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Isolation and Identification of *Brucella* Species from Dairy Cattle by Biochemical Tests: The First Report from Ethiopia

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ABSTRACT

Isolation of *Brucella* organism is considered as the gold standard diagnostic method for brucellosis since it is specific and allows biotyping of the isolate, which is relevant for control of brucellosis using vaccination. Serological studies revealed that brucellosis is endemic in bovines in Ethiopia. Even though seroprevalence of brucellosis is established in different species of animals, so far there was no successful attempt to isolate and identify *Brucella* spp. in dairy cattle at farm level in the country. Therefore, the endeavor of the present study was to isolate *Brucella* spp. from seropositive cattle with a history of abortion. A total of 570 dairy cattle from 35 herds were screened serologically by Rose Bengal plate test based on the history of abortion in the farm. Among the tested samples 13 (2.28%) were found positive by Rose Bengal plate test screening while 33 samples were found sero negative upon serological screening test but were collected from the cattle with history of recent abortion. Forty six clinical samples were cultured which were both from *Brucella* seropositive and seronegative (dairy cattle with history of abortion) upon Rose Bengal plate test screening. Three (6.52%) samples were *Brucella* culture positive and further characterization of all the three isolates based on biochemical tests result confirmed that the pathogen was *Brucella abortus*. *Brucella abortus* was isolated from placental cotyledon 1/9 (11.1%) and vaginal swab 2/23 (8.69%) while no isolate was obtained from milk and fetal abomasal contents (abomasal aspirate) of aborted fetus. Our finding revealed the occurrence of *B. abortus* in dairy cattle of Ethiopia through isolation of the organism for the first time from seropositive dairy cattle with a history of abortion. The organisms were isolated from placental cotyledon (one isolate) and vaginal swab (two isolates) while no isolate was obtained from milk and fetal abomasal contents (abomasal aspirate) of the aborted fetus. Hence, the bacteriological isolation and identification of *Brucella abortus* from dairy cattle indicates the importance of brucellosis in dairy cattle industry of the area and potential public health implication for human population in the study areas.

Key words: Isolation, Dairy cattle, *Brucella abortus*, Biochemical test, Ethiopia

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INTRODUCTION

Brucellosis is endemic in many developing countries and is caused by *Brucella* species that affect man, domestic and some wild animals, and marine mammals (Bhatia and Narain, 2010; Seleem et al., 2010 and Geresu et al., 2016). An estimated 500 000 new human *Brucella* cases were reported annually worldwide (Pappas et al., 2006). Brucellosis is the second most important zoonosis after rabies and has gained prominence over the years since its discovery on the island of Malta (Seleem et al., 2010 and Abubakar et al., 2012).

Brucellae species are gram negative cocci bacilli, which are classified into species by various techniques such as growth patterns on media and phage susceptibility. There are six "classical" recognized species; *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae* (Godfroid et al., 2005; Hadush and Pal, 2013). Recently, four new *Brucella* species have been recognized and classified, namely, *B. pinnipedialis*, *B. ceti*, *B. microti* and *B. inopinata* (Foster et al., 2007; Scholz et al., 2009).

The mode of transmission of the bacteria varies with the epidemiological area, the animal reservoir and the occupational exposed groups (Seleem et al., 2010 and Geresu et al., 2016). Sources of infection for the transmission of the bovine brucellosis are aborted fetuses, the fetal membranes after birth, and vaginal discharges and milk from infected animals (Tolosa et al., 2010 and Geresu et al., 2016). The most common route of transmission is the gastrointestinal tract following ingestion of contaminated pasture, feed, fodder, or water, and after birth, fetuses, and newborn calves, all of

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which may contain a large number of the organisms and constitute a very important source of infection. The bacteria can be transmitted to humans through direct contact with infected tissue via breaks in skin, ingestion of contaminated tissues or milk products, and inhalation or mucosal exposure to aerosolized bacteria (Radostits et al., 2007).

