Egyptian Journal of Veterinary Sciences

https://ejvs.journals.ekb.eg/

In Vitro Efficacy of Chitosan Nanoparticles against *Staphylococcus aureus* and *Klebsiella* Spp. Isolates From Bovine Mastitis Milk: Addressing Antibiotic Resistance and Exploring Alternative Treatments

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Abstract

THIS study evaluates the potential antimicrobial properties of chitosan nanoparticles (CNPs) against extended-spectrum beta-lactamases (ESBLs)-producing bacteria (*S. aureus* and *K. pneumoniae*) isolated from mastitic milk samples taken from bovine livestock within the El-Menoufia government. One hundred milk samples were tested using the California Mastitis Test, and the isolated bacteria were identified using traditional and molecular techniques. A scanning electron microscope, a Fourier transform infrared spectrometer (FTIR), and X-ray diffraction were utilized to characterize CNPs. The results show that *Klebsiella* spp. were found in 48% of clinical mastitis cows and 24% of buffaloes, and in 36% and 20% of subclinical cases, respectively. *Staphylococcus* spp. were found in 56% of clinical mastitis cows and 32% of buffaloes, and in 44% and 24% of subclinical cases. Antibiogram analysis showed *Klebsiella* spp. were most sensitive to quinolones (53%) and least to tetracycline (34%),while *Staphylococcus* spp. showed the highest sensitivity to ofloxacin (43.5%) and ampicillin/sulbactam (41.1%). The distribution of bla_{TEM} gene was observed at 41.8%, followed by bla_{SHV} gene and bla_{CTX-M} gene were 34.8% and 32.5% respectively. *K. pneumoniae* and *S. aureus* showed the highest multidrug resistance against the tested antibiotic discs. The CNPs were tested in vitro against isolated bacteria and exhibited inhibition zones ranging from 6 to 12 mm. Transfer electron microscopy (TEM) images showed that CNPs significantly altered cell morphology, leading to bacterial death. Therefore, nanoparticles could be a promising solution for controlling, preventing, and treating bovine mastitis.

Keywords: Bovine Mastitis, ESBL, *K. pneumoniae*, Antimicrobial Susceptibility, CNPs.

Introduction

Mastitis is a devastating infection that causes large financial losses as a result of the reduction in milk production, high culling rates, veterinary expenses, and occasionally even death in complex or untreated cases [1]. Mastitis costs reach approximately $147 per year per cow; these costs are estimated to be 11% to 18% of the gross margin for each cow annually [2]. Most dairy producers rely on subclinical early identification to enhance recovery prospects and minimize production losses. Several opportunistic pathogens have been associated with mastitis, including *Streptococcus* spp., *Staphylococcus* spp., *K. pneumoniae*, *E. coli*, and *Pseudomonas aeruginosa* [3-4]. Among the Gram-negative rods, *K. pneumoniae* is the most common bacteria causing mastitis. It is most frequently found in organic materials, including manure, damp soils, and animal bedding and is recognized as an environmental mastitis pathogen [5]. The udder tissue is thoroughly infiltrated with the bacterium, which causes damage to the glandular tissue. According to Pinzon Sanchez et al. [6], this infection becomes a chronic condition that limits milk production. *S. aureus* is a major pathogen in livestock, causing both subclinical and clinical intramammary infections (IMIs). Subclinical IMIs often go undetected and respond poorly to antibiotic therapy [7]. Certain strains, as noted by Anderson et al. [8] may act as sources, contributing

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(Received 31 August 2025, accepted 28 October 2025)
DOI: 10.21608/ejvs.2025.419155.3097
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to recurrent outbreaks in livestock populations. Antibiotics are typically employed to alleviate mastitis [9]. However, the uncontrolled and inappropriate use of antibiotics exposes animals and humans to multidrug resistance as well as residues in milk [10]. To date, the emergence of antimicrobial resistance has been attributed to conventional antibiotic treatment [11]. Numerous cases of antibiotic resistance have been reported in *K. pneumoniae*, including resistance to commonly used antibiotics such as penicillin, amoxicillin, amikacin, tetracycline, gentamicin, and erythromycin, the issue of antimicrobial drug residues in dairy environments is gaining increasing attention [12]. *K. pneumoniae* isolates exhibit resistance to various antibiotic classes, particularly those associated with extended-spectrum β-lactamase (ESBL) genes such as *blaCTX-M, blaSHV*, and *blaTEM* [13].

Nanoparticles have emerged as promising antibacterial agents in veterinary medicine [14]. Akmaz et al. [15] reported that chitosan nanoparticles (CNPs) effectively suppress a wide range of pathogens, including both Gram-positive and Gram-negative bacteria, as well as fungi. Notably, bacteria have not developed resistance to chitosan, which has contributed to its growing use as an antibacterial agent [16]. With the rise of green chemistry, CNPs have gained popularity as superior antibacterial agents [17]. According to Goy et al. [18], the antibacterial properties of chitosan make it an ideal candidate for oral antibiotic delivery, particularly for targeting Gram-negative organisms. This approach enhances the bioavailability and efficacy of antibiotics in treating infections [19]. The global challenge posed by extended-spectrum β-lactamase (ESBL)-producing bacteria continues to intensify.

There is limited scientific evidence regarding the relationship between antibiotic composition and the rate of extended-spectrum β-lactamase (ESBL) production in *K. pneumoniae* and *S. aureus* strains isolated from bovine mastitis [20]. Therefore, the aim of this study is to investigate the genetic diversity and antimicrobial resistance profiles of *K. pneumoniae* and *S. aureus* isolates from bovine mastitis cases, with a specific focus on ESBL production. Additionally, the study explores the potential association between resistance patterns and antibiotic usage, as well as the efficacy of alternative treatments such as chitosan-based nanoparticles.

Material and Methods

Sample collection

Between October 2023 and June 2024, a total of 100 milk samples were aseptically collected from private bovine farms located in El-Menoufia Governorate, Egypt. The samples were obtained from both cows and buffaloes during multiple field visits. Each sample was screened using the California Mastitis Test (CMT) to assess the presence and severity of mastitis. Based on the CMT results and clinical examination, the samples were categorized into two groups: subclinical mastitis (n = 50) and clinical mastitis (n = 50). The udder was physically examined, and the amount of somatic cells in the milk was estimated as part of the veterinarian's standardized clinical mastitis diagnosis. Milk with subclinical mastitis were selected from animals who did not have positive CMT findings and no clinical symptoms. Ten milliliters of milk were gathered aseptically from physically affected quarters in sterile containers and taken to the laboratory in a cool icebox for further examination. After sampling, bacterial analyses were conducted within 24 hours.

Bacterialisolation and identification of Klebsiella s pp. and Staphylococcus spp.

