Serological and molecular diagnostic surveys combined with examining hematological profiles suggests increased levels of infection and hematological response of cattle to babesiosis infections compared to native buffaloes in Egypt

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Abstract

Background: Babesiosis threatens the development of the cattle and buffaloes industries in Egypt and improved control is needed. The main objectives of this study are surveying the presence of bovine babesiosis in distinct selected bovine and buffalo populations in Egypt using novel molecular and previously validated serological methods, while also comparing the occurrence of hematological alterations among Babesia infected cattle and buffaloes.

Methods: A total of 253 and 81 blood samples from apparently healthy cattle and buffaloes, respectively, were randomly collected from diverse locations in Egypt. All samples were tested for Babesia bovis and B. bigemina infection using blood film examination, competitive ELISA (cELISA) and PCR. Novel semi-nested and nested PCR assays for the detection of B. bovis and B. bigemina respectively, were developed and used to analyze DNA extracted from bovine and buffalo samples. Hematological profiles were studied using a hematological analyzer.

Results: Blood films examination revealed 13.8 % and 7.4 % Babesia infection rates in cattle and buffaloes, respectively. However, in cattle, the cELISA detected 32.8 %, 21.3 % and 10.7 % infection rates with B. bigemina, B. bovis and mixed infection, respectively. In addition, cELISA identified 22.2 %, 22.2 % and 6.2 % infection rates with B. bigemina, B. bovis and mixed infection, respectively in buffaloes. The semi-nested PCR assay showed that 15 % of the tested samples were positive for B. bovis in cattle, but just 3 % in buffaloes. Infections with B. bigemina were also found in cattle (32.4 %), but not in buffaloes upon nested PCR analysis. Sequencing analysis confirmed the identity of the PCR amplicons and showed that Egyptian genotypes of B. bigemina and B. bovis highly resemble sequences previously deposited in GenBank. Hemograms performed on the sampled animals revealed macrocytic hypochromic anemia associated with reduced platelet counts in infected cattle with babesiosis. In addition, marked increases in total leukocyte and granulocytic counts and decreases in lymphocytic counts were found in infected cattle. In contrast, no such hematological anomalies were found in presumably Babesia-infected buffaloes.

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Background

Babesiosis is caused by intraerythrocytic protozoan parasites of the genus *Babesia* that infect a wide range of domestic and wild animals and occasionally humans [1, 2]. Bovine babesiosis is the most significant tick-borne disease of cattle worldwide threatening approximately 1.3 billion animals in vast areas of Asia, Africa, Australia, Central and South America, and Southern Europe [3]. The disease has the potential to cause large economic and sanitary disruptions compromising the livestock industry all over the world [4].

In Egypt, bovine babesiosis is caused mainly by *Babesia bovis* and *Babesia bigemina*. *B. bovis* parasites are known to be transmitted in this country by *Rhipicephalus microplus* whereas *B. bigemina* is known to be transmitted by both *R. microplus* and *Rhipicephalus annulatus* [5, 6]. Because of its significant economic impact on meat and milk production and on livestock management, it is currently considered as the most important endemic parasitic disease affecting cattle in Egypt [5]. Both *B. bigemina* and *B. bovis* are capable of causing acute disease resulting in life-threatening hemolytic anemia as well as other severe clinical manifestations [7, 8]. Often, especially in bovines younger than a year old, acute disease resolves into persistent disease and the animals become carriers of the parasites, thus ensuring transmission by competent ticks in zoonotic areas. In addition to hemolytic anemia, bovine babesiosis is known to cause other blood disorders including thrombocytopenia [9]. However, there is a paucity of information on the overall impact that these hematological disorders may have in the development of cattle and buffalo industries in Egypt. Assessing hematological disorders can contribute to our better understanding of the pathogenic mechanisms of the disease, but these data can also become a predictor for recovery and wellbeing for the animals in endemic areas, thus this information can be valuable for gauging efficiency upon the application of measures for the control of the disease. Previous studies suggest that there are large numbers of cattle in Egypt infected with subclinical babesiosis [5]. In addition, although clinical evidence suggests that buffaloes are likely more tolerant to *Babesia* infections [10], the hematological response of buffaloes to *Babesia* infection in Egypt remains poorly investigated [10, 11].

Accurate diagnosis of babesial infections plays an important role in monitoring, management and control [12]. A large diversity of diagnostic techniques including blood smear examination, serological tests, and molecular (DNA-based) assays exist currently, yet, each of these methods has limitations. It is increasingly recognized that accurate diagnosis requires a combination of distinct approaches. Blood smear examination is often considered to be the standard technique for routine diagnosis of babesiosis, particularly in acute cases, and it is considered by some as a gold standard [13]. It is less time consuming than most other methods and is relatively inexpensive [14]. However, the examination of the stained blood smears is insufficient for accurate detection and identification of *B. bovis* and *B. bigemina* during mixed infections and in particular for the detection of carrier cases or subclinical infections with low parasitemia [15].

The serological tests, including the indirect fluorescent antibody test (IFAT) and the enzyme-linked immunosorbent assay (ELISA) are capable of detecting antibodies in carrier animals; therefore they are often used for monitoring surveillance and export certification [16]. Moreover, specific and sensitive competitive ELISA (cELISA) methods have been developed for *B. bovis* and *B. bigemina*, and their use has been validated in several diagnostic laboratories around the world [17–19]. Drawbacks of serological tests include that antibodies can still be detected even years after recovery of infection though no active infection is prevalent, so these methods cannot help in revealing the exact picture of prevalence of infection at that particular point [20]. Nevertheless, the use of serological methods in conjunction with molecular methods aimed at detecting the *B. bovis* and *B. bigemina* DNA in the blood of infected animals can provide a more complete representation of the incidence of bovine babesiosis in endemic areas.

