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The Antibacterial Effect of Silver and Zinc Oxide Nanoparticles against Intracellular *Brucella melitensis*

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ABSTRACT

Background: Brucellosis is a zoonotic disease of worldwide importance among animal and humans. The most important virulence factor of *Brucella melitensis* is related to intra-macrophage survival. On the other hand, the side effects of the current brucellosis treatment regime, it is necessary to find out new antimicrobial agents to treat the disease.

Objective: To Isolate *Brucella melitensis* from local white cheese and raw milk by culture method with identification using PCR and determination of their susceptibility to classical antibiotics and silver and zinc oxide nanoparticles.

Materials and methods: A total of 150 local soft cheese and raw milk samples collected from different local markets in Erbil city and were examined for the presence of *Brucella melitensis* during a period of (6) months (November 2015 to April 2016).Selective media; such as, Brucella agar were used for the isolation, and identified according to standard biochemical tests and confirmed by PCR.Antibiotic susceptibility test determined by Kirby-Bauer disk diffusion method and the antimicrobial activity of silver and zinc oxide nanoparticles were carried out in Muller–Hinton agar with 1% sheep's blood by well diffusion method. Five mm diameter wells were prepared and loaded with Ag(20 nm size) and ZnO (20 nm size) nanoparticles dilutions, finally the combination of ampicillin/ cloxacillin with the nanoparticles were tested against isolates of *Brucella melitensis*.

Results: Out of the 150 food samples, 53(35.33%) were contaminated; 21(28%) of the total samples of local soft cheese and 32(42.6%) of raw milk was contaminated with *Brucella melitensis*. Twenty isolates were identified according to standard biochemical tests, and confirmed by PCR for detection of pure *Brucella* genomic DNA in PCR. *Brucella*-specific primer BgF/BgR, were evaluated for detection of pure *Brucella* genomic DNA, provided bands on agarose gel corresponding to a 208 base pair product when compared to molecular ladder, the specific primer IS711were detected for *Brucella melitensis*, and achieved by given bands on agarose electrophoresis corresponding to 731 base pair from the total isolates. Seventeen from 20 isolates were found positive for *Brucella* with BgR/BgF primers and 15 isolates were positive for IS711 primer. The sensitivity to antibioticsshowed that the resistance to ampicillin and ceftazidime were (85%), for aampicillin/cloxacillin was (45%), and for imipenem and piperacillin were 80% and 90% respectively. The inhibition zone of ZnO-NP against *Brucella melitenisis* ranged between 10-20 mm when the concentration was 10-1000 ppm. While the inhibition zone of Ag NP was 11.5-24.5 mm. Combination of amoxicillin/ cloxacillin with nanoparticles show a synergistic effect as demonstrated by the increase of inhibition zone to 20 mm fir ZnO-NP and 23 mm for AgNP nanoparticles.

Conclusion: *Brucella melitensis* contaminate local white soft cheese and raw milk and showed variable sensitivity to antibiotics and some isolates were resistant to ampicillin/ cloxacillin, ceftazidime, imipenem, piperacillin, and trimethoprim-sulphamethoxazole. The present study data show that silver and zinc oxide nanoparticles inhibit the growth of *Brucella melitensis*. The combination of silver nanoparticles and ampicillin/ cloxacillin showed a synergistic effect.

Key word: *Brucella melitensis*, BgF/BgR, IS711 gene, Ag and ZnO Nanoparticles

Introduction

Bacteria from genus *Brucella* are the causative agent of brucellosis. It is a serious public health problem worldwide [1-2].Species of *Brucella* are zoonotic and in humans the infection causes a weakening disease with relapsing fever and flu-like symptoms [3]. Human brucellosis is considered as a life-threatening debilitating disease [4]. An important aspect of *Brucella* infection, it ability to persist and replicate within phagocytic cells of the reticuloendothelial system as well as in non-phagocytic cells such as trophoblasts. Because of intracellular localization of *Brucella* and its ability to adapt to the environmental conditions encountered in its replicative niche[5], and this attribute to high rate of treatment failure and relapse. The optimal treatment for brucellosis is a combination regimen using two antibiotics, because mono-therapies with single antibiotics have been associated with increased relapse rates [6,7].

