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JIVS JOURNAL OF ISTANBUL VETERINARY SCIENCES

Investigation of bovine coronavirus and bovine rotavirus by rapid diagnosis kit and RT-PCR in diarrheic calf feces*

Research Article
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ABSTRACT

This study has investigated bovine coronavirus (BCoV) and bovine rotavirus (BRV), which are among the most important causes of diarrhea in calves leading to financial losses in Turkey and all over the world BCoV and BRV were detected by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), which is one of the most reliable method of diagnosis, The results obtained by RT-PCR were compared to the sensitivity of the commercial Rota-Corona Rapid Test Kits used by clinical veterinarians in fields. In this study, 96 fecal samples were examined from diarrheic calves in cattle farms in the cities of Konya and Afyon for BRV and BCoV firstly by BoviD-5 Ag rapid test kit, and then we applied the RT-PCR test. A comparison of the rapid test kit with the RT-PCR in terms of sensitivity and specificity revealed the 83% sensitivity and 100% specificity of the BRV and 7.6% sensitivity and 100% specificity of BCoV. In conclusion the practical and rapid diagnosis of the disease using of Rapid Diagnosis kit used by the clinician veterinarians may be useful, but the results must be interpreted with caution since the sensitivity of the test decreases due to the reduction in the number of viruses in the later stages of the infection.

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Introduction

Neonatal calf diarrhea caused by viral, bacterial and protozoon agents is one of the infections characterized by enteritis leading to weight loss and deaths in calves under one month of age (Murphy et al.,1999). Neonatal calf diarrhea is among the most important reasons for financial losses in the meat and milk industry all over the world (Boileau et al.,2010). Although its causes show variations depending on the regional and stable conditions, the role of rotavirus and coronavirus in the cases of calf diarrhea have been found to reach up to 50% and 80% respectively. Rotaviruses generally cause infections characterized by diarrhea in dairy calves (Al Mawly et al., 2015) and beef calves (Cho et al., 2013) up to 9-21 days old. Bovine group A rotavirus, bovine coronavirus, enterotoxigenic K99+ Escherichia coli (K99), Cryptosporidium parvum and Salmonella spp. are reported to be the most common enteric infection

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agents (Bartels et al., 2010; Izzo et al., 2012). Among the factors that affect the course of rotavirus and coronavirus infections are whether the newborns received colostrum, time of weaning, climate conditions, their immune conditions, and other present enteropathogenic agents. The main mode of transmission of rotaviruses is the fecal-oral route. Through the feces of the infected animals, a high level of viral particles (approximately 1011 particle/g) is shed around. This shedding reaches the highest level on the third and fourth days, and the virus can survive in the feces for several months (Murphy et al., 1999). Virus isolation (Mebus et al., 1969; Hasoksuz et al.,2002; Gulyaz et al., 2005), immunochromatographic rapid diagnostic assays (Al-Yousif et al., 2001; Uhde et al., 2008; Klein et al., 2009; Bartels et al., 2010; Altug et al., 2013), IEM (Immunelektron microscopy) (Saif et al., 1980), ELISA (Alkan, 1998; Murphy et al., 1999; Gulyaz et al., 2010) and RT-PCR (Cho et al., 2001; Hasoksuz et al., 2002; Aich et al., 2007; Decaro et al., 2008; Asano et al., 2010; Bok et al., 2015) are among the most preferred methods to diagnose rotavirus and coronavirus infections.

Materials and Methods

Specimens: The samples were collected from calves (1 to 30 days old) with acute diarrhea cases in stables in Konya and Afyon. Total of 96 fecal samples were collected for this study. The age distribution of the collected samples is presented in Figure 1. The fecal samples were collected from the rectums of the animals with sterile cotton swabs. One swab taken out of the rectum was put in the solution in the rapid test kit, and another was homogenized by putting it into Phosphate Buffer Saline (PBS), and stored at -20ºC until it was used for RT-PCR. The samples for the rapid test kits were examined under stable conditions, and their results were reported to the owners in 15 minutes. Necessary notes were taken and the positive samples were taken into account for the next examination.

Rapid Diagnostic Test: In this study, we used the Bionote BoviD-5 Ag (Cat. No: RG13-02) rapid diagnosis kit. We followed the test procedure of the producer. In line with the procedure, firstly the swab contaminated with the feces was placed in the solution included in the kit during the sampling and was homogenized. Then one drop of the solution was added onto the arrays and according to the change of color, coronavirus or rotavirus was interpreted as positive or negative.

RT- PCR Materials: Extraction of the Viral RNA: We used High Pure Viral RNA isolation kit of Roche (Roche, Cat. No: 11858874001). The fecal samples were suspended at a rate of 1/10 in PBS including 25000 U/ml Penicillin and 20 mg/ml Streptomycin, centrifuged at +4oC, 3000 rpm for 15 minutes, and then supernatant was transferred into a sterile tube. Following the centrifugation 200 µl of the supernatant was taken and transferred into a 1.5 ml RNase-free sterile tube. Each sample was mixed with working solution containing 4 µl Poly A and 400 µl Binding Buffer. The working solution and the sample mixture were treated with the Removal Buffer, Wash Buffer, Elution Buffer included in the kit by using the silica gel spin column. At the end of the extraction process, 50 µl of viral nucleic acid was isolated, and stored at -80^{o}C.

cDNA Synthesis: We used Reverse Transcription System (Promega A3500) to obtain cDNAs. In order to synthesize cDNA from the isolated viral RNA, we used the Promega Reverse Transcription System synthesis kit (Promega A3500), and followed the recommended protocols. 5 µl of the isolated viral RNA was transferred into the PCR tubes and was incubated at 70ºC for 10 minutes. Following the incubation, it was kept in ice for 2 minutes. Master Mix (15 µl for each sample) was prepared in a different PCR tube and 15 µl of Master Mix was added onto each of the 5 µl RNA samples.

Viral RNA and Master Mix mixture totaling to 20 µl was put into the Thermal Cycler and amplified at 22ºC for 10 minutes, at 42 ^{o}C for 15 minutes, at 95 ^{o}C for 5 minutes at 4^{o}C for 5 minutes, and then the cDNA was synthesized. The resulting products were stored at -20^{o}C.

Polymerase Chain Reaction (PCR): We used Promega Go Taq Flexi DNA Polymerase (Promega M8305) To detect the presence of bovine rotavirus in the fecal samples of the calves with the one-step RT-PCR method. The primers reported by Hasoksuz et al., (2008) and Chang et al., (1997) were used. These primers are specific to the VP7 gene region of the group A rotaviruses. We used the primers reported by Cho et al., (2013) for the detection of bovine coronavirus. These primers are specific to the N protein gene of the virus. We used S-Beg5-GGC TTT AAA AGA GAG AAT TTC-3, End-9, 5-GGT CAC ATC ATA CAA TTC TAA TCT AAG-3 primers of 1062 bp for bovine rotavirus and NOF-5-GCA ATC CAG TAG TAG AGC GT-3, NOR-5-CTT AGT GGC ATC CTT GCC AA-3 primers of 730 bp for bovine coronavirus.

BRV: We used the one-step RT-PCR method for the viral RNAs obtained. 0.8 µl DMSO, 0.6 µl End-9 of the rotavirus primers and 0.6 µl S-Beg were added onto each of the 5 µl RNA products, and they were mixed with a straw to homogenize. The mixture was incubated at 94°C for 5 minutes, and then kept in ice. Following the incubation, 43 µl Master Mix composed of Primer F (20 pmol), Primer R (20 pmol), and solutions of the Promega M8305 kit and the Promega A3500 cDNA kit was treated with 7 µl RNA and DMSO mixture. It was amplified at 42°C for 60 minutes, at 94°C for 3 minutes, (at 95°C for 1 minute, at 55°C for 2 minutes, at 72°C for 1 minute at 35 cycles), and at 72°C for 10 minutes.

BCoV: cDNAs of the samples were treated with the Master Mix mixture of the Promega M8305 kit including Primer F (50 pmol) and Primer R (50 pmol) and, for the PCR reaction, Go Taq Flexi DNA Polymerase, 5X buffer green flexi color, MgCl2 (25 mM) and dNTP. It was amplified at 94°C for 3 minutes (at 94°C for 1 minute, at 52ºC for 2 minutes and at 72°C for 1 minute at 35 cycles), at 72°C for 7 minutes.

To display the amplification products, 1.5% agarose gel containing ethidium bromide was prepared. The PCR products were run at 100 V for 30-45 minutes and the amplified DNA bands were controlled under UV light.

Statistical analysis: We used the chi-square test ($\chi 2$) for the statistical analysis of the diagnostic tests. We recorded $p<0.05$ as statistically significant.

Results

We examined 96 diarrheic fecal samples in total for BCoV and BRV by rapid diagnostic test and RT-PCR method. The collective results of the study are presented in Table 1. According to the results, the

Table 1: Number of positive samples identified by the rapid test kit and RT-PCR

Pathogen	Rapid Test Kit	RT-PCR
BRV (Group A)	15/96 (15.62%)	18/96 (18.75%)
BCoV	1/96 (1.04%)	13/96 (13.54%)
BRV (Group A)- BCoV	-	4/96 (4.16%)

rapid diagnostic test revealed 15 samples as BRV positive (15.62%) and 1 sample as BCoV positive (1.04%). The RT-PCR method detected 18 cases of BRV presence (18.75%), 13 cases of BCoV presence (13.54%), and 4 cases of both BRV and BCoV presence (4.16%). The electrophoresis images are presented in Figure 2 and Figure 3. According to these results, four samples were found to be both BRV and BCoV positive by RT-PCR method. However, only one sample was found to be BRV and BCoV positive with the rapid diagnostic test.

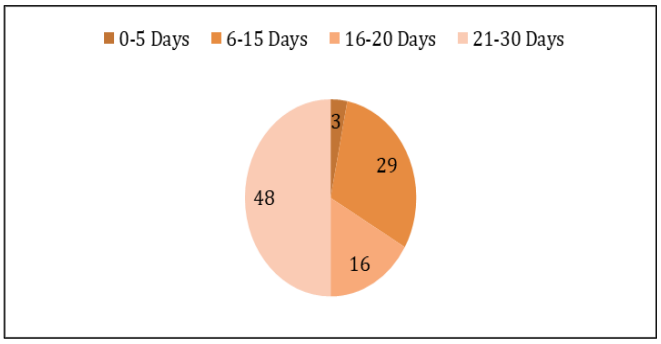

Figure 1: Distribution of the samples of calve feces by age (days)

This sample showed a quite strong DNA band appearance after the agarose gel electrophoresis using RT-PCR. (Figure 3). For the 0-5, 6-15, 16-20 and 21-30 days old calves found to be BRV positive by the rapid test kit, the rates of positivity were 67%, 24%, 0% and 13% respectively. Only 4% of the 6-15 days old calves were found to be BCoV positive. For the 0-5, 6-15, 16-20 and 21-30 days old calves found to be BRV positive by the RT-PCR method, the rates of positivity were 67%, 31%, 6% and 13% respectively. In terms of BCoV, while RT-PCR found no positive samples in the 0 -5 days old group, the other groups found to be 34%, 13% and 2% positive respectively (Figure 4).

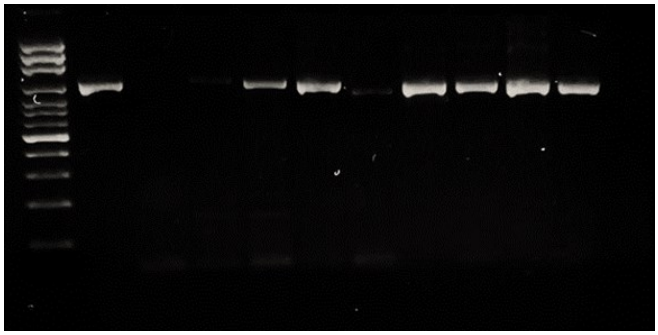

Figure 2: Electrophoresis image of the BRV (1062 bp) positive samples, DNA Ladder (100 bp Fermentas), PC (Positive Control), NC (Negative Control), DNA bands of the samples; 27, 28, 12, 13, 14, 15, 16 and 17

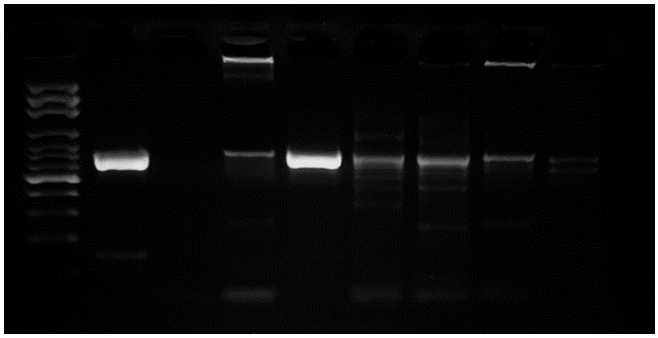

Figure 3: Electrophoresis image of the BCoV (730 bp) positive samples; DNA Ladder (100 bp Fermentas), PC (Positive Control), NC (Negative Control), DNA band of the samples 16, 17, 19, 22, 23 and 25

Table 2. Sensitivity, Specificity, PPV and NPV rates of the Rapid Test Kit versus RT-PCR in terms of BRV/BCoV

Reference Test	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
BRV Rapid Test BRV RT-PCR	83	100	100	96
BCoV Rapid Test BCoV RT-PCR	7.6	100	100	88

PPV: Positive Predictive Value, NPV: Negative Predictive Value

Discussion

Rapid and accurate diagnosis of BRV and BCoV infection is important for the control and eradication of the disease in newborn animals in cattle farms in many developed and developing countries. Therefore, it is important to diagnose BRV and BCoV rapidly in the field, and to detect it through rapid and effective test techniques in veterinary diagnostic laboratories. Among these methods, isolating the RNA of the virus and converting it into DNA (cDNA) and multiplying the cDNAs by using specific primers (RT-PCR) has the highest sensitivity and originality. However, as these techniques can only be applied under laboratory conditions and require time, clinical veterinarians need rapid test kits to diagnose the infection under field conditions. These rapid test kits are important in terms of determining the treatment process and avoiding wrong antibiotic use, but the use of rapid test kits is unfortunately behind the desired levels.

Table 3. Statistical comparison of the positive results by the tests applied

	Rapid Test Kit (n)	RT-PCR (n)
BRV Positive	15/96[a]	18/96[a]
BCoV Positive	1/96[b]	13/96[a]
BRV-BCoV Positive	0/96[b]	4/96[b]

a, b: Different letters within the same column are statistically different. ($p<0.05$) a is statistically higher than b.

The most important reason behind this is the righteous suspicion about the sensitivity and specificity of these rapid test kits. In recent years, the rapid immunochromatographic tests, which are more advantageous under filed conditions, it has become possible to diagnose different enteropathogens in the feces of calves in approximately such short time periods as 10 to 15 minutes, and to plan prophylaxis and treatment (Klein et al., 2009).

