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Original Article

Development of a Modified Paperfuge for Field Diagnosis of Anemia in Cebu, Philippines

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ABSTRACT

The packed cell volume (PCV) or hematocrit can be used as an indicator of an animal's health status. An animal with a low PCV value will usually have an anemia, which may be caused by tick-borne pathogens. PCV is routinely measured by equipment which can be expensive and difficult to bring in the field. Thus, developing an alternative method or tool that can be applied in the field to determine PCV would be advantageous. The present study aimed to develop a modified paperfuge, assess its reliability in different animals and evaluate its relevance to the diagnosis of Mycoplasma spp. infection in cattle and water buffalo. Initially, a modified paperfuge tool using locally available materials and its corresponding technique for usage was developed. Its reliability was assessed in different animal species (dog, cat, sheep, cattle, goat) along with other equipment, including an automated hematology analyzer and standard centrifuge. Results showed that the red blood cell count (RBC) was associated with PCV. Further statistical analyses showed that except in cats, there were highly significant correlations between the improvised paperfuge method and the other established methods. Field application of the modified paperfuge was performed in 12 water buffaloes and 40 cattle, which were tested for Mycoplasma spp. infection using polymerase chain reaction (PCR). Molecular detection for the presence of Mycoplasma spp. showed no correlation between the PCV values obtained. This study supports the reliability of the paperfugation technique and showed its applicability in field conditions. However, as results showed that PCV cannot be used as an indicator of the infection, paperfuge cannot be reliably used to diagnose hemoplasma infection, most especially in asymptomatic cases.

KEYWORDS: *anemia, livestock, paperfuge, PCV*

1 INTRODUCTION

Routine diagnosis and monitoring of the health of livestock is usually performed with the aid of laboratory equipment. Among these procedures is the complete blood count (Grimm *et al.*, 1985; Rosman, 2003; Ybañez *et al.*, 2016). A useful parameter is the Pack cell volume (PCV), which is the volume percentage of red blood cells (RBC) in the blood (Purves *et al.*, 2004). It is used as an indicator of health, especially in relation to anemia and dehydration. Anemia is referred to as the decrease of RBC or hemoglobin and is usually detected in those with PCV values below the normal range (Stedman's medical dictionary, 2006). Several livestock animal diseases can cause anemia that includes anaplasmosis, babesiosis and mycoplasmosis.

Clinical anaplasmosis is usually caused by *Anaplasma marginale*. On the other hand, babesiosis is usually caused by *Babesia bovis* and *Babesia bigemina*. These pathogens have been reported in the Philippines (Ybañez *et al.*, 2012, Ybanez *et al.*, 2013). Another pathogen is the Mycoplasma spp. (Ybañez, *et al.*, 2015), which is known to affect the dairy productivity. The hemotropic Mycoplasma, or otherwise known as hemoplasma, is a pathogen that attaches and grows on the surface of erythrocytes (Jones and Allison, 2007; Morrison *et al.*, 1981). It can induce acute hemolysis, associated with anorexia, lethargy, dehydration, weight loss, low-grade parasitemia, and mild anemia that may cause death (Willi *et al.*, 2008; Neimark *et al.*, 2001). Nevertheless, studies on hemoplasmosis are still limited in the Philippines. At present, information on hemoplasma is only limited to cattle (Ybañez *et al.*, 2015). Through time, complications may soon compromise the health of livestock animals including their milk yield and overall productivity affecting one of the country's production.

Among the different ways in evaluating the PCV, centrifugation is considered as the gold standard method (Myers and Browne, 2007). Centrifugation provides the direct measurement of the percent hematocrit in contrary to automated machines which

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calculate PCV based on RBC indices that yield different results (Gebretssadkan *et al.*, 2015). However, current diagnostic tools available are not readily accessible. The conventional equipment is bulky and expensive, and run on electricity that makes it difficult to use in field conditions. Thus, development or use of substitute devices capable of evaluating PCV is essential especially in the field and resource-poor settings. The present study generally aimed to evaluate the applicability of a modified paperfuge technique in estimating PCVs in Hemoplasma-infected and non-infected dairy water buffaloes and cattle. Specifically, it aimed to develop a paperfuge device using locally available materials and its corresponding modified technique, establish the reliability of the obtained PCV readings using the modified paperfuge technique in comparison with those obtained from a centrifuge equipment and/or a hematology machine (Mindray BCVET300) in dogs, cats, sheep, cattle and goats, detect the presence of Mycoplasma spp. in the selected cattle and water buffaloes using peripheral blood smear and polymerase chain reaction (PCR), evaluate the field applicability of the modified paperfuge technique in assessing the PCV of infected and non-infected cattle and water buffaloes, and determine if a relationship exists between PCV and Mycoplasma spp. positivity.

