Serological and Molecular Diagnosis of Bovine Brucellosis in Menoufia Province

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ABSTRACT

Brucellosis represents a serious problem in cattle relaying huge economic and reproductive losses. *Brucella meletensis* is one of the most brucella strains isolated from sheep and cattle which can infect human. The aim of present study was the detection of antibodies against brucellosis among cattle population in Egypt. Serologically brucella was detected with Rose Bengal Plate Test. Evaluation of the specificity and sensitivity of (RBPT, IC) assay and cELISA against CFT test were done. Molecular detection of brucella using PCR and bacteriological isolation followed by phenotypic and molecular typing of the isolated bacteria. Results revealed that the overall prevalence using RBT was 1.44%. Holstein Friesian cattle have highest prevalence (1.57%) while native cattle were (1.28%). Cattle over 3 years old have the highest seroprevalence (2.77%). The positive results for RBPT, cELISA, IC Assay and CFT were 91%, 60%, 91% and 88% respectively after examination 100 serum sample. *Brucella melitensis biovar 3* was isolated from the tissue specimens (uterus and/or lymph nodes). PCR targeting (Immuno-dominant antigen, gene bp26) generated product of 450 bp from (16/20) tissues specimens. These findings should be help in assisting future planning pragmatic control strategies against bovine brucellosis in Egypt and for reproductive herd health fertility.

Keywords: Cattle, Brucellosis, PCR, Serological tests.

INTRODUCTION

Brucellosis was a serious zoonotic disease affecting a wide range of domestic and wild animals all over the world (Ocholi *et al.,* 2005; Neha *et al.,* 2014; Verma *et al.,* 2014; Kumar *et al.,* 2016), leading to a potentially debilitating infection in man (Hosein *et al.,* 2010). Brucellosis in animals causes great economic losses in cattle due to abortion, premature birth, retained placenta, decreased milk production, and reduced reproduction rate. While, in bull it form orchitis and sterility (Muma *et al.,* 2007). Even though, the advances made in surveillance and control, the prevalence of brucellosis was increasing in many developing countries due to various sanitary, socioeconomic, and political factors (Pappas *et al.,* 2006; Gwida *et al.,* 2010;

Matope *et al.,* 2010; Seleem *et al.,* 2010). The disease was caused by *Brucella spp*., a gram-negative bacterium, which can infect several important livestock species, including cattle, water buffaloes, goats, sheep, and pigs (Pappas *et al.,* 2006; Di Giannatale *et al.,* 2008). The infected animals shed the organisms in the uterine discharges following abortion and subsequent parturition, and also in the colostrum and milk (FAO, 2003). Despite the application of the National Brucellosis Control Program in Egypt from many years ago (Refai, 2002), the disease was still endemic among ruminants and humans (Holt *et al.,* 2011). In Egypt, brucellosis control programs for bovines were based on a test and slaughter policy in combination with vaccination (El-Diasty, 2004; Refai, 2002). Bacteriological isolation of the causative organism in specific media remains the gold standard method for the diagnosis of brucellosis, despite they were not always successful and represent a great risk of infection for technicians and isolation of Brucella spp. was tedious, time consuming and difficult due to the intra-cellular and fastidious nature of the bacteria (Alton *et al.,* 1988; Kaynak- Onurdag *et al.,* 2016). *Brucella abortus* biovars and occasionally *Brucella melitensis* are the main cause of cattle brucellosis. (OIE, 2008). In Egypt, *Brucella melitensis* was recorded to be the common strain isolated from cattle as reported by (Shalaby *et al.,* 2003; Sayour, 2004; Shehata, 2004). In brucellosis control and eradication programs, detection and identification of Brucella species was generally based on phenotypic, biochemical and serologic tests (Alton *et al.,* 1988). Genotyping and identification of Brucella species based on molecular approaches which have been proved to be powerful tools to confirm the disease and to assess the genetic relationship among field isolates (Al Dahouk *et al.,* 2007; Minharro *et al.,* 2013; Dorneles *et al.,*2014; Mick *et al.,* 2014; Allen *et al.,* 2015). The common serological tests used for brucellosis routine diagnosis were Rose Bengal Plate Agglutination Test (RBPT), Serum Tube Agglutination Test (STAT), Milk Ring Test (MRT), Complement Fixation Test (CFT) and ELISA. Although these traditional serological assays were easy to perform, faster and reduce risk of laboratory acquired infection, but they suffer from a lower sensitivity and specificity in

diagnosing of the disease mainly due to cross-reactivity with other antigens (Mantur *et al.,* 2007). Recently the methods of molecular biology have been progressively used in the diagnosis and PCR was mainly useful in detection of brucella DNA in tissues and body fluids contaminated with non-viable or low numbers of brucella (Leal-Klevezas *et al.,* 2000; Neha *et al.,* 2014). PCR and indirect-ELISA give a significant advantage over others serological methods used in the diagnosis of brucellosis in endemic geographical region (Çiftci *et al.,* 2017; Saadat *et al.,* 2017). Several studies on brucellosis were done at the level of Menoufia province concerning test efficacy (El Shafey, 2017), seroprevalence and risk factors assessment (Al-Bukair, 2014) and seroprevalence in conjunction with trials for isolation and identification in two farms (Sherif, 2008).Therefore, the current study was aimed to estimate the seroprevalence of brucellosis in Menoufia province, comparison of sensitivity and specificity of different serological tests used for detection of Brucella spp. Molecular techniques for isolation and characterization of brucella isolates from serologically positive animals

Materials and Methods

Sample collection

The present study was carried out in the nine districts of Menoufia province. A total number of 6290 serum samples were collected from cattle from different districts of Menoufia province for serological survey. All of these animals were not vaccinated against brucellosis. The age, district and breed of each animal were recorded. About 10 ml of blood were collected from jugular vein of cattle. Collected samples were kept in refrigerator overnight giving chance for serum separation then centrifuged at 3000 r.p.m. for five minutes. Clear sera were siphoned off and stored in cryotubes at -20 C until its use for serological studies (RBT, IC assay, cELISA & CFT) (Alton *et al.,* 1988).

