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Seasonal changes in hemograms and *Theileria orientalis* infection rates among Holstein cattle pastured in the mountains in the Republic of Korea

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ABSTRACT

In the current study, we compared seasonal changes in complete blood counts (CBCs) and rates of infection with a tick-borne pathogen between Holstein cattle housed indoors and those maintained outside on pasture. There were differences in white blood cell (WBC) parameters, but the changes were not associated with seasons or the housing type. Analysis of red blood cell (RBC) parameters showed lower values in August and November versus March, and in the cattle maintained on pasture versus the housed cattle. In comparison with the RBC count of the housed cattle in March (10.1 M/μL), the RBC counts of the pastured cattle were significantly lower in August (7.8 M/μL; $p < 0.01$) and November (7.5 M/μL; $p < 0.01$). The hematocrit (HCT) also showed a decrease in March (33.5%), August (30.0%, $p < 0.01$) and November (28.5%, $p < 0.01$). According to PCR analysis, the *Theileria* infection rate among the pastured cattle in March was only 11%, but this rate increased to 22% and 60% in August and November, respectively. The RBC count (7.4 M/μL) and HCT (27.7%) values in *Theileria*-positive pastured cattle in November showed a dramatic decrease compared to those of cattle examined in March. Phylogenetic analysis revealed that these *Theileria* isolates correspond to *T. orientalis*. These results suggest that a remarkable increase in tick infestation in mountainous areas in the summer may cause increased rates of infection with *T. orientalis,* leading to significant changes in the RBC profile after grazing. Therefore, these hematological changes may be associated with *T. orientalis* infection caused by tick-biting; thus, additional studies on the pathogenicity of *T. orientalis* are needed.

1. Introduction

In the Republic of Korea (ROK), more than 3 million heads of cattle are raised in 0.13 million farmhouses (based on December 2013 statistics) (Korean statistical information system, 2013), and more than 95% of these animals are raised using conventional management in indoor housing; less than 3% of the cattle graze on pasture in mountainous areas. Conventional management provides crowded living conditions, but is less influenced by the seasons and the environment and is effective at control of a large number of cattle in limited spaces. Nevertheless, this type of non-grazing management is currently causing a variety of problems: livestock production and feed costs have increased, and there is environmental pollution around the housing areas (Hulbert and Moisá, 2016; Pieper et al., 2015), and rapid spread of acute infections such as foot-and-mouth disease.

Pasture-type management on grassy mountains has been shown to have advantages such as decreasing feed costs and increasing cattle activity. Grazing of cattle has advantages such as reduced production costs and improved animal welfare as compared to crowded housing (Barkema et al., 2015). Nevertheless, there are

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difficulties with management of nutrition and diseases. Regardless of temperature changes with seasons, there is a greater risk of tick-borne diseases transmitted by ectoparasites even in mountainous areas (Chae et al., 2008; Kamio et al., 1990; Onuma et al., 1998). To date, there have not been many studies on diseases associated with pasturing in mountainous areas in the ROK. No prior studies have evaluated hematological changes or rates of infection with tick-borne pathogens in dairy cattle in the same area or depending on breeds anywhere in the world (Hofmann-Lehmann et al., 2004; Savini et al., 1999; Wang et al., 1998).

Theileriosis is one of the most important tick-transmitted diseases of cattle worldwide. The clinical signs include fever, chronic anemia, anorexia, weight loss, reduced milk production, and icterus. *Theileria* parasites are classified into two groups: transforming (*Theileria parva, T. annulata* and *T. taurotragi*) and non-transforming (*Theileria orientalis*, *T. mutans* and *T. velifera*) by their ability to transform leukocytes in the infected hosts (Sivakumar et al., 2014). *Theileria parva* and *Theileria annulata* cause highly virulent lymphoproliferative diseases in cattle with high mortality and morbidity, commonly known as East Coast Fever and Tropical theileriosis, respectively (Aktas et al., 2006). The group *"Theileria buffeli, Theileria sergenti*, *Theileria orientalis"* is very similar, and the separate taxonomy of this group is controversial. On the basis of molecular studies, the three parasites have been classified as one species, *T. orientalis* (Sivakumar et al., 2014). Benign theileriosis is widespread in Southeast Asia and is caused by *T. orientalis* (Onuma et al., 1998; Kamau et al., 2011; Jirapattharasate et al., 2016). Generally, *T. orientalis* has been known as a low pathogenic parasite, but infected cattle can occasionally present with hemolytic anemia, jaundice, anorexia, and an abortion (Aparna et al., 2011; McFadden et al., 2011). This disease can also cause severe economic losses in the livestock industry. In addition, this parasite causes bovine theileriosis in the ROK (Ko et al., 2008; Jeong et al., 2010).

