Comparison between conventional and molecular methods for diagnosis of bovine babesiosis (*Babesia bovis* infection) in tick infested cattle in upper Egypt

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Abstract Ticks and tick-borne diseases are the main problems affecting the livestock production in Egypt. Bovine babesiosis has adverse effects on the animal health and production. A comparison of Giemsa stained blood smears, polymerase chain reaction (PCR) and nested PCR (nPCR) assays for detection of *Babesia bovis* infection in Egyptian Baladi cattle (*Bos taurus*) in reference to reverse line blot was carried out. The sensitivity of PCR and nested PCR (nPCR) assays were 65 and 100 % respectively. Giemsa stained blood smears showed the lowest sensitivity (30 %). According to these results using of PCR and nPCR target for *B. bovis*, [BBOV-IV005650 (BV5650)] gene are suitable for diagnosis of *B. bovis* infection. The 18Ss rRNA partial sequence confirmed that all the positive samples were *Babesia bovis* and all of them were deposited in the GenBank databases (Accession No: KM455548, KM455549 and KM455550).

Keywords *Babesia bovis* · Giemsa · 18Ss rRNA · PCR · nPCR

Introduction

Babesia bovis infection is a tick-borne parasite of cattle transmitted by *Rhipicephalus annulatus* ticks. The first description of this disease in Egypt was in 1947 by Nagati (1947). *B. bovis* infection is a worldwide distributed and considered as one of the most important destructive diseases of cattle (McCosker, 1981; Ibrahim et al. 2013 and Elsify et al. 2015). The clinical signs include fever, hemoglobinuria, acute anemia, and nervous signs (Mosqueda et al. 2012; Radwan et al. 2013). Low parasitemia carrier state is usually developed after survival of the animals; they serve as a reservoir of the parasite (Mahoney, 1969). The diagnosis of bovine babesiosis depends mainly on clinical and microscopic examination of Giemsa stained blood smear which is the conventional laboratory technique. Presence of piroplasms is the gold standard for the diagnosis, especially during the acute stage of the disease. Indeed, during this stage the number of parasites inside the erythrocytes is high enough to be detected easier by microscopical examination (Bose et al. 1995). In carrier animals this method is less sensitive since the parasitaemia is low. In this case, molecular tools using PCR and nested PCR increase the sensitivity. They are more sensitive and specific, providing rapid and accurate results (Oliveira-Sequeira et al. 2005; Costa-Junior et al. 2006; Martins et al. 2008). This study aim to compare four diagnostic techniques, namely Giemsa stained blood smears, PCR and nested PCR for detection of *Babesia bovis* infection in Egyptian Baladi cattle (*Bos taurus*). The Reverse line blot (RLB) was the reference technique.

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Materials and methods

Animals

Baladi breed cattle belong to different localities in Assuit governorate in Upper Egypt (Egypt) were admitted to the Veterinary Teaching Hospital. They were subjected to clinical examination according to Radostits et al. (2000).

Parasitological diagnosis

Blood samples were collected directly from the ear vein and used for preparation of blood smears (Coles, 1986).

Molecular diagnosis

Whole blood samples were collected directly from the jugular vein on vacutainer tube containing EDTA and stored at -20°C until used. DNA extraction was performed with *QIAamp* DNA blood Mini kit, (Qiagen, Ltd, UK) according to manufacturer's instruction. PCR and nested PCR (nPCR) amplifications were carried out by using specific primers to *B. bovis*, [BBOV-IV005650 (BV5650)] F, 5'-CCGGAATTCCAAATGGCAACA AAGGTTGA-3'R, 5'-CCGCTCGAGGGAGCAGCGTA TTACTTCCTCACGT-3', F1, 5'-CGAGGATTTGGTA GACCTCATC-3', R2 5'-CGTAAAATGTGTACAATA TTT-3', respectively (Figueroa et al. 1993; Aboulaila, et al. 2010a, b). RLB was performed with a set of 18Ss rRNA *Babesia* spp.-catchall primers (RLB-F2 5'-GAC ACA GGG AGG TAG TGA CAA G-3', Nested RLB-F 5'-GAC AAG AAA TAA CAA TAC RGG GC-3', RLB-R2 5'-CTA AGA ATT TCA CCT CTG ACA GT-3') (Gubbels et al. 1999). The products were subjected to electrophoresis in 2 % agarose gel and then visualized under ultraviolet (UV) light after staining with ethidium bromide (Sigma-Aldrich). Positive DNA products from 18Ss rRNA primer were purified and then cloned into pDrive Cloning vector using QIAGEN Kit (Qiagen, Ltd, UK) according to manufacturer's instructions. The amplicons were sequenced in both directions (Molecular Biology Research Unite, Assiut University, Egypt), and subjected to BLAST similarity searches.

Statistical analysis

Giemsa stained blood smears, PCR and nested PCR were compared with RLB as a reference test. Evaluation parameters included sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and combined predictive value (CPV) (Thrusfield, 2005).

