Antimicrobial susceptibility profile, Adherence and invasion to mammalian cells of *Brucella melitensis* isolates

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Abstract: Susceptibilities of 66 *Brucella* isolates were tested *in vitro*. All isolates were susceptible to doxycycline, gentamic in and streptomycin. In addition, propyl paraben, cresol and benzalkoniumchloride were found to be the most powerful tested preservative, disinfectant and antiseptic, respectively. All isolates adhered to and invaded into *Vero* cells by variable degrees. Adherence and invasion of most isolates were significantly reduced by: (1) pretreatment of test isolates with trypsin and sodium metaperiodate; (2) pretreatment of *Vero* cells with lipase, neuraminidase and sodium metaperiodate; (3) presence of Ca^{++}, Mg^{++} and 200mM mannose in the assay medium and (4) growth of test isolates in half MICs of different antimicrobial agents. On the other hand, pretreatment of *Vero* cells with trypsin increased the adherence and invasion of most test isolates. No significant change in adhesion and invasion by changing the temperature from 27°C to 42°Cor the pH from 6 to 8. Log phase cultures showed higher adherence and invasion than stationary phase cultures.

Keywords: **S**usceptibility, adherence, invasion, mammalian cells, *Brucella melitensis*.

INTRODUCTION

The genus *Brucella* is aerobic, facultative intracellular, Gram-negative non-motile, non-spore forming coccobacilli. The genus Brucella consist of seven species according to the antigenic variation and primary host which are *B. melitensis*, *B. suis*, *B. abortus*, *B. ovis*, *B. canis*, *B. neotomae and B. maris*. The main pathogenic species worldwide are *B. abortus*, *B. melitensis* and *B. suis*, which cause abortion in their natural hosts and account for most cases of human brucellosis (Corbel, 1997).

Diseased animals excrete Brucella through the urine, milk, placenta and the products of miscarriages. In this way, the bacteria disseminated and infect other animals and humans (Doganay and Aygen, 2003). Routes of transmission of the infection to humans include direct contact with infected animals and their secretions during septic abortion or at the time of slaughter through injered skin, inhalation or inoculation into the conjunctival sac of the eyes and more frequently via the ingestion of unpasteurized dairy products (Corbel, 1997) with high risk of acquiring the infection between laboratory workers handling Brucella cultures (Fiori *et al.,* 2000) and Veterinarians (Doganay and Aygen, 2003). Person to person transmission of human brucellosis have been reported in rare cases due to blood transfusion, bone marrow transplantation, sexual transmission (Ruben *et al.,* 1991) and from lactating mothers to their breastfed infants (Corbel *et al.,* 2006).

Brucellosis is a zoonotic disease that is widely distributed throughout the developing world causing decrease in reproductive efficiency and abortion in animals (Rijpens *et al.,* 1996) with half a million of new human cases reported annually worldwide (Pappas *et al.,* 2006) and considered as an important cause of acute febrile illness in Egypt (Jennings *et al.,* 2007).

As *Brucella* species are intracellular pathogens, the treatment requires combined regimens with agents that may efficiently penetrate macrophages (Trujillano-Martin *et al.,* 1999). *Brucella* isolates are considered susceptible to the recommended WHO antibiotics. Nevertheless sporadic cases of antibiotic resistance have been reported (Lopez-Merino *et al.,* 2004).

Brucella can infect a broad range of mammalian cell lines *in vitro* (Pizarro-Cerda *et al.,* 1998). In contrast to other pathogenic bacteria, no classical virulence factors have been described in *Brucella* organisms (Moreno and Moriyon, 2006), however, the involvement of non-fibrillaradhesions remains open (Del Vecchio *et al.,* 2002). The virulence factors in *Brucella* are those molecular determinants that allow the bacteria to invade (Guzman-Verri *et al.*, 2001), resist intracellular killing (Moreno and Moriyon, 2006) and reach their replicating niche in professional and nonprofessional phagocytes (Pizarro-Cerda *et al.,* 1998).

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Accordingly, this study aimed to recover *Brucella* isolates from some collected specimens. In addition, the adherence and invasion characteristics as well as the antibiotic susceptibility profile of some *Brucella* isolates will be also studied.

MATERIALS AND METHODS

Isolation and identification microorganisms

Blood specimens from 40 *Brucella* infected patients depending on the serological results were inoculated into bottles containing 100ml sterile trypticase-soya broth with 1-2% sodium citrate. The bottles were incubated in a humidified CO_2 incubator at 37°C and 5% CO_2. Subcultures were done every 3-7 days over a period of 35 days on *Brucella* selective media. Supramammary lymph nodes and spleen collected by veterinarians from killed animals infected with *Brucella* based on the serological results (32 buffalo, 55 cattle and 99 sheep) were homogenized, then two aliquots were separately streaked on 2 plates of *Brucella* selective media and the plates were incubated in humidified CO_2 incubator at 37°C and 5% CO_2.

Identification, differentiation and bio typing of species of the genus *Brucella* was carried out according to Alton *et al.* (Alton *et al.*, 1988).

Antimicrobial susceptibility test

The antimicrobial agents tested were: Doxycycline, rifampicin, streptomycin and povidone iodine, cotrimoxazole, gentamicin, ciprofloxacin, erythromycin, amoxicillin/clavulinic acid, ceftriaxone, chloramphenicol, sorbic acid, benzalkonium chloride and cetrimide, chlorohexidinegluconate, methylparaben and propylparaben, phenol, cresol, sodium citrate, citric acid.

The well broth micro dilution method was used using 96-well plates according to procedure described by Rubinstein *et al*. (Rubinstein *et al.*, 1991). The minimum inhibitory concentration (MIC) values of cotrimoxazole, doxycycline, gentamicin, streptomycin amoxicillin/ clavulanic acid, ceftriaxone, chloramphenicol, ciprofloxacin, erythromycin and rifampicin were interpreted according to the CLSI (CLSI, 2006).

