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Research Article

Comparative evaluation of florfenicol and polymeric nanoparticles loaded with florfenicol against bacterial strains isolated from chickens

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Abstract: Antimicrobial resistance (AMR) poses a significant threat to both human and animal health, necessitating the search for alternative antimicrobial agents and strategies. In this study, we aimed to identify and isolate clinical bacterial strains from chickens and evaluate their sensitivity to florfenicol, a common antimicrobial agent that is used exclusively in veterinary medicine, along with polymeric nanoparticles loaded with florfenicol at various concentrations. Three clinical bacterial strains (*Escherichia coli*, *Enterococcus faecalis* and *Enterobacter cloacae*) were successfully isolated and identified from chicken presenting clinical signs. In order to assess their susceptibility, the isolated strains were subjected to a standard disc diffusion assay using florfenicol. Subsequently, polymeric nanoparticles loaded with florfenicol were tested at six different concentrations and compared their efficacy against the bacterial strains. Our results demonstrated that all three clinical bacterial strains exhibited varying degrees of resistance to florfenicol. Interestingly, the use of polymeric nanoparticles loaded with florfenicol did not displayed enhanced antimicrobial activity compared to the free drug. Notably, the efficacy of the loaded nanoparticles did not significantly vary with different concentrations of active substance. This study highlights the importance of exploring novel therapeutic approaches to combat antimicrobial resistance. The use of polymeric nanoparticles loaded with florfenicol presents a promising avenue for overcoming resistance mechanisms and improving the efficacy of antimicrobial treatments both in human and veterinary medicine. Further investigations are needed to elucidate the underlying mechanisms and optimize the formulation of polymer nanoparticles for enhanced therapeutic outcomes in combating AMR.

Keywords: florfenicol; antibiotic-loaded nanoparticles; antimicrobial resistance;

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1. Introduction

The discovery and integration of antimicrobial substances in the twentieth century stands as a remarkable achievement in modern medicine. This category of active substances has revolutionized the treatment of infectious diseases, ranging from minor to life-threatening complex surgical procedures, feasible organ transplantation to more effective chemotherapy treatment protocols [1]. However, the alarming rise of antimicrobial resistance (AMR) on a global scale poses a significant threat, potentially reversing the progress made and returning us to a time similar to the pre-antibiotic era. Furthermore, the economic impact of AMR is staggering, with a significant loss of $3 trillion in gross domestic product [2]. AMR is an inevitable consequence of the evolutionary process, as organisms develop genetic mutations to evade the lethal selective pressures imposed by antibiotics [3]. As long as antimicrobial substances continue to be utilized against human, veterinary or agriculture pathogens, bacteria will persistently develop and employ resistance mechanisms. Currently, more that 70% of pathogenic bacteria display resistance to at least one antibiotic [4]. Being ubiquitous, microorganisms serve as a reservoir of AMR in various ecological niches. The intrinsic network of interactions among microbial communities in diverse environments facilitates the transfer of genetic material, thereby expanding the spread of AMR, leading to a global concern [5].

Many classes of antibiotics used in human infections are shared with the veterinary sectors and vice versa, exerting cumulative selective pressure on microorganisms and leading to reduced efficacy on antimicrobial based treatments [7]. Traditionally, antibiotics have been used in animal husbandry for the treatment of infectious diseases, as well as for preventive measures and as growth-promoting factors. The latter application is based on observations linking the administration of subtherapeutic doses of antibiotics to significant weight gain in treated animals [4]. Although the precise mechanism behind this phenomenon is not yet fully understood, is has been observed that prolonged administration of antibiotics at subtherapeutic doses affects multiple organs and physiological processes [4]. The later mentioned processes represent a reduced diversity of the intestinal microbiota and diminished competition for nutrients, a decrease in harmful bacteria, reduced immune stimulation or increased vitamin biosynthesis in the intestines. Collectively, these effects improve the net energy balance and enhance animal performance from a zootechnical standpoint [8]. Furthermore, sublethal doses of antibiotics act as selective pressure, stimulating bacterial evolutionary mechanisms to adapt to environmental stressors and allowing the survival and propagation of more resistant strains carrying AMR traits. Similar effects can be observed with the use of antibiotics for prophylactic purposes. In this context, antimicrobial compounds are commonly administered via drinking water or feed, ensuring prolonged exposure of animals to low antibiotic doses over an extended period. However, the protective effects are reversed once antibiotic administration is suspended, leaving the animals susceptible to infections [9], [10].