A 0.01 mL of milk from each sample was cultivated on a variety of bacteriological media, including (MacConkey agar, EMB agar media, Paired Barker agar media, and Mannitol salt agar). The samples were subsequently incubated at 37°C for 48 hours. The putative colonies were examined for morphological characteristics, such as colony size, shape, color, pigment production, colony texture (smooth or rough), and metabolic activity, on MacConkey agar (lactose fermenter or non-lactose fermenter). Gram-stained bacterial specimens were prepared from the colonies and subsequently examined under a microscope. Biochemical identification of *S. aureus* and *K. pneumoniae* was performed following the protocols described by Quinn et al. [21]. *S. aureus* is characterized by its Gram-positive cocci morphology, catalase positivity, and coagulase positivity. It also ferments mannitol and produces golden-yellow colonies on mannitol salt agar. In contrast, *K. pneumoniae* is a Gram-negative, non-motile bacillus, oxidase-negative, urease-positive, and indole-negative, and typically shows positive results for citrate utilization and Voges-Proskauer tests. All culture media used for biochemical testing were obtained from Oxoid Ltd., Basingstoke, UK.

Antibiogram analysis

Antibiotic susceptibility testing was performed using the disk diffusion method as described by Gloria et al. [22]. The most commonly isolated bacteria *K. pneumoniae* and *S. aureus* were tested for sensitivity to selected antibiotics. Commercial antibiotic discs were obtained from Hi Media Laboratories Pvt. Ltd. (India) and included ofloxacin (OFX, 5 µg), clindamycin (DA, 2 µg), ceftriaxone (CRD), Ampicillin/Sulbactam (SAM, 20 µg), and tetracycline (TE, 20 µg), all supplied by Oxoid. Bacterial suspensions were standardized to a 0.5 McFarland turbidity standard, equivalent to approximately 1.5×10^8 CFU/mL. Results were

interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [23] and classified as sensitive, intermediate, or resistant.

Molecular identification and ESBL resistance genes detection

Biochemically confirmed isolates of *Klebsiella* and *Staphylococcus* species were submitted to the Central Laboratory for Veterinary Quality Control on Poultry Production (CLQP-PCR Unit), Animal Health Research Institute, for molecular identification. Molecular detection was performed using PCR targeting the *16S rRNA* gene for *S. aureus* and the *16S–23S* internal transcribed spacer (ITS) region for *Klebsiella* species, including *K. pneumoniae* and *K. oxytoca*. Multiplex PCR was employed to screen these isolates for extended-spectrum β-lactamase (ESBL)-encoding resistance genes. Specific oligonucleotide primers targeting *blaTEM*, *blaSHV*, and *blaCTX-M* were used (Table 1). The PCR reaction mixture (25 µL total volume) included template DNA, EmeraldAmp MAX PCR master mix, primers, and nuclease-free water. Each run included a negative control and a positive control using Escherichia coli ATCC 25922. PCR products were electrophoresed on a 1.5% agarose gel, visualized under a UV transilluminator, and compared against a 100-bp DNA ladder (Jena Bioscience GmbH, Jena, Germany) to determine amplicon sizes.

Phylogenetic analysis:

Isolates of biochemically confirmed *K. pneumoniae* and *S. aureus* were subjected to molecular sequencing at the Animal Health Research Institute, Dokki, Giza Governorate. Genomic DNA was extracted from overnight bacterial cultures and amplified using universal primers targeting the highly conserved *16S rRNA* gene: forward (5′-AGAGTTTGATCMTGGCTCAG-3′) and reverse (5′-TACGGYTACCTTGTTACGACTT-3′) (Metabion, Germany), following the protocol described by Lagacé et al. [30]. PCR amplification was performed using a T3 Thermal Cycler (Biometra, Germany). Amplified products were visualized via agarose gel electrophoresis (1.5%) under UV illumination. Unassembled raw sequences were analyzed using BLAST, and nucleotide sequences were identified through GenBank. A phylogenetic tree was constructed using MEGA X.11 software, and bootstrap analysis with 1,000 replicates was conducted to assess the confidence levels of the phylogenetic relationships [31].

Bactercidal efficacy of chitosan against K. pneumoniae and S. aureus isolates

Preparation of chitosan Nanoparticles (CNPs): Chitosan, with a low molecular weight and 90% deacetylation degree, was obtained from Sigma-Aldrich. A chitosan solution was created by dissolving it in acetic acid and dissolved in sodium tripolyphosphate (TPP). The solution was mixed with TPP solution, resulting in the spontaneous production of chitosan-TPP nanoparticles. The nanoparticle suspension was stirred for 60 minutes before analysis [32]. CNPs were prepared by mixing chitosan with sterile normal saline, stirring until a consistent suspension was achieved, and stored at 4°C until consistent colloidal suspension with a final concentration of 1000µg/mL was achieved.

Characterization of CNPs: A suspension of CNPs was submitted to El Azhar Scientific Laboratory, Egypt, for physicochemical characterization. Fourier Transform Infrared Spectroscopy (FTIR) was employed to identify the functional groups present in the nanoparticles, while X-ray Diffraction (XRD) analysis was conducted using a Philips PW 1730 diffractometer to determine their crystalline structure. Scanning Electron Microscopy (SEM) was used to examine the size and surface morphology of the CNPs, utilizing a MIRA3 TESCAN microscope operated at an accelerating voltage of 15 kV. These analyses provided detailed insights into the structural and morphological properties of the synthesized nanoparticles.

Determination of Minimum Inhibitory Concentrations of CNPs: The MIC of CNPs was determined against *K. pneumoniae* and *S. aureus*, after overnight incubation in brain heart infusion broth which was adjusted to 0.5 McFarland turbidity. The tube dilution method was used to determine the minimum inhibitory concentration (MIC) of synthesized CNPs against various bacterial isolates in culture broth. This was achieved by preparing varying dilutions (10, 5, 2.5, 1.25, and 0.6 mg/ml) from the stock nanoparticle. Then, 50µL of 0.5 McFarland suspensions of *K. pneumoniae* and *S. aureus* were added to individual tubes in two distinct groups. After that, the tubes were incubated at 37°C for 24 hours. The MIC is the lowest concentration of CNPs that terminated visible bacterial growth after 24 hours of incubation. The Minimum Bactericidal Concentration (MBC) of synthesized CNPs was determined using the standard tube dilution method. Following MIC testing, 50 µL from each tube showing no visible bacterial growth or turbidity was inoculated onto Mueller-Hinton agar plates. These plates were incubated at 37°C for 24 hours. The presence or absence of bacterial colonies was then assessed. The MBC was defined as the lowest concentration of CNPs that resulted in complete bacterial killing, indicated by the absence of growth, corresponding to a 99.9% reduction in the initial bacterial population (33).

Well diffusion method:

A well-diffusion assay was conducted to evaluate the antibacterial activity of CNPs against *K.*

pneumoniae and *S.aureus* strains (34). Bacterial suspensions were standardized to 0.5 McFarland turbidity and uniformly spread onto Mueller-Hinton Agar plates using sterile cotton swabs. Wells of 6 mm diameter were then punched into the agar, and 50 µL of CNPs at varying concentrations (10, 5, 2.5, 1.25, and 0.62 mg/mL) were added to each well. The plates were incubated at 37°C for 24 hours. Following incubation, the diameters of the zones of inhibition surrounding each well were measured in millimeters to assess the antibacterial efficacy of the nanoparticles.

