MODIFIED buffy coat method (BCM) using microhematocrit tubes and acridine orange (AO) staining permits quick (~10 min/sample) and accurate diagnosis of common bovine hemoparasites compared to the light microscopy (LM) of Giemsa stained smears. Babesia, Theileria, Anaplasma, Ehrlichia and Trypanosoma parasites were detected. It was easy to see Babesia merozoites, trypomastigotes and epimastigotes of Trypanosoma parasite. Similarly, fluorescent microscopy (FLM), offers a method for detecting low parasitemia that are undetectable by light microscopy. Under LM, the agreement between the two diagnostic techniques using Cohen’s Kappa index was perfect for Trypanosoma (1.000), substantial for Anaplasma (0.615), moderate for Theileria, and Ehrlichia (0.459, 0.433 respectively). BCM was able to detect all positive cases (100%) of Babesia infections using LM and FLM. Modified buffy coat method using dark field microscopy (DFM) is recommended as a fast and dependable tool to detect Babesia and Trypanosoma parasites. BCM using AO stain can be set up directly with low cost using an ordinary LM. BCM is a valuable tool for diagnosing hemoparasites mixed infection. Buffy coat method can be used as an alternative technique for the dry stained smear for quickly screening of bovine blood samples.

**Keywords:** Hemoparasites, Buffy Coat, Acridine Orange, Bovine.

**Introduction**

Hemoparasites infections in ruminants are primarily caused by the protozoans such as Babesia spp., Theileria spp., Anaplasma spp., and Trypanosoma spp. These protozoans are transmitted by arthropod vectors such as ticks and flies. [1-5]. Bovine hemoparasites are relatively important and endemic infections, as such infections usually cause economic losses such as body weight loss, reduced milk production, decreased reproductive efficacy, abortion, and mortality [6]. Rapid diagnostic examinations have become an essential tool in the diseases control and management programs in the world [7]. Microscopic examination (ME) of Giemsa stained blood smear is the gold standard tool for the diagnosis of these hemoparasites, mostly mixed infections dominate in cattle [8]. None of the present molecular techniques can detect all mixed infections together, but microscopic examination offers chances for this to be made in Giemsa stained blood smears. However, the process of fixing, drying and staining of the blood smears in addition to their examination are laborious and require at least 60 minutes from specimen collection to the report of the result [9]. The diagnosis can be a difficulty, especially when parasitaemia are low or blood smear preparation is less than optimal [10]. To enhance and easediagnosis of hemoparasites, several methods are presented for the quick diagnosis of hemoparasites. The buffy coat method (BCM) was developed in malarialogy researches which depend on centrifugation of blood, for separation of blood cells and parasites in different layers [11].
The quantitative buffy coat (QBC) is one of the routine techniques used worldwide for detection and quantitation of malaria parasites in human [12, 13]. BCM is a direct and fast fluorescent method used for the identification of parasitemia depending on micro-centrifugation, fluorescence, and density of infected RBCs. This method involves centrifuging the layer of pressurized erythrocytes stained with acridine orange and then examining them under an ultraviolet light source. The whole process takes place in a thick Becton Dickson hematocrit [7]. fluorescent microscopy is sensitive particularly the quantitative buffy coat (QBC) examination. This method can rapidly screen many samples but needs special equipment and training [14]. Motile parasitic such as Trypanosoma spp. and microfilaria can be screened by QBC [11,15]. It is widely used for Trypanosoma and microfilariae detections in humans [10] and dogs [15,16]. Also, a similar method was used to identify Babesia spp. [14] and Plasmodium spp. [18, 19] in humans. However, few studies are present on BCM application for detecting parasites in birds [15, 17,20].

The quantitative buffy coat method imposes cost limitations in spite of its high performance [21]. As for modern techniques, such as the focusing technique for detecting hemoparasites, it requires special equipment and cost, in addition to the need of advanced microscopes such as fluorescent microscopy, as the technique for detection and quantitative enumeration of parasites in the buffy coat QBC. The objectives of this study are: (i) To modifying the BCM for a more practical procedure in cattle blood sampling throughout fieldwork; (ii) To evaluate the performance of modified buffy coat method (BCM) as a rapid test for diagnosis of hemoparasites in cow’s blood using the ordinary light microscope, fluorescent and dark-field microscopy in a comparison to the Giemsa stained blood smear as a gold standard.