In the present study, we analyzed and compared seasonal changes in complete blood counts (CBCs) and in rates of infection with tick-borne pathogens in cattle managed on pasture and in indoor housing. These results may provide new information about health conditions and the rate of infection with tick-borne disease among the cattle pastured in mountainous areas.

1.1. Materials & methods

1.1.1. Sample collection

The study was conducted on a Holstein cattle farm located 600 m above the sea level on Jiri Mountain in Jeolla Province, ROK. The farm uses both indoor housing and land grazing methods. Cattle 7 – 13 months of age were placed on the grassy mountains from spring to fall (April to November) and were maintained indoors in the winter. The test group (n = 18 in the summer; n = 15 in the fall) were randomly selected from the cattle that were allowed to graze on the grass from spring to fall; the comparison group (n = 10 in the summer; n = 15 in the fall) was randomly selected from the cattle raised only in indoor housing without pasturing. Ticks were collected from the grazing cattle. On the basis of microscopic examination, each tick species was identified and classified morphologically according to the developmental stage (Yamaguti et al., 1971).

1.2. Ethical statement

The managers of the surveryed farms were informed of the study and gave their approval for sampling of the cattle. All procedures were carried out according to ethics guidelines for the use of animal samples as permitted by Chonbuk National University (institutional animal care and use committee [IACUC] decision No. CBU 2014-00026).

1.3. Hematological analysis

We visited the farm three times; once in the spring before pasturing (March), the second time in the summer during pasturing (August), and finally, in the fall after pasturing (November). We collected 5 mL of blood from the jugular veins of the cattle into EDTA-supplemented tubes, which were delivered to the lab immediately after the blood collection. Hematological examinations included a red blood cell (RBC) profile consisting of an RBC count, hemoglobin (Hb), hematocrit (HCT), mean cell volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), and a white blood cell (WBC) profile comprising a total WBC count and differential counts (neutrophils, lymphocytes, monocytes, eosinophils, and basophils). The samples were processed on an automatic blood analyzer (Hemavet 960, Erba Diagnostics Inc., Miami, FL, USA) in the laboratory on the day of blood collection. After the analysis, the blood samples were immediately frozen at −80°C until DNA extraction.

1.4. Polymerase chain reaction (PCR)

Genomic DNA for PCR was extracted from 200 μL of whole blood using the DNeasy Blood & Tissue Kit (Qiagen Inc., Valencia, CA, USA). The AccuPower *Theileria* PCR Kit (Bioneer, Daejeon, Korea) was used to detect infection with *Theileria* spp. (forward primer [F], 5′-GTTATAAATCGCAAGGAAGTTTAAGGC-3′; reverse primer [R], 5′-GTGTACAAAGGGCAGGGACGTA-3′); this method amplifies a portion of the gene of 18S ribosomal RNA, which is a gene frequently used for phylogenetic analysis and a key marker for biodiversity screening by PCR. The predicted size of the amplicon was 239 bp, under the following cycling conditions: 94°C for 5 min, followed by 40 cycles of 94°C for 20 s and 65°C for 35 s, and then final extension of 72°C for 5 min. The AccuPower *Rickettsiales* 3-Plex PCR Kit (Bioneer) was used to detect *Anaplasma* spp. (F,5′-TACCTCTGTGTTGTAGCTAACGC-3′; R, 5′-CTTGCGACATTGCAACCTATTGT-3′), *Ehrlichia* spp. (F, 5′-CGGAATTCCTAGTGTAGAGG-3′; R, 5′-AGGAGGGATACGACCTTCAT-3′), and *Rickettsia* spp. (F, 5′-TAGGGGATGATGGAATTCCTA-3′; R, 5′-CCCCCGTCAATTCCTTTGAG-3′). These PCRs were performed with specific primer sets that targeted the gene of 16S ribosomal RNA, which is typically used for classification and identification of such pathogens. The anticipated sizes of the PCR products for *Anaplasma, Ehrlichia,* and *Rickettsia* were 429, 340, and 252 bp, respectively, under the following cycling conditions: 95°C for 15 min; followed by 40 cycles of 95°C for 10 s, 58°C for 30 s, and 72°C for 30 s; and then final extension of 72°C for 5 min. The amplicons were separated by electrophoresis in 1.5% agarose gels and visualized by staining with ethidium bromide.

1.5. Nucleotide sequencing and phylogenetic analysis

For further analysis, the amplified DNA was purified using the QIAquick PCR Purification Kit (Qiagen) and processed by means of the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster, CA, USA). The DNA sequencing data were analyzed in the ABI Prism software (Version 2.1; Thermo Fisher Scientific, Waltham, MA, USA) and in the Chromas software (Version 1.51; Technelysium Pty Ltd., South Brisbane, Queensland, Australia). A phylogenetic tree based on nucleotide alignments was constructed using the neighbor-joining method (Saitou and Nei, 1987). Bootstrap analysis was performed using 1000 replications, and the tree was visualized in the Treeview software (Page, 1996).