Original Article

Efficiency of Rifampin-loaded chitosan NPs in Killing of Brucella melitensis Isolated From Aborted Sheep in Wasit Province, Iraq

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Abstract

Brucellosis is consider one of the utmost common bacterial infectious illness that infect man and animals. unfortunately, A worldwide community in the developing country didn't provide active assistance for reducing it spread out globally. Therefore, the aim of this study is the synthesis of chitosan nanoparticles loaded with rifampin to overcome bacterial resistance and provide high intracellular penetration and the potential for potent intracellular antibacterial activity. The study was conducted from October 2019 to September 2021, Of 57 positive clinical specimens identified in the present study, 25 (65.78%), 20 (74.07%), and 12 (66.66%) were isolated from vaginal swabs, fetal membranes, and fetal abomasum contents, respectively. From the results of SEM images of NPs, it was clear that RIF-loaded CHNPs presented irregular surfaces or rough with spherical shape and presence of some particle aggregates. The RIF-CHNPs particle size observed using SEM images was 260.54 nm, while the diameter was 150.23 nm. In addition, the antibacterial activity were also measured and the results shows that the MIC values for RIF, CHNPs and RIF-CHNPs and they were 4, 2.5 and 0.625 ug/ml, respectively. While the EE % and DL % of RIF were measured spectrophotometrically. Loaded antibiotic formulation gave good percentage of entrapment efficiency and drug loading at concentration 50 mg/ml. Then the EE % at concentration 50 mg/ml was re-estimated in different pH values and the best EE% was at pH 5. In conclusion, the antibacterial activity of drug nanocarrier against B. melitensis found that it had a relatively low MIC 0.625 ug/ml. whereas the inhibitory concentrations were about 4 µg/ml and 2.5 µg/ml for the RIF and CHTNPs, respectively. Indicating there were 6.4 times reduction in MIC for RIF-CHTNPs and 1.6 time reduction for CHTNPs comparing to MIC of antibiotic alone.

Keywords: Bacterial resistance; Nanoparticles; B. melitensis; Chitosan NPs; Rifampin.

1. Introduction

Brucellosis is consider one of the utmost common bacterial infectious illness that infect man and animals. unfortunately, A worldwide community in the developing country didn't provide active assistance for reducing it spread out globally, however, it is rare in most built-up countries and additional common in low and middle-income countries [1]. The disease in Iraq [2] and Middle East is endemic and has terribly reported prevalence of brucellosis in the world [3]. Brucella melitensis and Brucella suis, which are known to become an issue of great importance recently due to the high virulence factors that affects humans and animals very dangerously [4].

These microorganism are facultative intracellular cocco-bacilli as well as immotile, aerobic and G-ve bacteria and therefore the most important etiological species of small ruminants is B. melitensis [5].

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Humans can contract brucellosis through contact with infected animal secretions and carcasses or by consuming their products, largely unpasteurized milk and dairy products [6]. In the Middle East and North Africa (MENA) region, Brucella is an outbreak communicable tropical disease. Consequently, associated failures in veterinary public health systems, and the unrestricted movement of animals across open borders between these countries have fueled the resurgence of brucellosis. the highest prevalence of brucellosis among cattle and sheep occurs in 2010 in Jordan, while goats have the highest infection rates in Iraq and Jordan, whereas the highest infection rate of Brucellosis in camels were recorded in Saudi Arabia, Egypt and Iran [7]. The drugs that are commonly recommended to treat brucellosis in humans, usually these drugs used as in combination therapies [8,9]. the foremost wide used and recommended therapies are those that combine doxycycline, rifampicin, aminoglycoside and/or co-trimoxazole, but monotherapy and other antibacterial combination have also shown some effectiveness [8,9]. In animals, a combination of antibiotics such as long-acting oxytetracycline, streptomycin, and intramammary oxytetracycline infusion is strongly recommended. However, B. abortus, B. melitensis or B. suis could potentially remove from livestock by using these combinations or other antibiotic combinations, but unfortunately the treatment with these combinations are recently not proven and unsafe, and the treatment is generally not recommended. Even if brucellae disappeared, the re-infection may occur because may remain in lymph nodes or other tissues. It is also unlikely that the treatment will be cost effective in many flocks [10]. However, at a time of fast emergence of bacterial resistance to most available Antibiotics, Controversies concerning the prolonged use of antibiotics with proven efficacy against Brucella pose particular problems .There are a number of issues with using antimicrobials widely, One of the biggest issues is the issue of Antimicrobial resistance (AMR) which may threatens the effective prevention and treatment of an increasing number of infections caused by pathogenic microorganism such as bacteria, parasites, viruses and fungi. It also threatens the worldwide public health that needs action across all governments and community [11]. A report from Egypt [12] detected relapse in 59.3% of patients with osteoarticular brucellosis treated with two antibiotics (rifampicin and doxycycline) for five months or less. In contrast, 7.9% of patients treated for more than five months were relapsed. This report also noted that although long-term treatment with streptomycin has been associated with ototoxic or nephrotoxic symptoms, moreover, patients receiving three antibiotics (rifampicin, streptomycin and doxycycline) in combination showed no relapse [13]. In 2012, B. melitensis isolates with a high rate (45.0%) of occurrence with decrease sensitivity to rifampicin was also reported in Egypt [14]. There are multiple potential explanations for these, one of the concerns raised the incidence of the emergence of multiple resistant Brucella spp. is that rifampicin is one of the antibiotic of choice for treating Brucella disease in humans [8,9]. Furthermore, there are a number of obstacles to antibiotic therapy for controlling of Brucella disease, one of most difficulty is that Brucella spp able to survive and replicate intracellularly such as macrophage and dendritic cells. Therefore, it is reasonable to expect that antibiotics must be active intracellularly. rather than searching for new antibiotics, For this reason, researchers are investigating alternative treatment methods such as drug delivery systems. Nanocarriers for example nanoparticles and liposomes, provide high intracellular penetration and the possible for potent intracellular antimicrobial activity over prolonged periods [15–17]. Chitosan is a derivative of glucan with chitin repeating units discovered by Rogat at the end of 1850s with a known active ingredient derived from chitin, a natural amino-polysaccharide compound with chemical structural formula ($C_8H_{13}NO_5$). In addition, this chemical substance has great biocompatibility with various materials, and because of its ease digestibility, non-toxicity, great absorption capability and accessibility this material is largely used as a drug carrier [16,18,19]. The polymers employed in the nanoparticles are depend on hydrophilic and hydrophobic properties. The hydrophilic nanoparticles like chitosan, are an appropriate choice for drug delivery systems. Therefore, the aim of this study is the synthesis of chitosan nanoparticles loaded with rifampin to overcome bacterial resistance and provide high intracellular penetration and the potential for potent intracellular antibacterial activity.