Materials and Methods

Ethical approval
The present study was approved by the College of Veterinary Medicine Committee.

Sample collection
A total of 60 cows attended the Veterinary Teaching Hospital, University of Mosul, Iraq, with a history of fever enlargement of lymph node, and pale mucous membrane. Two milliliters of the blood from each cow were collected from the jugular vein into an EDTA container and thoroughly mixed. Blood smears were prepared and the rest of the blood samples were also tested by modified BCM.

Laboratory processing of blood samples
Thick/thin smears were made on the same slide and labeled, stained with Giemsa technique according to Gajendra et al. (22), and examined under 1000X objective of a light microscope. A thick smear was used for the rapid screening while a thin smear was used for the identification of the parasite.

Modified Buffy Coat Method
A 0.1 ml of Acridine Orange (AO 0.01%, Sigma-Aldrich) was added to 0.9 ml of blood in a glass blood container pre-coated with potassium oxalate anticoagulant in place of the conventional Becton Dickson thick capillary tube used in QBC technique and mix gently. For obtaining enough amounts of buffy coat sample fill two microhematocrit capillary tubes (5 μl size) pre-coated with EDTA anticoagulant with the mixture of blood and AO stain. Seal one end of each micro-tube with clay to prevent blood leakage. After rotating it by hand for 10 seconds, the tubes were placed in a microhematocrit centrifuge, the samples were centrifuged at 12,000 rpm for two minutes instead of 6000 rpm for 5 minutes with QBC. The microcapillary tube was cut at the desired junction between the white blood cell layer, platelets and the red blood cell layer, and by lightly pressing the sharp edge of the slide on the microhematocrit tube, the buffy coat layer with a little plasma transferred to the other slide; followed by a gentle mixing through moving the slide, then placing the slide cover (size 18 x 18 mm). To allow blood cells to settle on the slide, the wet preparation was left for a minute, then examined within 10 minutes using an ordinary light microscope, fluorescent microscope (BX51 Olympus U-RFL-T-Japan), and dark field microscope (DK-Gundlach, Denmark), the results were marked with the presence (+) or absence (-) of parasites at the magnification power of 400 X (objective x eyepiece), but photography was done at 1000X (under oil).

Statistical Analyses
Data obtained from the study were input and analyzed with Statistical Package for Social Sciences software of computer using SPSS software version 21.0. Cohen’s kappa value (κ) was calculated to determine the compatibility.
between the modified buffy coat technique and Giemsa stained blood smear as a gold standard under the light microscope. The Kappa value $\kappa$ values were interpreted according to as: poor, $\kappa < 0$; light, $\kappa = 0.21–0.40$; moderate, $\kappa = 0.41–0.60$; substantial, $\kappa = 0.61–0.80$; and perfect, $\kappa = 0.81–1.00$ [23].

**Results**

Blood samples of a total of 60 cows were tested, 18 cases were infected with *Babesia*, 17 *Theliera*, 11 *Anaplasma*, 10 *Ehrlichia*, and 17 cases with *Trypanosoma* after pooling the results of Giemsa stained smears and BCM tests (Table 1). In buffy coat wet preparations, *Babesia* species had the most positive percentage, followed by *Trypanosoma* parasites. All cases (100%) of *Babesia* and *Trypanosoma* infections were detected under light microscope, fluorescent and dark-field microscopy compared to the ME using standard Giemsa stained blood smear which was able to detect only 61.11% of the cases. Regarding to *Theliera*, *Anaplasma*, and *Ehrlichia* parasites, BCM had a low positive rates. In ME of Giemsa stained smear, *Babesia* and *Trypanosoma* species had also the higher positive rates, followed by *Theliera*, *Ehrlichia*, and *Anaplasma* parasites (Table 1). The compatibility between the two diagnostic tools under LM was perfect for *Trypanosoma* ($\kappa = 1.000$), substantial for *Anaplasma* (0.615), moderate for *Theliera* and *Ehrlichia* (0.459, 0.433 respectively). The BCM technique using FLM showed moderate agreement for the detection of *Anaplasma* ($\kappa = .476$), *Ehrlichia* ($\kappa = .460$) parasites, and fair agreement ($\kappa = 0.209$) in detecting *Theliera* and *Trypanosoma* parasites in a comparison with the blood smears stained with Giemsa. Fluorescent and dark-field microscopy using BCM was able to diagnose all cases (100%) of *Trypanosoma* parasite infections compared with 64.70% using the Giemsa stained blood smear. Interestingly, the BCM technique of live blood samples using dark field microscopy was able to detect all 18 (100%) cases of *Babesia* infection and 17 (100%) trypanosomes. Compared with dry-stained blood smear scanning microscopy, DFM microscopy failed to detect cases of *Theileria*, *Anaplasma*, and *Ehrlichia* (Table 1).