Tissue samples

Tissue specimens from positive serologically animals including 20 slaughtered cows (20 supra-mammary lymph nodes and 20 uteri with

its surrounding fat) were obtained immediately after slaughtering, placed in sterile plastic bags and transferred to laboratory on ice **(Dhama *et al.,* 2013).** These specimens were subjected to culture for isolation of Brucella organisms, DNA extraction for PCR detection of Brucella spp. and characterization.

Brucella isolation and bacteriological examination

This study was performed according to the recommendations of the FAO/WHO Expert Committee on Brucellosis (Alton et al.*,* 1988). All materials were collected in sterile swabs and transferred on ice to laboratory for bacteriological examination. Brucella were cultivated on blood agar, Brucella agar and selective media at 37∘for 48 h. Identification and biotyping of brucella isolates was done by assessing colony morphology, biochemical reactions (oxidase, catalase, and urease), $CO2$ requirement, production of $H2S$, growth in the presence of the dyes thionine and fuchsine, reaction with mono specific antisera (A, Mand R), and phage lysis (F25, Tb, Wb). Agglutination with acriflavine and staining of colonies with crystal violet were used to assess the colonial morphology of the isolated strains and to differentiate between rough and smooth type colonies as previously described (Alton *et al.,* 1988).

Serological examination

One hundred serum samples were examined by 4 serological tests (RBT, IC assay, cELISA & CFT) for evaluation of the efficacy of these tests considering the CFT as the standard test. Rose Bengal Plate Test was conducted as per standard procedure (Alton *et al.,* 1975). Immuno-chromatographic rapid brucella assay was conducted as Lilli test rapid Brucella Ab test kit ® (Lillidale Diagnostic, Wimborne, England), Product code; VR-1007 Bach; LRB021501). competitive enzyme linked immunosorbent assay was performed as per the manufacturer's instructions, using kits A (COMPLISA ELISA PLATE) pre coated with B. melitensis LPS Antigen (Animal Health and Veterinary Laboratories Agency, UK (AHVLA). Complement fixation test was done as described in (OIE, 2013).

The sensitivity of different diagnostic tests; RBPT, IC Assay and cELISA were estimated against the results of CFT which is used as a gold standard test by OIE because of its high specificity. A total of 100 serum samples collected from suspected cases were examined by the 4 different serological tests and the test evaluation was carried out on Win Episcope with 95% confidence as explained in (Hsieh *et al.,* 2005).The agreement between the performance of PCR and bacterial isolation was carried out with 95% confidence on win Episcope and the Kappa coefficient for the degree of association was calculated (Sim and Wright 2005).

Molecular identification and genotyping analysis of isolates

A PCR was applied for detection of bovine brucellosis from twenty (20) serologically positive cases. This was performed by amplification product of 450 bp from the tissues specimens (uterus and supra-mammary lymph nodes). The genomic DNA of Brucella spp. was extracted and carried out according to the instruction manual of gSync TM DNA extraction kit, Geneaid (New Taipei City, 22180 Taiwan, Cat. No. GS 100). and Primers for conventional PCR (Garcia-Yoldi *et al.,* 2006) were prepared by Biosearch Technologies, South McDowell Boulevard, Petaluma, USA. Amplification of target gene (Immunodominant antigen, gene *bp26)* was carried out for molecular identification of Brucella in DNA extracts at the genus level.

Bruce-ladder PCR on DNA extract of one Brucella isolate:

INgene Bruce ladder was used in multiplex PCR for molecular typing of one Brucella isolate on species level. The PCR amplicons were analysed by running 10 μl of the PCR products in 1% agarose gel stained with ethidium bromide (0.5μg/ml). Thereafter, gels were photographed under UV illumination using gel documentation and analysis system.

Statistical analysis

Serological and molecular tests were compared with each other. Sensitivity, specificity, concordance percentage and the agreement

between the tests (kappa statistic) were evaluated (Thrusfield, 2008).

Table (1): Primers sequences used for PCR for detection of *Brucella spp*. (**Garcia-Yoldi *et al.*, 2006**)

Primers	Sequence (5'–3')	Amplicon size (bp)	DNA targets	Source of genetic Difference
BMEI0535f BMEI0535r	GCG-CAT-TCT-TCG-GTT-ATG-AA CGC-AGG-CGA-AAA-CAG-CTA-TAA	450	Immunodominant antigen, gene *bp26*	IS*711* insertion in BMEI0535-BMEI0536in Brucella strains isolated from marine mammals

Table (2): Primer sets for Bruce ladder multiplex PCR (**Garcia-Yoldi *et al.*, 2006).**

Primer	Sequence (5'–3')	Amplicon size (bp)
BMEI0997r	ATC-CTA-TTG-CCC-CGA-TAA-GG	1682
BMEI0997r	GCT-TCG-CAT-TTT-CAC-TGT-AGC	1071
BMEII0843f	TTT-ACA-CAG-GCA-ATC-CAG-CA	
BMEII0844r	GCG-TCC-AGT-TGT-TGT-TGA-TG	587
BMEII0428f	GCC-GCT-ATT-ATG-TGG-ACT-GG	
BMEII0428r	AAT-GAC-TTC-ACG-GTC-GTT-CG	272
BR0953f	GGA-ACA-CTA-CGC-CAC-CTT-GT	
BR0953r	GAT-GGA-GCA-AAC-GCT-GAA-G	218
BMEI0752f	CAG-GCA-AAC-CCT-CAG-AAG-C	
BMEI0752r	GAT-GTG-GTA-ACG-CAC-ACC-AA	

RESULTS

The overall prevalence of bovine brucellosis in Menoufia province using RBT as screening test was 1.44% (91 positive from 6290 serum samples). The overall prevalence of apparent and true bovine brucellosis in Menoufia province in relation to locality was 1.5% and 0.7 respectively. The highest prevalence of bovine brucellosis was recorded in Holstein Friesian cattle 1.57% (63 from 4007) while the lowest prevalence was recoded among native cattle 1.28% (28 from 2177). The highest seroprevalence percentage of bovine brucellosis were recorded in animals over 3 years old (2.77%) while the lowest one was recorded in animals of less than one year old with (0.35%). The age range between 1-3 years recorded (1.22%).