Adherence and invasion assays

Vero cell line was kindly provided by the Virology department, Animal Reproduction Research Institute, Egypt and supplied as confluent monolayer in micro titer plate ready for assay purposes (adherence and invasion).

Adherence assay was carried out principally as described by PlotKowski *et al.* (Plotkowsi *et al.*, 1994) as follows: The medium super merging *Vero* cells monolayer was first discarded and the cells were washed then aliquots of 100µl of the bacterial suspension were added to the wells which was prepared by standardization of 24 hours trypticase-soya broth culture to $3x10^8$CFU/ml using Dulbicco minimum essential liquid medium (Sigma, USA) supplemented with 5% fetal bovine serum (Sigma, USA). The count was adjusted using McFarland standard No.1 and verified by viable count. Following 4 hours of incubation at 37ºC, the bacterial suspension was removed and wells were washed three times with phosphate buffer saline (PBS) to remove non associated bacteria. Lysis of *Vero* cells was carried out by treating with 0.025% trypsin (Sigma, USA) in PBS for 30 minutes at 37°C. The associated bacterial cells (adherent and invaded cells), was determined by viable count method. The number of invaded bacterial cells was determined using gentamicin survival assay which was carried out as mentioned above except that after washing of the monolayer three times with PBS, aliquots of 150µl of gentamicin solution (200 µg/ml) in PBS were added to the wells and the plates were left at room temperature for 1 hour to allow killing of the extra cellular adherent bacteria. After removal of gentamicin solution by aspiration, the *Vero* cells were washed and treated with the lysis solution as mentioned above. Quantitative determination of the adherent viable bacteria was calculated depending on the difference between the total number of the associated bacterial cells (adherent and invaded) and the number of invaded bacterial cells.

Testing the influence of different factors on adherence and invasion of test isolates

Pretreatment of test isolates with some agents

The bacterial suspension was prepared as described before except that trypsin, lipase, neuraminidase and sodium metaperiodate were incorporated in Dulbicco minimum essential liquid medium at concentrations of 20 µg/ml, 5000U/ml, 1.8U/ml and 40mM, respectively. The resulting bacterial suspension was incubated for 30 min at 37ºC then cells were washed and used for adherence and invasion assays as mentioned before.

Pretreatment of vero cells with some agents

This was done as described by Szkaradkiewicz and Wal (Szkaradkiewicz and Wal, 2001) as follows: The medium super merging *Vero* cells monolayer was aspirated and the cells were washed twice with PBS. Aliquots of 100µl of trypsin, lipase, neuraminidase and sodium metaperiodate in Dulbicco minimum essential liquid medium were incorporated at concentrations of 10µg/ml, 2500U/ml, 0.9 U/ml and 20mM, respectively and the plates were incubated at 37ºC for 30 minutes. Then, the monolayer was washed and used for adherence and invasion assays as mentioned above.

Testing the influence of the growth phase of test isolates

Time course of growth (OD_{600}) and growth curves of different test isolates were carried out and constructed according to Burkhardt *et al*. (Burkhardt *et al*., 2005).

Adherence and invasion assays were carried out as described previously using bacterial suspensions prepared from both log and stationary phases.

Testing the influence of growth of test isolates in presence of sub MIC concentrations of different antimicrobial agents

The test isolate was grown in trypticase-soya broth in test tubes supplemented with 1/2MIC of different antimicrobial agents at 37°C for 24 hours. The resulting cultures were centrifuged, washed twice with PBS and then used in adherence and invasion assays as mentioned previously.

Testing the influence of pH and incubation temperature

The bacterial suspensions were prepared as previously described except that the pH of the assay medium was adjusted with either 1N of HCl or NaOH sterile solutions. The pH values studied were 6, 7 and 8 and the adherence and invasion assays were carried out as mentioned before. To study the effect of incubation temperature, the adherence and invasion assays were carried out as mentioned previously except that the plates were incubated at different temperatures (27°C, 32°C, 37°C & 42°C).

Testing the influence of different electrolytes and sugars added during adherence and invasion assays

The adherence and invasion assays were carried out as mentioned previously except that the test isolate was suspended in Dulbicco minimum essential liquid medium supplemented with 5% fetal bovine serum and the test agents were incorporated. $CaCl_2$ and $MgCl_2$ were added at the concentrations of 1,3 and 5mM while glucose, fructose, lactose, sucrose and mannose were added at concentrations of 20, 100 and 200mM.

STATISTICAL ANALYSIS

Experiments were carried out in duplicate and results represented means ± standard deviation. Statistical analyses were done using Graph Pad Prism software (version 5.01). Spearman's correlation coefficient (r_s) and its significance (P) were calculated and a value for $P< 0.05$ was taken to indicate a statistically significant correlation (Silva *et al.*, 2008).

RESULTS

Isolation and identification of microorganisms

A total of 66 isolates suspected for *Brucella* were recovered. Of these isolates, 9 were recovered from clinical blood specimens and 57 isolates were recovered from different investigated animals. The recovered isolates from animals comprised 7 isolates from buffalo, 19 isolates from cattle and 31 isolates from sheep. All isolates were identified as *Brucellamelitensis* biovar 3.

Susceptibility of Brucella melitensis biovar 3 isolates to different antimicrobial agents.

All isolates were susceptible to doxycycline, gentamicin and streptomycin. The order of susceptibility of the collected *Brucella* isolates to other tested antimicrobial agents took the following order amoxicillin/clavulanic acid > ceftriaxone = ciprofloxacin > cotrimoxazole> rifampicin > chloramphenicol > erythromycin. It is obvious that the highest percentage of antimicrobial resistance was recorded for erythromycin (9%). High prevalence of intermediate susceptibility was recorded for chloramphenicol (5 out of 66 isolates) and rifampicin (4 out of 66 isolates). This result could represent an intermediate phase of resistance development (table 1).

According to MIC50 values, the activity of tested non-antibiotics antimicrobial agents showed the order benzalkonium chloride > chlorohexidinegluconate > cetrimide > propyl paraben > methyl paraben = cresol >sorbic acid >phenol = citric acid > povidone iodine > sodium citrate (table 2).