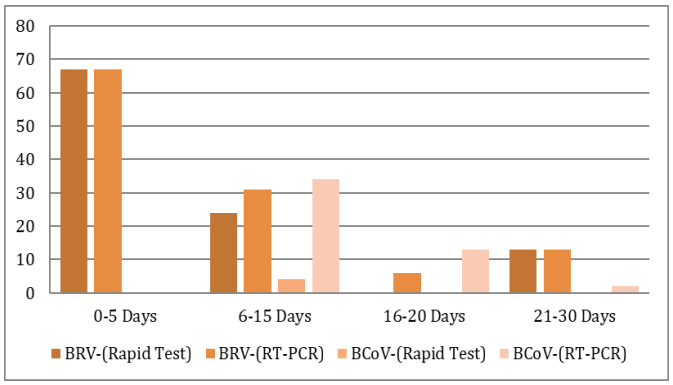

Figure 4. Percentage distribution of the BRV and BCoV positive calves by age (days)

Many investigators have reported that the immunochromatographic rapid test kits are a simple and easy-to-apply method for the diagnosis of enteropathogens in feces, and that they may be preferred by clinical veterinarians and investigators more often as they do not require specialist and fully-equipped laboratory, are cheap and rapid in comparison to other techniques, and can be applied under any laboratory or office conditions which can be found in each private clinic and even under field conditions (Thorns et al.,1992; De la Fuente et al., 2009; Klein et al., 2009). Klein et al. (2009) examined the fecal samples collected from 1 day to 42 days old 180 calves (98 of them had the symptoms of diarrhea) both with immunochromatographic rapid test kit and RT-PCR method. Compared to RT-PCR, the investigators (Klein et al., 2009) found the sensitivity of the rapid test kit for BRV as 71.9% and the specificity for the same as 95.3%, and its sensitivity as 60% for BCoV and its specificity as 96.4% for the same. In their study comparing the commercial rapid test kits with the multiplex PCR method, Cho et al. (2012) found the sensitivity of the rapid test kits as 60% for BCoV, and 42.3% for BRV, and they, therefore, stated that the rapid test kits had to be interpreted carefully in terms of originality and sensitivity. In a study

In a study comparing real-time RT-PCR, ELISA and immunochromatographic tests, Izzo et al. (2012) found the sensitivity of the rapid test kit as 32.7% for BRV and as 28.2% for BCoV in comparison to RT-PCR technique. The investigators reported that the sensitivity and specificity levels of the immunochromatographic rapid test kits were very low in comparison to real-time PCR, and that it was possible to interpret the course of the disease at the clinic since the viral RNA amount is known due to real-time PCR method. In their study on the rapid etiological diagnosis of neonatal calf diarrhea by immunochromatographic test kits, Altug et al. (2013) reported 14 cases of BRV (27.5%) and 1 case of BCoV (1.96%) among the samples from 51 diarrheic calves. In this study, among the samples examined by rapid test kit, we found 15.6% (15/96) to be BRV-positive and 1.04% (1/96) to be BCoV-positive. The same samples were tested using RT-PCR method and the positivity rates for BRV and BCoV were found 18.75% (18/96) and 13.5% (13/96), respectively. A combination of BRV and BCoV infections was detected in 4% of the diarrheic feces (4/96).The results obtained in this study were found to be compatible to those of Altug et al. (2013). Besides, in comparison to RT-PCR technique, the sensitivity of the immunochromatographic rapid test kits for BRV was 83% and the specificity of the same was 100%, its sensitivity for BCoV was 7.6% and specificity for the same was 100%. The results for bovine rotaviruses were found to be compatible with those of (Klein et al., 2009), who have previously contrasted the immunochromatographic rapid test kits to RT-PCR in terms of sensitivity and specificity, while they were determined to be higher than those of Cho et al. (2012) and Izzo et al. (2012). In terms of bovine coronavirus, our results were significantly lower than those of many other investigators (Klein et al. 2009; Cho et al., 2012; Izzo et al., 2012) who have studied the same subject matter. The possible reason for this might be the fact that the sampling is carried out in the late course of the disease when the level of virus shedding and the amount of viral particles are low. The rapid immunochromatographic diagnostic method is based on the attachment by the agent within the sample dropped on the test stripe to the conjugated specific antibodies. Therefore, it is essential to carry out the sampling during the peak time of virus shedding. It is necessary to collect the samples within 72 hours after the onset of the disease, because virus shedding decreases in time. However, it is possible to detect even very low levels of viruses by the RT-PCR method. The diagnostic ability of the rapid test kit can be inferior to that of RT -PCR in samples containing small amount of virus. “In this study, we identified both BRV and BCoV by RT-PCR in four samples. Only one sample was found positive in terms of BoCV using the rapid test kit. The electrophoresis images from the RT-PCR diagnosis of this positive sample presented/showed a stronger DNA stripe image in comparison to the other positive samples. This indicates that the rapid test kit determines positive results if there is high amount of coronavirus in the fecal samples. Therefore, it is necessary to support the results with a lot of samples. Examining the age ranges of the calves and the infection-positive results by RT-PCR for these age ranges, we see that the highest level of BRV-positivity was found as 67% in calves of 0-5 days of age. This rate was identified as 31% in the 6-15 days age group, 6% in the 16-20 days age group and 13% in the 21-30 days age group. In our investigations by RT-PCR for BCoV, we identified no positivity in the 0-5 days age group, but 34% in the 6-15 days age group, 13% in the 16-20 days age group, and 2% in the 21-30 days age group (Figure 4). While the rates identified for BRV by this study are close to those reported by Al Mawly et al. (2015) (20% in calves of 1-5 days of age, and 19% in calves of 9-21 days of age), but in terms of BCoV, the results of Al Mawly et al. (2015) (5.4% in calves of 1-5 days of age, 6.1% in calves of 9-21 days of age) are lower than those of this study. Alkan (1998) has pointed out that this situation can be associated with the colostrum that calves receive from their mothers. Alkan (1998) has reported that one of the most important factors affecting the average infection age is maternal immunity. In this study, we know that the calves from which we collected the samples had generally received colostrum from their mothers. Ellens et al., (1978) and Wood et al., (1975) have reported that there were no rotavirus specific antibodies in the second week after birth, but antibodies specific to coronavirus reached significantly high levels in the third week. Contemplating on the fact that the coronavirus antibodies are secreted for a long time in milk, Wellemans and Van Opdenbosch (1981) have explained it with the fact that mothers were considerably infected with coronavirus during the diarrheic periods, their immune systems were stimulated as they shed the virus through their feces on the day of giving birth, inducing the mammary gland to secrete Ig antibody. Therefore, the total rate of BRV-positivity in the first two weeks (0-15 days) in this study is 34% while it decreases to 6% and 13% in

the third and fourth weeks, respectively. With regards to BCoV, the positivity rate in the first 3 weeks is 26% while it decreases to as low as 2% in the fourth week. The distribution of positivity by the age groups identified in this study was found to support the ideas of Ellens et al. (1978), Wood et al. (1975) and Wellemans and Van Opdenbosch (1981). This fact shows that in this study colostrum received from the mothers of calves stimulated the maternal immunity, and affected the positivity rate by age specified in the study. Although this study identifies a low level of sensitivity for immunochromatographic rapid test kits, one might think that the most important advantage of these kits for clinical veterinarians with regard to BRV diagnosis is to avoid wrong treatment with antibiotics. Nevertheless, as the amount of virus decreases in the late course of the disease, one must not ignore the fact that it is not a very effective method. This might lead to an inaccurate interpretation of the disease. The presence of subclinical carrier animals is another important issue to bear in mind while evaluating the disease. This is a point a good veterinarian would not like to ignore while assessing the cases of diarrhea in calves posing a problem especially in big farms. As a permanent solution, molecular methods such as RT-PCR play an essential role in identification of these animals. İt is possible to identify even one virus particle in the feces by RT-PCR (Klein et al. 2009). Thus, veterinarians will be able to interpret the disease accurately, and to take such protective measures as vaccination. It is of paramount importance to identify the field strains of infections such as bovine rotavirus and bovine coronavirus present in Turkey. Apart from the group A rotavirus identified in this study, identifying the G and P type rotavirus strains present in Turkey is of significant importance for the effectiveness of vaccinations. The results of this study indicates that the rapid test kits used by veterinarians under field conditions to diagnose the diseases quickly can be beneficial, but a careful interpretation is advisable since the sensitivity of the rapid test kits has been found to be low (especially for BCoV) in comparison to RT-PCR. In order to be able to interpret diseases more effectively and to seek more permanent solutions, it is advisable to support the results through molecular techniques such as RT-PCR.

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Livestock policies and red meat sector: Republic period and ensuing years*

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ABSTRACT

Implementation of policies aimed at agriculture and animal husbandry, agricultural supports are used as an important tool. Since the proclamation of the Republic, livestock sector of Turkey has entered into a number of changes and improvements. The data used in this study were collected from Turkey Statistical Institute (TURKSTAT), reports of drafting convention in sector, the five-year development plans and literature. It has been determined that there is not enough share from the subsidies within the agricultural policies applied in the livestock sector in terms of periods. Support for the sector was also insufficient in terms of credit and financing supply in animal husbandry. It was observed that there was no significant development in animal production in planned development periods. In macroeconomic planning of the sector, input-output relationship could not be considered on a sectoral basis according to the development goals. It has been seen that technical and economic integration such as health, breeding, production, cultivation, fattening and product evaluation is not taken into consideration adequately in planning. Today, the most important problem of the sector is high quality, sufficient roughage and concentrate feed supply and low yield per unit animal. However, real policy in supporting livestock should ensure that product / feed price ratios to maintain profitability in production. In order to achieve a certain increase in animal production, the support provided by the state and the price policy are important. Price policy has an important share in increasing production both in quantity and quality. The enterprises operating in animal husbandry are under the economic grip of the sellers and buyers, who are limited in number during the production stage. In this respect, the organization of the producer and the development of the marketing infrastructure, support and encouragement by the state may play an important role in solving the problems.

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Introduction

There is an extensive and intensive intervention of the state in agriculture and animal husbandry both in underdeveloped and industrialized advanced economies. Product, price and income instability and low elasticity of demand in the sector have made policy measures widespread which don't differ from each other. In industrialized economies, the main issue in terms of long-term agricultural policy is to take the necessary measures to prevent the decreasing product prices and producer incomes in consequence of the increase in agricultural and animal production and imports. In developing countries such as Turkey, the development of policies aimed at increasing production and regulation of markets have priority (Kazgan, 1977).

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In addition to its important role in national nutrition, livestock sector in our country; has undertaken many economic functions such as providing raw materials to industry, increasing exports, achieving the stability between the region and sectors in balance, preventing hidden unemployment and migration in rural areas and creating new employment opportunities in industry-services sectors.

Livestock sector of our country has gone through several changes and developments in consideration of all the developments and changes in the world from the proclamation of the Republic until today. In this study, policies implemented in the livestock sector in Turkey were examined into periods as; 1923-1950, 1950-1960, 1960-1980 and after 1980.

Period of 1923-1950

In the first years of the Republic, Turkey was lagging behind in terms of industrial aspect in consequence of war, and about 80% of the country's population were living in villages and towns. At the same time, many sectors (trade, transportation, banking, etc.) were monopolized by foreign capital (HAYGEM, 2015). In this period, Mustafa Kemal Atatürk, who was aware of the fact that political independence could be achieved by obtaining economic independence, convened the I. Economic Congress on February 17, 1923, to determine the financial measures to be taken in the process of transition to an independent economy. In congress, restructuring decisions have been taken aimed at integrating our country with contemporary world conditions which were extremely important in terms of the national economy and history. An important consequence of this congress was the establishment of industrial branches which supply raw materials domestically and so that initiation of development based on equities (TUİK, 2019).

In the period following the proclamation of the Republic, it is seen to be a current number of animals in Turkey were decreased by reason of war and outbreak of animal diseases experienced in previous years. For example, in the agricultural census of 1912 year, 7 million head of cattle, calculated as 4 million head in 1927 and 19 million head of sheep had fallen to 10 million head (TUİK, 2019).

It is obvious that there are specific measures to be taken in this regard in a country whose economy is largely based on agriculture and animal husbandry. Therefore, the necessity of establishing an organization came to the fore in order to redevelop the animal husbandry, which has declined due to various periodical elements in this time. Accordingly, the Branch Office of Animal Production was established primarily within the scope of the Ministry of Economy in 1923. In 1924, the Ministry of Economy was divided into two sections as the Ministry of Agriculture and the Ministry of Trade, so studies related to animal husbandry were began to carry out by the Ministry of Agriculture. Moreover, it is understood that the public services concerning the rural sector should be carried out in a sectoral public organization structure. In this context, since it was aimed to increase the efficiency of services and reduce costs by determining clearly the authorities and responsibilities in public services, the General Directorate of Agricultural Affairs, Veterinary Affairs and Forestry were established in 1937 with the Law No. 3202 named as Duties and Organization of Agriculture Ministry of Agriculture (Cevger et al., 2011).

Considering the importance of the livestock sector in terms of Turkey's economy, studies focused on improvement of animal breeding on existing animals have also begun in this period. It is aimed to produce and distribute qualified breeding animals to the public by improving the quality of the existing animal potential with the Animal Breeding Law No. 904 enacted in 1926. In this period, 15 animal breeding institutions were established, such as stud farms, pens, and trial farms. Besides, breeding animals' imports have been done by these institutions for the purpose of using them in cattle, sheep and horse breeding studies and distribution to the public (TUİK, 2019).

The economic crisis, which emerged with the end of World War I, affected the whole world, later on, especially Germany and the USA. Agricultural product prices decreased in Turkey, and also currency depreciation occurred in Turkish Lira, and the foreign trade deficit increased, especially between the years 1928-1932 like many other countries. As a result of the economic crisis, a step has been taken towards supporting agricultural products in Turkey for the first time, and with the ‘‘Wheat Protection Law'' enacted in 1932 support purchases have been made by Ziraat Bank. While these subsidies continued in agricultural products such as other cereals, tobacco and cotton in the following years, unfortunately, no subsidies were implemented for the livestock subsectors dealing with similar problems (Cevger et al., 2011).

At the Industrial Congress held in 1936, it was planned to establish a public institution to operate in the meat industry. For this purpose, a step was taken for the establishment of the Meat and Fish Institution, which became operational in 1953, and the technical and economic infrastructure and establishment

studies were started which were required by the institution.

In spite of the global crisis experienced throughout the world, the average increase in the production of animal products (milk, meat and wool) was above 3% in this period.

In the early years of the Republic, multidimensional studies were conducted to fight against epidemic animal diseases. Modern animal husbandry methods started to be used and also modern methods of fighting against animal diseases were added to curriculum in Veterinary Education due to the intensive pressure of the European countries. A significant amount of allowance has been allocated in the budget in order to increase vaccine production for prophylactic purposes, additions have been made to the number of occupational staff who would work in the fight against diseases, experts have been brought from abroad to carry out studies in our country and also some Turkish experts have been sent to international congresses and meetings about animal breeding and health (Temel, 2015).

Memberships were done to Office International des Epizooties (OIE) and World Veterinary Association. Within the frame of "5-year Veterinary Services Program" between 1924-1929, ‘‘Animal Breeding Law'' in 1926 and ‘Animal Health and Surveillance Law'' in 1928 enacted. Veterinarians have achieved superior success fighting against many diseases, especially in the fight against rinderpest by implementing these practices (Erk, 1973).

Another important development in the sector in this period was enacting the ‘‘Agricultural Sales Cooperatives Law’’ in 1935. Atatürk also gave particular importance to the cooperatives which had an important share in the organization of producers in rural areas.

It is thought that the decrease in production increase rate occurred from 1939 to 1950 was because of the negative environment caused by World War II.

Period of 1950-1960

Several innovations in terms of Turkey's economic and political structure has occurred as at the beginning of 1950. One of these innovations was the introduction of tractors in Turkey's agriculture sector following World War II. Marshall assistance, which was effective between 1946 and 1950, provided the necessary assistance to the farmers of our country in the point of mechanization in agriculture. Although significant increases in agricultural production have been achieved by this means, unfortunately, these increases have not been mainly due to the increase in yield per decare, but by the destruction and transformation of meadows and pastures to agricultural land which is the main input of animal husbandry and the cheapest source of roughage. Meadow and pasture areas; were 44.3 million hectares in total in 1935, decreased by 15% to 37.8 million hectares in 1950 and decreased to 28.6 million hectares with a decrease of 35% in 1960. So significant deterioration has been shaped in the rural economic structure (TUİK, 2019).

The Meat and Fish Institution was established in 1952 under the leadership of the state and started its activities on January 1, 1953, with the important organizational goals and macro objectives such as rationalization of animal production and economic organization of producers, establishing a stable market structure and leading the establishment of the national meat industry.

Another state economic enterprise named Fleece and Angora Inc. was established in 1955 for the purposes of protecting the benefit of the producers in the fleece and angora marketing in our country, making direct purchases from the producer when necessary and encouraging the production of fine-grained fleece. And Feed Industry Inc. was established in 1956, to produce feed, which is one of the most important input elements of the livestock sector, and to promote the production of raw feed materials (Cevger et al., 2011).

