한국가축위생학회지 제42권 제4호 (2019)
Korean J Vet Serv, 2019, 42(4), 265-274
ISSN 1225-6552, eISSN 2287-7630
https://doi.org/10.7853/kjvs.2019.42.4.265

Korean Journal of
Veterinary Service
Available online at http://kjves.org

< Original Article >

Assessment of bovine blood sample stability for complete blood count and blood gases and electrolytes analysis during storage

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(Received 11 December 2019; revised 16 December 2019; accepted 17 December 2019)

Abstract

Delayed arrival of blood samples from the field and a large number of samples delivered often causes delay in sample analysis leading to inaccurate measurements. Therefore, this study aimed to assess whether prolonged storage in refrigerator could influence the stability of cattle blood samples and to establish an optimal time limit for complete blood count (CBC) parameters and blood gas and electrolyte (BGE) parameters analyses. Samples collected from healthy cows were tested immediately for CBC and BGE using automated hematology, blood gas and electrolyte analyzers. Samples were kept in refrigerator at 4°C and analyzed after 6 h, 12 h, 24 h, 48 h, 72 h, 120 h, and 192 h of storage. Mean differences between observations were assessed at 5% significance level using ANOVA and Duncan's multiple range test. Total CBC parameters and the platelet profile remained stable for 192 h, except for MCHC. Among leukocyte-related counts, NEU and EOS remained stable for 192 hours. WBC and LYM, and MONO values produced inconsistent measurements which recovered its initial measurement after 12 h and 24 h of storage, respectively, then remained stable until 120 h. Among the blood gas indices, PCO_2, PO_2, tCO_2, and BE showed declining and significant changes over time, but pH, tHb, and SO_2 remained stable for 192 h. Electrolyte status in the blood showed that ions are unstable and tend to change in as early as 6 h of storage. This study established that cattle blood specimens for CBC analysis can be stored for 120 h at 4°C, but specimens for BGE analyses must be tested within 6 to 24 h.

Key words : Blood stability, Blood cell count, Blood gas analysis

INTRODUCTION

Animal health monitoring is a highly crucial routine used to determine the current status of health and welfare of animals and to reduce disease incidence. Testing of physiological parameters is an essential implementation in monitoring the health status of animals. The most accepted test in assessing the physiological state of animals is blood testing. There are many inhibiting and stimulating factors that affect the circulatory system of animals, and those factors may influence blood components (Radkowska and Herbut, 2014). Maintenance of the body's physiological balance is one of the main functions of blood, and hematological indicators such as complete blood count (CBC), and biochemistry indicators such as electrolyte and blood gas levels are some of the main determinants of the animals (Anderson et al, 1999; Sattar and Mirza, 2009; Radkowska and Herbut, 2014). Evaluation of these hematological indicators is fundamental in the routine assessment of healthy and diseased animals in veterinary clinics and field situations.

An essential tool in evaluating the overall health of and disease presence in an animal is the CBC (Margallo and Jia, 2018). The CBC is particularly advantageous because it can suggest certain disease manifestations when physical examinations are too vague for diagnosis,

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and it is very useful when establishing a prognosis (Margallo and Jia, 2018). Evaluations of blood gas levels and the acid–base balance are also important in the diagnosis and treatment of diseases that affect animals (Jones and Allison, 2007). Several metabolic and respiratory diseases affect the venous blood gas composition and acid–base values in animals (Hussein and Aamer, 2013). Moreover, evaluation of blood electrolytes is an important part of assessing the blood chemistry profile in cattle as changes in blood electrolytes concentrations can occur in different diseases (Constable et al, 2013). As blood is the main carrier of toxins and other fermentation by-products in the body, identification and monitoring of blood gases and electrolytes is a valuable and essential means to efficiently and effectively detect the early onset of a disease (Médaille et al, 2006; Constable et al, 2013). However, inaccurate results for such parameters could lead to misdiagnoses. It has been estimated that up to 70% of laboratory sample errors may occur before the sample is subjected for analysis (Zaninotto et al, 2012; Daves et al, 2015; Tendulkar et al, 2015; Buoro et al, 2016). Factors ranging from the materials and chemicals used during collection and storage, the handling methods used during processing the blood (Tendulkar et al, 2015; Nnamdi et al, 2019), as well as the length of storage and temperature level, can directly influence the stability of blood parameters before sample analysis (Hedberg and Lehto, 2009; Buoro et al, 2016). Management of blood samples, as well as the method of storage, can considerably affect the results of hematological determinations (Tendulkar et al, 2015).