In vitro bactericidal activity of CNPs against K. pneumoniae and S. aureus isolates:

The two bacterial isolates were subcultured in separate falcon tubes filled with tryptic soy broth using 20 µg/mL of CNPs. After being incubated for 24 hours at 30°C, the supernatants were extracted by centrifuging for 15 minutes at 3000 rpm. At the EM unit of Assiut University in Egypt, the precipitate was collected for TEM (JEOL JEM-2100, Japan) according to Kinner et al. (35).

Statistical Analysis:

The prevalence rate of *Klebsiella* and *Staphylococcus* spp. in bovine animals was determined using non-parametric tests using SPSS, with a probability level of $p \leq 0.05$.

Results

As shown in Table 2, bacteriological analysis of milk samples obtained from bovine animals in El-Menoufia Governorate revealed the presence of *Klebsiella* and *Staphylococcus* spp. The prevalence of *Klebsiella* was 48% in cows and 24% in buffaloes with clinical mastitis, and 36% and 20% in subclinical mastitis cases, respectively with no significant difference in prevalence between species or mastitis types ($p > 0.05$). Similarly, *Staphylococcus* spp. were isolated in 56% of cows and 32% of buffaloes with clinical mastitis, and in 44% and 24% of subclinical cases, respectively, also with no statistically significant difference ($p > 0.05$). Overall, the chi-square test indicated no significant variation in the total isolation rates of *Klebsiella* and *Staphylococcus* spp. between clinical and subclinical mastitis cases.

According to the antibiogram results, quinolones exhibited the highest sensitivity against the 32 *Klebsiella* spp. isolates recovered from cows and buffaloes, with a susceptibility rate of 53%. In contrast, tetracycline showed lower efficacy, with only 34% of isolates being sensitive. Table 3 presents the antimicrobial susceptibility profiles of 39 *Staphylococcus* spp. isolates. Among these, the highest sensitivity was observed for ofloxacin (43.5%), followed by ampicillin/sulbactam (41.1%), tetracycline (38.5%), and ceftriaxone (35.6%). Despite moderate sensitivity, a notable proportion of *Staphylococcus* isolates also exhibited resistance to tetracycline (38.5%), indicating variable responses to this antibiotic.

The molecular confirmation of *Klebsiella* spp. and *S. aureus* from collected milk samples revealed a total of 14 samples tested positive for *K. pneumoniae*, 7 samples for *K. oxytoca*, and 22 samples for *S. aureus*. These results stressed the presence of multiple pathogenic species associated with mastitis in bovine populations. The prevalence and variance of resistance mediated by ESBL in a range of *Klebsiella* and *S. aureus* isolates, as well as the antibiogram analysis obtained from a variety of animal origins (cow and buffalo), were documented in Table 4. bla_{SHV} was detected in 15 of the 43 molecularly identified isolates (34.8%), however, the bla_{TEM} and $bla_{CTX\text{-}M}$ genes were reported in 18 of the 43 isolates (41.8%) and 14 of the 43 isolates (32.5%), respectively. The statistical analysis did not reveal any significant differences between the individual genes bla_{TEM} ($X^2 = 8.57$, $p = 0.072$) and bla_{SHV} ($X^2 = 1.08$, $p = 0.896$). However, there was a significant difference in $bla_{CTX\text{-}M}$ across the isolates ($X^2 = 8.14$, $p = .043$). our results show the distribution of ESBL-producing genes in different animal species. The distribution of bla_{TEM} was 41.8% followed by bla_{SHV} (34.8 %), and $bla_{CTX\text{-}M}$ (32.5 %).

Two representative bacterial isolates *K. pneumoniae* and *S. aureus* were subjected to PCR amplification and *16S rRNA* gene sequencing. The resulting *16S rRNA* gene sequences were submitted to the GenBank database and assigned accession numbers PP430293 (*K. pneumoniae*) and PP430371 (*S. aureus*) (Figure 1A). A neighbor-joining phylogenetic tree was constructed for the *K. pneumoniae* isolate based on its *16S rRNA* sequence (Figure 1B). Additionally, the sequence divergence of the *S. aureus* isolate was analyzed using Lasergene software, and the results are presented in Figures 2A and 2B.

The FTIR spectra of CNPs (Fig. 3A) exhibited distinctive peaks, such as the symmetric C-H stretching at 2850 cm− 1 and the asymmetric C-H stretching at 2912 cm− 1. The band occurring at approximately 1412 cm− 1 confirmed the presence of the C-N elongation of amide III. Fig. 3.B. illustrates that the XRD pattern of CNPs exhibited two prominent peaks at $2\theta = 11.6°$ and $2\theta = 20.09°$, which suggest the presence of a crystalline structure. The synthesized CNPs were analyzed using SEM to determine their size and morphology. The NPs presented a spherical appearance and a relatively homogeneous morphology, as indicated by the SEM images (Fig. 3.C).

CNP nanoparticles exhibited a minimum inhibitory concentration (MIC) of 5 mg/mL against *K. pneumoniae* and 2.5 mg/mL against *S. aureus*. The minimum bactericidal concentrations (MBCs)

were 10 mg/mL for *K. pneumoniae* and 5 mg/mL for *S. aureus* (Table 5). Colony-forming units (CFU) of *K. pneumoniae* and *S. aureus* were quantified after overnight incubation with varying concentrations of CS nanoparticles.

The antibacterial efficacy of CNPs against various isolates (*K. pneumoniae* and *S. aureus*) was evaluated using the agar well diffusion test. CNPs exhibited potent inhibitory effects, resulting in a growth inhibition zone that extended from 6 to 12 mm, as indicated by the results. The visible clear zone formed by CNPs against *K. pneumoniae* and *S. aureus* strains is depicted in Fig. 4A - B. Furthermore, Figure 4. C illustrates the diameters of the inhibition zones that encircle the wells. Transmission Electron Microscopy (TEM) analysis was conducted to investigate the ultrastructural alterations induced by CNPs in *K. pneumoniae* and *S. aureus* strains. The examination focused on morphological changes resulting from nanoparticle exposure, providing insights into the bactericidal effects of CNPs at the cellular level. Fig. 5 A-B illustrates the untreated *K. pneumoniae* strain's normal cell morphology and intact cell membrane. Following exposure to CNPs, the cell morphology underwent substantial changes, and the cell size increased significantly in comparison to the untreated cells. (C-D). The intact *S. aureus* bacteria was depicted in Fig. 6 A-B, and it appeared to be in its typical shape. After being exposed to CNPs, the CNPs were dispersed throughout the bacterial cells and adsorb into the cell wall, resulting in a change in the permeability of the cell wall, Intracellular cytosolic components leaked. This resulted in the loss of architecture and the death of bacterial cells (Fig. 6 C-D).