2. Material And Methods

2.1. Study Area and Animals

This study was conducted in different cities of the Iraqi province of Wasit. Wasit Province is located in central Iraq about 180 km south of Baghdad. The study was conducted from October 2019 to September 2021. This area was chosen intentionally based on previous experience within the research team which had found such as availability of a good number of small ruminants and easiness for transporting samples for microbiological diagnosis. Aborted sheep and those that had recently aborted (within the last 7 days at the time of sampling), The subjects of interest for the purpose of this study were those sheep with uterine discharges and/or retained placenta. The sampling methods were decided on the basis of the medical history. Therefore, A total of 83 biological specimens were included in our analysis (38 vaginal swabs, 18 abomasum contents, and 27 fetal membranes) were collected from 50 sheep for further bacteriological studies.

2.2. Sample Collection and Bacterial Isolation

Vaginal swab samples were collected into a vial of Ames transport medium (HiMedia, Mumbai, India) using a sterile applicator stick. Tissue samples were also collected using a sterile 50 mL screw-cap tube containing sterile saline. All samples were stored at -20°C in the laboratory of microbiology department at College of Veterinary Medicine,

Wasit University and processed for microbiological diagnosis. All specimens were handled under [Biosafety level Two (BSL2)] with extraordinary personal protection [20]. Briefly, tissue specimens were processed aseptically by eliminating the foreign material and mincing it into little pieces and macerating it with a small volume of sterile phosphate buffered saline (PBS) using a stomacher or tissue mincer. Then the samples were inoculated onto Brucella Selective Agar with Antibiotics Added (FD005) and incubated at 37°C in both the lack and existence of 5-10% CO2 and the cultured plates were checked for growth of Brucella spp. on day 4 and then checked every day for two weeks. Vaginal swabs were streaked and incubated on solid media similar to that of the clinical specimen mentioned above. Initially, the Rose Bengal Test (RBT) [21] was used. Further checks are carried out to such as CO2 demand as well as biochemical assays including urea hydrolysis, oxidase catalase, H2S production and others, and growth measurement for thionine and basic fuchsin dyes present in trypticase soy agar at various concentrations were performed as described previously [22,23].

2.3. Synthesis of Chitosan (CHNPs) and Rifampin-loaded Chitosan (RIF-CHNPs) Nanoparticles

Chitosan (average molecular weight, 108 kDa) was obtained in the highest purity from (Sigma Aldrich Company, Germany). Sodium tripolyphosphate (STPP), ethyl alcohol, sodium chloride, sodium hydroxide, Tween 80, and acetic acid were purchased from (Merck Company, Germany). All chemical materials used were analytical grade. Rifampin (Sanofi, France) was used as the active pharmaceutical ingredient. According to Ebrahimi et al. [24], with some modifications, one gram of chitosan powder was dissolved in (100) ml of acetic acid solution (1% v/v). Then 50 ml of NaCl solution (3 g/L) were added and stirred at 3000 rpm for 30 minutes. A series of diluted (10, 20, 50 and 100) mg/ml concentrations of the chitosan stock solution were then prepared. It is important to note that the pH of solution provided was adjusted to approximately 5 with 0.5M NaOH for this sample. Insoluble chitosan were removed by using (Millipore 0.45 µm filters). Six gram of rifampin was dissolved in one hundred milliliter of ethyl alcohol at 80°C and 1 ml of Tween 80 was added to prevent aggregation of rifampin. Then this solution was added to the prepared chitosan solutions. Furthermore, A solution contain 5 ml of sodium tripolyphosphate (STPP) (1% v/v) was slowly (0.25 ml/min) added to the sample under magnetic stirring at 1000 rpm for 5 h using ultrasonic waves in a water bath (25°C). The solutions were centrifuged for 20 minutes at 10,000 rpm (IKAT18, Germany). Thereafter, The absorbance (Spectrophotometer; Cecil ,France) of the filtrate was measured. Meanwhile, the sediment was washed with distilled water and filtered three times to remove any residues. The final product was dried in an oven (Memmert, Germany) at 55°C for 12 hours which is used for analysis.

2.4. Measurement of Entrapment Efficiency (EE%) In Different pH Values.

First, the drug entrapment efficiency was calculated by measuring the absorbance at wavelength (334 nm) for rifampin using different concentration of NPs (10, 20, 50 and 100 mg/ml) according to the following equation [25].

EE%= total drug added – free non-entrapped drug) divided by the total drug added x 100. Eq. (1). Then the best EE % was chosen and re-estimated in different pH values (3, 5, 7 and 8).

2.5. Measurement of Drug Loading Efficiency (DL%)

About 0.01 g of RIF-CHNs was weighed and dissolved in (1% v/v) of acetic acid using ultrasonic waves in water bath (25 oC) to get complete dissolution. The absorbance of solution was measured at wavelength (334 nm) by using the following equation [25]:

DL%= Total amount of drug – Free drug in supernatant / Total amount of drug x100%. Eq. (2)

2.6. In Vitro Drug Release Study In Different pH Values

The kinetic release study of the nanoparticles was performed in triplicate using a dialysis membrane. 0.01 g of the dried mixture was weighed and loaded into standard Spectra/Por 3 regenerated cellulose dialysis tubing (Repligen, Waltham, MA, US) with a MWCO of 3.5 kDa and fitted with standard caps (Repligen, Waltham, MA, US). locked. The dialysis tubing was immersed in three 500 ml separation media with different pH values (5, 7 and 8) stir at 75 rpm with a magnetic stirrer at 37°C. At 15, 30, 60, 120, 240, 90, 480, 600 and 1440 min, three milliliters of fresh medium were replaced each equal volume of dissolution medium was removed. The absorbance of the withdrawn solutions was measured at a wavelength (334 nm).

The drug releasing percentage at each time point was determined depend on the following [26]:

Drug release(%) = [Drug in solution (mg/mL)] / [Initial drug in particles (mg/mL)] = (mg/mL)×100 Eq. (3)

2.7. Size of NPs, Zeta potential and Polydispersity Index (PDI)

The mean particle size, zeta potential (ZP) and polydispersity index (PDI) of the prepared NPs were measured by using The [Malvern Nano ZS 90 (Malvern Instruments, UK)] after a proper dilution with distilled water (DW) [24].

2.8. Surface Morphology Determination

Surface morphology of the nanoparticles were analyzed by a [(scanning electron microscope (SEM), Hitachi, Japan)]. A 5 ml of DW was used to dispersed and sonicated for 3 minutes (Misonix, USA). A few drops of the prepared samples were attached with double sided tape attached to a metal stub [27].