Results

Giemsa stained blood smears confirmed the infection in 7.9 % (6/76) of the examined animals. The positive animals showed specific clinical signs of babesiosis, including fever ($>40^{\circ}\text{C}$) and hemoglobin urea with different intensities of *Boophilus (Rhipicephalus) annulatus* ticks infestations. PCR and nested PCR detected the DNA target of *Babesia bovis* in 17.1 % (13/76) and 26.3 % (20/76) of examined cattle respectively. RLB assay confirmed the infection in 26.3 % (20/76) of the animals (Table 1, Figs. 1, 2, 3, 4, 5). The PCR showed 100 % specificity for

Table 1 Infection rates according to Giemsa stained blood smears, PCR, nPCR and RLB for detection of *Babesia bovis* in tick infested cattle

Diagnostic assays	Positive	$\pm\text{SD}$ (%)
Blood smear	6/76	7.89 ± 25.32
BV5650 PCR	13/76	17.11 ± 25.23
BV5650 nPCR	20/76	26.31 ± 26.41
RLB	20/76	26.31 ± 26.41

Fig. 1 Giemsa stained blood smear showing *Babesia bovis* piroplasms

Fig. 2 *Babesia bovis* PCR products. Lane M: DNA ladder 100 bp, Lanes 1, 3, 5, 7 and 8: positive PCR with bands of 720 bp

Fig. 3 *Babesia bovis* nested PCR products. Lane M: DNA ladder 100 bp, Lanes 1, 3, 5, 7, 8 and 10: positive PCR with bands of 561 bp

Fig. 4 *Babesia bovis* RLB-PCR products. Lane M: DNA ladder 100 bp, Lanes 1, 4, 6, 7 and 10: positive PCR

Fig. 5 *Babesia bovis* nested RLB-PCR products. Lane M: DNA ladder 100 bp, Lanes 1, 2, 4, 6, 7 and 10: positive PCRs both of them gave bands of 460 and 520 bp

detection of *Babesia bovis* infection but their sensitivity varied between 30 and 100 % (Table 2). Partial sequences were deposited in the GenBank databases under accession numbers (KM455548, KM455549 and KM455550).

Discussion

Bovine babesiosis is one of the most important diseases in Egypt. It has an adverse effect on both production and reproduction of cattle. *B. bovis* and *B. bigemina* are the most common parasites in cattle (Ibrahim et al. 2013; Elsify et al. 2015). The prevalence of them usually becomes different according to the investigated locality. The prevalence of *B. bovis* is higher than *B. bigemina* in Dakahlia Governorate, Egypt it was 7.3 and 1.2 %, respectively according to El-Ashker et al. (2015) while Ibrahim et al. (2013) reported that the prevalence of *B. bigemina* is higher than *B. bovis* it was 5.30 and 3.97 %, respectively in Beheira and Faiyum, Governorates Egypt. In Upper Egypt PCR and nPCR of BV5650 gene are recently used in detection of *B. bovis* infection in randomly collected field samples from baladi breed cattle. The molecular assay, specially nested PCR of BV5650 gene was the most sensitive test (100 %) followed by BV5650 gene PCR (65 %) and blood smears examination (30 %) this came in agreement with El-Ashker et al. (2015) and Aboulaila, et al. (2010a, b) the first study reported that molecular assay was more sensitive than conventional method and both of them should be in combination to detect both clinically infected animals and carrier state; the second study reported that using of PCR and nPCR of gene BV5650 had the highest sensitivity rate when applied on field samples collected from cattle in Ghana, Mongolia and Brazil, respectively. This could be attributed to the presence of several gene copies in the genome; also the sequence conservation among strains may be playing an important role in these findings according to Aboulaila, et al. (2010a, b). Giemsa stained blood smears, PCR and nPCR of BV5650 gene revealed high PPV (100 %). These

Table 2 Evaluation of Giemsa stained blood smears, PCR and nPCR against RLB as a reference test for *Babesia bovis* detection in tick infested cattle

Diagnostic assays	TP	TN	FP	FN	Sensitivity	Specificity	PPV	NPV	CPV
Blood film	6	56	0	14	30	100	100	80	81.58
BV5650 PCR	13	56	0	7	65	100	100	88.89	90.79
BV5650 nPCR	20	56	0	0	100	100	100	100	100

TP true positive, TN true negative, FP false positive, FN false negative

findings indicate that during further investigations, these tests will have the ability to detect the infected animals correctly. The NPVs of Giemsa stained blood smears and PCR were relatively low as 80 and 88.9 %, respectively; if compared with nPCR BV5650 which revealed 100 % NPV. These results indicate the ability of nPCR BV5650 to detect true negative if compared with Giemsa stained blood smears and BV5650 PCR assay. The combined predictive values were 81.6, 90.8 and 100 % for Giemsa stained blood film and BV5650 PCR and nPCR assays.

In conclusion, PCR and nPCR methods based on BV5650 gene detection should be recommended for the detection of *B. bovis* infection in cattle under field conditions and for epidemiological studies.