The sensitivity of different serological testes used for serodiagnosis of bovine brucellosis

Evaluation of the efficacy of different serological testes used for serodiagnosis of bovine brucellosis revealed the results of examined 100 serum samples were 91%, 60%, 91% and 88% as examined by (RBT, IC assay,

cELISA and CFT) respectively as presented in Table (3).

Molecular detection of brucella Spp from uterus and supra mammary lymph nodes of serologically positive cases

Molecular detection of brucella Spp from uterus and supra-mammary lymph nodes of serologically positive cases which slaughtered in abattoirs revealed as in Fig. (1). Molecular detection of brucella species directly from the tissue samples (uterus and/or lymph nodes) using PCR targeting (Immunodominant antigen, gene bp26) generated product of 450 bp from the tissues specimens and (16) samples were positive by PCR.
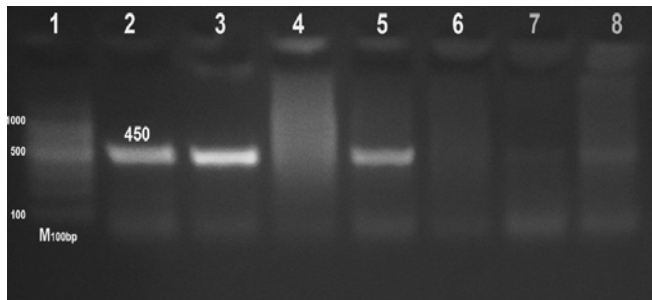
Fig. (1): PCR amplification products generated by *Brucella* genus-specific primers. PCR was performed with 5 DNA samples: lanes 1, 2, and 3 represent 100 bp ladder, Br.melitensis (control positive), and RB51 (control positive), Lane 4: DNA of (lymph node), Lane 5: DNA (uterus), Lane 6: DNA (lymph node), Lane 7: DNA (lymph node), Lane 8: DNA (uterus).

Only 5 cases had previous history of abortion. The highest prevalence of bovine brucellosis was observed in animals over 3 years in 15 cases while only 5 cases in animals of less than 3 years old and all affected cattle breed were Holstein Friesian.

Isolation and typing of Brucella organism

Brucella organisms could be detected from six samples. Brucella culture showed typical characteristics for the genus Brucella. Colonies were smooth elevated, transparent, and convex, with intact borders, brilliant surface and have a honey color under transmitted light.

The Phenotypic characteristics of Brucella isolate in this study was summarized in table (4) Indicated that the isolate was *Brucella melitensis biovar 3.*

Bruce-ladder PCR on DNA extract of one Brucella isolate

Brucella species was determined according to molecular size of the amplified products using DNA ladder indicating the Brucella isolate recovered in this study was Brucella melitensis biovar 3. There is agreement between bacterial isolation and PCR which estimated at 50% and the kappa coefficient was estimated at 0.2. (This is in fact good agreement).

Fig. (2): Result of Bruce-ladder PCR on DNA extract of one Brucella isolate. Lane 1:100bp DNA ladder; Lane 2: Rev1 (Bruce-ladder kit control); Lane 3: RB51 (Bruce-ladder kit control); Lane 4: Suis (Bruce-ladder kit control); Lane 5: brucella isolate; Lane 6: negative control.

Table (3): Different serological test used for diagnosis of bovine brucellosis.

Test	Examined sample No.	Positive sample	
		No.	%
RBPT	100	91	91%
IC Assay	100	60	60%
cELISA	100	91	91%
CFT	100	88	88%

Statistical analysis showed that the sensitivity of RBPT, cELISA and IC Assay in comparison with CFT as gold standard test was estimated at 100%, 100% and 68.2%, respectively. On the other hand, the specificity for the 3 serological tests was estimated as 75%, 75% and 100%, respectively.
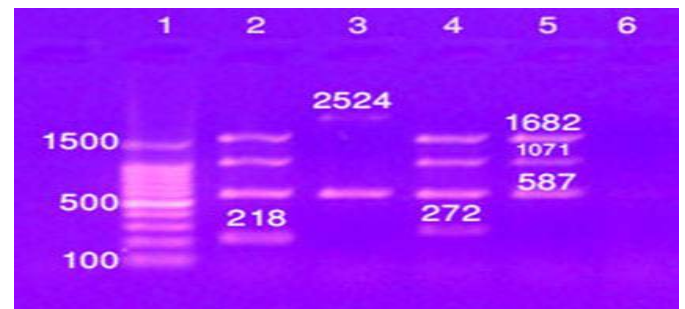
Table (4): Phenotypic characteristics of Brucella isolate (Brucella melitensis biovar 3) recovered from one tissue specimen. RTD: routine test dilution Tp: Tbilisi (Tb) a: 1:50000 b: 1:100000 A: anti *Brucella abortus* M: anti *Brucella melitensis*   R: rough brucella antiserum