There are only limited investigations on the stability of blood parameters reported to date (Tendulkar et al, 2015) and only a few of these studies were based on veterinary blood samples. According to the International Council for Standardization in Hematology (ICSH), in order to obtain accurate hematological parameter results, blood specimens stored at 4℃ must be tested within 24 h or up to a maximum 72 hours (Briggs et al, 2008; Briggs et al, 2014). But the testing situation in veterinary practice can vary, especially in field sampling situations in which, oftentimes, a delay of sample arrival at a central diagnostic laboratory is expected and may be more than the ICSH recommended period. Also, the arrival of a large number of field samples could defer the analysis due to the associated prolonged waiting time. Therefore, this study aimed to assess whether prolonged storage, up to 192 hours, at 4°C could influence the stability of bovine blood samples and to establish optimal time limits for measurement of CBC parameters, and determination of blood gas and electrolyte (BGE) status indices that can serve as guidelines for veterinary practitioners and farmers, as well as laboratory technicians, and result in improved animal health monitoring and blood sample management practices.

MATERIALS AND METHODS

Study design

Blood samples were obtained from six healthy female Holstein-Friesian dairy cows (*Bos taurus* L.), ranging from three to five years of age. Blood samples were collected via venipuncture in the jugular vein during a routine animal health monitoring check-up at the Sunchon National University experimental farm. K2 Ethylenediaminetetraacetic acid (EDTA) (Vacutainer®, BD and Company) and Lithium Heparin tubes (Vacutainer®, BD and Company) were used for CBC and BGE analyses, respectively. After blood collection, sample tubes were immediately transferred to an insulated cooler box with ice and transported to the laboratory. Analysis of CBC and BGE parameters were performed on each sample immediately after arrival at the laboratory, with the interval not exceeding one hour. These measurements were marked as the initial observations (0H). Afterwards, the samples were stored in a refrigerator at 4°C and repeated measures were obtained from each sample after 6 h, 12 h, 24 h, 48 h, 72 h, 120 h, and 192 h. Refrigerator temperature was monitored every 12 h daily to ensure there was no temperature fluctuation during the study period.

Analysis of complete blood count

Analysis of the CBC parameters was performed during the designated observation time using an IDEXX

Procyte DxTM hematology analyzer (IDEXX Laboratories, Inc., USA). The CBC parameters measured were as follows: total red blood cell count parameters – erythrocyte count (RBC), hemoglobin count (HGB), hematocrit value (HCT), as well as basic CBC parameters – mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and red cell distribution width (RDW); leukocyte count (WBC) and its extended differential counts includes neutrophil (NEU), eosinophil (EOS), basophil (BASO), lymphocyte (LYM) and monocyte (MONO) counts; platelet profile parameters including platelet count (PLT), plateletcrit value (PCT), mean platelet volume (MPV), and platelet distribution width (PDW).

Blood gas and electrolyte analysis

The blood gas and electrolyte analyses were done using IDEXX VetStat^{TM} blood gas and electrolyte analyzer (IDEXX Laboratories, Inc., USA) during the designated observation periods. Indices for the determination of acid-base status in blood are as follows: pH, partial pressure of carbon dioxide (PCO_2), base excess (BE), and total carbon dioxide (tCO_2), partial pressure of oxygen (PO_2), total hemoglobin (tHb), and oxygen saturation (SO_2); and parameters for electrolyte status were sodium (Na^+), potassium (K^+), bicarbonate (HCO_3^-), and chloride (Cl^-) ions, as well as anion gap (AnGap).

Statistical analysis

Variation in the means of variables was computed using ANOVA at the 5% level of significance. Post-hoc testing was used to determine the mean differences in variables between observation times by applying Duncan's multiple range test using SAS 9.4 software (SAS Institute Inc., USA). Data are presented as line graphs denoted by including the plotted values along with the estimated regression line for statistically significant variables to elucidate the trend of the parameter changes over time.