The diagnosis of brucellosis is based on serological, bacteriological, allergic skin reaction, and molecular methods (Simsek et al., 2004). The most important confirmatory method of *Brucella* infection is bacteriological diagnosis since its specificity is much higher than that of other diagnostic methods and it is used as a gold standard diagnostic method. The existence of different *Brucella* biotypes among the *Brucella* spp. and their identification is important to confirm the infection and trace the source of the infection (Guler et al., 2003). Because of the complications involved in the diagnosis of the disease, including the difficulties in distinguishing between infected and vaccinated animals by conventional serological tests, bacteriological isolation and identification of biotypes of the etiological agent are necessary steps in the design of epidemiological and eradication programs (Refai, 2002; Zinstag et al., 2005). Molecular diagnostic methods are also currently being used for the detection of *Brucella* spp. in various samples (Şahin et al., 2008).

Samples for *Brucella* spp. isolation from cattle include fetal membranes, particularly the placental cotyledons where the number of organisms tends to be very high. In addition, fetal organs such as the lungs, bronchial lymph nodes, spleen and liver, as well as fetal gastric contents, milk, vaginal secretions and semen are samples of choice for isolation (Poester et al., 2006 and Lage et al., 2008). Vaginal secretions should be sampled after abortion or parturition, preferably using a swab with transporter medium, allowing isolation of the organism up to six weeks post parturition or abortion (Poester et al., 2010). Milk samples should be a pool from all four mammary glands. Non pasteurized dairy products can also be sampled for isolation (Lage et al., 2008; Poester et al., 2010).

Since the first report of brucellosis in the 1970s in Ethiopia, the disease has been noted as one of the important livestock diseases in the country (Ibrahim et al., 2010; Kebede et al., 2008; Geresu et al., 2016). A large number of studies on bovine have been reporting individual brucellosis seroprevalence ranging from 1.1% to 22.6% in intensive livestock management systems (Tolosa et al., 2010; Tesfaye et al., 2011) and 0.05% -15.2% in extensive management systems (Degefa et al., 2011; Megersa et al., 2011).

Though serological survey for *Brucella* antibodies revealed that brucellosis is known to be endemic in the country, there was no successful attempt to isolate and identify *Brucella* spp. in dairy cattle at farm level. Therefore, the present study aimed to isolate *Brucella* spp. from dairy cattle with a history of abortion for the first time in Ethiopia by using standard cultural methods in order to establish an epidemiological base for studies on the control and prevention of brucellosis in the country.

MATERIALS AND METHODS

Study areas and design

The study was conducted in two purposely selected sites in central Ethiopia, Bishoftu, East Shewa zone and Assela, East Arsi zone. These study areas were selected based on the abundance of dairy farms that constituted the known milk sheds (Land O'Lakes Inc, 2010; Geresu et al., 2016). Bishoftu is located at 47kms south east of Addis Ababa. The area is located at 9°N latitude and 40°E longitudes at an altitude of 1850 meters above sea level in the central high land of Ethiopia. It has an annual rainfall of 866mm of which 84% is in the long rainy season (June to September). The dry season extends from October to February. The mean annual maximum and minimum temperatures are 26°C and 14°C respectively, with mean relatively humidity of 61.3% (ADARDO, 2007). Farmers in the vicinity of Bishoftu town use a mixed crop and livestock farming system. Moreover, Bishoftu and its surrounding have variable and yet representative agroecologies of the country. These agroclimatic zones are inhabited with different plant and animal species (Conway and McKenzie, 2007).

The second study area was Asella, which is located at 175 km south east of Addis Ababa and the altitude and annual rainfall of the area ranges from 502-4130 meters above sea level and 200-400mm, respectively with mean annual temperature of 22.5^{o}C. It is one of the highly populated area in Ethiopia with estimated human population of 2521349 and livestock population of cattle, 82190; sheep, 51292; goat, 811479; poultry, 562915 and equine, 22055 (Deselegn and Gangwar, 2011; Geresu et al ., 2016).

Definitions

In Ethiopia dairy cattle production systems are classified into rural smallholder (mixed crop-livestock) production, pastoral and agro pastoral production, urban and peri-urban smallholder dairy production, and commercial dairy production systems (Land O'Lakes Inc, 2010; Asmare et al., 2013). This study focuses on the latter two production systems.

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Study population

The target populations were dairy cattle in urban and peri-urban dairy (both smallholder and commercial) farms of Asella and Bishoftu towns which are composed of Holstein Friesian, their crosses and local breeds established in the major milk sheds of the study sites (Asmare et al., 2013; Geresu et al., 2016).

