Assessment of different bovine blood collection methods

Avaliação de diferentes métodos de coleta de sangue em bovinos

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ABSTRACT
The criteria for choosing the best technique to be used in bovine blood collection must surpass their costs, thus taking into consideration the easiness of handling and the efficiency of the method. The aim of this study was to assess three different bovine blood collection methods. The collection methods used were: the Traditional, the Vacuum-tube and the Krev. Nellore and Girolando (¾ Holstein x ¼ Gir) animals were used, twenty-five of each genetic group were submitted to two groups of operators, skilled and unskilled in blood collection. The reactivity of the animals in the containment trunk during blood collection was determined by heart and respiratory rate. The time spent for blood collection and the hematoma degree caused by the venipuncture in the coccygeal and jugular veins was measured. The microbiological contamination was measured by the Total Bacteria Counting and the presence of Staphylococcus aureus and Escherichia coli after blood collection was determined. The data were submitted to the variance analysis following a completely randomized design. The group means were compared by the Tukey Test with a maximum significance level of 5%. The Nellore animals were more reactive and presented a higher level of stress during blood collection. The Krev method was fastest and caused less severe hematomas. The Krev and the Vacuum-tube methods had significantly lower levels of microbiological contamination. The Krev method is preferable due to its characteristics.

Keywords: animal behavior, Krev, microbiological contamination, reactivity, venipuncture.

RESUMO
Os critérios para a escolha da melhor técnica a ser utilizada na coleta de sangue bovino deve ir além dos custos, considerando-se assim a facilidade do manuseio e a eficiência do método. O objetivo foi avaliar diferentes métodos de coleta de sangue em bovinos. Os sistemas de coleta utilizados foram: os Métodos Tradicional, à Vácuo e Krev. Foram utilizados 25 animais de cada grupo genético, sendo Nelore e Girolando (¾ Holandês x ¼ Gir), submetidos a dois grupos de manejadores, indivíduos sem e com experiência em coleta de sangue. A reatividade dos animais no tronco de contenção durante a coleta de sangue, foi determinada através da avaliação subjetiva do comportamento (escores), frequência cardíaca e respiratória. O tempo de coleta de sangue na veia cocígea e jugular foi medido nos diferentes métodos, assim como, a formação de hematomas após punção venosa. Foi determinado a Contagem Bacteriana Total, Staphylococcus aureus e Escherichia coli. Os dados foram submetidos à análise de variância em um delineamento inteiramente casualizado. As médias dos grupos foram comparadas pelo Teste de Tukey, ao nível de 5% de significância. Os animais Nelore foram mais reativos e apresentaram maior nível de estresse durante a coleta de sangue. O tempo desprendido para a coleta de sangue foi menor pelo método Krev, além disso, este causou hematomas menos severos. Os métodos Krev e à Vácuo tiveram níveis significativamente mais baixos de contaminação microbiológica. O método Krev é mais eficiente para coleta de sangue em bovinos.
1 INTRODUCTION

Cattle production in Brazil is an important economic activity accounting for a bovine herd of 220 million of heads, distributed through the whole country. Nellore and Girolando are the main breeds used to produce beef and milk, respectively, and they present very different behaviour when being handled.

In this context, the efficiency increase in production systems has driven research investments to focus on enhancing herd productivity without losses to the environmental sustainability and animal welfare. Nonetheless, bovines are often exposed to stressful conditions during herd management practices such as blood sampling and this affects farm economic performance. Therefore, the adoption of available techniques and introduction of new procedures to collect blood can potentially reduce animal stress and improve farm's and processors' profitability. Labour training is one of the main procedures in order for bovine stress to be reduced during blood collection yet, very few efforts to develop and compare new and current technologies have been observed.

The quality of the blood sample is another concern when it comes to the diagnosis of diseases and physiologic and metabolic assessments. The most relevant factors that may affect quality is the operators' skill (phlebotomist), the collection method, the animal stress level during blood collection and the environmental contamination of the sample. Besides, incorrect transportation methods can also reduce quality and sometimes make laboratory analysis unfeasible (Ashavaid et al., 2008).

There are two methods of blood sampling, the open and the closed systems. The most widely used method in Brazil is the open system in which the needle and the container consist of two distinct pieces. The Vacuum-tube is an example of closed blood collection system that allows for collecting several samples in only one venipuncture, however, it may require training and skills in order for errors to be prevented and for the necessity of exchanging the tube and the needle frequently to be avoided. Krev is an alternative closed method system composed by a flexible and a disposable container made of plastic in which the needle is attached.
Thus, we aim at assessing the effect of the bovine breed, the venipuncture site and operators' skills over three blood collection methods: Traditional, Vacuum-tube and Krev.