RESULTS

RBC count and basic CBC parameters

The RBC count and the basic parameters for the CBC exhibited stability until the 192 h observation samples, except for the MCHC which, based on the computed mean differences, showed a statistically significant decreasing trend after the 72 h observation as shown in Fig. 1 (and Supplemental Table 1).

WBC and extended differentials parameters

The WBC results and the extended leucocyte differential counts are presented in Fig. 2 (and Supplemental

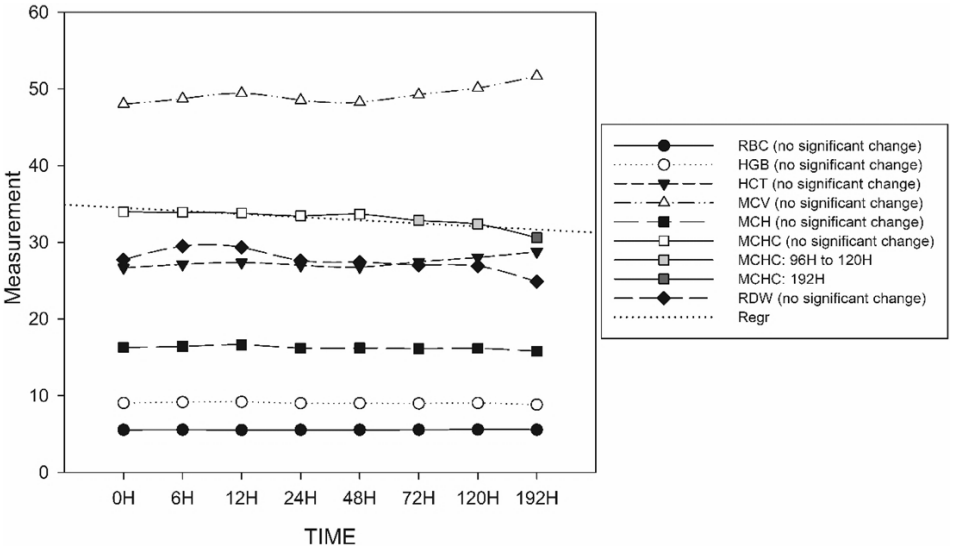

Fig. 1. Graphical presentation of the mean values of total RBC and basic CBC parameters from 0 h through the subsequent 192 h. A regression line is shown for MCHC as it was the only basic CBC parameter with significant changes at certain times, which are indicated by a change of shade on the plotted values.

Table 1). Both NEU and EOS counts remained stable for up to 192 h of storage, whereas the BASO count remained stable for 48 h but started to significantly increase at the 72 h observation. Concurrently, the WBC, LYM, and MONO values showed statistically significant inconsistent fluctuations throughout the observation period. The inconsistencies observed in the WBC, LYM, and MONO values resulted in a significant decrease in their measurements in the 6 h analysis, but they returned to the initial measurement in the 24 h observation, then remained stable until the 120 h observation.

Platelet profile

The platelet profile parameters showed stability up to 192 h (Supplemental Table 1). The apparent trends observed in the PLT, MPV, PDW, and PCT values over time were insignificant throughout the observation periods (Fig. 3).

Blood gases and electrolytes parameters

The blood gas indices measured from 0 h to 192 h are presented in Fig. 4A and 4B. Among the blood gas indices, the pH, tHb and SO_2 levels remained stable