Adherence and invasion of Brucella melitensis biovar 3 isolates to vero cells

Most isolates showed adherence ranged between > 0.02 to <0.3%, and these comprised 31.81% in the range >0.02 to < 0.1%, 46.96% in the range >0.1 to <0.2% and 19.69% in the range of > 0.2 to <0.3%. However, only one isolate which represent 1.5% of total isolates showed adherence level >0.3%. Statistical analysis showed no significant difference in adherence of isolates depending on isolate source. All test isolates were able to invade into *Vero* cells but by variable degrees ranged between 0.016 to 0.174% of initial count. Most isolates from different sources (87.88%) showed invasion level ranged between >0.01 to <0.1% while only 8 isolates (12.12%) showed higher invasion level ranged between >0.1 to <0.2%. Also statistical analysis showed no significant difference in invasion of *Brucellamelitensis* biovar 3 isolates depending on isolate source.

The correlation between adherence and invasion of test isolates to *Vero* cells was determined by calculating the Spearman's correlation coefficient (r_s). Spearman's correlation coefficient (r_s) value was found to be 0.733 ($P =0.031$), 0.607 ($P=0.034$), 0.626 ($P=0.004$) and 0.683 ($P < 0.0001$) for isolates recovered from human, buffalo, cattle and sheep, respectively. These values indicated an intermediate significant positive relationship between adherence and invasion of all test isolates.

Testing the effect of different factors on adherence to and invasion into Vero cells by Brucellamelitensis biovar 3 test isolates

This was carried out using eight isolates with intermediate adherence and invasion properties, which included 2 human isolates (H3 and H6), 2 buffalo isolates (B1 and B3), 2 cattle isolates (C1 and C14) and 2 sheep isolates (S4 and S17).

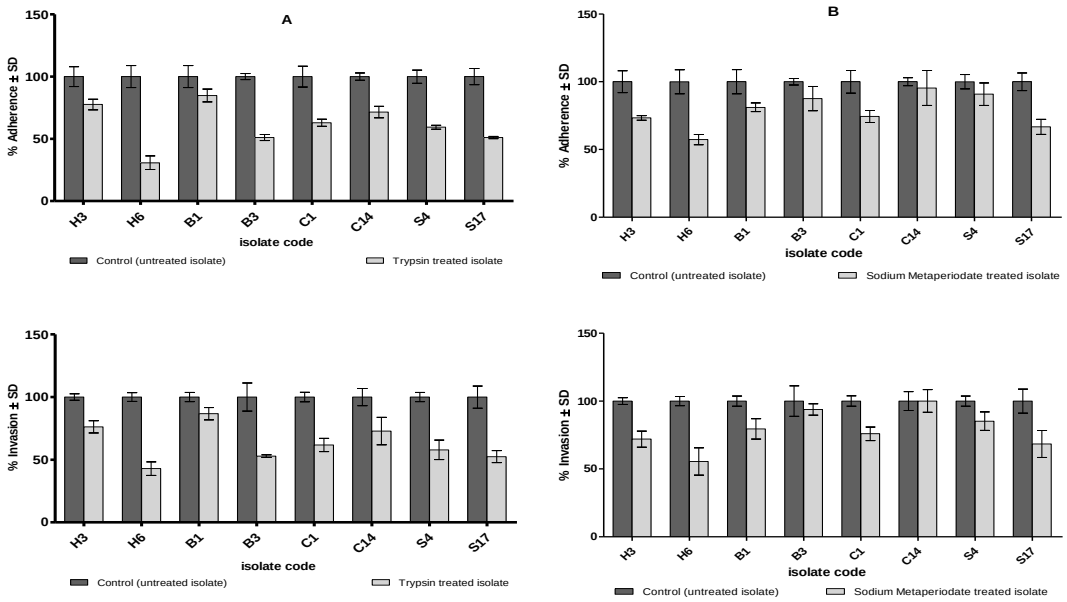

Fig. 1: Effect of pretreatment of *Brucella melitensis* biovar 3 isolates with (A) trypsin and (B) sodium metaperiodate on adherence to and invasion into *Vero* cells.

Table 1: MICs ranges, MIC50, MIC90 and susceptibility profile of different antibiotics and other antimicrobial chemotherapeutic agents against the tested *Brucella melitensis* biovar 3 isolates

Antibiotic / antimicrobial chemotherapeutic agent.	MIC range (µg/ml)	MIC50 (µg/ml)	MIC90 (µg/ml)	No. of isolates that are *		
				Susceptible	of intermediate susceptibility	Resistant
Amoxicillin/ clavulanic acid	0.125/0.063-8/4	0.5/0.25	2/1	65		1
Ceftriaxone	0.094-4	0.5	1.5	64		2
Chloramphenicol	0.25-4	1	2	61	5	0
Ciprofloxacin	0.016-4	0.25	0.94	64		2
Cotrimoxazole(trimethoprim / sulfamethoxazole)	0.079/1.49 - 4/76	0.5/9.5	1.5/28. 5	63		3
Doxycycline	0.031 - 1	0.094	0.5	66		0
Erythromycin	0.75-16	2	4	60		6
Gentamicin	0.031-2.5	0.25	1	66		0
Rifampicin	0.047-2	0. 375	1	62	4	0
Streptomycin	0.094-3	1.25	2.5	66		0

*As stated in CLSI (M100-S16), there are two limits for few antibiotics to distinguish susceptible and resistant isolates and for most antibiotics there is only one limit to distinguish the susceptible isolates. In the former but not in the later case, isolates of intermediate susceptibility could be determined.

Table 2: MICs ranges, MIC 50 and MIC90 of different non-antibiotic antimicrobial agents against the tested *Brucella melitenis* biovar 3 isolates.