2 MATERIALS AND METHODS

All experimental procedures were approved by the Animal Use Ethic Committee of the Universidade Estadual de Mato Grosso do Sul (protocol 007/2016). The experiment was conducted in the beef and milk cattle sector of the Universidade Estadual de Mato Grosso do Sul (Unidade Universitária de Aquidauana/MS) and in the Microbiology Laboratory of the Universidade Federal de Mato Grosso do Sul (Campus de Aquidauana-MS).

2.1 BLOOD COLLECTION PROCEDURES

We used 50 bovines from two genetic groups, 25 Nellore and 25 Girolando (¾ Holstein x ¼ Gir). Every three days the animals were submitted to venipuncture in two distinct body parts, the coccygeal and the jugular veins, performing 1,200 blood collection tests. One group was composed by unskilled operators who had never collected blood before and did not repeat the blood collection during the experiment and the other group in which the operators performed blood collection routinely (skilled operators). Prior to every blood collection, we used paper towel to clean the venipuncture site. The animals were immobilised in a containment trunk in a shaded area during the blood collection.

We compared the three blood sampling methods by using the same needle size (40-12 mm). In the Traditional method (TRAD) we used a needle to puncture the vein and the blood was stored in a sterile test-tube (T-tube) made of plastic and with a rubber stopper. The tube was opened only at the very moment of the blood collection. On the Vacuum-tube method (VACU) the vein was punctured with a double needle and the blood stored in a sterile plastic tube with a vacuum and a screw stopper made of plastic. The needle and the tube were disposable. On the Krev method (KREV) the vein was punctured with a disposable needle and the blood stored in an attached disposable sterilised flexible plastic tube.

During the venipuncture process we simultaneously measured the heart rate (HR) and the respiratory rate (RR) to determine the stress level of the animals. The HR was expressed in number of beats per minute and measured by using a stethoscope for 30 seconds. The RR was obtained by counting the flank movements of the animal for 30 seconds and recorded in minutes.
The hematomas after puncturing the coccygeal and jugular veins were subjectively evaluated through the usage of the following score: 1 for no hematoma formation; 2 for a blood spot; 3 when a swelling was observed; 4 for blood spot along with swelling; and 5 if heavy bleeding together with swelling were observed.

The behaviour of the animals during the blood collection was also evaluated subjectively by using a score scale adapted from Silveira, Fisher and Soares, (2006) ranging from 1 to 5 in which: 1 the animal makes small movements within the containment trunk (most of the time remaining still), and with occasional relaxed tail movements; 2 the animal is more active not remaining in the same position for more than a few seconds and with occasional vigorous tail movements; 3 frequent movements within the containment trunk, vigorous and abrupt movements with frequent vigorous tail movements; 4 frequent displacements within the containment trunk, vigorous and abrupt movements and the animal tries to lie down in the trunk; 5 continuous displacements, jumps, the exit grid is forced with the head, continuous and vigorous tail movement, and the animal falls in the containment trunk.

The time spent for blood collection was also recorded. We started counting at the moment in which the animal was contained in the containment trunk and stopped when the blood sample was placed in the thermal box.

2.2 MICROBIOLOGICAL ANALYSIS

The blood analysis of red (erythrocyte, hematocrit, hemoglobin and hematimetric indices) and white (leukocytes, lymphocytes, monocytes and platelets) components were performed prior to the microbiological tests to assure that biological factors do not affect the outcomes. All parameters were within the normal range (Table 1). To perform the microbiological analyses, 10 animals from each genetic group were randomly selected and 10 blood samples per method were collected. The samples were identified by the number of the animal then diluted into flasks with 10 mL of Hemoprov reagent (New Prov, Pinhais, Paraná, Brazil), and inoculated into the specific culture medium. Then, the samples were stored in an oven at 35°C.
Table 1: Hemogram of the blood samples used in the microbiological analyses.