During BCM examination, it was possible for the first time to observe *Babesia* spp. merozoites moving in the buffy coat wet preparations of cow blood. *Babesia* merozoites were particularly easily identified even using LM (Fig. 1: A, B). *Babesia* piroplasm was seen with orange coloration inside red blood cells, while the yellowish merozoites were seen outside RBCs. *Trypanosoma* parasites were also easy to detect with bright yellow color under the FLM (Fig. 2: C, D). All parasites were also seen inside and outside red blood cells under a fluorescent microscope in a bright golden color such as *Theliera*, *Anaplasma*, and *Ehrlichia* infections were also detected using BCM but the motile parasites such as *Babesia* merozoites and *Trypanosoma* (Trypomastigotes and Epimastigotes) are quickly seen during the rapid screening of the modified buffy coat BCM technique. Especially because of its concentration under the buffy coat layer as a result of centrifugation of the blood sample, and its movement, as well as its coloration with AO stain (Fig. 3:E, F).

**TABLE 1.** Comparison of Kappa value ($\kappa$) and the compatibility between modified buffy coat method and Giemsa-stained blood smear in its ability to detect hemoparasites of cow’s blood.

<table>
<thead>
<tr>
<th>Blood parasites</th>
<th>Total No. cases</th>
<th>Standard Giemsa (GS)</th>
<th>Modified buffy coat (BCM)</th>
<th>Modified buffy coat (BCM)</th>
<th>Modified buffy coat (BCM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+ (%)</td>
<td>+ (%)</td>
<td>(k)</td>
<td>+ (%)</td>
</tr>
<tr>
<td>Babesia</td>
<td>18</td>
<td>11 (61.11)</td>
<td>18 (100.0)</td>
<td>.000</td>
<td>18 (100.0)</td>
</tr>
<tr>
<td>Theileria</td>
<td>17</td>
<td>10 (58.8)</td>
<td>6 (35.29)</td>
<td>.459</td>
<td>3 (17.64)</td>
</tr>
<tr>
<td>Anaplasma</td>
<td>11</td>
<td>6 (54.54)</td>
<td>4 (36.36)</td>
<td>.615</td>
<td>3 (27.27)</td>
</tr>
<tr>
<td>Ehrlichia</td>
<td>10</td>
<td>7 (70.00)</td>
<td>5 (50.00)</td>
<td>.443</td>
<td>7 (70.00)</td>
</tr>
<tr>
<td>Trypanosoma</td>
<td>17</td>
<td>11 (64.70)</td>
<td>11 (64.70)</td>
<td>1.000</td>
<td>17 (100.0)</td>
</tr>
</tbody>
</table>

Fig. 1. *Babesia* parasites inside and outside of RBC in buffy coat layer wet preparation (A) under light microscope, (B) *Babesia* merozoites outside RBC under fluorescent microscope. Oil immersion (1000X).

Fig. 2. The buffy coat layer wet preparation in which (C) *Babesia* merozoites appear inside the red blood cells in a reddish-orange color of the cytoplasm with the brilliant yellow nucleus. (D) *Trypanosoma* outside erythrocytes. Fluorescent microscope magnification power 1000 X.

Fig. 3. (E) The buffy coat layer wet preparations show the brightness of *Babesia* parasites inside red blood cells. (F) *Trypanosoma* outside erythrocytes. Under dark field microscope magnification power 1000 X.

Based on the microscopic examination of the concentrated live blood samples using dark-field microscopy, detailed observations were made about the processes of invasion or egress from red blood cells mediated by *Babesia* parasites. Many unexpected results related to the dynamics of their ability to deal with the red blood cells of cows under laboratory conditions, and despite the speed with which these processes occur and the small size of the parasites, infective merozoites were moving fast and making surprising abnormalities in the erythrocytes membrane during the active invasion. Large quantities of merozoites in a short period of time.