Non-antibiotic antimicrobial agents	MIC range (mg/ml)	$\frac{\text{highest MIC}}{\text{Lowest MIC}}$ ratio	MIC50 (mg/ml)	MIC90 (mg/ml)
Benzalkonium chloride	0.023-0.125	5.3	0.031	0.063
Cetrimide	0.094-0.5	5.3	0.25	0.5
Chlorohexidinegluconate	0.031-0.5	16	0.125	0.25
Citric acid	1-8	8	2	4
Cresol	0.5-2	4	1	2
Methyl paraben	0.25-2	8	1	1.5
Phenol	0.5-4	8	2	3
Povidone iodine	1-8	8	6	8
Propyl paraben	0.25-1	4	0.375	0.5
Sodium citrate	8->128	>16	128	>128
Sorbic acid	1-3	3	1.5	2

Effect of pretreatment of test isolates with different agents

Tryp sin pretreatment resulted in reduction in adherence and invasion of all test isolates to *Vero* cells. The reduction was significant and ranged between 22.41 to 69.29% and 23.76 to 57.07% for adherence and invasion, respectively for all test isolates except for B1 isolate (fig. 1A). Sodium metaperiodate pretreatment resulted in reduction in adherence and invasion of all test isolates to *Vero* cells. The reduction was significant and ranged between 25.58 to 42.67% and 24.07 to 44.51% for adherence and invasion, respectively for 4 out of 8 isolates (H3, H6, C1 and S17). However, the adherence and invasion of the four isolates B1, B3, C14 and S4 was insignificantly reduced (fig. 1B). Lipase and neuraminidase pretreatment of test isolates did not result in significant change in adherence or invasion of all the test isolates to *Vero* cells (results not shown).

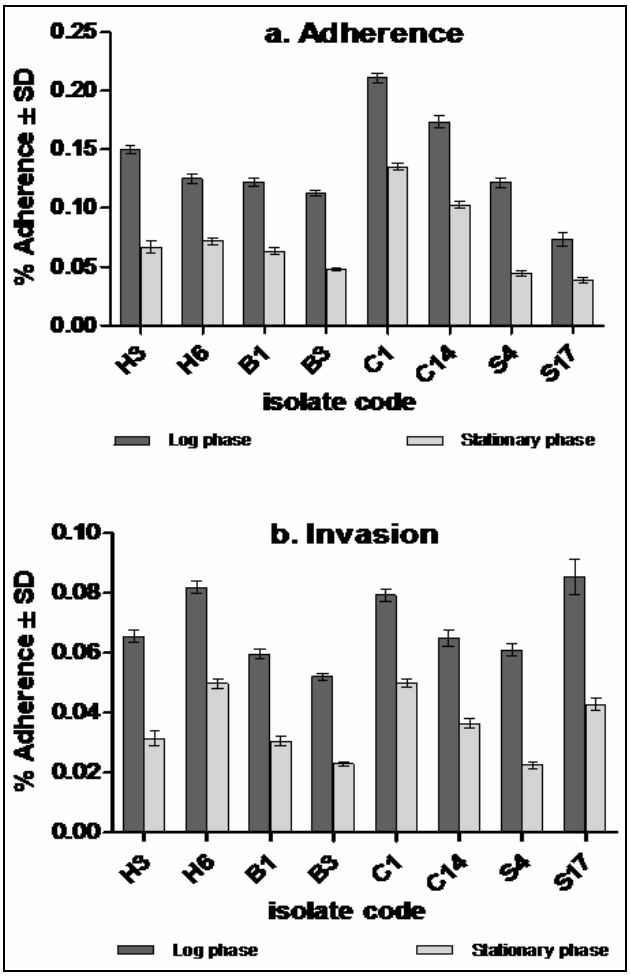

Fig. 2: Effect of growth phase of *Brucellamelitensis* biovar 3 test isolates on adherence to and invasion into *Vero* cells.

Effect of growth of test isolates in presence of sub MIC concentrations of different antimicrobial agents

The results showed that the adherence (table 3) and invasion (table 4) of all test isolate were affected when the test isolates were grown in presence of 1/2 MIC of different antimicrobial agents. Except for erythromycin, all antimicrobial agents significantly reduced the adherence and invasion of all test isolate to *Vero* cells. Erythromycin significantly reduced the adherence and invasion of only three isolates (H3, B1 and C1)

Effect of the growth phase of test isolates

The results (fig. 2) indicated that cultures of *B. melitensis* in the log phase of growth showed higher adherence and invasion to *Vero* cells than cultures at stationary growth phase. The difference in adherence and invasion of both growth phases ranged between 0.034 to 0.084% and 0.028 to 0.043% of the initial count, respectively which was found to be significant ($P<0.001$).

Effect of pretreatment of Vero cells with different agents

Trypsin pretreatment of *Vero* cells increased the adherence and invasion of all test isolates (fig. 3A). This increase was significant and ranged between 24.6 to 53.8% and 21.95% to 37.33% for adherence and invasion, respectively of most isolates. The adherence and invasion of C14 isolate were increased by only 15.93% and 7.48%, respectively and this increase was insignificant. In addition, the increase of invasion of C1 and S17 isolates were found to be also insignificant. Lipase pretreatment of *Vero* cells reduced the adherence and invasion of all test isolates (fig. 3B). This reduction was significant and ranged between 23.45 to 56.25% for adherence and between 33.33 to 48.04% for invasion for most isolates. However, the reduction in adherence and invasion was insignificant for B1 and C1 isolates. In addition, the reduction in invasion of C14 was found to be insignificant. Neuraminidase pretreatment of *Vero* cells reduced the adherence and invasion of all test isolates (fig. 3C). This reduction was significant and ranged between 30.95 to 67.19% for adherence and between 30.08 to 60% for invasion for all isolates except for C14 isolate. Sodium metaperiodate pretreatment of *Vero* cells reduced the adherence and invasion of all test isolates (fig. 3D). This reduction was significant and ranged between 23.31 to 45.83% for adherence and between 18.67 to 38.24% for invasion for most isolates. However, the reduction in invasion was insignificant for B3 and C14 isolates.

Testing different conditions during adherence and invasion assays

Compared to the original incubation temperature (37°C) and pH (pH 7), no significant change in adhesion and invasion of all test isolates to *Vero* cells was observed at the tested incubation temperatures and pH values (data not shown).