Discussion

Despite its economic impact and implications for animal health, bovine mastitis remains a significant challenge for the global dairy industry (36). Misuse and overuse of antibiotics contribute to the development of antimicrobial resistance through a variety of mechanisms, including enzymatic drug modification, alteration of target sites, activation of efflux pumps, metabolic adaptation, and acquisition of resistance genes. ESBL-producing *Enterobacteriaceae* have been isolated from mastitic milk, bulk-tank milk, and even healthy livestock in several countries [37]. A variety of alternative therapeutic approaches are being investigated to mitigate the limitations of conventional antibiotics, such as the use of chitosan, a biodegradable polymer widely used to deliver drugs [38].

In the present study, *S. aureus* was detected in 39% of milk samples collected from cows and buffaloes with clinical and subclinical mastitis. This prevalence aligns with the 32.95% reported by Poli et al. [39], and lower than the 94% reported by Kamal et al. [40], indicating regional and methodological variability in detection rates. *Klebsiella* species were isolated from cows and buffaloes in 32% of the examined cases, indicating a notable prevalence in dairy animals. These findings are consistent with those reported by Wu et al. [41], who identified *K. pneumoniae* in 26.94% (239/887) of milk samples using biochemical methods. In contrast, Yang et al. [42] reported a lower detection rate, with *Klebsiella* spp. found in only 9.78% (104/1,006) of samples. Similarly, Tsuka et al. [43] observed *K. pneumoniae* in 7.83% (65/830) of samples collected from farms in Jiangsu Province, they also demonstrated a positive correlation between the presence of *K. pneumoniae* and California Mastitis Test (CMT) scores, suggesting a potential link between bacterial infection and subclinical mastitis. *K. pneumoniae* infections are associated with significant economic losses in the dairy industry due to reduced milk production and increased veterinary costs. According to Haxhiaj et al. [44] and Fuenzalida and Ruegg [5], these infections are characterized by severe clinical manifestations and low therapeutic success rates with antibiotic treatment, highlighting the challenges in managing *K. pneumoniae*-related mastitis.

Antibiogram analysis in the current study revealed that *S. aureus* isolates exhibited high sensitivity to ofloxacin (70%) and ceftriaxone (60%). However, they showed marked resistance to tetracycline (80%), clindamycin (70%), and ampicillin/sulbactam (70%). These findings align with those reported by Narmeen et al. [45], who also observed multidrug resistance in *S. aureus* isolates. Similarly, Wang et al. [46] documented a tetracycline resistance rate of 38.2% in *S. aureus*, supporting the variability in resistance patterns across regions and strains. In contrast, *Klebsiella* isolates demonstrated high susceptibility to ceftriaxone (70%) and moderate susceptibility to clindamycin (50%), while showing strong resistance to tetracycline (80%) and ampicillin/sulbactam (70%). These results are consistent with the findings of Wu et al. [41], who reported complete resistance of *Klebsiella* to ampicillin and a high resistance rate to tetracycline (47.28%).

Among the 43 molecularly identified isolates, the *blaTEM* gene was detected in 18 isolates (41.8%), *blaCTX-M* in 14 isolates (32.5%), and *blaSHV* in 15 isolates (34.8%). Statistical analysis revealed no significant differences in the distribution of *blaTEM* ($\chi^2 = 8.57$, $p = 0.072$) and *blaSHV* ($\chi^2 = 1.08$, $p = 0.896$) genes. However, a statistically significant variation was observed in the presence of the *blaCTX-M* gene ($\chi^2 = 8.14$, $p = 0.043$), suggesting its potentially higher epidemiological relevance in the studied population. The findings of this study are consistent with those reported by Yang et al. [42], who identified the β-lactamase genes *blaSHV* and *blaCTX-M* in both *S. aureus* and *Klebsiella* spp.,

supporting the widespread occurrence of these resistance determinants among pathogenic bacteria. The prevalence of β-lactamase genes observed in this study was comparatively lower than that reported in several previous investigations conducted in other regions. This may be attributed to regional differences in antibiotic usage, farm management practices, and environmental conditions that influence bacterial resistance development [47]. In India, Kaza et al. [48] reported the presence of extended-spectrum β-lactamase (ESBL) genes in all *K. pneumoniae* isolates examined.

Phylogenetic analysis using maximum likelihood methods revealed that the *K. pneumoniae* isolate clustered with other Gram-negative bacteria, showing 98.4–100% sequence identity with strains such as *K. pneumoniae* strain KP-MILK-23 (GenBank accession:CP141987). Similarly, the *S. aureus* isolate from cattle demonstrated close genetic relatedness to Gram-positive bacteria from both animal and human sources, including *S. aureus* strain SA-MILK-17 (GenBank accession: KU922499), with a sequence identity range of 98.4–100%. This clustering pattern raises concerns about the potential zoonotic transmission of multidrug-resistant (MDR) pathogens through the dairy production chain.

This study assessed the antibacterial efficacy of chitosan through susceptibility testing against two bacterial strains (*K. neumoniae* and *S. aureus)* isolated from milk samples. The results demonstrated that chitosan nanoparticles exhibited minimum inhibitory concentrations (MICs) of 5 mg/mL for *K. pneumoniae* and 2.5 mg/mL for *S. aureus*, with corresponding minimum bactericidal concentrations (MBCs) of 10 mg/mL and 5 mg/mL after overnight incubation. These findings are consistent with previous studies showing a correlation between chitosan's antibacterial activity and its MW. However, variability in outcomes may arise from differences in chitosan sources and polymerization levels. For example, Zheng and Zhu [49] reported reduced activity against *Klebsiella* due to excessive polymerization, while Islam et al. [50] found that 1.2 mg/mL of chitosan exhibited strong inhibitory effects against *S. aureus*. Transmission electron microscopy (TEM) analysis in this study revealed the presence of CNPs on the surfaces of bacterial cells, forming distinct "vacuole-like" structures beneath the cell wall. This observation aligns with the findings of Raafat et al. [51], who reported similar morphological changes. Chitosan has been shown to induce cell membrane lysis in both Gram-positive and Gram-negative bacteria. In the present study, TEM imaging showed significant morphological alterations in bacterial cells following exposure to CNPs, including increased cell size and disrupted cell shape compared to untreated controls. For example, *S. aureus* cells (Fig. 6A–B) retained their typical morphology prior to treatment, but post-exposure, CNPs were visibly adsorbed onto the cell wall, altering its permeability. The bactericidal effect of chitosan was more pronounced in Gram-positive bacteria, such as *S. aureus*, than in Gram-negative bacteria like *Klebsiella* spp. In Gram-negative bacteria, chitosan disrupted the outer membrane, compromising its barrier function and forming vesicular structures. Additionally, chitosan's interaction with lipopolysaccharides (LPS) may have increased membrane permeability, facilitating the uptake of non-protein nitrogenous compounds and promoting LPS release. Campos et al. [52] and Huang et al. [53] proposed that chitosan's antimicrobial mechanism involves membrane damage and leakage of intracellular contents, ultimately leading to cell death.