Future study

A recent type of molecular assay like Loop-Mediated isothermal amplification (LAMP) test is more applicable, not expensive and not need sophisticated instruments. This molecular assay is more sensitive than conventional method, especially in the carrier state. We will start to use it for confirmation of infection in randomly collected field samples and evaluate its validity to use in epidemiological studies.

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Compliance with ethical standards

Conflict of interest Author confirmed that there is no conflict of interest.

References

- AbouLaila M, Yokoyama N, Igarashi I (2010a) Development and evaluation of a nested PCR based on spherical body protein 2 genes for the diagnosis of *Babesia bovis* infection. *Vet Parasitol* 139:45–50
- AbouLaila M, Yokoyama N, Igarashi I (2010b) Development and evaluation of two nested PCR assays for the detection of *Babesia bovis* from cattle blood. *Vet Parasitol* 172:65–70
- Bose R, Jorgensen WK, Dalglish RJ, Friedhoff KT, De vos AJ (1995) Current state and future trends in the diagnosis of babesiosis. *Vet Parasitol* 57:61–67
- Coles EH (1986) *Veterinary clinical pathology*, 4th edn. W B Saunders Company, London and Toronto Philadelphia, pp 46–56
- Costa-Junior LM, Rabelo EML, Filho OAM, Ribeiro MFB (2006) Comparison of different direct diagnostic methods to identify *Babesia bovis* and *Babesia bigemina* in animals vaccinated with live attenuated parasites. *Vet Parasitol* 139:231–236
- El-Ashker M, Hotzel H, Gwida M, El-Beskawy M, Silaghi C, Tomaso H (2015) Molecular biological identification of *Babesia*, *Theileria*, and *Anaplasma* species in cattle in Egypt using PCR assays, gene sequence analysis and a novel DNA microarray. *Vet Parasitol* 207:329–334
- Elsify A, Sivakumar T, Nayel M, Salama A, Elkhtam A, Rizk M, Mosaab O, Sultan K, Elsayed S, Igarashi I, Yokoyama N (2015) An epidemiological survey of bovine *Babesia* and *Theileria* parasites in cattle, buffaloes, and sheep in Egypt. *Parasitol Int* 64:79–85
- Figueroa JV, Chieves LP, Johnson GS, Buening GM (1993) Multiplex polymerase chain reaction based assay for the detection of *Babesia bigemina* *Babesia bovis* and *Anaplasma marginale* DNA in bovine blood. *Vet Parasitol* 50:69–81
- Gubbels JM, de Vos AP, van der Weide M, Viseras J, Schouls LM, de Vries E, Jongejan F (1999) Simultaneous detection of bovine *Theileria* and *Babesia* species by reverse line blot hybridization. *J Clin Microbiol* 37:1782–1789
- Ibrahim HM, Adjou Moumouni PF, Mohammed-Geba K, Sheir SK, Hashem IS, Cao S (2013) Molecular and serological prevalence of *Babesia bigemina* and *Babesia bovis* in cattle and water buffaloes under small-scale dairy farming in Beheira and Faiyum Provinces, Egypt. *Vet Parasitol* 198:187–192
- Mahoney DF (1969) Bovine babesiosis: a study of factors concerned in transmission. *Ann Trop Parasitol* 63:1–14
- Martins TM, Pedro OC, Caldeira RA, do Rosario VE, Neves L, Domingos A (2008) Detection of bovine babesiosis in Mozambique by a novel seminested hot-start PCR method. *Vet Parasitol* 153:225–230
- McCosker PJ (1981) The global importance of babesiosis. In: Ristic M, Krier JP (eds) *babesiosis*. Academic Press, New York, pp 1–24
- Mosqueda J, Olvera-Ramírez A, Aguilar-Tipacamú G, Cantó GL (2012) Current Advances in Detection and Treatment of Babesiosis. *Curr Med Chem* 19:1504–1518
- Nagati HE (1947) Some new and rare records of piroplasmosis with a list of the species of *Babesia* and *Theileria* so far recorded from Egypt. *Vet Rec* 59:145–147
- Oliveira-Sequeira TC, Oliveira MC, Araujo JP, Amarante AF (2005) PCR-based detection of *Babesia bovis* and *Babesia bigemina* in their natural host *Boophilus microplus* and cattle. *Int J Parasitol* 35:105–111
- Radostits OM, Gay CC, Blood DC, Hinchcliff KW (2000) *A text book of veterinary medicine*, 9th edn. Baillier, Tindal and Cox, pp 1328–1329
- Radwan AM, Ibrahim MA, Abdalla AA, AL-Hosary AA, Ahmed LS (2013) Conventional detection of *Babesia bovis* in tick infested cattle and its effect on the hematological profile. In: 16th ISAH congress. Nanjing, China, 5–9 May 2013, pp 170–172
- Thrusfield M (2005) *Veterinary epidemiology*, 3rd edn. Blackwell science publishing, New Jersey, pp 313–317