al., 2012). Those results are compatible with our study.

Conclusion

As a result, although analysis with NIRS has advantages such as being very fast and not using chemicals, there are important disadvantages of analyzing with NIRS, such as the cost of the device and requiring advanced knowledge and experience for calibration. It has been demonstrated that the NIRS method is a fast, reliable and good estimation method for quantitatively determining the metabolic energy value in ruminant mixed feeds.

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JIVS JOURNAL OF ISTANBUL VETERINARY SCIENCES

Exploring bacterial pathogens and risk factors associated with the occurrence of navel ill in calves

Research Article

Volume: 4, Issue: 2
August 2020
Pages: 37-42

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ABSTRACT

The study mentioned here was designed to investigate both bacterial pathogens and risk factors associated with the occurrence of naval ill in calves. A total of 350 calves diagnosed to have navel ill formed the population in our study. Incidence density reports representing the period between 2009 and 2018 were obtained from Veterinary Teaching Hospital (VTH), Bangladesh Agricultural University where information about age, sex, breed, seasonal effect was also included. Umbilical swab from each individual suffering from confirmed navel ill was collected for microbiological study. The occurrence of navel ill in the age of <30 days (n = 244, 69.71%) was noted higher compared to the age of ≥30 days (n = 106, 30.29%). Male calves were highly susceptible (n = 192, 54.86%) compared to females (n=158, 45.14%). In cross breed individuals, the occurrence was higher (n = 330, 94.29%) in regard of indigenous calves (n=20, 5.71%). The occurrence of the illness in summer (March-June) was more common (n = 159, 45.43%) in comparison to both rainy (n = 111, 31.71%) and winter (n = 80, 22.86%) seasons. In term of microbiological study, *Staphylococcus aureus*, *E. coli* and *Proteus* spp. were identified and isolated from the infected areas of the calves with the disease. In conclusion, navel ill occurred more commonly in male calves with the age of less than one month. The appearance of navel ill was noted to be more frequently in summer season (March-June) in cross breed calves. The results also demonstrated that the condition is mediated by mixed bacterial infection formed by gram-positive and gram-negative agents which induce the initial inflammation.

Article History

Received: 18.04.2020
Accepted: 25.05.2020
Available online: 26.05.2020

Keywords: calf, navel ill, *Staphylococcus aureus, E. coli*, *Proteus* spp.

DOI: 10.30704/http-www-jivs-net.722788

To cite this article: Rassel, M. G. R., Mishra P., Rahman M., Alam, M. M. (2020). Exploring bacterial pathogens and risk factors associated with the occurrence of navel ill in calves. *Journal of Istanbul Veterinary Sciences. 4*(2), 37-42, **Abbreviated Title:** *J Ist Vet Sci*

Introduction

Navel ill is a disease of young calves, usually less than a week of age. The infection enters via umbilical cord or soon after birth with a prevalence of 5-15% (Mee, 2008a). In Bangladesh, 6.14 million cattle of a total sum of 24.09 million, is dairy cattle (DLS, 2019). Mortality in calves less than one year of age is about 9% (Debnath et al., 1990). Most of the animals die at young age due to different infectious diseases and surgical disorders like avel ill (Samad et al., 2002). The facultative myiasis producing flies can be a responsible agent for navel infection. There is usually a mixed bacterial flora including *E. coli*, *Proteus* spp., *Staphylococcus* spp., *Actinomyces pyogenes* etc. (Sherif et al., 2017). This infection can result in a wide range of signs depending on where the

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bacteria spread to. In some calves, infection spreads from the navel to the liver causing a liver abscess (Mee, 1990; Biss et al., 1994). Though individual antibody levels alter the course of the infection, the health situation remains also highly dependent to postnatal management (Waldner and Rosengren, 2009). The occurrence of this condition is strongly associated with poor hygienic maintenance of both calf and shed as well as with the intake of low-quality colostrum (Waltner-Toews et al., 1986).

In Bangladesh, several studies have been conducted in calves with neonatal diseases and their recurrence such as Schistosomus reflexus infection and umbilical hernia while on the other hand only few works about the healing of navel ill have been arranged and very few of these studies were mostly confined to revealing the prevalence of the disease (Hoda et. al., 2018; Jaman et al., 2018; Mishra et al., 2020). Therefore, the present research work has been implemented to harvest an incidence data of calves diagnosed with navel ill-treated at Veterinary Teaching Hospital (VTH), Bangladesh Agricultural University (BAU), Mymensingh and belonging to a decade between 2009-2018 in order to explore the bacterial pathogens and risk factors responsible for the occurrence of the disease.

Material and methods

Collection of occurrence data: Incidence data of navel ill was collected from clinical data records of Veterinary Teaching Hospital (VTH), Bangladesh Agricultural University (BAU), Mymensingh. The temporal period contained approximately a decade between 2009 and 2018 while details such as age, sex, breed, and occurrence season were also taken into consideration.

Experimental animals: Calves with confirmed navel ill at Veterinary Teaching Hospital (VTH), Bangladesh Agricultural University (BAU), Mymensingh were adopted as the individuals of the research study mentioned here and umbilical swab for microbiological study was collected from each one, between January 2019 and May 2019.

Collection of bacteriological samples: All of the samples were collected aseptically from the deep navel region with the use of sterile cotton buds. Before the procedure, the area was soaked with saline solution and swab samples were collected by circling the cotton bud into the infected region before quickly dipping it into screwed capped test tubes containing nutrient broth.

Culture of navel ill causing bacteria: The collected broth samples were incubated at 37 °C for 2 hours for enrichment. The samples were inoculated into different selective medias such as Mannitol Salt agar (MSA) and MacConkey agar and incubated at 37 °C overnight. Next day bacterial growth in various medias were observed and pure culture of each bacteria were obtained by repeated culture preparations of a single colony.

Identification of isolated bacteria by Gram´s staining: The pure cultures of isolated bacteria were subjected to Gram´s staining for an observation of bacterial morphology, arrangement and staining characteristics under light microscope at 10x magnification.

Statistical analysis: Descriptive analysis was performed. Data were collected and calculated to determine the occurrence of navel ill in the above -mentioned species. The connecting link between the disease's occurrence and variables such as age, sex, breed and seasonal differences was evaluated and presented in percentage for each group.

Results

Effects of different variables on the occurrence of navel ill: A total amount of 350 calves diagnosed with naval ill were recorded from 2009 to 2018 at VTH, BAU, Mymensingh. Different attributes on which the occurrence of the condition was based on are shown below.

Occurrence of navel ill based on age:

The effect of age on navel ill in calves is presented in Figure 1. Occurrence was higher (n = 244, 69.71%) at the age of less than 30 days in comparison with the occurrence at the age higher than or equal to 30 days (n = 106, 30.29%).

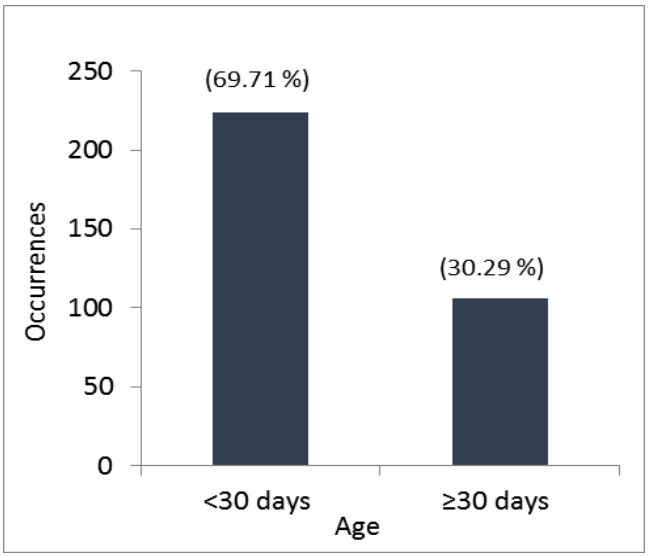

Figure 1. Occurrence of navel ill in calves based on age.

Occurrence of navel ill based on sex: The effect of sex on navel ill occurrence in calves is presented in Figure 2. Occurrence was higher (n = 192, 54.86%) in males than in females (n = 158, 45.14%).

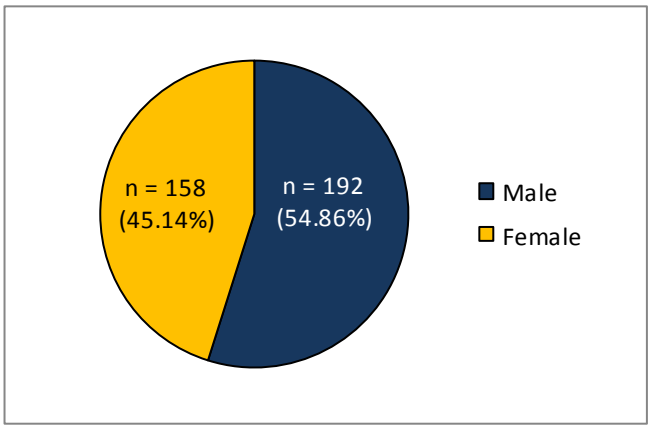

Figure 2. Graphical presentation of occurrence of navel ill based on sex in calves.

Occurrence of navel ill based on breed: The effect of breed on navel ill appearance in calves is shown in Figure 3. Occurrence was higher (n = 330, 94.29%) in cross bred calves than in indigenous calves (n = 20, 5.71%).

Occurrence of navel ill based on season: The effect of season on navel ill appearance in calves is presented in Figure 4. Highest occurrence of the disease was noted (n = 159, 45.43%) in summer season (March-June) while the lowest level was detected (n = 80, 22.86%) in winter season (July-October).

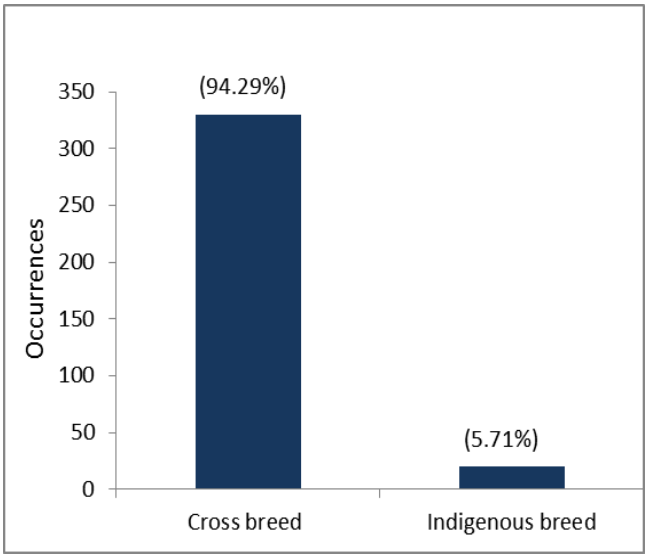

Figure 3. Occurrence of navel ill based on breed in calves.

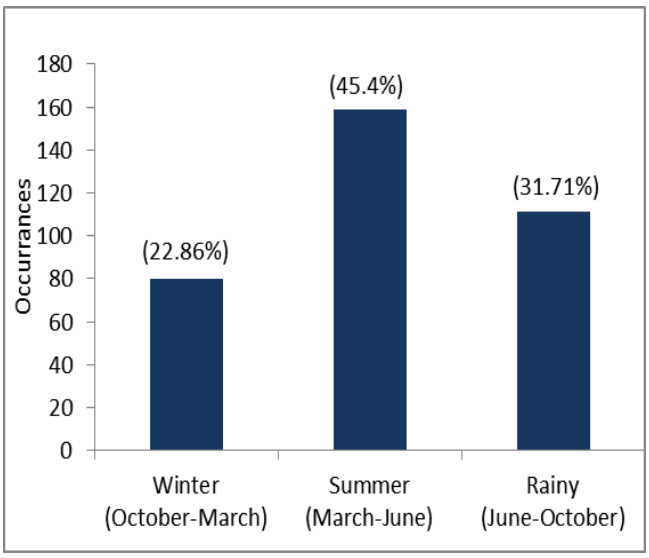

Figure 4. Graphical representation of occurrence of navel ill in calves based on season

Bacteriological findings

Spreading of swab on agar plate: On the basis of cultural characteristics and staining properties, *Staphylococcus* spp., *E. coli* and Proteus spp. were identified from the samples of all calves suffering from navel ill. On mannitol salt agar (MSA), *Staphylococcus* spp. produced a golden yellow colony due to the fermentation of Mannitol indicating that the isolates were *Staphylococcus aureus* (Figure 5A). On MacConkey Agar, *E. coli* caused only pink apparition and *Proteus* spp. produced colorless colonies (Figure 5B).

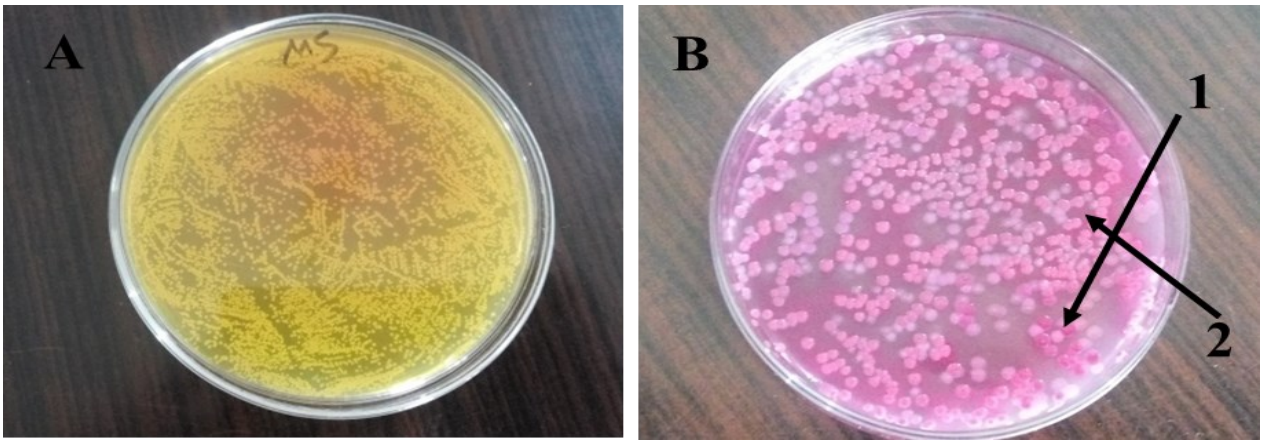

Figure 5. Isolated bacteria from navel ill patient. (A) Growth of *Staphylococcus aureus* in MSA (B) Growth of suspected (1) *E. coli* and (2) *Proteus* spp. on MacConkey Agar.

Pure culture finding: A single colony from each plate were spread on the same agar plate respectively. On MSA, a golden yellow colony were found; which was suspected to be *Staphylococcus aureus* (Figure 6A) and on MacConkey Agar, a pinkish colony was found with metallic sheen, which was thought to be *E. coli* (Figure 6B1) and a colony with lose color were found, which was suspected to be Proteus spp. (Figure 6B2).

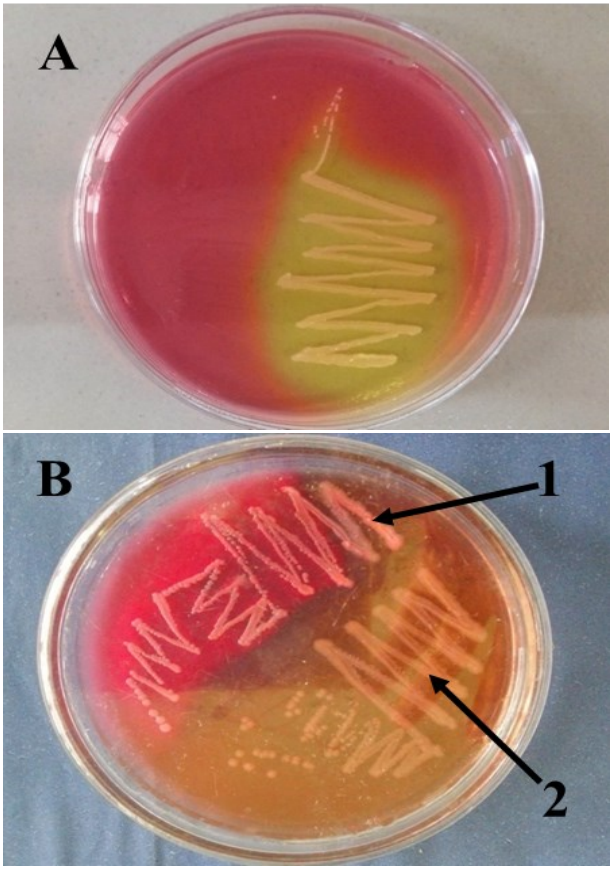

Figure 6. Colony characteristics of bacteria. (A) A Golden yellow color colony was found which was the characteristics of *Staphylococcus aureus*, (B1) A characteristics pinkish colony with metallic sheen was detected and the colony was evaluated as *E. coli* and (B2) a pale color colony was found which are usually recognized as the colony characteristics of *Proteus* spp.

Characteristics of isolated bacteria: On Gram´s staining, The *Staphylococcus aureus* showed Gram positive, spherical shaped in cluster form (Figure 7A), the *E. coli* showed Gram negative rod-shaped organisms arranged in single or short chain like (Figure 7B) and the Proteus spp. showed Gram negative short rod arranged with periodic cycles of migration producing concentric zones or spread in a uniform film (Figure 7C).

Discussion

During the study period it was found that among 350 of infected calves, 244 were brought to VTH earlier than thirty days and 106 calves were of ≥30 days of age. Samad et al. (2001) reported that the infection rate was higher (73.03%) in those aged between 0-30 days compared to individuals aged between 31-90 days (24.72%) while the same rate was measured very low (2.25%) for those aged >90 days. Blowey and Weaver (2011) also reported that navel ill usually occurs in calves younger than one weeks of age, as a result of inflammation, due to umbilical tissue infection following parturition in insanitary environment; which was compatible with our study.

