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Diagnosis of Brucella Infection in Sheep and Goat and Evaluation of the associated Practices in Animal Contacts

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Abstract Brucellosis is major zoonosis that affects health and economy in many parts of the world. Brucellosis remains an uncontrolled problem especially in several regions of high endemicity such as the Mediterranean, Middle East. Transmition of Brucellosis in humans is strongly related to contact with infected animals. Diagnosis of brucellosis is based on microbiological and serological laboratory test. **Methodology:** A cross-sectional study was conducted between January 2016 to June 2016 to estimate the seroprevalence of brucellosis in the small ruminants. A total of 315 blood samples collected from sheep, goats and human contacts with animals in the age from 15 to 58 yearswere tested by Rose Bengal test. Also, bacteriological examination was done for 171 available milk samples small ruminants (134 sheep and 37 goats), one sample of stomach and visceral content from aborted fetus of goat and five blood samples of human contact with a current history of fever. PCR was employed. **Results:** The prevalence in human was (16) 15.2% and in small ruminants was (40)19% and the risk of infection was associated with increasing age, assistance in labour, presence of infected animal with brucellosis and who giving their animal males for fertilization other flocks. **Conclusion:** There were 56 positive Rose Bengal test in sheep, goats and human. All recovered isolates of brucella were B. melitesisbiovar 3 and confirmed by PCR. It is probable that they share the sameorigin of infection. The general public, especially where brucellosis present has to be made aware of the danger to health.

Keywords: *diagnosis, brucellosis, sheep, goat, risk factors*

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1. Introduction

Brucellosis is one of the major zoonosis that affects health and economy in many parts of the world. Although brucellosis in livestock and human has been decreased through the prevention programmes in many parts of the world, and it has been eradicated from several countries of the world, however it remains an uncontrolled problem in many regions especially of high endemicity such as the Mediterranean, Middle East, Africa, Latin America and parts of Asia [1,2,3].

Brucellosis is an infectious disease caused by bacteria of genus *Brucella;* it is an animal disease in origin and is a disease of the sexually mature animals with preference of placenta, fetal fluids and tests of male animals. Brucellosis has been known as a global problem of wild and domestic animals, especially cattle, sheep and goats [4].

In small rumnants, brucellosis is mainly caused by *Brucella melitensis*, a Gram–negative coccobacillus or short rod. It is a facultative intracellular organism. *B. melitensis* includes three biovars (biovars 1, 2 and 3). All three biovars give rise to a disease in sheep and goats, but their geographic distribution differs. Although *Brucella abortus* and *Brucella suis* infections occur occasionally in small ruminants, but the clinical disease sounds to be scarce [5].

Brucellosis may give rise to significant economic losses. In livestock, brucellosis results in decreased productivity, abortions and weak progeny and is a major barrier for commerce and export. Regarding to human, brucellosis is a severely debilitating disease that requires prolonged treatment, compliance of the patient, and results in considerable medical expenses in addition to loss of income due to loss of working hours [6].

In Egypt, the seroprevalence of human brucellosis was reported to be as high as 8 % in high-risk populations [7]. Actually, the true incidence of human brucellosis in Egypt couldn't be recognized as several cases call for the medical care in private clinics and many of these patients are not informed to the public health authorities. For example, in Fayoum governorate depending on caring out a hospital-based surveillance the incidence of brucellosis was estimated by less than 6% of the human brucellosis cases [8]. Transmition of Brucellosis in humans is strongly related to contact with infected animals [9].So, farmers, shepherds, abattoir workers and veterinarians are recognized as being the highest occupational risk groups [10].

Currently, diagnosis of this zoonosis is based on microbiological and serological laboratory tests [11]. Nucleic acid amplification methods such as PCR can overcome the limitation of conventional detection methods as they are rapid, sensitive, high specific and low cost [12,13]. We aimed in this study to determine the prevalence of brucellosis in small ruminants and human in rural area atfayoum governorate and associated risk factors

2. Methodology

2.1. Study Design

A cross-sectional study was conducted between January 2016 to June 2016 to estimate the seroprevalence of brucellosis in the small ruminants and human population and collect information on different practices of animal contacts that may lead increase risk of brucellosis in animal and human at Fayoum governorate, Egypt.