Conclusion

This study evaluated the antimicrobial efficacy of chitosan nanoparticles (CNPs) against *K. pneumoniae* and *S. aureus* strains isolated from bovine milk samples. The findings highlight the potential of CNPs as a promising alternative to conventional antibiotics in the treatment of antibiotic-resistant pathogens associated with bovine mastitis. The nanoparticles demonstrated strong inhibitory activity, effectively suppressing bacterial proliferation. Compared to traditional antibiotics such as ampicillin and gentamicin, CNPs exhibited superior performance, suggesting their utility in controlling, preventing, and treating mastitis. Moreover, the application of chitosan-based treatments supports the global effort to reduce antibiotic usage in livestock production, thereby mitigating the risk of antimicrobial resistance transmission through the food chain.

Acknowledgments

Not applicable.

Funding statement

This study didn't receive any funding support

Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical of approval

There are no experimental studies on either animals or human data in the manuscript.

TABLE 1. Primers and their base pair (bp) sizes for molecular detection of *Klebsiella, K. pneumoniae, K. oxytoca, S. aureus*, and *ESBL* resistance genes.

Bacteria	Target gene	Primer sequence (5′–3′)	Amplicon size (bp)	Reference
***Klebsiella* spp.**	***gyrA***	F: CGC GTA CTA TAC GCC ATG AAC GTA R: ACC GTT GAT CAC TTC GGT CAGG	**441**	**[24]**
Klebsiella pneumoniae	***16S-23S ITS***	F: ATTTGAAGAGGTTGCAAACGAT R: TTCACTCTGAAGTTTTCTTGTGTTC	**130**	**[25]**
Klebsiella oxytoca	***pehX***	F: GAT ACG GAG TAT GCC TTT ACG GTG R: TAG CCT TTA TCA AGC GGA TAC TGG	**343**	**[24]**
Staphylococcus aureus	***16S rRNA***	F: CCTATAAGACTGGGATAACTTCGGG R: CTTTGAGTTTCAACCTTGCGGTCG	**791**	**[26]**
***ESBL*s encoding genes**	***bla*$_{SHV}$**	F: AGGATTGACTGCCTTTTTG R: ATTTGCTGATTTCGCTCG	**237**	**[27]**
	***bla*$_{TEM}$**	F: ATCAGCAATAAACCAGC R: CCCCGAAGAACGTTTTC	**445**	**[28]**
	***bla*$_{CTX-M}$**	F: ATG TGC AGY ACC AGTAAR GTK ATG GC R: TGG GTR AAR TAR GTS ACC AGA AYC AGC GG	**593**	**[29]**

TABLE 2. Prevalence of *Klebsiella* and *Staphylococcus* spp in mastitis cows and buffaloes

Sample	***Klebsiella* species**			***Staphylococcus* species**		
	Clinical mastitis (n = 18) (36%)	**Subclinical mastitis (n = 14) (56%)**	***p-value***	**Clinical mastitis (n = 22) (44%)**	**Subclinical mastitis (n = 17) (34%)**	***p-value***
Cows	**12(48%)**	**9(36%)**	0.391	14(56%)	11(44%)	0.305
Buffaloes	6(24%)	5(20%)		8(32%)	6(24%)	
X2	2.439	1.32		2.09	1.77	
p-value	0.118	0.249		0.147	0.183	

X^2 :Chi square *p <0.05 is significant

TABLE 3. Antimicrobial susceptibility patterns of *Klebsiella* spp. And *Staphylococcus* isolated from cows and buffaloes

				***Klebsiella* spp. (32)**			***Staphylococcus* (39)**		
Antimicrobial class	**Antimicrobial agents**	**Disc**	**Conc.**	**Resistant (%)**	**Intermediate (%)**	**Sensitive (%)**	**Resistant (%)**	**Intermediate (%)**	**Sensitive (%)**
Quinolones	**Ofloxacin**	Ofx	5	7(21.8)	8(25)	17(53.1)	9(23)	13(33.3)	17(43.5)
Tetracycline	**tetracycline**	TE	2	9(28.1)	12(37.5)	11(34.4)	15(38.5)	14(35.8)	10(25.6)
Cephalosporins	**Ceftriaxone**	CRD	-	11(34.4)	9(28.1)	12(37.5)	11(28.2)	14(35.8)	14(35.8)
Lincomycin	**Clindamycin**	DA	2	10(31.2)	9(28.1)	13 (40.6)	14(35.8)	14(35.8)	11(28.2)
B –lactams	**Ampicillin/Sulbactam**	SAM	20	8(25)	10(31.2)	14(43.7)	9(23)	14(35.8)	16 (41)

TABLE 4. Incidence of ESBL resistance genes and phenotypes to antibiotics in *K. pneumoniae, K. oxytoca,* and *S. aureus* isolates from cows, and buffalos' milk samples

	Source	No. of examined samples	No. positive* (%)	*ESBL* resistance genes			Resistance phenotypes to antibiotics
				bla_{TEM}	bla_{SHV}	bla_{CTXM}	
K. pneumoniae	**Cows**	50	9(18)	7(77.7)	5(55)	7(77.7)	SAM, CRD, TE
	Buffaloes	50	5(10)	2(40)	0(0)	2(40)	DA, CRD, SAM
K. oxytoca	**Cows**	50	5(10)	2(40)	2(40)	0(0)	Ofx, TE, CRD
	Buffaloes	50	2(4)	0(0)	0(0)	0(0)	DA, SAM
S. aureus	**Cows**	50	13(26)	2(15.4)	5(38.5)	3(23)	Ofx,TEM, DA
	Buffaloes	50	9(18)	4(44)	3(33)	2(22)	SAM, DA,
Total			43/100(43)	18(41.8)	15(34.8)	14(32.5)	
X^2			11.21	8.57	1.08	8.14	
p value			0.024	0.072	0.896	0.043*	

X^2: Chi-Square. *p-value* is significant ($p \leq 0.05$).

TABLE 5. Minimum inhibitory concentration (MIC) of CS nanoparticles (mg/ml) against *K. pneumoniae* and *S. aureus*

Concentration (mg/ml)	*K. pneumoniae*	*S. aureus*
0.6	Growth	Growth
1.25	Growth	Growth
2.5	Bacteriostatic	Bacteriostatic
5	Bacteriostatic	Bactericidal
10	Bactericidal	Bactericidal