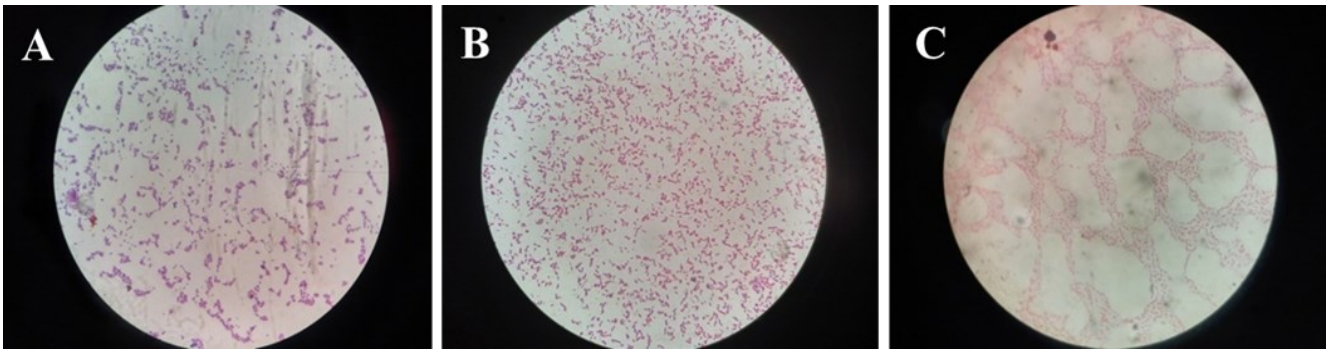

Figure 7. Staining characteristics of bacteria. (A) Smooth, convex and grape like clusters were found which is the characteristics of *Staphylococcus aureus*, (B) Rod shaped, irregular arrangement and pink in color was found which is the characteristics of *E. coli* and (C) Rod shaped with ranging in length from coccoid rods to long wire forms was detected which is the characteristics of *Proteus* spp.

In the present study, we found that the infection rate was higher in males (54.86%) than in females (45.14%); which was also in concordance with Radostits et al. (2007) having also reported that, male calves were more susceptible to the infection than the females. Since urethral opening was placed nearer to the navel area compared to the location of same opening in females, the navel area of male calves exhibit a higher possibility of being soiled by urine and getting infected.

In cross breed calves, the rate of infection was higher than in indigenous breed. However, Rweyemamu et al. (2008) reported that in local breeds, the infection rate was higher than in crossbred calves. This breed-related variation is estimated to be correlated to genetic predisposition.

In our study we observed that the rate of infection (45.43%) augmented in summer season. Watson (2004) also reported that the rate of incidence for navel ill and conjunctivitis were significantly ($p<0.05$) higher in summer season compared to other seasons. As the condition is commonly seen during summer months, it is suspected that the odds are in favor of ultraviolet light, dust and flies acting as predisposing factors.

In bacteriological study we found *Staphylococcus aureus*, *E. coli* and *Proteus* spp. in samples obtained from navel region of the calves infected with naval ill. Sherif et al. (2017) reported that there is usually a mixed bacterial flora including *E. coli*, *Proteus* spp., *Staphylococcus* spp. *Actinomyces pyogenes* etc. According to Blood and Radostits (1989), bacteria commonly involved include *E. coli, Streptococcus* spp., *Staphylococcus* spp., *Actinomyces* (*Corynebacterium*) *pyogenes* and other soil and fecal contaminants. A wide range of opportunistic bacteria were found in samples taken from the umbilical lesions (Jaman et al., 2018).

In conclusion, based on the findings of our foregoing study, navel ill occurred mostly in calves of less than one month of age and more common in male calves than that in female calves. The higher occurrence of navel ill encountered in the cross bred calves in contrast to indigenous calves. This disorder is more prevalent in summer season (March-June).
The results also demonstrated that the condition is mediated by mixed bacterial infection (gram-positive and gram-negative bacterial infection) which induce the initial inflammation. Therefore, after birth the navel area should manage properly, calves should keep in dry and hygienic environment where light and air can easily enter.

Acknowledgement

We thank National Science and Technology (NST) of Bangladesh for providing financial support to conduct the research.

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JIVS JOURNAL OF ISTANBUL VETERINARY SCIENCES

Use of a dynamic axial external fixator combined with internal cross pins for metacarpal fracture treatment in calves

Research Article
Volume: 4, Issue: 2
August 2020
Pages: 43-50

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ABSTRACT

This study aimed to investigate the effectiveness of using dynamic axial external fixator combined with cross pins in the treatment of distal metacarpal fractures in calves. The results of distal metaphyseal metacarpal fractures treated with this method in six calves (four females, two males) were examined. The ages of the patients were 17.16 ± 13.22 and their weight was 52 ± 9.48. The calves were born by pulling during labor and they could not stand up after birth. The clinical and radiological examinations found a distal metaphyseal metacarpal fracture. Dynamic axial external fixator with cross pin support was applied to all patients in the operative treatment. The patients' foot postures, stepping, walking and running were found to be smooth and adequate in the postoperative examination. Therefore, cross-pin supported dynamic axial external fixation was found to be a strong enough, well-tolerated and easy-to-apply technique which can be used especially in the treatment of open and infected metacarpal fractures.

Article History
Received: 05.05.2020
Accepted: 29.06.2020
Available online: 29.06.2020

Keywords: calf, cross pin, dynamic axial external fixator, fracture healing

DOI: https://doi.org/10.30704/http-www-jivs-net.732607

To cite this article: Yurtal, Z., Altuğ, M.E., Deveci, M.Z.Y., İşler C.T., Alakuş, İ. (2020). Use of a dynamic axial external fixator combined with internal cross pins for metacarpal fracture treatment in calves. *Journal of Istanbul Veterinary Sciences. 4*(2), 43 -50, **Abbreviated Title:** *J Ist Vet Sci*

Introduction

Limb fractures are more common than other fractures (skull, spine, pelvis, sacrum, etc.) in cattles (Akın, 2017). Fractures may occur in the long bones of calves due to traumatic reasons such as incorrect interventions during labor, mother's stepping on the calf, falling or kicking (Gülaydın and Sarıerler, 2018). Metacarpal fractures rank first in long bone fractures in calves, and the most common cause is excessive pulling at birth (Belge et al., 2016). Metacarpal and metatarsal fractures approximately comprise 50% of the fractures in calves (Tulleners, 1986; Auer et al., 1993; Bilgili et al., 2008). Metacarpal fractures are twice the incidence rate of metatarsal fractures, and higher than the total incidence rate of radial and tibial fractures (Ferguson, 1982; Steiner et al., 1993; Bilgili et al., 2008). Metacarpal fractures are most commonly observed in the distal epiphysis and metaphyseal part of the bone in calves, and the Salter - Harris Type I fracture is the most common

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fracture type (Belge et al., 2016). The type and localization of the fracture, the state of fracture (open or closed fracture), economic value of the animal, cost of treatment and care conditions during the recovery period are considered when determining the treatment option (Aksoy et al., 2009). The economic status of the patient owner plays a crucial role in consideration of the treatment option as well as the shape and location of the fracture (Oztas and Avki, 2015).

There are conservative and surgical treatment options for long bone fractures in cattles (Belge et al., 2016). Rest and bandage applications are carried out in a restricted area in the conservative treatment (Yanmaz et al., 2014). Plaster is the most commonly used method for simple transverse and mid-diaphyseal bone fractures (Ferguson, 1982; Tulleners, 1986; Bilgili et al., 2008). Additionally, treatment of closed fractures is often performed using bandage techniques combined with Thomas split or bandage, as well as bandaging techniques supported by certain materials such as polyvinylchloride (PVC) and aluminum (Auer et al., 1993; Nuss et al., 2011; Arican et al., 2014). Internal and external fixation methods are used for surgical treatment (Yanmaz et al., 2014). Internal fixation methods are recommended for the dislocated, multifragmental or complicated fractures. Techniques such as intramedullary nailing, cerclage, screw, dynamic compression plate (DCP), interlocking pins are applied for internal fixation (Arican et al., 2014). The main purpose of external fixation is to maintain normal joint mobility and return the patient to normal life as soon as possible without losing weight after surgery (Anderson and Jean, 1996). This fixation method can be especially useful in multifragmented, open and infected fractures (Singh et al., 2007). Materials and tools such as transfixation pinning and bandages, dynamic axial external fixator and circular external fixator are used for external fixation methods (Singh et al., 2007; Bilgili et al., 2008).

Internal (cross pin) and external fixation (dynamic axial external fixator) methods were used in combination for the surgical treatment of metacarpal fractures in six calves and the effectiveness and long-term results of this treatment were evaluated in this study.

Materials and Methods

The study material consisted of six calves brought to Hatay Mustafa Kemal University Veterinary Health, Practice and Research Hospital and diagnosed with distal metaphyseal metacarpal fracture. Demographic data on calves are presented in Table 1. The reason for the fractures was found to be the irregular extraction force applied in difficult labor conditions. The results of distal metaphyseal metacarpal fractures treated with this method in six calves (four females and two males) were examined. The ages of the patients were 17.16 ± 13.22 and their weight was 52 ± 9.48. Two of our cases were open fractures and four of them were closed fractures. The patient owners were informed about anesthesia and surgical complications. Consent was obtained from all patient owners. Then, the routine procedures mentioned below were applied to the cases.

Anesthesia: 0.1 mg / kg dose xylazine (Alfazyne % 2; Egevet, Turkey) and 5 mg / kg ketamine (Alfamine %10; Egevet, , Turkey) was allowed to be given as anesthesia induction. Following intubation, 100% oxygen was administered and anesthesia was maintained with a concentration of 1-3% sevoflurane (Sevorane; Aesica Queenborough Ltd, UK).

Preoperative preparation and planning: Anterioposterior and mediolateral radiographs of the broken limb were taken after the affected extremities and surrounding tissues were carefully examined. The dynamic axial fixator to be applied according to the shape and length of the bone fracture (TIPS, Turkey) size was determined by radiography.

Surgical method: Operative procedures in all of our patients started with a horizontal incision over the fracture line (Figure 1. A). Fragments of the fracture were reduced and internal fixation was achieved by cross-nailing after reaching the broken bone (Figure 1B-C). Following this, the incision line was closed using no.1 absorbable suture material (Ethicon, Johnson&Johnson, Belgium) (Figure 1D). Six threaded pins (3 mm and 4 mm in diameter) were inserted into the proximal and distal of the fracture line and fixed

Table 1. Demographic data and clinical findings in calves

Case number	Age / Weight	Sex	Breed	R/L	Open / Closed	Bone/ Localization / Shape	Cause	Complications	Result
1	1 d/40 kg	F	Holstein	R	Closed	Bone : Metacarpus Location: Distal metaphyseal Shape: Transverse	During birth	Pin tract infection and soft tissue inflammation	Excellent
2	20 d/52 kg	M	Belgian blue	R	Closed	Bone : Metacarpus Location: Distal metaphyseal Shape: Transverse	During birth	Pin tract infection and soft tissue inflammation	Excellent
3	30 d/61 kg	F	Holstein-Simmental hybrid	R	Open	Bone : Metacarpus Location: Distal metaphyseal Shape: Transverse	During birth	The bone was broken again as a result of trauma. Pin tract infection and soft tissue inflammation	Moderate
4	1 d/42 kg	F	Holstein	L	Closed	Bone : Metacarpus Location: Distal metaphyseal Shape: Transverse	During birth	2 pins sent to the proximal fragment were broken. Pin tract infection and soft tissue inflammation	Excellent
5	21d/54 kg	F	Simmental	L	Open	Bone : Metacarpus Location: Distal metaphyseal Shape: Fragmented	During birth	Anesthesia intolerance	Dead (Post-op 1st day)
6	30 d/63 kg	M	Simmental	L	Closed	Bone : Metacarpus Location: Distal metaphyseal Shape: Transverse	During birth	Anesthesia intolerance	Dead (Post-op 1st day)

R: right, **L:** left**, d:** days, **M:** Male, **F:** Female

on the dynamic axial external fixator in the external fixation processes except in that of case three (Figure 1E). Proximal pins were placed in the metaphysis and distal pins in the first phalanx due to the lack of sufficient bone tissue in the distal fragment in all cases. Similar to the other cases, only the third patient was applied two threaded pins to the proximal of the fracture line and three threaded pins to the first phalanx. Since the structure of this patient was smaller than other cases, there was enough space to place two threaded pins in the proximal fragment.

Postoperative maintenance: After the fixation was completed, the radiographs were taken for the check of fixation. Antibiotics containing benzyl penicillin and dihydrostreptomycin (intramuscular 5.7 mg benzyl penicillin and 10 mg dihydrostreptomycin / kg / day, Reptopen, Ceva, Turkey) were postoperatively used. Additionally, 10% povidin iodine was used for 14 days for pin tract care, rifamycin (Rif ampul 250 mg; Koçak- Pharma, Turkey), and flunixin meglumine (2.2 mg/kg) (Fulimed, Alke, Turkey)

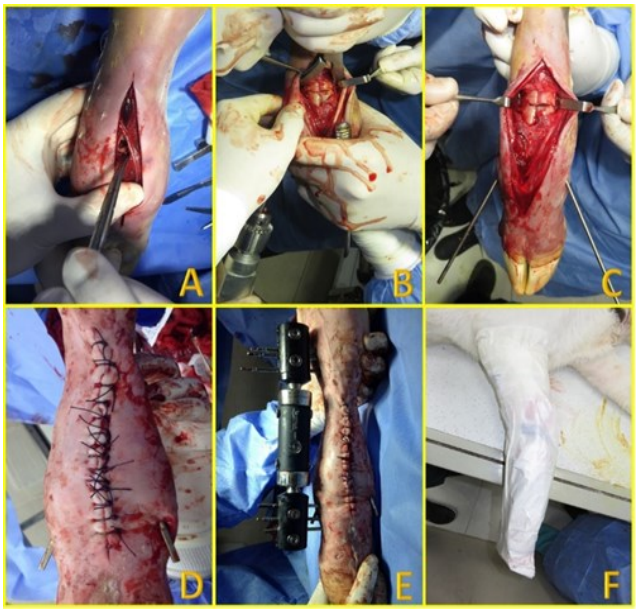

Figure 1. A: Horizontal incision above the fracture line (Case 6), B–C: Reduction of the broken line with a cross pin (Case 4), D: Closing the incision line with sutures (Case 2) E: Dynamic axial fixator application (Case 2) F: Covering a breathable perforated sack over the bandage (Case 3).

for analgesia and anti-inflammatory activity. Patient owners were informed about possible postoperative complications and pin tract care. A protective bandage was applied to patients

and a perforated sack was attached to this bandage to keep the operation area cleaner (Figure 1F). The affected limb was evaluated at short intervals for edema, swelling, color change, pain, increase in temperature and loss of function. Calves were called for routine postoperative controls with two weeks intervals and callus formation was followed by radiography. During the removal of the external fixators, the screws were loosened and the external fixators and pins were removed on the 78th day. The holes where the pins came out were cleaned with antiseptic solution. Bandages were not applied again. The activities of the animals were restricted for two weeks.

Results

The clinical information of the calves used in the study are shown in Table 1. According to the anamnesis information received from animal owners, the fractures were formed as a result of excessive force application during delivery. Distal metaphyseal metacarpus fracture was detected in the clinical and radiographic examination (Figure 2).

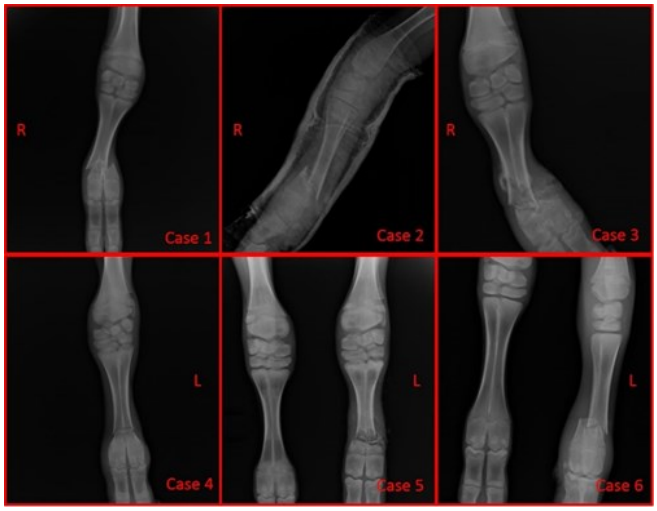

Figure 2. Preoperative radiography images of cases.

Dynamic axial external fixator with cross pin support was used in all cases. Closed fractures did not have infections while the open fractures were infected. Based on the control radiographs taken postoperatively, the fractures reduced (Figure 3). The first case was initially applied a synthetic plaster bandage. Upon this, the procedure of operation was determined and the operation was performed in accordance with the procedure

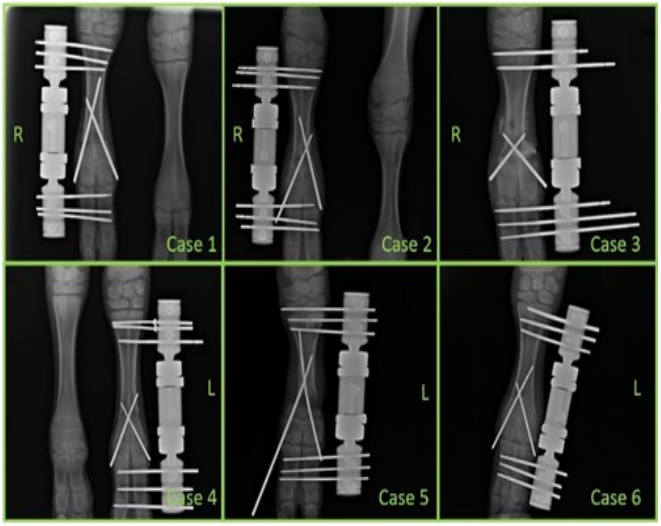

Figure 3. Postoperative radiography images of cases

described above. However, the foot swell and limped. It was prescribed both ceftriaxone (30 mg / kg / day, two times a day intramuscularly, Unacefin; Yavuz Medicine, Turkey) and flunixin meglumine (2.2 mg / kg, Fulimed, Alke, Turkey) systemically and 10% povidone iodine and rifamycin locally. According to the radiograph taken at the third month of follow-up, the healing between fractures was sufficient; so, the external fixator was removed (Figure 4).