2.2. Sampling

We targeted some of the sheep and goat population with a recent history of abortion, and the nearby flocks in (El -Tesin, Allam, Sawiris El Kbera and Rubil) villages in Tamia district, Fayoum governorate. Also, human contacts of different animal groups were examined for brucella.

The sampling was 10 groups including (10 flocks of sheep and goats (165 sheep and 45 goats) and (105 human contacts with animals in the age from 15 to 58years) excluding children below 15 years. A total of 315 blood samples collected from studied animals and human, serum was separated and tested by Rose Bengal test. Also, culture was done for 171 available milk samples of small ruminants (134 sheep and 37 goats), one sample of stomach contact and visceral content from aborted fetus of goat and five blood samples of human contact with a current history of fever belong to group1. PCR was employed in the identification and typing of 3 DNA extracting of pure isolate cultures from sheep, goat and human samples for group1.

Data concerning practices of animal contacts was collected using an interviewed structured questionnaire, developed in English and translated Arabic. The questionnaire was used to collect data on Sociodemographic data age, sex, education, marital status, practices of animal breeders; breeding places, mixing species, cleaning, waste disposal, helping in parturition.

Table 1. Distribution Of The Studied Groups Of Animals And Human

Groups	Sheep			Goat			Human	
	Total	blood for serology	Milk	Total	blood for serology	Milk	Blood for serology	Blood for culture
Group 1	21	21	11	11	11	9	15	5
Group 2	25	25	20	-	-	-	10	-
Group 3	24	24	20	-	-	-	8	-
Group 4	27	27	25	-	-	-	6	-
Group 5	20	20	18	3	3	3	6	-
Group 6	20	20	15	-	-	-	10	-
Group 7	2	2	2	8	8	5	13	-
Group 8	12	12	10	9	9	8	15	-
Group 9	4	4	4	6	6	5	10	-
Group10	10	10	9	8	8	7	12	
Total	165	165	134	45	45	37	105	5

2.3. Methods for Brucella Detection in Sheep and Goats

2.3.1. Serological Tests Methods

2.3.1.1. Rose Bengal plate test (Alton *et al.*, 1988)

The sera under test and antigen Rose Bengal were obtained from veterinary serum and vaccines Research Institute (VSVRI), Abbasia, Cairo, Egypt.) were brought to room temperature before testing using antigen micropipette a drop (30 µl) of the serum was placed on dry white enamel plat then one drop of RB antigen (30 µl) was added then antigen and serum where thoroughly mixed in a circular movement using tooth pick or glass red. The plate was rocked by hand for four minutes and any agglutination that appeared within this time was recorded as a positive reaction.

2.3.2. Bacteriological Examination

Direct culture of milk under aseptic conditions was conducted as follows: ≈20 mL of milk was centrifuged at 1,620 × *g* for 10 min, and the sediment cream mixture was culture onto *Brucella* spp. agar plates containing an antimicrobial drug supplement. Tissue specimens obtained from internal organs and stomach content from abortus goat fetus were cultured onto the same media and incubated at 37°C in an atmosphere of 10% CO2. Although the isolation of brucellae from blood patient normally sterile sites may be achieved by using routine culture techniques. Cultured plates were examined for *Brucella* spp. growth on day 4 and daily for 4 weeks. Suspected colonies were further identified and subcultured onto *Brucella* spp. agar slants. We identified *Brucella* spp. isolates according to morphologic characteristics, microscopic appearance, and reactions with positive sera. *Brucella* spp. isolates were typed according to their CO2 requirement, H2S production, growth in the presence of dyes, reaction with monospecific sera (immunoglobulin [Ig] A and IgM), and bacteriophage typing (Tiblisi phage; Central Veterinary Laboratory, Wybridge, UK) (14).

2.3.3. PCR Method

2.3.3.1. DNA Extraction

DNA extraction from 3 the purified isolates was performed using the QIAamp DNA Mini kit (Qiagen,

Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, bacterial pellet was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

2.3.3.2. Oligonucleotide Primers

Primers used were supplied from biobasic (Canada) and are listed in Table 2.