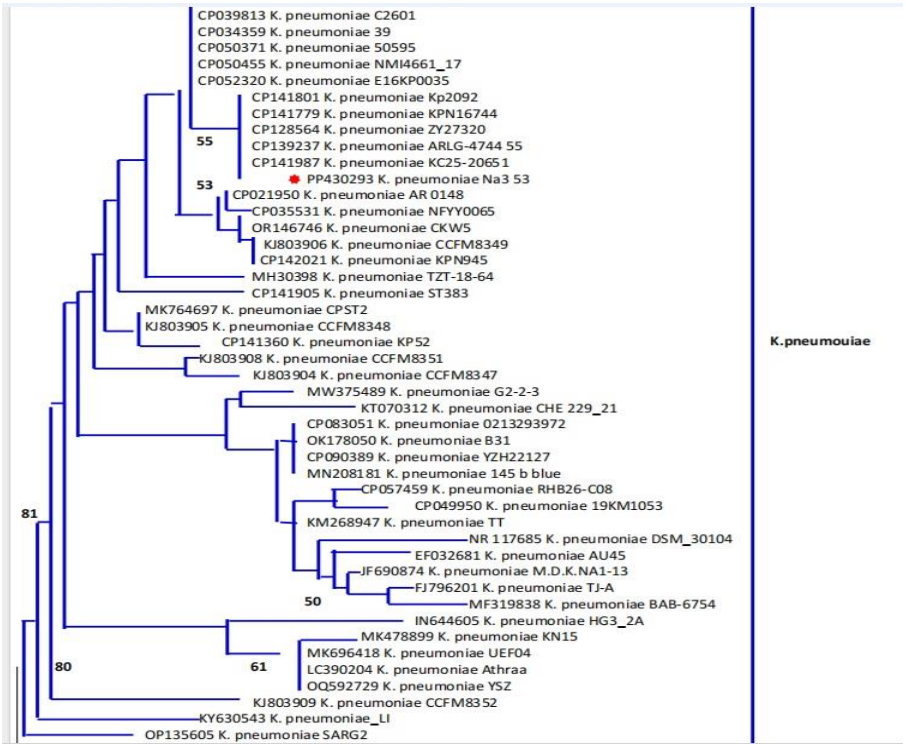

Fig. 1-A. The phylogenetic tree of *K. pneumoniae* was created using MEGA X 11.0 software using the neighbor-joining method based on 16S rRNA nucleotide sequences.

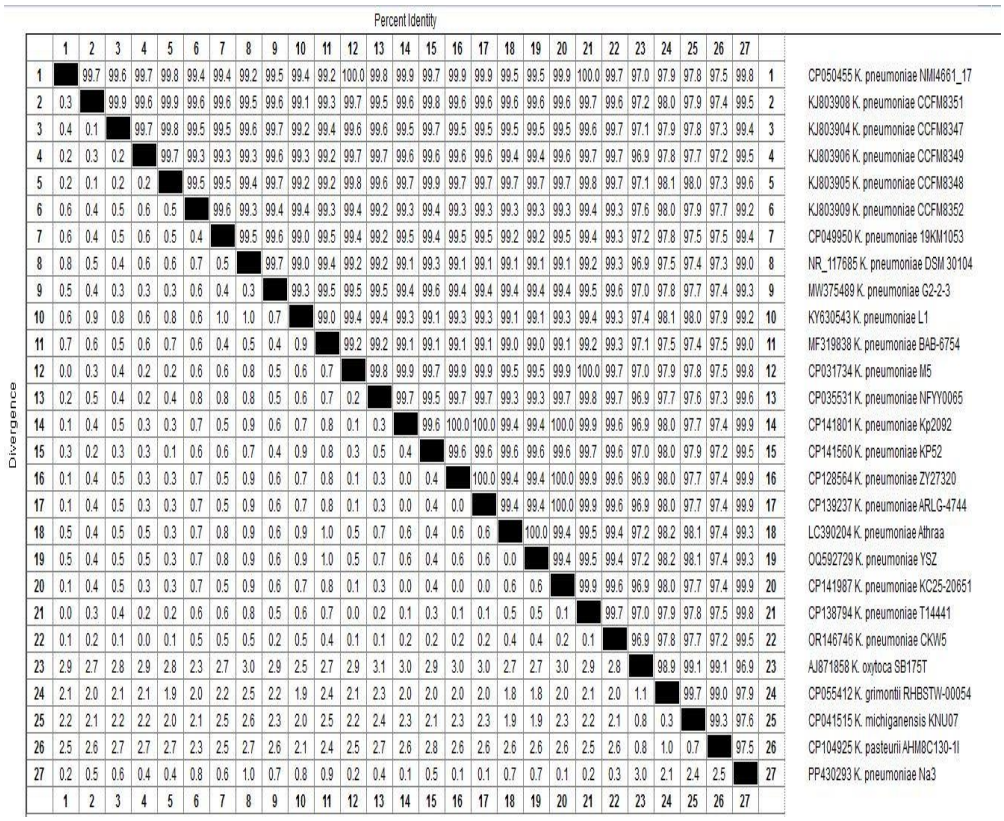

Fig. 1-B. The *K. pneumoniae* sequence distance of a test isolate, generated by Lasergene, demonstrates an identity range of 98.4-100% with various test isolates from clinical and subclinical mastitis.

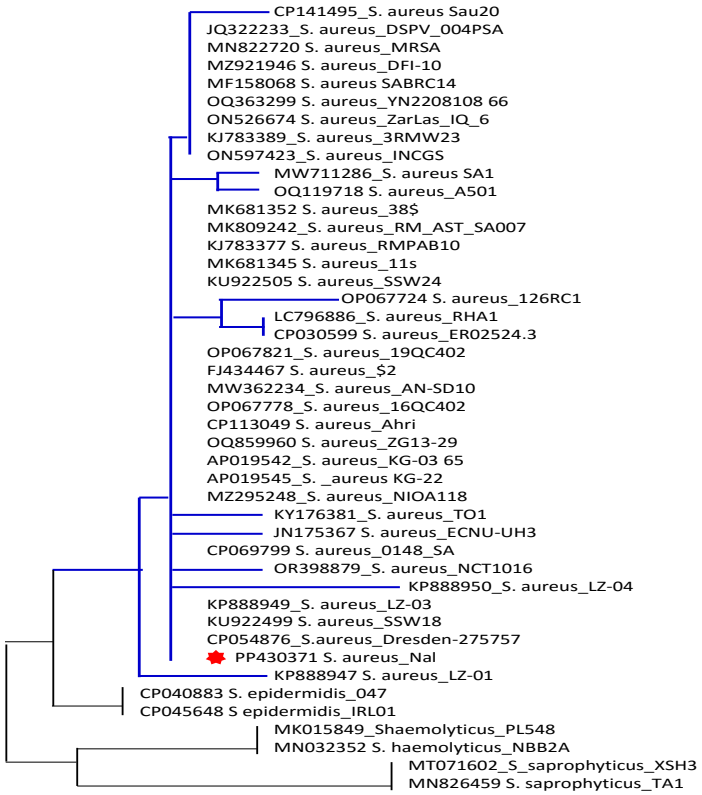

Fig. 2-A. The phylogenetic tree of *S.aureus*, identified using the Unrooted tree, was generated using MEGA X11.0 software using 16S rRNA nucleotide sequences.