2.9. Antibacterial Activity of RIF-CHNPs, CHNPs and Free Drug (RIF)

For assessing the antibacterial activity of the RIF, CHNPs and

RIF-CHNPs against B. melitensis, Zones of bacterial growth inhibition were determine by using well diffusion methods. While MICs were determined by using broth dilution assay method (Asghari et al., 2006; NCCLS, 2000). In the tube dilution assay, 1 ml standard bacterial suspension (1.5 ×108 cfu/ml) was added to tubes containing 9 ml Nutrient broth, then different concentrations (ranging from 5 ug/mL to 0.156 ug/mL with a total volume of 75 µL of CHNPs and RIF-CHNPs) were added. Three tubes without bacteria containing CHNPs and RIF-CHNs served as negative control and nutrient broth served as positive control. Simultaneously, Rifampicin RIF (free drug) was used to determine sensitivity of B. melitensis by using the serial concentrations of 0.5, 1, 2, 4, 8 and 16 mcg/ml. All tubes were incubated at 37oC under 5% CO2 for 72 hours. The tests were performed in triplicate. The MIC of NPs and free drug was taken as the lowest concentration that showed no growth.

In well agar diffusion method 5 ml of standardized bacterial stock suspensions (1.5 ×108 cfu/ml) of B. melitensis was thoroughly mixed to each 500 ml of sterile Mueller Hinton agar supplemented with 1% sheep hemoglobin (Hb), 20 ml of the inoculated Mueller Hinton agar was distributed into sterile Petri dishes of each, then 6 mm diameter wells were prepared. the wells were loaded with 1/2 MIC, 1 MIC and 2 MIC of RIF, CHNPs and RIF-CHNPs . The plates were incubated at 37oC under 5% CO2 for 72 hours. The activity was determined by measuring the diameter of inhibition zone around each well by millimeter against the tested organism. The tests were performed in triplicate. As MIC breakpoints for clinically used antimicrobials are not yet established for Brucellae, the guidelines for slow-growing bacteria (Haemophilus influenzae) were used as an alternative [28,29].

2.10. Statistical Analysis

The data was collected and reported as mean ± SE. Data were analyzed statistically using the Microsoft Program, SAS (Statistical Analysis System - version 9.1) [30]. Continuous and numerical values were analyzed by Student's t-test. Differences were considered significant at P <0.05.

3. Results

3.1. Bacterial Isolation and Identification:

According to standard laboratory investigations, such serological, cultural characteristics and biochemical tests showed that from 83 different types of samples, 57 (68.67%) were found positive for the presence of B. melitensis by the Rose Bengal test. Of 57 positive clinical specimens identified in the present study, 25 (65.78%), 20 (74.07%), and 12 (66.66%) were isolated from vaginal swabs, fetal membranes, and fetal abomasum contents, respectively. Results were summarized in Table (1, 2 and 3).

Table 1: Seroprevalence of Brucellosis (B. Melitensis) in sSheep by Rose Bengal Test

Type of sample	No. of sample	Positive No. (%)	Negative No. (%)
Vaginal swab	38	25(65.78%)	13(34.21%)
Fetal membrane	27	20(74.07%)	7(25.92%)
Fetal abomasal contents	18	12(66.66%)	6(33.33%)
Total	83	57(68.67%)	26(31.32%)

Table 2: Brucella isolates recovered from clinical samples of seropositive sheep (RBT +ve) using bacteriological tests

Type of sample	No of sample	Positive cultural isolation	Percentage %
Vaginal swab	25	6	24 %
Fetal membrane	20	3	15 %
Fetal abomasal contents	12	2	16.6 %
Total	57	11	19.29

Table 3: Basic biochemical tests of clinical samples of seropositive sheep (RBT +ve)

Brucella isolates	No of sample 25	Biochemical tests						CO2 req.	Grow in dyes	
		Cat	Oxi	Ure	Ind	VP	H2S Pro.		Thn	BF
Vaginal swab	25	+	+	+	-	-	-	-	+	+
Fetal membrane	20	+	+	+	-	-	-	-	+	+
Fetal abomasal contents	12	+	+	+	-	-	-	-	+	+

Cat = Catalase, Oxi = Oxidase, Ure = Urea hydrolysis (All isolate positive within 2 hours of culture),

Ind = Indole production, VP = Voges Proskauer, H2S pro = H2S production, CO2 req.= CO2 requirements,

Thn= Thionin, BF= Basic Fuchsin.

3.2. Physicochemical Characterizations

Table (4) shows the particle size, zeta potential (ZP) and polydispersity index (PDI) for the formulations prepared. The particle size values were 150.23±3.4 nm for CHNPs and 260.54±2.3nm for RIF-CHNPs. While A PDI for CHNPs and RIF-CHNPs was 0.17±0.03 and 0.26±0.05, respectively. The ZP is a charge on the molecular surface. ZP for CHNPs was found to be +27.31±0.32 mV and +24.14±0.12 mV, showing that the formulations prepared are stable.

Table 4: Physicochemical characterizations of freshly prepared suspensions (SUS) of RIF-CHNPs and CHNPs formulations.

Formulation	ZP (mv)	Particles Size (nm)	PDI
CHNPs	+27.31±0.32	150.23±3.4	0.17±0.03
RIF-CHNPs	+24.14±0.12	260.54±2.3	0.26±0.05

PDI: polydispersity index, ZP: zeta potential

3.3. Surface Morphology

The results of SEM images of NPs (Fig. 1, A and B) appear to make sense and to be compatible with our expectations. From image A, it was clear that RIF-loaded CHNPs presented irregular surfaces or rough with spherical shape and presence of some particle aggregates. The RIF-CHNPs particle size observed using SEM images was 260.54 nm, while the diameter was 150.23 nm.

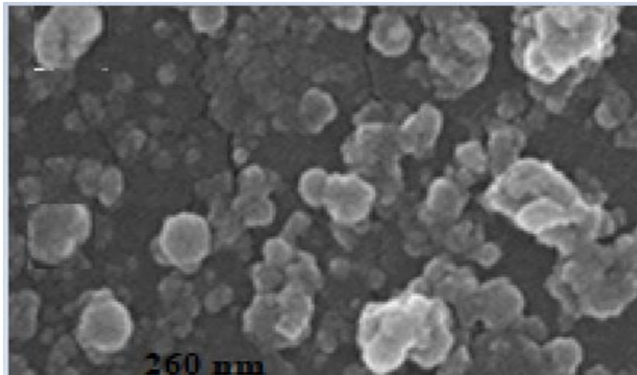

Figure 1: A. SEM graphs of RIF-CHNPs formulation (average particle size of ~260 nm)

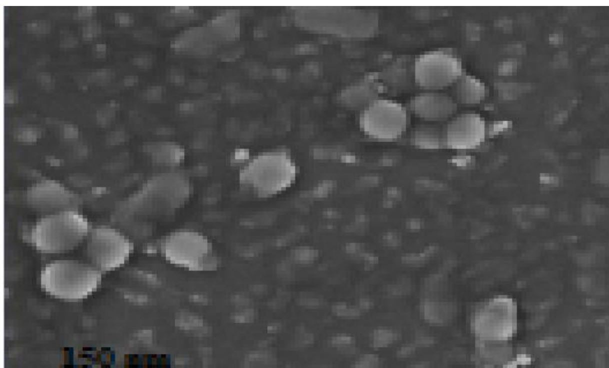

Figure 1: B. SEM graphs of CHNPs formulation (average particle size of ~150 nm)

3.4. Entrapment Efficiency (EE%) and Drug Loading (LD%)

EE % and DL % of RIF was measured spectrophotometrically. Loaded antibiotic formulation gave good percentage of entrapment efficiency and drug loading at concentration 50 mg/ml as shown in (Table 5). Then the EE % at concentration 50 mg/ml was re-estimated in different pH values and the best EE% was at pH 5. As shown in (fig. 2).