Table 2. Primers Sequences, Target gene and Cycling Conditions for PCR

Target gene	Target agent	Primers sequences	Amplified segment (bp)	Primary Denaturation	Amplification (35 cycles)			References
					Secondary denaturation	Annealing	Extension	
1S711	*B. abortus*	1S711-specificPrimer TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT B. abortus-specific Primer GAC-GAA-CGG-AAT-TTT-TCC-AAT-CCC	498	94˚C 5 min.	94˚C 30 sec.	55˚C 45 sec.	72˚C 45 sec.	Bricker and Halling, 1994 (15)
	B. melitensis	1S711-specificPrimer TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT B. melitensis-specific Primer AAA-TCG-CGT-CCT-TGC-TGG-TCT-GA	731	94˚C 5 min.	94˚C 30 sec.	55˚C 45 sec.	72˚C 45 sec.	

2.3.3.3. PCR Amplification

Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler.

2.3.3.4. Analysis of the PCR Products.

2.3.3.4.1. Conventional PCR Products

The products of PCR were separated by electrophoresis on 1 % agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the products was loaded in each gel slot. A generulert 100 bp DNA Ladder (Fermentas) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software (15).

3. Statistical Analysis

Statistical analyses were performed using the Statistical Package for Social Sciences Version 16.0 for Windows. Comparison of variables distribution across different categories was done using Chi-square test of significance. A probability value of less than 0.05 was considered statistically significant.

4. Results

4.1. Animal Contacts Characterization

There were 105 animal contacts participated in the study during the period from January 2016 to June 2016 excluding children <=15 years. All of them were living in different villages with different characteristics Table 3.

The majority of the animal contacts were males and 34.3 % were females from 15 to 58years, only 6.7 % were with higher education and 80% were married.

Table 3. Demographic Characteristics Of The animal Contacts

Characteristics	N(%)
Age	
Less than <=20	42(40)
>20	63(60)
Mean ±SD	27±11.6
Sex	
Males	69(65.7)
Females	36(34.3)
Education	
Illiterate and read and write	90 (85.7)
Primary and secondary	8(7.6)
High	7(6.7)
Residence	
Villages	105(100)
Marriage	
Yes	84(80)
N0	21(20)

Table 4. Practices of Animal Contacts

Practices	%
keeping place of the animal	
Inside a room within the house	67.6%
Beside the house	32.4
Keeping all animals in the same place aborted or not	
Yes	79%
No	21%
Veterinary do seminars to increase the awareness about abortion or infections	
Yes	93.3%
No	6.7%
Wearing glove or masks on assisting in parturition or dealing aborted or dead fetus	
No	100%
Disposal of animal waste	
-used as fertilizers	96.2%
-disposed in ponds or canals	3.8%
Disposal of aborted, placenta or dead fetus	
-disposed in water canals and ponds	22.9%
-eating for dogs	77.1%
What do you do when you have aborted animals	
Selling the aborted animal	29%
Continue breeding	71%
Who you call first, when the animal became diseased	
Veterinarian doctor	29%
The worker	71%
Used goat milk for lactation	
Yes	30%
No	70%

These questions directed to the owners of the sheep and goat flocks, (n=14).

4.2. Animal Contacts Practices

Many of the owners of the sheep and goats keep their animals in rooms within their houses and the majority of them were keeping all animals in the same place and all of them not wearing gloves.

The majority used the animal waste as fertilizers and many of them feeding the placenta and aborted material for their dogs (77.1%) and 41% of them could sell aborted animals while (71%) of them call the veterinary worker first when the animal became diseased.

4.3. Identification of Risk Factors of Brucella Infection

The participants older than 20 years were more exposed to Brucella infection than <=20 years (Pvalue=0.015) and the study showed that,

Table 5. The Associated Risk Factors Of Brucella Infection

Risk factors	Positive sera (16)	Negative sera (89)	P value	unadjusted odds ratio	Upper limit	Lower limit
Age		40(44.9)				
≤20	2(12.5)	49(55.1)	(0.015)	5.714	1.226	26.637
>20	14(87.5)	40(44.9)				
Sex		58(65.2)				
Males	11(65.2)	31(34.8)	(0.781)	0.850	0.271	2.669
Females	5(31.2)	58(65.2)				
Educated	1(6.2)	19(21.3)	0.157	0.246	0.030	1.979
Uneducated	15(93.8)	70(78.7)				
Helping in labor						
Yes	15(93.8)	12(13.5)	(0.000)	96.250	11.267	796.806
No	1(6.2)	77(86.5)				
Eating home made cheese						
Yes	13(81.2)	55(61.8)	(0.134)	2.679	0.711	10.090
No	3(18.8)	34(38.2)				
consuming raw milk						
Yes	1(6.2)	13(14.6)	(0.365)	2.342	0.294	20.147
No	15(93.3)	76(85.4)				
Presence of animal with positive of sera						
yes	13(81.2)	45(50.6)	(0.023)	4.237	1.129	15.899
no	3(18.8)	44(49.4)				
Giving their animal male for fertilization other flocks		37(41.6)				
Yes	13(81.2)	52(58.4)	(0.003)	6.090	1.620	22.895
No	3(18.8)	37(41.6)				