Most of the DNA based methods employed in the diagnosis of *B. bovis* and *B. bigemina* babesiosis are based on standard polymerase chain reaction (PCR) techniques [21, 22]. Appropriate targets for PCR assays include well conserved and species specific genes. PCR techniques permit the identification of parasites at levels far below those identified by the commonly used conventional parasitological techniques for *B. bovis* and *B. bigemina* [20], which is critical for the diagnosis of subclinical and latent infection. Consequently, DNA amplification for the diagnostic detection of babesiosis is considered as a powerful tool both in the early phase of
infection and in carrier animals. PCR results for *Babesia sp.* can be rapidly made available and as few as three parasites in 100 µl of blood can yield a positive result [23]. Additionally, the sensitivity of a PCR assay can be increased several fold by performing a semi-nested (snPCR) or nested PCR (nPCR), where two different sets of amplification primers are used [2]. This feature not only improves the sensitivity of the assay, but also confirms the specificity of the first round product [24].

The aim of the present study is to estimate the presence of bovine and buffaloes babesiosis in distinct populations in Egypt using blood smear examination, competitive inhibition ELISA and novel PCR procedures based on the amplification of the recently identified and highly conserved rhoptry associated protein (rap)-1c gene of *B. bigemina* [25] and rap-1 related antigen (rra) gene of *B. bovis* [26]. We also compared *Babesia*-associated hematological alterations in the cattle and buffalo populations sampled in this study.

**Methods**

**Collection and processing of blood samples**

Blood samples were collected from clinically healthy 253 cattle and 81 buffaloes examined for diagnosis of babesiosis from Kafr El-Sheikh, El-Behaira and Cairo governorate. The blood collected without anti-coagulants were processed for serum. Sera were stored in -20 °C until used in serological tests. The blood collected with anti-coagulant EDTA (ethylenediaminetetraacetic acid tripotassium) was used for PCR and hematology. For PCR, blood was washed three times with PBS pH 7.2 and stored at -20 °C. Blood from *B. bigemina* experimentally infected animals used in the PCR comparative studies was kindly provided by Dr. Massaro Ueti (ADRU-USDA), and the *B. bovis* Mo7 experimentally infected samples were derived from a previously published experiment [27]. The protocol of infection and all animal handling, was approved by the Institutional Animal Care and Use Committee, IACUC, Washington State University (#03735-008 approved on 12/9/2009).

**Analysis of samples**

**Blood film examination**

Blood smears stained with DIFF-3 Rapid Staining kit were prepared from all animals included in this study and examined by light microscopy at 1000x magnification.

**Competitive ELISA**

The *B. bovis* and *B. bigemina* Antibody Test Kits (cELISA) were kindly donated by VMRD Inc. (Pullman, WA, USA). Optical density (O.D.) values were determined at 650 nm using EL-X 800 Universal microplate reader Bio-TEK instruments, INC, USA. The results were expressed as a value of the percent inhibition (%I) according to the following formula:

\[
\%I = 100 - \left( \frac{\text{sample O.D.}}{\text{mean negative control O.D.}} \right) \times 100
\]

Samples were classified as positive if the %I value was above 40 % and negative if the %I value was less than 40 %. These cut-off values were established by the manufacturer of the kit.

**Extraction of DNA**

The DNA was extracted from the blood samples according to the instructions of the manufacturer using FTA® Elute cards, Whatman Cat. No.WB120410.

**Novel PCR methods for the molecular diagnostic of bovine and buffalo babesiosis**

Selection of the *B. bovis rra* and *B. bigemina rap-1c* genes as targets for the development of novel PCR molecular diagnostic tools for the detection of bovine babesiosis was based on their high degree of species-specific conservation among distinct strains, and their limited levels of expression in merozoites [25, 26]. Semi-nested and nested PCR techniques were developed to amplify regions of the *rra* gene [26] (GenBank accession number: XM_001610900) from *B. bovis* DNA and the *rap-1c* gene [25] (GenBank Accession number: AY146983.1) from *B. bigemina* DNA. Sequences derived from the *rra* (bp 114-937) and *rap-1c* (bp 1-602) genes were amplified from *B. bovis* and *B. bigemina* genomic DNA using the set of primers described in Table 1, and cloned into pCR® 2.1-TOPO™ (Life technologies) plasmids. Taking into consideration the size of each resulting plasmid, it was estimated that 1 ng of *B. bovis rra* 2.1-TOPO plasmid contains 2.1 × 10⁸ molecules of the target gene and 1 ng of *B. bigemina rap-1c* 2.1-TOPO plasmid contains 2.2 × 10⁸ molecules of the target gene. To estimate the sensitivity of the assays, we performed serial dilutions of known amounts of plasmid that we then amplified by PCR using the protocol described below. The presence and size of the final PCR products was determined in 1.5 % agarose gels and stained with SYBR Safe (Invitrogen). All PCR amplicons were sequenced for confirming specificity. PCR reactions were performed in a final volume of 25 µl containing (12.5 µl JumpStart RED Taq Ready Mix PCR reaction mix (Sigma-Aldrich), 10 pmol of each primer and 9.5 µl water). All primers were synthesized by Integrated DNA technologies Inc. (California, USA). 2 µl of template DNA was used for the primary PCR. The snPCR and nPCR utilized 0.5 µl of primary PCR product as template.