In this period, breeding animal imports continued in order to continue cattle breeding activities, but sheep breeding studies could not be carried out with necessary diligence. In the same way, it is understood that the necessary importance was not given to cooperatives.

If the process is evaluated until planned development periods in terms of animal husbandry policies; it can be said that the sector hasn't developed enough to the extent of its potential due to ceiling price application. Nevertheless, in this decade, there was an average annual production increase in products following as; 4.1% in milk, 6.5% in meat and 4.6% in fleece. It is understood that the production increase was basically in consequence of Atatürk's period until 1939 (TUİK, 2019).

Period of 1960-1980

State Planning Organization was established in 1961 with the objectives as; presenting Turkey's social and economic problems and accordingly conducting applications for national and regional development within the framework of democratic principles desired

results could not be achieved from I., II. and III. I Five-Year Development Plans prepared by the State Planning Organization in terms of achieving the targeted production levels in livestock and mobilizing the existing potential, and realizations fell behind the plan targets.

One of the key developments occurred in the sector in this period was launching Dairy Industry Institution of Turkey in 1965 with the main objectives such as; development of dairy industry and providing production-industry integration in the sector.

Significant changes greet the eye when numbers of animals examined from the establishment of the Republic of Turkey until today. There was an increase in the number of bovine and ovine animals until World War II. During the war years, the rate of increase has decreased, and the number of some species have decreased, and in the following, period there have been increases in the number of some animals. In the 1980s and beyond, unplanned livestock and meat exports and liberal policies implemented in animal husbandry led to decreases in the number of animals and withdrawal from production (TİGEM, 2018).

Period after 1980

The period which starts with economic stabilization measures in Turkey implemented on Jan 24th 1980 and transition to the free market economy has led to major changes in both the national economy and policies implemented for agriculture and livestock sectors. In particular, animal husbandry has been the sector that was considerably affected by these changes (Yalçınkaya, et al., 2006, Cevger et al., 2011).

A number of crops and animal products covered by price support in our country in the 1970s was around 20, and it decreased to 10 with the effect of the policies implemented in the 1980s. In this context, exclusion of animal products from the scope of support, especially slaughtering animals and meat has led to the further deepening of problems for production and consumption which was continuing for many years in Turkey. However, the decision of determining the production, supply and demand by the price which would form in market economy conditions, have negatively affected livestock sector in need of support policies (Cevger et al., 2011, TUİK, 2019).

In the livestock sector, the producers faced a semi -monopoly market condition in which no fully competitive market was established without an institutional infrastructure to ensure the functioning of the free market economy system (Cevger et al., 2011). Rural animal breeders were generally small-scale enterprises with poor bargaining power. Therefore evaluation of Turkey's livestock and meat market in free-market conditions was not possible. Thus, both in the 1980s period and today, animal products' price in rural areas forms in oligopsony market conditions (TUİK, 2019).

Withdrawal of state support from animal products, the formation of animal product prices in the incomplete competition market and additionally continuation of giving supports to plant products caused a further deterioration of socio-economic structure of rural areas and also prevented the interaction between sectors which was an important factor in development (Cevger et al., 2011, TUİK, 2019).

After the year 1980, insufficiency in animal production, the increase in input prices which results in price increase in red meat due to inflation meat has been accepted as a partial scarcity and speculative increases in livestock sector and imports were made in order to balance the market against rising prices, to ensure price stability and to nurture the producer and trade sector. Despite the implementation of this import policy, supply-demand balance and price stability in animal products could not be achieved, sector has entered an "economic vicious circle" and the damage of imports to the sector was understood after years (Cevger et al., 2011, TUİK, 2019).

One of the other wrong practices in Turkey was in the organization of public services. With the Decree-Law No. 183 issued in 1983 and the Law no. 3202 in 1937, the law regulating agriculture and animal husbandry services with a sectoral approach was abolished, and rural services were reorganized with a functional public organization approach. But since the operational structures, the characteristics of the services and the differences between the sectors are not taken into consideration in this regulation, the reorganization of the Ministry of Agriculture and Forestry has been constantly brought to the agenda since the year 2000 and after.

Initiated liberalization policies within the framework of globalization had significant effects on the livestock sector in Turkey in the early 1990s. State economic enterprises such as Meat and Fish Institution, Dairy Industry Institution and Feed Industry Inc., which are the locomotives and socio-economic balance elements of the production sector were included in the scope of privatization.

Most of the combines and dairy factories affiliated to the Meat and Fish Institution and the Dairy Industry

Institution were closed down, and production could not be sustained, and also problems occurred in terms of employment.

Import tax rates were raised in 1997, and the importation of live animals and meat was banned, considering the risk of mad cow disease (BSE).

Pasture law enacted in 1998, which was addressing identification of pasture areas and their protection.

The economic crisis in 2001 caused negative consequences in agriculture and animal husbandry, and as a result of decreasing public supports in the policies implemented, both the number of animals and animal products' production decreased in this period. Credits and financing needs, which are very important elements for the rationalization of production in animal husbandry, could not be adequately met, and incentive measures could not be taken to meet the investment financing need and propel the private sector to production in this field. The share of the livestock sector in total agricultural loans remained around 10% on average.

The important developments and changes in the agriculture and animal husbandry sector after the year 2003 are as follows;

•Supporting budget was prepared for the development of animal husbandry, and the Agricultural Law No. 5488 was enacted, which was the main source of the policies to be applied in the livestock sector.

•Producer Association Law No. 5200 has been enacted. The aims of this law were to deliver products in conformity with the norms and standards in the market, to take measures to increase the marketing power of the products on the international scale, to plan production according to demand and to improve the product quality.

•Bee Producers Association, Sheep-Goat Breeders Association and Cattle Breeders Association were established with the law No. 5996.

•Practices focusing on the development of the ovine breeding such as supporting, breeding animals by public and herd manager projects were implemented, and rams and goats were distributed in order to increase the productivity in sheep and goats. And also 80% grant support was provided for animal purchases.

•Subsidized loans were extended to meet the financing needs of enterprises, and subsidies were provided as female animals, calves, milk premium, milk quality premium, feed crops and fattening material support (HAYGEM, 2015).

The Meat and Fish Institution, which was included in the scope of privatization as of 1995, was removed from this scope and started to serve as the supporting organization in the year 2005. However, the effectiveness of the institution as an intervention agency in the red meat market decreased comparing the period before 1980. In the following years, Institution has been taken away from its purpose and duties in order to regulate the market with an importer approach.

With the global economic crisis and drought in 2008, rising input prices (oil, feed, etc.) increased production costs of enterprises operating in the agriculture and livestock sector. In parallel with this, increases in general prices of crops and animal products have occurred, but the production sector made losses as a result of not being able to reflect this increase in sales prices.

As a result of the decrease in milk prices and increasing costs, producers disposed of their breeding livestock animals, on the other hand, the price and market instability resulted in an increase in red meat prices and the sector was pressed for money.

The policies implemented in our country until 2010 were generally in the form of import restrictions, tariffs and direct market interventions. As of 2010; the decision was taken for the importation of livestock after prohibition of imports for 14 years showing the reasons such as; increased red meat prices as a result of the decrease in red meat supply, providing red meat at reasonable prices to consumers, raw material supply of the livestock-based industry and the concessions given to the European Union in the previous periods about bovine meat. Within this framework, customs tax rates were reduced and notably imports of butchery animals, breeding animals, fattening animals, ovine animals, carcasses and meat have been made. Imports in the livestock sector which is increasingly ongoing for nine years is being discussed by public opinion in terms of sustainability by the reasons for the destruction caused on the sector and spent foreign currency (Figure 1, Figure 2).

Importer approach in Turkey, which eliminates the production dynamics of agriculture and animal husbandry is extremely wrong for the future of the country. It is seen that tested policies in the past are now on the agenda again (as of 2010); livestock products, which were only $ 24 million in 1982, increased 15 times to $ 353 million over time. Again, fifteen years later, imported animal products (excluding breeding animals) increased approximately tenfold and reached $ 2.857.531.911 today (TİGEM, 2018). These numbers show that Turkey is a net importer country in animal products and in some crops and there's no self-sufficiency.

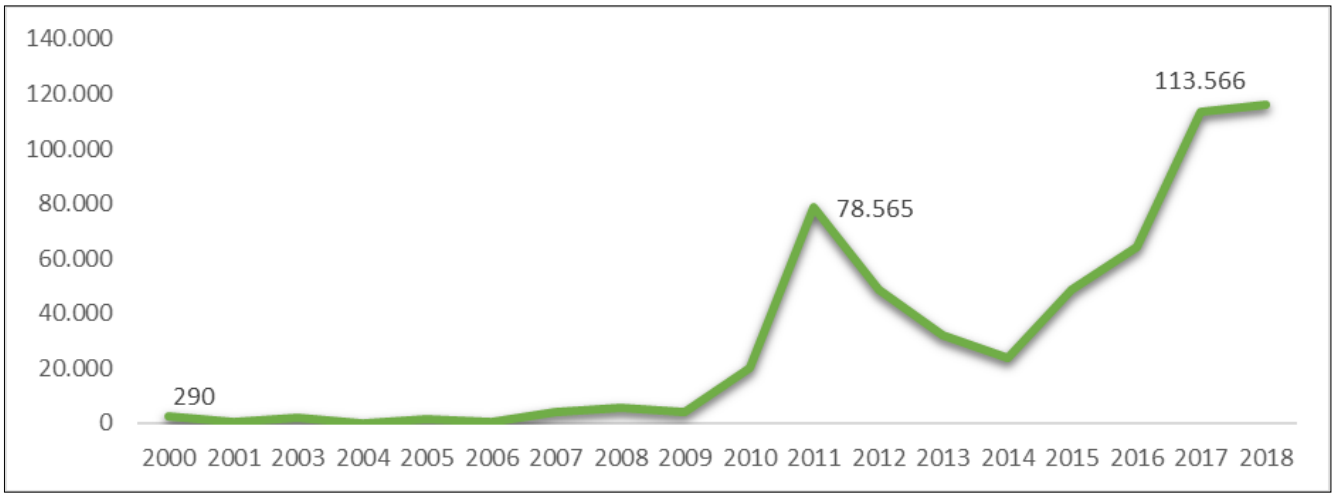

Figure 1. Butchery cattle import by years (heads)

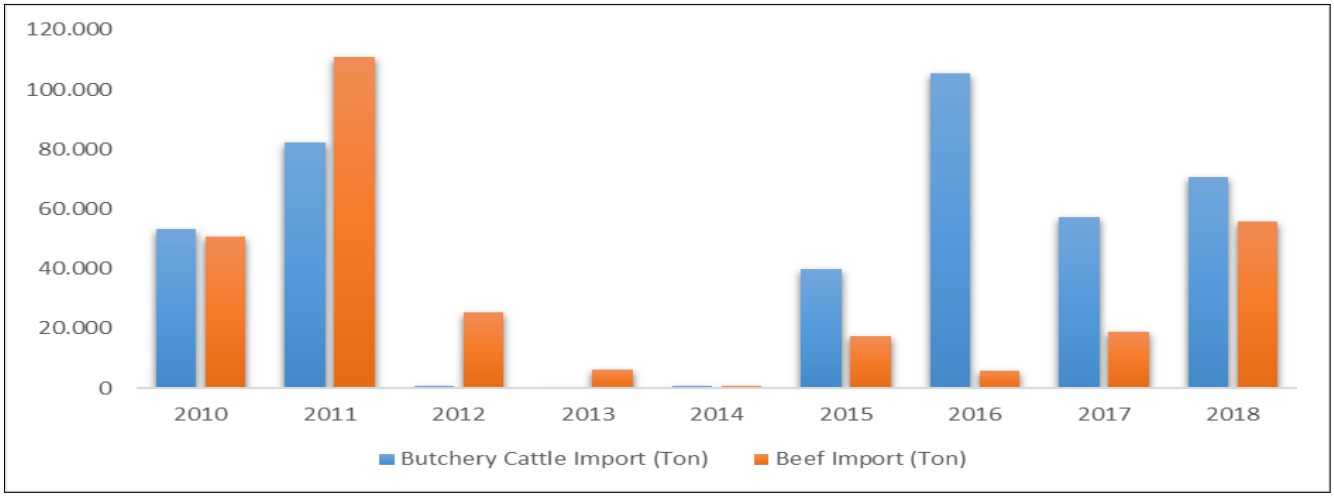

Figure 2. Butchery cattle and beef import by years (ton)

National Dairy Council was established in in 2013. As of 2015, a new regulation has been published which revised the provisions of the pasture law, which was enacted in 1998, and so that the pastures were opened for construction.

When the developments in the livestock sector mentioned in the five-year development plans are examined, it is seen that the targets and realizations in animal production (except poultry meat and eggs) in the 8th and 9th plans are insufficient, the productivity per unit animal cannot be increased sufficiently and the developments aimed at pasture and animal breeding did not occur.

Production and Future of Red Meat

The production of animal products is an indispensable production area that must be handled within the scope of strategic products. Especially in milk and red meat production, as the main sectors; ensuring self-sufficiency, sustainability and food safety are among the priorities of many countries, particularly in developed countries. On the other hand, it is observed that the policies aimed at preserving the level of living (welfare) of the producers compared to the other sectors (industry and service) and the consumers to consume healthy and high-quality products at the most reasonable price have been sustained and gained importance.

The meat and meat products sector is considered as one of the fastest-growing sub-sectors in the global agriculture and food sectors. In addition to the increase in global meat demand, productivity in production, processing and transportation were also effective in this development.

The contribution of fattening activities to the economy is not limited to red meat production, the by-products obtained from this sector can be used as raw materials in the production process of many sectors (Çiçek, 2002).

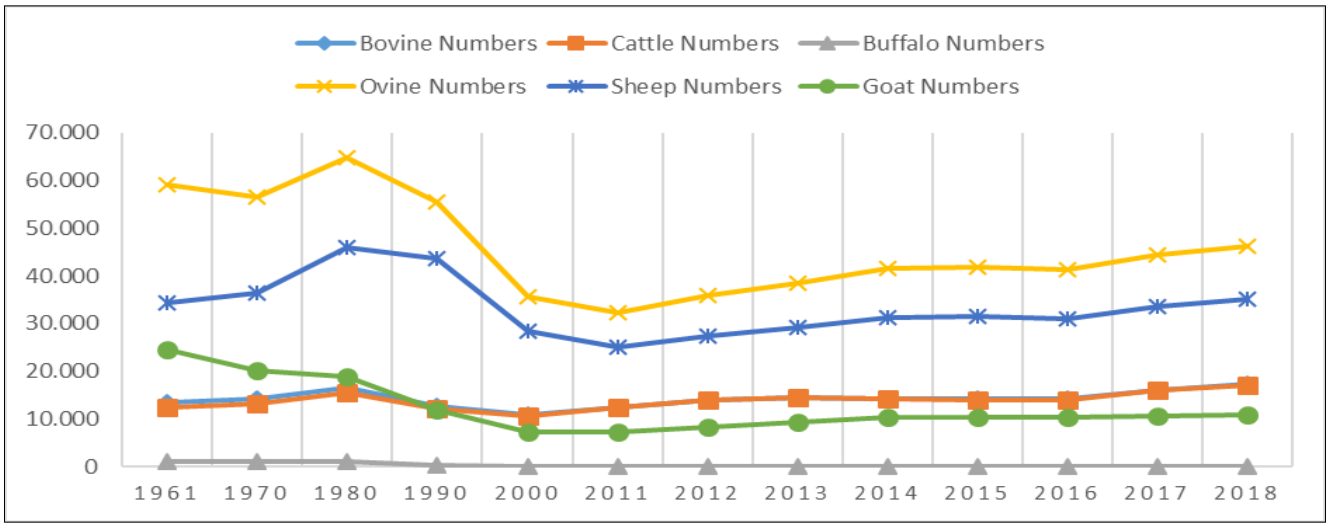

Figure 3. Presence of animal numbers by species and by years (1961-2018)

In our country, the red meat sector meets the demand of meat of approximately 82 million inhabitants, as well as tourists who reach 37 million people annually and approximately 5 million immigrants. The sector is critical because of its place in nutrition and its potential in production.