<table>
<thead>
<tr>
<th>Blood components</th>
<th>Hemogram</th>
<th>Reference Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes x10³/μl</td>
<td>12.4 - 8.7</td>
<td>5.0 - 16.0</td>
</tr>
<tr>
<td>Lymphocyte x10³/μl</td>
<td>4.3 - 7.4</td>
<td>1.5 - 9.0</td>
</tr>
<tr>
<td>Monocytes x10³/μl</td>
<td>0.8 - 1.2</td>
<td>0.3 - 1.6</td>
</tr>
<tr>
<td>Granulocytes x10³/μl</td>
<td>3.6 - 5.3</td>
<td>2.3 - 9.1</td>
</tr>
<tr>
<td>Lymphocyte (%)</td>
<td>46.7 - 55.1</td>
<td>20.0 - 60.3</td>
</tr>
<tr>
<td>Monocytes (%)</td>
<td>8.4 - 9.4</td>
<td>4.0 - 12.1</td>
</tr>
<tr>
<td>Granulocytes (%)</td>
<td>36.4 - 44.0</td>
<td>30.0 - 65.0</td>
</tr>
<tr>
<td>Erythrocyte x10⁶/μl</td>
<td>6.1 - 7.1</td>
<td>5.0 - 10.1</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>9.8 - 10.4</td>
<td>9.0 - 13.9</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>28.0 - 31.2</td>
<td>28.0 - 46.0</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>44.6 - 75.0</td>
<td>38.0 - 53.0</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>14.9 - 17.1</td>
<td>13.0 - 19.0</td>
</tr>
<tr>
<td>CHCM (g/dL)</td>
<td>33.2 - 36.1</td>
<td>30.0 - 37.0</td>
</tr>
<tr>
<td>Platelet (L) x10⁹</td>
<td>143 - 220</td>
<td>120 - 820</td>
</tr>
</tbody>
</table>

* Values based on Júnior et al (2006); Da Silva et al. (2008) and Conceição et al. (2019)

Source: Authors

The microbiological analyses were performed in five times: zero (immediately after blood collection), 24, 48, 72 and 96 hours after the blood collection. All microbiological analyses followed the appropriate asepsis recommendations. The plates with Tryptic Agar (TSA) (New Prov, Pinhais Paraná, Brazil), Azide Agar (Kasvi, Curitiba, PR, Brazil), Blood Agar (New Prov, Pinhais Paraná, Brazil) and Mac Conkey Agar (New Prov, Pinhais Paraná, Brazil) for Staphylococcus aureus and Escherichia coli were purchased ready in order for the inoculation to be proceeded.

To calculate the CBT, an agar medium for standard counting was prepared, diluted in distilled water, autoclaved and then distributed in sterile Petri plates (15x100), finally, the plates were stored for solidification. For the inoculation process, we diluted 0.05 mL of blood to the Hemoprov reagent in the plate. After that, the samples were incubated in an oven at 35°C for 24 hours, and then, the plates that had bacterial development were observed and classified as Gram-positive and Gram-negative.

A staining method was used to differentiate the species in two groups, Staphylococcus aureus (Gram-positive) and Escherichia coli (Gram-negative). Thus, slides that presented rod-
shaped bacteria with pink staining were identified as *Escherichia coli*. Slides with spherical purple-coloured bacteria were identified as *Staphylococcus aureus*. We used plates with soybean Tryptic Agar culture media as a control method. It was inoculated and evaluated in the same way, however, without the Gram procedures.

The strains of *Staphylococcus aureus* were isolated by using Petri plates containing Azide Agar and Blood Agar. For each plate, 0.05 mL of blood diluted in the Hemoprov reagent was added. Plates containing 20 to 200 of these typical *Staphylococcus aureus* colonies were selected for counting, which were submitted to biochemical, coagulase, catalase and oxidase tests. Inoculation with Azida blood in a Blood Agar culture medium was used as a control.

Faecal coliforms were determined by the observation of *Escherichia coli* with 0.05 mL of blood diluted in the Hemoprov reagent seeded in a Petri plate containing Mac Conkey Agar media. Plates with pink or purplish bacteria were submitted to the Gram staining test, after 24 hours of inoculation.

2.3 STATISTICAL ANALYSES

The data were submitted to the variance analysis following a completely randomized design. The group means were compared by the Tukey Test with a maximum significance level of 5%. For Heart Rate and Respiratory Rate, the range (higher values – lower values) were used and the microbiologic results were expressed in percentage. All data analyses were performed in the “R” software version 3.3.1 (R Development Core Team 2016).