Questions directed to all participants there was significant difference (p value <5%) between the persons with positive sera and with negative sera regarding age (p=0.015), their assistance in labor, presence of infected animals (p=0.023), giving their animal male for fertilization of other flocks

4.4. Serological Diagnosis of Brucellosis

By using Rose Bengal Test, the seroprevalence of brucellosis among sheep, goat and human was 16.4%, 28.9% and 15.2% respectively.

Table 6. The Seroprevalence of Brucella Infection By Rose Bengal test in Different Groups among Sheep, Goats And Human

Groups	Sheep (165)		Goat (45)		Total	Human (105)		Total
	Positive	Negative	Positive	Negative		+ve	-ve	
Group 1	10(6.1)	11(6.7)	5(11.1)	6(13.3)	32(15.2)	3(2.9)	12(11.4)	15
Group 2	5(3.0)	20(12.1)	-	-	25(11.9)	2(1.9)	8 (7.8)	10
Group 3	-	24(14.5)	-	-	24(11.4)	-	8(7.6)	8
Group 4	-	27(16.4)	-	-	27(12.9)	-	6(3.8)	6
Group 5	2(1.2)	18(10.9)	3(6.7)	-	23(11)	4(3.4)	2(1.9)	6
Group 6	7(4.2)	13(7.9)	-	-	20(9.5)	3(2.9)	7(6.7)	10
Group 7	-	2(1.2)	-	8(17.8)	10(4.8)	-	13 (12.4)	13
Group 8	-	12(7.3)	3(6.7)	6(13.3)	21(10)	2(1.9)	13 (12.4)	15
Group 9	-	4(2.4)	-	6(13.3)	10(4.8)	-	10(9.5)	10
Group 10	3(1.8)	7(4.2)	2(4.4)	6(13.3)	18(8.6)	2(1.9)	10(9.5)	12
Total	27(16.4)	138(83.6)	13(28.9)	32(71.1)	210	16(15.2)	89 (84.8)	105

All positive groups from sheep and goats associated with positive human.

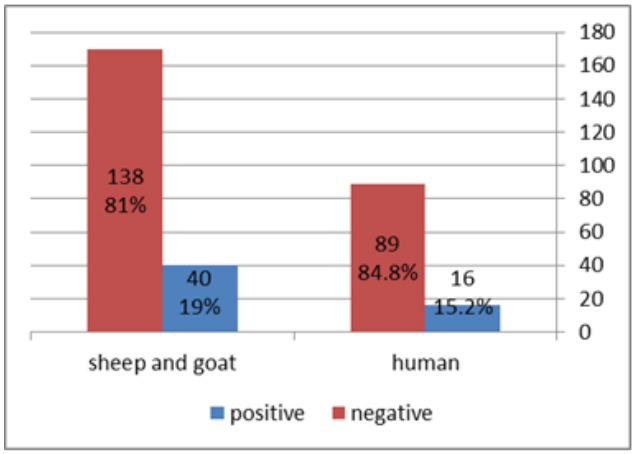

Figure 1. Seroprevelance of brucellosis in small ruminants (210) and human (105)

4.5. Bacteriological Diagnosis of Brucellosis

A total of 34 isolates (20 milk sheep, 11 milk and 1 aborted fetus goat and 2 human blood).

The entire seropositive animals give positive with milk culture positive.