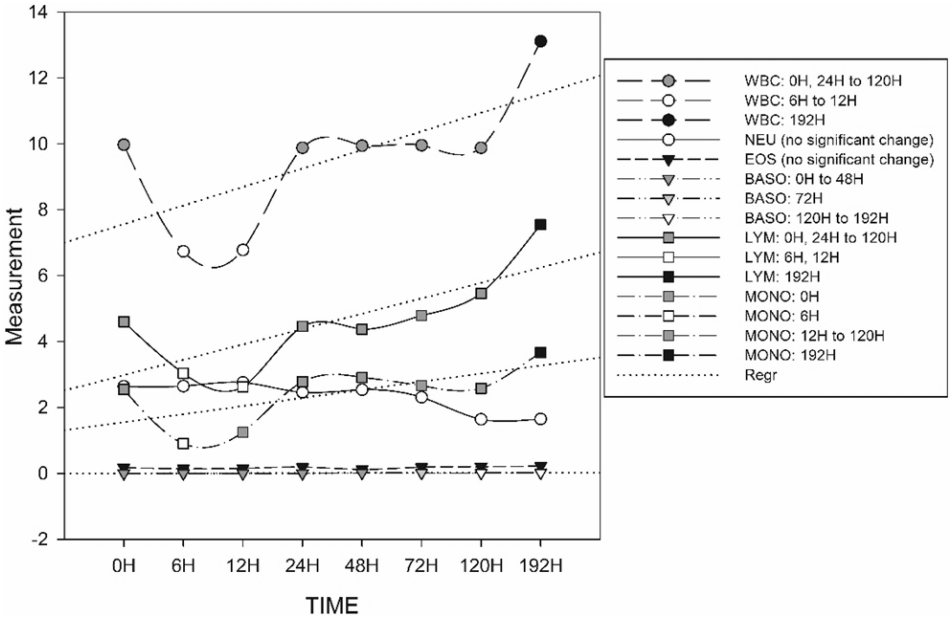

Fig. 2. Graphical presentation of mean WBC and its extended differential parameter values from 0H to 192 h with regression lines shown for parameters with significant changes at certain times. Significant changes indicated by a change of shade of the plotted value.

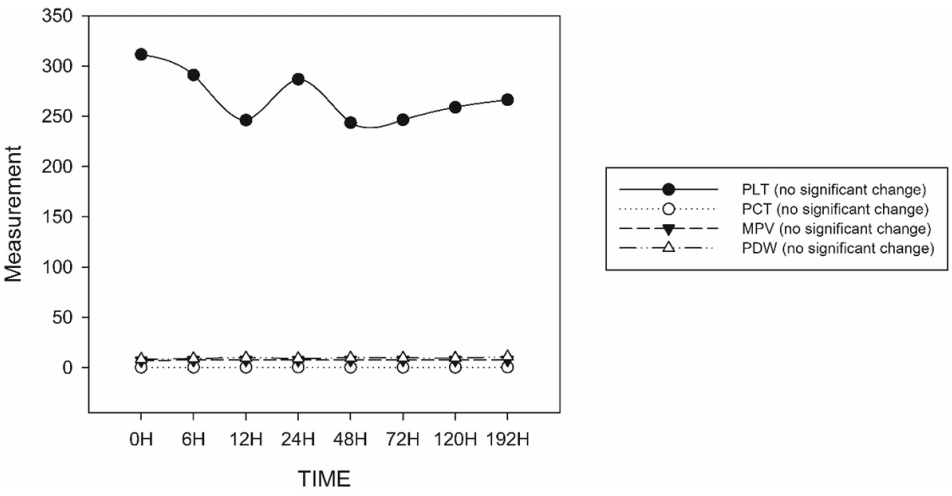

Fig. 3. Graphical presentation of mean platelet profile values showing the stability of measured parameters and the absence of significant changes from 0H to 192 h.