Study design and sample size determination

A cross-sectional study design was conducted to isolate *Brucella* spp. infecting dairy cattle by tracing back RBPT sero screening and abortion history of the animals. Dairy cattle above six months of age were selected for this study. The sampling was performed using a two level approach, selecting first individual farms with abortion history and then randomly selecting individual animals systematically inside each farm, while all animals in each farm with recent history of abortion were sampled purposely for bacteriological culture and isolation. A list of dairy farm was prepared for each of the two study areas in collaboration with the respective district livestock health departments.

The sample size for sero screening of cattle in Asella was calculated on the basis of previous report of 14.14% sero prevalence of bovine brucellosis in Arsi Zone (Deselegn and Gangwar, 2011). Therefore to determine the sample size of dairy cattle in this area, 14.14% was used as pexp and 95% confidence interval and 5% required precision (Thrusfield, 2007).

$$n = \frac{1.96^2 \times P_{exp} x 1\text{-}P_{exp})}{d^2}$$

$$n = \frac{3.84 x .1414\ (1\text{-}0.1414)}{(0.05)^2} = 186$$

In Bishoftu, since there was no previous study done in the area, by considering 50% expected prevalence, 95% confidence interval and 5% required precision, 384 cattle were selected for this study (Thrusfield, 2007). Hence, a total of 570 dairy cattles (186 from Asella and 384 from Bishoftu) were considered for this study from 35 farms in the study areas.

Bacteriological sample collection

Fetal and placental cotyledon: Aborted cattle fetuses (aspirate of stomach content) and placental cotyledon were collected during the visits to the farms after the report of bovine abortion cases and transported to National Animal Health and Diagnostic Center (NAHDIC) in ice packs and stored at -20 ˚C until processed.

Milk: Initially the cattle in the dairy farm were screened serologically using RBPT and positive animal's milk samples were collected. Samples of milk were collected cleanly after washing and drying the whole udder and disinfecting the teats. The samples were containing milk from all quarters, and 10 ml of milk was taken from each teat. The first streams were discarded and the sample was milked directly into a sterile vessel and transported to NAHDIC in ice packs and stored at +4 ˚C until processed.

Vaginal discharge: A vaginal swab was taken after abortion or parturition in Stuart medium and transported to NAHDIC in ice packs and stored at -20 ˚C until processed.

Bacteriological isolation of *Brucella*

Brucella isolation from fetal and placental cotyledon was performed according to Farrell method (Farrell, 1974). Approximately 1ml of fetal abomasal contents and placental cotyledon collected were rubbed on to *Brucella* medium base supplemented with 5% horse serum (Oxoid, CM 0169) and onto Farrell's medium, selective medium, which is prepared by the addition of *Brucella* selective supplement (Oxoid,SR0083A) containing [polymyxin B (as SO4) = 2,500IU, bacitracin =12,500IU, cycloheximide = 50.0mg, nalidixic acid = 2.5 mg, nystatin =50,000 IU, vancomycin (as HCL) = 10.0 mg)], 5% horse serum , 50% methanol and 50% dextrose on *Brucella* medium base and tryptic soy agar. Milk samples for isolation of *Brucella* were processed according to Tantillo et al. (2003).

The milk samples were centrifuged at 3000 rpm for 10 minutes to obtain the sediment-cream mixture which then was cultured on both basal media (*Brucella* medium base supplemented with 5% horse serum) and Farrell's medium (*Brucella* selective medium). Vaginal swabs were streaked on to solid media similar to that of above mentioned clinical specimen and incubated.

The inoculated plates from different clinical specimen were incubated at 37ºC both in the absence and presence of 10% CO_2 for up to 2 weeks. After the incubation, the suspected colonies were examined for *Brucella* spp. growth. *Brucella* suspected colonies were characterized by their typical round, glistening, pinpoint and honey drop-like appearance and examined for Gram stain and modified Ziehl-Nelsen stain (MZN) initially. Subsequent biochemical tests for oxidase, catalase, urease production, methyl red, voges proskauer test, acid production on media containing

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glucose, citrate utilization, indole test, motility (at both 37°C and 20°C) were carried out. Absence of growth on MacConkey agar and non-hemolytic appearance on blood agar were also conceded.