From the analysis of Figure 3, the number of cattle is 17.043.000 heads, and the number of ovine is 46.117.000 heads, including goats as of 2018. In the cattle population, the percentage of culture breeds is 49%, 41% includes hybrids, and 10% consists of native breeds.

When Figure 4 is analyzed, the number of slaughtered cattle reached 3.426.178 heads as of the end of 2018, while 89.7% (1,003,859 tons) of the total red meat production (1.118.695 tons) was bovine meet at the end of the year.

When red meat consumption per capita in Turkey is compared to developed countries, it appears to be at a lower level. When the consumption of red meat in the last five years is examined, it is seen that the per capita consumption which was 12.4 kg in 2012 was 12.9 kg in 2014 and increased to 14.1 kg in 2017. In 2018, per capita, red meat consumption amounted to 14.84 kg, of which 13.3 kg was veal. Annual per capita consumption of beef in the USA is 25.8 kg, 41.2 kg in Argentina, 43.2 kg in Uruguay and 10.1 kg in Russia (TEPGE, 2019).

When the average carcass productivity of cattle was examined, it was 215.6 kg in 2007 and reached 274 kg in 2017 (UKON, 2019). It should not be overlooked the role of live slaughter animals and fattening animals imported to the country in recent years in the increasing of cattle carcass productivity with breeding activities together.

Development of cattle breeding and cattle meat production is not the solution for red meat supply in the short term. Projects and supports to increase lamb

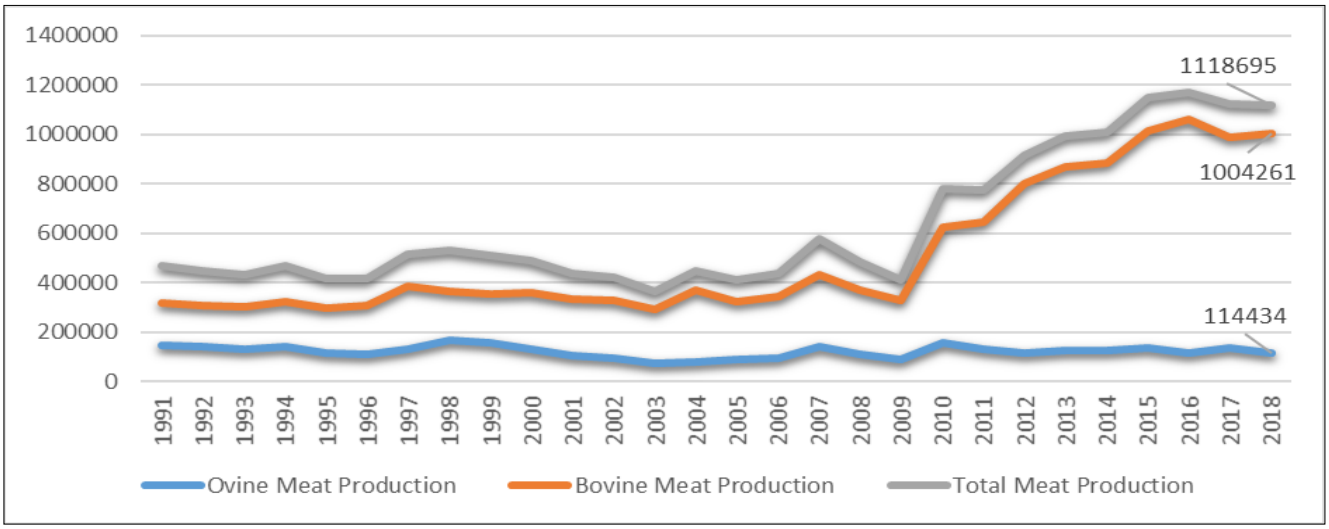

Figure 4. Meat production according to slaughtered animal species and their share in total red meat production

Table 1. Scale of Cattle Fattening Enterprises in Turkey (2017)

Cattle fattening farm-scale (head)	Number of enterprises (number)	Distribution of enterprises by scales (%)	Animal numbers by scales	Distribution of animal existence by scales (%)
1-5	119.077	45,07	359.542	9,01
6-9	50.077	18,95	380.802	9,54
10-19	48.740	18,45	705.809	17,69
20-49	32.942	12,47	1.011.701	25,35
50-99	9.731	3,68	672.333	16,85
100-199	2.683	1,02	378.362	9,48
200-499	775	0,29	229.429	5,75
500 and above	205	0,08	252.440	6,33
Total	264.230	100	3.990.418	100

production, which is a substitute product, will play an important role in covering this production gap. In this context, it should be aimed to increase the share of ovine livestock in red meat production to 20-25% in the short and medium-term. On the other hand, studies to create demand for lamb meat; publicity advertising, public service announcements, the proportion of calf, lamb and goat meat in public tenders, as well as the export target within the framework of the research aimed to reduce production costs gain importance.

In red meat production, within the consideration of the supply characteristic of the "production cycle"; it is important to develop necessary livestock policies in other production sub-sectors; such as turkey, duck, goose, rabbit etc. in order to create demand and to encourage consumption of meat.

It is observed that the supports applied to animal husbandry have increased, especially in the last ten years. The share of animal husbandry from total agricultural support was 4.4% in 2002, and this ratio reached 28.3% in 2018, which is a positive development for the sector, and is expected to be around 31% in 2019 (TEPGE, 2019).

Despite all these supports and regulations related to the animal husbandry, it is observed that targeted production increases cannot be achieved. The involvement of more than one institution in the implementation of the support policies, the difference of the programs and objectives of the institutions and the lack of coordination in the policies couldn't cause the foreseen developments in animal husbandry. On the other hand, the reason why the support for animal husbandry is insufficient and the effectiveness is not at the desired level is the failure to solve the structural problems in the sector with the wrong agricultural policies.

After the economic crisis in 2008, the bottleneck in the meat and milk sector, red meat supply shortage due to increase of population and demand level in recent years and increasing red meat prices in market has led to the importation of fattening and slaughter animals and carcass meat. These were palliative measures for the purpose of meeting the raw material demand for industry and provide cheap meat for consumers. However, after the import red meat prices have not been stabilized with the deliberate policies, the supply of cheap meat to the consumer has been failed.

There are around 3 million enterprises operating in the agricultural sector, 67% of them are specialized in polyculture (plant and animal production), 30% in agriculture and the remaining 3% are specialized in animal husbandry. The fact that the specialization in the farms is inadequate, in general small-scale, disorganized family farms with traditional production reduces production increase and efficiency.

In the examination of the Table 1, in total, there are 264.230 cattle fattening enterprises in Turkey. 64.02 % of these enterprises are small scale enterprises with the animal number under ten heads. In cattle fattening, the share of 50 heads and above enterprises in the total farms is only 5.07%. On the other hand, these enterprises, which are classified as large-scale, have 38.41% of total fattening cattle with 1.532.564 head cattle.

Animal production models suitable for economies of scale should be considered as opportunities, especially in areas where the country has a comparative advantage.

On the other hand, it is known that exporting countries that are producing low-cost meat have the advantage of competing in world trade. In Turkey, meat production takes place at a high cost. Due to this, it is unable to find a chance to compete on a global scale, and Turkey falls into countries that import red meat and butchery fattening animals.

Especially in recent years, the increase in production costs due to inflation, foreign exchange and input prices could not be reflected in sales prices. Farmers, whose profitability levels drop due to uncertainty and unfair competition in the market, are withdrawn from the production area, and this situation creates a negative environment in terms of sustainability in production. The purchase prices of butchers announced by Meat and Dairy Institution did not take into consideration the production costs and meat-feed parities and the red meat prices were suppressed by the deliberate policies.

In parallel with the increase in the scope and quantity of imports, the instability and unfair competition in the meat market has been reported that around 20 thousand cattle fattening enterprises have shut down or stopped the operation in the last two years. In this process, it is necessary to determine the needs of the enterprises that stopped operation and to encourage and support them in order to start fattening and production under the controlled conditions of flocks that will consist of right animal breeds.

When we look at the fattening system in our country, it is seen that it is based on coarse feed consisting mainly of cereals, pulp and other by-products. The 40-45% of compound feed produced in Turkey depends on imported feedstuffs. According to this situation, feed costs increase, and this adversely affects the cost of meat production. The fattening method based on concentrated eating is expensive. In Europe, Australia and some South American countries, the main reason why the cost of animal production is cheap compared to our country is the availability of abundant and cheap roughage. Red meat production costs can be lowered as long as the roughage rate in animal fattening is increased according to the concentrate rate. For a rational fattening and cheaper meat production, the ratio of roughage used in fattening must be increased, and the dependence on import should be reduced, and self-sufficiency must be ensured.

If sufficient success is not achieved in a country in terms of race/genotype, healthy environment, proper feeding and herd management, neither the desired targets in milk or meat production can be achieved. Since the desired success cannot be achieved in these four important issues, red meat production costs are very high in our country. A production model to specialize in the production of red meat has also not been adopted. Current technical and economic data are insufficient in order to calculate feed cost and production planning for enterprises.

When the beef carcass/feed parity which is an important ratio in cattle fattening in line with the profitability and costs of the producer is examined, in 2015, the farmer received 30.2 kg fattening feed for 1 kg carcass; in 2016, it was able to take 30.7 kg and 28.3 kg in 2017. In 2018, 23.89 kg of fattening feed could be obtained for 1 kg of calf carcass. At this point, the high rate of increase in feed prices reduces this parity to below 20 levels. The reason for the decrease in carcass/feed parity is that the increase in feed prices is higher than the increase in carcass prices. Currently, since the cost of unit carcass production in cattle fattening is higher than the carcass sales income, the profit takes the negative value, and the producer makes a loss in the current conditions (Aral, 2019, TEPGE, 2019).

General Evaluation

Animal husbandry has become an industry in developed countries and has become an integral part of the economy. This situation reveals that agriculture and therefore, animal husbandry is a strategic sector that needs to be developed at the national level (TİGEM, 2018).

In our country, a structure based on imports in red meat has some disadvantages in terms of rural economic development and sustainability in production and creates unfair competition by disrupting animal product markets.

Considering the national interests; measures should be taken in the short term, to protect the domestic producer and prevent unfair competition in the importation of animal and animal products, feed raw materials and additives. However, customs duties should be regulated within this framework, and the decision to import live animals and red meat should be completely abandoned in the medium term (HAYGEM 2018a, UKON, 2019).

Policies and practices that will allocate confidence in the sector in terms of market balance, price formation, marketing structure that considers production and sales costs, industry-production integration, stability and competitiveness should be implemented effectively and quickly to increase red meat production, to ensure self-sufficiency and sustainability in Turkey.

With the policies to be taken on a macro basis to solve the technical and economic problems of the livestock sector; structural changes such as ensuring market stability and increasing productivity have to take place. By this time various supports have been made to the sector in different forms, periods and amounts. However, the supports implemented without solving the structural problems were not sufficient and effective in reaching the targeted level of the sector.

The classification of the enterprises operating in the agricultural sector according to their production forms and characteristics may reveal which product production is supported in particular. It will be possible to make comparative analyses of different sized enterprises in order to determine where market trends, policies, and economic conditions are more influenced than where and at what point of a particular entity and to help researches to monitor market conditions and evaluate the possible impact of policymakers.

The real policy to support animal husbandry should be to ensure that product/feed price ratios continue to maintain profitability in animal husbandry activities. In the modern sense, livestock enterprises in an optimum scale, which are integrated with forage crops will be inevitable for our country in the future.

In order to improve the technical and economic efficiency of fattening enterprises, a general production planning and periodic cost calculations will be able to help to see the way through the entire production period. It is important to focus on practices such as intervention purchases and contractual fattening with the industry in order to continue its activities with guaranteed purchase prices including production costs and profit to not have any problems in the marketing of butchery animals and meat (Aral, 2019).

Due to the contribution of the livestock sector to the national economy, a large number of people have demand for services from the public sector. Meeting these demands effectively will enable the livestock sector to develop and solve most problems. There is a need for a new restructuring and reorganization of the veterinary services provided by the public, especially in rural areas.

It is obligatory to consider the livestock services in the public sector on a sectoral basis by taking into account the EU harmonization and developed country examples. Considering the nature of animal-oriented services, an effective structuring with a chain of command should be established in the ministry. In this framework, the quality and efficiency of veterinary services to be taken to rural areas will increase, and service cost will be reduced, effectiveness in the struggle against animal diseases will be ensured, and multi-headed decisions will be avoided in animal husbandry.

In order to increase red meat production, it would be an effective approach to link organized animal husbandry regions with public sector and to take similar initiatives to the previous projects (contract model) of the Meat and Milk Institution. Within the framework of this model, it will be possible to achieve market stability and sustainability by creating purchase prices that take into account the producer's production costs

In the improvement of the meat market; the development of marketing infrastructure and the encouragement of producer organization will contribute to the solution of the problems. On the other hand, studies and researches aimed at increasing productivity and quality in these markets, determining product and cutting standards, increasing product diversity and establishing the quality-price relationship will also increase competitiveness.

The milk and red meat sub-sectors are complementary to each other and have a strong relation (input-output). Therefore it is also important to establish a rational production and marketing organization in the dairy sector, to prevent periodic price fluctuations, to cooperate with the administrative structures operating in the meat and milk markets, and to ensure and maintain the regulation of market conditions effectively.

The enterprises operating in the field of animal husbandry are under the economic grip of the sellers and buyers, who are limited in number and dominate the prices, both in the production stage (feed, medicine, etc.) and in the supply stages. In this respect, it is necessary to support the producer organization by the state to solve the problems.

In Turkey, the production of some crops has decreased, and agricultural areas have not been planted in recent years. Incentives should be given to increase the production of forage crops and roughage as an alternative product to these areas which are out of production. On the other hand, in regional projects (GAP, DAP, KOP, etc.), supporting the production of forage crops in order to cover the roughage gap may make an important contribution.
Required precautions should be taken to extend and develop insurance services in order to protect the producer by providing adequate amounts of agricultural insurances, especially about animal diseases and economic losses.

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Investigation of bovine coronavirus and bovine rotavirus by rapid diagnosis kit and RT-PCR in diarrheic calf feces*

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ABSTRACT

This study has investigated bovine coronavirus (BCoV) and bovine rotavirus (BRV), which are among the most important causes of diarrhea in calves leading to financial losses in Turkey and all over the world BCoV and BRV were detected by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), which is one of the most reliable method of diagnosis, The results obtained by RT-PCR were compared to the sensitivity of the commercial Rota-Corona Rapid Test Kits used by clinical veterinarians in fields. In this study, 96 fecal samples were examined from diarrheic calves in cattle farms in the cities of Konya and Afyon for BRV and BCoV firstly by BoviD-5 Ag rapid test kit, and then we applied the RT-PCR test. A comparison of the rapid test kit with the RT-PCR in terms of sensitivity and specificity revealed the 83% sensitivity and 100% specificity of the BRV and 7.6% sensitivity and 100% specificity of BCoV. In conclusion the practical and rapid diagnosis of the disease using of Rapid Diagnosis kit used by the clinician veterinarians may be useful, but the results must be interpreted with caution since the sensitivity of the test decreases due to the reduction in the number of viruses in the later stages of the infection.