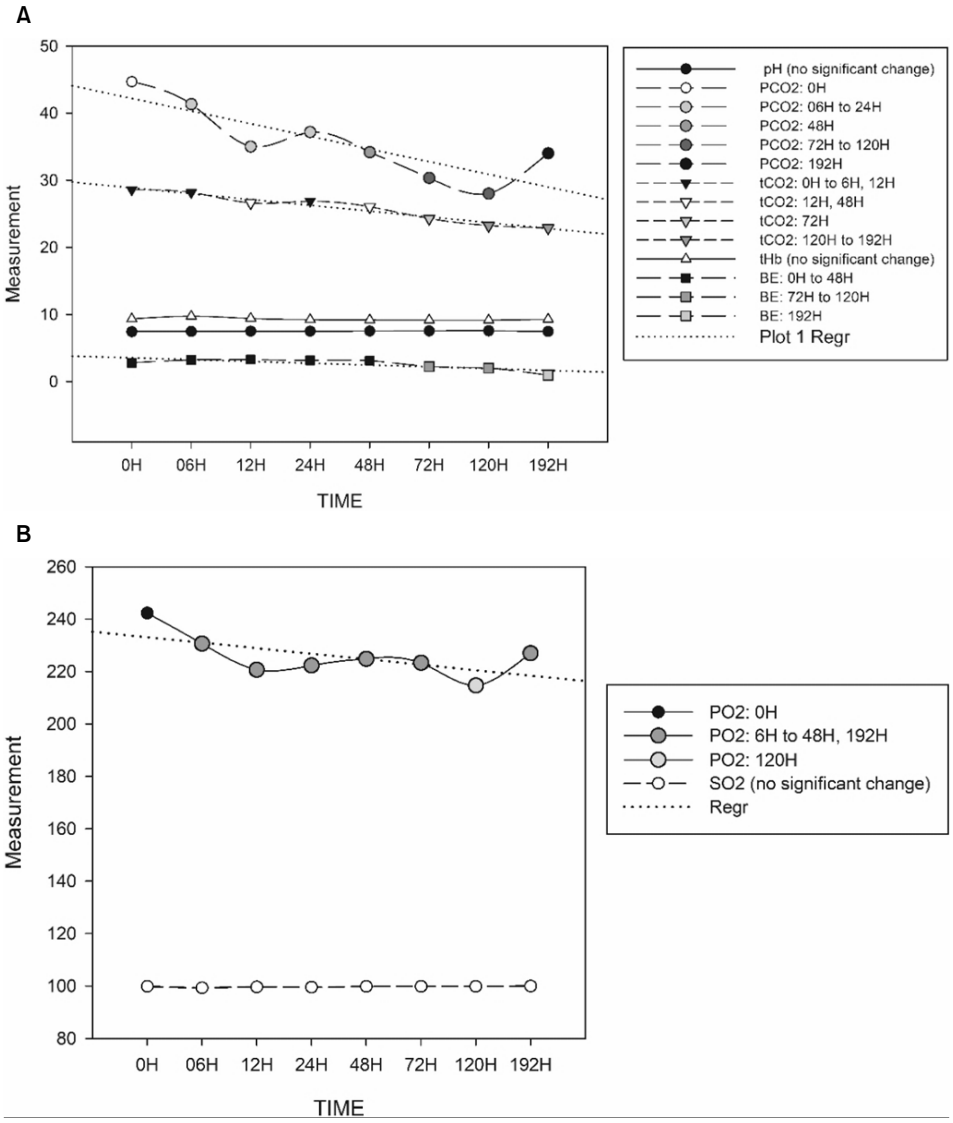

Fig. 4. Graphical presentation of mean values of (A) pH, total hemoglobin (tHb), base excess, partial pressure of carbon dioxide (PCO_2), and total carbon dioxide (PO_2), and (B) partial pressure of oxygen (PO_2) and saturation of oxygen (SO_2). Regression lines are included for parameters with significant changes at certain times. Significant changes indicated by a change of shade of the plotted value.

throughout the observation period. The tCO_2 and PCO_2 indices significantly declined in the 12 h and 6 h observations, respectively. The PO_2 also significantly declined in the 6 h observation. On the other hand, BE remained stable before decreasing significantly in the 72 h observation. Although the results of the 6 h observations showed that the levels of PO_2 and PCO_2 rapidly and significantly decreased, the pH level remained stable for 192 h.

All parameters related to electrolyte status showed significant differences over time (Fig. 5). Intracellular anion HCO_3- and extracellular cation Na^+ displayed inconsistent decreasing trends throughout the observation period. On the other hand, the extracellular anion Cl- showed a significant inconsistent increase, while the intracellular cation K^+ displayed significant increases in each period starting from the 24 h observation.