RESULTS

Among 570 tested samples, 13(2.28%) were found positive by RBPT. The higher sero screening result was observed in and around Asella town (5.38%) compared to Bishoftu (0.78%). Thirty three different samples were included from dairy cattle with a history of recent abortion but were seronegative upon sero screening by RBPT (Table 1). Of 46 clinical samples cultured in the present study, an overall rate of 6.52% (3/46) isolation was found. All the three isolates were obtained from the RBPT positive samples while no isolate was obtained from dairy cattle with a history of abortion which are sero negative upon RBPT screening. All the three isolates were from Asella while no isolate was obtained from Bishoftu.

The result of biochemical tests revealed that all the three isolates were *B. abortus*. *Brucella abortus* was isolated from placental cotyledon 1/9 (11.1%) and vaginal swab 2/23 (8.69%) while no isolate was obtained from milk and fetal abomasal contents (abomasal aspirate) of dairy cattle (Table 2). Three colonies of *B. abortus* were observed on *Brucella* selective media (Farrell's medium) after 4 days of incubation, as pinpoint, round, convex with smooth margin, translucent and pale honey in color (Figure 1). All the colonies were grown in 10% CO_2 supplied incubator and agglutinated with positive control serum for *B. abortus* while negative with the negative control serum (slide agglutination test with an anti-*Brucella* polyclonal serum). The culture smear showed Gram negative coccobacilli in Gram's staining (Figure 2) and red stained coccobacilli in modified Ziehl-Neelsen staining (Figure 3). The isolated colonies were not grown on MacConkey agar (non-lactose fermenter) (Figure 4) and non hemolytic on blood agar (Figure 5). Growth was noticed in plate with basic fuchsin (Figure 6). The detailed result of basic biochemical and metabolic profiles of field *B.abortus* isolated from the study area were depicted in Table 3.

Table 1. Culture results of RBPT seropositive (aborted or not) and samples from dairy with abortion history (RBPT seronegative) from Asella and Bishoftu towns in 2014

Origin	Total sample	RBPT vs. Culture			Samples from dairy cattle with abortion history (RBPT seronegative)		
		RBPT +ve	Culture		N	Culture	
			+ve	-ve		+ve	-ve
Bishoftu	384	3(0.78)	0	0	14	0	14
Asella	186	10(5.38)	3	7	19	0	19
Total	570	13(2.28)	3	7	33	0	33

N = number of samples cultured, +ve = positive, -ve = negative, RBPT = Rose Bengal plate test, vs. = versus

Table 2. *Brucella* isolates recovered from clinical samples of seropositive animals from Asella dairy farm in 2014

Sample type	No. of examined samples	+ve culture isolation	%
Foetal abomasal content	1	0	0
Placental cotyledon	9	1	11.11
Milk	13	0	0
Vaginal swab	23	2	8.69
Total	46	3	6.52

No = number, +ve = Positive, % = Percent

Table 3. Basic biochemical and metabolic profiles of field *B. abortus* isolated from cattle of Asella dairy farm in 2104

Brucella isolate	Biochemical properties										Growth on dyes
	Cat	Oxi	[a]Ure	Mot	Ind	MZN	Cit	MR	VP	Glu	BF
Placental cotyledon	+	+	+	-	-	+	-	-	-	-	+
Vaginal swab 1	+	+	+	-	-	+	-	-	-	-	+
Vaginal swab 2	+	+	+	-	-	+	-	-	-	-	+

Cat = Catalase, Oxi = Oxidase, Ure = Urea hydrolysis, Mot = Motility test [+ , motile, - , non-motile], Ind = Indole production, MZN = Modified Zeihl Neelsen stain, Cit = Citrate Utilization; MR = Methyl Red, VP = Voges Proskauer, Glu = Glucose, BF = Basic Fuchsin, TSA = Tryptic Soy Agar, + = Positive test result, - = Negative test result; [a]Urea hydrolysis = All isolate positive within 2 hours of culture (Figure 7)