2 MATERIALS AND METHODS

Research Subject and Blood collection

Twenty dog samples were used in determining the PCV values obtained from three different methods. An additional 20, 6, 19, and 10 samples of cats, sheep, goats, and cattle respectively, were used in comparing PCV using standard centrifugation and paperfugation. Dog, cat and cattle blood samples were from Cebu, Philippines, while sheep and goat samples were from Leyte, Philippines. Blood samples were collected from the jugular vein and saphenous veins of the livestock and companion animals using a BD K3EDTA Vacutainer® tube (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Aliquot of the blood samples were used for PCV determination and other haematological procedures. The remaining blood samples were stored at −20°C until further use. In the field application of the modified technique, blood samples from 12 dairy water buffalos and 40 cattle from the PCC Dairy Buffalo Multiplier Farm and the National Dairy Authority in Ubay, Bohol were collected.

Paperfuge Development and Technique

From the original paperfuge design, modifications were made using materials that are locally available. An increase of the disc size and the addition of more capillary tubes-slots was applied in the improvised model. Specifications include the following: disc (illustration board) with 21 cm in diameter, six capillary slots (plastic reed) with 7.7 cm length and 0.4 cm diameter, a central circular steel plate with 5.0 cm diameter and 0.2 cm thickness, and twine (nylon) with 1.3 m length. Hook and loop fastener (Velero®) were also placed evenly in between tube slots (Figures 1 and 2). The technique in using the paperfuge was also modified. Instead of spinning the disc by pulling it sideways, the paperfuge was hanged, making the pull direction downwards and spinning horizontal. This procedure positions the disc parallel to the ground. Paperfugation was performed for 15 minutes (Bhamla *et al.*, 2017).

Figure 1. The spinning modified paperfuge

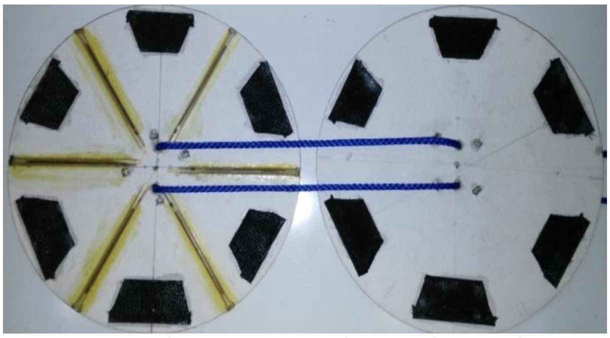

Figure 2. The modified internal set-up of the modified paperfuge

DNA Extraction and PCR Amplification

DNA extraction was performed as previously described (Ybañez *et al.*, 2013). PCR amplification of Hemoplasma species was also performed as previously described (Ybañez *et al.*, 2013; Ybañez *et al.*, 2015). PCR amplification was performed using a primer pair F2 (5'-ACGAAAGTCTGATGGAGCAATA-3') and R2 (5 ACGCCCAATAAATCCGRATAAT-3) (Jensen *et al.*, 2001) in a 25 µl reaction mixture containing 5 µl of each DNA template. The primers used can amplify the 16S rRNA genes of most Hemoplasma species (Jensen *et al.*, 2001; Tagawa *et al.*, 2008). The negative control was double distilled water (DDW). The amplification products were visualized under UV light using 1.5 % agarose gel in Tris-borate-EDTA (TBE) buffer after migration for 30 minutes and staining with ethidium bromide.

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Data Processing and Analysis

Raw data was manually recorded and transferred to Microsoft Excel software. Encoded data were imported to a statistical software. Simple linear regression analysis was used to evaluate the correlation of obtained PCV values from paperfugation, standard centrifugation and hematology analysis with each other, and with the RBC count. Analysis of variance was performed on the obtained values from different methods. Chi-square test was performed between the PCV status and positivity of Mycoplasma.

Ethical consideration

The procedures performed in this study were guided by the principles of animal welfare, Animal Welfare Act of the Philippines (RA 8485) and Administrative Order # 45 of the Bureau of the Animal Industry of the Philippines.

3 RESULTS AND DISCUSSION

The paper centrifuge used in the study was based on the original paperfuge model (Bahmla *et al.*, 2016). The disc size was increased to provide enough space to the longer capillary tubes available. Additional tube slots were installed to accommodate more samples within a single run. All modifications followed the same proportions of the previous model. A thin circular steel plate was firmly attached to the center to provide holes that can withstand high tension during the spin. Edges of the holes are refined to provide a smooth surface interaction between the disc and the twine. A thicker type of cotton twine was used instead of a fishing twine. The string length has been extended to provide more twists and total rotation during paperfugation. The paper centrifuge spinning was done in a horizontal way to mimic the spin of a standard centrifuge which is considered as the gold standard method for determining PCV values (Myers and Browne, 2007).

The PCV of different species was evaluated for the validation of the technique. Paperfugation was done along with either the standard centrifuge or the hematology analyzer to establish the reliability of the technique. PCV obtained from different methods were associated with the RBC counts from the hematology analysis. Even though blood values including PCV obtained from automated hematology analyzers may yield different results from the direct hematocrit percentage measurements obtained using the gold standard (Gebretsadkan *et al.*, 2015), the hematology analyzer still was proven to have reliable results (Harris *et al.*, 2004). Red blood cell values from the hematology analyzer were used as the baseline data for the RBC counts as this method has already been established and has an excellent correlation coefficient range of 0.8 to 0.99 in its hematocrit levels with the gold standard, centrifugation (Bienzle *et al.*, 2000). Dog blood samples were used in the primary validation which includes running hematology analysis and centrifugation along with paperfugation.