Table 5: Entrapment Efficiency % and Drug Loading % of the Nanoparticles

NPs concentrations	Entrapment Efficiency %	Drug loading %
10 mg/ml	12 %	16 %
20 mg/ml	23 %	32 %
50 mg/ml	61 %	42 %
100 mg/ml	42 %	37 %

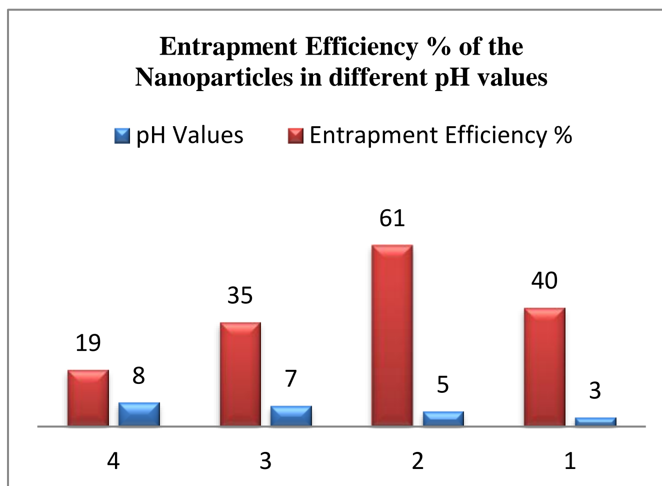

Figure 2: Entrapment Efficiency % of the Nanoparticles in different pH values

3.5. Drug Release Kinetic study in Vitro

The concentrations of drug release was plotted against time to obtain the drug release profile. (Fig. 3) shows the release behavior of RIF from RIF-CH nanoparticles over 24 hr in release media with different pH. Accumulative release of the antibiotic followed a steady, continued-release pattern.

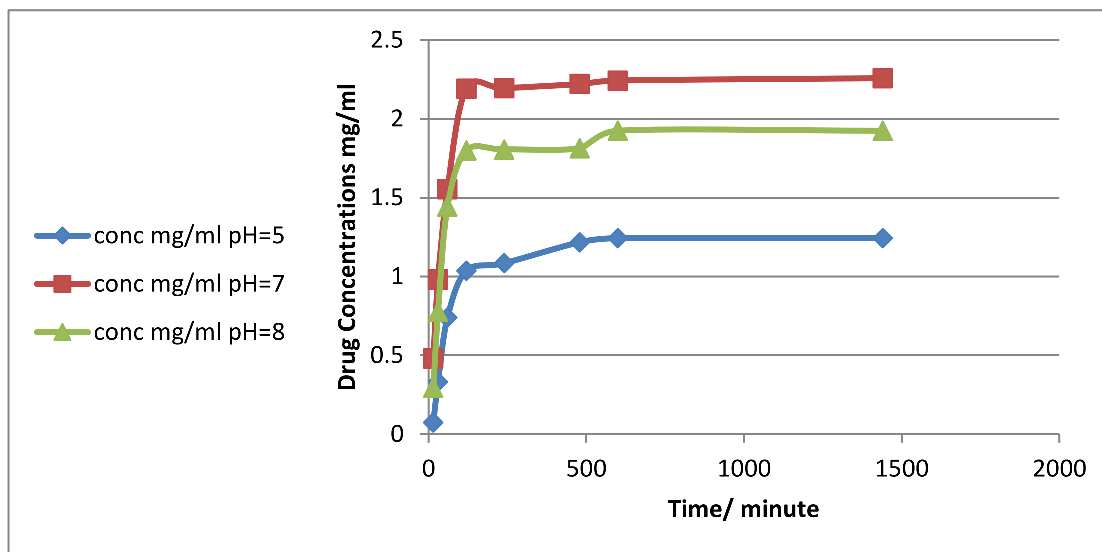

Figure 3: In Vitro Drug Release Study in different pH values.

3.6. Antibacterial Activity of RIF-CHNPs, CHNPs and Free Drug (RIF)

Different serial concentrations of drug and nanoparticles were used to determine the MIC by using broth dilution method. (Table 6) shows the MIC values for RIF, CHNPs and RIF-CHNPs and they were 4, 2.5 and 0.625 ug/ml, respectively. Furthermore, the 1MIC, 1/2MIC and 2MIC for each one were used to determine the diameter of inhibition zones for Rifampin, nanoparticle and drug nanoparticle using well agar diffusion method, results summarized in (Table 7).

Table 6: MIC of CHNPs, RIF-CHNPs suspension and RIF against B. melitensis

Formulation	MIC (ug/ml)
RIF	4
CHNPs	2.5
RIF-CHNPs	0.625

Table 7: Inhibition zone diameter of CHNPs, RIF-CHNPs suspension and RIF against B. melitensis

Formulations	MIC Conc. and Zones of inhibition	2MIC	1MIC	1/2MIC
RIF	MIC (ug/ml)	8	4	2
	Inhibition Zone diameter (mm)	10	8	0
RIF-CHNPs	MIC (ug/ml)	1.25	0.625	0.312
	Inhibition Zone diameter (mm)	24	22	20
CHNPs	MIC (ug/ml)	5	2.5	1.25
	Inhibition Zone diameter (mm)	14	10	7