Brucella isolates	CO_2	H_2S	Urease	Growth on dyes				Lysis by Tb phage		Non-specific sera			Conclusion
				Thionin		Fuchsin		RTD	RTD 10^4	A	M	R	
				a	b	a	B						
One Brucella isolate	-	-	+in 20 hrs	+	+	+	+	-	-	+	+	-	***B. melitensis* 3**
Reference strains													
***B. melitensis* Ether**	-	-	+ in 18-24 hrs	+	+	+	+	-	-	+	+	-	*B. melitensis* 3
***B. abortus* 544**	-	+	+ in 2 hrs	-	-	+	+	+	+	+	-	-	*B. abortus* 1
***B. Suis* 1330**	-	+++	++ in < 15 min.	+	+	-	-	-	+	+	-	-	*B. Suis* 1

DISCUSSION

Bovine brucellosis was a disease with a significant economic and public health importance due to losses occurred as a result of infertility in animals and extensive chronic morbidity in humans (Gwida *et al.,* 2016).

The overall seroprevalence of bovine brucellosis in Menoufia province among cattle population using RBPT as screening test was 1.44% (91 positive out of 6290), while the apparent prevalence was 1.5%. This result higher than recorded by (Hegazy *et al.,* 2011) the prevalence of bovine brucellosis in Upper Egypt by RBPT was 0.79% and (Wareth *et al.,* 2014) in 2011 was 0.33% but lower than that reported by many authors such as Montasser *et al.,* 2011 (4.5%), Salem *et al.,* 2014 (6%), Selim *et al.,* 2015 (8.4%), AL-Habaty *et al.,* 2015 (10.23%) and Ahmed *et al.,* 2016 (8.91%).

The result of seroprevalence in this study was lower than that reported in Menoufia province in other studies such as (Sherif, 2008) who reported that seroprevalence of brucellosis among cattle using RBPT was 6% (412 were positive out of 6780) and (Al-Bukair, 2014) who reported that apparent and true seroprevalence of brucellosis were 1.67% and 1.74% (119 were seropositive out of 7133) respectively. Although, the seroprevalence of brucellosis in some local areas was >30% or even >50%, indicating that Brucella infection was highly endemic in dairy herds (Ran *et al.,* 2019). This difference in the seroprevalence of the disease could be attributed to the difference in breeds, sensitivity of test kits, farming system, and sample size. In human, the transmission of Brucella infection and its prevalence in a region depends upon several factors like food habits, milk processing methods and milk products, social customs, husbandry practices, climatic conditions, socioeconomic status, and environmental hygiene (Mantur and Amarnath, 2008).

The seroprevalence of brucellosis among native breed of cattle was 1.28% (28 out of 2177) and among Holstein Friesian cattle was 1.57% (63 out of 4007). Frisian breed revealed the highest prevalence of bovine brucellosis than native breeds. This disagrees by that reported by (Nanveen 2013, Farouk 2015) they reported that Frisian breed has low prevalence than local breeds this may be due to the larger number of locals tested compared to the foreign. There is need for undertaking studies on the genetic resistance of different cattle breeds to brucellosis (Mittal *et al.,* 2018). In general differences in breed susceptibility are uncommon with brucellosis and breed susceptibility may depend on management system (Radostits *et al.,* 2007). Mittal et al. (2018) recorded that a significant breed differences in abortions due to B. abortus infection (p < 0.01), among 24 PCR positive cases, 20 samples were from the Frieswal breed and four were from Crossbred cows. None of the

abortions among the Sahiwal dams were found positive for B. abortus by PCR.

The highest seroprevalence percentage of bovine brucellosis were recorded in animals over 3 years old was (2.77%) while the lowest one was recorded in animals of less than one year old with (0.35%). It was found that old animals were at higher risk of getting brucellosis infection. Similar results in accordance with that obtained by (Alton 1981; Nanveen 2013; Farouk 2015) brucellosis occurs in animals of all ages but was highest in age group 2- 5years. This is due to older cattle are more susceptible to infection due to recurrent exposure chance, hormonal changes after sexual maturity and latent infection. But disagree with Matope *et al.,* (2011) reported decreased frequency of brucellosis with increasing age, with 24–48 months old cattle having higher odds of being seropositive compared to those older than 84 months. They concluded that some older cows may not exhibit detectable antibody titers possibly due to latency or self-limiting infection which is common in chronic brucellosis. The accuracy of serodiagnosis depends on the presence of antibodies in the serum, therefore an infected animal with a low antibody concentration or no circulating antibody will not be detected resulting in false negative results (Bercovich, 1998). However, they are very likely to be infectious (Arellano-Reynoso*et al.,* 2013).

A total of 100 serum samples collected from suspected cases were examined by the 4 different serological tests RBPT, cELISA, IC Assay and CFT; with 91%, 60%, 91% and 88% positive results respectively. These results were supported with results recorded by (Corrente *et al.,* 2015).

The sensitivity of RBPT, cELISA and IC Assay in comparison with CFT was estimated at 100%, 1000% and 68.2%, respectively. On the other hand, the specificity for the 3 serological tests were estimated at 75%, 75% and 100%, respectively. This agrees with many authors as (Farouk 2015, Ahmed *et al.,* 2016; El Shafey, 2017). 82.6% of samples were positive based on the Indirect ELISA, the sensitivity and specificity of ELISA (95.83% sensitivity and 65% specificity) (Saadat *et al.,* 2017). While,

Mittal *et al.,* (2005) reported that RBPT was more sensitive than ELISA, when applied to buffalo sera. Serological screening by STAT detected, 16 positive cases (18.18%) and 15 doubtful cases (17.05%). This may be due to fact that not all infected animals produce detectable level of circulating antibodies and factors such as cross-reacting organism, calf-hood vaccination and endemic condition of the brucellosis in this country further impairs the serological diagnosis (Mittal *et al.,* 2018).