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Introduction

Neonatal calf diarrhea caused by viral, bacterial and protozoon agents is one of the infections characterized by enteritis leading to weight loss and deaths in calves under one month of age (Murphy et al.,1999). Neonatal calf diarrhea is among the most important reasons for financial losses in the meat and milk industry all over the world (Boileau et al.,2010). Although its causes show variations depending on the regional and stable conditions, the role of rotavirus and coronavirus in the cases of calf diarrhea have been found to reach up to 50% and 80% respectively. Rotaviruses generally cause infections characterized by diarrhea in dairy calves (Al Mawly et al., 2015) and beef calves (Cho et al., 2013) up to 9-21 days old. Bovine group A rotavirus, bovine coronavirus, enterotoxigenic K99+ Escherichia coli (K99), Cryptosporidium parvum and Salmonella spp. are reported to be the most common enteric infection

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agents (Bartels et al., 2010; Izzo et al., 2012). Among the factors that affect the course of rotavirus and coronavirus infections are whether the newborns received colostrum, time of weaning, climate conditions, their immune conditions, and other present enteropathogenic agents. The main mode of transmission of rotaviruses is the fecal-oral route. Through the feces of the infected animals, a high level of viral particles (approximately 1011 particle/g) is shed around. This shedding reaches the highest level on the third and fourth days, and the virus can survive in the feces for several months (Murphy et al., 1999). Virus isolation (Mebus et al., 1969; Hasoksuz et al.,2002; Gulyaz et al., 2005), immunochromatographic rapid diagnostic assays (Al-Yousif et al., 2001; Uhde et al., 2008; Klein et al., 2009; Bartels et al., 2010; Altug et al., 2013), IEM (Immunelektron microscopy) (Saif et al., 1980), ELISA (Alkan, 1998; Murphy et al., 1999; Gulyaz et al., 2010) and RT-PCR (Cho et al., 2001; Hasoksuz et al., 2002; Aich et al., 2007; Decaro et al., 2008; Asano et al., 2010; Bok et al., 2015) are among the most preferred methods to diagnose rotavirus and coronavirus infections.

Materials and Methods

Specimens: The samples were collected from calves (1 to 30 days old) with acute diarrhea cases in stables in Konya and Afyon. Total of 96 fecal samples were collected for this study. The age distribution of the collected samples is presented in Figure 1. The fecal samples were collected from the rectums of the animals with sterile cotton swabs. One swab taken out of the rectum was put in the solution in the rapid test kit, and another was homogenized by putting it into Phosphate Buffer Saline (PBS), and stored at -20ºC until it was used for RT-PCR. The samples for the rapid test kits were examined under stable conditions, and their results were reported to the owners in 15 minutes. Necessary notes were taken and the positive samples were taken into account for the next examination.

Rapid Diagnostic Test: In this study, we used the Bionote BoviD-5 Ag (Cat. No: RG13-02) rapid diagnosis kit. We followed the test procedure of the producer. In line with the procedure, firstly the swab contaminated with the feces was placed in the solution included in the kit during the sampling and was homogenized. Then one drop of the solution was added onto the arrays and according to the change of color, coronavirus or rotavirus was interpreted as positive or negative.

RT- PCR Materials: Extraction of the Viral RNA: We used High Pure Viral RNA isolation kit of Roche (Roche, Cat. No: 11858874001). The fecal samples were suspended at a rate of 1/10 in PBS including 25000 U/ml Penicillin and 20 mg/ml Streptomycin, centrifuged at +4oC, 3000 rpm for 15 minutes, and then supernatant was transferred into a sterile tube. Following the centrifugation 200 µl of the supernatant was taken and transferred into a 1.5 ml RNase-free sterile tube. Each sample was mixed with working solution containing 4 µl Poly A and 400 µl Binding Buffer. The working solution and the sample mixture were treated with the Removal Buffer, Wash Buffer, Elution Buffer included in the kit by using the silica gel spin column. At the end of the extraction process, 50 µl of viral nucleic acid was isolated, and stored at -80^{o}C.

cDNA Synthesis: We used Reverse Transcription System (Promega A3500) to obtain cDNAs. In order to synthesize cDNA from the isolated viral RNA, we used the Promega Reverse Transcription System synthesis kit (Promega A3500), and followed the recommended protocols. 5 µl of the isolated viral RNA was transferred into the PCR tubes and was incubated at 70ºC for 10 minutes. Following the incubation, it was kept in ice for 2 minutes. Master Mix (15 µl for each sample) was prepared in a different PCR tube and 15 µl of Master Mix was added onto each of the 5 µl RNA samples.

Viral RNA and Master Mix mixture totaling to 20 µl was put into the Thermal Cycler and amplified at 22ºC for 10 minutes, at 42 ^{o}C for 15 minutes, at 95 ^{o}C for 5 minutes at 4^{o}C for 5 minutes, and then the cDNA was synthesized. The resulting products were stored at -20^{o}C.

Polymerase Chain Reaction (PCR): We used Promega Go Taq Flexi DNA Polymerase (Promega M8305) To detect the presence of bovine rotavirus in the fecal samples of the calves with the one-step RT-PCR method. The primers reported by Hasoksuz et al., (2008) and Chang et al., (1997) were used. These primers are specific to the VP7 gene region of the group A rotaviruses. We used the primers reported by Cho et al., (2013) for the detection of bovine coronavirus. These primers are specific to the N protein gene of the virus. We used S-Beg5-GGC TTT AAA AGA GAG AAT TTC-3, End-9, 5-GGT CAC ATC ATA CAA TTC TAA TCT AAG-3 primers of 1062 bp for bovine rotavirus and NOF-5-GCA ATC CAG TAG TAG AGC GT-3, NOR-5-CTT AGT GGC ATC CTT GCC AA-3 primers of 730 bp for bovine coronavirus.

BRV: We used the one-step RT-PCR method for the viral RNAs obtained. 0.8 µl DMSO, 0.6 µl End-9 of the rotavirus primers and 0.6 µl S-Beg were added onto each of the 5 µl RNA products, and they were mixed with a straw to homogenize. The mixture was incubated at 94°C for 5 minutes, and then kept in ice. Following the incubation, 43 µl Master Mix composed of Primer F (20 pmol), Primer R (20 pmol), and solutions of the Promega M8305 kit and the Promega A3500 cDNA kit was treated with 7 µl RNA and DMSO mixture. It was amplified at 42°C for 60 minutes, at 94°C for 3 minutes, (at 95°C for 1 minute, at 55°C for 2 minutes, at 72°C for 1 minute at 35 cycles), and at 72°C for 10 minutes.

BCoV: cDNAs of the samples were treated with the Master Mix mixture of the Promega M8305 kit including Primer F (50 pmol) and Primer R (50 pmol) and, for the PCR reaction, Go Taq Flexi DNA Polymerase, 5X buffer green flexi color, MgCl2 (25 mM) and dNTP. It was amplified at 94°C for 3 minutes (at 94°C for 1 minute, at 52ºC for 2 minutes and at 72°C for 1 minute at 35 cycles), at 72°C for 7 minutes.

To display the amplification products, 1.5% agarose gel containing ethidium bromide was prepared. The PCR products were run at 100 V for 30-45 minutes and the amplified DNA bands were controlled under UV light.

Statistical analysis: We used the chi-square test ($\chi 2$) for the statistical analysis of the diagnostic tests. We recorded $p<0.05$ as statistically significant.

Results

We examined 96 diarrheic fecal samples in total for BCoV and BRV by rapid diagnostic test and RT-PCR method. The collective results of the study are presented in Table 1. According to the results, the

Table 1: Number of positive samples identified by the rapid test kit and RT-PCR

Pathogen	Rapid Test Kit	RT-PCR
BRV (Group A)	15/96 (15.62%)	18/96 (18.75%)
BCoV	1/96 (1.04%)	13/96 (13.54%)
BRV (Group A)- BCoV	-	4/96 (4.16%)

rapid diagnostic test revealed 15 samples as BRV positive (15.62%) and 1 sample as BCoV positive (1.04%). The RT-PCR method detected 18 cases of BRV presence (18.75%), 13 cases of BCoV presence (13.54%), and 4 cases of both BRV and BCoV presence (4.16%). The electrophoresis images are presented in Figure 2 and Figure 3. According to these results, four samples were found to be both BRV and BCoV positive by RT-PCR method. However, only one sample was found to be BRV and BCoV positive with the rapid diagnostic test.

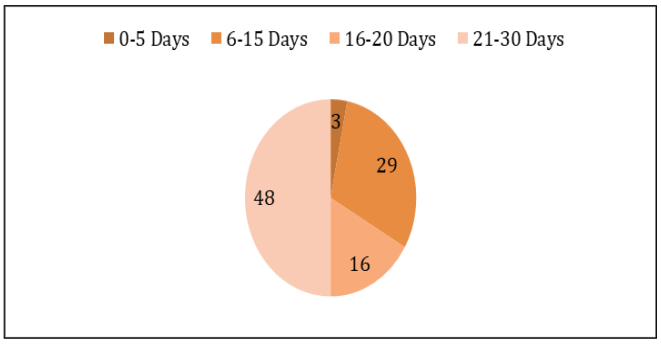

Figure 1: Distribution of the samples of calve feces by age (days)

This sample showed a quite strong DNA band appearance after the agarose gel electrophoresis using RT-PCR. (Figure 3). For the 0-5, 6-15, 16-20 and 21-30 days old calves found to be BRV positive by the rapid test kit, the rates of positivity were 67%, 24%, 0% and 13% respectively. Only 4% of the 6-15 days old calves were found to be BCoV positive. For the 0-5, 6-15, 16-20 and 21-30 days old calves found to be BRV positive by the RT-PCR method, the rates of positivity were 67%, 31%, 6% and 13% respectively. In terms of BCoV, while RT-PCR found no positive samples in the 0 -5 days old group, the other groups found to be 34%, 13% and 2% positive respectively (Figure 4).

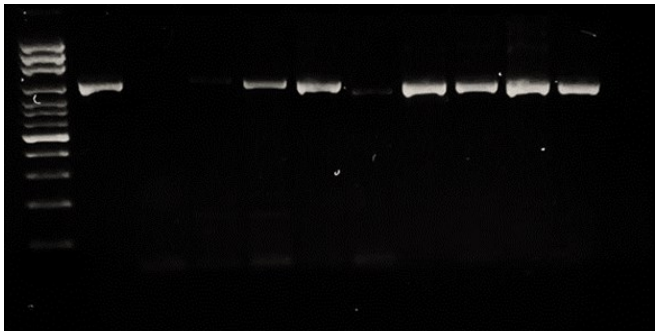

Figure 2: Electrophoresis image of the BRV (1062 bp) positive samples, DNA Ladder (100 bp Fermentas), PC (Positive Control), NC (Negative Control), DNA bands of the samples; 27, 28, 12, 13, 14, 15, 16 and 17

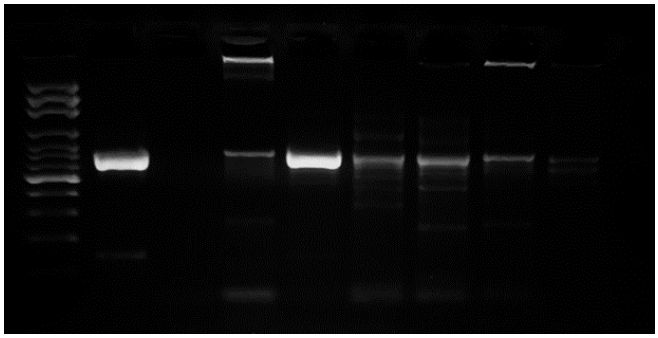

Figure 3: Electrophoresis image of the BCoV (730 bp) positive samples; DNA Ladder (100 bp Fermentas), PC (Positive Control), NC (Negative Control), DNA band of the samples 16, 17, 19, 22, 23 and 25

Table 2. Sensitivity, Specificity, PPV and NPV rates of the Rapid Test Kit versus RT-PCR in terms of BRV/BCoV

Reference Test	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
BRV Rapid Test BRV RT-PCR	83	100	100	96
BCoV Rapid Test BCoV RT-PCR	7.6	100	100	88

PPV: Positive Predictive Value, NPV: Negative Predictive Value

Discussion

Rapid and accurate diagnosis of BRV and BCoV infection is important for the control and eradication of the disease in newborn animals in cattle farms in many developed and developing countries. Therefore, it is important to diagnose BRV and BCoV rapidly in the field, and to detect it through rapid and effective test techniques in veterinary diagnostic laboratories. Among these methods, isolating the RNA of the virus and converting it into DNA (cDNA) and multiplying the cDNAs by using specific primers (RT-PCR) has the highest sensitivity and originality. However, as these techniques can only be applied under laboratory conditions and require time, clinical veterinarians need rapid test kits to diagnose the infection under field conditions. These rapid test kits are important in terms of determining the treatment process and avoiding wrong antibiotic use, but the use of rapid test kits is unfortunately behind the desired levels.

Table 3. Statistical comparison of the positive results by the tests applied

	Rapid Test Kit (n)	RT-PCR (n)
BRV Positive	15/96[a]	18/96[a]
BCoV Positive	1/96[b]	13/96[a]
BRV-BCoV Positive	0/96[b]	4/96[b]

a, b: Different letters within the same column are statistically different. ($p<0.05$) a is statistically higher than b.

The most important reason behind this is the righteous suspicion about the sensitivity and specificity of these rapid test kits. In recent years, the rapid immunochromatographic tests, which are more advantageous under filed conditions, it has become possible to diagnose different enteropathogens in the feces of calves in approximately such short time periods as 10 to 15 minutes, and to plan prophylaxis and treatment (Klein et al., 2009).

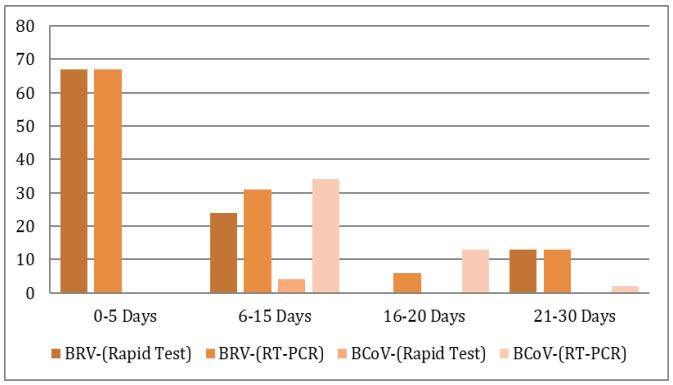

Figure 4. Percentage distribution of the BRV and BCoV positive calves by age (days)

Many investigators have reported that the immunochromatographic rapid test kits are a simple and easy-to-apply method for the diagnosis of enteropathogens in feces, and that they may be preferred by clinical veterinarians and investigators more often as they do not require specialist and fully-equipped laboratory, are cheap and rapid in comparison to other techniques, and can be applied under any laboratory or office conditions which can be found in each private clinic and even under field conditions (Thorns et al.,1992; De la Fuente et al., 2009; Klein et al., 2009). Klein et al. (2009) examined the fecal samples collected from 1 day to 42 days old 180 calves (98 of them had the symptoms of diarrhea) both with immunochromatographic rapid test kit and RT-PCR method. Compared to RT-PCR, the investigators (Klein et al., 2009) found the sensitivity of the rapid test kit for BRV as 71.9% and the specificity for the same as 95.3%, and its sensitivity as 60% for BCoV and its specificity as 96.4% for the same. In their study comparing the commercial rapid test kits with the multiplex PCR method, Cho et al. (2012) found the sensitivity of the rapid test kits as 60% for BCoV, and 42.3% for BRV, and they, therefore, stated that the rapid test kits had to be interpreted carefully in terms of originality and sensitivity. In a study