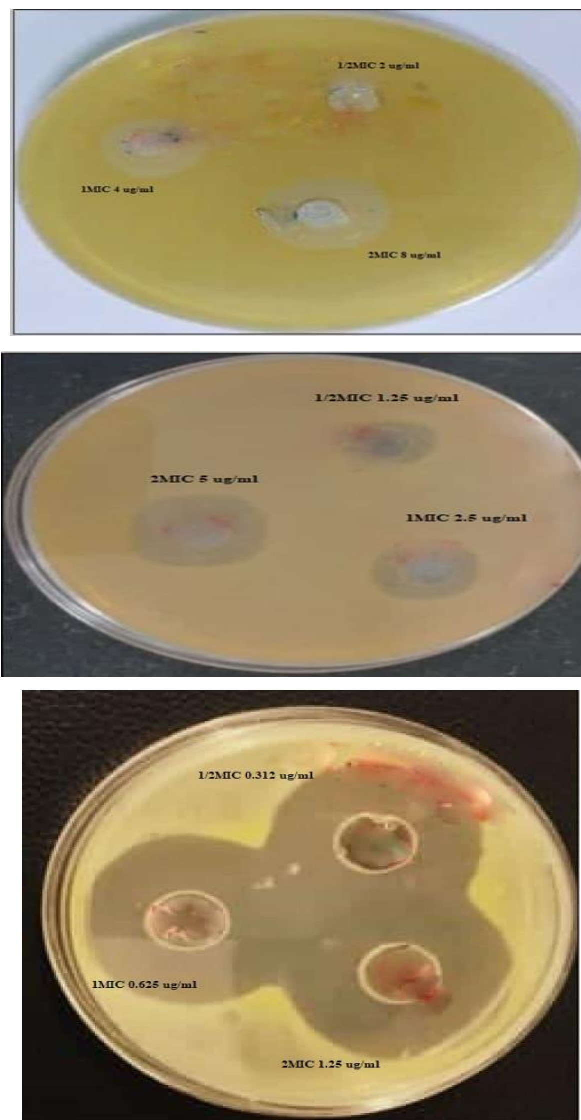

Figure 4: Inhibition zone diameter of free RIF (A) CHNPs (B), RIF-CHNPs (C) suspension against B. melitensis.

4. Discussion

Among all Brucella species, B. melitensis is mainly responsible for brucellosis in animals and is widespread in Asia, including Iraq in line with the findings of this study, we reported the isolation of B. melitensis from aborted sheep and characterized the isolates by conventional cultural, biochemical, and serological methods. The experiments were carried out in laboratories of veterinary medicine college, Wasit university. Initially, Seroprevalence of Brucellosis in sheep by Rose Bengal Test which is consider as an important screening and rapid test to investigate brucella [31]. Out of 83 samples, 57 (68.67 %) were Brucella positive as follow; 25(65.78 %) were isolated from vaginal swabs, 20(74.67 %) from fetal membrane and 12(66.66 %) were isolated from fetal abomasal contents. Then a confirmatory isolation of Brucella isolates, fifty seven specimens recovered from clinical specimens of seropositive sheep (RBT +ve) using bacteriological tests. The vaginal swabs of clinically aborted sheep were 6 (24%), while the prevalence of B. melitensis from fetal membrane were 3 (15%) and 2 (16.6%) of fetal abomasum contents were positive as B. melitensis. The high incidence of Brucella infections were consistent with 2018 CDC reports and the finding of Berger (2020) who reported that in Africa and Asia and the average prevalence of Brucella in animals was: in sheep and goats 088.8 %, cattle 068.8%, camels 0.420%, pigs and dogs 012.9%. People were at high risk exist due to occupational exposure (11%) and hospital patients (7%) [32,33]. In addition, in Egypt in 2008 there was a real prevalence in sheep (41.3%) and goats (32.2%). In Saudi Arabia, individual is 3 to 3.8 times more likely to be seropositive for Brucella antibodies on serology. in Jordan, the seroprevalence in sheep was predictable at 2.2% at the individual level and 45% at the flock level [32,33]. The different parameters like physiochemical properties, morphology, drug release in vitro and antibacterial activity in vitro are the ranges over which to test the different parameters, the formulations of RIF-CHNPs, CHNPs were prepared and evaluated. Chitosan is a biodegradable polymer that can be broadly defined as a class of polymers with antibacterial activity [34,35]. We have prepared chitosan nanoparticles containing rifampin by the ionic gelation method to evaluate the possible changes in the antibacterial agent's effectiveness. The preparation of rifampin-loaded nanoparticles has been performed in a few studies, but there have been very few studies on the antibacterial activity of chitosan-loaded rifampin against B. melitensis. And according to our knowledge this is a first time in Iraq. The typical parameters used to study this is the polydispersity index (PDI) and the ZP, these are the most important parameter to be considered here due to when Manufacture of food or pharmaceutical grade products. These properties of nanocarriers can affect bulk properties, process ability, stability, product performance and final product appearance. The PDI value can vary from monodisperse systems to highly disperse systems with a values ranging from 0.00 to 1.00, respectively with a relatively larger size distribution indicating a PDI value greater than 0.5. However, for all formulations, especially for the CHNP formulation a small size distribution was observed [24,36,37]. In our results Positive overall of the values of Zeta Potential for CHNPs owing to the carboxyl group of chitosan (CH) [38]. Based on the results, we make the following observations, the ZP values increased for formulations of CHNPs. As much as the free concentration of CH and amine groups on the surface of NPs, the stronger the electrostatic repulsion between the particles [39]. Another interaction was observed when the volume ratio of tripolyphosphate (TPP) increased and the zeta potential decreased in. This confirmed that is largely attributed to the dependence of the formation of NPs on the TPP concentration that crosslinked with free NH2 groups [24]. Positive surface charges due to functional groups in CH chains are taken into account in prepared formulations. In addition, It has been shown that positively charged NPs can be adsorbed on the bacterial surface and are closely associated with bacteria [40]. The roughly spherical and subspherical in shape and positive surface charges of the NPs could be a possible explanation for the stabilization of the NPs [24]. The adding of Tween 80 and the surfactants to the CH solution modified the surface equilibrium to viscous forces to allow a smooth particle surface and improved particle sphericity. Also, CHNPs showed a rigid surface with higher fidelity and it is suspected that this may be due to the interfacial interaction between the polymer chain and TPP. Good agreement was found when comparing the results of the TEM images against published data [27,38]. Antibiotic loading affected the PDI and zeta potential values which is increased the size of the NPs. While the Zeta Potential dropped from +27.31 to +24.14 and the PDI switched from 0.17 to 0.26. Loaded antibiotic formulation gave good percentage of entrapment efficiency (61%) and drug loading (42%) at concentration 50 mg/ml. Then the concentration 50 mg/ml of NP re-estimated in different pH values and the best EE% was at pH 5. A low aqueous solubility of the product increased the efficiency of drug loading and this is indicated by a good EE% of RIF in CHNPs. The result showed that the value of EE% increases more rapidly until it reaches a peak value at a concentration of 50 mg/ml, but when the concentration was increased to 100 mg/ml, EE% and DL% reduced, possibly due to a continuous increase in CH concentration contributing to a reduction in encapsulation due to insufficient TPP for crosslinking. Therefore, at a constant CH concentration of 50 mg/mL, as the TPP ratio increased, encapsulation increased due to increased crosslinking, The observations also agree with the results reported by [24,41]. The results of the in vitro release profile of RIF from NPs at different pH values at 37 °C (Figure 3), pH 7 was an appropriate medium to give an efficient release of the antibiotic from the NP. Two-phase kinetics were observed for the RIF-CHNPs with a rapid and massive initial burst release equivalent to almost 90% of RIF in the first 4 hours. Such a burst release of antibiotic allows concentrations above the MIC to be reached quickly. Nanoparticles formulations and