3 RESULTS AND DISCUSSION

Significant differences between the two breeds regarding animal behaviour during blood collection were found. The animals from the Girolando group were less stressed than the Nellore group while being managed inside the containment trunk (Table 2).
Table 2: Animal behaviour based on breed collection method and group of operators.

<table>
<thead>
<tr>
<th>Breed</th>
<th>Methods</th>
<th>Operators</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TRAD</td>
<td>VACU</td>
</tr>
<tr>
<td>Girolando</td>
<td>2.78</td>
<td>2.85</td>
</tr>
<tr>
<td>Nellore</td>
<td>3.36a</td>
<td>2.76</td>
</tr>
</tbody>
</table>

Means followed by different lower-case letters in the same lines for the same variable differ from each other by the Tukey test (p<0.05). Methods: TRAD (Traditional), VACU (Vacuum-tube), KREV (Krev).

Source: Authors

Animals from the Nellore group showed more reactive behaviour in the containment trunk during blood collection than the Girolando group. Differences between breeds were similarly described by Silveira, Fisher and Soares, (2006) when assessing the interaction of genetic groups and temperament. The authors found differences for the time and distance of escaping for groups of different breeds. They concluded that the Nellore or Nellore cross-breed are more reactive and excitable than the European breeds. Hotzel, Gomes and Machado Neto, (2008) concluded that dairy cows from the European breed do not present behavioural changes when subjected to aversive and sporadic procedures.

In general, reactivity has a stimulatory effect on the animal behavioural response, becoming more intense as the animal is more reactive (Maffei, 2009). Differences in temperament are physiologically based, so reactive animals tend to have a lower adaptive capacity to changes and are more susceptible to stress while restrained (Costa, Sant’Anna and Silva, 2015).

The Nellore group had a higher heart rate (HR) variation during the blood collection in the coccygeal and jugular veins when compared to the Girolando group (Table 3). The respiratory rate (RR) of the Nellore group was also significantly higher than the Girolando group. Mean differences in HR, RR were not significant at 5% of probability when the three blood collection methods and the operators' skills were compared.
Table 3: Heart rate (HR) range during the blood collection.

<table>
<thead>
<tr>
<th>Breed</th>
<th>Methods</th>
<th>Operators</th>
</tr>
</thead>
<tbody>
<tr>
<td>Girolando</td>
<td>Nellore</td>
<td>TRAD</td>
</tr>
<tr>
<td>Coccygeal</td>
<td>-1.11b</td>
<td>-5.53a</td>
</tr>
<tr>
<td>Jugular</td>
<td>1.32b</td>
<td>-1.98a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VACU</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KREV</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Skilled</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unskilled</td>
</tr>
</tbody>
</table>

Means followed by different lower-case letters in the same lines for the same variable, differ from each other by the Tukey test (p<0.05). Methods: TRAD (Traditional), VACU (Vacuum-tube), KREV (Krev).

Source: Authors

The Nellore animals presented a restless behaviour inside the containment trunk resulting in an increased heart rate. According to Luchiari Filho and Mourão (2006), higher values of heart rate in the Nellore breed can be explained by the more active temperament of these animals during management.

Therefore, physiological changes and short-term responses such as the increase in heart and respiratory rates are directly linked to fear and excitement responses. Nevertheless, it is worth noticing that the increase or reduction of rate depends on the intensity of stress, and on the animal's adaptive capacity. According to Arzamendia, Bonacic and Vilá, (2010), the sudden exposure to stimulus to which the animals are not adapted can trigger stress or alarm reactions characterised by a set of responses such as the ones found in this study for the Nellore breed group.

Interactions between the collection method and the genetic group for the respiratory rate (Figure 1) were observed. The Girolando group presented lower RR for the Vacuum-tube and Krev methods when compared to the Traditional method. The Nellore group did not present a significant difference (p>0.05) between the collection methods and the RR.
Boissy and Bouissou (1988) assessed the effect of different intensities of herd management on animal reactivity. They found that animals submitted to longer and more intensive treatment presented higher heart rate values during behavioural tests. This evidence reinforces the need for the use of techniques that reduce human impact over animal behaviour and highlights the importance of studies such as ours, which assess the efficiency of alternative methods like blood sampling, for instance. According to Rivera (2002), when a behavioural response does not relieve stress, the animal needs to change its biological state by evoking the autonomic system that has rapid and specific responses, such as increased heart and respiratory rates.

The Traditional method of blood collection caused more severe hematomas in the coccygeal vein when compared to the Vacuum-tube and Krev methods (Table 4).
Table 4: Means for the hematoma degree in the coccygeal vein.