There were many reasons to believe that a new comeback of brucellosis may occur in near future and this expectation was supported by the recent discovery of new atypical Brucella species with new genetic properties (El-Sayed and Awad, 2018), mixed livestock farming strategy enables cross species infections (Xavier *et al.,* 2009), newly discovered Brucella species display a great genetic diversity (Al Dahouk *et al.,* 2017). Bacteriological isolation of brucella species from the tissue specimens (uterus and/or lymph nodes) had been done successfully from 6 tissue specimens out of 20 examine samples. Phenotypic and bruce ladder typing of brucella isolates indicated that the isolates were *Brucella melitensis biovar 3*. Our results similar to that reported in Egypt by Ammar ,2000; Montasser *et al.,* 2001; El Sherif and El Sheary, 2002; El Diasty, 2004; Abdel Hamid *et al.,* 2012; AL-habaty *et al.,* 2015; Ahmed *et al.,* 2016) all of them isolated *Brucella melitensis biovar 3* from tissue specimens.

In dairy animals, Brucella spp. replicate in the mammary gland and supra-mammary lymph nodes, and these animals continually excrete the pathogen into milk throughout their lives (Refai, 2003). Also the result of isolation and typing was in agreement with previous trials of isolation from Menoufia province done by (Sherif, 2008) who try to isolate and identify of brucella from stomach contents, uterine discharge and fetal membrane of 6 aborted feti, also 18 tissue samples from supra-mammary lymph node, retro-pharyngeal lymph node, pre-scapular lymph node, liver, spleen, uterus and udder of slaughtered cows. Results were 100% from aborted feti and 20% from the other tissue samples. All isolates were *Brucella melitensis biovar3*.

The low isolation rate of brucella organism from tissue samples in this study agreed with (Seleem *et al.,* 2010 and De Jong & Tsolis 2012) they reported that brucella isolation was challenging. Brucella spp. is a fastidious bacterium and requires rich media for primary cultures. Furthermore, its isolation requires a large number of viable bacteria in clinical samples, proper storage and quick delivery to the diagnostic laboratory. *Brucella abortus* could be isolated (4.54%) from only four clinical cases out of the total 88 abortions screened (Mittal *et al.,* 2018). The main reasons for the low percent of isolation may be due to quantum of the bacteria in the clinical sample or the use of antibiotics in treatment of clinical cases and inherent difficulty of *B. abortus* to adapt to the culture milieu. Further, the contaminating bacteria present in some of the samples might have hindered in the successful isolation.

PCR being ten times more sensitive assay than bacteriological isolation (Kaushik *et al.,* 2006) is more suitable assay for determining possible cases of brucellosis during abortions in cattle. Molecular detection of brucella species directly from the tissue samples (uterus and/or lymph nodes) using PCR targeting (Immunodominant antigen, gene bp26) generated product of 450 bp from the tissues specimens and (16) samples were positive by PCR. This was in agreement with successful molecular characterization done by (Abdelhamid *et al.,* 2012) who use multiplex PCR for characterization of brucella isolates isolated from different animal's species from 7 Egyptian provinces during 2010-2011.Also, (Amin *et al.,* 2012) who use P1 and P2 primers specific for Brucella melitensis; resulted in 18 were positive out of 20 samples (as indicated by the size of the PCR product in agarose gel (approximately 282 bp) with 90% sensitivity for infected cows.

The PCR assay was shown to be a valuable tool for the detection of Brucella organisms from organs as reported by (Quahrani-Bettache *et al.,* 1996). The major advantage being the time taken compared to conventional methods which require several days to isolate and identify the organism (Fekete *et al.,* 1992; Ouahrani-Bettache et al. 1996; Ewalt and Bricker 2000). These findings indicate that PCR can be valuable for laboratory diagnosis of chronic infections or very early stage when antibodies could not be diagnosed (Ghorbani *et al.,* 2013).

CONCLUSION

The prevalence of brucellosis using RBT was 1.44% and Holstein Friesian cattle have highest prevalence (1.57%) than balady breeds. There is need for undertaking studies on risk factors and the genetic resistance of different cattle breeds to brucellosis. These points will definitely help in control of bovine brucellosis, which in turn is directly linked to control of human brucellosis in developing countries like Egypt. *Brucella meltiness biovar 3* was isolated from the tissue specimens. PCR and indirect-ELISA offers a significant advantage over conventional serological methods in the diagnosis of brucellosis in endemic geographical region.

Conflict of interest

The authors declare that they have no conflict of interest.

REFERENCES

Abdel-Hamid N., Ebeid M., Arnaout F., Elgarhy M. and Elbauomy E., 2012. Serological and bacteriological monitoring of ruminant brucellosis in seven provinces with control program follow-up in three cattle farms. Benha Veterinary Medical Journal, Vol. 23, 254-263.

Al Dahouk, S., Köhler S., Occhialini, A., de Bagüés, M., Hammerl, J., Eisenberg, T., 2017. Brucella spp. of amphibians comprise genomically diverse motile strains competent for replication in macrophages and survival in mammalian hosts. Sci Rep, 7:44420

Ahmed M. El-Hady, Mohamed Sayed-Ahmed, Mohamed E. Saleh, and Emad E. Younis, 2016. Seroprevalence and Molecular Epidemiology of Brucellosis in Cattle in Egypt. J. Adv. Dairy Res 4: 153. doi:10.4172/2329-888X.1000153.

Al Dahouk, S., Fleche, P.L., Nockler, K., Jacques, I., Grayon, M., Scholz, H.C., Tomaso,H., Vergnaud, G., Neubauer, H., 2007. Evaluation of Brucella MLVA typing for human brucellosis. J. Microbiol. Methods 69, 137–145.