designing consider an important factor influences the success of nanopharmacology which can overcome physiological barriers for controlled delivery of bioactivies to yield therapeutic outcome [42], therefore, which bodes well for preventing the emergence of B. melitensis resistance induced by subinhibitory antibiotic concentrations. while the sustained release pattern could play a supportive role in the maintenance of the drug dose [43]. Despite the fact that studies on nanodrugs targeting intracellular bacteria are still in the early stages, several nanocarriers with the ability to load antimicrobial drugs have been synthesized [15]. In fact, The efficacy of antibiotic treatment for brucellosis in humans depends in part on bacterial resistance. Over the years, Bacteria have learned to avoid the killing mechanisms of not only their host immune system however similarly the antimicrobial agents [44]. In many cases, Efforts have been made to overcome the problem of antibiotics-resistant bacteria by synthesize potent antibacterial. However, these approaches have met with limited success and, in some cases, even greater resistance [45,46]. Rifampicin is an antibiotic that commonly used for both brucellosis and a variety of diseases, as well as tuberculosis [47]. The reduced number of bacteria treated with rifampicin alone is due to the fact that some extracellular bacteria and those that are transferred to other cells over life cycles are eradicated [48]. The potential benefits of CHNPs as antibiotic carriers to increase the effectiveness of antibiotics in treating an intracellular bacterium such as Brucella was investigated in this study. The antibacterial activity of the RIF, CHNPs and RIF-CHNPs against B. melitensis were evaluated by calculation of minimum inhibitory concentrations and thereafter, 1/2 MIC, 1 MIC and 2 MIC were used to determine the zone of growth inhibition following the guidelines for slow-growing bacteria (Haemophilus influenzae) which were used as an alternative. Table 6 showed the compare MIC mean for Rifampin and nanoparticles. As it can be seen there were significant differences between inhibitory zone diameters. The drug nanoparticles killed B. melitensis with a relatively low MIC 0.625 ug/ml. whereas the inhibitory concentrations were about 4 µg/ml and 2.5 µg/ml for the RIF and CHTNPs, respectively table 7. Indicating 6.4 times reduction in MIC for RIF-CHTNPs and 1.6 time reduction for CHTNPs comparing to MIC of antibiotic alone, these findings were compatible with Azhdarzadeh et al., [29]. The zone of growth inhibition results showed a large zone for RIF-CHTNPs when using MIC and 2 MIC comparing to blank nanoparticles and the antibiotic alone. A recent study conducted in Brazil by Barbosa et al. (2015) concluded an emergence bacterial resistance towards rifampicin was detected in 54 out of 147 (36.73%) of the B. abortus. This incidence of reduced susceptibility is higher to rifampicin among isolated B. abortus, this is probably point to the emergence of strains resistant to this drug. Therefore, according to the guideline for interpreting MIC and zone diameters, the Brucella isolate was resistant to rifampin [49]. Previously, a high rate of reduced susceptibility to rifampicin 45.0% (158/355) among B. melitensis isolates with was also observed in Egypt [14]. One concern about these approaches is that related to the bacterial resistant emergent among Brucella spp is that rifampicin is used world widely as a drug of choice to treat brucellosis in humans [4,8,9]. Therefore, an alternative treatment for brucellosis using nanoparticles has become an important means of overcoming resistance with less toxicity [50]. Marianelli et al. [51] described the resistance mechanism of rifampicin in Brucella spp. strains associated with mutations occur in the rpoB gene, which encodes the β-subunit of DNA-dependent RNA polymerase, contain the rifampicin site of action. his antibacterial activity of CNPs is clearly demonstrated the consistency with the previous studies on the antibacterial activity of CNPs against a broad spectrum of gram-negative bacteria [52]. The main practical problem that confronts us to combating Brucella spp. infections is the resistance, particularly B. melitensis. Nanoparticles have recently been viewed as encouraging model to overcome the brucellosis issue. The nanoparticles can be assembled with single or more than one drugs without affecting the delivery regimen and increasing the therapeutic efficacy of the preparations. CHS is a co-polymer composed of N-acetylglucosamine and glucosamine units [53]. Numerous mechanisms describe the antibacterial activities of CHS, a very satisfactory one is that chitosan attached to the bacterial cell membrane, rupturing it and change its permeability, then bounds to DNA, causing stopping of DNA replication and eventually cell death [54]. Another expected mechanism is the selectivity CHS binding behavior as a chelating agent to cellular trace elements, resulting in toxicity and inhibition of bacterial growth [55]. These mechanisms are no doubt a major contributor for bactericidal activity of NPs, The mechanism behind this can be attributed to the larger CHNPs surface area, which can be firmly adsorbed on the bacterial cell membrane to disturb it, CHNPs offer superior affinity within bacterial cell membrane for a quantum size effect which cause the intracellular contents to escape, leading to bacterial death [38]. Based on these results, it can also be assumed that chitosan possesses biosensing properties and can detect multiple communication signals between bacteria. This property causes bacteria to be unable to communicate between bacteria to form quorum sensing in biofilm formation [56]. Also, chitosan has high levels of dissolved oxygen, which interferes with communication between bacteria, mainly through the respiratory oxygen species (ROS) [57] Nanocarriers loaded with antibiotics could reach the intracellular place of the bacteria, where they can release their contents close to their targets, the nanoparticles had exhibited a good activity on bacteria at lower concentration [58]. It well-known that potent antibiotics have been synthesized to solve the issue of antibiotic-resistant bacteria. However, these approaches have met with limited success and, in some cases, even greater resistance [45,46]. These issues stand in the way of obtaining excellent performance of antibiotics As a result, the synthesis of more potent antibiotics to overcome resistant bacteria may fail due to new mechanisms by which bacteria escape the

therapeutic method. The ineffectiveness of antibiotics also leads to taking of high doses and hence cytotoxic effects occur. Rifampicin is a commonly used antibiotic for both brucellosis and a variety of diseases, including tuberculosis [47]. The reduced number of bacteria treated with free rifampicin is due to the eradication of some extracellular bacteria and those that are transferred to other cells over life cycles [48]. In this study, the antibacterial activity of free rifampicin was reduced, as confirmed by relatively small zones of inhibition. In contrast, Rif-CTNPs prevent it from being inactivated due to drug encapsulation and together with its controlled release over time act on bacteria more efficiently than free rifampicin.