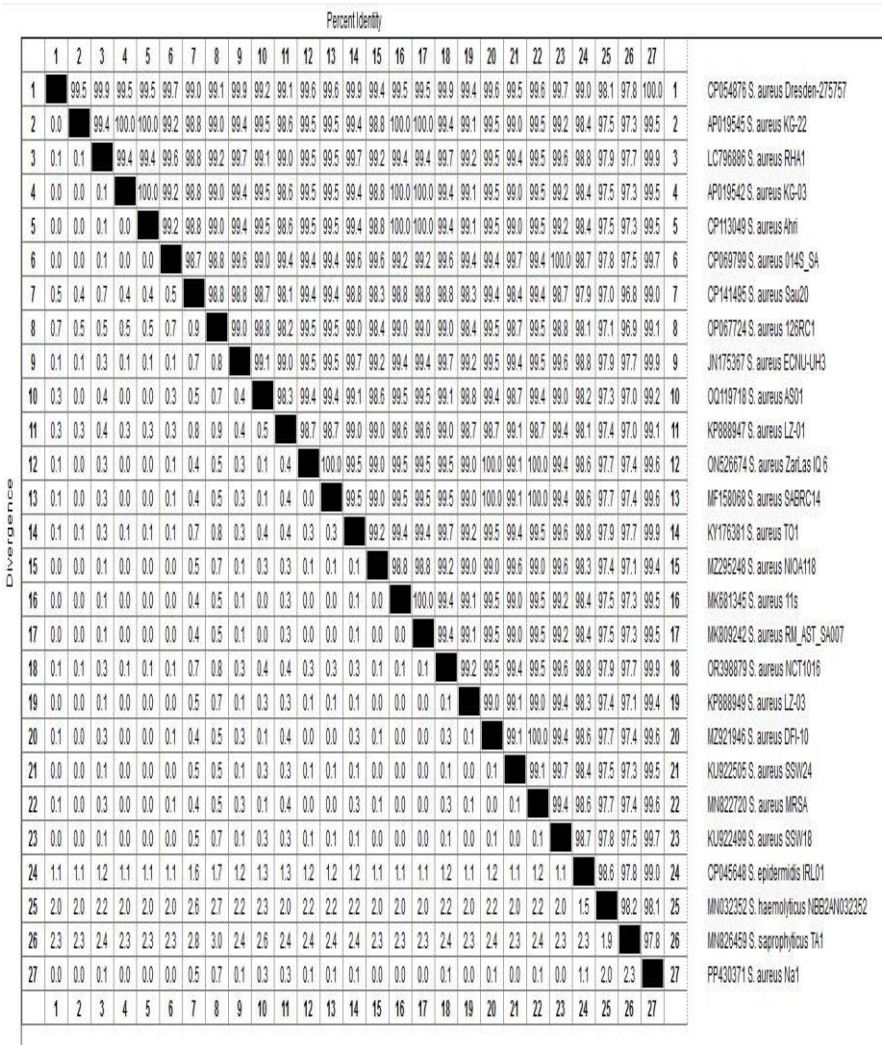

Fig. 2-B. The *S.aureus* sequence distance of a test isolate from Lasergene demonstrates an identity range of 97.8% - 99.5% with various test isolates from clinical and subclinical mastitis.