The combination of doxycycline with streptomycin (SD) is currently the best therapeutic option with fewer side effects, especially in cases of acute and localized forms of brucellosis [8]. Although the SD combination is accepted to be the most effective regimen, therapeutic failure and adverse drug reactions due to prolonged use and relapse related to the pharmacokinetic and pharmacodynamics properties of the antibiotics are common among brucellosis patients [9]. These problems have led to investigations of new drugs and improved drug carrier strategies for treating brucellosis, including antibiotics loaded into nanoparticles, due to their small size and target-specific localization properties, are being actively investigated for preferential drug delivery to various disease sites in the body, including intracellular bacterial and viral infections [10].Nanoparticles exhibit attractive properties like high stability, and the ability to modify their surface characteristics easily [11]. Exerting their antibacterial properties, nanoparticles attach to the surface of the cell, this interaction causes structural changes and damage, markedly disturbing vital cell functions, such as permeability, causing pits and gaps, depressing the activity of respiratory chain enzymes, and finally leading to cell death [12]. So the need to classic antibiotics has been made the major goal for the drug industry nanoparticles in low concentration has proven less toxic to humans [13].

The use of nanoparticles is gaining impetus in the present century as they possess defined chemical, optical and mechanical properties. Among them, the metallic nanoparticles are most promising as they contain remarkable antibacterial properties due to their large surface area to volume ratio, which is of interest to researchers due to the growing microbial resistance against antibiotics, and the development of resistant strains [14]. Nanoparticles of silver have been studied as a medium for antibiotic delivery, and to synthesize composites for use as disinfecting filters, and coating materials [15]. Nano-silver antibacterial properties are an effective killing agent against a broad spectrum of Gram-negative and Gram-positive bacteria [16,17]. It has also long been established that silver can kill microorganisms. As an antimicrobial, silver has offered the ability to disinfect while seemingly presenting few, if any, short-term harmful effects to human beings, other than in large doses [18]. Brucellosis are wildly distribute among Kurdistan Region peoples due to consuming dairy products especially local white cheese, milks, cream as well as yogurt; these disease become chronic in some infected persons.

Material and methods

A total of 150 samples from local white soft cheese (75) and raw milk (75) were collected from different locations in Erbil city. About twenty five grams from each sample were obtained in sterilized condition [19]. Brain heart infusion broth tubes were inoculated and incubated for 48hr. at 37˚C. Loop full of each tube was streaked on Brucella selective agar plates supplemented with 5% of human serum and polymyxin B sulphate (5 mg); bacitracin (25 mg); kanamycin (50 mg); nalidixic acid (5 mg); nystatin (100,000 units); vancomycin (20 mg) (20), incubated at 37˚C for 3 – 5 days. The plates were maintained under micro-aerobic conditions (10% CO2). Colonies suspected of *B.* Species were subjected to Gram stains, and conventional biochemical characterization based on requirement for CO_2, catalase, oxidase, urease, citrate [21,22].

Molecular Identification of bacteria by PCR

A 731 bp fragment was amplified with *Br. melitensis*-specific primers described before [23,24], Table 1.All amplifications were achieved in a total reaction volume of 50 µl containing 5 µl of 10 × PCR buffer, 6 µl of 25 mmol l^{-1} $MgCl_2$, 1 µl (10 mmol l^{-1}) each of the four dNTPs, 1 µl of *Taq* DNA polymerase (5 U μl^{-1}), 0·5 µl of each primer (50 pmol ml^{-1}) and 5 µl of template DNA.

PCR was performed in a DNA thermo cycler (PX2 Thermal Cycler; Thermo Electronic Corp., Milford, MA, USA) and the temperatures of denaturation and annealing for each primer amplifications were done as shown in Table 2. Negative controls containing all PCR reagents except template DNA were used to monitor cross contamination. The positive control used was DNA isolated from *Br. melitensis* reference strain, kindly supplied by the Veterinary Research Institute in Erbil city. To check the reliability of the results and to detect any external contamination, all samples were processed in duplicate.

The amplified products were analyzed by electrophoresis on 1·5% (w/v) agarose gel at 80–90 V for 1·5–2 h and stained with ethidium bromide (0·5 μg ml^{-1}). Amplified products were visualized by a computerized image analysis system (Gl-5000; Spectronics Co., Westburg, NY, USA). PCR products with molecular size 731 bp were considered positive for *Br. Melitensis*[23].