5. Conclusion

Loaded antibiotic formulation gave good percentage of entrapment efficiency and drug loading at concentration 50 mg/ml. Then the EE % at concentration 50 mg/ml was re-estimated in different pH values and the best EE% was at pH 5. It was studied the antibacterial activity of drug nanocarrier against B. melitensis and found that it had a relatively low MIC 0.625 ug/ml. whereas the inhibitory concentrations were about 4 µg/ml and 2.5 µg/ml for the RIF and CHTNPs, respectively. Indicating there were 6.4 times reduction in MIC for RIF-CHTNPs and 1.6 time reduction for CHTNPs comparing to MIC of antibiotic alone.

Conflict Of Interest

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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References

1. Franc KA, Krecek RC, Häsler BN, Arenas-Gamboa AM. Brucellosis remains a neglected disease in the developing world: a call for interdisciplinary action. BMC Public Health. BioMed Central; 2018;18:1–9.
2. Shareef JM. A review of serological investigations of brucellosis among farm animals and humans in northern provinces of Iraq (1974–2004). J Vet Med Ser B. Wiley Online Library; 2006;53:38–40.
3. Pappas G, Papadimitriou P, Akritidis N, Christou L, Tsianos E V. The new global map of human brucellosis. Lancet Infect Dis. Elsevier; 2006;6:91–9.
4. Corbel MJ. Brucellosis in humans and animals. World Health Organization; 2006.
5. Şahin M, Unver A, Otlu S. Isolation and biotyping of Brucella melitensis from aborted sheep fetuses in Turkey. Bull Vet Inst Pulawy. 2008;52:59–62.
6. Köse Ş, SENGER SS, Akkoçlu G, Kuzucu L, Ulu Y, Ersan G, et al. Clinical manifestations, complications, and treatment of brucellosis: evaluation of 72 cases. Turkish J Med Sci. 2014;44:220–3.
7. Hotez PJ, Savioli L, Fenwick A. Neglected tropical diseases of the Middle East and North Africa: review of their prevalence, distribution, and opportunities for control. PLoS Negl Trop Dis. Public Library of Science San Francisco, USA; 2012;6:e1475.
8. Ariza J, Bosilkovski M, Cascio A, Colmenero JD, Corbel MJ, Falagas ME, et al. Perspectives for the treatment of brucellosis in the 21st century: the Ioannina recommendations. PLOS Med. Public Library of Science San Francisco, USA; 2007;4:e317.
9. Skalsky K, Yahav D, Bishara J, Pitlik S, Leibovici L, Paul M. Treatment of human brucellosis: systematic review and meta-analysis of randomised controlled trials. Bmj. British Medical Journal Publishing Group; 2008;336:701–4.
10. Vekemans J, Hasso-Agopsowicz M, Kang G, Hausdorff WP, Fiore A, Tayler E, et al. Leveraging vaccines to reduce antibiotic use and prevent antimicrobial resistance: A World Health Organization action framework. Clin Infect Dis. Oxford University Press US; 2021;73:e1011–7.
11. Organization WH. Antimicrobial resistance and primary health care. World Health Organization; 2018.
12. El Miedany YM, El Gaafary M, Baddour M, Ahmed I. Human brucellosis: do we need to revise our therapeutic policy? J Rheumatol. The Journal of Rheumatology; 2003;30:2666–72.
13. Roland PS, Wright CG. Topical aminoglycoside cochlear ototoxicity. Ototoxic 1st ed London BC Decker Inc. 2004;114–9.
14. Abdel-Maksoud M, House B, Wasfy M, Abdel-Rahman B, Pimentel G, Roushdy G, et al. In vitro antibiotic susceptibility testing of Brucella isolates from Egypt between 1999 and 2007 and evidence of probable rifampin resistance. Ann Clin Microbiol Antimicrob. BioMed Central; 2012;11:1–4.
15. Abed N, Couvreur P. Nanocarriers for antibiotics: A promising solution to treat intracellular bacterial infections. Int J Antimicrob Agents. Elsevier; 2014;43:485–96.
16. Usković V, Desai TA. Simultaneous bactericidal and osteogenic effect of nanoparticulate calcium phosphate powders loaded with clindamycin on osteoblasts infected with Staphylococcus aureus. Mater Sci Eng C. Elsevier; 2014;37:210–22.
17. Huh AJ, Kwon YJ. "Nanoantibiotics": a new paradigm for treating infectious diseases using nanomaterials in the antibiotics resistant era. J Control release. Elsevier; 2011;156:128–45.
18. Zakeri-Milani P, Loveymi BD, Jelvehgari M, Valizadeh H. The characteristics and improved intestinal permeability of vancomycin PLGA-nanoparticles as colloidal drug delivery system. Colloids Surfaces B Biointerfaces. Elsevier; 2013;103:174–81.
19. Kalhapure RS, Mocktar C, Sikwal DR, Sonawane SJ, Kathiravan MK, Skelton A, et al. Ion pairing with linoleic acid simultaneously enhances encapsulation efficiency and antibacterial activity of vancomycin in solid lipid nanoparticles. Colloids Surfaces B Biointerfaces. Elsevier; 2014;117:303–11.
20. Mohammed NA. The Prevalence of Brucellosis for Exported Sheep In Alkadaru Quarantine Khartoum State–Sudan. Sudan University of Science and Technology; 2018.
21. Al Dahouk S, Tomaso H, Nöckler K, Neubauer H, Frangoulidis D. Laboratory-based diagnosis of brucellosis--a review of the literature. Part I: Techniques for direct detection and identification of Brucella spp. Clin Lab. 2003;49:487–505.
22. Quinn PJ, Carter ME, Markey B, Carter GR. Clinical veterinary microbiology. Mosby. Elsevier, London, Sec. 2004;2:130.
23. Ebrahimi A, kanluye Milan JS, Mahzoonieh MR, Khaksar K. Shedding rates and Seroprevalence of Brucella melitensis in lactating Goats of Shahrekord, Iran. Jundishapur J Microbiol. Brieflands; 2014;7.
24. Ashvini HM, Balla A, Mutta SK. Clarithromycin-loaded chitosan nanoparticles: preparation, characterisation and antibacterial activity on Streptococcus pneumonia. Indian J Pharm Sci. OMICS International; 2019;81:302–8.
25. Chow SF, Sun CC, Chow AHL. Assessment of the relative performance of a confined impinging jets mixer and a multi-inlet vortex mixer for curcumin nanoparticle production. Eur J Pharm Biopharm. Elsevier; 2014;88:462–71.
26. Weng J, Tong HHY, Chow SF. In vitro release study of the polymeric drug nanoparticles: development and validation of a novel method. Pharmaceutics. MDPI; 2020;12:732.
27. Shende P, Yadava SK, Patil PS. Development and characterization of chitosan nanoparticles containing erthromycin estolate. Int J Pharm Appl. 2014;5:1–7.
28. Turkmani A, Ioannidis A, Christidou A, Psaroulaki A, Loukaides F,

Tselentis Y. In vitro susceptibilities of Brucella melitensis isolates to eleven antibiotics. Ann Clin Microbiol Antimicrob. Springer; 2006;5:1–4.