<table>
<thead>
<tr>
<th>Methods</th>
<th>Hematoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Traditional</td>
<td>2.96a</td>
</tr>
<tr>
<td>Vacuum-tube V</td>
<td>2.44b</td>
</tr>
<tr>
<td>Krev</td>
<td>2.37b</td>
</tr>
</tbody>
</table>

Source: Authors

Stöber (1993) found that the Traditional method of blood sampling causes more severe hematomas than the Krev and Vacuum-tube methods because it requires longer time and more ability to locate and puncture the vein. It is worth noticing that the blood collection is a painful method and needs to be done fast and safely.

The time spent to collect blood using the Krev method was more than 50% faster than the Vacuum-tube and almost 61% faster than the Traditional method. The group of skilled operators collected blood 27% faster than the unskilled operators. The collection from the coccygeal vein was significantly faster than the the blood collection from the jugular vein (Table 5). Therfore, we recommend that the venipuncture be made through the coccygeal vein once it takes less time despite the method used, the operators' skills and the animal breed.

Table 5: Time spent (in seconds) during the blood collection based on the collection site, methods and operators' skills.

<table>
<thead>
<tr>
<th>Site</th>
<th>Methods</th>
<th>Operators</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC</td>
<td>CJ</td>
</tr>
<tr>
<td></td>
<td>55.52b</td>
<td>67.66a</td>
</tr>
</tbody>
</table>

Means followed by different lower-case letters in the same lines for the same variable, differ from each other by the Tukey test (p<0.05). CC (coccygeal vein), CJ (jugular vein), Methods: TRAD (Traditional), VACU (Vacuum-tube), KREV (Krev).

Source: Authors

We found that Krev is the fastest and most efficient method because it has a flexible structure made of plastic that works with a “memory system” – the plastic returns to its original form after having been folded. Thus, if an error occurs during the puncture, the operator can refold the plastic container and form a new vacuum, thus preventing it from being discarded as it usually happens to the Vacuum-tube. Besides, the Krev method was easier to manipulate than the other methods because the needle is permanently attached to the plastic container.

The Traditional method presented a higher contamination level measured by the TBC. The methods Vacuum-tube and Krev had similar contamination levels at the end of the test period, nevertheless, the bacteria counting was slightly lower for the Krev method in the first 48 hours (Figure 2). Already, the contamination by *Staphylococcus aureus* for Traditional method
was observed since the initial time of sample incubation. The bacteria started growing in the Vacuum-tube and Krev samples after 24 hours and until 48 hours the contamination level of these samples were about half (56%) of the Traditional method. The Vacuum-tube and Krev samples were not contaminated by *Escherichia Coli* while in the Traditional method bacteria was observed after 24 hours of incubation.

![Figure 2: Total bacteria counting (TBC) in the blood samples collected in the coccygeal vein by the three different methods](image)

Regardless of the collection method used and the time spent, contaminations that occur during the procedure may interfere with the correct analysis of blood parameters. In this regard, the Traditional collection method has allowed greater bacterial growth in the blood samples as it is an open system where the needle is not coupled with the collection flask. Consistent support for our results has been found in the previous literature concerning sample contamination.

Wiwanitkit (2003) analysed 100 blood samples and found that closed collection systems such as the Vacuum-tube, have lower probability of microbial growth in the tube when compared to the open systems. Ashavaid et al. (2009) also assessed the contamination in blood samples collected by an open and a closed system and reached the conclusion that the latter presented
lower contamination levels (3 of 10,000 blood samples studied) right after the collection. Washington (1977) analysed the contamination of the collection tubes of 20 laboratories and concluded that 14% of the 1,433 tubes examined contained micro-organisms, such as Pseudomonas and Enterococcus. It was therefore found that with the Krev method the chance of previous contamination at the zero time is reduced.

4 CONCLUSIONS

In conclusion, the Nellore animals are more reactive and presented a higher level of stress in the containment trunk during blood collection when compared to the Girolando ones. The Krev method is faster and causes less hematomas in the coccyeal and jugular veins when compared to the Vacuum-tube and Traditional methods. Furthermore, the Vacuum-tube and Krev methods have significantly lower levels of microbiological contamination when compared to the Traditional method. Therefore, the Krev method is preferable to the others due to its characteristics and observed vantages, mainly if the operator does not have any experience.

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