Effect of different electrolytes added during adherence and invasion assays

All concentrations of $CaCl_2$ caused significant increase in adherence and invasion (fig. 4A) of all test isolates. The

increase in adherence ranged between 29.67 to 86.16%, 63.74 to 143.3% and 106.4 to 207.1% for 1mM, 3mM and 5mM $CaCl_2$ concentrations, respectively while the increase in invasion ranged between 18.42 to 81.58%, 62.82 to 157.9% and 104.8 to 209.2% for the same$CaCl_2$ concentrations, respectively. $MgCl_2$ at concentration of 1mM resulted in minimum effect on the adherence and invasion of all test isolates. However, 3 mM$MgCl_2$ caused significant increase in adherence and invasion of the two isolates H6 and S17 by 49.36% and 31.16% for adherence and 46.99% and 40.24% for invasion, respectively. Moreover, at 5mM, most isolates showed significant increase in adherence ranged between 28.02 to 57.79% and significant increase in invasion ranged between 22.45 % and 57.32% except for B3 and C14 isolates (fig. 4B).

Effect of different sugars added during adherence and invasion assays

The results showed that all test sugars (except mannose) had nearly no effect on the adherence and invasion of all test isolates to *Vero* cells at the three tested concentrations (data not shown). Mannose at 20 and 100mM had nearly no effect on adherence and invasion of all test isolates. On the other hand, 200mM mannose caused significant reduction ranging between 20.36% to 29.11% for adherence and 19.78 to 28.43% for invasion for all test isolates except for the three isolates H6, B1 and C14 (fig. 4C).

DISCUSSION

The recovery percent from clinical specimens was found to be 22.5% (9 isolates) while the recovery percentage of isolation from supramammary lymph nodes and spleen specimens collected from different animals were 21.88%(7 isolates), 34.54% (19 isolates) and 31.3% (31isolates) from buffalo, cattle and sheep specimens, respectively.

All isolates were identified as *Brucellamelitensis* biovar 3. Available information indicates that *B. melitensis* infection is mostly widespread in Egypt, Sudan, Syria, Morocco, Turkey, Greece, Spain and Italy. The transmission of *B. melitensis* to cattle and buffaloes was considered to be the predominant cause of brucellosis in animals and humans in most Middle Eastern countries. This situation increases the importance of *B. melitensis* as the causative agent of brucellosis (Refai, 2002).

Our results should give warning signs for emerging resistant isolates for certain commonly prescribed antibiotics. Several factors that may influence the ability of various antibiotics to act on intracellular organisms have been suggested (Schwab and Mandel, 1989) including the intracellular concentration of antibiotic which could be less than that necessary to kill the pathogen which may be among the reasons for lack of *in vivo* effectiveness of β-lactam antibiotics indicating that the low MIC values of amoxicillin/clavulanic acid do not correspond to any therapeutic effect (Turkmani *et al.,* 2006). Eng *et al*. (Eng *et al*., 1991) reported decreased bactericidal capacity of quinolones against slowly replicating and non-replicating bacteria. Chloramphenicol cannot be considered as an appropriate therapy for human brucellosis due to high rate of relapse, serious side effects and the availability of safer and more effective drugs. The MIC values of erythromycin indicated its reduced activity.

Parabens, sorbic acid, citric acid and sodium citrate are widely used preservatives. Organic acids activity, such as citric and sorbic acids, decreases with increasing the pH as the lower pH values keep these agents in the non ionizable form that easily uptaken by the microbial cell. Alkyl esters of *p*-hydroxybenzoic acid, such as methyl and propyl parabens, exhibit good preservative activity even at pH levels of 7-8. Sodium citrate was reported to inhibit several human pathogens at low concentrations. However, the presence of $MgCl_2$ and$CaCl_2$ increased the MIC and MBC values of sodium citrate (Nagaoka *et al.,* 2010). These findings suggesting that parabens are preferred for preservation of dairy products (natural source of Ca^{++} which may be contaminated with *Brucella*) because they are effective at the slightly alkaline pH of milk and not affected by calcium.

Cresol had MIC50 and MIC90 values half that of phenol suggesting that it is better to use cresol in disinfection of *Brucella* infected farms. Quaternary ammonium compounds (QACs), chlorohexidine and povidone iodine are widely used antiseptics. According to MIC50 and MIC90 values, the activity order of these agents was benzalkonium chloride > chlorohexidinegluconate > cetrimide > povidone iodine. The MIC values of povidone iodine were high; this may be attributed to volatilization of iodine from culture media during the long incubation period of assay (48 hrs.). In addition, aqueous iodine solutions are generally unstable; in solution, at least seven iodine species are present in a complex equilibrium, with molecular iodine (I_2) being primarily responsible for antimicrobial efficacy (Gottardi, 1991).

Vero cell line was used as a model for mammalian cells to screen the adherence and invasion of the collected isolates because it is one of the most popular cell cultures (Draganov *et al.,* 2005) and was previously used as non-professional phagocytes to study *Brucella* adherence and invasion (Pizarro-Cerda *et al.,* 1998). All isolates were able to adhere to and invade into Vero cells but with variable degrees with an intermediate significant positive relationship between adherence and invasion of all test isolates indicating that adherence to *Vero* cells is a prerequisite for *Brucella* invasion. Thus, adherence is considered an important virulence trait, because it enables bacterial pathogens to interact closely with the cell membrane favoring intracellular penetration (Castaneda-Roldan *et al.,* 2004).