DISCUSSION

Complete blood count analysis

Red blood cells or erythrocytes are mainly responsible for carrying oxygen from the lungs to the cells of tissues and carry carbon dioxide from those cells back to the lungs. CBC measures usually assessed are total RBC, HCT, HGB, and RDW values. In addition, erythrocyte

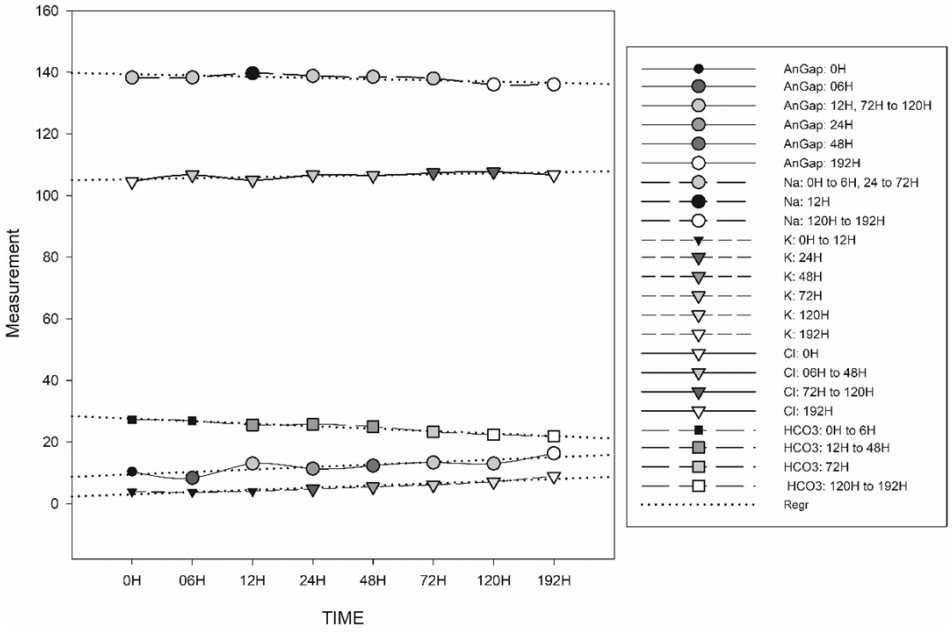

Fig. 5. Graphical presentation of measured electrolyte indices: An-Gap, HCO_3-, Na^+, Cl-, and K+. All parameters display significant changes at specific times, which are indicated by a change of shade of the plotted value.

indices including MCV, MCH, and MCHC are typically assessed as CBC basic parameters (Roland et al, 2014). In this study, with the exception of MCHC which only remained stable up to 72 h, the RBC and other basic CBC parameters were stable for up to 196 h. Another study reported that human blood parameters, RBC, HGB, HCT, MCV, MCH, and MCHC remained stable for 48 h at 4°C (Gunawardena et al, 2017). Insignificant changes in CBC parameters were observed in a previous study when bovine blood samples were stored at 4°C until 72h (Abd Ellah et al, 2011). It has been reported that cattle blood has a long erythrocyte lifespan ranging from 130～160 days under normal conditions (Roland et al, 2014). However, it is unclear whether the life span of erythrocytes within the normal physiological condition affects their stability ex vivo in long term refrigerated storage. In the present study, these blood parameters were observed over an extended period of time, and most showed stability for up to 192 h. Among the CBC parameters, the MCHC level decreased after 72 h of storage in this study. One of the most commonly associated implications of a slightly decreased MCHC is strongly regenerative anemia, which can be distinguished as normal (normochromic), decreased (hypochromic), or increased (hyperchromic) (Roland et al, 2014). The range of normal reference values used by the IDEXX Procyte® hematology analyzer for MCHC was 30.2～33.5 g/dL for bovines. Statistically, when the range of normal reference value is narrow, a slight increment or decrement could imply significant changes in the value measured (Médaille et al, 2006).

White blood cells play an essential role in immune defense. A complete leucocyte count includes the number of leukocytes, the relative differential blood counts, and the absolute differential blood count which includes neutrophil, lymphocytes, monocytes, eosinophils, and basophils (Roland et al, 2014). The evaluation of all WBC parameters is referred to as a leukogram. In cattle, indications for a leukogram include diagnostic, general assessment, monitoring of a disease, or monitoring of therapeutic action (Roland et al, 2014; Gunawardena et al, 2017). Among the leukocytes assessed in this study, NEU and EOS were stable until 192 h, while BASO, LYM, and MONO counts showed inconsistent fluctuations throughout the observation periods. These results are in agreement with a previous study in which they reported that the WBC count in human and veterinary samples undergo no significant changes when stored at 4°C for 5 days (Wu et al, 2017). Imeri et al found that storing a blood sample can prolong its stability when stored between 4°C and 8°C (Imeri et al, 2008), and Butarello et al concluded that WBC counts are stable for up to 72 h when stored at 4°C (Buttarello, 2004). Gunawardena et al determined that differential WBC parameters remain-