The merozoites of *Babesia bovis* enter red blood cells and invasion into the membrane of red blood cells through their gliding movement, as the merozoites recognize red blood cells through their apical end, which is the guiding part of *Babesia* parasite during the process of invasion into the red blood cell, and cause an initial decline in the membrane of the host cell at the entry location, a tight connection is initiated between the plasma membrane of the red blood cell and the free merozoite membrane, with clear entry from the front pointed end, which contains the apical complex. *Babesia* merozoites appeared with different instantaneous morphology moving forcefully in all directions to invade suitable RBCs. Their movement was in different styles, such as straight, curvilinear, zigzag, front and back…etc.

Different intraerythrocytic dynamic stages were observed such as single pyriforms, combined pyriforms, two paired pyriforms, tetrads, and multiple parasite stages resulting from exit of a large number of merozoites within relatively short period (Fig. 4 G, H).

**Discussion**

For a reliable diagnostic method, the higher the positivity percentage of known cases the more useful method. The opposite is commonly the case for negativity percentage which is predictable to be attentive towards zero for good diagnostic techniques. In the study, the positivity rate for *Babesia* cases was 100% by modified buffy coat method while Giemsa staining method recorded it as (61.11%). Whereas the negativity rates were 0% and 38.89% respectively. One of the most interesting notes is that the agreement between the modified buffy coat and Giemsa stained smear using LM was considered as perfect for *Trypanosoma* detection using the Cohen’s kappa value. It is commonly preferred to decrease the time required for sample screening in the fieldwork, substituting the time-consuming techniques with rapid ones, which undoubtedly is the case with the BCM. However, there is less...
agreement between BCM and ME in detecting parasites other than *Trypanosoma* species. This work indicates that the present method is more reliable in the detection of Babesia and *Trypanosoma* infections. This technique could also be used for the detection of *Theileria, Anaplasma*, and *Ehrlichia* infections under LM and FLM, but it is not recommended for *Theileria, Anaplasma* and *Ehrlichia* species diagnostics using DFM. This conclusion is significant for clinical and field work when infected animals should be rapidly screened for the assessment of treatment and prevent the complications of the infections in an endemic area. Accurate and quick diagnosis represents the essential weapon in the fight against parasitic infections [7]. This method is a simple and rapid diagnostic tool and can be used at every laboratory with a simple LM microscope and microhematocrit centrifuge equipment. The main modification of the present BCM used for diagnoses of hemoparasites is that the new protocol does not require specialized capillary tube, ordinary microhematocrit tube was used after adding acridine orange stain to blood sample and concentration with high speed centrifugation at 12,000 rpm for two minutes instead from 6000 rpm for 5 minutes with commercial QBC. The modified BCT is based on using only the staining buffy coat wet preparations with the fluorescent vital stain acridine orange (AO) to make parasites more visible in blood samples and to improve the image contrast, as exemplified by the QBC technique. In a comparison with previous modifications of buffy coat method, the protocols used for the detection of malaria in human [19, 24] was applying capillary tubes containing acridine orange stain and required fluorescence microscopy for parasite observation, several fluorochrome particularly the nucleic acid binding stain acridine orange has been used for the rapid detection of Apicomplexa, Rickettsia, and *Trypanosoma* parasites in blood smear using fluorescence microscopy [25,26,27]. In the current study; a conventional laboratory microscope (LM) is suitable to permit quick diagnosing of the parasites. *Babesia, Theileria, Anaplasma, Ehrlichia*, and *Trypanosoma* parasites were detected easily in AO staining buffy coat using the light microscopic examination because the parasites are rapidly absorbed the stain and appeared with luster inside or outside red blood cells which facilitate the rapid detection of parasites in even cases with low parasitaemia in the comparison to the Giemsa method. Detection of *Trypanosoma* parasite depends on the volume of the blood sample examined. Centrifugation of blood samples with high speed cause separation of the buffy coat, where most trypanosomes concentrate [28], and their detection is mostly dependent on their motility because they are translucent and colorless [15].