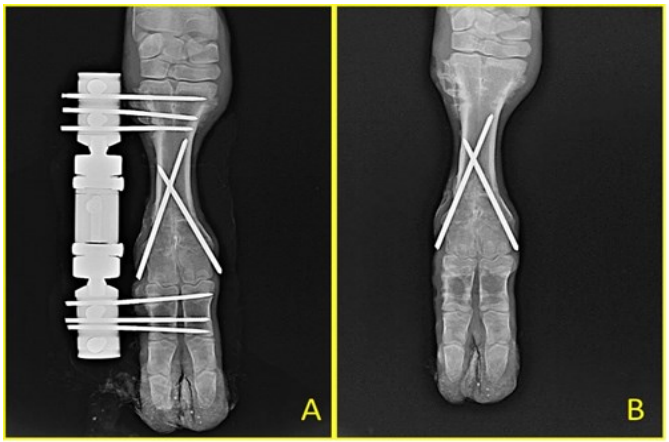

Figure 4. A: Radiographic view before dynamic axial external fixator removal (case 1) B: Radiographic view after dynamic axial external fixator removal (case 1)

The patient's sixth month postoperative control revealed that there was no difficulty in pressing his foot and he used his foot very well. Another physician applied bandage to the second case. The information obtained from the anamnesis revealed that the second case had his foot bandaged. The radiography found distal metacarpal fracture and there was no improvement in the broken bone. The operation

was carried out with the same procedure. The case was completely recovered in the postoperative fourth month follow-up. The third case was infected and open fractured. Infection was controlled using rivanol bandage. Then, a cross pin was inserted internally and a dynamic axial external fixator was attached externally in accordance with the operation procedure. It was supported by medical treatment against the risk of infection. This case had a broken the bone at the same location as a result of the pressure on the door of the external fixator applied to the limb on the 56th postoperative day. Following the control, the patient was referred to be cut at the request of the patient owner. In the fourth case, two of the pins sent to the proximal fragment of the fracture were broken at the third week postoperative control (Figure 5A). Fixation was achieved by sending new ones instead of the broken pins (Figure 5B).

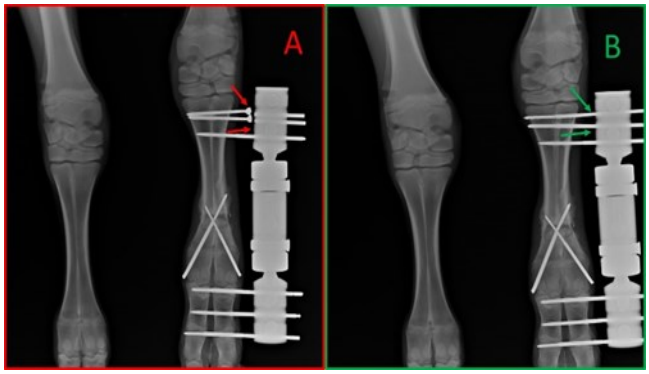

Figure 5. A: The postoperative 3rd week control revealed that 2 of the pins sent to the proximal fragment of the fracture were broken B: Fixation was achieved by sending a new one in place of the broken pins (case 4).

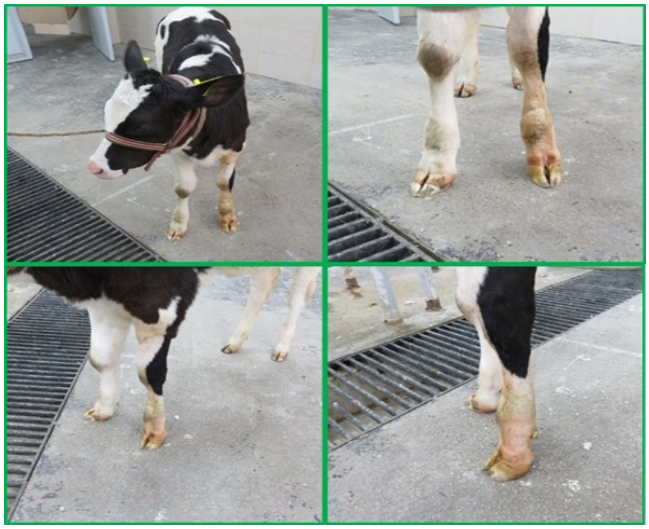

Figure 6. Photographs taken from different angles in the third month postoperative control (case 4).

The patient used his foot very well in the third month postoperative control (Figure 6). The most important postoperative finding was pin tract infection and the lameness related to it. These clinical symptoms were overcome with medical treatment.

Discussion

Based on our clinical experience, when carpal fractures are not treated in calves, they mostly result in death due to infection or cutting without commercial carcass value. Under market conditions, considering that a 20 days old calf is sold at around 500-600 $ it causes significant economic loss for the enterprise if proper treatment is not performed. Various treatment techniques for metacarpal fractures in calves are applied (Akın, 2017). Although bandage applications are generally preferred in fractures for some ruminants due to economic reasons, as it was also observed in our study, it is accepted as an imperative to treat metacarpus fractures and to treat the fracture with external and internal methods (Denny et al., 1988; Anderson and Jean, 1996). In our study, two metacarpus fractures were bandaged by some private veterinary clinics before admission to our hospital; however, the callus was not shaped and one turned into an open fracture (Figure. 2, second case). We achieved improvement by applying dynamic axial external fixator in both cases. We have considered the application of dynamic axial external fixator in calves to be a suitable and successful technique in clinical practice for the distal metaphyseal metacarpal fractures that cannot be stabilized by bandages.

External fixators are commonly used in angular deformity treatments and bone lengthening surgeries in cats and dogs (Thommasini and Betts, 1991; Marcellin-Little et al., 1998; Stallings et al., 1998). Internal fixation technique can be used in the treatment of all closed or open metaphyseal, diaphysis and especially epiphysis fractures and external fixation in closed fractures that cannot be stabilized with traction and bandages, periarticular fractures extending to the diaphysis, and long bone fractures (Gülaydin et al., 2019). The present study found the use of external

fixator was found to be very important in calves, especially in the treatment of distal extremity fractures.

Podolski and Chao reported that the thickness of the pin diameter increased in line with the rigidity of the external fixator (Podolsky and Chao, 1993). Circular external fixators are most frequently used in humans with polyaxial and 1.5-2 mm diameter pins (Kummer, 1992) and 1-1.6 mm pins in cats and dogs (Ferretti, 1991; Aithal et al., 2007). The fixators used in current study were the dynamic axial external fixator and it is monoaxial. Therefore, thicker pins were preferred to provide sufficient durability. Edgerton et al. reported that the thickness of the pins selected should not exceed 20% of the affected bone diameter in general to not decrease bone strength (Edgerton et al., 1990). The pin diameters selected in our study were 10% of the affected bone diameter.

The pins must be fixed to the external fixator system from two points at least while fixing fracture fragments in external fixator applications (Cakmak and Bilen, 1999; Gülaydin et al., 2019). The weakest part of using circular external fixators is that the pin diameters are small (Bianchi-Maiocchi, 1994; Aithal et al., 2004). We used three pins for each of the proximal and distal parts of the fracture line in five patients in our study. We applied two pins on the proximal line and three pins on the distal line in one of our cases. The application and diameter of the pin may vary according to the case and proper diameter and number of pins that provide sufficient fixation should be used.

Anderson and St Jean reported that implant failures such as clamp or sidebar malfunctions, pin bending or pin breakage may occur in circular external fixators and limit the use of external fixators in large animals (Anderson and Jean, 1996). We used dynamic axial external fixators in our study to prevent these reported adverse conditions. The use of clamps, nuts or rods used in this technique for circular external fixators was considered to be advantageous while pin breakage was considered to be caused by the owner's irresponsibility. However, the fixation of the fracture could be impaired if the external fixator was not supported with cross pins.

Aithal et al. stated that the animals placed a load on their feet immediately after the surgery since their fixation in the circular external fixator was stiff enough (Aithal et al., 2004). In our study, animals were able to put a load on their feet immediately after the surgery, therefore; the use of dynamic axial external fixators is sufficient for fixation.

Pin tract infections and lameness were reported as complications in circular external fixator applications (Aithal et al., 2004; Aithal et al., 2007; Gülaydin et al., 2019;). In our study, pin tract infection and lameness occurred in four cases due to this infection, in line with the literature. Singh et al. reported that pin path infections decrease gradually by regularly washing them with sterile saline solution containing ciprofloxacin (Singh et al., 2007). In our study, this complication was eliminated by using local 10% povidone iodine solution, local injection of rifamycin and systematic administration of third generation cephalosporin and flunixin meglumine.

Heo et al. emphasized that complications such as pin migration and poor strength of the transcortical pins are common in newborn calves due to the low density of bones and thin cortexes (Heo et al., 2012). As threaded pins were used in the transcortical pinning process, no complication such as pin migration was encountered in our study.

Conclusions

Dynamic axial external fixation technique can be used as it is easy to apply and well tolerated by patients. This was considered to be an important advantage especially in the treatment of open and infected metacarpus fractures near the joint. Consequently, dynamic axial external fixation combined with cross pin was a successful technieque in fixation and treatment of distal extremity fractures in calves.

Acknowledgement

The authors would like to thank Halil ALAKUŞ and Ömer KIRGIZ (Hatay Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Surgery) for their support in the clinical work of the study.

Conflicts of interest

The authors declared that there are no conflicts of interest.

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JIVS JOURNAL OF ISTANBUL VETERINARY SCIENCES

Surgical correction and outcomes of persistent right aortic arch in two dogs

Case Report
Volume: 4, Issue: 2
August 2020
Pages: 51-56

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ABSTRACT

The case report was consisted of two puppies with the complaint of chronic vomiting after feeding. The dogs were vomiting after eating and their abdominal areas were tense. Also, growth retardation was present. Accumulation of the contrast material was seen at the thoracic part of the esophagus with indirect radiographs in both cases. The cranial and caudal widths of the stricture in the esophagus were measured before and after surgery. The cranial and caudal parts of the stricture were found both dilated. The ligament that causing stricture was dissected by surgery and the stricture area was enlarged by balloon esophagoplasty. Case 1 was died on 2nd hour postoperatively, while Case 2 fully recovered and healed. In Case 2, the diameter of the prestenotic and poststenotic esophagus found decreased at 16th month postoperatively. The long-term results of Case 2 were fairly well.

Article History
Received: 20.07.2020
Accepted: 12.08.2020
Available online: 18.08.2020

Keywords: persistent right aortic arch, vascular ring anomaly, dog

DOI: https://doi.org/10.30704/http-www-jivs-net.772064

To cite this article: Yurtal, Z., İşler, C.T., Deveci, M.Z.Y., Altuğ, M.E., Kırgız, Ö. (2020). Surgical correction and outcomes of persistent right aortic arch in two dogs. *Journal of Istanbul Veterinary Sciences. 4*(2), 51-56, **Abbreviated Title:** *J Ist Vet Sci*

Introduction

Vascular ring anomalies (VRA) occur as a result of abnormal development of embryonic aortic arches. It is a congenital disease caused by the trachea and esophagus being surrounded by blood vessels and adjacent structures (House et al., 2005; Fossum, 2013). The large vessels from the heart that developed abnormally, result in the compression of the esophagus and rarely the trachea (Yalçin et al., 2009). The cause is not fully known in animals. However, maternal infections, vitamin A deficiency, genetic or teratogenic factors are thought to play a role in the pathogenesis (Karabagli and Bahadir, 2017). The most common type of VRA is the permanent right aortic arch (PRAA), also known as the fourth permanent right aortic arch (Loughin and Marino, 2008, Yalcin et al., 2009). The permanent right aortic arch, accounts for 95% of all VRAs diagnosed in dogs (Buchanan, 2004). Although it can be seen in all breeds, it is mostly seen in German shepherd and

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Irish setter dogs, and in Persian and Siamese cats. There is no gender predisposition. Neutering of the affected animals is recommended. Vascular ring anomaly is usually diagnosed before 6 months old. It is rarely diagnosed in adults (Karabagli and Bahadir, 2017). Clinical symptoms usually start in the 2nd-6th months with vomiting at the changeover to solid feeding (Muldoon et al., 1997; Menzel and Distl, 2011). The diagnosis is provided by clinical and radiographic findings. Although medical treatment does not result satisfactory, it may be useful to prevent secondary diseases caused by VRA (Karabagli and Bahadir, 2017). The definitive treatment of VRA in animals, is to ligate and cut off the vascular ring causing constriction (Helphrey, 1979). Surgery should be performed at the earliest stage to prevent irreversible disruptions.

Cases

The aim was to share the clinical presentation, diagnosis and results of surgical treatment of vascular ring anomalies in two dogs. In case 1, a 1.5 months old male Chow Chow puppy was presenting with the vomiting immediately after feeding for 15 days. Clinical examination revealed a rubbing sound from lungs, abdomen was swollen and tympanic, but general findings such as body temperature and pulse were normal. In radiographic examination, direct radiographs (Laterolateral and ventrodorsal) were taken first. The stomach was filled with gas and then indirect radiography was taken. Barium sulfate was observed to accumulate in the front of the stricture in the esophagus and esophageal dilatation (ED) was measured on radiography (Figure 1a). Vascular ring anomaly was diagnosed and the surgery planned.

In case 2, the patient was a male, 2 months old Malinois-Siberian husky crossbreed puppy. The patient had been vomiting undigested content immediately after eating and or drinking for 20 days. Direct radiographs (laterolateral and ventrodorsal) were taken first. Indirect radiography taken with barium sulfate revealed a stricture in the esophagus and an excess filling in the anterior aspect of the stricture (Figure 1 b). The diagnosis was vascular ring anomaly, which was typical in appearance. Surgical operation was considered appropriate and planned.

On the indirect radiographic images taken laterolateral position (LL), there was esophageal dilatation in the cranial of the structured area in both cases. The esophageal dilation measurements of two cases are reported in Tables 1 and 2. The result in case 1 was prestenotic ED, 2.931, and considered as moderate esophageal dilation (Table 1). Poststhenotic esophageal dilation was 4.424 and considered advanced esophageal dilation (Table 2). Pre and post-stenotic areas are shown in the preoperative radiograph of Case 1 in Figure 1 a. In case 2, the degree of prestenoic dilation was measured as 5.761 and it was observed that there was an advanced degree of esophagus dilation (Table 1). Poststhenotic esophageal dilatation was 2.315 and considered as moderate esophageal dilation (Table 2). Pre and post-stenotic areas are shown in the preoperative radiograph of Case 2 in Figure 1b. On the contrast radiograph taken 16th month postoperatively,

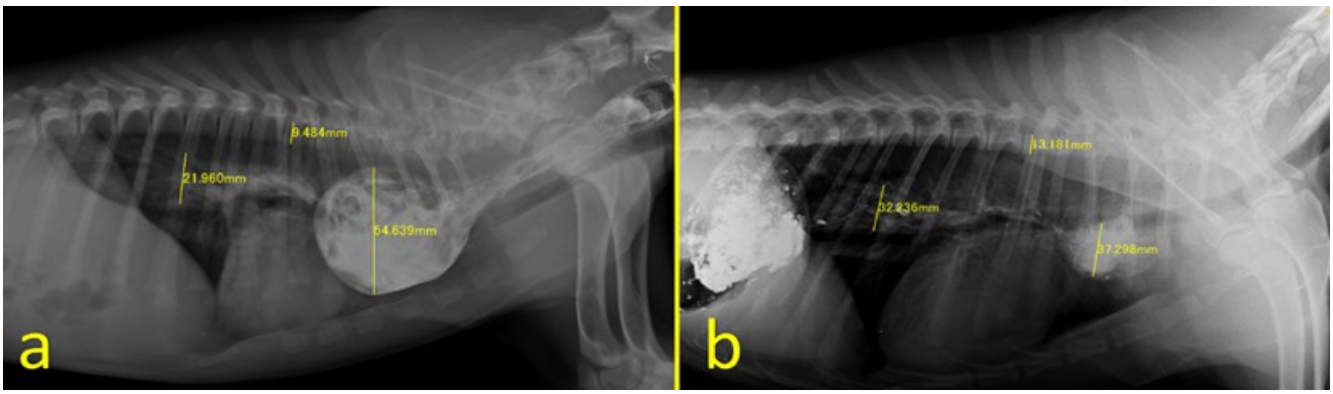

Figure 1. Preoperative and poststenoic radiographic images of case 1 (a) and case 2 (b)

Table 1. Prestenotic dilatation degrees of case 1 and case 2 before the surgery

Case	T5- height (mm)	Esophageal dilation (ED) (mm)	Dilation value (Dilatation / T5)	Dilation degree
Case-1	5.869	17.205	2.931	Moderate
Case-2	9.484	54.639	5.761	Severe

Table 2. Poststenotic dilatation degrees of case 1 and case 2 before the surgery

Case	T5- height (mm)	Esophageal dilation (ED) (mm)	Dilation value (Dilatation / T5)	Dilation degree
Case-1	5.869	24.791	4.424	Sever
Case-2	9.484	21.960	2.315	Moderate

Table 3. Postoperative (16th month) prestenotic and poststenotic dilatation degrees of case 2

Case	T5- height (mm)	Esophageal dilation (ED) (mm)	Dilation value (Dilatation / T5)	Dilation degree
Prestenotic	13.181	37.298	2.829	Moderate
Poststenotic	13.181	28.594	2.169	Moderate

pre-stenotic esophageal dilatation was 2.829 mm, post-stenotic esophageal dilatation was 2.169 mm. Therefore, esophageal dilatation found to be regressed (Table 3). The postoperative 16th month prestenoic and poststenoic radiography image is given in Figure 2.