Table 1:Primersof *Brucella melitensis*

Primer	Sequences of primers	Length of Amplicon	Reference
IS711	5′-AAA TCG CGT CCT TGC TGG TCT GA-3′ 5′-TGC CGA TCA CTT AAG GGC CTT CAT-3′	731bp	23
BgF BgR	5’-CAATCTCGGAACTGGCCATCTCGAACGGTAT-3’ 5’-ATGTTATAGATGAGGTCGTCCGGCTGCTTGG-3’	208bp	24

Table 2: Amplification condition for target genes

primers	Base pair	Initial Denaturation Temp C°	Annealing Temp C°	Extension C°
BgF BgR	208bp	95	68	72
IS711	731bp	94	58	72

Antibiotic susceptibility test

Microbial susceptibility to antibiotics was determined by Kirby-Bauer disk diffusion method [22]. The tested bacterium was from an overnight culture approximately 10^5 CFU/ml., a bacterial lawn was cultured on Mueller-Hinton agar. Discs of antibiotics were used to observe antibiotic susceptibility patterns against 14 antibiotics (Himedia) concentration of antibiotic per disc in microgram (μg); ampicillin (Amp10 μg),ampicillin/cloxacillin (Amc 10 μg), tetracycline (Tet 30 μg), gentamycin (Gen10 μg), erythromycin (Ery 15 μg), chloramphenicol (Chlo 30 μg), streptomycin (Strep.10 µg), pipracillin (Pip. 10 µg), ciprofloxacin (Cip.10 µg), ceftazindime (Cef. 30 µg), and imipenem (Imp.10 µg), and trimethoprim-sulphamethoxazole/(Sxt30 µg).

Antimicrobial activity of silver and zinc oxide nanoparticles

Using of silver nanoparticles (AgNPs) size (20) nm, ZnONPs) size (20) nm. According to the manufacturer's characterization of the silver nanoparticles measuring 20 nm molecular weight of 107.87 g / mol, density of 10.49 g / ml, purity 99.95%, powder gray color and shape of spherical product chemical way and space superficial density of 20 g / ml. ZnONPs was in the form of powder, reaching the size (20) nm and purity 99.9% white color powder, molecular weight 81.39 g / mol, density 5.610 g / ml and for the TiO_2NPs measuring 10 nanometers has been received in the form of nano powder, and an area of superficial grave of 225 g / ml, the degree of purity of 99%, moisture 0.48%. stock solution prepared according to Ansari et al., [25] methods. A 100 mg of nanoparticles were added to 10 ml of deionized water and shacked vigorously for 5 minutes to break the mass and get a homogeneous solution and then sterilized at 121°C for 20 minutes, cooled at room temperature to get a final concentration of stocks of 10 mg / ml.

Muller–Hinton agar was supplemented with 1% sheep's blood. Five mm. diameter wells were prepared and 1.5×10^8cfu/ml of *Br. melitensis* isolates suspension was cultivated in plates with a sterile swab and then, the wells were loaded with Ag and ZnO nanoparticles dilutions (70 µl of 2, 5, 10, 20, 50, 100, 250,500, 1000 and 2000 ppm). The plates were incubated at 37 °C. under 7%–10% CO2 for 72 hr. The zone of growth inhibition was measured by a ruler[26,27].

Synergistic effect of silver and zinc oxide nanoparticles with antibiotics

The MIC solution of ampicillin/cloxacillin(Amc 10µg) antibiotic mixed with the MIC of each nanoparticles separately shacked well for 24hr. by using vortex at room temperature.Well diffusion method was used as spreading (0.1) ml of bacteria (24) hr. old, which contain 10^7 CFU according to standard McFarland on the surface of Muller Hinton agar , wells with diameter 5 mm. were filled the 100 µml. of MIC selected from the previous step for each nanoparticles alone, as well as other well filled with 100µl ,50 µl of each, of mixed antibiotic with nanoparticles,and incubated at 37°C for 24 hr. The diameters of inhibition around hole measured in mm.[28-30].

Result and discussion

In this study one hundred and fifty food samples were collected from different markets in Erbil city centre for examining the occurrence of *Brucella* *melitensis*, included 75 samples from each of local white soft cheese and raw milk, out of the total (150) samples, 53(35.33%) were contaminated with *Brucella* species divided as 21(28%) and 32(42.6%)of white soft cheese and raw milk respectively, Table 3.

Suspected *Brucella melitensis* isolates were identified according to biochemical tests, all the isolate(20, 100%)were catalase and urease positive while11(55%)were oxidase and indol positive. The isolates were Gram negative, non-motile, gelatinase and blood haemolysis negative, Table 4.