Allen, A., Breadon, E., Byrne, A., Mallon, T., Skuce, R., Groussaud, P., Dainty, A., Graham, J., Jones, K., Pollock, L., Whatmore, A., 2015. Molecular epidemiology of Brucella abortus in Northern Ireland-1991 to 2012. PLoS One 10, e0136721.

Alton, GG; Jones, LM and Piezt, DE.,1975. Laboratory techniques in brucellosis. 2nd Edn., Monograph Series No. 55, World Health Organization (WHO) Geneva. PP: 112- 113.

Alton G.G. 1981. The control of bovine brucellosis. Recent Development. World Anim. Rev. FAO 39:17-24.

Alton G.G., Jones L.M., Angus R.D., and Verger J.M., 1988. Serological methods In: Techniques for the Brucellosis Laboratory. Institut National de la Recherche Agronomique (INRA) Paris: 17-60.

Amin M., Ahmed S., Zaki, A., and Ismail R., 2012. Serological and Molecular Studies on the Diagnosis of Bovine Brucellosis. Nature and Science. 10 (11): 68.

Ammar K. M.; 2000. Some epidemiological aspects of bovine and caprine brucellosis in Egypt. SCVMJ, 111(1), 145-155.

Arellano-Reynoso, B., Suárez-Güemes, F., Estrada, F., Michel-GómezFlores, F.,Hernández-Castro, R., Acosta, R., Díaz-Aparicio, E., 2013. Isolation of a fieldstrain of Brucella abortus from RB51-vaccinated- and brucellosis-seronegative bovine yearlings that calved normally. Trop. Anim. Health Prod. 45, 695–697, http://dx.doi.org/10.1007/s11250-012-0252-8.

Aznar, M.N., Samartino, L.E., Humblet, M.F., Saegerman, C., 2014. Bovine brucellosisin Argentina and bordering countries: update. Transbound. Emerg. Dis. 61,121–133.

AL-Bukair, A. A. A., 2014. Epidemiological Studies on Brucellosis in Farm Animals in Minufyia province. PhD thesis (Animal Hygiene), Faculty of Veterinary Medicine, El Sadat City University, Minufyia, Egypt.

AL-Habaty S. H., Abou-Gazia, K. A. and Ammar M. A., 2015. Prevalence Study on Brucellosis in some Ruminants Slaughtered out of Abattoirs in Assuit Province. Assuit veterinary Medical Journal. Vol. 61(144): 65-72.

Bercovich, Z., 1998. Maintenance of Brucella abortus-free herds: a review with emphasis on the epidemiology and the problems in diagnosing brucellosis in areas of low prevalence. Vet. Q. 20, 81–88.

Çiftci, A; Ica, T; Savaşan, S; Sareyyupoglu, B; Akan, M and Diker, KS (2017). Evaluation of PCR methods for detection of Brucella strains from culture and tissues. Trop. Anim. Health Prod., 49: 755-763.

Corrente M., Desario C., Parisi A., Grandolfo E., Scaltrito D., Vesco G., Colao V., and Buonavoglia D., 2015. Diagnosis of Bovine Brucellosis Using B. Melitensis Strain B115. J. Microbiol. Methods 119, 106-109.

De Jong, M.F. and Tsolis, R.M., 2012. Brucellosis and type IV secretion. Future Microbiol. 7(1):47-58. doi: 10.2217/fmb.11.136.

Dhama,K., Karthik K., Chakraborty S., Tiwari R., Kapoor S., Kumar A., and Thomas P., 2013. Loop-mediated isothermal amplification of DNA (LAMP) - a new diagnostic tool lights the world of diagnosis of animal and human pathogens; a review. Pakistan Journal of Biological Sciences 17: 151-166. DOI: 10.3923/pjbs.2014.151.166.

Di Giannatale, E., De Massis, F., Ancora, M., Zilli, K., Alessiani, A., 2008. Typing of Brucella field strains isolated from livestock populations in Italy between 2001 and 2006. Vet. Ital. 44, 383–388.

Dorneles, E.M., Santana, J.A., Alves, T.M., Pauletti, R.B., Mol, J.P., Heinemann, M.B., Lage, A.P., 2014. Genetic stability of Brucella abortus isolates from an outbreak by multiple-locus variable-number tandem repeat analysis (MLVA16). BMC Microbiol. 14, 186.

Ewalt D. R., and Bricker B. J., 2000. Validation of the abbreviated Brucella AMOS PCR as a rapid screening method for differentiation of 97 Brucella abortus field strain isolates and the vaccine strains, 19 and RB51. J. Clin. Microbiol. 38:3085–3086.

El shafey Ali. 2017. Studies on using of immunochromatographic assay for detection of Brucella infection in animals. Ph.D. thesis (bacteriology). Faculty of Veterinary Medicine-University of Sadat City.

El-Diasty M. M., 2004. Epidemiological and immunological studies on bovine brucellosis. M. V. Sc. Thesis. Fac. Vet. Suez-Canal Univ.

El-Sherif A.M. and El-Sheary M.N., 2002. Role of stray dogs and rats as carriers for brucella infection for cattle. 10th Sci. Cong. Fac. Vet. Med. Assiut Univ. Egypt.

El-Sayed, A., Awad, W. 2018. Brucellosis: Evolution and expected comeback. International Journal of Veterinary Science and Medicine 6 : S31–S35.

FAO. 2003. Guidelines for coordinated human and animal brucellosis surveillance". FAO Animal Production and Health Paper 156, 3-4.

Farouk, M. 2015. Studies on brucellosis in cattle in Jigawa state, Nigeria. Ph.D. Thesis (Veterinary Medicine). Department of Veterinary Medicine - Faculty of Veterinary Medicine - Ahmadu Bello University, Zaria, Nigeria.