Results revealed high degree of correlation and significance between the RBC count and each of the methods used obtaining p values of 0.000, 0.005, and 0.000 using the hematology analyzer, centrifuge, and the paperfuge respectively (see Table 1.1). On the other hand, analysis of variance (ANOVA) of the PCV values obtained from different methods revealed no significant differences (F value=0.595, p value=0.556).

Table 1.1 Correlation of the red blood cell count with the different methods in dogs (n=20)

Assessed Method with RBC	R	R Square	Model	p value
Hematology analyzer	0.985a	0.971	HA=(RBC*16.196)-(.231)	0.000**
Standard centrifugation	0.667a	0.445	CF=(RBC*15.125)-(.324)	0.005**
Paperfuge	0.812a	0.659	PF=(RBC*15.792)-(.745)	0.000**

**highly significant

The modified paperfuge technique was further validated and tested in field conditions using other species. Paperfugation PCV values of each species were associated to its standard centrifugation counterpart to evaluate the closeness of their value. Linear regression analysis showed accepted p values on both methods in all the species tested proving that paperfuge is a good predictor of centrifuge values.

Correlation between the paperfuge technique and standard centrifugation was assessed (Table 1.2). Results revealed a strong and highly significant correlation between the values obtained from the two techniques, indicating that paperfuge values can predict those values obtained from centrifugation regardless of the species. Centrifugation is the reference method or gold standard for estimating the true value of the PCV (Jensen and Hansen, 2006). It is a commonly used reference and baseline for PCV readings in several studies (Becker *et al.*, 2008; McDaniel *et al.*, 2013; Bienzle *et al.*, 2000). On the other hand, PCVs of different species obtained from the paperfuge and standard centrifuge were statistically compared, which revealed no significant differences (data not shown).

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Table 1.2 Correlation of Packed Cell Volume (PCV) values obtained from paperfugation and centrifugation in differentanimal species

Species	Number of samples	R	R Square	Model	p value
Goat	19	0.97	0.941	CF=(PF*0.994)-(0.044)	0.000**
Cat	20	0.488	0.238	CF=(PF*0.623)-11.854	0.029*
Sheep	6	0.912	0.831	CF=(PF*0.839)-.014	0.011*
Cattle	10	0.879	0.772	CF=(PF*0.917)-.067	0.001**
All species	55	0.938	0.879	CF=(PF*0.957)-0.071	0.000**

CF, centrifuge; **PF**, paperfuge; *Significant, **Highly significant

Hemoplasma has been recently detected in cattle in the Philippines from the studies of Ybanez *et al.* (2015). This imposes the same threat for other closely related bovine species. Earlier known as Haemobartonella and Eperythrozoon and formerly classified as rickettsial organisms, Hemoplasma caused acute hemolytic anemia to asymptomatic infection to livestock animals (Barnett, 1974; Pitcher and Nicholas, 2005). Hemoplasmosis can be asymptomatic, and even if anemia is a common abnormality (Jones and Allison, 2007), hematopoietic diseases can be associated with inflammatory and renal disease, malignancy and hepatic failure (Willard and Tvedten, 2011).

PCV of water buffaloes and cattle were evaluated using the paperfuge technique and were tested if a correlation with the presence of Mycoplasma exists. From the chi-square analysis, most of the tested animals have normal to high PCV values (Table 2) based on the reference values by AbdEllah *et al.* (2013). Results from the analysis between the PCV values obtained from paperfugation and the Mycoplasma spp. positivity (Table 2) showed no significant association (p = 0.736). While anemia can be a characteristic of Mycoplasma spp. infection, this clinical sign is not always observed especially in sub-clinical cases. Another possible explanation is a condition called macrocytosis, where red blood cells are larger than normal (Aslinia *et al.*, 2006) which can normal to high PCV values despite the low RBC count that can be caused by the infection. In this condition, PCV levels may appear normal despite low RBC counts. A recent study (Mcfadden *et al.*, 2015) has showed these similar results in their investigation of bovine hemoplasma with anemia in New Zealand. Therefore, PCV values alone may not be useful in diagnosing Mycoplasma spp. infection.

Table 2. Pack Cell Volume of water buffaloes and cattle using "paperfugation" technique

Animal	Normal- High PCV Value		Low PCV Value	
	Freq	Relative %	Freq	Relative %
Water Buffalo Mean 42.07 SD 7.07	10	83.3	2	16.7
Cattle Mean 49.33 SD 10.42	36	90	4	10
Total	46	88.5	6	11.5

4 CONCLUSION

The modified paperfuge technique is a good estimator of the PCV and can be reliably used in field conditions. However, the PCV or the results obtained from paperfugation cannot be used as an indicator for Mycoplasmaspp. positivity. The use of this technique in the diagnosis of other anemia-causing diseases can be explored.

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