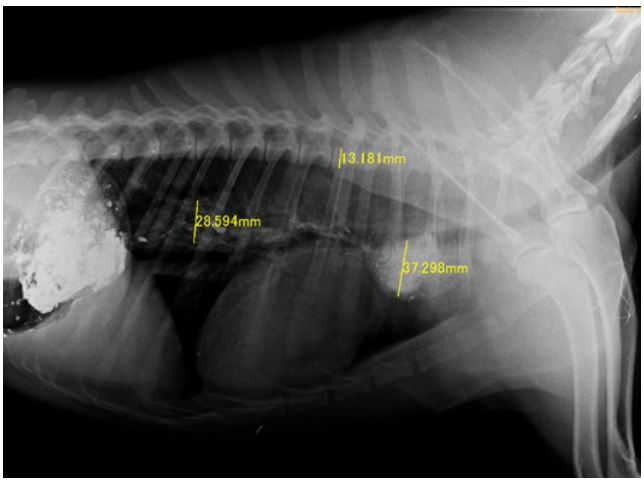

Figure 2: Poststenotic radiographic image of Case 2 (16th month postoperatively)

Both patients were operated on with the same procedure. Metamizol sodium 10 mg / kg (Geralg-M, Text chemist, Turkey) intramuscularly (IM) and cefazolin sodium 30 mg / kg of (Cefazolin, MN, Turkey) IM, and xylazine HCl 2 mg / kg (IM) (20 mg / ml (Alfazyne® EgeVet, Turkey) were applied preoperatively. Endotracheal intubation tube was placed in both patients after premedication. Induction was performed with ketamine HCl 20 mg / kg (100 mg / ml Alfamine® EgeVet, Turkey) IM and maintenance of the anesthesia provided by inhalation of sevoflurane 2-5% (Sevorane Liquid 100%, Abbot, USA). Routine preparations were completed by placing the patients on the operation table in the right lateral recumbency (Figure 3 a-b). Surgical site was reached by thoracotomy approach from the 4th lateral intercostal space (Figure 3 c). The right aortic arch was confirmed (Figure 3 d). The peri-esophageal fibrous bands and ligamentum arteriosum, which compress the esophagus, were carefully dissected. The ligamentum arteriosum was then ligated with double ligature at 1 cm spacing using absorbable (No:0) surgical suture (P.G.AÒ, Çetin Chemistry, Turkey) (Figure 3 e). It was cut through the ligatures (Figure 3 f). Then, a Foley (14 Fr) catheter was then advanced to the distal esophagus and the

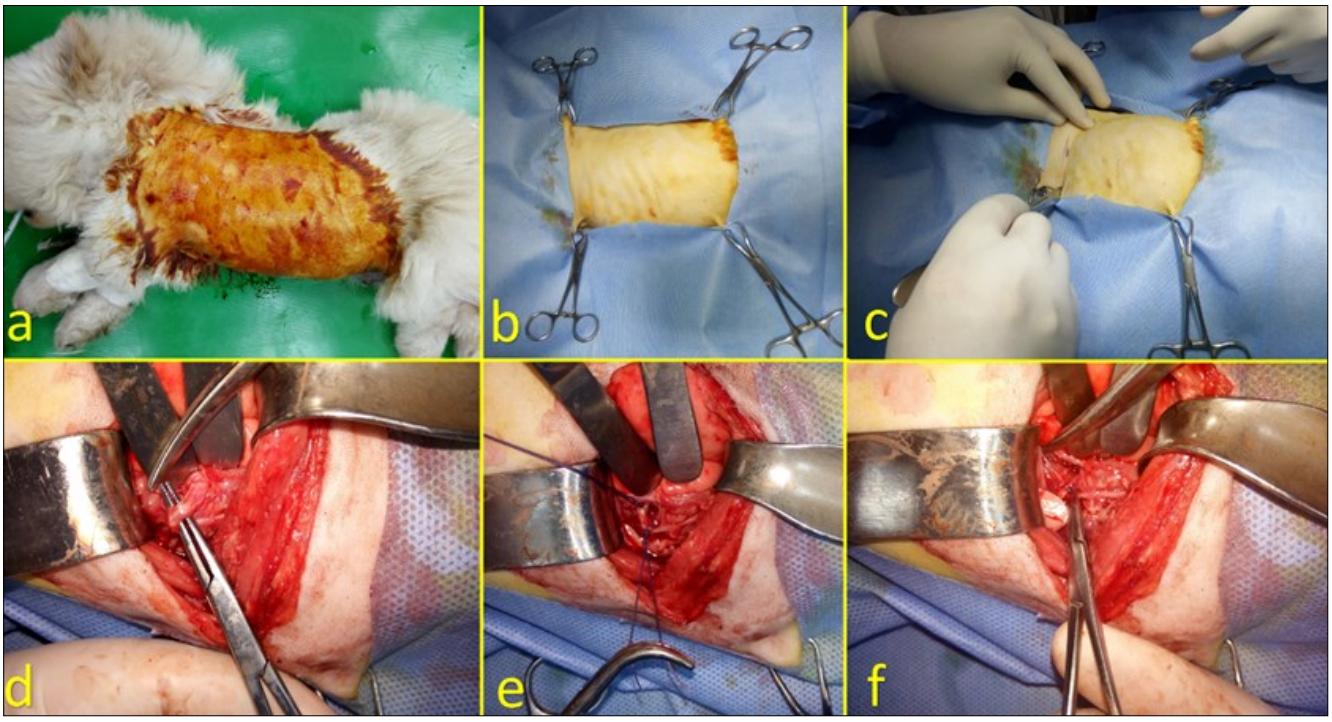

Figure 3. Surgical approach of case 1 diagnosed with vascular ring

balloon was inflated with 5 ml of saline. The catheter was pulled back and its passage from the caudal part to the cranial was tested. The Foley catheter was easily slipped in the esophagus and the passage was open. Following the elimination of the contraction, the operation was completed. Intercostal muscles, subcutaneous connective tissues and skin were closed with absorbable sutures routinely. The operation was ended with providing intra-thoracic negative pressure. Recovery of the cases were normal after the surgery. During surgery, 0.9 % saline (Poliflex, Polifarma, Turkey), 10 ml/kg /h was administered intravenously. Cefazolin sodium (cefazolin, MN, Turkey) 25 mg/kg IM was administered every 12 hours for 5 days, postoperatively.

In case 1, it was tried to stabilization of the patient firstly, and intensive care measures were taken. Although it was recovered completely from anesthesia, it died 2 hours after recovery. In case 2, it was discharged without any problem and recommended to feed with small amounts and frequent meals in vertical position for first month of postoperative period. Controls were performed at 1st, 4th, 9th, and 16th months postoperatively. Control radiographs revealed that the esophageal dilatation caused by the vascular ring regressed in the first month postoperatively, the animal gained weight, its growth improved and clinical complaints resolved.

Discussion

Breeds reported with the diagnosis of vascular ring include German Shepherd, Labrador Retriever, Irish Setter, Mixed-breeds, Brittany Spaniel, Beagle, Great Dane, Miniature Schnauzer and Yorkshire Terrier in dogs (Macphail et al., 2001; Buchanan, 2004; Vianna and Krahwinkel, 2004; House et al., 2005; Du Plessis et al., 2006; Loughin and Marino 2008). The breeds that reported in this case report, Chow Chow and the Malinois-Siberian husky cross-breed were not included previous reports of VRA. Therefore, it is understood that the literature data are not sufficient for dogs with this anomaly. In our article searches, Malinois-Siberian husky cross-breed was not found among the breeds reported as mix breeds. The most common type of VRA is the permanent right aortic arch (PRAA) (Yalcin et al., 2009). In addition, persistent left subclavian artery with persistent right aortic arch, persistent left ligamentum arteriosum and left subclavian artery with persistent right aortic arch, double aortic arch, persistent right ligamentum arterium with normal left aortic arch, persistent arteria subclavia sinistra with normal left aortic arch and arteria subclavia sinistra and normal left aortic

arches have also been reported (Buchanan, 2004; Fossum, 2013). Although PRAA and left ligamentum arteriosum are common in dogs and cats, other vascular ring anomalies have been reported rarely (Koc et al., 2004). In both cases, the cause of vascular ring anomaly was peri-esophageal fibrous bands and ligamentum arteriosum.

One of the main pathophysiological causes of esophageal-associated vomiting has been reported to be vascular ring anomaly in dogs and cats. It can successfully treated by surgery (Cave, 2013). The loss of time with symptomatic treatment generally affects the success of surgery adversely. It has been confirmed once more in our cases that detailed clinical examinations are important for correct diagnosis, treatment and survival. It has been reported that vascular anomalies are also observed with PRAA and barium sulfate radiography is not sufficient for the diagnosis of vascular ring anomaly (Buchanan, 2004). We could not identify the type of vascular ring anomaly in our cases, but it was determined that vascular ring anomaly could be diagnosed with clinical experience and good radiographic information.

Cranial thoracic esophageal dilatation and esophageal stenosis on the base of the heart are the most important findings of radiography. Caudal thoracic esophageal dilatation (poststenotic dilatation) is rarely seen with vascular ring anomalies (Ellison, 1980). Radiography reveals the dilated esophagus, and after contrast radiography the strictured part of esophagus (Yalcin et al., 2009). Air, water and food accumulate in the dilated part of the esophagus. Indirect radiography with liquid barium sulphate shows the accumulation of contrast material in the dilated area (Fossum, 2013). Liquid barium sulphate (8 ml / kg PO, 30%) is given for the diagnosis of esophageal dilatation. Following, the ratio of the maximum esophageal dilatation area to the narrowest height of the fifth thoracic vertebrae (T5) should be ≤1 in normal dogs and cats. It should be considered mild dilatation if ≤ 2.5, moderate if ≤ 4, and advanced if > 4 (Fossum, 2013). In the present study, prestenotic esophageal dilation values were evaluated (Dilatation / T5) similarly to Fossum (Fossum, 2013). In case 1, indirect laterolateral (LL) radiographs of the esophagus showed the presence of a moderate prestenotic dilatation (Dilatation / T5: 2.931) consistent with the thoracic permanent right aortic arch, whereas case 2 showed severe poststenotic esophageal dilatation (ED> 4).

In persistent right aortic arched VRAs, the best surgical approach is performed through the left fourth intercostal space. After ligation, a folley catheter is introduced orally into the strictured area of the esophagus (Ellison, 1980). Our surgical approach was similar to Ellison. Differently, after dissection of the persistent right aortic arch, we checked the flexibility of the esophagus and the elimination of the contraction factor by Foley catheter. The thinness of the esophagus and the high risk of perforation require very careful dissection (Ellison, 1980). These complications are possible because dilatation disrupts the normal structure in the region and makes it fragile. However, with a careful surgery, possible complications were prevented. However, it was concluded that early and accurate diagnosis of patients is as important as operative care to ensure the well-being of the patient. Calıskan et al. reported that the studies were limited due to the very rare occurrence of vasculer ring anomalies (Caliskan et al., 2018). Therefore, two cases were evaluated in this study.

Conclusions

As a result; symptoms such as vomiting, flatulence and abdominal tension must be examined with detailed radiographs and possibility of the vascular ring anomaly should not be overlooked, or confused with other digestive system diseases. Early diagnosis is important. The surgical treatment may have a better prognosis in early treated cases. In addition to the surgical treatment, the diet is also required to be rearranged.

Acknowledgement

The authors would like to thank Halil ALAKUŞ and İbrahim ALAKUŞ for their support in the clinical work of the study.

Conflict of Interest

The authors declared that there is no conflict of interest.

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JIVS JOURNAL OF ISTANBUL VETERINARY SCIENCES

Sero-prevalence and associated risk factors of bovine brucellosis in selected districts of Benadir Region, Somalia

Research Article

Volume: 4, Issue: 2
August 2020
Pages: 57-63

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ABSTRACT

A cross-sectional sero-prevalence study using random sampling was conducted from June to November 2019 in the selected districts of the Benadir Region of Somalia (Karaan District) and (Deyniile District) to determine the seroprevalence of bovine brucellosis and assess the potential risk factors. In this study, a total of 395 animals aged from 7 months and above were screened for Brucella antibodies using the Rose Bengal plate test, and positive sera were confirmed by complement fixation test. Four sera samples out of the 395 (0.7%) reacted positively for the Rose Bengal plate test and one of them reacted positively for the complement fixation test (0.2%). In the present result of this study, the prevalence in female and male and age were not significantly related to the seroprevalence of brucellosis ($P>0.05$). But there is a significant relationship of seropositivity of brucellosis with a record of abortion ($P<0.05$).The current cross-sectional study of bovine brucellosis in the Benadir region of Somalia showed is very low. There is a need to institute control measures of brucellosis through vaccination education on control to the public and conducting serosurveys and those animals testing positive culled.

Article History

Received: 24.06.2020
Accepted: 17.08.2020
Available online: 18.08.2020

Keywords: bovine brucellosis; Benadir Region; seroprevalence.

DOI: https://doi.org/10.30704/http-www-jivs-net.757196

To cite this article: Mohamud, A. I., Mohamed, Y. A. & Mohamed, M.I. (2020). Sero-prevalence and associated risk factors of bovine brucellosis in selected districts of Benadir Region, Somalia. *Journal of Istanbul Veterinary Sciences. 4*(2), 57-63, **Abbreviated Title:** *J Ist Vet Sci*

Introduction

Somalis are one of the most livestock production populations and they contribute greatly to the family in cash profits (Nega et al., 2009). Sheep and goats, camels, and dairy cattle are the domesticated animals kept in Somalia. And they kept under agro-pastoralist (in southern and western regions) or pastoralist production system (central and north regions) (Ombui et al., 2014). Brucellosis is approved as one of the ignored tropical zoonotic diseases with a serious global public health importance caused by the genus Brucella (WHO, 2006 and OIE, 2018) and affects humans and animals (Corbel, 1997). Bovine Brucellosis is primarily a reproductive disease in a female is characterized by abortion in the very last trimester and epididymitis and orchitis with common infertility in male animals (David et al., 2001).

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https://dergipark.org.tr/tr/pub/http-www-jivs-net

Brucellosis can transmit by direct or indirect contact with diseased animals. The Brucella organism is most commonly acquired by ingestion, skin contamination and genital inoculation, Respiratory route, intrauterine and venereal transmissions are other possibilities of the organism (Walker, 1999) and (Corbel,2006).
Brucellosis can also cause a huge economic loss in farm animals due to abortion, sterility, dead offspring and the birth of weak, increased calving interval, and drop off or the decrease in milk yield (Rahman, 2019).

Brucellosis is the second most important zoonotic disease after rabies as ranked by the WHO, OIE and FAO cause an estimated half-million human cases yearly in the world (Schelling et al., 2003 and Pappas et al., 2006). The disease affects almost wild animals, domestic animals, as well as humans (Ghanem et al., 2009).

Husbandry system and environmental factors, herd size, type of breed of animal (dairy or beef), characteristics of the animal, management practices, and biology of the disease are Risk factors associated with brucellosis. Abortion of the cows is the main important source of risk for the spread of the infection (Ebrahim et al., 2016). The different livestock share water, pasture, and housing which increases direct contact between animals hence facilitating transmission of brucellosis. The lack of vaccination and immunization in the pastoral community is also a major factor that perpetuates brucellosis transmission and outbreaks in rural areas (Kabagambe et al., 2001).

Brucellosis is endemic in many countries in Africa (Cadmus et al., 2006, Tijjani et al., 2009 and kaltungo et al., 2013).

In Somalia, the prevalence of brucellosis estimates ranged from 2.8% to 5.6% (Flade and Hussein, 1979). Sero-prevalence of Bovine Brucellosis in Mogadishu is reported by 10% (Afrah et al., 2020), in serosurvey of Brucellosis in Kismayo, the prevalence estimates ranged from 7.2% in sheep and 5.3% in goats (Andreani et al., 1982). The prevalence reported in Jijjiga Zone of Somalia regional state estimated 1.38% (Geresu et al., 2016; Gumi et al., 2013).

The specific economic losses of Somalia related to the diseases include loss of milk, sterilized infertility and, cost of vaccine and droplet value of animals culled because of the disease (Bale et al., 1991). The level and effects of brucellosis in Somalia remains unidentified but the problem is likely to be great particular because of the large number of livestock and pastoral production practices.

For screening or confirming of brucellosis serological tests are used (Navarro et al., 2004). Indirect Enzyme-Linked Immunosorbent Assay (I-ELISA), Standard Agglutination Test (STAT), and Rose Bangle Plate Test (RBPT) are mostly used serological tests for the diagnosis of brucellosis in animals. (Rahman et al., 2014).

The objective of this study is to determine sero-prevalence and associated risk factors of bovine brucellosis in the Benadir Region.

Materials and Methods

Study area and period: The study was conducted in the Benadir region which lies among latitude 2.046934 and longitude 45.318161. The normal yearly temperature ranges between 28.7˚C - 37˚C. Benadir region is the capital city it has the largest population across Somalia and it is probable to have about 2.3 million people and covers an area of around 96,878 km (Wikipedia, 2018). The study was conducted from June to November 2019 at two Districts were Karaan and Deyniile which was located in Benadir Region. The criteria of selected herds purposively for the study because it is a huge population of Bovine and we were selected the suspected animals in herds respectively.

Study design: The cross-sectional study was conducted to determine the seroprevalence of Bovine Brucellosis by selecting two Districts of the Benadir Region.

Study population: A total of 395 indigenous breeds of cattle of different ages and sexes reared under the semi-intensive management system was sampled. An open system was used to determine the seroprevalence of Brucellosis.

Sample size determination: For sample size determination expected prevalence of 50% and the level of confidence of 95% was used using the formula of Thrusfield (Thrusfield, 2005).
The sample size used in the study was 395

indigenous breeds of cattle. N=1.962 pq/d2 where, N=sample size; p=expected prevalence; q=1-p; d=standard error (Thrusfield, 2005).

Blood sample collection: Whole blood samples from 395 indigenous breeds of Cattle, samples were collected from 5 ml of blood by jugular venipuncture of each animal using 10 ml syringe and 21G needle following proper restraint and allowed to clot overnight at room temperature. Serum was separated from clotted blood by transferring to other tubes and transported immediately to the Kasmo University laboratory for processing.

Rose Bengal plate test (RBPT): The presence of Brucella antibodies in serum samples were determined by the Bengal test (Alton et al., 1988). For a short time, enough antigen, test sera, Negative and positive manage sera for a day's testing was removed from the refrigerator and kept back at the room temperature for 90 minutes previous to the test; 30 µl of RBPT antigen and 30 µl of test serum was put beside on plate, and then combine thoroughly. (Concentrated suspension serological analysis, equal volumes 30 of *B. abortus* biotype 1, Instituto de Salud Tropical Universidad de Navrra, Spain) were mixed and rotated on a glass plate for four minutes. If agglutination was observed after 4 minutes, samples were considered positive, otherwise, they were considered negative for brucellosis.

Complement fixation test (CFT): Complement fixation test was used to all sera tested positive by Rose Bengal Plate Test (RBPT) for more confirmation *B. abortus* antigen for CFT was used to mark the presence of anti-Brucella antibody in the sera like RBPT. The test was prepared according to the standard method mentioned by OIE (OIE, 2004).