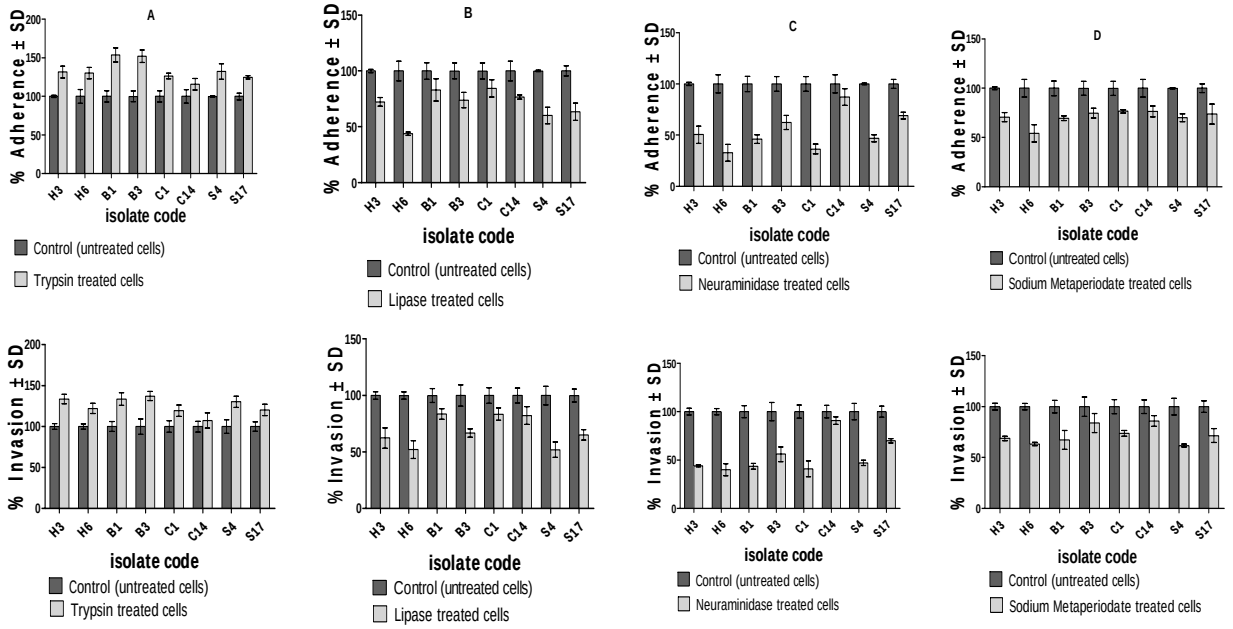

Fig. 3: Effect of pretreatment of *Vero* cells with (A) trypsin, (B) lipase, (C) neuraminidase and (D) sodium metaperiodate on adherence and invasion of *Brucellamelitensis* biovar 3 isolates.

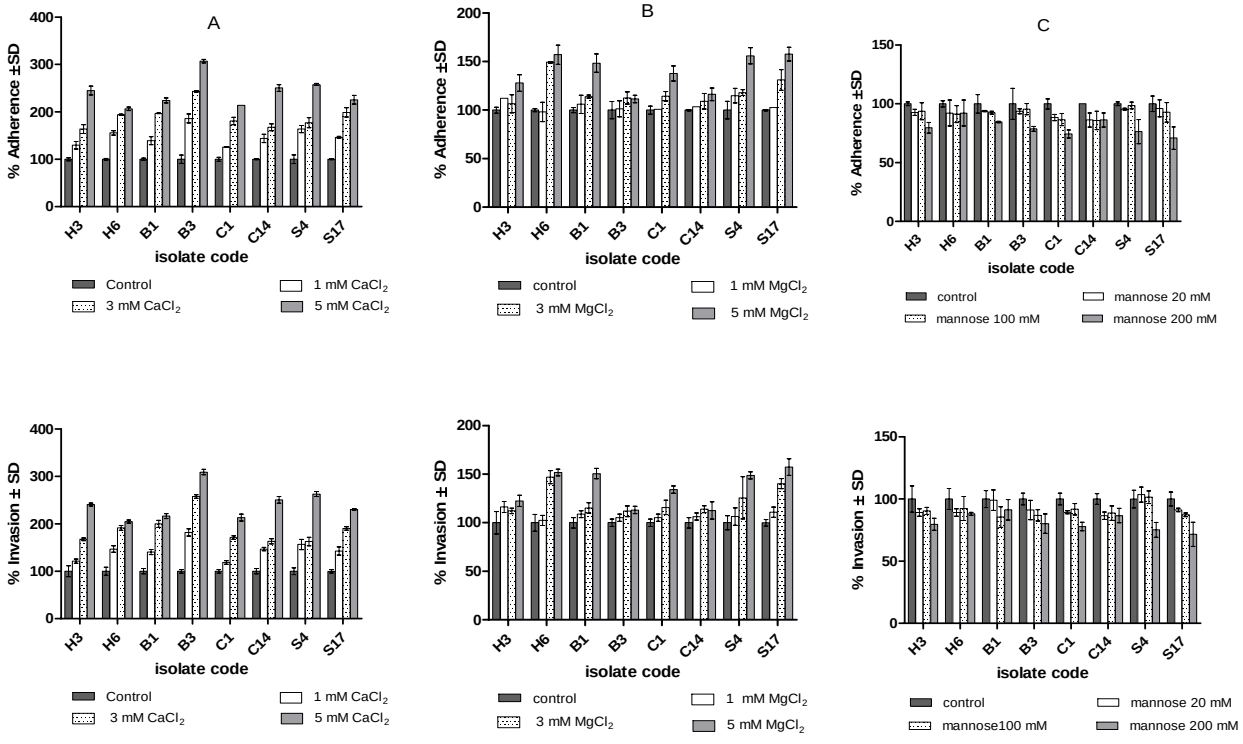

Fig. 4: Effect of (A) $CaCl_2$, (B) $MgCl_2$ and (C) mannose on the adherence and invasion of *Brucellamelitensis* biovar 3 isolates to *Vero* cells.