In a study comparing real-time RT-PCR, ELISA and immunochromatographic tests, Izzo et al. (2012) found the sensitivity of the rapid test kit as 32.7% for BRV and as 28.2% for BCoV in comparison to RT-PCR technique. The investigators reported that the sensitivity and specificity levels of the immunochromatographic rapid test kits were very low in comparison to real-time PCR, and that it was possible to interpret the course of the disease at the clinic since the viral RNA amount is known due to real-time PCR method. In their study on the rapid etiological diagnosis of neonatal calf diarrhea by immunochromatographic test kits, Altug et al. (2013) reported 14 cases of BRV (27.5%) and 1 case of BCoV (1.96%) among the samples from 51 diarrheic calves. In this study, among the samples examined by rapid test kit, we found 15.6% (15/96) to be BRV-positive and 1.04% (1/96) to be BCoV-positive. The same samples were tested using RT-PCR method and the positivity rates for BRV and BCoV were found 18.75% (18/96) and 13.5% (13/96), respectively. A combination of BRV and BCoV infections was detected in 4% of the diarrheic feces (4/96).The results obtained in this study were found to be compatible to those of Altug et al. (2013). Besides, in comparison to RT-PCR technique, the sensitivity of the immunochromatographic rapid test kits for BRV was 83% and the specificity of the same was 100%, its sensitivity for BCoV was 7.6% and specificity for the same was 100%. The results for bovine rotaviruses were found to be compatible with those of (Klein et al., 2009), who have previously contrasted the immunochromatographic rapid test kits to RT-PCR in terms of sensitivity and specificity, while they were determined to be higher than those of Cho et al. (2012) and Izzo et al. (2012). In terms of bovine coronavirus, our results were significantly lower than those of many other investigators (Klein et al. 2009; Cho et al., 2012; Izzo et al., 2012) who have studied the same subject matter. The possible reason for this might be the fact that the sampling is carried out in the late course of the disease when the level of virus shedding and the amount of viral particles are low. The rapid immunochromatographic diagnostic method is based on the attachment by the agent within the sample dropped on the test stripe to the conjugated specific antibodies. Therefore, it is essential to carry out the sampling during the peak time of virus shedding. It is necessary to collect the samples within 72 hours after the onset of the disease, because virus shedding decreases in time. However, it is possible to detect even very low levels of viruses by the RT-PCR method. The diagnostic ability of the rapid test kit can be inferior to that of RT -PCR in samples containing small amount of virus. “In this study, we identified both BRV and BCoV by RT-PCR in four samples. Only one sample was found positive in terms of BoCV using the rapid test kit. The electrophoresis images from the RT-PCR diagnosis of this positive sample presented/showed a stronger DNA stripe image in comparison to the other positive samples. This indicates that the rapid test kit determines positive results if there is high amount of coronavirus in the fecal samples. Therefore, it is necessary to support the results with a lot of samples. Examining the age ranges of the calves and the infection-positive results by RT-PCR for these age ranges, we see that the highest level of BRV-positivity was found as 67% in calves of 0-5 days of age. This rate was identified as 31% in the 6-15 days age group, 6% in the 16-20 days age group and 13% in the 21-30 days age group. In our investigations by RT-PCR for BCoV, we identified no positivity in the 0-5 days age group, but 34% in the 6-15 days age group, 13% in the 16-20 days age group, and 2% in the 21-30 days age group (Figure 4). While the rates identified for BRV by this study are close to those reported by Al Mawly et al. (2015) (20% in calves of 1-5 days of age, and 19% in calves of 9-21 days of age), but in terms of BCoV, the results of Al Mawly et al. (2015) (5.4% in calves of 1-5 days of age, 6.1% in calves of 9-21 days of age) are lower than those of this study. Alkan (1998) has pointed out that this situation can be associated with the colostrum that calves receive from their mothers. Alkan (1998) has reported that one of the most important factors affecting the average infection age is maternal immunity. In this study, we know that the calves from which we collected the samples had generally received colostrum from their mothers. Ellens et al., (1978) and Wood et al., (1975) have reported that there were no rotavirus specific antibodies in the second week after birth, but antibodies specific to coronavirus reached significantly high levels in the third week. Contemplating on the fact that the coronavirus antibodies are secreted for a long time in milk, Wellemans and Van Opdenbosch (1981) have explained it with the fact that mothers were considerably infected with coronavirus during the diarrheic periods, their immune systems were stimulated as they shed the virus through their feces on the day of giving birth, inducing the mammary gland to secrete Ig antibody. Therefore, the total rate of BRV-positivity in the first two weeks (0-15 days) in this study is 34% while it decreases to 6% and 13% in

the third and fourth weeks, respectively. With regards to BCoV, the positivity rate in the first 3 weeks is 26% while it decreases to as low as 2% in the fourth week. The distribution of positivity by the age groups identified in this study was found to support the ideas of Ellens et al. (1978), Wood et al. (1975) and Wellemans and Van Opdenbosch (1981). This fact shows that in this study colostrum received from the mothers of calves stimulated the maternal immunity, and affected the positivity rate by age specified in the study. Although this study identifies a low level of sensitivity for immunochromatographic rapid test kits, one might think that the most important advantage of these kits for clinical veterinarians with regard to BRV diagnosis is to avoid wrong treatment with antibiotics. Nevertheless, as the amount of virus decreases in the late course of the disease, one must not ignore the fact that it is not a very effective method. This might lead to an inaccurate interpretation of the disease. The presence of subclinical carrier animals is another important issue to bear in mind while evaluating the disease. This is a point a good veterinarian would not like to ignore while assessing the cases of diarrhea in calves posing a problem especially in big farms. As a permanent solution, molecular methods such as RT-PCR play an essential role in identification of these animals. İt is possible to identify even one virus particle in the feces by RT-PCR (Klein et al. 2009). Thus, veterinarians will be able to interpret the disease accurately, and to take such protective measures as vaccination. It is of paramount importance to identify the field strains of infections such as bovine rotavirus and bovine coronavirus present in Turkey. Apart from the group A rotavirus identified in this study, identifying the G and P type rotavirus strains present in Turkey is of significant importance for the effectiveness of vaccinations. The results of this study indicates that the rapid test kits used by veterinarians under field conditions to diagnose the diseases quickly can be beneficial, but a careful interpretation is advisable since the sensitivity of the rapid test kits has been found to be low (especially for BCoV) in comparison to RT-PCR. In order to be able to interpret diseases more effectively and to seek more permanent solutions, it is advisable to support the results through molecular techniques such as RT-PCR.

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JIVS

Archaea and their potential pathogenicity in human and animal diseases

Review Article

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ABSTRACT

There are hundreds of organisms that infect and cause disease in humans and animals. These organisms can be bacteria and single-celled eukaryote, as well as a few parasites. Archaea, one of the three domain of life, immensely diverse group of prokaryotes and includes a number of “extremophiles” that develop in such environments as hot springs, salt lakes, human and animal gut, volcanic submarines and low, high pH habitats. It is puzzling that despite being one of the most numerous and ubiquitous life forms on earth, no member of the domain Archaea has been described as human or animal pathogen. The absence of pathogenic Archaea in the taxonomy database is statistically highly significant. The aim of this article is to display a brief overview of what is currently known about archaea and archaeal potential pathogenicity in and on human being and animals.

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Introduction

Archaeabacteria are single-celled organisms that can survive in extreme conditions and they have challenged the way scientists classify life. They are believed to be the oldest form of organisms, being about 3.5 billion years old. In the past, they were placed under the Kingdom monera along with bacteria. However, this classification is no longer followed. Since Archaeabacteria are biochemically and genetically different from bacteria and possess unique evolutionary history, they have a separate domain in the three-domain system of biological classification. In fact, Archaeabacteria are no longer called so, they are instead known as *Archaea*. The term *achaio* is a Greek word, which means 'ancient'. The meaning of the word aptly describes the Archaeabacteria who are thought to have a common ancestor like the bacteria and eukaryotes. Archaeabacteria is similar in structure (biochemical and genetic features) to eukaryotes than bacteria (Balch et al., 1979; Madigan et al., 2000).

The First Findings

Several scientist groups and institutions have not yet to find powerful evidence of an Archaeal pathogen, although some Archaeal phenotypes inhabit the human body and share commensal and symbiotic relationships with many species of animals and single-celled eukaryotes. Several theories have been published

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according to Archaea and their relationship to diseases. Since the publication of the Cavicchioli's (2003) article, meta-genomic studies have revealed more about the diversity of microbial life and prevalence of Archaea domain (Cavicchioli et al., 2003; Eckburg et al., 2003).

According to Cavicchioli et al. (2003) they are (Archaea) a highly diverse domain of life that is present in highly numbers in the environment that would afford them the opportunity to cause disease. They are able to interact with eukaryotic cells in symbiotic relationships, suggesting that pathogenic relationships may be possible. Archaea subspecies are present in many animals, especially in ruminants 'digest system or in human oral cavity, vagina, and they are recognized by the immune system (Cavicchioli et al. 2003). Cavicchioli et al. (2003) have written that it is likely that there are archaeal pathogens, but they have not yet been discovered. So far, as the publication of the Cavicchioli et al. (2003) released, meta-genomic studies have revealed more about the diversity of microbial life and prevalence of Archaea species. Unfortunately, such methods are worthless tools for illuminating the causes of unknown illnesses (Cavicchioli et al., 2003).

Another researcher Martin (2004) postulates that, "There are two possible reasons why no pathogenic archaea are known. First, they do not exist or second is they have not been identified yet". He also wrote that, Archaea are not pathogens because they use different co-factors in their biochemical reactions compared to Eukarya and Bacteria. For instance, vitamins like co-enzyme M, cobamides, factor F430, co-enzyme B. That is why, Archaea do not parasitize since they have enough food to survive. As how we know, pathogens are looking for a meal for infection and growth (Martin, 2004).

Archaea at the present

Moreover, Gill and Brinkman (2011) published different hypothesis that Archaea may contribute to disease caused by other organisms indirectly. They presume Archaea may facilitate the growth of disease-causing organisms rather than causing disease directly by themselves. It can be possible by removing H2 from microbiota where complex microbial communities exist. H2 inhibits the growth of some disease-causing bacteria, and once removed, these species could flourish. In addition to that, it has known that methanogenic Archaea has been connected with various human disease, such as periodontal disease, gastrointestinal ailments, and colon cancer; however no causative relationships have been established exactly. For these reasons, this hypothesis requires further study and to be confirmed.

Also it was assumed that disease causing bacteria may have receive some of their virulence factors from Archaea through the transfer of "Pathogenic" genes from species that engage in symbiotic or commensal relationships with Eukarya. Nevertheless, gene transfer has not been inferred between Bacteria and Archaea exactly until today and the mechanisms that provide the transfer have not been classified yet. Currently anyone definitively pointed pathogenesis with any gene that has been transferred from Archaea to a bacterium. Furthermore, there is hypothesized that virulence bacteriophages could not interact with Archaea, in this way hindering the ability of Archaea to become pathogens. The lack of gene exchange from bacteriophages to Archaea may explain why so few (if any) archaeal pathogens exist (Lawrence, 2005; Bennewies et al., 2006; Gill and Brinkman, 2011).

Despite hypothesis discussed above Aminov (2013) from the Technical University of Denmark, National Veterinary Institute has published an article in which was stated role of Archaea in human disease. He looked into many potential clinical cases connecting with the Archaeal species. By him clarified that, patients with Crohn's disease, ulcerative colitis and primary pneumatosis intestinalis displayed a significantly lower incidence of methane excretion compared to healthy subjects. This work was the first suggesting an association of the Archaea with human gastrointestinal disease published in 1985. Since that time, there have been a number of studies, more recent ones using molecular ecology markers, which have confirmed these two initial observations (McKay et al., 1985; Aminov, 2013). Also Scanlan (2008) was written how the diarrheal conditions of human gastrointestinal disease result in the opposite trend with lower incidences of Methanogenic Archaea and lower rates of methane production. In addition to that, chemotherapy-induced diarrhea in cancer patients have also resulted in the decrease of Methanogenic Archaea in parallel with the loss of beneficial bacteria in 2013 (Scanlan et al., 2008).

Reassessments by many scientists show the role of methane production among patients with gastrointestinal disturbances have clearly associated the elevated methane production with alterations in intestinal motility, such as constipation, but not with other conditions. In this regard, alternative generation of highly toxic hydrogen sulfide. As a result, sulfate reduction in the gut may impose much higher health risks compared to more inert methane (Franck at al., 2012).

Archaea in oral cavity

In another study, Lepp (2004) observed several clinical cases, which have been detected by PCR in up to 36% of periodontitis patients. So compared to periodontitis patients, the supragingival plaque of healthy subjects harbors a lower total microbial load, and the hydrogenotrophic group is represented exclusively by acetogenic bacteria at lower numbers too. On the other hand, the subgingival plaque from periodontitis patients harbors a larger number of total bacteria, and the hydrogenotrophic group includes methanogenic archaea and sulphate-reducing bacteria (SRB). The latter two groups are absent in healthy control subjects but present in 65% of periodontitis patients, alone or in combination (Lepp et al., 2004). However, the role of the Archaea in periodontal disease cannot be understand within the frames of a typical host-pathogen interaction, and it has to acknowledge that these are not bona fide pathogens (Bennewies et al., 2006; Liu et al., 2012).

Scientist states that, their involvement in disease can still be interpreted from the point of view of polymicrobial diseases that has recently gained considerable attention. Due to interface malfunction, a subset of usually symbiotic bacteria can display potentially pathogenic properties; they thus have been called “pathobionts” to be differentiated from the “classical,” opportunistic pathogens. Then it was questioned if could the commensal methanogenic Archaea be considered as “pathobionts”? In polymicrobial diseases, such as periodontitis however, taxonomic signatures are less effective as disease predictors, although some attempts are being made to identify the key players within certain pathobiota (Chow et al., 2011).

The role of hydrogenotrophic microbiota is interchangeable and can be played by SRB, methanogenic Archaea, or acetogenic bacteria. Briefly, comparing of metagenomes of healthy and diseased microbiota may help to identify the sets of genes differentially represented in these two conditions and point to the enrichment or reduction of genes specific for pathologies. Signatures of periodontal disease indicate the enrichment by genes encoding metabolic functions that are consistent with a parasitic lifestyle and anaerobic metabolism, as well as by genes encoding virulence factors and the biosynthesis of toxic factors (Bartold et al., 2006; Liu et al., 2012; Aminov, 2013).

Methanogenic archaea in diseases

In a study by Convey and Makario (2008) were investigated an attitude of Methanogenic Archaea in health and disease. They collected many laboratory works and cases about Methanogens Archaea. For instance, it was suggested that we have to look at their pathogenicity from a various angle in comparison to classic pathogens that occupy tissues and release toxins. Instead, these organisms (methanogens) seem to share their pathogenicity indirectly, helping the growth of other microbes, which are directly involved in pathogenesis. Their data show that methanogens are more abundant in adults than in children (confirming results from other laboratories, discussed in their study). The study also shows that methanogens interact not only with SRB (sulphate-reducing bacteria) in the human and animal intestine but also with other bacteria. They presumed that, could it be that non-bacterial sources of H2 provide it for H2-using methanogens to grow? This is a possibility that deserves attention, for instance in colon cancer, in most of which M. *smithii* is more abundant than in healthy controls. Data from this study support also the concept that pathology of the abdominal aorta with impact on the colon vascular circulation is accompanied by a high incidence of breath-methane excretion. (Conway et al., 2008; Maczulak et al; 2000). Also reported that the frequency of methane breath excretors was upper among patients with malignant or pre-malignant colonic pathology, usually higher than in matched controls without colonic disease or with non-malignant pathology. The same result was in colonic diverticulosis as well (Pique et al., 1984; Weaver et al., 1986).

Methanogens emerge in the intestinal tract of humans at about 2 years of age and then increase continuously with age reaching their highest concentrations in the elderly. This result was also, observed in the laboratory animals. It was obvious that the higher the percentage of methanogens in oral cavity, the more severe the periodontitis, and colon cancer is accompanied by a growth in colonic methanogen archaea (McKay et al., 1983).

Recently, a role for intestinal methanogens in obesity was noticed. Presence of methanogens in the colon contributes calorie and adiposis in laboratory mice, accordingly contributing to the progress of obesity. It was questioned if would M. *smithii* enhance growth or activity of the fiber-consumer polysaccharide-digesting bacteria and thus increase the utilization of fiber and caloric intake? If that were so, dietary fiber would be digested more efficiently and its caloric yield per unit weight would be greater than in the absence of M. *smithii*. It may also lead to aggregation of fat, obesity, especially in individuals who are on a high-fiber diet (Conway et al., 2008; Samuel and Gordon, 2006).