The thermo-cycling conditions used for the *B. bovis* primary PCR were: 95 °C for 3 min followed by 25 cycles, each consisting of denaturation at 94 °C for 30 sec,
Table 1. Sequences of the oligonucleotide primers used in the PCR assays and expected PCR product sizes

| Parasite      | Primer name | Sequence                 | Product Size | Source gene and reference |
|---------------|-------------|--------------------------|--------------|---------------------------|
| Babesia bovis | BoF         | 5’ ATTGCGATCTGGGCTAAAGTG 3’ | 823 bp       | B. bovis Rhoptry associated protein related antigen (rap-1c) gene |
|               | BoR         | 5’ CAGCCCATTACAGGTTTT 3’ |              |                           |
|               | BoNF        | 5’ TGGTCTGAGCCGCTATCTT 3’ | 387 bp       |                           |
|               | BoNR        | 5’ CAGCCCATTTACAGGTTTT 3’ |              |                           |
| Babesia bigemina | BIF        | 5’ ATGATTCACTACGCTTGCCTC 3’ | 236 bp       | B. bigemina Rhohtyp associated protein (rap-1c) gene |
|               | BiR         | 5’ GTCTGTATATATGGCGGCATG 3’ |              |                           |
|               | BiNF        | 5’ TCTCGAAGACAGCGAACAGA 3’ |              |                           |
|               | BiNR        | 5’ GTGAAAGCTGTTAGGGGTC 3’  |              |                           |

The primer sets used for the primary reaction were: BoF and BoR for the amplification of the rra gene of B. bovis, and BIF and BiR for the amplification of the rap-1c gene of B. bigemina. The primer sets used for semi-nested PCR reaction were: BoNF and BoNR for amplification of the rra gene of B. bovis and BiNF and BiNR for amplification of the rap-1c gene of B. bigemina.

annealing at 60 °C for 1 min, and extension at 72 °C for 1 min. The program also included a final extension step at 72 °C for 5 min. For the semi-nested PCR the same conditions were used except the number of cycles used was 35.

The thermocycling conditions used for the B. bigemina primary PCR consisted of: 95 °C for 3 min followed by 25 cycles, each consisting of denaturation at 95 °C for 30 sec, annealing at 61.2 °C for 30 sec, and extension at 72 °C for 30 sec, followed by a final extension step at 72 °C for 5 min. The thermocycling conditions used for the B. bigemina semi-nested PCR were: 95 °C for 3 min followed by 35 cycles, each consisting of denaturation at 95 °C for 30 sec, annealing at 63.1 °C for 30 sec, and extension at 72 °C for 30 sec, followed by a final extension step at 72 °C for 5 min.

The B. bovis and B. bigemina genomic DNA derived from cultured T3Bo B. bovis and Puerto Rico B. bigemina strains respectively used as positive controls were provided by the Animal Disease Research Unit, Agricultural Research Service, USDA, WSU, Pullman. A negative control with no DNA template was always included for PCR amplification. Bovine DNA extracted from blood from non-infected calves was also used as a control for the nested and semi-nested PCRs. Amplified DNA samples were electrophoresed on 1.5 % agarose gels and stained with SYBR Safe. The length of the amplified products was estimated using a 100 base pair (bp) DNA ladder and the amplified products were visualized with an UV trans-illuminator (Bachofer D7410) and photographed using gel documentation system (BioDoc-Analyze, Biometra Analytic GmbH). Amplicons presenting visible unique bands of approximately 387 base pairs (bp) and 236 bp in gel electrophoresis analysis were considered positive for B. bovis rra and the B. bigemina rap-1c genes, respectively. The relative sensitivities of the B. bovis and B. bigemina nested and semi-nested-PCR reactions of the tests based on the B. bovis rra and the B. bigemina rap-1c genes were compared with standardized nested PCR tests described previously [28]. The two new PCR methods were initially tested on gDNA extracted from B. bovis and B. bigemina in vitro cultures and from B. bovis and B. bigemina experimentally infected calves in order to compare their performance with previously standardized nested PCR methods for the detection of B. bovis and B. bigemina in bovine samples by Figueroa et al. 1993 [28]. The previously reported nested PCR for B. bovis, based on the rap-1 gene as the target of amplification [28] was compared with a semi-nested PCR targeting the B. bovis rra gene, using serial dilutions of genomic DNA extracted from in vitro cultured B. bovis parasites from the T3Bo strain. Similar comparative analysis was performed for the novel rap-1c gene based nested PCR for the detection of B. bigemina.

Sequencing of PCR products

The PCR products were purified for sequencing using the QIA quick Spin PCR Purification kit (Qiagen, Courtaboeuf, France) according to the manufacturer’s instructions. Sequencing reactions were performed using oligonucleotide primers that were used in the PCR (Table 1). Sequencing of the PCR products was performed by the GATC Company with an ABI 3730xl DNA sequencer, using the nested forward and reverse primers. Each sequencing reaction was repeated three times in both the forward and reverse directions before being accepted for analysis. Sequences derived from Babesia sp. were assembled using ChromasPro 1.49 beta (Technelysium Pty. Ltd., Tewantin, QLD, Australia).

Hematological investigations

Complete blood analysis was performed on 133 blood samples (107 cattle and 26 buffaloes) from the three Egyptian governorates using a hematological analyzer (MEDONIC CA620, Sweden). The criteria for the selection of the blood samples for hematological examinations were based on: breed consistency; cross-bred cattle and water buffaloes; gender (all animals studied...
are male); and age range (all animals tested are between 2-3 years old). The analysis included erythrocyte count (RBC), hematocrit (HCT), hemoglobin (Hb) concentration and red cell indices, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width absolute (RDWA), red cell distribution width percentage (RDW%), leukogram (including white blood cell count (WBC), lymphocytes, granulocytes and mid cells (MID) and platelet count (PLT)), mean platelet volume (MPV), platelet distribution width (PDW), plateletcrit (PCT), and large platelet concentration ratio (LPCR).

**Statistical analysis**

All data were subjected to statistical analysis including the calculation of the mean and standard error. Differences between infected groups detected by different techniques and non-infected groups were tested for significance using a one-way analysis of variance followed by Duncan’s multiple range test. Differences were considered significant at \( P < 0.05 \) level [29] using SPSS software version 15.

**Results**

**Diagnosis of bovine babesiosis using direct blood film microscopic examination**

We examined a total of 253 cattle and 81 buffaloes’ microscopic stained slide samples. Because of the difficulties in objectively providing differential diagnosis among *B. bovis* and *B. bigemina* parasites using this method, added to the possibility of frequent double *B. bovis* and *B. bigemina* infections in bovines, we decided to report our microscopic examination findings as “*Babesia* sp.” Direct microscopic examination resulted in the detection of *Babesia* infected erythrocytes in 13.8 % and 7.4 % samples of cattle and buffaloes respectively.