Table 3: Frequency of *Brucella species* in food samples

Type of Food	Total No. of Examined Samples	No. and (%) of Contaminated Food Samples
White soft cheese	**75**	**21(28%)**
Raw milk	**75**	**32(42.6 %)**
Total	**150**	**53(35.33%)**

Table 4: Number and percentage of positive isolates of *Brucella melitensis* according to biochemical Tests.

Biochemical tests	Number of positive isolate (%)
Catalase	**20 (100)**
Urease	**20(100)**
Oxidase	**11(55)**
Indol	**11(55)**
Gram stain	**Negative**
Haemolysin	**Negative**
Motility	**Non motile**

The low recovery rates of *Brucella* are mainly attributed to the failure of traditional culture techniques to isolate *Brucella* specially in the presence of other contaminating bacteria, as well as the fastidious properties of *Brucella* in their natural necessities [31]. The present study agree with other studies performed in Turkey, the presence of *Brucella* species was found to be 3.33- 19.33 % [32,33]. It was thought that the source of the milk, the vaccination status of the animals, the geographic region, heat treatment and the usage of salt in the production might influence the presence of *Brucella* species in cheeses. In this study, the low number of presence *Brucella* might be explained by, boiling of the milk resulted in killing large number of bacteria, and the origin of isolated bacteria may be the contamination post manufacturing.

Suhail and Israa [34] in Baquba city showed that out of fifty cheese samples; only six *Brucella* isolates were found at rates of 12%. Pamuk et al.[35] found *Brucella* species at a rate of 14.2 % from collected aged skin bag cheeses samples. The present study results are approximately similar to that of Kara et al.[29] who isolated *Brucella* species in a rate of 9% from white cheese samples. Additionally, Abbas et al. [36] isolated the bacteria at a rate 8% from collected cheese samples in Basrah city. The variation in isolation rate of *Brucella* species from diary samples might be due to differences in source of the milk used, the level of the contamination, use of raw milk, collection of milk from infected animal, workers hands contamination, contaminated tool used in making cheese, dusts and flies or by using unpasteurized milk [37,38].

The most contaminated kind of white fresh cheese with *Brucella* species are produced and presented under improper hygienic conditions. In order to eliminate brucellosis in Kurdistan, hygienic precaution must be applied to reduce contamination and effective pasteurization of milk that used in cheese preparation [39].

Identification of *Brucella melitensis* by amplification BgF / BgR and IS711 genes

Brucella-specific primer BgF/BgR, were evaluated for detection of pure *Brucella* genomic DNA, the sensitivity was achieved with the BgR/BgF primer pair provided bands on agarose gel corresponding to a 208 base pair product when compared to molecular ladder as shown in Figure 1, as well as the specific primer IS711were detected for *Brucella melitensis,* and achieved by given bands on agarose electrophoresis corresponding to 731 base pair, Figure 2. From the total 20 isolates; 17 (85%) were found positive for *Brucella* with BgR/BgF primers and 15 (75%) isolates were positive for IS711 primer.Kumar *et al.*,[24], found that BGF/BGR primer pair was sensitive primer for the detection of *Brucella* species. Due to the disadvantages of culture and serological methods, the uses of molecular methods increased in practice as diagnostic approach for *Brucella* infection. Although, there are different reports about sensitivity of primers for PCR detection of *Brucella* species, Navarro et al. [40], reported that B4/B5 primers were more sensitive than other genes. Detection methods based on nucleic acids are promising tools for the diagnosis and eradication of disease [41]. The possibility of contamination in molecular biological methods is very low than traditional methods such as cultures and the results can be achieved in a very short period [42]. High specificity and sensitivity and ability to perform faster procedures in the molecular detection lead to increase in uses of PCR for diagnosis of *Brucella* infections [43]. However, many factors constrains PCR uses in general practice such as the cost-effectiveness, funding and staff training.