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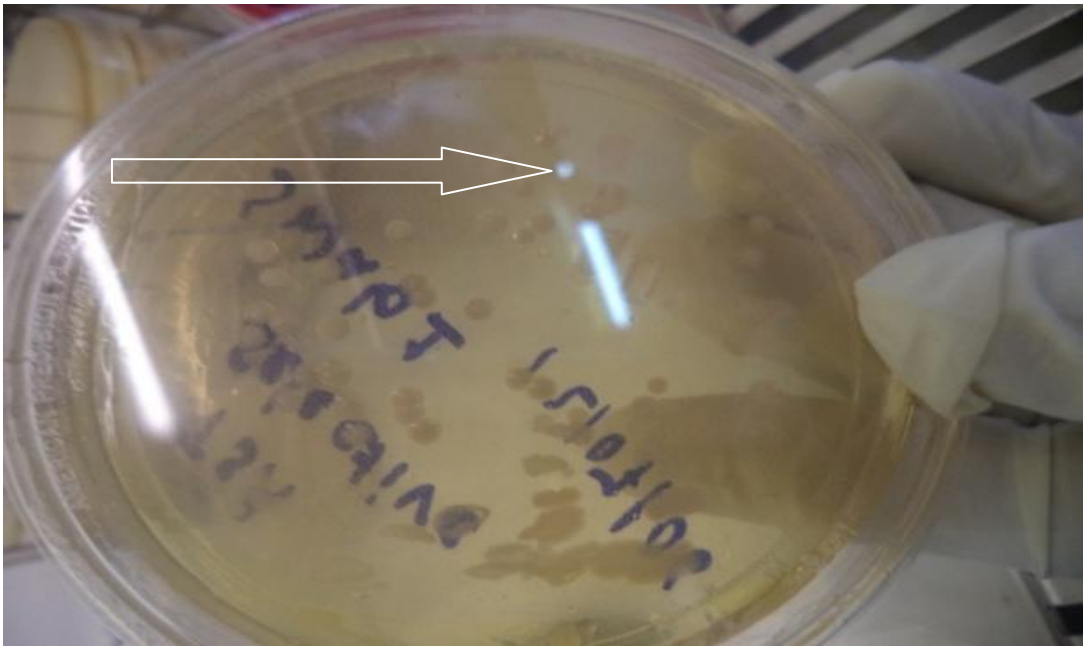

Figure 1. *Brucella* spp growth (morphology) on Farrell's medium from placental cotyledon of aborted dairy cattle in Asella town dairy farm

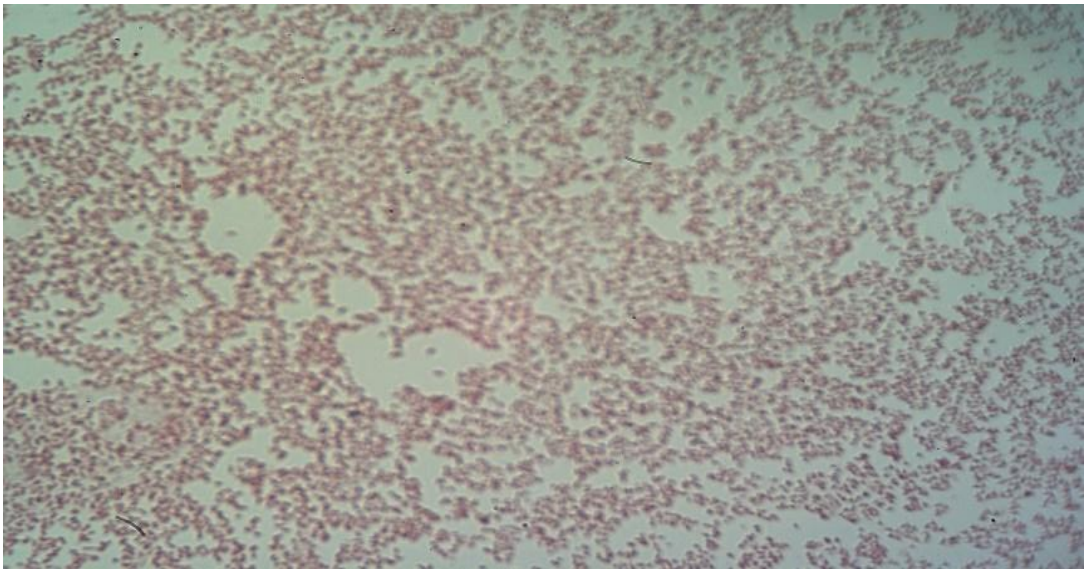

Figure 2. Gram's stain (coccobacilli) result of the isolated colony of *Brucella* ssp isolated from dairy cattle