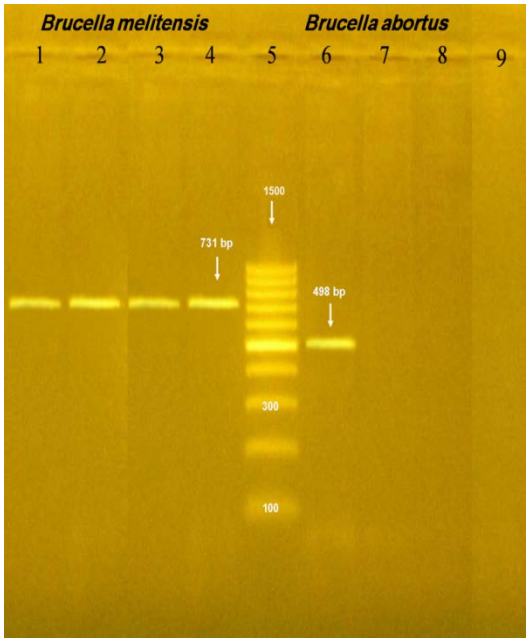

Photo 1. By using PCR amplification for detection of *B. melitensis* and *B.abortus* three DNA extracted from sheep milk culture, abortus goat feti culture and human blood culture showed positive for *B.melitensis* at 731bp in lane 1, 2, and 3 respectively while negative for *B. abortus* at 498bp in lane 7, 8, and 9 respectively. Lane 4: Control Positive *B. Melitensis* Lane 5: Marker 100 bp. Lane 6: Control Positive *B. abortus*

Table 7. Culture and Isolation of Brucella from Milk, Aborted Fetus and Human Blood

Groups	Sheep (134 milk samples)		Goat (37 milk+1 aborted fetus)			Human (5 blood)	
	positive	negative	positive		Negative	Positive	negative
			milk	Aborted fetus			
Group 1	4	7	3	1	6	2	3
Group 2	5	15	-	-	-	-	-
Group 3	-	20	-	-	-	-	-
Group 4	-	25	-	-	-	-	-
Group 5	2	16	3	-		-	-
Group 6	6	9	-	-	-	-	-
Group 7	-	2	-	-	5	-	-
Group 8	-	10	3	-	5	-	-
Group 9	-	4	-	-	5	-	-
Group 10	3	6	2	-	5	-	-
Total	20	114	11	1	26	2	3

5. Discussion

Brucellosis has been considered as a global problem of wild and domestic animals, especially cattle, buffaloes, sheep and goats causing a decline in reproductive efficiency and abortion [16].

The standard Rose Bengal (RB) and Complement Fixation Test (CFT) are the prime serological tests used to find out antibodies against *B. abortus* and *B. melitensis* infections. In Egypt the serological test used to determine the prevalence of brucellosis in different animal species and human was Rose Bengal plate test as recorded by different studies **[17,18]**.

In the present work we found that, out of 315 examined serum samples of animals and human by Rose Bengal test for the present of *Brucella* spp. 56 (17.8%) samples were positive; sheep 27/165 (16.4%), goat 13/45 (28.9%) and human 16/105 (15.2%) were positive for *Brucella*.

Rose Bengal test is one of both tests (RBT and CFT) has been used for many decades, confirming to be successful for eradicating bovine brucellosis in some countries, and presentaly used in the European for the *B. melitensis* infection eradication in small ruminants. However, both tests are evidenced significantly less effective for the diagnosis of brucellosis in sheep and goats than in cattle [19,20].

In the present work 171 Milk samples (134 sheep and 37 goats) are used for diagnosis of brucellosis in alive sheep and one cultured from a aborted goat fetus (stomach content, spleen and lung). Also 5 blood samples were cultured from human contact with a current history of fever belong to group1 used for diagnosis of brucella.

Isolation of the causative agent is yet the standard diagnostic method for brucellosis [21]. Thus, for definitive and confirmative diagnosis of serologically reactive animal's bacteriologic isolation and identification were performed however, some microorganisms, of brucella spp can escape identification by not causing important

serologic responses can be isolated from milk samples. In our results of bacterial isolation from milk and tissues all animals are shown in Table 7. A total of 34 isolates of brucella spp. were identified; all isolates were *B.melitensis* biovar 3. Isolation of brucella spp. confirmed active brucellosis in the animals tested (Table 7).

The PCR assay described has several advantages over the current microbiological methods used to identify Brucella species. A major advantage is the speed and safe with which the assay can be performed, the assay is unaffected by contamination by other microbes that might be present in the tissue samples used for isolation. After collection, the bacteria can be killed and sent for identification [15]. In our results, field strains of *B. melitensis* and *B.abortus* for three DNA extracted from sheep milk culture, abortus goat feti culture and human blood culture showed positive for *B.melitensis* at 731bp. respectively while negative for *B. abortus* at 498bp. Photo [1].