**Serological diagnosis of bovine and buffalo babesiosis using cELISA**

We performed cELISA tests for the detection of *B. bovis* and *B. bigemina* antibodies on serum samples obtained from the 253 bovines and 81 buffaloes as described above. The cELISA data performed on cattle sera (Table 2) revealed 32.8 %, 21.3 % and 10.3 % infection with *B. bigemina*, *B. bovis* and mixed infection, respectively. The cELISA test performed on buffaloes sera (Table 3), detected 22.2 %, 22.2 % and 6.2 % infection with *B. bigemina*, *B. bovis* and mixed infection, respectively.

**Molecular detection of *B. bovis* and *B. bigemina* in infected bovines and buffaloes using PCR**

We first determined the optimal conditions and sensitivity of nested and semi-nested PCR reactions for the detection of *B. bigemina* and *B. bovis*, respectively. When these two methods were compared using plasmid DNA encoding for the target genes, both reactions have similar levels of sensitivity, and are able to clearly detect down to 0.1 fg for the *B. bigemina* rap-1c and *B. bovis* rra genes (Fig. 1a and b) using nPCR and snPCR respectively. In addition, the performances of the novel rra based semi-nested PCR and rap-1c tests were compared with previously established *B. bovis* rap-1- based nPCR and *B. bigemina* nPCR tests respectively, before applying them for the analysis of the field samples. Results on the application of the rap-1 nested PCR on genomic DNA (gDNA) from *in vitro* cultured parasites are shown in the upper panel of Fig. 2a, and results for the rra semi-nested PCR are shown in the lower panel. Both tests are capable of producing PCR products reliably down to at least 5×10^{-7} ng of gDNA, and so, they appear to have comparable sensitivity. Importantly, the rra-based PCR method generates single PCR final products upon gel analysis. When similar comparisons were performed using 10 fold serial dilutions of gDNA extracted from persistently and experimentally *B. bovis* Mo7 strain infected bovines with unknown parasitemia (less than 0.5 % PPE) (Fig. 2b), both tests again appear to perform similarly and to have a comparable level for detection of *B. bovis* DNA. In addition, sequencing of the PCR products of 291 bp (rap-1 targeted PCR) and 387 bp (rra targeted PCR) demonstrated the specificity of the amplicons as true rap-1 and rra sequences. Importantly, none of the tests amplify bovine gDNA (data not shown), the sequences of the rra gene were previously shown to be identical among the T3Bo and the Mo7 strains [26], and sequencing demonstrates that the novel PCRs only amplify their expected target genes. Taking together the comparisons of both PCR tests performed on DNA extracted from *in vitro* grown cultures and *in vivo* *B. bovis* infected calves, suggests that both tests have similar performances in terms of sensitivity and specificity. However, the rra-based test seems to have a slight advantage in terms of specificity since it invariably generated a single product upon PCR.

The data shown in the upper panel of the gel in Fig. 3a depicts nested PCR amplification using the rap-1c primer sets performed on 5 ng of *in vitro* cultured *B. bigemina* gDNA, where lanes 2-9 represent identical PCR amplifications performed on serial 10-fold dilutions from this material. The lower panel of the gel shows the data obtained from nested PCR amplifications performed on identical ten-fold dilutions *in vitro* cultured infected erythrocytes, but using the previously described by Figueroa *et al* primer set [28]. All experiments were performed in duplicate. Overall, the data suggests that both PCR methods have similar limits for the detection of *B. bigemina* in *in vitro* cultured parasites, consistently
Table 2: Results of cELISA and PCR tests for the diagnosis of *B. bovis* and *B. bigemina* performed on sera from cattle from Kafr El-Sheikh, El-Beheira and Cairo governorates in Egypt.

| Governorate  | ELISA                  | PCR                   |
|--------------|------------------------|-----------------------|
|              | *B. bigemina*          | *B. bovis*            | *B. bigemina and B. bovis* | *B. bigemina* | *B. bovis* | *B. bigemina and B. bovis* |
|              | No. of positive animals | Infection (%)         | No. of positive animals | Infection (%) | No. of positive animals | Co-infection (%) | No. of positive animals | Infection (%) | No. of infected animals | Infection (%) | No. of infected animals | Co-infection (%) |
| Kafr El-Sheikh | 147                    | 47                    | 31.9                  | 38            | 25.9                  | 18                  | 12.2                  | 64            | 43.5                  | 33            | 22.4                  | 17                  | 11.6                  |
| El-Beheira    | 88                     | 31                    | 35.2                  | 10            | 11.4                  | 3                   | 3.4                   | 0             | 0                    | 2             | 2.3                   | 0                   | 0                    |
| Cairo         | 18                     | 5                     | 27.8                  | 6             | 33.3                  | 5                   | 27.8                  | 18            | 100                  | 3             | 16.7                  | 5                   | 27.8                  |
| Total         | 253                    | 83                    | 32.8                  | 54            | 21.3                  | 26                  | 10.3                  | 82            | 32.4                  | 38            | 15.0                  | 22                  | 8.7                   |
Table 3 Results of cELISA and PCR tests for the diagnosis of *B. bovis* and *B. bigemina* performed on sera from buffalo sera from Kafr El-Sheikh and El-Beheira governorates in Egypt