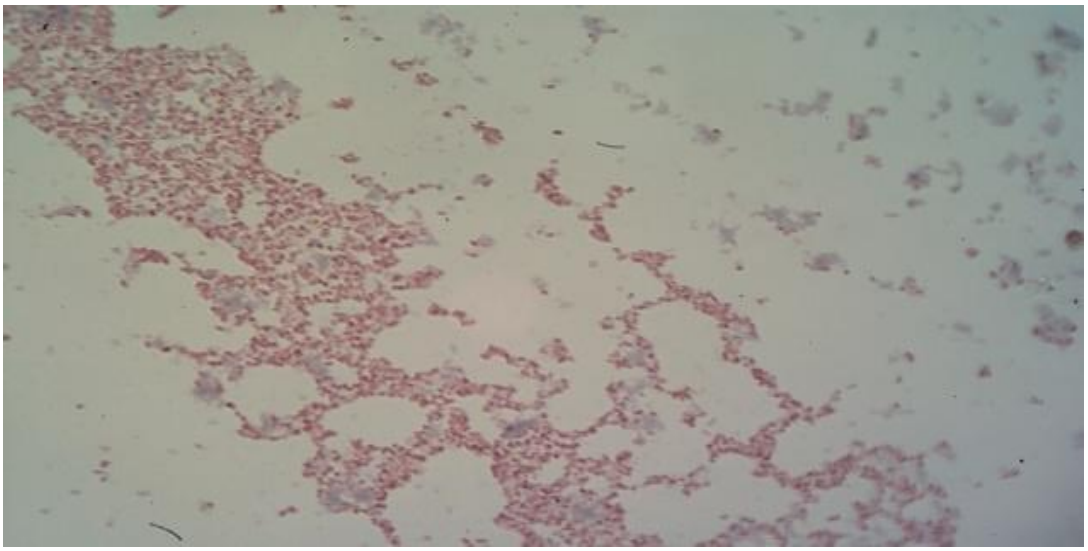

Figure 3. Modified Zeihl Neelsen (Red coccobacilli against blue back ground) stain of *Brucella* isolated from dairy cattle

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Figure 4. No growth of the isolated *Brucella* spp isolated from dairy cattle on Mac Conkey agar


Figure 5. Non-heamolytic appearance of the colony of *Brucella* spp isolated from dairy cattle on blood agar

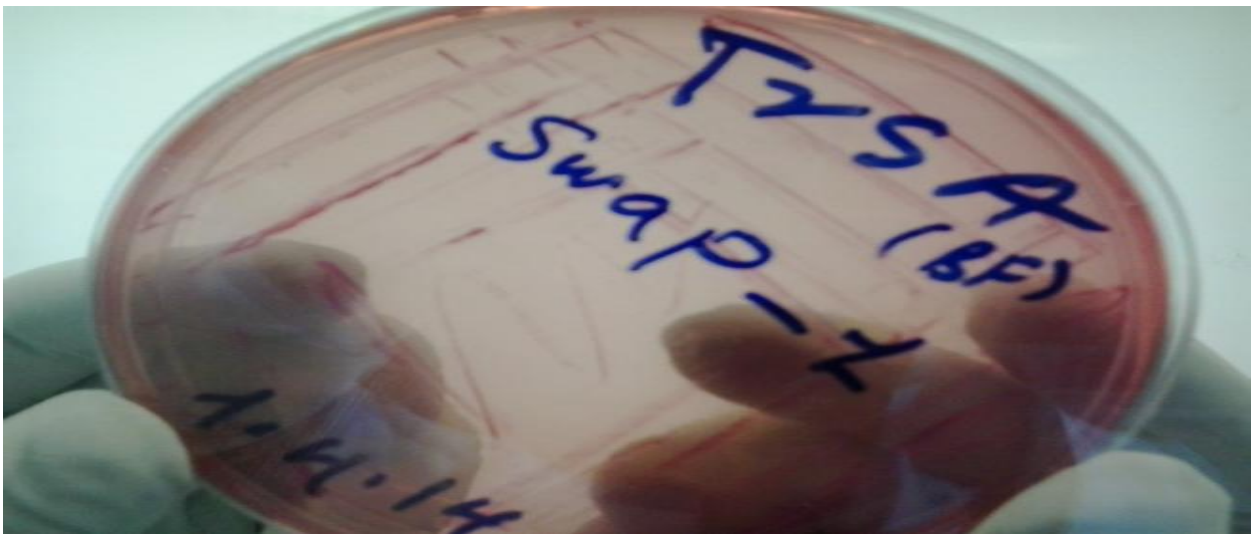

Figure 6. Growth of *Brucella* spp. isolated from dairy cattle on media containing basic fuchsin dye