Sheep are considered the main source of *Brucella mellitensis*, which is the most pathogenic *Brucella* sp. In humans and the predominant strain spreading in Middle East, including Egypt [22,23]. Human mostly gets infected through contact with the infected animal and eating of contaminated animal products [24,25,26]. So the prevalence of brucellosis in humans tends to correspond to that in animal. The present work showed that out of 105 persons seriologically investigated 16(15.2%) persons were positive for brucellosis and all the positive human sera was associated with positive sheep and or goat serum (Figure 1 and Table 6) this prevalence was similar to [27], reported, that the seroprevalance of Brucella infection in Assiut governorate of 108 veterinarians and 51 animal attendants were working in infected farm was 16.7% among veterinarians and 9.8% among animal attendants and more than reported in Suez Canal area**,** by [28], who found that the seroprevalance of Brucella infection of human sera was 5.1% this difference may be due the studied populations in our work were a higher risk group.

Although there was no significant difference (p value> 0.5), between males and females however, males were more affected than females this was in line with many studies reported that males affected by brucellosis more than females [29,30,31]. This distribution may be due to that male are more concerned in activities such as slaughter and handling carcass, and as a consequence they are at a greater risk of exposure to infection especially in contact of sheep and goats. However other reported that females affected more than males [32], this because in the study areas, those milking the cows were females.

Although human brucellosis can occur in any age group, but the majority of cases are found in young age group. The common age group of brucellosis infection from15 to 50 years [5,33] in our finding, the persons in the age group >20 years were more at risk of brucellosis infection (Table 3).

Dogs are important source of brucellosis infection they play a role in mechanical transmission of the infection when they pull aborted material across the ground [34]. In the present work the majority of the animal contacts (77.1%) fed the placenta and aborted material for their dogs and all of them not used wearing protective masks or gloves when dealing with animals this was similar to [35].

Regarding the practices of animal contacts in the present work the majority (79%) were keeping their animals, whether aborted or not in the same place and this gives chances for contacts in between animals and facilitates transmission of brucellosis in between animals. Also (18) 29% of the participants sold sheep and goats with repeated abortion or dead fetus in the markets while the majority 71% continue breeding them to be slaughtered or sold on aid and none of them take any precautions on animal contact such as, wearing gloves or masks (Table 2), these findings, were similar to [35,36], they reported that, Most shepherds kept aborted animals in their flocks, while a few numbers of them reported that they might sell them at the market, only one shepherd remembered slaughtering as a possible action. Also, the shepherds, not wearing protective gloves or masks when helping in animal labour.

So in our findings the risk of infection was associated with increasing age, assistance in labour, presence of infected animal with brucellosis and who giving their animal males for fertilization other flocks (Table 3) however, Kavi et al [37], Afifi et al [38], Jennings et al [39], reported that, The risk of developing brucellosis was attributed to poor animal handling practices or close animal contact and consumption of unpasteurized milk., while Hassanian [40] found that, the males, animal and animal products handlers and rural residence were the risk factors this may reflect the wide animal exposure in many regions in Egypt

Eating fresh milk or dairy products prepared from unheated milk is the main source of infection for many populations [5,41]. The resedence of these villages in timia not using goat milk for Consumption or making cheese but they may use the goat milk for lactation of their babies.

In our study 30% of the families used goat milk as a complementary feeding for their children, this may increases the risk of brucella transimation

6. Conclusion and Recommendation

There were 56 positive Rose Bengal test in sheep, goats and human in 10 groups from 4 villages in Tamia district-El fayoum Governorate. It is probable that they share the same origin of infection. Infection with 34 isolates *B. melitensis* were bacteriologically confirmed.

Increased prevalence of brucellosis in human in Egypt can be related to rearing sheep and goats in villages. Most sheep or goat herds in Egypt are moving. Movement of infected sheep or goats can pollute grass and spread brucellosis to other animals (e.g., cattle or buffaloes) in other flocks or areas. This movement is a great risk factor for failure of brucellosis eradication programs.

The general public, especially where brucellosis present, has to be made aware of the danger to health and of the economic importance of the disease and motivate the role of health education to be an essential pillars of any programme or intervention implanted by the governmental or the non governmental organizations.

There is must be acollaboration between public health and veterinary medical managements to train both, the physicians and veterinary doctors to increase the health awareness of people especially in rural community.

7. Ethical Consideration

A verbal consent was obtained from the animal contacts after they were informed about the study purpose and procedure.

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