Statistical analysis: The entire data collected was entered into a Microsoft Excel spreadsheet and coded appropriately. The coded data were replaced into SPSS version 19 software. For data analysis, descriptive statistics were used but to test the association of the hazard factors with the disease chi-square test were used and the association was considered if P-value is less than 0.05.

Results

The general prevalence of the current finding was 0.2% with the rates of 0.2% in Karaan and 0.7% in Deyniile districts in Benadir Region of Somalia using the RBPT test while during the confirmation of the sera-positive animals using CFT it was 0% in Karaan and 0.2% in Deyniile with no statistical significance variation between these two Districts (P>0.05) as indicated in Table 1.

The prevalence of brucellosis in female and male was 1%, 0.0% when tested using RBPT while the CFT test indicated that it was 0.2% and 0% in female and male respectively. But there was no statistical variation in the rate of the disease between male and female (P>0.05) as indicated in Table 2.

Age grouping prevalence of brucellosis disease indicated that is was upper in those cattle, it is age ranged from 7 months to 5 years old compared to the rest of the age groups with no statistical variation between age groups (P>0.05) as indicated in Table 3.

The prevalence of brucellosis was associated with their history of abortion on those examined animals (P<0.05) as indicated in Table 4.

Discussion

During this study, we used the RBPT screening test because of it's fastest, easy, and susceptible (97.9%), and allows processing lots of samples per day (PAHO/WHO, 2001). Those RBPT sera test positives were retested using the CFT test having a specificity of 100% (Dohoo et al., 1986). İn order to maximize the specificity of the tests. Animals were considered as positive if it was positive by both RBPT and CFT.

The overall prevalence of study findings was 0.2%, this indicated that only a limited number of dairy cows were affected by the disease, but this doesn't mean that the disease is insignificant as it is a very serious disease responsible for reproduction failure of the dairy industry in the area and its zoonosis important.

The overall prevalence of the current finding was lowered compared to the study conducted Afrah who reported a prevalence of 10% in Mogadishu, Somalia, Similarly, the prevalence

Table 1: The prevalence of bovine brucellosis in Karaan and Deyniile districts

Districts	Number of examined animals	RBPT Positive	CFT positive	*P*-value
Karaaan	162	1 (0.2%)	0 (0%)	
Deyniile	233	3 (0.7%)	1 (0.2%)	0.52

Table 2: Prevalence of bovine brucellosis in female and male.

Sex	Number of examined animals	RBPT Positive	CFT positive	*P*-value
Female	297	5 (1%)	1 (0.2%)	
Male	233	0 (0%)	0 (0%)	0.251

Table 3: Prevalence of bovine brucellosis in different age group

Sex	Number of examined animals	RBPT Positive	CFT positive	*P*-value
7 month-5 years	282	4(1%)	1(0.2%)	
6 year-9 years	113	0(0%)	0(0%)	0.229

Table 4: Association of brucellosis with abortion

Sex	Number of examined animals	RBPT Positive	CFT positive	*P*-value
Aborted	87	2(0.5%)	1(0.2%)	
Non aborted	308	2(0.5%)	0(0%)	0.042

reported in Jijjiga Zone of Somalia regional state 1.38% (Geresu et al., 2016 and Gumi et al., 2013), relatively higher seroprevalence was reported in other African countries. İn central Ethiopia 11.0% (Megersa, et al., 2011), in Uganda 3.3% (Magona et al., 2009), in Sudan 24.5% (Angara et al., 2004), in Tanzania 5.3 % (Swai and Schoonman, 2010), in Algeria 31.5% (Aggad and Boukraa, 2006).

The reasons for the low seroprevalence of our study of Bovine Brucellosis might be attributable to well hygienic practices in the study area and use of maternity pen and separation of cows for the period of parturition, cleaning and disinfection actions, the killing of diseased animals, depending on their own farms for replacing supply and farm owners understanding of disease in these intensive farms.

The prevalence of the disease in male was zero compared to female having a prevalence of 0.25%. In accord with the present result, Tesfay and Yayeh reported that only female cattle were well-thought-out as positive reactors (Tesfaye 2003; Yahyeh, 2003). This was as well explained by Radostits et al. who stated evidently that sex has been one of the risk factors causing susceptibility of cattle to Brucella abortus infection (Radostits et al., 2000). Age-wise produce of the current finding as well determined that the rate of the disease in this study was 0.2% in those cattle its age ranging from seven months to five years old. In line with the recent finding recorded that the superiority (97.87%) of sero-reactors notice were in animals above two years of age in both the extensive and intensive management systems (Kassahun

et al., 2007). Additionally, Radostits et al. explained that effective of cattle to Brucella abortus disease is helpful by the age of the individual animal (Radostits et al., 2000).

Further confirmed that while younger animals tend to be additional resistant to infection and often clear infections, a latent infection does happen (Walker, 1999). In the present finding, there was a statistical association of the history of abortion and the presence of infection in those animals (P<0.05). (Radostits et al., 2000). Also showed that in extremely vulnerable unvaccinated pregnant cattle, Abortion after the late months of pregnancy is an important characteristic of the infection.

The study suggested warranting immediate preventive measures in these areas. To avoid spreading out of brucellosis disease, it is essential to pay attention to aborting animals and supplies associated with abortion. Further study for isolation, identification, and typing of *Brucella spp.* epidemiological relatedness among animals or between animals and humans in Somalia is recommended.

Conclusion

The current cross-sectional study of Bovine brucellosis in the Benadir region of Somalia showed is very low, At the same time, the low prevalence of the disease was observed in different sex of animals and age groups of cattle. Though it's seroprevalence is low, it can still be a potential risk for both susceptible animals and humans. and perhaps in other areas of Somalia where nomadic pastoralism is practiced. There is a need for an institute to control measures of brucellosis through vaccination, education on control to the public, and conducting serosurveys and those animals testing positive culled.

Acknowledgments

We would like to appreciate Kasmo University, Mogadishu, Somalia for their overall guidance during the research activity. For providing the necessary facilities and support throughout the investigation.

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JIVS JOURNAL OF ISTANBUL VETERINARY SCIENCES

Effect of yeast *Saccharomyces cerevisiae* feed supplement on milk production and its composition of lactating Holstein Friesian cow from Northern Algeria

Research Article

Volume: 4, Issue: 2
August 2020
Pages: 64-74

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ABSTRACT

The objectives of the present study were to determine the effect of adding *Saccharomyces cerevisiae* (SC) yeast culture commercial on milk production, milk composition of lactating Holstein Friesian cows under Algerian conditions. A total of 16 lactating Holstein Friesian cows were used. The control group received basal diet without feed additives and the yeast, SC group received daily 2, 5 and 10 g/day per head. Individual milk samples were collected for analysis of milk composition and bacterial. The results of this experiment showed a significant high milk production in treated groups compared to control group ($P<0.05$). There is a significant difference between the control and the cows fed different treatments in somatic cells ($P<0.001$). It revealed that higher of milk protein in three treated groups was recorded than in the control group. The milk lactose content seems to be stable in cows supplemented SC and was practically similar to control group. On the other hand, the content milk fat was low in SC supplemented cows. The bacterial milk analysis was lower for the cows fed supplemented SC than the control group. The obtained results showed that the SC from Algeria can improve the milk production in dairy cows during the postpartum.

Article History

Received: 19.06.2020
Accepted: 23.08.2020
Available online: 24.08.2020

Keywords: cows, milk yield, milk composition, *saccharomyces cerevisiae*

DOI: https://doi.org/10.30704/http-www-jivs-net.755144

To cite this article: Besseboua, O., Benbarek, H., Hornick, J. N., & Ayad, A. (2020). Effect of yeast *Saccharomyces cerevisiae* feed supplement on milk production and its composition of lactating Holstein Friesian cow from Northern Algeria. *Journal of Istanbul Veterinary Sciences. 4*(2), 64-74, **Abbreviated Title:** *J Ist Vet Sci*

Introduction

Over the two decades, the will to reduce the adding antibiotics in animal diet to enhance production efficiency has strongly agreed. Therefore, many researchers have been aimed to develop alternatives with particular emphasis on the potential use of natural feed additives, one of which is yeast. Yeast culture and yeast by-products (e.g. *Saccharomyces cerevisiae*), as microbial additives, are widely used and approved in livestock nutrition. It has been found a positive effect on the rumen fermentation and production performance in ruminants.

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There are several *Saccharomyces cerevisiae* (SC) products on the market with different recommendation of dosage and in their manufacturing processes that may have an effect on animal performance (Wallace, 1994; Newbold et al., 1996; Stone, 2002). However, research results of the inclusion of SC in ruminants dietary have been variable. The beneficial effects of yeast culture and yeast by products on the rumen environment and production performance of ruminants have been well documented in literature (Dobicki et al., 2007; Phondba et al., 2009), and it has aroused particular scientific interest in feed industry.

Several researchers reported that the dietary inclusion of yeast in animal ration improves the feed conversion ratio, body gains, fattening performance, carcass quality, milk yield and lowers disease incidence (Fuchs et al., 2007; Milewski & Sobiech, 2009). Likewise, Kudrna and collaborators (2007) noted that yeast supplementation significantly improved the milk yield despite reducing the dry matter intake. In another study, the researchers concluded that characteristic, sources, production of probiotics on animal production have increased potentially milk yield and composition under Indian condition (Chandrasekharaiah et al., 2007).

Many studies have shown that the yeast supplementation feed could improves gut health of the animals, which results in increased digestion rate and better growth performance (Frizzo et al., 2010; Kawakami et al., 2010; Frizzo et al., 2011; Ghazanfar et al., 2015; 2018). The use of yeast culture as a dietary supplement has been suggested as source of improvement milk production and composition, and body condition in cows during the peripartum period (Vibhute et al., 2011; Ayad et al., 2013). Thus, Milewski and co-workers (2012) reported that the inclusion of SC yeast in the diet could enhance the quality of milk proteins in lactating ewes.

In the Algeria, the low productivity of dairy cattle is primarily due to forage unavailability, nutritive quality and probably loss or poor genetic potential. Hence, in order to improve the milk production of cows, the breeders distributes the high concentrate quantity during the lactating period. In the context, the adoption of scientific approach in feeding is more necessary. Keeping in view, the objectives of the present study were to determine the effect of adding *Saccharomyces cerevisiae* yeast culture commercial on milk production, milk composition of lactating Holstein Friesian cows under Algerian conditions.

Materials and Methods

As regards to the ethical aspects, the experimental protocol was approved by the scientific committee of the University Hassiba BenBouali (Chlef, Algeria).

Yeast: The supplemented SC (CNCM I-1077, Lallemand Animal Nutrition) marketed by Vetam Company (Mostaganem, Algeria) and contains of live yeast ($20x10^9$ CFU/g).

Animals and feed: The experiment was conducted in a private farm which is located in Chlef province, Algeria (36°10' N, 1°19' E). This study was conducted from 15 May to 15 July 2013 with 15 days of adaptation period. A total of 16 lactating Holstein Friesian primiparous cows were used. All cows calved during a two month period. The animals were divided in four groups as Group-0 (Control), Group-2, Group-5, and Group-10. All of the animals received a same feed ration during the experimental.

The cows in the control group were fed a basal diet without yeast. 2, 5 and 10 grams of yeast per cow per day was added to the feed of the cows in the Group-2, Group-5 and Group-10, respectively. The yeast additives was given in the morning after milking.

Milk production and sampling: Cows were milked two times daily (morning, at 06:30 and evening, 16:30 p.m) during the experimental period. Individual milk samples (100ml) were also collected from afternoon milking (1 p.m.) and kept at 4 °C for further analysis

Analysis of milk composition: Milk viscosity, pH, acidity, fat, protein and lactose contents were analyzed. In addition, somatic cell count (SCC) was determined. Viscosity and pH analysis were performed using Brookfield Viscometer and pH meter (model WTW, Weilheim, Germany), respectively. The acidity was measured according the method described previously by Caric et al. (2000). Fat content is usually based on a butyric

acid determination (Glaeser, 2002). Protein and lactose content were analyzed according to Kjeldahl method (AOAC, 2003) and Bertrand method (Budsławski, 1973), respectively. The somatic cell count (SCC) was determined every three weeks using the Malassez Counting Cells.

Bacterial milk analysis: Milk samples were collected in sterilized milk tubes and transferred in an icebox to laboratory. 0.1 ml sample was cultivated on various culture media including Chapman, mannitol, salt agar, at a temperature 37 °C during a lapse time of 48 h. Bacterial colonies were identified, based on colony growth, morphology and appearance, catalase reaction, and gram staining. In addition, coagulase positive isolates were identified based on hemolytic activity, acetoin production (Voges -Proskauer test) and anaerobic fermentation of mannitol (Koneman et al., 1992; Roberson et al., 1992).

Statistical analysis: Data were analyzed using a mixed model for repeated measurements (Statview Software, Version 4.55). Statistical analysis was performed using t-test to compare treated and control groups. The data were expressed as mean ± SD, and P<0.05 was considered significant.

Results

The effects of SC supplementation on dairy milk production is illustrated in Figure 1. The production of daily milk recorded in cows received of yeast cell supplement increased slowly and continuously over the time points investigated, however this difference was not statistically significant (P<0.05). Control cows (Group-0) had the lowest levels of dairy milk production in period 20-45 days post-partum.

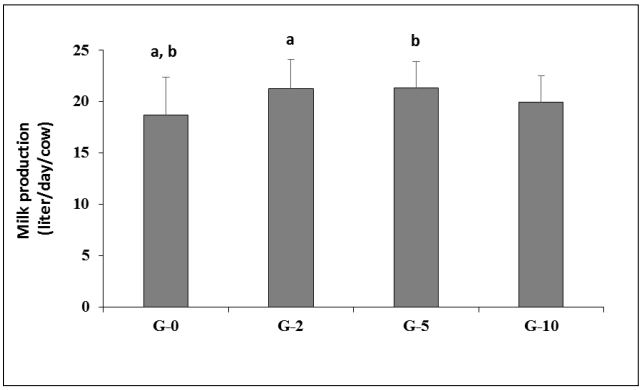

Figure 1. Effect of SC levels on milk production (liter/day/cow) of Algerian dairy cows during the first 60 days post-partum. A significant difference in mean milk production between the SC level treated groups (2, 5 and 10%) and the control group (0%) during the first 60 days post-partum is indicated by letters (a,bP < 0.05).

The results of this experiment showed a significant (P<0.05) high milk production in Group-2, Group-5 and Group-10 (21.3 ± 2.9, 21.3 ± 2.6 and 19.9 ± 2.6 liter/day, respectively) compared to control group (18.7 ± 3.7 liter/day) (Figure 2).

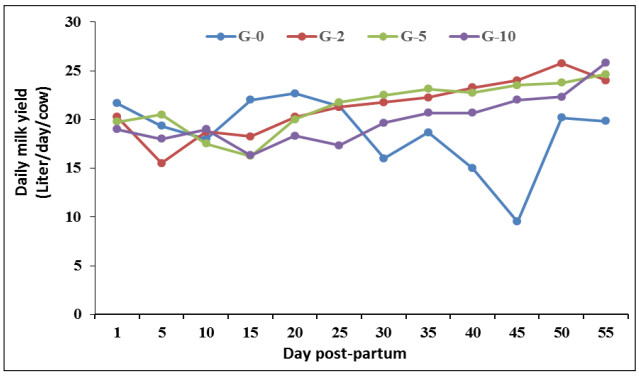

Figure 2. Effect of SC levels (2, 5 and 10%) on daily milk yield (liter/day/cow) and the control group (0%) during the first 60 days post-partum in Algerian dairy cows.

Chemical composition of milk collected in different groups of cows is summarized in Table 1. Milk viscosity was significantly (P<0.02) lower in cows supplemented with different SC concentrations than control group. In addition, SC supplemented cows (Group 2%) had a lower somatic cell than group 5% and 10%. There is a significant difference between the control and the cows fed different treatments in somatic cells (P<0.001). The cows fed ration containing 10% SC recorded an increased milk acidity in comparison to the control and all other diets treatment.

The milk fat, protein and lactose content according to the concentrations of SC supplemented cows is shown in Table 2. It revealed that higher of milk protein in three treated groups was recorded than in the control group. The milk lactose content seems to be stable in cows supplemented SC and was practically similar to control group. On the other hand, the content milk fat was low in SC supplemented cows during the two months post -partum. The bacterial milk analysis was lower for the cows fed supplemented SC than the control group.

Table 1. Effect of graded levels of SC on chemical composition of milk (pH, viscosity, acidity and somatic cells) in Algerian dairy cows. A significant difference in chemical composition of milk between the control group (0% SC level) and the treated groups (2, 5 and 10% SC level) is indicated by letters (a, b $P < 0.05$).

	Group-0 (n=12)	Group-2 (n=14)	Group-5 (n=16)	Group-10 (n=12)	*P*
pH	6.68±0.18	6.72±0.07	6.71±0.15	6.78±0.15	NS
Viscosity	0.23±0.02[a]	0.25±0.03[b]	0.26±0.03[a,b,]	0.25±0.21	< 0.02
Acidity (°D)	10.7±2.21[a]	13.0±3.04[b]	12.75±4.61	15.44±6.69	< 0.02
Somatic cells (10^2 / mm^3)	7.51±0.83[a]	1.18±0.14[b]	1.73±0.17[b]	2.12±0.11[b]	< 0.001

Table 2. Effect of graded levels of SC on the milk composition and bacterial in Algerian dairy cows. A significant difference in fat (g/L), proteins (g/L), lactose (g/L), lactate bacteria (colony/50µL) and *Staphylococcus aureus* (colony/50µl) between the control group (0% SC level) and the treated groups (2, 5% and 10% SC level) is indicated by letters (a, $P < 0.05$).