Archaea on skin

There were several studies dedicated to the skin archaea although, there were not found much Archaea species. In a study by (Caporaso et al., 2011) periodically sampled the left and right palm of a male and female over few month, from the male was observed only transient Thaumarchaeota, while from the female observed persistent, albeit low, presence of these Archaea on her right palm. It has been stated that, different members of the *Thaumarchaeota phylum* are thought to be chemolithotrophic ammonium oxidizers and encode characteristic amoA gene homologs. Thus, people who sweat or exercise often could harbor larger communities of the Archaea. The scientists wrote also that, people use statin to control their cholesterol, thus they are cholesterol- lowering. However, at the same time this medical drug is anti - archaeal agent. That is why for today no exist exact information about how it would be harmful or beneficial for human microbiota disappearing Archaea Domain. Thus, some researchers suggest to think adequately before Archaea become part of "disappearing human microbiota" (Caporaso et al., 2011; Hulcr et al., 2012; Probst et al., 2013; Moissl-Eichinger et al., 2017).

Eckburg et al. (2003) published a study which was stated as "Archaea and Their Potential Role in Human Disease". They pointed about difficulties in the isolation and incubation of Archaea. Also, contribute to a relative lack of knowledge. It is puzzling that despite being one of the most numerous and ubiquitous life forms on earth, no member of the domain Archaea has been described as a human pathogen. These scientists tried to answer for this puzzle with its (archaea) cell structure difference. Archaea have ether-linked lipid and liposome (archaeosome) which play a role as potent immune adjuvants in vitro and in vivo. As an example, they inoculated an experimental vaccine to the mice. They vaccinated the mice with archaeasom which interrupted given listeriolysine. After several time, they noticed that this vaccination supposed prolonged specific immunity against *Listeria monocytogenes*. The explanation was like below: in immunology, archaeaosome activate APCs' by increasing expression of MHC class II and evoke strong antigen-specific responses to entrapped antigens when injected into the mice (Krishnan et al., 2000; Krishnan et al., 2001; Eckburg et al., 2003; Conlan et al., 2011).

Effects of antibiotics on archaea

According to some researches, utilizing of antibiotics may suppress the growth of some archaea. Upper levels of breath methane have been observed in patients with precancerous conditions and cancer of the colon than in healthy patients. A study by (Gijzen et al., 1991) suggests that antibiotics may effect to some archaea species. For example, "defaunation" of cockroaches with low concentrations of metronidazole results in a rapid drop in methane production, presumably due to methanogen eradication from the hindgut. It is unknown that these antibiotics directly kill methanogens in the gut, kill their ciliate protozoal hosts, or effect the local anaerobic bacterial population indirectly by altering the concentrations of coexistent methanogens. In a study from Spain a survey for antibiotic resistance within the genus *Halobacterium* showed that most extreme halophiles were resistant to lactams and aminoglycosides however were sensitive to many other antimicrobials, including macrolides, chloramphenicol, novobiocin, rifampin, bacitracin, and fluoroquinolones (Bonelo et al., 1984).

Unsolved difficulties

Currently, at least 16 Archaeal genome sequences have been studied. The completed genomes of Archaea, however, may still provide clues to the presence of possible virulence factors. For instance, a survey of genes that codify transcriptional regulators in four archaeal genomes revealed possible members of the bacterial Lys R and sensor transduction regulator families. In addition, some of the members of these families are known to be related with controlling of virulence factors in bacterial pathogens. Unfortunately, there is no clear virulence phenotype as well as obvious animal model systems in which to estimate virulence. Methanogens are the only archaea that have been identified in humans. The default to identify other non-methanogens in humans might be in large part due to the lack of any molecular and biotechnological methods to define the abundance or diversity of archaea in human and animal microbiota. Methanogen Archaea may also follow virulence policy in eukaryotic organisms similar to those of the known anaerobic bacteria (Cavicchioli et al., 2003; Aminov, 2013).

Each scientist overviewed above conclude their studies about Archaea almost the same. No any clear or exact connection between archaea and disease has been clarified to date in human and animals, in part thanks to limitations in our ability to detect, identify, and isolate Archaea species. If Archaea are take part in human disease, it is likely that such participation will be illuminated using new molecular methods, knowing difficulties of their cultivation. When identified Archaea species the first time as a separate domain of taxonomy, its subspecies were dated as

extremophiles and for these reasons unfamiliar to the human environment and microbiota. However, this discover was not a true reflection of archaeal physiology and we aware that their failure result emerge because of limited datasheets and narrow scientific opportunities. With the passing of days, molecular approaches have disclosed Archaea in decidedly non-extreme environments. It is hopeful that the appearance of application of similar and high techniques to the biology science may expand our perspective soon again. (Relman et al., 1990; Wilson et al., 1991; Relman et al., 1992).

Conclusion

No conclusive virulence genes or details have been described in Archaea to date. Nonetheless, Archaea may have the means, and they undouble have the chance, to cause disease. Whether or not members of the Archaea Domain possess virulence factors as commonly defined is questionable. Most scientist discussed human diseases in which archaea species may play a role as well as potential virulence characteristics of these organisms. Feasible elucidation for the current absence of information about Archaea as pathogens, and molecular methods that might be utilized in the search for such pathogens. Who knows it may discovered pathogenic Archaea after decade or nearly future accompanied by fresh molecular technologies and equipment in life sciences.

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JIVS

Successful treatment of cutaneous solid type adenocarcinoma with cryosurgery in a Pekingese dog

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ABSTRACT

The aim of this study is to defined the treatment of cutaneous solid type adenocarcinoma in a Pekingese dog using cryotherapy. A 3.5 years old intact female Pekingese dog was referred to the small animal surgery clinic. The first neoplasm localization was dorsal to the vulva and the others were bilateral to the vulva. The shape of the nodules were circular. The nodule diameters were 1.8, 1.5, and 1.2 cm. In cross section, the nodules were whitish-yellow in colour, of solid consistency, and characterized by thickening of the skin. The probe-based cryosurgical system was used for cryoablation, using local anesthetic as the interface for uniform freezing. Based on histopathological features, the dog was diagnosed with solid type perianal adenocarcinoma. In conclusion, cryosurgery can be a potent alternate treatment for pleasant, nodular perianal cutaneous adenocarcinoma in animals, particularly those not suited for operation, or whose owners refuse to have them undergo operation.

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Introduction

Cryosurgery has been used for different benign, malignant, and premalignant cancers of dermis lesions by different practice methods, such as superficial spray, cotton-tipped applicator, intralesional, and cryoprobe (Kuflik and Kuflik, 2012; Lee et. al., 2016). When tissue is refrigerated, it is scarred by ice creation within the cells, vascular stasis and thrombosis, and the delivery of toxins and electrolytes. Extermination of malignant mass requires a resent tissue temperature of –60°C (Kuflik, 2004; Ethan et. al., 2012).

Perianal glands in the dog are non-secretory, altered sebaceous glands indwelled around the anus and predisposed to tumor Moulton, 1990; Banks, 1993). The breeds most susceptible to perianal adenomas and carcinomas are the Siberian Husky, Cocker Spaniel, Pekingese and, for adenocarcinomas, the Bulldog and the Siberian Husky (Merck, 2006). The aim of this study is to defined the treatment of cutaneous solid type adenocarcinoma in a Pekingese dog through cryotherapy.

Case

3.5 years old, intact female Pekingese dog weighing 6 kg observed to have three different sized nodules on her perianal and vulva region for 2 months was referred to the small animal surgery clinic (Figure 1). Blood values were normal (Table 1). The first neoplasm localization was dorsal to the vulva and the others were bilateral to the vulva, ruby-coloured, of firm consistency, and of luminous mucosal appearance. The shape of the nodules were circular. The nodule

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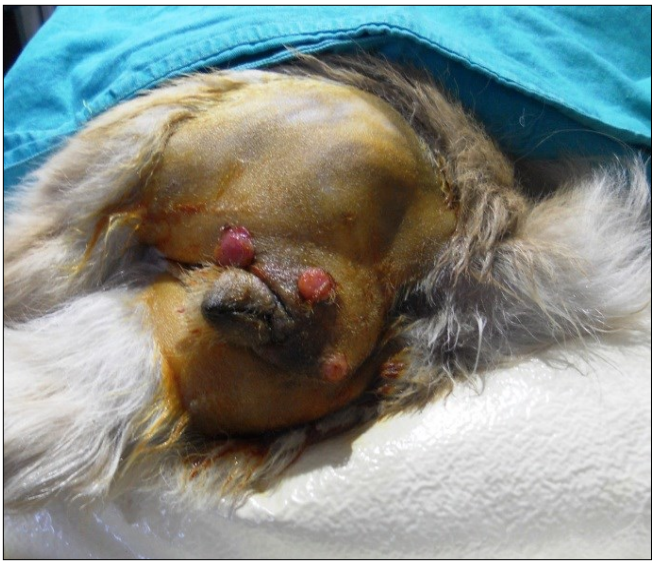

Figure 1. View of nodules in perianal region.

diameters were 1.8, 1.5, and 1.2 cm. In cross section, the nodules were whitish-yellow in colour, of solid consistency, and characterized by thickening of the skin. The dog had not previously undergone surgical removal of tissue.

Table 1. Dog's blood values

Parameters	Values
WBC (1 x10^3/µL)	11.3
Lymphocyte (1 x10^3/µL)	5.1
Granulocytes (1 x10^3/µL)	3.4
HGB (g/dL)	17.2
HCT (%)	54.3
RBC (1 x10^6/µL)	7.77
MCV (fL)	70.0
PLT (1 x10^3/µL)	613

The dog was premedicated with xylazine HCl (2 mg kg-1, IM, Alfazin®, Turkey). The probe-based cryosurgical system (Üzümcü, Istanbul, Turkey) was used for cryoablation using a local anesthetic (Industrial Ave, Molendinar, Australia) as the interface for uniform freezing. This system was comprised of a tube of liquid nitrogen (-195 °C) and a probe (Figure 2).

Figure 2. View of criosurgery equipment.

A probe with cytotherapeutic zones of 2 cm diameter was used in the study. The probe delivered liquid nitrogen, which did not come into contact with the ablated tissue. Being closed to liquid nitrogen flow allows for the thawing effect in the frozen cavity. Three cycles of freezing and thawing were induced, with the skin area being observed throughout (Figure 3).

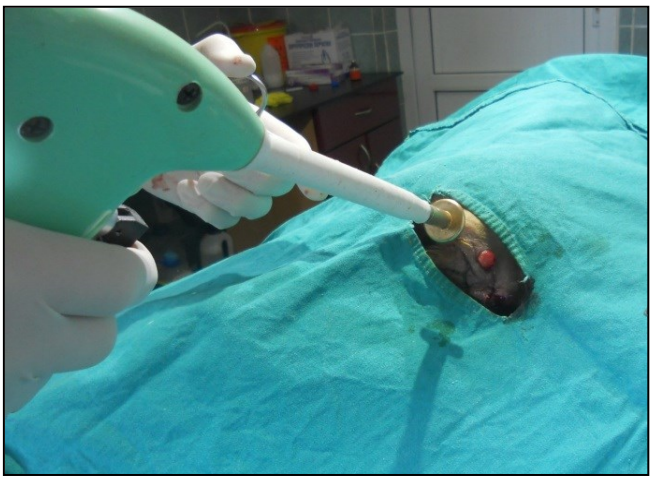

Figure 3. Application of cryoprobe to mass.

Freezing time was about 30 to 60 seconds. This was accomplished by ice creation over the whole tumor with at minimum 5-mm of independent margins (Figure 4). Technical achievement was noted as an elongation of the ice ball going further the tumor margin and post-ablation views indicating no contrast rise in the field of the original tumor 6 months following the primary procedure. Postoperatively, the patient was administered intravenous antibiotics for 72 hr. No visible tumor was defined by day 45, and no recurrence was observed till 2 years.

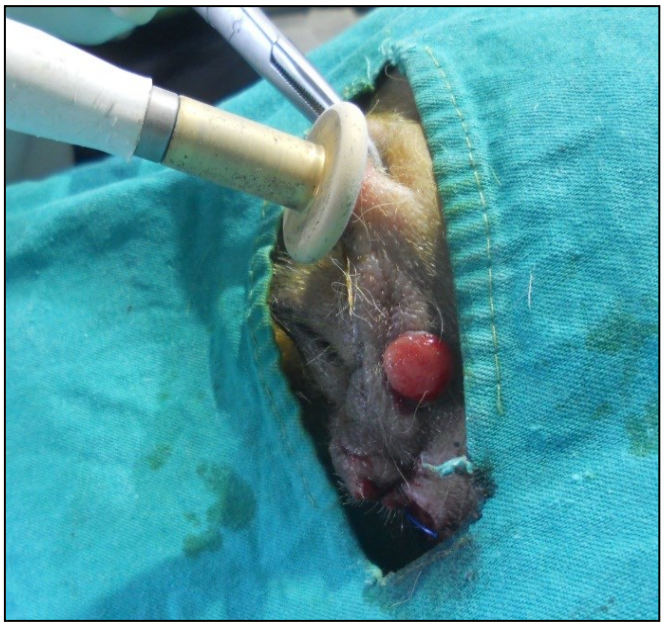

Figure 4. View of the ice formation of the whole tumor.

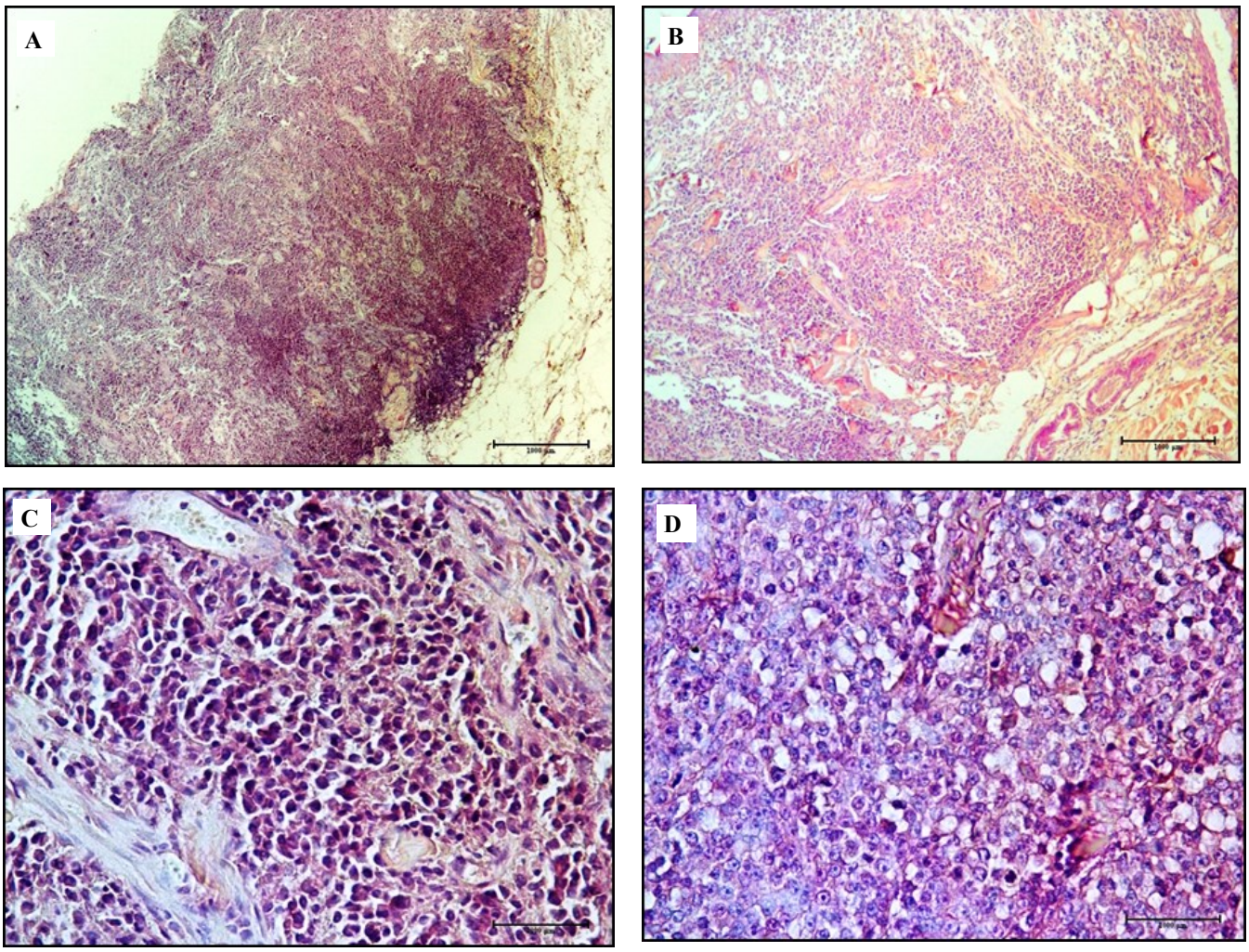

Figure 5. Histopathological sections of circumanal nodules in female Pekingese dog.