Fekete A., Bantle A., Halling S.M., and Stich W., 1992. Amplification fragment length polymorphism in Brucella strains by use of polymerase chain reaction with arbitrary primers. Journal of Bacteriology 174, 7778-7783.

García-Yoldi D., Marín C.M., de Miguel M.J., Muñoz P.M., Vizmanos J.L., and & López-Goñi I., 2006. Multiplex PCR assay for the identification and differentiation of all Brucella species and the vaccine strains Brucella abortus S19 and RB51 and Brucella melitensis Rev1. Clinical Chemistry, 52(4): 779-781.

Ghorbani, A., Khorasgani, M.R., Zarkesh-Esfahani, H., Sharifiyazdi, H., Kashani, A.D., Emami, H., 2013. Comparison of serology, culture, and PCR for detection of brucellosis in slaughtered camels in Iran. Comp. Clin. Pathol., 22: 913-917.

Gwida M, Al Dahouk S, Melzer F, Rösler U, Neubauer H, Tomaso H.2010. Brucellosis-regionally emerging zoonotic disease? Croat Med J. 51(4):289-95.

Gwida M., El-Ashker M., Melzer F., El-Diasty M., El-Beskawy M., and Neubauer H., 2016. Use of serology and real time PCR to control an outbreak of bovine Br. at a dairy cattle farm in the Nile Delta region. Egypt. Irish Vet. J.; 69(1):1.

Hegazy Y. M., Molina-Flores B., Shafik H., Ridler A. L., and Guitian F. J., 2011. Ruminant brucellosis in Upper Egypt (2005–2008). Prev. Vet. Med. J. 101(3-4): 173-181.

Holt H, Eltholth M, Hegazy Y, El-Tras W, Tayel A, Guitian J., 2011. Brucella spp. infection in large ruminants in an endemic area of Egypt: Cross-sectional study investigating seroprevalence, risk factors and livestock owner's knowledge, attitudes and practices (KAPs). BMC Public Health 11: 341-350.

Hsieh Y. C., Chen S. H., Chou C. S., Hsiao H. W. , Chen S. Z. , Lee Y. F., and Liu H. J., 2005. Development of a reliable assay protocol for identification of diseases (RAPID) bioactive amplification with probing (BAP) for detection of bovine ephemeral fever virus. J Virological Methods 129: 75-82.

Hosein H. I., Ghobashy H., Azzam R. A., Elsherif A. M., and Sassi M. F. 2010. Nitric oxide and lysozyme activities as early monitors of the immune response of Brucell; 3-5 July 2010.

Kaushik, P., Singh, D.K., Tiwari, A.K., Kataria, R.S., Kumar, P., 2006. PCR based detection of Brucella species in the aborted cattle fetus samples, J. Immunol. Immunopathol. 8:71–73.

Kaynak-Onurdag, F; Okten, S and Sen, B., 2016. Screening Brucella spp. in bovine raw milk by real-time quantitative PCR and conventional methods in a pilot region of vaccination, Edirne, Turkey. J. Dairy Sci., 99: 3351-3357.

Kumar, A; Gupta, VK; Verma, AK; Sahzad; Kumar, V; Singh, A and Reddy, NCP.,2016. Seroprevalence and risk factors associated with bovine brucellosis in western Uttar Pradesh, India. Ind. J. Anim. Sci., 86: 131-135.

Leal-Klevezas D. S., Martinez-Vazquez I. O., Garcia-Cantu J., Lopez-Merino A., and Martinez-Soriano J. P.; 2000. Use of polymerase chain reaction to detect Brucella abortus biovar 1 in infected goats. Vet. Microbiol. (75): 91-97.

Mantur, B.G., Amarnath, S.K., Shinde, R.S., 2007. Review of clinical and laboratory features of human brucellosis, Indian J. Med. Microbiol. 25:188–202.

Mantur, B.G. and Amarnath, S.K. 2008. Brucellosis in India-a review, J. Biosci. 33 :539-547.

Matope G., Bhebhe E., Muma J .B., Lund A., and Skjerve E., 2010. Herd-level factors for Brucella seropositivity in cattle reared in small holder dairy farms of Zimbabwe. Preventive Veterinary Medicine, (94): 213-221.

Matope G., Bhebhe E., Muma J. B., Oloya J., Madekurozwa R. L., Lund A. & Skjerve E., 2011. Seroprevalence of brucellosis and its associated risk factors in cattle from smallholder dairy farms in Zimbabwe. Tropical animal health and production. (43): 975-982.

Mick, V., Le Carrou, G., Corde, Y., Game, Y., Jay, M., Garin-Bastuji, B., 2014. Brucella melitensis in France: persistence in wildlife and probable spillover from Alpine ibex to domestic animals. PLoS One 9, 94168.

Minharro, S., Mol, J.P.S., Dorneles, E.M.S., Pauletti, R.B., Neubauer, H., Melzer, F.,Poester, F.P., Dasso, M.G., Pinheiro, E.S., Soares, P.M., Santos, R.L., Heinemann,M.B., Lage, A.P., 2013. Biotyping and genotyping (MLVA16) of Brucella abortus isolated from cattle in Brazil, 1977 to 2008. PLoS One 8.

Mittal, V; Kumar, M and Ambwani, T., 2005. Sero-epidemiological pattern of brucellosis among livestock of district Udham Singh Nagar in Uttaranchal. Ind. J. Vet. Med., 25: 28-32.

Mittal, M., Sharma, V., Nehra, K., Chakravarti, S., Kundu, K., Bansal, V.K., Churamani, C.P., Kumar, A.2018. Abortions in an organized dairy farm from North India reveal the possibility of breed susceptibility to Bovine Brucellosis. One Health 5 : 1–5.