ed stable for 48 h, except for the basophil count which increased in their 6 h observation when stored in a refrigerator (Gunawardena et al, 2017). Differences in WBC differential counts can lead to clinical misinterpretations, especially when the results are close to the limits of the reference interval (Abd Ellah et al, 2011); therefore, establishment of guidelines to determine how long the stability of these parameters can be maintained would support better management of bovine blood samples.

Platelets are anuclear cytoplasmic fragments of megakaryocytes that function in the formation of the initial hemostatic plug of damaged vasculature and in maintaining vascular integrity (Jones and Allison, 2007; Roland et al, 2014). The most common tendencies observed in platelet profile evaluations are petechia and mucosal bleeding which can be assessed accurately from blood samples at 4 to 6 h after collection (Jones and Allison, 2007). In this study, platelet profiles were stable, although they did show insignificant increases and decreases until 192 h of storage. This is because prolonged exposure of samples to anticoagulants, especially EDTA, can cause an artifactual decrease in platelet count, which has been previously reported in cattle blood (Jones and Allison, 2007). This may also lead to a falsely elevated platelet count which can commonly occur because small RBCs can be misread as platelets by some automated analyzers (Jones and Allison, 2007). Also, earlier studies on PLT counts have determined that sample storage can affect platelet volume due to clumping, but this can be remedied by intermittent mixing of samples to provide consistent results (Hussein and Aamer, 2013; Tendulkar et al, 2015). Consequently, this mixing method was practiced in all blood samples in this study prior to analysis. Although some of these concerns do not have significant relevance in this study due to the exhibited stability of the platelet profile parameters over a prolonged period, these also poses potential complications on addressing platelet stability in the management of bovine blood samples.

Blood gases and electrolytes

Based on the results, even though the level of PCO_2 and PO_2 immediately decreased significantly after 6 h of storage, the pH level remained stable for 192 h. The same finding was reported previously in cattle blood in which PCO_2 and PO_2 decreased significantly through time without affecting the pH and SO_2 levels (Gokce et al, 2004). The pH level is considered to be the single most valuable factor in the evaluation of the acid–base status of an animal patient. Its determination depends on the level of concentration of oxygen and carbon dioxide in the blood. A decline in the PCO_2 may be due to reduced cellular activity ex vivo related to exposure to low temperatures (4°C), thereby reducing the glycolysis in erythrocytes (Gokce et al, 2004). On the other hand, PO_2, which also decreased through time, may be affected by the consumption of oxygen in the aerobic metabolism of leukocytes (Gokce et al, 2004), particularly because leukocytes are responsible for most of the aerobic metabolism occurring in blood (Hussein and Aamer, 2013). It was also reported that materials used (such as plastic syringes) and handling procedures (such as intermittent mixing and opening the tubes) could alter the blood gas level in blood samples before and during analysis (Gokce et al, 2004). Such factors could be the reasons why the PO_2 tended to decrease over time as the leucocyte count increased in this study. Additionally, the decrease in the BE and tCO_2 did not affect the pH level of the samples at up to 192 h of storage.

The AnGap is a measure of the difference between the concentrations of commonly measured cations minus the concentrations of commonly measured anions and is commonly expressed as AnGap=$(Na^+ + K^+) - (Cl^- + HCO_3^-)$. Some of the main pathophysiologic causes of an increased AnGap are metabolic acidosis (Gomez et al, 2015) and alkalemia (Dhondup and Qian, 2017), while a decreased AnGap is usually due to hypoalbuminemia, because of a decrease in albumin (Dhondup and Qian, 2017). In the present study, AnGap was automatically calculated using the above formula and was shown to increase with time in the blood stored at 4°C. An increase in AnGap is usually due to an increase in unmeasured organic anions and is generally associated with decreased HCO_3^- concentration (Lee et al, 2006). HCO_3^- has a mutual role with Cl^- in maintaining the electrical balance outside and within the cell. This combined role affects the concentration of these anions; for example,