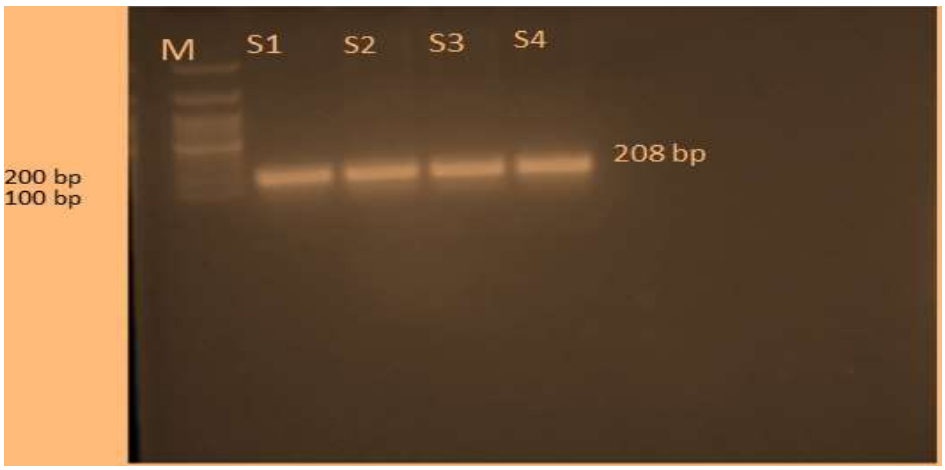

Figure 1: PCR detection of primer RgF/RgR208 bp.; Lane M:1000-100-bp marker,S1and S2 S3 and S4shows positive amplification of gene.

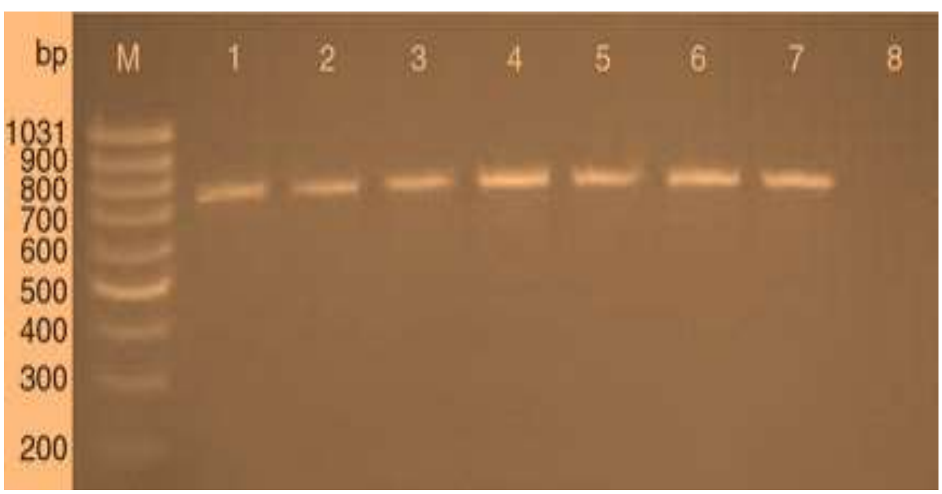

Figure 2. PCR detection of IS711 specific primer for *Brucella melitensis.* Lane M: 3000-bp marker, lane 8: Negative control, lane 1-7: 731-bp band of IS711 gene, lane 4:poisitive control

Susceptibility of *Brucella melitensis* to antibiotics

Susceptibility to antibiotics was tested according to disc diffusion method using Muller-Hinton agar, Table 5. All tested isolates were sensitive to ciprofloxacin chloramphenicol, gentamicin, streptomycin and trimethoprim/sulphamethoxazole. However, these isolates showed variable sensitivity to other antibiotics. The resistance rate to ampicillin and ceftazidime was 85%, while the resistance rate to imipenem and pipracillin was 80% and 90% respectively.

The present study results agreed with that reported by Abbas and Talei [36] who showed that all isolated *Brucella* were sensitive to streptomycin, trimethoprim, gentamycin, rifampicin, trimethoprim/sulamethaoxazole. Also the results were similar to that reported by other research group [44], who found that all *Brucella* isolates were sensitive to streptomycin, tetracycline, gentamycin, chloramphenicol. In contrast, Al-Abbasi et al., [45]reported different results as they found that all *Brucella* isolates were resistance to streptomycin and cotriamoxcizol with variable resistance to cefotaxim, ampicillin and erythromycin.