Montasser A. M., Hamdy M. E., EL-Biomy E. M., and Khoudier R., 2001. Bacteriological profile of brucella isolated from cattle in Egypt. 6th Sci. Cong., Egyptian Society for Cattle Diseases. 4-6 Nov: 163-170.

Muma J. B., Godfroid J., Samui K. L., and Skjerve E., 2007. The role of Brucella infection in abortions among traditional cattle reared in proximity to wildlife on the Kafue flats of Zambia. Rev. Sci. tech. Off. Int. Epiz. 26 (4), 721–730.

Nanven, A. M., 2013. Bacteriological and Serological Studies of Bovine Brucellosis in Obudu Cattle Ranch, cross River state, Nigeria. A Master of Science in Veterinary Microbiology Thesis. Ahmadu Bello University, Zaria.

Neha; Ahmed, W; Verma, AK; Jain, U and Bist, B., 2014. Brucellosis in organized dairy farm: an investigation. Asian J. Anim. Sci., 8: 29-33.

Ocholi R. A., Kwaga J. K. P., Ajogi I., and Bale J. O. O. 2005. Abortion due to Brucella abortus in sheep in Nigeria. Revue scientifique et technique International Office of Epizootics, vol. 24, no. 3, pp. 973–979.

OIE. 2008. Manual of Diagnostic Tests and Vaccines for Terrestrial

Animals. Chapter 2.4.3: Bovine Brucellosis. OIE, Paris, France.

OIE. 2013. Manual of Diagnostic Tests and Vaccines for Terrestrial

Animals. Chapter 2.4.3: Bovine brucellosis. OIE, Paris, France.

Pappas G., Papadimitriou P., Akritidis N., Christou L. & Tsianos E. V., 2006. The new global map of human brucellosis. The Lancet infectious diseases. (6): 91-99.

Ouahrani-Bettache S., Soubrier M.P., and Liautard J.P., 1996. IS6501 anchored PCR for the detection and identification of Brucella spp. and strain. J. Appl. Bacteriol. 81: 154-160.

Radostitis O. M., Gay C. C., Blood D. C. and. Hinchcliff K. W., 2007. Veterinary Medicine. 9th Ed., ELBS Bailliere Tindall, London, UK, pp: 870-871.

Ran, X., Cheng, J., Wang, M., Chen, X., Wang, H., Ge, Y., Ni, H., Zhang, X.X., Wen, X., 2019. Brucellosis seroprevalence in dairy cattle in China during 2008–2018: A systematic review and meta-analysis. Acta Tropica 189:117–123.

Refai, M., 2002. Incidence and control of brucellosis in the Near East region. Vet.Microbiol. 90, 81–110.

Refai M., 2003. Application of biotechnology in the diagnosis and control of brucellosis in the Near East Region. World J Microbiol Biotechnol 19: 443-449.

Saadat, S., Mardaneh, J., Ahouran, M., Mohammadzadeh, A., Ardebili, A., Yousefi, M., Mansouri, M.2017. Diagnosis of Cattle Brucellosis by PCR and Serological Methods: Comparison of Diagnostic Tests. Biomedical & Pharmacology Journal. Vol. 10(2), 881-888.

Salem L. M. A., Khoudair M. R., and Osman S. A., 2014. Sero Diagnosis of Brucellosis by Using Simple and Rapid Field Tests with Emphasis on Some Possible Risk Factors in Humans. Global Veterinaria 12(3): 320-325.

Sayour A., 2004. The use of recent bacteriological techniques in the differentiation of Brucella group of micro-organisms. PhD. thesis, Department of Microbiology; Fac of Vet Med, Cairo University; Egypt.

Selim A., Gaber A., and Moustafa A., 2015. Diagnosis of Brucellosis in Ruminants in Kafr El-Sheikh province, Egypt. International Journal of Advanced Research 3(1): 345-350.

Seleem, M.N., Boyle, S.M., Sriranganathan, N., 2010. Brucellosis: A re-emerging zoonosis.Vet. Micriobiol., 140:392-398.

Sherif H., 2008. Serosurvey on Cattle Brucellosis at Menoufia Province. M. Sc.V. Thesis (Infectious Diseases). Faculty of Veterinary Medicine – Menoufia University – Sadat City Branch.

Shalaby M. N., Ghobashy H. M., EL-Bauomy E. M., and Saleh W. M., 2003. Prevalence of brucellosis among farm animal species in some provinces in Egypt. Proceedings of the 7th Scientific Congress, Egyptian Society for Cattle Diseases. Assiut. Egypt. 271-282.

Shehata A. E., 2004. Immunological studies on Brucella microorganisms in farm animals in Beni-Sueif province. M.V.Sc. Thesis (Micro.), Fac. Vet. Med., Cairo University, Beni-Sueif Branch.

Sim J. and Wright C. C., 2005. The kappa statistic in reliability studies: use, interpretation, and sample size requirements. Phys. Ther. 85 (3): 257-268.

Thrusfield, M (2008). Veterinary epidemiology. 3rd Edn., UK, Blackwell Publishing Ltd., PP: 311-316.

Verma, AK; Dhama, K; Chakraborty, S; Kumar, A; Tiwari, R; Rahal, A; Mahima and Singh, SV., 2014. Strategies for combating and eradicating important infectious diseases of animals with particular reference to India: present and future perspectives. Asian J. Anim. Vet. Adv., 9: 77-106.

Wareth G., Melzer F., Elschner M. C., Neubauer H. and Roesler U., 2014. Detection of Br. melitensis in bovine milk and milk products from apparently healthy animals in Egypt by real-time PCR. J. Infect. Dev. Ctries. 8(10): 1339-1343.

Xavier, N. M., Costal, E., Paixaol, T., Santos, I., R., 2009. The genus brucella and clinical manifeststion of brucellosis. Cienc. Rural, 39(7): 2252-2260.