Table 3: Effect of growth at sub MIC concentrations of different antimicrobial agents on adherence of test

	Relative adherence percentage of test isolates when treated with*:									
Brucella isolate	Doxycycline	Rifampicin	Gentamicin	Streptomycin	Ceftrixaone	Ciprofloxacin	Cotrimoxazole	Chloramphenicol	Amoxicillin/ clavulanic acid	Erythromycin
H3	37.71 ±0.70	17.84 ±0.40	20.35 ±0.50	36.47 ±3.44	42.04± 0.28	34.62 ±0.49	27.54 ±1.80	15.74 ±0.37	44.73 2.09	71.69 ±3.14
H6	67.39 ±3.85	40.47 ±1.30	55.02 ±5.43	68.54 ±8.38	72.12 ± 4.08	58.73 ±1.97	57.12 ±2.99	26.95 ±1.17	68.11 ±1.13	96.15 ±8.16
B1	48.97 ± 3.22	41.59 ±1.56	29.55 ±1.77	50.92 ±5.40	33.29 ± 1.39	54.27 ±0.73	46.62 ±0.16	33.68 ±1.23	52.75 ±1.91	80.55 ±1.70
B3	61.21 ±0.92	39.02 ±1.35	67.94 ±3.56	59.51 ±2.50	68.25 ±0.29	63.35 ±2.24	70.64 ±2.82	41.46 ±0.56	76.55 ±3.28	93.14 ±3.40
C1	27.72 ±0.98	26.03 ±0.77	15.86 ±1.02	16.50 ±0.57	22.08 ±1.56	48.16 ±0.41	13.02 ±1.53	9.339 ±0.47	56.13 ±0.68	48.11 ±1.26
C14	44.27 ±1.53	35.37 ±0.79	66.09 ±0.83	54.38 ±0.47	49.98 ±1.26	60.48 ±0.91	50.53 ±1.99	39.65 ±0.59	74.43 ±0.56	99.10 ±1.63
S4	58.22 ±1.83	46.31 ±1.56	72.07 ±2.68	69.27 ±1.51	74.83 ±0.98	68.23 ±1.93	59.14 ±1.29	46.12 ±0.96	78.81 ±2.23	91.18 ±2.35
S17	74.71 ±1.96	41.73 ±1.44	68.37 ±2.42	76.43 ±3.28	71.80 ±1.64	63.92 ±3.93	70.29 ±1.88	11.29 ±1.60	83.10 ±4.52	100± 3.08

Adherence percentages were calculated relative to the controls (untreated isolates). The results were represented as means ± standard deviations.

Table 4: Effect of growth at sub MIC concentrations of different antimicrobial agents on invasion of test isolates to *Vero* cells.

	Relative invasion percentage of test isolates when treated with*:									
Brucella isolate	Doxycycline	Rifampicin	Gentamicin	Streptomycin	Ceftrixaone	Ciprofloxacin	Cotrimoxazole	Chloramphenicol	Amoxicillin / clavulanic acid	Erythromycin
H3	35.77 ±1.36	18.35± 1.18	17.64± 1.92	36.84± 1.09	40.12 ±1.23	33.83± 0.71	26.67± 1.99	15.02± 0.71	44.89 ±2.05	69.40± 1.16
H6	60.61 ±1.19	37.15± 0.91	49.90± 4.20	62.46± 5.00	67.97 ±0.47	56.99± 1.65	49.60± 3.47	23.70± 1.63	62.50 ±6.32	91.74± 6.63
B1	47.28 ±8.15	41.73± 4.22	31.18± 1.19	50.26± 5.47	32.22 ±0.70	51.98± 2.47	45.45± 0.99	31.80± 1.00	46.10 ±3.23	82.77± 6.89
B3	58.07 ±0.90	35.44± 5.28	62.47± 4.65	56.01± 2.45	65.07 ±4.00	58.98± 2.20	65.32± 2.77	37.41± 1.10	69.72 ±7.15	86.90± 8.57
C1	25.85 ±2.44	24.28± 1.91	14.35± 2.54	15.01± 1.42	19.22 ± 3.88	44.73± 1.02	13.49± 3.82	8.296± 1.17	51.31 ±1.69	44.32± 3.13
C14	41.56 ±3.92	33.14± 2.04	60.03± 2.12	49.96± 1.22	50.39 ±3.24	56.13± 2.34	46.83± 5.09	36.41± 1.51	68.07 ±1.44	91.66± 4.18
S4	52.95 ±3.26	43.17± 2.78	64.13± 4.77	62.59± 2.68	66.59 ±1.74	60.72± 3.44	53.43± 2.29	41.04± 1.71	70.12 ±3.97	88.51± 4.17
S17	75.60 ±3.56	40.79± 2.62	71.60± 4.40	75.93± 5.97	71.71 ±2.98	65.81± 7.16	70.05± 3.42	10.29± 2.91	81.50 ±8.23	99.00± 5.60

Invasion percentages were calculated relative to the controls (untreated isolates). The results were represented as means ± standard deviations.

Trypsin pretreatment of test isolates caused proteolytic removal of extra cellular protein structures. The results obtained after pretreatment of the bacterial cells with trypsin strongly suggested that the bacterial compounds involved in adhesion are proteins and/or glycoproteins. The effect of sodium metaperiodate may be due to the re-arrangement and blockage of carbohydrate structures on the bacterial cell surface (Thornley *et al.,* 1996) indicating that bacterial surface carbohydrates play an important role in binding to *Vero* cells. Pretreatment with lipase and neuraminidase did not result in any significant change in adherence or invasion of all test isolates to *Vero* cells suggesting that no role of lipidic groups on the bacterial surface as well as absence of mechanisms other than that mediated by sialic acid for neuraminidase.

Cultures of *B. melitensis* biovar 3 test isolatesin the log phase of growth showed higher adherence and invasion to *Vero* cells than stationary phase cultures indicating that the expression of adhesins and invasins on the bacterial surface is growth phase dependent character. It was reported that 454 genes were significantly differentially expressed between the log and stationary phase cultures of *Brucellamelitensis (*Rossetti *et al.,* 2009). In additions, significant number of genes directly involved in cell envelope/ outer membrane biogenesis were differentially expressed in both growth phase indicating the presence of growth-phase regulated *Brucella* virulence genes that are involved in the host-pathogen interactions.