Table 5:- Percentage of Sensitivity of *Brucella melitensis* to some antibiotics

Antibiotics	Number (%)		
	Resistant	Intermediate	Sensitive
Ampicillin(Amp10µg)	17(85)	-	3(15)
Ampicillin/ Cloxacillin(Amc10 µg)	17(85)	-	-
Ciprofloxacin(Cip10 µg)	-	-	20(100)
Chloramphenicol(Chl30 µg)	-	-	20(100)
Ceftazidime(Cef 30 µg)	17(85)	-	3(15)
Tetracycline(Tet 30 µg)	-	-	20(100)
Gentamicin(Gen10 µg)	-	-	20(100)
Erythromycin(Ery30 µg)	-	-	20(100)
Imipenem(Imp10 µg)	16(80)	-	2(10)
Pipracillin(Pip30 µg)	18(90)	2(10)	-
Streptomycin(Sterp10 µg)	-	-	20(100)
Trimethoprim/Sulphamethoxazole /(Sxt30 µg)	9(45)	9(45)	2(10)

Antibacterial effect of nanoparticles against *Brucella melitensis.*

The antibacterial activity of Ag and ZnO nanoparticles against *Br. Melitensis* determined by agar well diffusion methods. The results are shown in Table 6. The pattern of zone inhibition was correlated with the concentration of nanoparticles for both Ag and ZnO preparations. For Ag nanoparticles the inhibition zone increased from 11.5 mm at concentration of 5 ppm to 24.5 mm for concentration of 1000 ppm. While the corresponding values were 10 mm and 20 mm for ZnO nanoparticles. Both nanoparticles preparations show doubling of the inhibition zone at the higher concentration when compared to the lower concentration, indication that bacterial inhibition was dose dependent.

Table 6: The Inhibition zone (mm.) of different concentration of Ag and ZnO nanoparticles against *Brucella melitensis*

Nanoparticles	Concentration of nanoparticles(ppm.)								
	5	10	15	20	50	100	250	500	1000
ZnO(20nm)	10	12.5	13	13	14	14.5	17	18	20
Ag(20nm)	11.5	13	14.5	16	16	18.5	20	22	24.5

Synergism effect of nanoparticles with ampicillin/ cloxacillin against *Brucella melitensis*

The results of present study showed that silver nanoparticles and ZnO NP have an antimicrobial effect against *Brucella melitensis* and the antibacterial effect increased when the nanoparticles mixed with the antibiotics. *Brucella melitensis* were resistance to ampicillin/ cloxacillin(10µg),but when this antibiotics combined with nanoparticles, the inhibition diameter increased from 9 mm to 20 mm for ZnONP and 23 mm for AgNP nanoparticles combination, Table 7.

The increasing of drug resistance of *Brucella* bacteria has created many problems. So trying to find the new antibacterial agent for inhibiting the growth of *Brucella* is one of the important goals of the World Health Organization in order to control brucellosis [46].Silver nanoparticles have emerged up as the novel antimicrobial agents owing to their high surface area to volume ratio and its unique chemical and physical properties [47]. Previously, the highest synergistic antibacterial activity was observed with silver nanoparticles combined with some antibiotics such as penicillin G, amoxicillin, erythromycin, clindamycin, and vancomycin [48].

Table 7.The synergistic effect of nanoparticles with ampicillin/ cloxacillin on the growth of *Brucella melitensis*

The isolates	**Inhibition zone in mm**		
	ampicillin/ cloxacillin	**Ampicillin/cloxacilin plus AgNP**	**Ampicillin/cloxacillin plus ZnONP**
Br. 1	**9.6**	**23.5**	**20**
Br. 2	**9.2**	**20.5**	**18**
Br. 3	**9**	**19**	**18.5**
Br. 4	**9.5**	**22**	**20**
Br. 5	**9**	**23**	**19**
Br. 6	**9.4**	**22**	**18**

Antimicrobial effects of silver nanoparticles on *Br. abortus* have not been studied so far, Alizadeh et al.[47] found that silver nanoparticles have an antimicrobial effect on intra- macrophage *Br. abortus* and significant effect on the elimination of intra-macrophage *Br. abortus* over a period of 24 hours. Therefore, silver nanoparticles are able to penetrate into the macrophage cells and kill *Br. abortus* , in addition to their ability to inhibiting the growth in broth dilution and agar-well diffusion. It can be concluded that silver nanoparticles may be useful for the treatment of diseases, caused by intracellular microorganisms such as *Brucella*, while there are some antibiotics that are not effective *in vivo* conditions, this feature can be useful in the rapid control of *Brucella* infections.

In conclusion, *Brucella melitensis* contaminate local white soft cheese and raw milk and showed variable sensitivity to antibiotics and some isolates were resistant to ampicillin/ cloxacillin, ceftazidime, imipenem, piperacillin, and trimethoprim-sulphamethoxazole. The present study data show that silver and zinc oxide nanoparticles inhibit the growth of *Brucella melitensis*. The combination of silver nanoparticles and ampicillin/ cloxacillin showed a synergistic effect.

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