	Group-0 (n = 12)	Group-2 (n = 14)	Group-5 (n = 16)	Group-10 (n = 12)
Fat (g/L)	19.5 ± 20.35	14.5 ± 1	13 ± 3.56	20 ± 7.07
Proteins (g/L)	26.73 ± 5.25[a]	35.8 ± 2.08[a]	35.18 ± 3.8	33.98 ± 5.84
Lactose (g/L)	55.55 ± 0.35[a]	54.8 ± 2.82	54.55 ± 0.49	53.25 ± 0.07[a]
Lactate bacteria (colony/50 µl)	205.000	85.445	7480	5485
Staphylococcus aureus (colony/50 µL)	260.400	89.000	2000	1490

Discussion

The results of the present study shown that supplement of SC in diets of dairy cows increased the milk production (Figure 1), this in agreement with the results previously described (Bruno et al., 2008; Desnoyers et al., 2009; Moallem, et al., 2009). A significant increase in production of milk associated with the yeast supplementation has already been reported in dairy cows (Evans et al., 2012; Bayram et al., 2014; Bakr et al., 2015; Anjum et al., 2018; Rossow et al., 2018). Likewise, Ayad et al. (2013) demonstrated that supplementation with SC had statistically significant effect on milk production over under conditions of field Algerian, which mean daily milk yield was 32.7 ± 1.39 kg/d for the experimental group and 30.7 ± 5.3 kg/d for the control group. On the other hand, Kalmus et al. (2009) reported that cows receiving SC having numerically higher milk yield than the controls, although not statistically significant. In the other hand, Numerous studies have reported an increase in milk yield, but the effects have not been significant (Eramus et al., 1992; Dann et al., 2000; Lima, et al, 2012). Likewise, experiments have noted a response to Saccharomyces cerevisiae (SC) supplementation only in early lactation cows (Wohlt et al., 1991; Robinson & Garret, 1999; Dann et al., 2000). Also, trials annule the effect of yeast on the milk production (Kung et al., 1997; Erasmus et al., 2005). The variation of response of probiotics in previous investigation might be attributed to different

reasons such the variability associated with the distributed diets, the types and concentration of SC yeast used and the test animals (Williams et al., 1991). Also, It may be due to the magnitude of improvement of milk production depends on the stage and number of lactation (Majdoub-Mathlouthi et al., 2009). Moreover, the increase in production of milk is in most case associated with increases of dry matter intake (Degirmencioglu et al., 2013). However, This relative increase in milk production is not due to consumption of dry matter intake but related to the complementation of SC in food, reported by Wallace (1994) and Dann et al. (2000). According to Alugongo et al. (2017), the Saccharomyces cerevisiae tended to enhance rumen fermentation by increased butyrate production; and rumen papillae growth. This allows maintenance of the cellulolytic flora and enhances the degradation of plant fibers and therefore the diet digestibility (Wallace 1994). In addition, probiotics increases gastrointestinal absorption of nutrients by the intake of vitamin B1 e.g. thiamine, promotes the colonization of plants by rumen microbes and improves benefit the digestibility of the ration (Erasmus et al., 1992).

Data presented in Table 2 demonstrated that the fat and protein content of milk was different in the treated groups compared to the control group. In this study, the results of fat content in milk were lower in cows receiving graded SC diets than those the control group. This is in accordance with the previously results showed that the use of SC in dairy cows diet would impact on fat content (Putnam et al., 1997; Wohlt et al., 1998; Kalmus et al., 2009; Moallem et al., 2009). Yalçın et al. (2011) have found that the average fat percentage was 6.1% higher in group of the yeast culture than the controls. According to the literature, several authors have not found influence of yeast SC in fat milk (Wohlt et al., 1991; Soder & Holden, 1999, Bayram et al., 2014 and Rossow et al., 2018). This positive effect on fat content may be due to stimulation of cellulolytic bacteria and preferred orientation of fermentation to acetic acid production, especially rich diet on concentrate or contain low degradable forages (Piva et al., 1993). The diversity of results may be due to the chemical composition of the diet, body condition score and lactation field conditions (Masek et al., 2008). Also, many other factors can influence the food characteristics (e.g. the content of starch, net energy, and physically effective fiber), the state physiological (Haimoud-Lekhal et al., 1999), or administrative practices (Beauchemin et al., 2003).

Our results are similar of those obtained by Bruno et al. (2009) who reported that addition of S. cerevisiae induces a significant reduction in the protein content. Also, sevreal studies observed that protein yield from cows fed live yeast culture is higher than the cows control (Kalmus et al., 2009;Yalçın et al., 2011; Bakr et al., 2015; Rossow et al., 2018). However, Szucs et al. (2013) and Lima, et al. (2012) observed that no significant in milk of group SC comparable to the control group. Moreover, Hadjipanayiotou et al. (1997) reported no beneficial effects in milk production, as well as in fat or protein content after feeding yeast to lactating goats in a high concentrate diet. On the other hand Desnoyers et al. (2009) observed that the SC supplementation induced a significant decrease in milk protein. In other investigation, Temim et al. (2009) reported that the protein content of milk from cows of group supplemented with yeast are similar to those measured in controls, except at day 28 of lactation where lower value protein is measured in the group treated compared to control group. Other recent studies have shown that the yeast has no effect on the composition of milk of dairy cows (Degirmencioglu et al., 2013; Aye et al., 2016). This increased of the protein content milk in the experimental group could be the well -known impact of yeast on ruminal fermentation and nutrient digestibility, which improves the absorption of ammonia and enhances the production of microbial protein (Jouany et al., 2007). Note that the supplement of Saccharomyces cerevisiae improved the proportions of casein fractions in total milk (Milewskiet al., 2012).

Lactose reduction rate obtained in milk content with a SC level were similar for all groups, and in agreement with those reported by numerous studies (Lima, et al., 2012; Degirmencioglu et al., 2013; Bayram et al., 2014). However, Bal & Göksu (2013) indicated that the addition of a yeast supplement in a diet 50% concentrate increase the lactose percentages in milk cow. In other investigation, Yalçın et al. (2011) reported a significant improvement in the percentage of lactose in milk of dairy cows when supplemented with yeast culture. The divergency of results could be due to stage of lactation, feeding strategy, the conditions of the environment, the diet composition, type of feed, type and dose yeast incorporated in food (Yalçın et al., 2011). Also, the yeast products could be more effective under stress rather than during normal conditions (Moallem et al., 2009). When compared with the control group, diets supplemented with different levels SC resulted in a remarkable decrease in the number of pathogenic bacteria such as *Staphylococcus aureus* and Lactate bacteria. Several bacterial pathogens can cause mastitis, *Staphylococcus aureusis* one of the most important etiologic agents in mastitis of cows, goats, and sheep (Rich, 2005; Moon et al., 2007; Li & Zhang, 2014; Sağlam et al., 2017).

According to Lapointe-Vignola (2002), the pH of normal milk is ranged from 6.6 to 6.8. On the other hand, pH above 7.0 is due to mastitis milks associated with high cell counts (Marschke & Kitchen, 1985; Rao, 1990). In the present experiment, the pH of cow milk was similar between all groups, and correspond to the pH values physiological. The determination of the pH gives a first idea of the quality of the product and the presence of germs which one can possibly find there (Siousarran, 2003). Our results are in agreement with those of Aye et al. (2016) and Maamouri et al. (2014) that show the complementation in yeast food has no significant difference in pH milk in cow. The feed supplemented with graded SC had effect statistically but slightly on the milk viscosity compared to the control group. The viscosity varies not only with changes in physical (T °C) and also with the hydration of proteins. Moreover, the increased viscosity is observed when the fat globules are broken down by homogenization (Kebchaoui, 2012). Noted that the viscosity of the milk is a complex property that is particularly affected by the emulsified particles and colloids dissolved. In addition, the content of fat and casein has the most important influence on milk viscosity (Ghaoues, 2011).

In this study, the results of titratible acidity of milk were significantly higher in cows receiving graded SC diets than those the control group. This increase in acidity may be due to a raise in lactic milk flora (Kuczaj et al., 2014). Carole (2002) indicated that the acidity of fresh milk can varied between 15 and18 °D. Our results that all mean values of titratable acidity of milk in diets group are similar to that cited previously, which could be explained by increase of acid components in diet supplementation. In the other hand, Hossain et al. (2014) showed that there was no significant ($P>0.05$) variation in acidity of milk between control group and treatment group. The mean of somatic cells count (SCC) recorded was similar in cows group supplemented with 2 and 5 g of SC diet. A significant decrease in somatic cell count in cows receiving different SC level compared to control cows ($P<0.05$). The results of this study correspond to those previously published, which demonstrated that the yeast application has significantly reduced the SCC in milk ($P <0.05$) for SC groups when compared to the control (Degirmencioglu et al., 2013; Kuczaj et al., 2014; Bakr et al, 2015). In 2016, Aye et al. indicated that feeding live SC could significantly reduce the incidence of subclinical mastitis in lactating cows. Otherwise there was no significant treatment effect on acidity (Lima, et al., 2012). The reduction of SCC in yeast-treated cows may be attributed to a better health status of their udder (Albenzio et al., 2015). The CSC determination is used worldwide in dairy practice to describe the hygienic control of milk (Wellnitz et al., 2009), and could be considered as a good indicator of intramammary infection (Petzer et al., 2017). The low somatic cell account is very important and required for a

good production of cheese and decrease the milk conservation in the transformation unities. The differences between trials may be due to the other ingredients of the diets (Dawson, 1989), the physiological stage of the lactating (Williams & Newbold, 1990), species, sex and environmental conditions of animals, which can influence the availability of nutrients (Lima, et al., 2012).

Conclusion

The results obtained under the conditions of this experiment showed that the SC from Algeria can improve the milk production in dairy cows. Also, the inclusion of a level (2%) of SC in diets is more effective on the milk yield and protein content. However, supplementation of grade SC at 5% level was found beneficial in ameliorating the milk yield. Thus, dietary inclusion of SC had effect on viscosity, acidity and somatic cell values in dairy cows at the end experiment. Based on the results of this investigation, an effect was found on milk protein and fat production in incorporating the probiotic yeast, Saccharomyces cerevisiae, in the diet of the cows during the postpartum.

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JIVS JOURNAL OF ISTANBUL VETERINARY SCIENCES

Virus imaging tools

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Research Article

Volume: 4, Issue: 2
August 2020
Pages: 75-83

ABSTRACT

Viruses are very small physical particles that can not be observed with a normal microscope. Only the largest virus, the poxvirus can be seen in the light microscope. Other tools are required for a detailed examination of viruses in ultrastructural size. Especially for the last 20 years, these advanced and ongoing tools have been used in the investigation of the biological molecules and biological processes of viruses. With the help of virus imaging tools, viruses can be identified in clinical samples, thereby explaining the detailed life cycle of the virus. Thus, these tools help the creation of new and safe vaccines and antiviral medicine. In this review, electron microscopy (EM) tools were given as scanning electron microscopy (SEM), transmission electron microscopy (TEM), cryo-electron microscopy (Cryo-EM) since generally used tools based on EM. Besides EM-based tools, X-ray crystallography which is the basis for cryo-EM and atomic force microscopy (AFM) that is relatively economic and easy to apply compared to other microscopies were also described.

Article History

Received: 10.06.2020
Accepted: 25.08.2020
Available online: 28.08.2020

Keywords: virus, visualisation, equipments

DOI: https://doi.org/10.30704/http-www-jivs-net.750638

To cite this article: Sareyyüpoğlu B.(2020). Virus imaging tools. *Journal of Istanbul Veterinary Sciences, 4*(2), 75-83,

Abbreviated Title: *J Ist Vet Sci*

Introduction

Ultrastructural dimensions about viruses is an important step to get information about morphology, viral entry, replication dynamics, changes in a virus infected-cell. Thus, these tools help the development of new diagnostic and treatment methods against the viruses. The basis of ultrastructural studies was laid in the 1930s by the filtration of viruses through colloid membranes. The first virus imaging method is the electron microscopy (EM) (Risco and Carracosa, 1999; Gelderbloom and Hazelton, 2000; Wagner and Hewlett, 2003; Bartenschaler and Romea Brey, 2015). There are varieties of EM such as SEM, TEM, and Cryo-EM. After EM; other tools were developed as X-ray crystallography, nuclear magnetic resonance, cryo-EM, bioluminescent imaging, and lastly positron emission tomography (Cherry and Gambhir, 2001; Cook et al., 2003). In this review, EM techniques, X-ray crystallography, cryo-EM, and AFM tolls were described.

Electron Microscopy (EM)

Eectron microscopy (EM) has a greater resolution than light microscopy because electrons have a shorter wavelength than light. Therefore, EM allows the image to grow ×10.000 more than a light microscope. It gives information about the virus topography, morphology, composition using the high energy electron beam. Topography

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describes the surface properties of an object, how it looks, its texture, and the direct relationships between these properties and material properties (tightness, reflectability). Morphology, on the other hand, explains the direct relationships between the shape and size of the particles forming the object and the material properties (flexibility, durability) while the composition explains the direct relationships between the elements and building blocks forming the object and the material properties (melting point, reactivity, tightness) (Gelderbloom and Hazelton, 2000; Bartenschaler and Romea Brey, 2015). The first EM developed by Max Knoll and Ernst Ruska in 1930 (Risco and Carracossa, 1999; Goldsmith, 2009; Bartenschaler and Romea Brey, 2015). Then SEM and TEM were developed (Risco and Carracossa, 1999; Goldsmith, 2009; Bartenschaler and Romea Brey, 2015). In general, EM works with a mechanism similar to that of the light microscope, which is its optical equivalent but uses the focused electron beam instead of light to examine the sample. The electron flow, first shaped by the electron source, is accelerated towards the sample using a positive electrical medium. Then, with the help of metal, the finely focused monochromatic light is dropped directly on the sample using magnetic lenses, and then various interactions occur in the sample, affecting the electron beam. These interactions are determined and converted into an image format (Risco and Carracosa, 1999; Gelderbloom and Hazelton, 2000; Wagner and Hewlett, 2003; Bartenschaler and Romea Brey, 2015).

The sizes of viruses are below the visible rays. Therefore, shorter wavelengths are required for viruses to be observed in EM. EM accelerates electrons to give high energy and focus them magnetically. High energy gives electrons a short wavelength, making them smaller in size than viruses (Wagner and Hewlett, 2003; Bartenschaler and Romea Brey, 2015).

The sample preparation steps for EM can be briefly listed as detection, washing, dehydration, embedding, cutting, dying. The purpose of all these processes is to protect the specimen in its current state, to protect the specimen against the procedures to be performed, and to thin the specimen. Thus, sufficient contrast provided so that electrons with limited penetration characteristics can easily pass through the sample (Bartenschaler and Romea Brey, 2015). The electron acceleration and focusing process performed when the sample can be examined and observed under a vacuum. To achieve this, the sample must be completely dry and fixed. During the sample preparation, the image may be lost or altered during the subsequent effects of protein hydration. In other words, works under in vacuum and staining process destroy the sample. This is the main limitation of EM (Wagner and Hewlett, 2003; Bartenschaler and Romea Brey, 2015). EM can be used imaging of many viruses like parapoxviruses, herpesviruses, rotaviruses (Roingeard, 2008). EM was also the first tool used for COVID-19 virus imaging. In that study (Min Kim et al., 2020) after the virus isolated in Vero cells, it was visualized with EM, and confirmed by sequencing.

Scanning electron micrroscopy (SEM)

SEM is another EM type for the visualization of viruses. SEM scans the 3D-high resolution image of virus surfaces. It can achieve sub-nanometer spatial resolution, revealing topological and compositional features. It magnifies an object × 200.000 (Raza, 2012; Haan et al., 2019).

The image of SEM is created by sending electron beams into the sample. Electrons sent on the sample to give charge to the sample. The sample preparation for SEM must have the following properties; the sample must be fixed, dried, have a certain conductivity, and the charge must not be collected regionally on the sample. For conductivity, it is coated with a metal like gold, palladium, or aluminum. For SEM microscopy, it is not necessary to prepare the samples as thinly as in EM. However, in both microscopies, the sample prepared by cold or freezing (Raza, 2012; Haan et al., 2019).

The SEM technique was used to display retroviral gag proteins involved in the cell binding process of retroviruses including HIV, and the result of the study, it was determined that each virus population showed different variations (Parker et al., 2001; Raza, 2012; Haan et al., 2019).

The parts that make up the image in SEM are the electron gun that works as an electron source. The condenser lenses that form a thin electron beam by compressing the electrons coming out of the gun, the beam deflector that allows the electrons to scan the sample inline form, the lenses, the detectors that collect the reflected rays after hitting the sample. The electron beam is better compressed and denser by the photomultiplier tube (PMT) (Haan et al., 2019). A schematic diagram of the SEM are shown in Figure 1 (Raza, 2012).

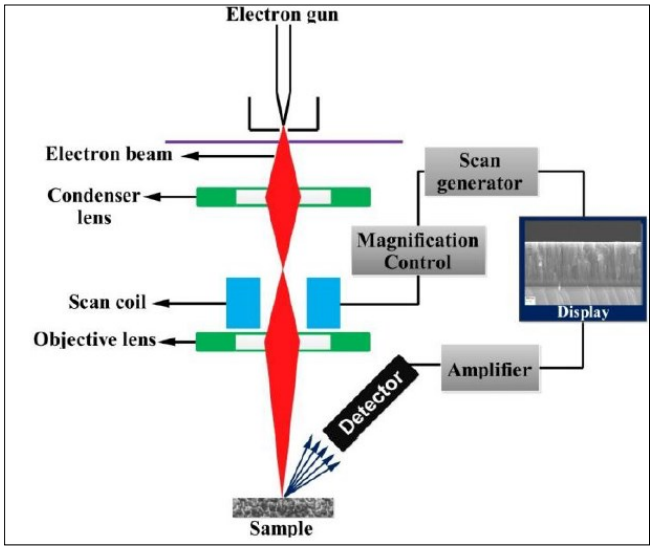

Figure 1. A schematic diagram of SEM (Raza, 2012)

Transmission electron microscopy (TEM)

The first TEM was demonstrated by Max Knoll and Ernst Ruska in 1931, Ruska was awarded the Nobel Prize in physics for the development of TEM contributed to the discovery of many types of viruses and served as a diagnostic tool for interior virus imaging. In the 1990s, the diagnosis of viral infections gained a lot of momentum with the help of enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR), and via TEM. TEM supported diagnostic methods by performing direct imaging without prior knowledge of the infected agent being investigated. Thus, it is still used today for the detection of infectious agents in situations where the molecular diagnosis is insufficient (Roingeard et al., 2019).