Staining of different forms of *Babesia* and *Trypanosoma* parasites exhibits light scattering when BCM wet preparations are viewed by dark-field microscopy due to their brightly illuminated appearance. Dark-field microscopy fails to detect *Theileria, Anaplasma,* and *Ehrlichia* infections in modified BCM preparations mainly because some of these hemoparasites are immotile and most of them are intracellular which remain in the erythrocyte layer beneath the buffy coat [29]. Using light microscopy, Babesia merozoites were easily visible moving inside and outside RBCs for the first time in BCM preparations of cattle blood samples. Some aspects of the process of *B. divergens* and *B. bovis* invasion in humans have been previously observed in culture medium by optical, fluorescent and vector electron microscopy [30,31]. Egress and invasion are important processes in the *Babesia* life cycle, where the merozoites are vulnerable and can be a very good therapeutic target. Thus transition was made from the dry and silent blood smear to parasite kinematic dynamics. These improved techniques will greatly facilitate epidemiological studies, as well as studying these parasites from many aspects, such as following up on the effect of different treatments on them and their different stages inside and outside red blood cells. In addition to studying some aspects of the pathogenesis and immunology of these parasites. Some previous studies indicated that the buffy coat method is highly sensitive in detecting human malaria parasites [18,31,32,33]. Whereas others showed a low sensitivity [34,35]. Adeoye & Nga [14] suggested that it is a valuable protocol in early human malaria therapeutic interposition owing to there duced time of diagnostics. The present work supports this conclusion, mainly because modified BCM can provide possibility to rapid diagnosis of hemoparasites, permits starting of treatment and/or control measures to be taken as soon as possible and to prevent further spread of hemoparasites infections.
Conclusion

Modified BCM is a sensitive and recommended method which is quick and reliable in detecting Babesia and Trypanosoma parasites in blood samples of cattle using light, fluorescent, and dark-field microscopy during fieldwork. BCM is a helpful method in detecting hemoparasites with low parasitemia. Its application can be drawn-out to studies of hemoparasites in other animals through field conditions.

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Competing interests

The authors declare that they have no competing interests.

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USES OF MODIFIED BUFFY COAT METHOD AS A TOOL FOR DIAGNOSIS OF BOVINE...


استخدام طريقة المعطف المعدلة كوسيلة للتشخيص المجئي لطفيليات دم الأبقار

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فرع الطب الباطني والوقائي - كلية الطب البيطري - جامعة الموصل - الموصل - العراق.

تمكنت طريقة المعطف المعدلة باستخدام أنابيب ميكروهيماتوكتر وصبغة أكردين البرتقالي من التشخيص السريع (10 دقائق/عينة) والدقيق لطفيليات الدم الشائعة في الأبقار، مقارنة بالفحص المجهري لمسح الدم الجافة المصبوغة بكيمزا. تم تشخيص طفيليات البابيزيا، الثايليريا، والإيرليخيا، والإلاغفا، والابنابلازما على الكشف بسهولة عن المراحل المتحركة وغير المتحركة لطفيليات الدم بوساطة المجهر الضوئي. بالمثل، كما وفر المجهر المقلّ للكشف عن وجود أعداد صغيرة جداً من الطفيليات التي لا يمكن كشفها بوساطة الفحص المجئي التقليدي. وفقًا لمؤشر كاهن كابا، فإن الاتفاق بين التكنولوجيا في تشخيص طفيليات دم البقر تحت المجهر الضوئي كان مثاليًا لطفيلي التريبانوسما (1000)، وحقيقي في الأ반ابلازما (0.875) (0.05). تمكنت تقنية المعطف الأبيض المعدلة من اكتشاف جميع الحالات الإيجابية (100%) من عدوى البابيزيا والإيرليخيا والثايليريا باستخدام المجهر الضوئي والمقلّ. يمكن إجراء تقنية المعطف المعدلة باستخدام صبغة الأكردين الرتيني بسهولة وبتكلفة منخفضة باستخدام مجهر ضوئي عادي. أظهرت طبقية المعطف المصفوفة (BCM) المحمولة (BCM) من طبقة المعطف المصقلة تأثيرها إيجابيًا لتشخيص العدوى المختلطة لطفيليات الدم ويمكن استخدامها كأسلوب بديل للمسحة الجافة المصبوغة بصبغة الكيمزا للفحص السريع لعينات دم البرق تحت المجهر الضوئي.

 cầm سارة محمد حامد 1 و باسمة عبد الفتاح البدراني 2

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