H&E. Barr=500 µm. (A) Epidermis, dermis and hypodermis structures destroyed. (B) Polymorphic, polyhedral cells organized in solid pattern and surrounded with massive connective tissue. (C, D) Tumour lobules observed well and they are separated with vast connective tissue. The lobes consist of the cells form solid sheets; have not abundant cytoplasm, quite pale, the nuclei are round with coarse chromatin and existence of one or more, but regular nucleoli. There is not a lot of anisocytosis, anisokaryosis and anisonucleoliosis and presence of frequent mitosis.

The obtained nodular tissues was fixated in 10% of neutral formalin. The tissue was processed and established in paraffin wax. Edges 4-5 µm thick were cut from the block and mounted with entellan Neu (Merck, Darmstat, Germany). For histopathological examination, edges were painted with routine hematoxylin and eosin.

This study describes the removal of 3 nodular adenocarcinoma in a patient who was successfully treated by cryoablation without complication. The histopathological examination showed that the layers of dermis and hypodermis were disturbed, and there was a bounded tumor nodule surrounded by massive fibrovascular stroma. Based on histopathological features, it was diagnosed as solid type perianal adenocarcinoma (Figure 5).

Discussion and Conclusion

The tumor varied from rosette, solid, to tubular types by histologically. The solid subtype consisted of sheets of neoplastic cells subdivided into thin bands of fibrous tissue, but lacked glandular structures (Schulman, 1998). According the histopathological results, this case demonstrated characteristics of solid type adenocarcinoma with vast connective tissue, grid -type reticulation, and frequent mitosis. Tumor cells originated from the glandulocytes of the derma.

This type of tumor is most often seen in dogs between ages 5 and 14 (median 9 years) (Schulman, 1998; Turek et. al., 2003). However, in this case, the dog was 3.5 years old; according the literature, affected dogs tend to be older. The breeds most susceptible to perianal adenomas are the Siberian Husky, Cocker Spaniel, Pekingese and, for adenocarcinomas, the Bulldog and Siberian Husky (Merck, 2006).

There are several great-scale studies display elevated therapy rates of cryosurgery for nonmelanocytic cancers of dermiş (Lee et. al., 2016).

In the report by Kuflik (2004), most cases were cured by curettage before use of the open spray technique, with a treatment rate of 99% in 522 cases. The 5-year therapy rate in squamous cell carcinoma lower than 2 cm (n = 134) was 100% (Lee et. al., 2016).

Our research is restricted to one case. However, cryoablation seems simple, suitable, and safe for advanced nodular tumors. All tumor masses ablated after 2 cryoprobe treatments in the patient. Cryosurgery may ensure long-term remission as integrate clinical alleviation was defined in the case till 2 years of follow-up. Also, the esthetic outcome was too extremely satisfactory. However, it should be determined that cryosurgery may not be acceptable for patients with tumors found in locations and conditions where cryosurgery is not useful, such as the eyelash region or medial canthus, to avoid damage of ocular tissue or canaliculus, acral part in patients with poor circulation or poor healing, or in locations where the nerves are quite outcrops, as this provides the potential risk of nerve wound. Moreover, cryosurgery is excellent to radiotherapy for its plainness, and as it is free of complications such as lymphedema, secondary cancer, and radiodermatitis (Samstein et. al, 2014).

In conclusion, cryosurgery can be an influential alternative cure for pleasant, nodular perianal cutaneous adenocarcinoma in animals, particularly those not suited for operation, or whose owners refuse to have them undergo operation Larger, controlled, and multi-disciplinary studies are required to approve its efficacy and safety.

Conflict of interest

The authors declare that there was not any conflict of interest.

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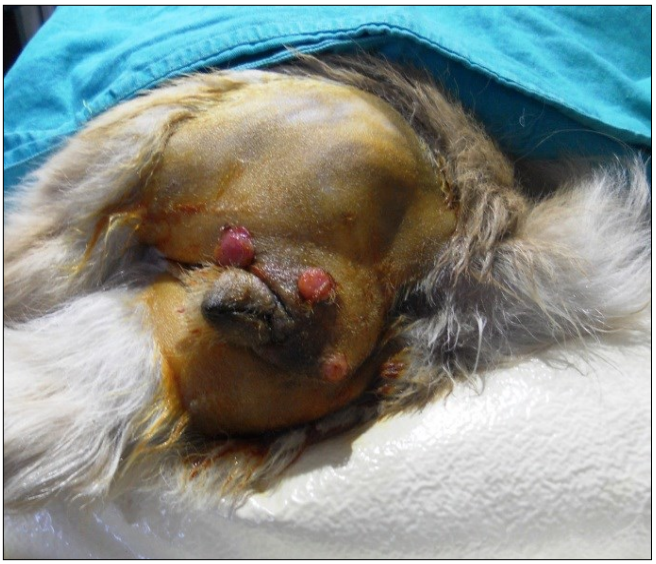

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Figure 2. View of criosurgery equipment.

A probe with cytotherapeutic zones of 2 cm diameter was used in the study. The probe delivered liquid nitrogen, which did not come into contact with the ablated tissue. Being closed to liquid nitrogen flow allows for the thawing effect in the frozen cavity. Three cycles of freezing and thawing were induced, with the skin area being observed throughout (Figure 3).

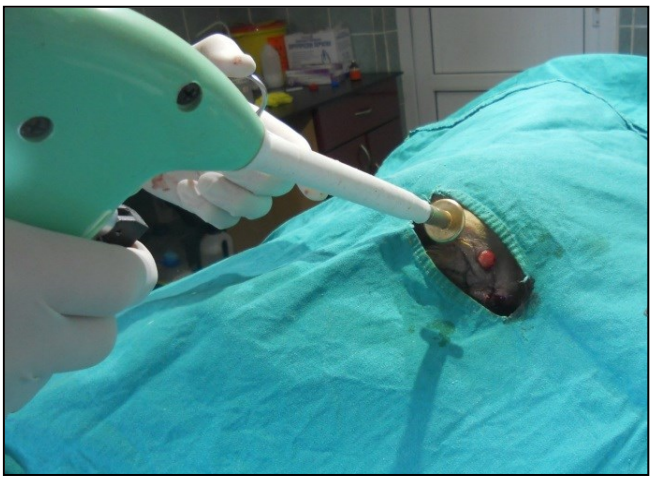

Figure 3. Application of cryoprobe to mass.

Freezing time was about 30 to 60 seconds. This was accomplished by ice creation over the whole tumor with at minimum 5-mm of independent margins (Figure 4). Technical achievement was noted as an elongation of the ice ball going further the tumor margin and post-ablation views indicating no contrast rise in the field of the original tumor 6 months following the primary procedure. Postoperatively, the patient was administered intravenous antibiotics for 72 hr. No visible tumor was defined by day 45, and no recurrence was observed till 2 years.

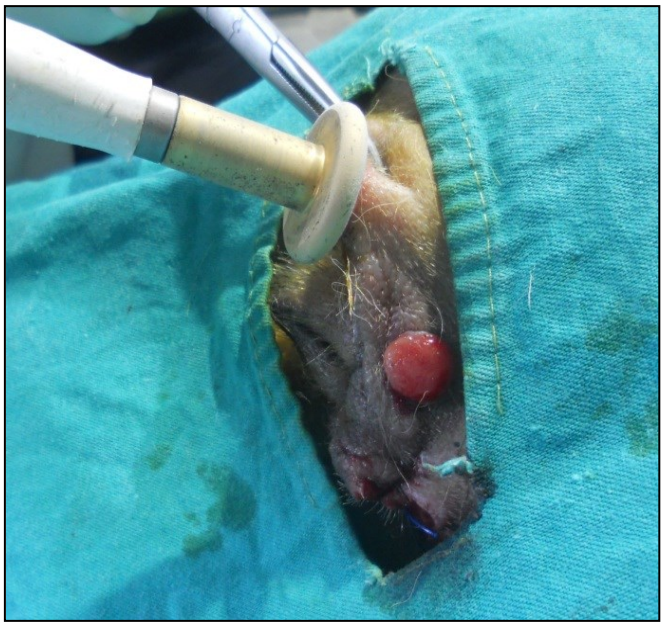

Figure 4. View of the ice formation of the whole tumor.

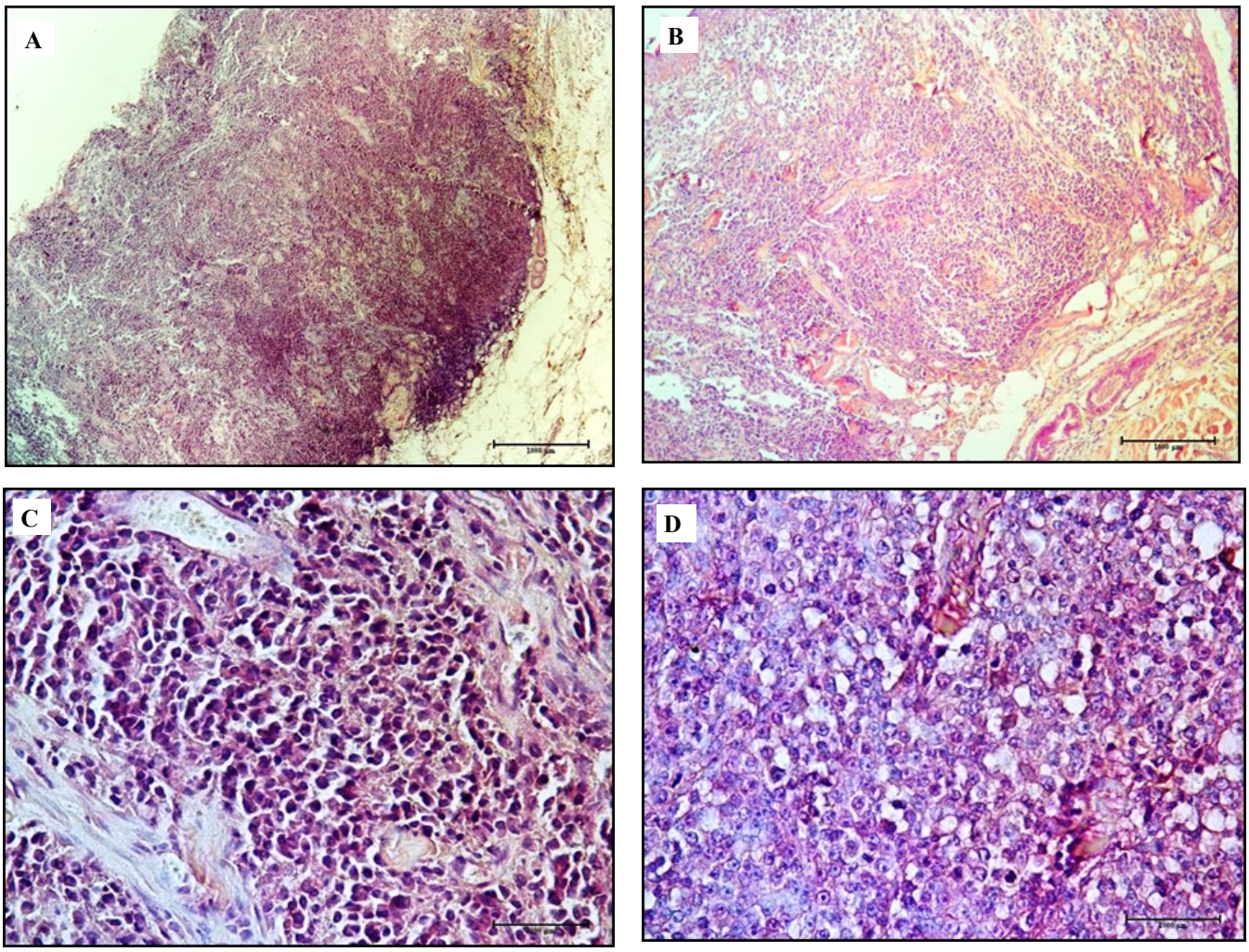

Figure 5. Histopathological sections of circumanal nodules in female Pekingese dog.

H&E. Barr=500 µm. (A) Epidermis, dermis and hypodermis structures destroyed. (B) Polymorphic, polyhedral cells organized in solid pattern and surrounded with massive connective tissue. (C, D) Tumour lobules observed well and they are separated with vast connective tissue. The lobes consist of the cells form solid sheets; have not abundant cytoplasm, quite pale, the nuclei are round with coarse chromatin and existence of one or more, but regular nucleoli. There is not a lot of anisocytosis, anisokaryosis and anisonucleoliosis and presence of frequent mitosis.

The obtained nodular tissues was fixated in 10% of neutral formalin. The tissue was processed and established in paraffin wax. Edges 4-5 µm thick were cut from the block and mounted with entellan Neu (Merck, Darmstat, Germany). For histopathological examination, edges were painted with routine hematoxylin and eosin.

This study describes the removal of 3 nodular adenocarcinoma in a patient who was successfully treated by cryoablation without complication. The histopathological examination showed that the layers of dermis and hypodermis were disturbed, and there was a bounded tumor nodule surrounded by massive fibrovascular stroma. Based on histopathological features, it was diagnosed as solid type perianal adenocarcinoma (Figure 5).

Discussion and Conclusion

The tumor varied from rosette, solid, to tubular types by histologically. The solid subtype consisted of sheets of neoplastic cells subdivided into thin bands of fibrous tissue, but lacked glandular structures (Schulman, 1998). According the histopathological results, this case demonstrated characteristics of solid type adenocarcinoma with vast connective tissue, grid -type reticulation, and frequent mitosis. Tumor cells originated from the glandulocytes of the derma.

This type of tumor is most often seen in dogs between ages 5 and 14 (median 9 years) (Schulman, 1998; Turek et. al., 2003). However, in this case, the dog was 3.5 years old; according the literature, affected dogs tend to be older. The breeds most susceptible to perianal adenomas are the Siberian Husky, Cocker Spaniel, Pekingese and, for adenocarcinomas, the Bulldog and Siberian Husky (Merck, 2006).

There are several great-scale studies display elevated therapy rates of cryosurgery for nonmelanocytic cancers of dermiş (Lee et. al., 2016).

In the report by Kuflik (2004), most cases were cured by curettage before use of the open spray technique, with a treatment rate of 99% in 522 cases. The 5-year therapy rate in squamous cell carcinoma lower than 2 cm (n = 134) was 100% (Lee et. al., 2016).

Our research is restricted to one case. However, cryoablation seems simple, suitable, and safe for advanced nodular tumors. All tumor masses ablated after 2 cryoprobe treatments in the patient. Cryosurgery may ensure long-term remission as integrate clinical alleviation was defined in the case till 2 years of follow-up. Also, the esthetic outcome was too extremely satisfactory. However, it should be determined that cryosurgery may not be acceptable for patients with tumors found in locations and conditions where cryosurgery is not useful, such as the eyelash region or medial canthus, to avoid damage of ocular tissue or canaliculus, acral part in patients with poor circulation or poor healing, or in locations where the nerves are quite outcrops, as this provides the potential risk of nerve wound. Moreover, cryosurgery is excellent to radiotherapy for its plainness, and as it is free of complications such as lymphedema, secondary cancer, and radiodermatitis (Samstein et. al, 2014).

In conclusion, cryosurgery can be an influential alternative cure for pleasant, nodular perianal cutaneous adenocarcinoma in animals, particularly those not suited for operation, or whose owners refuse to have them undergo operation Larger, controlled, and multi-disciplinary studies are required to approve its efficacy and safety.

Conflict of interest

The authors declare that there was not any conflict of interest.

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