TEM magnifies the examined object ×5000.000. It can obtain information about morphology, crystallographic information, and structure of the virus. Similar to SEM, the electron gun works as an electron source. TEM works just like a slide projector. The projector reflects light through the slide. As the light passes through the slide, it is affected by the structures and objects on the slide. These effects are observed in certain parts of the light beam transmitted by certain parts of the slide. The image is projected on the screen to magnify this transmitted light. The screen made of phosphor (Roingeard et al., 2018; Bartenschaler and Romea Brey, 2015). Samples for TEM should be very thin and it usually stained neural acetate. Then, image transferred a fluorescent screen. Similar to SEM, The lenses of TEM are electromagnetic. This is the main difference from the light microscope. The image is formed around this electromagnetic field. The rest of the microscope suspended in the air except for its anode and cathode. The rays penetrate more easily through the sample when the voltage increased. However, in this condition, the contrast is diminished. The voltage used varies depending on the sample. Variable charge grits control the electron density, in other words, the image brightness. Image brightness can be changed by changing the cathode temperature (Bartenschaler and Romea Brey, 2015; Roingeard, et al., 2018). The layout of optical components in a basic TEM is shown in Figure 2 (Williams and Carter, 2009).

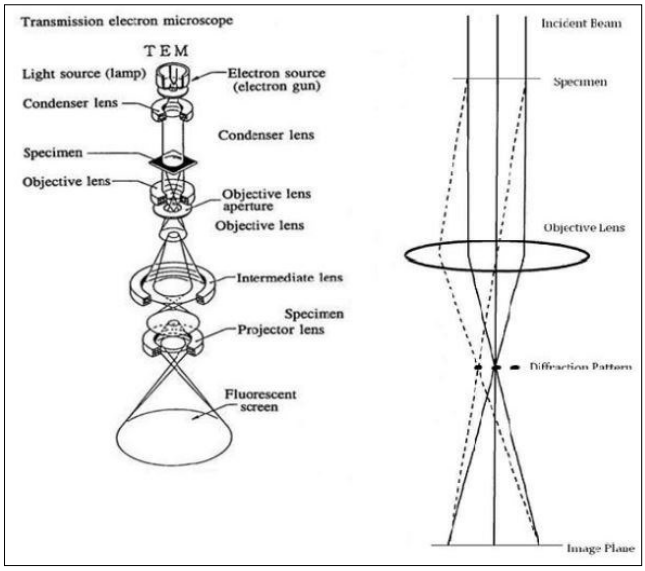

Figure 2. The layout of optical components in a basic TEM (Williams and Carter, 2009).

TEM and SEM are generally used two EM tools for virus imaging. Therefore, the differences between both should be explained in this review. The main difference between the TEM and SEM is the electron scattering feature. SEM uses scattered electrons, but TEM uses transmitted electrons. Therefore, SEM focuses on the sample surface like EM and its composition, whereas TEM provides more details about its internal composition. TEM magnified an object 5000.000x while the SEM 100.000x. In other words, SEM shows only the morphology of samples. TEM can show many characteristics of samples like morphology, crystallization, or even magnetic domains (Bartenschaler and Romea Brey, 2015; Haan et al., 2019). The main limitation in both techniques is that they are both expensive, required trained experts, toxic staining used, and like EM, sample dead during sample preparation because of vacuum and toxic staining.

X-ray crystallography

X-ray crystallography is a basic method to get information about the high resolution of a protein structure. X-ray crystallography provides atomic-resolution structures of proteins and small viruses in order to see the object. Visible rays have a wavelength greater than the distance between atoms. These rays are not enough to see molecules. Therefore, X-rays using electromagnetic radiation have a wavelength that is small enough to see atoms (Carter et al., 1997).

In 1953, Watson and Crick studied the X-ray diffraction model of crystallized DNA. The technique, which records the diffracted X-ray density and spatial distribution emitted from 3-D crystals, makes it possible to investigate many biological specimens. This diffraction model of the structure is measured by a mathematical method called the Fourrier transform. It is important to get the crystals to sufficient size and to be able to form a regular 3D crystal structure and obtain isomorphous heavy-atom derivatives for initial phasing of diffraction data. The main approach in a crystallization trial is to reduce protein solution ability by mixing protein solution with a precipitating solution. If the proteins are protected from amorphous precipitation and/or denaturation, a periodic 3D crystal structure will form (Auer, 1997; Carter et al., 1997; Hewat et al., 1997). X-ray tubes, anode tubes, goniometer, and X-ray microscopes are required with this tool. Moreover, the crystals that can break the X-ray beam are needed, which turns into a diffraction model that can be interpreted by a mathematical operation calculated on the computer. With the computer, the image data is captured when looking at the monitored molecules. X-ray refraction of a unit/ single cell is not evident. Repeating and replicating a unit cell with the help of crystals needs to transform the image. In short, crystals are regular 3-dimensional structures and are important because they are reproducible units of the cell in which they reside. The most difficult and crucial step in X-ray technique is the stage of enlarging crystals. The mostly used technique for enlarging crystals is the dynamic light scattering (DLS) method (Wery et al., 1997). In addition, solution concentration is important to enlarge crystals. In other words, crystals are enlarged changing the saturation degree of the solution (Carter et al., 1997). For example, there is no crystal forming in a non-dense solution. In the low-density solutions, crystals grow, but no new crystals are formed. In the high-density solutions, several crystals begin to grow in a high degree of solution. Then, they grow until when the crystals enclosed in less saturated solutions. The main drawback of X-ray crystallography examined protein must be crystallized. It is a very difficult process besides not all biomolecules suitable for this. Thus, only a very small number of membrane protein structures can be determined by X-ray crystallography. Often a combination of X-ray crystallography and Cryo-EM are used. For this reason, Cryo-EM was developed after X-ray crystallography. Schematic diagram of X-ray crystallography is shown in Figure 3 (Bütner et al., 2015)

Acharya et al. (1990) examined the molecular structure of Foot and Mouth Disease Virus (FMDV) by X-ray crystallography technique. In the study, crystals were formed and diffracted with weekly periods. The X-ray beam produced

by the synchrotron radiation source has helped to unravel the structure of the virus. X-ray crystallography also used for adenovirus imaging (Stewart et al., 1993).

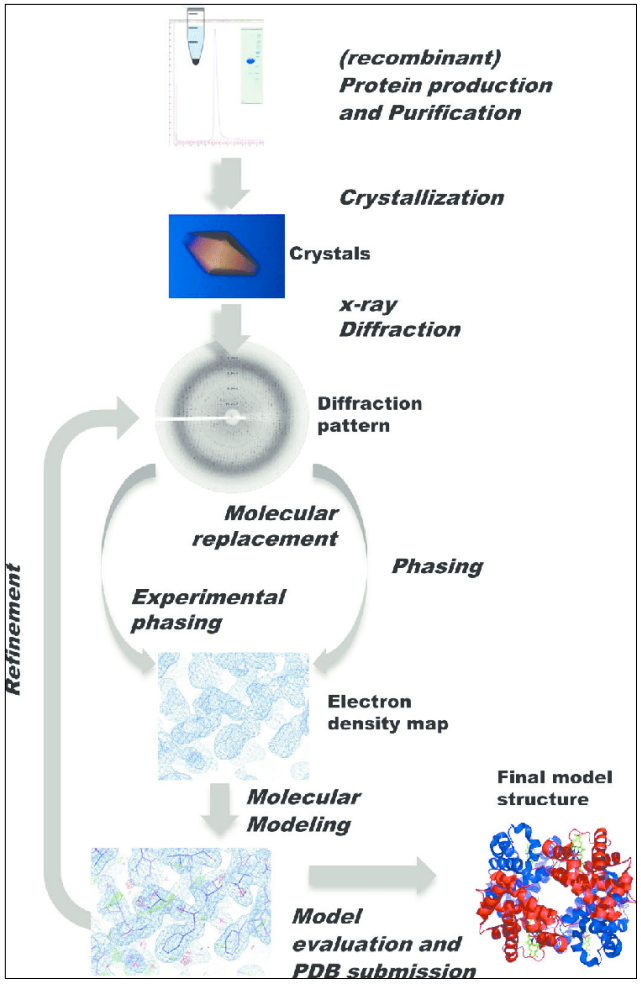

Figure 3. Schematic diagram of X-ray crystallography (Stewart et al., 1993).

Cryo-elektron-mikroskopy (CryoEM)

The Cryo-EM developed to prevent structural deformations that may occur when preparing a sample in a classical EM. Cryo-EM is a powerful tool and it is called a non-crystallography or single-particle reconstruction technique (Yu and Bajaj, 2005). Samples must be cooled by preventing the formation of ice crystals in the Cryo-EM technique. Because ice crystallography damages the ultrastructural structures of proteins (Risco et al., 2002). In 2017, Richard Henderson won Nobel prize with his work in developing detectors for cryo-EM. This technique allows the virus to be observed without loss, as there is no staining, shading, or coating process with heavy metals. In this method, the virus particle is quickly filled into the EM grits. The virus is enclosed in a glassy film (vitrified) layer consisting of thin ice. Since the dye is not used, the particles that receive water are well displayed by taking advantage of the differences between the electron density of the protein or lipid in the structure of the virus and the matrix that is surrounded by a layer of water (Wagner and Hewlett, 2003; Baker et al., 2004).

Vitrification can be achieved by cryosectioning or milling-substitution methods. Cryosectinoning is the removal of sections of vitrified tissue at liquid nitrogen temperatures. However, this approach is technically quite difficult. In the milling substitution, the sample is painted after being physically fixed, dried, and placed well by burying the water at temperatures well below the freezing temperatures. This second approach is quite simple compared to the first one. Nevertheless, due to staining and chemical application in the milling substitution, there are unwanted artifact formations (Auer, 1997).

The main problem with the Cryo-EM is the unexplained displacements of particles floating in the glassy ice environment. Therefore, many of the macromolecules studied with this technique remain dependent on the symmetry of the structures studied (Yu and Bajaj, 2005). In other words, a high concentration of homogeneous virus particles is required to create the image. Therefore, cryo-EM used particularly in the study of icosahedral viruses (Commike and Chiu 2000). The problem is Cryo-EM is that it needs an extraordinary expensive microscope. Similar to TEM and SEM, Cryo-EM also required trained experts. For this aim, in recent years, robots have been used the sample preparation.

In a study (Hewat et al., 1997), X-ray and Cryo-EM techniques were used to investigate the interaction between Foot and Mouth Disease Virus (FMDV) and monoclonal antibody SD6, a strongly neutralizing monoclonal antibody. Accordingly, the structure of the Fab part of the antibody against the virus and the monoclonal antibody was determined using Cryo -EM. In another study (Briggs et al., 2004), a beta

virus and mouse breast carcinoma virus was examined with the Cryo-EM technique, glycoprotein structure, and core formation of the virus was obtained.

Cryo-EM is also used for COVID-19 imaging (Gao et al., 2020). Cryo-EM used for imaging viral RNA polymerases for COVID-19 viruses to develop new coronavirus vaccines and therapeutics. In the study, the viral polymerase NSP12 looks an excellent target for new therapeutics, especially given that lead inhibitor already exists in the form of compounds such as Remdesivir.

Principally, an electron beam sends at a frozen protein solution. Scattered electrons pass through a lens to occur a magnified image on the detector. Therefore, multiple images of protein molecules in different orientations can be collected. A schematic diagram difference between the TEM and Cryo-EM is shown below. As to be seen in the diagram, the main difference to prepare the vitrified sample in Cryo -EM and detector covert the images 3D analysis. A schematic diagram difference between the TEM and Cryo-EM are shown in Figure 4 (Szatanek et al., 2017).

Atomic Force Microscopy (AFM)

AFM is a high-resolution technique used in the imaging of proteins, nucleic acids, and nucleoprotein complexes. Unlike the EM, it is a technique that allows the study of biological structures at a single molecule level and in their natural environment (Kiselyova et al., 2001; Kienberger et al., 2004; Kuznetsov et al., 2001; Kuznetsov and Mcpearson, 2011). AFM developed in 1986 (Binning et al., 1986), which is a better resolution than EM, as well as being in the sample (solid, liquid, gas, etc.) works independently and does not require a long-term sample preparation process. The AFM is a highly suitable tool for surface analysis/topography studies. A probe in the technique is mounted on a retaining arm (spring cantilever) with very precise flexibility. There is also a trestle, optical deflection (installed from a laser diode and photodetector) system where it is placed. The retaining arm is made of silicon or silicon nitride and its dimensions are 100-300 µm in length, 10-30 µm in width, and 0.5-3 µm in thickness. Pressing on the sample with a constant force, this arm moves down and up depending on the topological changes the probe encounters on the sample surface. The x-y-z values recorded during the scan are sent directly to the computer screen and memory using a digital signal processor (DSP). After this stage, image processing and analysis are done with appropriate software (Binning et al. 1986; Colton et al. 1997; Kuznetsov et al. 2001; Kuznetsov and Mcpearson 2011).

The main difference of AFM from other microscopes is that a continuous controlled force is applied to the sample. It provides direct height measurements and surface features without covering, compared to SEM, and provides a topographic contrast easily. When compared to SEM and TEM, it is very practical that a sample preparation process is not as there is no need to cover the sample required in AFM (Colton et al. 1997; Kuznetsov et al. 2001; Kuznetsov and Mcphearson 2011). A schematic diagram of AFM is illustrated in Figure 5 (Balthazar et al., 2013).

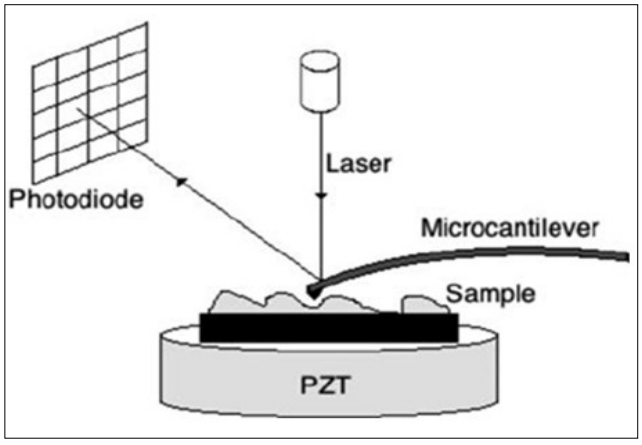

Figure 5. A schematic diagram of AFM (Balthazar et al., 2013).

Kienberger et al. (2004) examined human rhinoviruses within their physiological environment with the help of AFM. Topographic examination of the virus revealed several polygonal areas on the surface of the particle. It has been reported that RNA release occurs at low pH levels and that the length of released RNA is related to capsid length (ranging from 40 to 330 nm), while fork-like extensions may be

related to the characteristic multi-stem loop formation of RNA molecules.

There are several AFM models: constant force, contact, intermittent-contact mode, non-contact, and tapping mode. Topographic imaging can be performed with contact-model AFM. In this model, the tip and sample are in contact. During scanning, the gripper arm continuously touches the sample. However, in this model, imaging is not good if the sample is too weakly attached to the stand. Because during screening, biomolecules are pushed by AFM situluses. Also, the tip should touch the sample with a continuous up-and-down movement. The sideways movement will damage the sample and the tip. Manually controlled styluses can be used to eliminate this problem. Another approach is by tapping-mode AFM or magnetic AC mode models, where the sample is soft and weakly attached to the stand. In this model, the oscillator of the holder arm touches the sample only at intervals in its downward motion. Thus, contact time is reduced and the friction force is minimized. The cryo-AFM technique has also been developed where the sample can be monitored at low temperatures to increase the resolution (Colton et al. 1997; Kienberger et al. 2004; Kuznetsov et al., 2001; Kuznetsov and Mcpearson, 2011).

AFM allows direct imaging of viruses when they are in a watered-down position and can uniquely examine surface tomography. The technique is also used in revealing structural dynamics in vitro. However, it allows the display of surface parts of their internal structures. It is a very successful technique used in the imaging of large viruses, especially in asymmetric structure. In a study conducted (Malkin et al. 2003), an asymmetric and large-structure mature vaccinia virus was investigated by AFM technique.

The interaction of the virus with the cell is one of the fundamental issues of virology. Adsorption of the virus to the cell membrane, penetration, and infection of the cell constitutes the basic steps of virus infection. The AFM technique is a technique that helps to examine the first two steps. However, AFM is used only for the study of simple plant viruses. There is only one study of an animal virus with a cell membrane. In that study (Zaitsev et al. 2002), the interaction of a virus with an erythrocyte blood cell was studied. The erythrocyte membrane has always been a good model for the study of cell-virus interaction. Erythrocytes, which are well defined at the biochemical level, also remain stable during sample preparation in the AFM technique. The physical properties of the erythrocyte membrane were altered as a result of the effects of erythrocyte and virus and these changes were determined by AFM technique. In the study, the AFM technique was reported as an effective technique in the study of cell-virus interaction.

In conclusion, although most commonly used tools are EM, TEM, and Cryo-EM for virus imaging, SEM, X-ray crystallography, PET, bioluminescent imaging, and NMR can be mentioned among the other tools. Sometimes the combination of a few tools is preferred. The study of viruses in ultrastructural dimensions is a significant impact because it provides information about the viral entry, morphology, replication dynamics, changes in a virus infected -cell. Thus, the development of new diagnostic and treatment methods against viruses is only possible by examining them in more detail.

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JIVS JOURNAL OF ISTANBUL VETERINARY SCIENCES

Effects of cervical ripening treatments on pregnancy rates following transcervical artificial insemination in ewes: A preliminary study

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Research Article

Volume: 4, Issue: 2
August 2020
Pages: 84-89

ABSTRACT

The study was aimed to identify the effective cervical ripening treatment to increase the pregnancy rates following transcervical artificial insemination (TC-AI) in indigenous ewes of Bangladesh. Three treatments schedules were compared, G1 (n = 6, control), G2 [n = 6, prostaglandin E1 (PGE1) as Misoprostol-Cytomis®] and G3 (n = 6, estrogen as Oestriol-Ovestin® + oxytocin as Linda-S® Vet). Ewes (n=18) were synchronized for estrus using two doses of 100 µg prostaglandin (PGF2α; Ovuprost®) 9 days apart. The cervical ripening treatments were administered just after the onset of estrus and left for 12 h before TC-AI. The cervical ripening was reflected with the depth of cervical penetration. The depth of cervical penetration in G1, G2 and G3 both before and after cervical ripening treatments were 0.33 ± 0.10 cm vs 0.43 ± 0.07 cm, 0.41 ± 0.09 cm vs 3.50 ± 0.26 cm and 0.43 ± 0.09 cm vs 1.54 ± 0.14 cm, respectively. The depth of cervical penetration increased significantly ($p<0.01$) in G2 and ($p<0.05$) in G3 compared with G1. Comparing the pregnancy rates, no significant difference was observed among the groups. However, the pregnancy rates were increased (66.67%) in G2 near to significant compared to G1 (33.33%). In conclusion, the depth of cervical penetration and pregnancy rates was deeper and higher with PGE1-Misoprostol treatment. This preliminary study enlightens the development of effective TC-AI techniques in indigenous ewes for its satisfactory pregnancy rates and, to accept by the Bangladeshi sheep farmers. Furthermore, this study provides some important results that suggest room for further research with a greater number of ewes.