| Governorate      | No. of tested animals | ELISA   |         |         |         |         |         |         |         |
|------------------|-----------------------|---------|---------|---------|---------|---------|---------|---------|---------|
|                  |                       | *B. bigemina* | *B. bovis* | *B. bigemina* and *B. bovis* | PCR | *B. bigemina* | *B. bovis* |
|                  |                       | No. of positive animals | Infection (%) | No. of positive animals | Infection (%) | No. of positive animals | Co-infection (%) | No. of positive animals | Infection (%) | No. of infected animals | Infection (%) |
| Kafr El-Sheikh   | 7                     | 5       | 71.4    | 3       | 42.9    | 2       | 28.6    | 0       | 0       | 2             | 28.6           |
| El-Beheira       | 74                    | 13      | 17.6    | 15      | 20.3    | 3       | 4.1     | 0       | 0       | 1             | 1.4            |
| Total            | 81                    | 18      | 22.2    | 18      | 22.2    | 5       | 6.2     | 0       | 0       | 3             | 3.7             |
detecting up to $5 \times 10^{-4}$ ng of gDNA. We also compared the performance of these two *B. bigemina* nPCR amplification methods on an identical set of target genomic DNAs extracted from a *B. bigemina* infected bovine with an unknown parasitemia. None of these PCR methods generated PCR products when used on purified bovine gDNA and sequencing of the nPCR products of 236 and 170 bps confirmed specificity of the assays. However, the data in Fig. 3b shows that the PCR products generated using the *B. bigemina rap-1c* nPCR primer set on the DNA extracted from the experimentally infected animals were better visualized than the previously published *B. bigemina* primers set [28]. Overall, the two PCR procedures tested for the amplification of *B. bigemina* DNA showed similar levels of sensitivity and specificity. Yet, because better PCR product visualization in gels was obtained using the *rap-1c*-based PCR method, we decided to use this method for the molecular detection of *B. bigemina* field samples derived from cattle from distinct areas in Egypt, as described below.

The *rap-1c* *B. bigemina* and *rra* *B. bovis* based PCR methods described above were used to detect the presence of *B. bigemina* and *B. bovis* on total genomic DNA blood samples of cattle (n = 253) and buffaloes (n = 81). Fifteen percent of the cattle samples and 3.7 % of the buffaloes samples tested were found positive for *B. bovis* using snPCR. The percent of infection with *B. bigemina* calculated in cattle using nPCR was 32.4 %, whereas none of the buffalo samples were positive in nPCR for *B. bigemina*. In addition, mixed infection with *B. bigemina* and *B. bovis* was found in 8.7 % of cattle samples (Tables 2 and 3).

Sequencing of the 236 nucleotides long nPCR amplicons originating from the samples positive for *B. bigemina* revealed 100 % identity with the expected *B. bigemina rap-1c* gene of the strain S1A. In addition, the 283 nucleotide snPCR products positive for *B. bovis* were also 100 % identical to the sequence in the *B. bovis rra* gene. Sequences originating from the Egyptian positive samples were deposited in GenBank with accession number: KM212998 for *B. bigemina*, and KM213000 for *B. bovis*.

**Hematological findings**

The data for the hematological analysis performed on control non-infected and naturally *Babesia* sp.-infected cattle and buffaloes detected by different techniques are illustrated in Tables 4 and 5.

Compared to the control non-infected group, there was a significant decrease in the mean levels of RBCs, HCT%, Hb and MCHC in the samples derived from *Babesia* sp.-infected cattle, regardless of the method of detection (blood film, cELISA, PCR, or all of them combined). In contrast, MCV values were found increased in the infected group compared to the control group regardless of the method of detection. The result suggests that *Babesia* caused macrocytic hypochromic anemia in the persistently infected animals. In addition, the *Babesia* sp.-infected animals had, on average, significant reduced platelet counts (Table 4).

In infected groups of cattle with *Babesia* sp., as detected by blood film, total leukocyte count (WBCs) and granulocytic counts (neutrophils, eosinophils and basophils) were markedly increased compared to the control non-infected group. On the other hand, there was a decrease in lymphocytic count in infected groups with *Babesia* sp. detected by PCR and all other diagnostic methods (Blood film + cELISA + PCR) compared to the control group. Additionally, no significant changes in the count of MID cell were recorded in all groups (Table 4).

Interestingly, no significant differences were found in any of these blood parameters among buffaloes naturally infected with *Babesia* sp. and the control non-infected group (Table 5).
Discussion

Bovine and buffalo babesiosis remains neglected in Egypt, since its incidence and overall impact remains poorly assessed, and no preventive control measures, including the use of attenuated vaccines, are applied currently.

Our direct blood film microscopic examination data is generally consistent with previous reports of the presence of bovine babesiosis in distinct regions in Egypt, such as 12.5 % in Cairo [5], 11.1 % in Gharbia Governorate [30], 13 % in Giza Governorate [31], 13 % in Port Said Governorate [32]. Abd-El-Gawad [33] and Mazyad and Khalaf [34] reported 9.9 % in Beni-Suef Governorate and 8.1 % in North Sinai Governorate.

Competitive ELISA is an adequate serological tool for the epidemiological surveillance of the spread of bovine babesiosis, as it can be easily standardized, is less subjective and less time-consuming than the traditionally used IFAT. In addition, cELISA has the potential to display higher specificity than an indirect ELISA [35]. In
the current study, the cELISA assays detected a higher number of infected animals than the PCR assays; this could be explained by differences in the timing of the parasite presence and the antibody responses in the infected animals as well as the stage of infections [36, 37]. Consistently, similar studies such as those by Ibrahim et al. [38], Silva et al. [39] in Portugal, Terkawi et al [40] in Thailand and Dominguez et al [35] in Argentina, recorded higher babesiosis infection rates when using ELISA assays compared to that using nPCR methods. Yet,
the combination of serologic testing and PCR is considered to offer the greatest sensitivity for babesiosis diagnosis especially through the sub-clinical phase.