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Figure 7. *Brucella* spp isolated from dairy cattle hydrolyzing urea within two hour

DISCUSSION

Sero prevalence studies in animals show that brucellosis is endemic in Ethiopia (Tolosa et al., 2010; Degefa et al., 2011; Megersa et al., 2011Tesfaye et al., 2011; Asmare et al., 2013; Geresu et al., 2106). However, the *Brucella* species and their biovars endemic in Ethiopia are unknown.

In the present study, the isolation of *B. abortus* from sero positive cattle with history of abortion was carried out in Ethiopia for the first time. This confirmatory isolation of *B. abortus* was from clinically aborted cattle placental cotyledon (11.1%) and vaginal swab (8.69%) while no isolate was obtained from milk and fetal abomasal content. The low isolation rate (6.52%) of *B. abortus* obtained in the present study from sero positive animals with a history of abortion was in agreement with previous report of 6.4 % (Çelebi and Otlu, 2011). This might be because of the slow growing and fastidious nature of the pathogen (Seleem et al., 2010).

In contrast to this result, a higher rate of isolation of *B. abortus* was reported by Gülhan et al. (2011) (26.7%), Ali et al. (2014) (40%) and Ünver et al. (2006) (55.6%) from aborted cattle fetuses. This difference may be related to the usage of more than one selective culture media in their study while in the present study only Farrell's medium was used. Isolation of *B.abortus* can be improved if more than one selective culture medium is used (Ali et al., 2014). In the present study, bacteriological cultural, morphological and biochemical tests confirmed that all the three isolates obtained from the cases of placental cotyledon and vaginal swabs of aborted cattle were *B. abortus*. Similar to the earlier reports, all the *Brucella* isolates found in this study were positive for catalase, oxidase and urea hydrolysis and negative for indole production, citrate utilization, methyl red, and Voges-Proskauer tests revealing them to be *Brucella* spp. (Koneman et al., 1997). Other reports have also indicated that on the basis of cultural, morphological, and biochemical characteristics, it is possible to identify *Brucella* spp. (Alton et al., 1988; Koneman et al., 1997).

Shedding of *Brucella* in the milk of infected animals is an important source of transmission of disease to humans if the raw milk is consumed (Tantillo et al., 2003). Ocholi et al. (2004) isolated *Brucellae* from milk (7.2 %) in Nigeria while Ali et al. (2014) recovered *B. abortus* (3.2%) from milk samples in Pakistan.

In contrary to these authors' findings, there was no recovery of *Brucella* spp. from 13 milk samples of RBPT positive cows in the present study. This result might be due to the secretion of an organism in the milk a few days (2 to 5 days) after abortion, small number of sample cultured and use of only Farrell's medium in the present study. The isolation of *Brucella* from milk samples may be improved if more than one culture medium is used (Ali et al., 2014). Hence, the result should not underestimate the risk of consuming raw milk as source of *Brucella* infection in the study area.

The fact that *Brucella* species were isolated from milk of cattle from different studies with different rate in Nigeria (Ocholi et al., 2004), Turkey (Çelebi and Otlu, 2011) and Pakistan (Ali et al., 2014) illustrates the need for further investigation in Ethiopia.

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CONCLUSION

Bovine brucellosis caused by *B.abortus* has a major impact on human health, besides causing significant economic losses in dairy industry. In Ethiopia, despite of a number of research reports on sero prevalence of brucellosis in cattle and widespread occurrence of brucellosis in different production system, there is no bacteriological isolation and identification of *B.abortus* from dairy cattle. In the present study, *B.abortus* was isolated for the first time in Ethiopia from seropositive dairy cattle with history of recent abortion. The organisms were isolated from placental cotyledon (one isolate) and vaginal swab (two isolates). Hence, it is of practical importance to isolate *Brucella* spp. to design and utilize effective *Brucella* vaccines in Ethiopia.

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Competing interests

The authors declare that they have no competing interests.

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