Article History

Received: 25.06.2020
Accepted: 27.08.2020
Available online: 28.08.2020

Keywords: cervical ripening, cervical penetration depth, indigenous ewes, pregnancy

DOI: 10.30704/http-www-jivs-net.757791

To cite this article: Bari, F. Y., Islam, M. M., Jha P. K., & Juyena, N. S. (2020). Effects of cervical ripening treatments on pregnancy rates following transcervical artificial insemination in ewes: A preliminary study. *Journal of Istanbul Veterinary Sciences, 3*(2), 84-89. **Abbreviated Title:** *J Ist Vet Sci*

Introduction

Nowadays, sheep farming is gaining popularity among Bangladeshi farmers. Due to poor genetics, farmers are looking for sustainable and locally appropriate reproductive techniques to upgrade their sheep genetics. Artificial insemination (AI) in conjugation with estrus synchronization is extensively used in sheep breeding programs to disseminate genetic traits of interest and increase production. Achieving acceptable pregnancy rates is very important for uptake to AI technology by sheep farmers. To establish an AI program in Bangladeshi ewes, studies on estrus synchronization, AI techniques using chilled and frozen

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Journal home page: www.jivs.net
http://dergipark.gov.tr/http-www-jivs-net

semen have been accomplished (Roy et al., 2014; Jha et al., 2020). However, the pregnancy rates reported 60 - 70% with fresh semen and 11 - 26% with frozen-thawed semen following transcervical insemination (TC-AI; Azizunnesa, 2016; Rekha et al., 2016, 2018; Jha et al., 2020), which is not acceptable for field AI purpose. This lower pregnancy rates in ewes are due to complex cervical anatomy (long, convoluted funnel-shaped cervix) that prevents the passage of AI pipette into the body of uterus, as a result, there is the reduction in a high proportion of the spermatozoa when they arrive the place of fertilization (Wulster-Radcliffe et al., 2004; Kershaw et al., 2005; Leethongdee, 2010). To overcome these difficulties, laparoscopic artificial insemination (LAP-AI) is practiced in ewes. In this insemination technique, semen is directly deposited into the uterine lumen and thereby results in higher pregnancy rates (75 - 80%). However, this technique is expensive, needs minor surgery, and skilled surgeon and, therefore it has limited acceptance in developing countries (Bari and Haresign, 1998; Yamaki et al., 2003; Anel et al., 2005; Leethongdee, 2011). For acceptable pregnancy rates, researchers are trying to increase the depth of cervical penetration using hormonal preparations for cervical ripening. Some common hormones like; estrogen, oxytocin, and prostaglandin were studied for cervical ripening to increase the depth of cervical penetration and, to deposit semen deep into the cervical canal to increase pregnancy rates (> 50%; Rickords and White, 1988; Khalifa et al., 1992; Salamon and Maxwell, 1995; Donovan et al., 2004; Leethongdee et al., 2007). No study on pregnancy rates in Bangladeshi ewes after using cervical ripening treatments in the TC-AI technique has been reported, therefore, the objective of this study was to identify the effective cervical ripening treatments on pregnancy rates following TC-AI in indigenous ewes.

Materials and Methods

The study was conducted at the Department of Surgery and Obstetrics (DSO), Bangladesh Agricultural University (BAU), Mymensingh during the period of January - December 2015.

Animal and management: Altogether ewes (n = 18; age 24 to 36 months, BCS 3 - 4, bodyweight 14 - 16 kg) and rams (n = 3; age between 26 - 32 months, BCS between 3 - 4, bodyweight 24 - 26 kg) were selected from Sheep Research Farm, DSO, BAU for this study. The animals were kept in a semi-intensive system and supplied 200 gm of concentrates per animal per day. The health management, feeding, grazing, watering, and housing remained as routinely done by Sheep Research Farm, DSO, BAU.

Experimental design: Ewes (n = 18) were synchronized for estrus using prostaglandin (PGF2α) before underwent cervical ripening treatment. Ewes were randomly allocated into 3 groups for cervical ripening treatments; G1 [n = 06, control), G2 (n = 06, prostaglandin E1 (PGE1) as Misoprostol-Cytomis®, Incepta Pharmaceuticals Limited, Bangladesh], and G3 (n = 06, estrogen as Oestriol-Ovestin® + oxytocin as Linda-S® Vet). The cervical ripening treatments were administered just after the onset of estrus and left for 12 h before TC-AI. The ewes were inseminated transcervically at 12 - 24 h of onset of estrus using fresh semen. Cervical penetration was measured before cervical ripening treatment and after cervical ripening treatment i.e. at the time of insemination using a modified iron rod graded in centimeter. The cervical ripening was reflected with the depth of cervical penetration.

Semen collection and evaluation: Best selected indigenous ram (n = 3) were used as semen donors to inseminat in ewes. To prove the ram's fertility, six ejaculates of each ram were collected at an interval of 7 days and evaluated for semen quality. Semen was collected using an artificial vagina (especially for sheep and goat, Minitub, Germany) as described by Jha et al. (2018). Before collection, the prepuce of the ram was wiped clean to prevent semen contamination. Rams were allowed at least 1 - 2 false mounts before the collection of each ejaculation. Soon after semen collection, the graduated collecting tube was separated and kept at 35°C for semen evaluation as described by Jha et al. (2018).

Semen volume was recorded from a graded collection tube. To evaluate the mass activity, a drop (0.5 µl) of semen was placed on a pre-warmed slide (35°C) without coverslip and examined under a microscope equipped with phase-contrast optics (100×). The mass motility was scored into 4 scales: 0 = no motion, 1 = motion is mostly weak and oscillatory, not progressive. 2 = movements are vigorous, but no waves and eddies, 3 = motion is vigorous but waves and eddies formed slowly across the field, 4 = waves and eddies are formed rapidly but not so vigorous as in excellent grade and 5 = movements are so vigorous that it is impossible to observe individual spermatozoon in undiluted semen. The sperm concentration (spermatozoa 106/ ml) was calculated by using a hemocytometer. Briefly, semen samples were diluted with distilled water (1:400) to fix the spermatozoa. A drop of diluted semen was placed on the hemocytometer counting chamber from the edge of pipette and spermatozoa were allowed to settle for 5 - 6 min before placing the chamber on the stage of the microscope and calculated. The motility was determined by eye-estimation of the proportion of spermatozoa moving progressively straight forward.

Briefly, a drop (0.5 µl) of semen diluted at 1:4 ratio with TRIS was placed on a clean pre-warmed slide (37° C) and covered with a coverslip and observed at higher magnification (400×) and expressed as a percentage. The mean values of semen parameters; volume, density, mass motility, concentration, and progressive motility % are shown in Table 1. The mean volume, mass motility, concentration and motility were 0.75 ± 0.14 ml, 3.4 ± 0.65, 3.5 ± 0.47, 3398.6 ± 562.47 × 106/ml and 80.10 ± 5.70%, respectively.

Estrus synchronization: All ewes were synchronized for estrus using two doses of prostaglandin F2α (Ovuprost®, Cloprostenol, Bayer New Zealand Ltd., Auckland, New Zealand) 0.5 ml deep im per ewe at an interval of 9 days (Jha et al., 2020). Animals were closely observed for estrus behavior using a teaser ram.

Cervical ripening treatment: The synchronized estrus ewes; G2 received prostaglandin E1 analog (PGE1; Misoprostol-Cytomis®, Incepta Pharmaceuticals Ltd., Bangladesh) @ 400 µg per ewe intra-vaginally in the form of cream. For doing this, two tablets were grinded using pastel and mortar until becoming fine powder and mixed with 1 - 2 drops of glycerin (for diluting grinded powder and embedding into foam). A sterile piece of sponge was modified according to the size of vaginal diameter and embedded in powdered misoprostol saturated with glycerin. The medicated vaginal sponge then inserted just in front of the cervical opening using an applicator. Similarly, G3 received estrogen analog (Ovestin® 1 mg/ gm vaginal cream; Organon Ltd., Dublin, Ireland) @1 mg/ ewe intravaginally and, after 20 min before insemination, all the ewes were treated with synthetic oxytocin (Linda-S® Vet 10 IU/ml; Nuvista Pharma Limited, Gazipur, Bangladesh) @ 50 IU/ ewe through iv route.

Cervical Penetration Measurement: In all groups, cervical penetration was measured at the time at first detected estrus detection (before cervical ripening treatment) and just before insemination using a modified iron rod graded in cm with the modification of Leethongdee et al. (2007), who used AI gun plunger for measuring penetration depth. The effectiveness of treatment was measured by recording the depth of cervical orifice during TC-AI in term of easiness of AI gun passes.

Transcervical artificial insemination (TC-AI): All estrus ewes were inseminated following the measurement of cervical penetration, using fresh semen. A commercially available AI gun (especially for sheep and goat, Minitub, Germany) was used for trans-cervical insemination. The evaluated semen was loaded into 0.25 ml mini straws and then loaded into the AI gun. The estrous ewes were kept dorso-ventrally on the laparoscopic cradle at 30°. The vulva of the ewe was cleaned with a dry paper towel and lubricated with non-spermicidal water-soluble lubricant. A vaginal speculum was introduced into the vagina and forwarded to locate the cervix guided through a torchlight, and the cervix was grasped with a modified vulsellum forceps. The inseminating gun was then inserted into the cervix and pushed forward as much as it easily passes through the rings. After deposition of semen, the gun was kept in position for a period of 2 min before withdrawn from the cervix. A similar procedure was applied for insemination in all heated ewes.

Pregnancy diagnosis: All inseminated ewes were monitored for non-return rates to estrus by the aid of a vasectomized teaser ram on the 15 - 17 days following insemination. The ewes which were in non-return to estrus were allowed to trans-rectal ultrasonography within 40 - 50 days of post insemination for confirmation of pregnancy.

Statistical analysis: The paired t test and ANOVA was used with a confidence interval of 95% and p values less than 0.01 and 0.05 were considered as a significant difference. The analysis was done using the SPSS software (IBM SPSS data editor, Version-12).

Results

Effects of Cervical Ripening Treatment on Penetration of AI Gun in TCAI in Ewes: The effects of cervical relaxation treatment are shown in Table 2. The Mean ± SE values for depth of cervical penetration of different groups were 0.33 ± 0.10, 0.41 ± 0.09 and 0.43 ± 0.09 cm before cervical ripening treatment and 0.43 ± 0.07, 3.50 ± 0.26 and 1.54 ± 0.14 cm following

Table 1. Characteristics of ram semen used in the TCAI program (Mean ± SE)

Ram ID	Volume (ml)	Mass motility (1 - 4)	Concentration ($\times 10^6$ spermatozoa/ml)	Progressive motility (%)
21	0.86 ± 0.11	3.80 ± 0.25	3877.5 ± 244.40	81.25 ± 4.78
23	0.73 ± 0.09	3.25 ± 0.50	3309.0 ± 341.27	81.50 ± 6.02
HM06	0.55 ± 0.07	3.25 ± 0.35	2620.0 ± 395.97	75.00 ± 7.07
Pooled	0.75 ± 0.14	3.50 ± 0.47	3398.6 ± 562.47	80.10 ± 5.70

Table 2. Depth of cervical penetration (before and after cervical ripening treatments)

Group	Drug and dose of treatment	Number of ewes	Before treatment; time at first detected estrus (cm; Mean ± SE)	After treatment; during TC-AI (cm; Mean ± SE)
A	Not received any treatments	6	0.33 ± 0.10^{a}	0.43 ± 0.07^{c}
B	PGE1; Misoprostol-Cytomis®	6	0.41 ± 0.09^{a}	3.50 ± 0.26^{a}
C	Estrogen; Ovestin® + Oxytocin; Linda-S®	6	0.43 ± 0.09^{a}	1.54 ± 0.14^{b}

cervical ripening treatment, respectively. There was no significant variation in AI gun passes (depth of penetration) among the two different treatment and control groups before treatment, however, mean values of cervical penetration were significantly different following treatment ($p<0.05$). Cervical penetration depth was significantly increased ($p<0.01$) in G2 and G3 ($p<0.05$) treatment compared with G1.

Effects of Cervical Ripening Treatment on Pregnancy Rates and Sex Ratio: Pregnancy rates and numbers of different sex's lamb kids in different treatment and control groups are presented in Table 3. Though there was a different ratio of pregnancy rates between groups, but this difference was not statistically significant ($p>0.05$). However, the pregnancy rates of PGE-Misoprostol (66.67%) was near to significant difference compared to control groups (33.33%). Furthermore, the pregnancy rates of estrogen + oxytocin (50%) were higher than the control group (33.33%). In this study, all kids born under control groups were female (n = 03), However, all kids born under PGE-Misoprostol were males (n = 05. Interestingly, the ratio of male and female kids was more or less similar (2 vs 3). But in terms of the total number of kids, the sex ratio of different kids were not significant (male vs female: 7 vs 6).

Discussion

In sheep, AI is a commonly used technique to speed up the genetic gain and increase production in a short time. The success of TC-AI depends on semen deposition into the cervix as deep as possible for increased pregnancy rates (>50%; Leethongdee, 2011). However, it is challenging due to the complex cervical anatomy of the ovine cervix. In regular TC-AI methods; without using cervical ripening hormonal treatments, the pregnancy rates results lower and not supposed to be acceptable for farmers. To increase pregnancy rates, hormones like; estrogen, oxytocin, and prostaglandin are used for cervical ripening and thereby increase the depth of cervical penetration and pregnancy rates (Salamon and Maxwell, 1995; Donovan et al., 2004; Leethongdee et al., 2007).

In this study, we had planned to evaluate the effect of cervical ripening treatments, depth of cervical penetration, and its effect on pregnancy rates. The depth of cervical penetration before cervical ripening treatments were similar among the groups, however, differed significantly following cervical ripening treatments. We observed pregnancy rates 33.33%, 66.67%, and 50% in the G1, G2, and G3, respectively. The pregnancy rates 33.33% in G1 are comparable to that of King et al. (2004) who found 20 - 40%. Another report showed 68.18% pregnancy rates in ewes with frozen semen after 3 - 4 h intracervical administration with 50 - 100 µg of PGE1 analog (misoprostol) for each ewe (Rashidi and Cedden, 2013) which is comparable to our result.

In G2 (PGE1 as Misoprostol-Cytomis), the penetration depth was deeper than other two groups (3.50 ± 0.26 cm vs 0.43 ± 0.07 cm and 1.54 ± 0.14 cm). The cervical lumen of G3 (estrogen + oxytocin) was comparatively more dilated (1.54 ± 0.14 cm) to pass an AI gun than G1 (0.43 ± 0.07 cm). Misoprostol (Cytomis 400 µg/ ewe) was applied locally as intra-vaginal embedding for 12 h before TC-AI and found cervical penetration 3.50 ± 0.26 cm; however, Leethongdee et al. (2007) found 5 - 6 cm cervical penetration with misoprostol treatment (1 mg/ ewe dose). The dose difference of misoprostol in the present study (400 µg/ ewe) and a previous study (Leethongdee et al., 2007; 1 mg/ ewe) might be due to breed variation. Leethongdee et al., (2007) used Welsh Mountain Sheep, comparatively larger breed whereas we used indigenous ewes which are smaller sized sheep. Post-treatment cervical penetration is significantly differing from pre-treatment cervical penetration within the groups. Moreover, in G2, a highly significant difference is shown in our study. In the control group, this change might be due to an increase of estrogen level and increased cervical mucus flow, but which was not sufficient to pass an AI gun for achieving a satisfactory result. The local intra-vaginal application of the PGE1 analog, misoprostol to non-pregnant sheep (Leethongdee et al., 2010) reported evidence of a PGE-mediated mechanism of

cervical relaxation during the periovulatory period. We used PGE1 analog (Misoprostol®) @400 µg/ ewe for dilatation of cervical lumen in G2.

In fact, the PGE1 in misoprostol increases cervical ripening by inducing collagen breakdown and softening of the cervical tissue structure, a mechanism known to be associated with local production of PGE and glycosaminoglycans (Ellwood et al., 1980; Ledger et al., 1983). The recent hypothesis proposed that PGE2 selectively binds to EP2 and EP4 (PGE2 receptors), stimulating hyaluronan (HA) synthesis, which may cause remodeling of the cervical extracellular matrix and culminating in cervical relaxation (Kershaw-Young et al., 2009). The effect of misoprostol is short-lived and suggested that the length of time for cervical ripening after misoprostol application is 4 - 6 h (Goldberg et al., 2001). For this reason, misoprostol was administered after the onset of estrus and kept for 12 h before AI. Estrogen (Oestriol 1mg/ ewe, ie. Ovestin®) for 12 h and followed by Oxytocin (Linda S-Vet) 40 IU iv/ ewe just 20 min before AI in G3.

In the present study, cervical ripening with estradiol and oxytocin treatment, the AI gun was able to penetrate through the cervical canal in comparison to control group. Estrogen had no effect on cervix dilation but the injection of estrogen in the combination of oxytocin can completely dilate the cervix of ewes, which partially goes with the present study. Exogenous oxytocin can dilate the cervix of the ewe (Khalifa et al., 1992; Stellflug et al., 2001); however, it is not with the agreement of another study (King et al., 2004), who found no significant dilation with oxytocin treatment.

The present study identified an effective cervical ripening treatment protocol for increasing pregnancy rates following TC-AI in indigenous ewes of Bangladesh. However, the experiment was not conducted in a field assay. The field trial would reveal the sustainability of the results of the present study to be used for farmer's ewe AI.

Acknowledgements

We are grateful to the Ministry of Science and Technology, Government of the People's Republic of Bangladesh for the financial support of the research work.

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