Results in the current study, suggests that *B. bigemina* infections in bovines are more frequent than *B. bovis*, which is consistent with previous surveys performed in other locations in Africa. Furthermore, a similar finding has been observed by Ibrahim *et al* [38], who reported that *B. bigemina* is more prevalent than *B. bovis* in Egypt. It is possible that *B. bigemina* is more prevalent than *B. bovis* due in part to the pattern of geographic distribution of the *R. annulatus* tick vectors [41], added to the fact that the prevalence of *B. bigemina* is higher than *B. bovis* in this vector in Egypt [5]. We also found a significant number of animals that were co-infected with *B. bovis* and *B. bigemina*. This is not surprising, since similar co-infections are commonly reported in endemic areas, and is in part justified by both parasites sharing the same tick vectors. However, transmission of these two parasites by *Rhipicephalus* sp. ticks is by different mechanisms. While *B. bovis* is transmitted by the larval stage of the ticks, *B. bigemina* is transmitted by the nymph tick stages. Some previous reports suggested that priming the bovine immune system by infection with *B. bigemina* could protect animals of future co-infections with *B. bovis*. Because ticks can also be co-infected, it can be speculated that co-infected animals receive both parasites simultaneously, before they were able to mount cross-protective immune responses that would prevent super-infections [42].

In this study we developed novel snPCR and nPCR methods based on the recently described *B. bovis rra* gene [26] and *B. bigemina rap-1c* gene [25], respectively. Sequence comparisons demonstrated that these two genes are highly conserved among distinct strains worldwide, and, importantly, as these two genes are species-specific, they may be better candidates for PCR-based diagnosis of bovine babesiosis. In addition, these two genes generate transcripts in the blood stages of the parasites in a tightly regulated fashion, yet they appear to be expressed in minute amounts [25, 26]. This feature, at least in theory, minimizes the possibility for sequence variation due to immune pressure, and further supports the use of these two genes as ideal targets for developing PCR reactions with diagnostic applications. Additionally, comparisons performed using the new tests with the  

| Groups Parameters | Control | Non-infected | Method for the detection of Babesia sp. used in cattle | Sig. |
|-------------------|---------|-------------|------------------------------------------------------|------|
|                   | No. of Animals | 23 | 5 | 28 | 43 | 8 |
| Red blood cell count (x10^6/μl) | 7.09 ± 0.18a | 6.03 ± 0.10bc | 6.31 ± 0.14b | 5.45 ± 0.14a | 6.13 ± 0.19bc | *** |
| Hematocrit (%) | 27.73 ± 0.32a | 25.34 ± 0.41ab | 25.58 ± 0.44ab | 24.10 ± 0.59b | 23.90 ± 0.96b | *** |
| Hemoglobin (g/dl) | 11.16 ± 0.23a | 9.14 ± 0.49ab | 9.00 ± 0.13b | 8.42 ± 0.19b | 8.48 ± 0.32b | *** |
| Mean corpuscular volume (fl) | 39.53 ± 0.86b | 42.08 ± 0.95ab | 40.87 ± 0.78b | 44.43 ± 0.60a | 38.95 ± 0.76b | *** |
| Mean corpuscular hemoglobin (pg) | 15.86 ± 0.35a | 15.19 ± 0.91ab | 14.40 ± 0.22bc | 15.53 ± 0.19ab | 13.82 ± 0.29f | *** |
| MCHC (mg/dl) | 40.20 ± 0.58a | 36.03 ± 1.59a | 35.26 ± 0.21b | 34.97 ± 0.09b | 35.48 ± 0.21b | *** |
| Red blood cell distribution width (fl) | 31.51 ± 0.73b | 43.74 ± 5.00a | 31.38 ± 1.14b | 33.54 ± 0.69b | 31.66 ± 0.73b | *** |
| Red blood cell distribution width (%) | 21.01 ± 0.42b | 26.96 ± 2.35a | 19.44 ± 0.30ac | 18.20 ± 0.30bc | 19.93 ± 0.36bc | *** |
| White blood cell count (x10^3/μl) | 10.93 ± 0.40bc | 13.48 ± 0.37a | 12.20 ± 0.70ab | 9.63 ± 0.39f | 11.56 ± 0.21abc | ** |
| Lymphocytes (x10^3/μl) | 5.6 ± 0.28a | 6.73 ± 0.54a | 5.58 ± 0.39a | 3.51 ± 0.29b | 3.72 ± 0.71b | *** |
| Lymphocytes (%) | 52.38 ± 1.04a | 50.44 ± 5.05a | 45.77 ± 1.80a | 37.20 ± 2.53c | 32.38 ± 6.29c | *** |
| Granulocytes (x10^3/μl) | 4.08 ± 0.14a | 5.60 ± 0.94ab | 5.52 ± 0.39ab | 5.26 ± 0.30b | 6.90 ± 0.77a | * |
| Granulocytes (%) | 37.89 ± 1.34a | 40.96 ± 5.46a | 44.90 ± 1.76c | 53.63 ± 2.61ab | 59.49 ± 6.42a | ** |
| MID cells (%) | 1.08 ± 0.07a | 1.15 ± 0.06a | 1.10 ± 0.06a | 0.86 ± 0.03b | 0.94 ± 0.05c | * |
| MID cells (%) | 9.73 ± 0.30 | 8.60 ± 0.55 | 9.33 ± 0.46 | 9.17 ± 0.31 | 8.14 ± 0.45 | NS |
| Platelet count (x10^9/μl) | 269.78 ± 14.93a | 232.00 ± 2.86b | 214.75 ± 16.22bc | 158.16 ± 8.40b | 224.63 ± 29.66c | *** |
| Mean platelet volume (fl) | 6.47 ± 0.07a | 6.30 ± 0.09abc | 6.44 ± 0.07ab | 6.17 ± 0.04bc | 6.10 ± 0.09f | * |
| Platelet distribution width (fl) | 10.43 ± 0.12a | 10.14 ± 0.18ab | 10.44 ± 0.12a | 10.00 ± 0.08ab | 9.78 ± 0.12b | ** |
| Plateletcrit (%) | 0.18 ± 0.01f | 0.16 ± 0.00a | 0.14 ± 0.01f | 0.10 ± 0.01b | 0.17 ± 0.02f | ** |