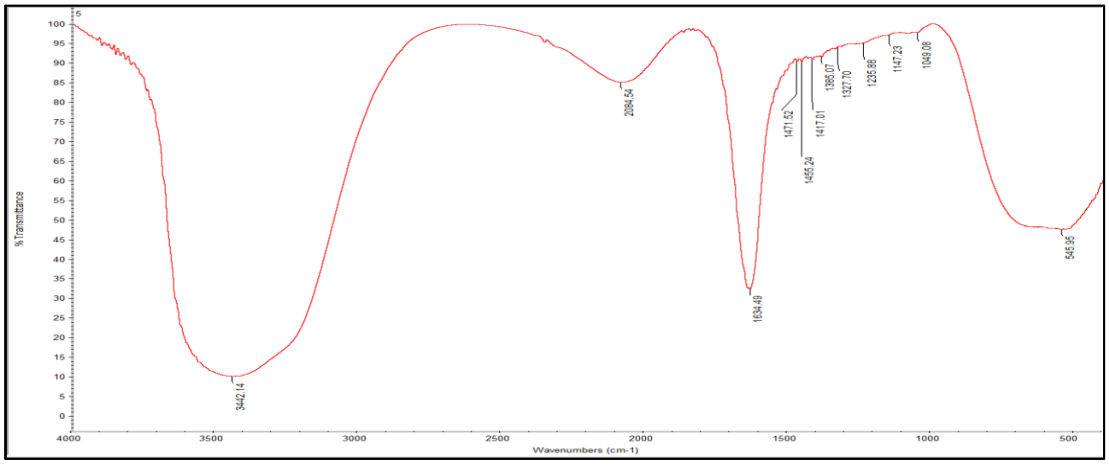

Fig. 3. A. FTIR of Chitosan nanoparticles

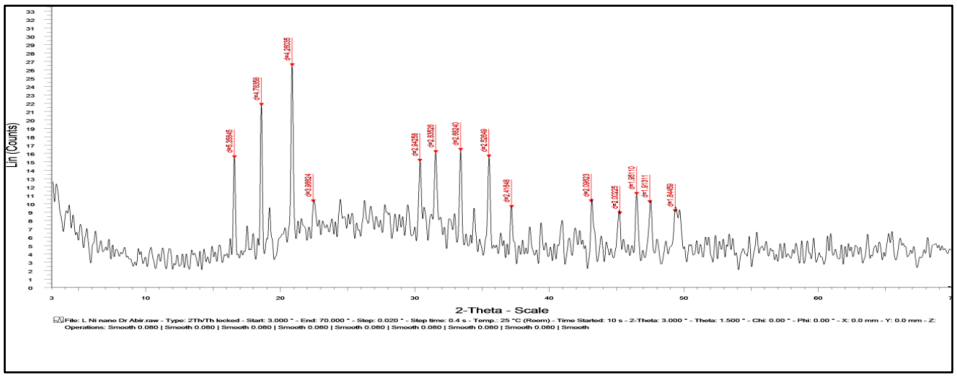

Fig. 3. B. XRD pattern of CNPs

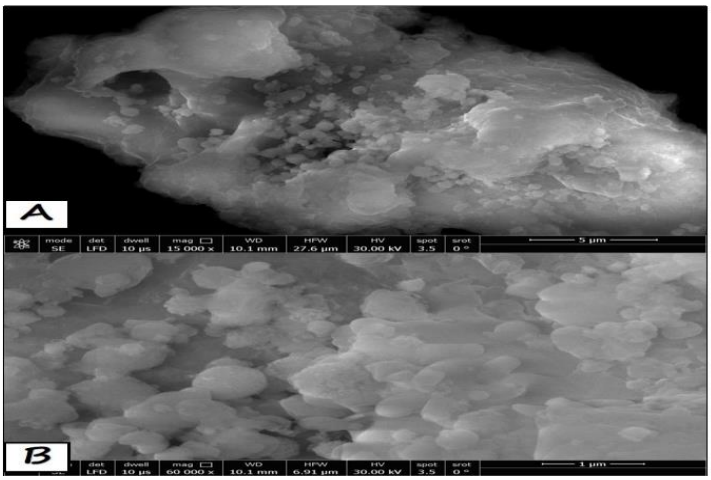

Fig. 3. C. Scanning Electron Microscopy image of chitosan nanoparticle

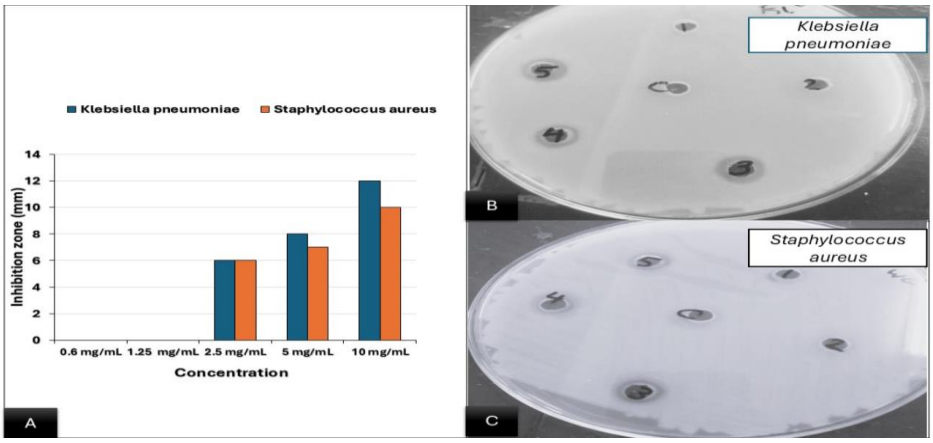

Fig. 4. The study evaluated the antibacterial activity of CNPs **at different concentrations using the agar well diffusion method against *K. pneumoniae* and *S. aureus* strains, with the results expressed as (1, 2, 3, 4, and 5), with c representing the negative control.**

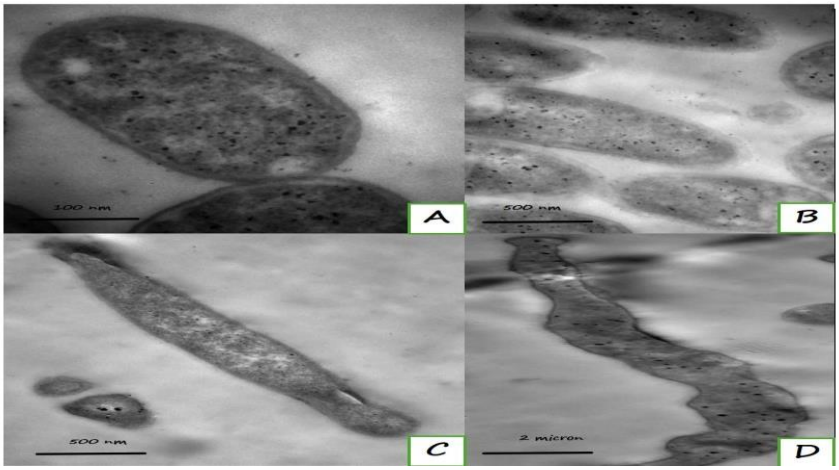

Fig. 5. TEM analysis revealed distinct morphological differences between untreated and CNPs treated *K. pneumoniae* cells. A-B) Untreated, the intact *K. pneumoniae* bacteria, and appeared to be in its typical shape. C-D)Treated and elongated *K. pneumoniae*

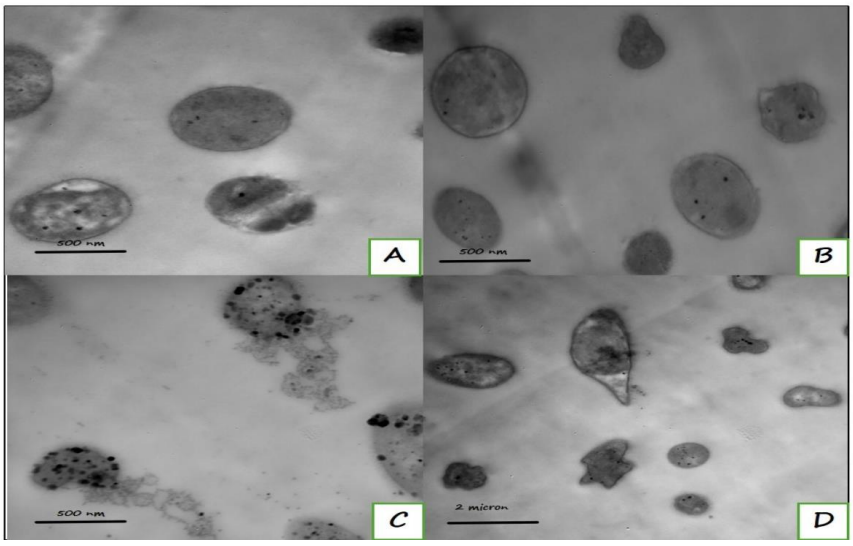

Fig. 6. TEM analysis revealed distinct morphological differences between untreated and CNPs treated *S. aureus* cells. A-B) The untreated cells exhibited intact morphology with well-defined cell walls and typical coccoid shape. C–D) CNP-treated cells showed widespread nanoparticle dispersion across the bacterial surface, with evident adsorption onto the cell wall.

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التقييم المعملي لجزيئات الكيتوزان النانوية ضد عزلات المكورات العنقودية الذهبية وجراثيم الكلبسيلا من حليب التهاب الضرع البقري: معالجة مقاومة المضادات الحيوية واستكشاف العلاجات البديلة

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الملخص

تُقيّم هذه الدراسة فعالية جزيئات النانو من الكيتوسان (CNPs) ضد البكتيريا المنتجة لإنزيمات ESBL مثل *Klebsiella pneumoniae* و *Staphylococcus aureus* المعزولة من عينات حليب مصابة بالتهاب الضرع في الأبقار والجاموس بمحافظة المنوفية .تم اختبار 100عينة باستخدام اختبار كاليفورنيا، وتحديد البكتيريا باستخدام تقنيات تقليدية وجزيئية .تم تصنيع جزيئات الكيتوسان وتحليلها باستخدام SEMو FTIRو XRD. .أظهرت النتائج انتشار *Klebsiella* بنسبة أعلى في الأبقار مقارنة بالجاموس، سواء في الحالات السريرية أو تحت السريرية، وكذلك *Staphylococcus*. أظهر تحليل الحساسية أن *Klebsiella* كانت أكثر حساسية للكينولونات، بينما كانت *Staphylococcus* أكثر حساسية للأوفلوكساسين والأمبيسيلين/سلباكتام .تم الكشف عن جينات المقاومة blaTEMو blaSHVو blaCTX-M بنسب متفاوتة، وكانت *K. pneumoniae* و *S. aureus*الأكثر مقاومة للمضادات الحيوية. أظهرت جزيئات الكيتوسان فعالية مضادة للبكتيريا، حيث شكلت مناطق تثبيط من 6إلى 12مم، وأظهرت صور TEMتغيرات واضحة في شكل الخلايا، مما أدى إلى موتها .تشير النتائج إلى أن الكيتوسان النانوي قد يكون بديلاً واعدًا للمضادات الحيوية التقليدية في علاج التهاب الضرع البقري.

الكلمات الدالة: التهاب الضرع، نانو كيتوزان، ميكروب الكلبسيلا، مقاومه المضادات.