It was previously reported that sub inhibitory concentrations (sub MICs) of various antibiotics are able to modify the molecular structure of the external surface of bacteria and some bacterial functions, such as the ability to adhere to the host cells; thus, influencing bacterial virulence (Braga, 1994). The inhibition of adhesion and invasion induced by antimicrobial agents reflects the phenomenon that could occur *in vivo* as it shows the real effect of very low concentrations of antibiotics. Thus, the ability of an antibiotic to interfere with bacterial virulence factors may be one of the main criteria for choosing one drug over another. The effect of antibiotic on the adherence and invasion of test isolate has clinical significance as they reduce the adherent and invasive ability of the bacteria (Balague *et al.,* 2003). Our finding suggested that the great effect of using combined regimen for brucellosis treatment that reported by WHO as the combined drugs could control infection not only through killing bacteria but also by inhibiting their adherence and invasion to host cells; thus, preventing dissemination of *Brucella* within human tissues. In accordance with these findings, it was reported that the ability of bacteria to adhere to cells is weakened by exposure of bacteria to sub MICs of antibiotics (Shibl *et al.,* 1995). The effect of antibiotics on adhesion may be strain specific and various antibiotics may have varying action on this propriety. Sub MICs of antibiotics may exert their inhibitory effect on adherence and invasion by (i) inhibition of synthesis of certain bacterial adhesins and invasins; (ii) release of adhesisns and invasins from the bacterial cell surface or (iii) modify bacterial shape in such a way as to interfere with the ability of the bacteria to approach receptors on cell surface (Lorian and Ernst, 1987).

The adherence and invasion enhancing effect of trypsin pretreatment of *Vero* cells may be probably due to elimination of superficial proteins leading to unmasking of cell surface receptors, allowing the bacteria to interact more efficiently with the cell surface membrane (Rocha-Gracia *et al.,* 2002; Castaneda-Roldan *et al.,* 2004). This effect was previously reported for *Brucella* adherence to human and animal red blood cells (Rocha-Gracia *et al.,* 2002) as well as to human epithelial cells and macrophages (Castaneda-Roldan *et al.,* 2004).

Pretreatment with lipase reduced the adherence and invasion of all test isolates which provide evidence for contribution of lipid molecules as cellular receptors for binding of *Brucella* to the *Vero* cells. This effect was previously reported for *Escherichia coli* adherence to bladder cells (Davis *et al.,* 1981) and *Pseudomonas aeruginosa* adherence to mouse corneas in organ culture (Singh *et al* 1990).

Pretreatment with neuraminidase reduced the adherence and invasion of all test isolates. It was reported that the reduction in adherence resulting from neuraminidase treatment support the involvement of sialic acid in the interaction of the *Brucella* with epithelial and nonepithelial cells (Rocha-Gracia *et al.,* 2002; Castaneda-Roldan *et al.,* 2004).

Pretreatment with sodium metaperiodatereduced the adherence and invasion of all test isolates indicating the involvement of carbohydrate moieties in the interaction between *Brucella* and *Vero* cells. In accordance with these findings, it was reported that carbohydrate moieties are involved in the interaction between *B. abortus* and eukaryotic cells (Traving *et al.,* 1998).

The adherence and invasion of *Brucellamelitensis* biovar 3 test isolates to *Vero* cells is temperature and pH independent within the tested temperature and pH ranges. Divalent cations may play a non-specific role in adherence and invasion of *Brucella* to *Vero* cells. Calcium binding proteins are often involved in bacterial adhesion to a surface and can be important for cell–cell aggregation suggesting that calcium promote non specific as well as specific adhesive interactions with protein and polysaccharide adhesion molecules at the cell surface (Geesey *et al.,* 2000). Rittig *et al*. (Rittig *et al*., 2001) reported that there are possibly two types of adhesion molecules promoted uptake of *Brucella*by human monocytes, one being Ca^{+2} and Mg^{+2} dependent and the

other not, and that both types participate in the uptake of wild-type bacteria suggesting that the presence of external Ca^{+2} or Mg^{+2} might be required for some receptor (s) mediating the uptake of *Brucella*. Our results indicate that the presence of divalent cations (particularly calcium) in milk and other dairy products or in dietary supplement may enhance the biofilm formation on the surface of intestinal epithelial cells following ingestion with consequently increase in the adherence and invasion ability of *Brucella.*

Microbial lectins are glycoproteins that are known to be important virulence factors involved in specific interactions with host carbohydrate cell membrane receptors. They can interact with corresponding sugar moieties located on cell surfaces. Furthermore, several studies suggested that occupation of lectins on the bacterial surface by exogenous sugars can prevent bacterial adherence to epithelial cells of different tissues (Thomas and Brooks, 2004).

Vemulapalli *et al*. (Vemulapalli *et al*., 2006) reported that the 14 kDa surface protein of *Brucella* possesseslectin like properties and has affinity towards mannose and is essential for the virulence. In this respect, non metabolizable mannose analogues (such as 2-deoxyglucose) can be applied instead of mannose and this may allow reduction of used effective concentration. As a result, mannose represents the carbohydrate moiety on mammalian cell surface glycoprotein (Ofek, *et al.,* 2003). The use of mannose analogues can interfere with the microbial binding to this receptor and this provides an effective approach for preventing *Brucella* interaction with mammalian.

CONCLUSION

In this study *Brucella* isolates exhibited sensitivity to protein synthesis inhibitor antibiotics such as doxycycline, gentamicin and streptomycin. Fortunately contamination of food, surfaces and animate objects by thses isolates can be controled by propyl paraben (as a preservative), cresol (for sufaces disinfection) and benzalkoniumchloride (as an antiseptic) as these agents showed high activity against the tested isolates. Adherence and invasion of most isolates to *Vero* cells were significantly reduced by pretreatment with trypsin, sodium metaperiodate, lipase and neuraminidase, incorporation of Ca^{++}, Mg^{++} and mannose and growth in half MICs of different antimicrobial agents. Log phase cultures showed higher adherence and invasion to *Vero* cells than stationary phase cultures.

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