Hematogram values expressed as mean ± SE (Standard error) for each technique. Means followed by different superscripts (a, b, c) within the same row are significantly different at (P < 0.05). Two means that are not follow by the same letter (a, b, or c) are significantly different (P < 0.05). Two means followed with the same letter implies that they are not significantly different. Sig.: represents statistical significance. * P < 0.05, ** P < 0.01, *** P < 0.001. NS = non- significant. MCHC = mean corpuscular hemoglobin concentration.
previously developed nPCR methods, show similar performances in terms of sensitivity and specificity; however, the PCR products are more clearly visualized upon performing both novel PCR tests. Furthermore, searches of the buffalo and bovine genomes demonstrate lack of significant identity of the nPCR primers and full target genes with any bovine or buffalo genes (data not shown), thus diminishing the risk of potential interferences and/or non-specific PCR amplifications. The PCR results obtained regarding the presence of B. bovis in cattle in our study are similar to those of Adam et al [5], Rania [43] and El-Fayomi et al [31] who reported 20 %, 25.33 % and 23 % infection rates in Egypt, respectively. Also, Silva et al [39] in Portugal, Chaudry et al [44] in Pakistan and Longzheng et al [45] in Philippines used nPCR methods for diagnosis of Babesia sp. in cattle, and found infection rates of 78.5 %, 29 % and 18.8 %, respectively. However, percentages of B. bovis infected buffaloes in our dataset, as detected by PCR, were higher than previously published for samples analyzed from other Egyptian herds. These variations in the prevalence rates are expectable due to geographic diversity and other environmental factors that can influence the population densities of the tick vectors [46]. It is well known that increases in temperature and changes in environmental humidity may influence the migration of vectors into new areas, and/or allow significant development of parasites [31, 47].

Interestingly, despite the identification of buffaloes infected by B. bigemina using the cELISA serological method and the presence of infected erythrocytes in the stained slides, B. bigemina infections in buffaloes were not detected by the PCR method. On one hand, these observations suggest that the parasites found in the direct microscopy analysis of the buffalo samples are likely B. bovis. On the other hand, these data also suggests that the numbers of B. bigemina parasites circulating in infected buffaloes may be below the level of detection of the PCR methods, or alternatively, that these B. bigemina infected buffaloes successfully cleared the parasites at the time of the samplings. Consistently, and in contrast to B. bovis, B. bigemina parasites do not sequester in capillaries, and bovines are able to clear B. bigemina faster than B. bovis. Also, these data is in agreement with a recent survey performed in Egypt using a PCR method which failed to identify a single B. bigemina infected

Table 5 Hematological profiles in non-infected and Babesia sp infected buffaloes as detected by different methods

| Groups Parameters | Control Non-infected | Method for the detection of Babesia sp. used in buffaloes | Sig. |
|-------------------|----------------------|----------------------------------------------------------|------|
| No. of Animals    | 10                   | 4                                                        | 10   | 2    |
| Red blood cell count (x10^6/μl) | 7.01 ± 0.38 | 6.49 ± 0.62 | 6.70 ± 0.38 | 7.88 ± 0.24 | NS |
| Hematocrit (%)    | 34.04 ± 1.48         | 31.78 ± 0.88  | 33.22 ± 1.53 | 40.45 ± 4.55 | NS |
| Hemoglobin (g/dl) | 11.32 ± 0.43         | 10.50 ± 0.40  | 10.89 ± 0.50 | 13.20 ± 1.00 | NS |
| Mean corpuscular volume (fl) | 48.81 ± 0.74 | 49.75 ± 2.85 | 50.13 ± 1.75 | 51.25 ± 4.25 | NS |
| Mean corpuscular hemoglobin (pg) | 16.29 ± 0.35 | 16.40 ± 0.80 | 16.42 ± 0.49 | 16.75 ± 0.75 | NS |
| MCHC (mg/dl)      | 33.40 ± 0.34         | 32.98 ± 0.38  | 32.85 ± 0.27 | 32.85 ± 1.25 | NS |
| Red blood cell distribution width (fl) | 20.11 ± 0.43 | 17.58 ± 0.38 | 19.53 ± 0.77 | 20.50 ± 1.50 | NS |
| Red blood cell distribution width (%) | 42.22 ± 0.74 | 40.65 ± 2.65 | 42.96 ± 1.60 | 45.40 ± 3.30 | NS |
| White blood cell count(x10^9/μl) | 8.74 ± 0.30 | 7.20 ± 2.94  | 7.30 ± 0.43 | 7.00 ± 0.70 | NS |
| Lymphocytes (x10^9/μl) | 2.14 ± 0.22 | 1.70 ± 0.58  | 2.34 ± 0.56 | 1.80 ± 0.40 | NS |
| Lymphocytes (%)   | 24.85 ± 2.32         | 28.45 ± 3.84  | 30.95 ± 5.49 | 25.90 ± 3.00 | NS |
| Granulocytes (x10^9/μl) | 5.92 ± 0.28 | 4.85 ± 2.17  | 4.00 ± 0.37 | 4.35 ± 0.45 | NS |
| Granulocytes (%)  | 67.94 ± 2.32         | 62.90 ± 4.10  | 56.60 ± 4.93 | 61.75 ± 0.45 | NS |
| Mid cells (%)     | 0.68 ± 0.03          | 0.65 ± 0.20   | 0.96 ± 0.08  | 0.85 ± 0.15 | NS |
| Mid cells (%)     | 7.21 ± 0.16b         | 8.65 ± 0.26ab | 12.45 ± 1.40a | 12.35 ± 3.45a | * |
| Platelet count (x10^11/μl) | 158.10 ± 8.83a | 151.75 ± 11.62ab | 148.80 ± 6.40bc | 115.50 ± 29.50cd | NS |
| Mean platelet volume (fl) | 6.90 ± 0.12 | 7.00 ± 0.11  | 6.91 ± 0.07  | 6.75 ± 0.25 | NS |
| Platelet distribution width (fl) | 10.78 ± 0.16 | 10.98 ± 0.17 | 10.84 ± 0.11 | 10.95 ± 0.35 | NS |
| Plateletcrit (%)  | 0.11 ± 0.01          | 0.11 ± 0.01   | 0.10 ± 0.01  | 0.08 ± 0.03 | NS |

Hemogram values expressed as mean ± SE (Standard error) for each technique. Means followed by different superscripts (a, b, c) within the same row are significantly different at (P < 0.05). Two means that are not follow by the same letter (a, b, or c) are significantly different (P < 0.05). Two means followed with the same letter implies that they are not significantly different. Sig.: represents statistical significance. * P < 0.05. NS = non-significant. MCHC = mean corpuscular hemoglobin concentration.
buffalo using PCR assays in a sample of 50 animals from different locations [11]. It follows that *B. bigemina* infections in buffaloes might perhaps be more accurately performed using serological, rather than molecular methods, and that, in contrast to *B. bovis*, buffaloes might not be highly significant as potential reservoirs of *B. bigemina* in Egypt.