29. Azhdarzadeh M, Lotfipour F, Zakeri-Milani P, Mohammadi G, Valizadeh H. Anti-bacterial performance of azithromycin nanoparticles as colloidal drug delivery system against different gram-negative and gram-positive bacteria. Adv Pharm Bull. Tabriz University of Medical Sciences; 2012;2:17.
30. Cary N. Statistical Analysis System, User's Guide. Statistical. Version 9. SAS. Inst. Inc USA. 2012;
31. Mousa ST, Saleh SS, Mohammed NT. Prevalence of Brucellosis antl Salmonellosis in goats. Res J Pharm Technol. A&V Publications; 2019;12:3690–2.
32. Prevention C for DC and. Emergency preparedness and response: bioterrorism agents/diseases. US Centers Dis Control Prev Atlanta, GA http//www bt cdc gov/agent/agentlist-category asp[Google Sch. 2014;
33. Berger S. Brucellosis: Global Status. GIDEON Informatics. Inc, Los Ángeles. 2019;
34. Divya L, Raju MB, Raut SY. Chitosan-based micro and nanoparticles: A promising system for drug delivery. Res J Pharm Technol. Research Journal of Pharmacy and Technology; 2014;7:1463–71.
35. Sowjanya NT, Dhivya R, Meenakshi K, Vedhanayakisri KA. Potential applications of chitosan nanoparticles as novel support in enzyme immobilization. Res J Eng Technol. A&V Publications; 2013;4:14.
36. Fan W, Yan W, Xu Z, Ni H. Formation mechanism of monodisperse, low molecular weight chitosan nanoparticles by ionic gelation technique. Colloids surfaces B Biointerfaces. Elsevier; 2012;90:21–7.
37. Arafa MG, Mousa HA, Afifi NN. Preparation of PLGA-chitosan based nanocarriers for enhancing antibacterial effect of ciprofloxacin in root canal infection. Drug Deliv. Taylor & Francis; 2020;27:26–39.
38. El-Assal MI, El-Menofy NG. Chitosan nanoparticles as drug delivery system for cephalexin and its antimicrobial activity against multiidrug resistent bacteria. Int J Pharm Pharm Sci. 2019;
39. Dhamane SP, Jagdale SC. Development of Rifampicin loaded Chitosan nanoparticles by 32 full Factorial design. Res J Pharm Technol. 2020;13:2545–50.
40. Wang L, Hu C, Shao L. The antimicrobial activity of nanoparticles: present situation and prospects for the future. Int J Nanomedicine. Dove Press; 2017;12:1227.
41. Shinde AJ, More HN. Lovastatin loaded chitosan nanoparticles: preparation, evaluation and in vitro release studies. Res J Pharm Technol. Research Journal of Pharmacy and Technology; 2011;4:1869–76.
42. Balaji EV, Selvan A. Nanopharmacology: A novel approach in therapeutics. Asian J Res Pharm Sci. A & V Publications; 2019;9:9–16.
43. Lebeaux D, Ghigo J-M, Beloin C. Biofilm-related infections: bridging the gap between clinical management and fundamental aspects of recalcitrance toward antibiotics. Microbiol Mol Biol Rev. Am Soc Microbiol; 2014;78:510–43.
44. Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, et al. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. Clin Infect Dis. The University of Chicago Press; 2009;48:1–12.
45. Gold HS, Moellering Jr RC. Antimicrobial-drug resistance. N Engl J Med. Mass Medical Soc; 1996;335:1445–53.
46. Walsh C. Molecular mechanisms that confer antibacterial drug resistance. Nature. Nature Publishing Group; 2000;406:775–81.
47. Patel BK, Parikh RH, Aboti PS. Development of oral sustained release rifampicin loaded chitosan nanoparticles by design of experiment. J Drug Deliv. Hindawi; 2013;2013.
48. Jain-Gupta N, Pothayee N, Pothayee N, Tyler R, Caudell DL, Balasubramaniam S, et al. Efficacies of gentamicin-loaded magnetite block ionomer complexes against chronic Brucella melitensis infection. J nanoparticle Res. Springer; 2013;15:1–13.
49. Barbosa Pauletti R, Reinato Stynen AP, Pinto da Silva Mol J, Seles Dorneles EM, Alves TM, de Sousa Moura Souto M, et al. Reduced susceptibility to Rifampicin and resistance to multiple antimicrobial agents among Brucella abortus isolates from cattle in Brazil. PLoS One. Public Library of Science San Francisco, CA USA; 2015;10:e0132532.
50. Kumari GD, Raksha G, Deepak K, Anjana G, Mary CS. A Review on Chitosan Nanoparticle as a Drug delivery system. Asian J Pharm Res. A & V Publications; 2020;10:299–306.
51. Marianelli C, Ciuchini F, Tarantino M, Pasquali P, Adone R. Genetic bases of the rifampin resistance phenotype in Brucella spp. J Clin Microbiol. Am Soc Microbiol; 2004;42:5439–43.
52. Divya K, Vijayan S, George TK, Jisha MS. Antimicrobial properties of chitosan nanoparticles: Mode of action and factors affecting activity. Fibers Polym. Springer; 2017;18:221–30.
53. Zhou K, Li C, Chen D, Pan Y, Tao Y, Qu W, et al. A review on nanosystems as an effective approach against infections of Staphylococcus aureus. Int J Nanomedicine. Dove Press; 2018;13:7333.
54. Monack DM, Mueller A, Falkow S. Persistent bacterial infections: the interface of the pathogen and the host immune system. Nat Rev Microbiol. Nature Publishing Group; 2004;2:747–65.
55. Cuero RG, Osuji G, Washington A. N-carboxymethylchitosan inhibition of aflatoxin production: role of zinc. Biotechnol Lett. Springer; 1991;13:441–4.
56. Abbas HA, Serry FM, EL-Masry EM. Biofilms: The microbial castle of resistance. Res J Pharm Technol. A&V Publications; 2013;6:2.
57. Saha D, Tamrakar A. Xenobiotics, oxidative stress, free radicals vs. antioxidants: dance of death to heaven's life. Asian J Res Pharm Sci. 2011;1:36–8.
58. Ramesh T, Thangaraj M, Kumaran R, Annadurai D, Subramanian J, Purushothaman S, et al. Synthesis, characterization and efficacy of antibiotic coated chitosan nanoparticles on human pathogens. Res J Pharm Technol. Research Journal of Pharmacy and Technology; 2020;13:3903–8.