when Cl- concentration outside the cell increases, HCO_3- concentration inside the cell decreases (Wieth et al, 1982). Also, K^+ and Na^+, as the main intracellular and extracellular cations, respectively, act as major buffers inside and outside of cells, serve to chemically neutralize the acid–base status, and maintain the osmotic pressure of cells in the body (Aronson and Giebisch, 2011). In this study, the K^+ concentration tended to increase as storage time increased. This is because blood for use in electrolyte and blood gas tests was not allowed to coagulate due to the presence of heparin sodium; thus, the blood was stored in a liquid state over a prolonged period, allowing K^+ to leak out of the cells (Asirvatham et al, 2013). Another reason could be due to a drop in Na^+ concentration, as was observed in this study, leading to a decrease in the osmotic pressure in the blood stored in the sample tubes, and/or due to the intermittent mixing of sample tubes before each analysis, which could lead to hemolysis allowing the K^+ ions to spread to the plasma (Freedman and Hoffman, 1979).

In summary, this study was conducted in order to establish recommendations for the management of veterinary field samples of blood, especially bovine blood. These recommendations are needed because some cattle herds are located in remote areas, which can result in long field-to-laboratory delivery periods of samples that are being stored in insulated boxes with a limited supply of ice coolant. In addition, occasionally, multiple samples collected from several farms may be sent in a single bulk batch for storage. Such an occurrence can result in long periods in the storage refrigerator, thus, reducing the freshness of the sample. Therefore, this study aimed to assess the influence of prolonged refrigeration at 4°C on hematological, blood gas, and blood electrolyte parameters of cattle blood specimens. Hematological test results showed varying stability of CBC parameters. Total RBC count and basic CBC parameters are stable for 192 h, but MCHC was only stable for 72 h. Platelet profile parameters are also stable for 192 h. Among the other CBC parameters, NEU and EOS remained stable for 192 h but the WBC extended differential parameters, BASO, LYM, and MONO produced inconsistent measurements. LYM and MONO showed statistically significant recovery of the initial measurement after being stored for 12 and 24 h, respectively, and then remained stable for 120 h. Such RBC and WBC changes can be associated with the availability of blood gases in vitro because cellular metabolism of blood cells remains active post-collection and mainly depends on the remaining blood gases in the sample tube, which, in turn, is influenced by the level of temperature to which the samples are exposed. In addition, blood gas indices showed significant changes over storage time. PCO_2, PO_2, tCO_2, and BE showed significant changes with a declining trend during the course of the storage period. Although changes in these parameters were observed, it did not affect the stability of pH, which is one of the main parameters used to assess the acid–base status of animal blood. Moreover, measuring the electrolyte status in the blood showed that blood ion levels are unstable and tend to change after as little as 6 h. Also, changes occurring in the major electrolytes varied from ion to ion, depending on their cellular localization. This suggests the use of portable equipment to immediately test the blood samples when measuring the BGE to assess the acid-base and electrolyte status of animals in field situation. Although this study is limited only to small population size due to limited resources, this study is time bound requiring minimal time for analysis, therefore small sample size would allow rapid measurement of all the samples within a short period of time in each of the designated observation time points, preventing the prolonged waiting for samples to be analyzed. However, bigger sample size, as well as inclusion of other breeds, is recommended in future studies for a more conclusive data analysis and interpretation.

In conclusion, this study established that cattle blood specimens for use in CBC analyses can be stored for 120 h (5 days) at 4°C when considering the leucocytes and the extended leucocyte differential count parameters, whereas, considering the highly unstable electrolyte parameters, blood specimen for assessing blood gas and blood electrolyte status must be tested immediately or within 6 to 12 h. Handling, storage, and the urgency of testing the specimen must all be considered since hematological and biochemical aspects of blood can be easily affected by certain physicochemical factors. Small variations in results for parameters that are very close to the

reference interval limits, especially parameters with a small range of reference values, can lead to erroneous interpretation.

ACKNOWLEDGEMENTS

This work was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry (IPET) Program, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA) (319015-01-1-HD030).

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