Hematological findings in this study revealed a marked decrease in the mean levels of RBCs, HCT%, Hb and MCHC and increase in MCV values in cattle infected with *B. bovis* and/or *B. bigemina* regardless of the method of detection (Blood film, cELISA, and PCR) used in this study, and even assuming that these parameters can be affected in these animals due to other unknown reasons different from *Babesia* infections. However, this is an unlikely occurrence, since the non-infected population consistently shows hematological values that are in the normal range. Collectively, these results revealed that *B. bovis* and/or *B. bigemina* likely caused macrocytic hypochromic anemia, indicative of severe intravascular hemolysis of red blood cells in cattle affected with persistent babesiosis [38, 48, 49]. These may be due to the fact that although *Babesia* sp. may cause direct damage on some erythrocytes, immune-mediated injury of parasite may be more important in the pathogenesis of anemia [7]. Yet, the increase in erythropagocytosis by activated macrophages [50, 51] and the production of anti-erythrocyte antibodies [8] may also contribute to the development of anemia.

The reduced platelet counts (thrombocytopenia) were noted in *B. bovis* and/or *B. bigemina* infected cattle with acute, chronic and sub-acute stages of babesiosis, as detected by all the diagnostic methods used in this study (blood film, cELISA, PCR, Blood film + cELISA + PCR). Thrombocytopenia is usually present with protozoal infections of erythrocytes. The increased platelet consumption may be attributed to an immune-mediated process, or it can result from intravascular disseminated coagulation in severe disorders [52, 53]. However, thrombocytopenia is more often associated with increased phagocytosis of platelets in response to antibodies on their surfaces and/or because of macrophage activation by inflammatory cytokines such as macrophage colony-stimulating factor (M-CSF) and Interferon gamma (IFN-γ) [54]. Consistently, splenomegaly, which is concurrent with many parasitic tick-borne diseases, may also be associated with increased platelet sequestration and destruction by splenic macrophages [9].

The increase in total leukocyte count (WBCs) and granulocytic counts found in cattle infected with *B. bovis* and/or *B. bigemina* as detected by blood film (acute stage of babesiosis) compared to control group together with decrease in lymphocyte counts in the groups infected with *Babesia*, when compared to the control group are in agreement with results previously reported by Court *et al.* [51]. These authors also propose that the significant increase in neutrophils of cattle infected with *B. bovis* could be attributed to their role as active mediators in the innate immune response. Taken together the observations derived from the hematological studies collected in this study can help to assess the overall impact of babesiosis on the health of the cattle and buffalo populations exposed to babesiosis in Egypt.

Remarkably, no such significant hematological changes were recorded in buffaloes naturally infected with *B. bovis* and/or *B. bigemina* in acute, chronic and sub-acute stages of babesiosis. Similar results were reported by Mahmmod [55], who recorded that buffaloes infected with *B. bovis* have less significant hematological changes than cattle infected with the same parasite. These observations, added to the inability of the nPCR to detect *B. bigemina* infections in buffaloes are consistent with the perceived decreased susceptibility of buffalos to *Babesia* infections.

**Conclusions**

We estimated high presence of babesiosis caused by *B. bovis* and *B. bigemina* in a diverse population of bovines in Egypt using distinct diagnostic classic, serological and novel molecular approaches. The cELISA data suggests that both parasites are able to circulate among both, bovines and buffalo herds in Egypt, perhaps due to sharing vectors competent for the transmission of both *B. bovis* and *B. bigemina* parasites. Hematological profiles in bovines indicate that infected populations present hematological disorders compatible with intravascular hemolysis and thrombocytopenia regardless of the stage of the disease. However, hematological changes were less evident in buffaloes naturally infected with *Babesia* sp. when compared to cattle.

**Abbreviations**

*B. bovis*: Babesia bovis; *B. bigemina*: Babesia bigemina; EDTA: Ethylene diamine tetra-acetic acid; IFAT: Indirect fluorescent antibody test; ELISA: Enzyme-linked immune-sorbent assay; cELISA: competitive ELISA; gDNA: Genomic DNA; PCR: Polymerase chain reaction; nPCR: Nested PCR; snPCR: Semi-nested PCR; rap-1c: rhoptry associated protein-1c; ra: rap-1 related antigen; fg: femtograms; O.D.: Optical density; RBC: Red blood cell count; HCT: Hematocrite; Hb: Hemoglobin; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; RDWA: Red blood cell distribution width absolute; RDW%: Red cell distribution width percentage; WBC: White blood cell count; MD: Mid cells; PLT: Platelet count; MPV: Mean platelet volume; PDW: Platelet distribution width; PCT: Plateletcrit; LPCR: Large platelet concentration ratio; ME: Microscopy examination; PPE: Percentage of parasitized erythrocytes.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

MM, CES and MGS designed the study and wrote the manuscript. MM, OK, SAN, SHH, SH and DM collected the blood samples from the cattle and buffaloes (Cairo, Behaiera and Kaf El-Sheikh governments). OK, SHH and SH performed the cELISA and the DNA extractions. OK and DM performed the
PCR. SAN performed the hematological analysis and statistical analysis. All authors read and approved the final version of the manuscript.

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