In the context of poultry farming, the use of antibiotics has been a common practice with far-reaching consequences, particularly concerning AMR. Antibiotics have been employed in poultry production for therapeutic purposes, and they are typically administered through drinking water [11]. Penicillins, aminoglycosides, tetracyclines, macrolides, and a combination of sulfonamide/trimethoprim are among the commonly used classes of antibiotics in this sector [12]. However, the extensive use of antibiotics in poultry farming raises concerns about the development and spread of antimicrobial resistance. The repetitive and widespread use of antibiotics in poultry production contributes to the selection and proliferation of resistant bacteria [13]. As a result, various resistance genes emerge, compromising the effectiveness of antibiotics not only in poultry sector but also in human medicine [14]. Florfenicol, a broad-spectrum antibiotic, is frequently employed in poultry to combat respiratory, enteric or septicemic infectios. In addition, it has beed utilized for prophylaxis and growth promotion, due to its lower risk of promoting resistance development when compared to other amphenicols. [15]. However, studies have identified resistance genes associated with florfenicol in poultry populations, that poses a significant challenge for public health. These genes, when transferred to human pathogens, can diminish the effectiveness of antibiotics used for treating human infections [16]. Therefore, the emergence and dissemination of resistance genes in poultry population warrant careful monitoring and intervention strategies to mitigate the spread of antimicrobial resistance. Understanding the impact of antibiotic usage in poultry and the prevalence of resistance genes, such as those linked to florfenicol, is crucial for implementing effective control measures [17]. Is is essential to develop alternative strategies that promote responsible antibiotic use in poultry farming, prioritize animal welfare, and minimize the risk of antimicrobial resistance transmission between animals and humans. By addressing these issues, we can safeguard the efficacy of antibiotics and ensure the continued protection of both animal and human health [18].

In order to determine the antimicrobial resistance of clinical bacterial strains, we employed a technique involving a previous isolation and identification of three strains using chemical identification. Our focus was on evaluating the sensitivity of these bacterial strains for florfenicol, as well as for six different concentrations of florfenicol-loaded nanoparticles. The antibiotic was chosen based on the interest in poultry farming, since florfenicol is an antibiotic commonly employed to combat bacterial infections in this species [19]. The methodology consisted in testing the susceptibility of the bacterial strains by using a diffusimetric method, hence the inhibitory effect of the antimicrobial agents was assessed by measuring the diameter of the inhibition zones around the wells. The obtained results were then interpreted by comparing the inhibiting diameters of the different concentrations of florfenicol and florfenicol-loaded nanoparticles. This analysis provided insights into the effectiveness of these agents against the tested bacterial strains and allowed for the determination of the minimum inhibitory concentration (MIC) required to inhibit bacterial growth. By employing the diffusimetric method and measuring the inhibiting diameter, we could evaluate the antimicrobial resistance of the clinical bacterial strains to florfenicol and assess the potential enhancement of its efficacy through the use of florfenicol-loaded nanoparticles. These findings contribute to our

understanding of the susceptibility patterns of bacterial strains and aid in the development of more effective antimicrobial strategies to combat antimicrobial resistance.

2. Materials and Methods

2.1. Sample Collection: Swab sampleswere collected from 10-day-old chickens exhibiting non-specific clinical signs such as weight loss and decreased appetite. A total of 10 chickens were selected for this study. The birds were carefully examined, and samples were collected using aseptic techniques to avoid contamination.

2.2 Isolation and identification of bacterial strains: upon sample collection, the specimens were inoculated onto nutrient agar plates using the streaking method. The plates were then incubated at 37°C for 24 hours to allow bacterial growth. Following incubation, individual bacterial colonies were isolated based on their morphological characteristics. The isolated bacterial strains were subjected to identification using the API 20 E biochemical rapid test (Biomérieux SA). This test utilizes a panel of biochemical reactions to identify the bacterial species. Each bacterial strain was inoculated into the API 20 E strip and incubated according to the manufacturer's instructions. The results obtained from the test were recorded and used for further analysis.

2.3. Preparation of Bacterial Cultures: to prepare 24-hour cultures of the identified bacterial strains, a loopful of each isolate was streaked onto nutrient agar plates. The plates were then incubated at 37°C for 24 hours. After incubation, a single colony from each plate was selected and inoculated into Mueller-Hinton broth at a reference scale of 0.5 McFarland. The broth cultures were incubated under optimal conditions for the respective bacterial strains.

2.4. Sensitivity testing: a plate containing 12 ml of Mueller-Hinton agar was used to perform the sensitivity testing for the three isolated bacterial strains. The tests aimed to evaluate the ability of florfenicol to inhibit bacterial growth using the diffusion method recommended by the Clinical and Laboratory Standards Institute (CLSI). Commercial susceptibility disks loaded with 30 μg of florfenicol were employed in the testing. Additionally, polymeric nanoparticles loaded with florfenicol were used as an alternative formulation. The nanoparticles were reconstituted at a concentration of 30 μg/ml and subjected to successive dilutions to obtain concentrations of 15 μg/ml, 7.5 μg/ml, 3.75 μg/ml, 1.875 μg/ml, and 0.937 μg/ml. The reconstituted nanoparticles were prepared in 38 ml of sterile saline solution in Wheaton scintillation vials made of borosilicate glass.

Table 1. Inhibition zone diameters of florfenicol and nanoparticle-loaded formulations against isolated bacterial strains

BACTERIAL STRAIN	FLORFENICOL DISK (30 μG)	FLORFENICOL-LOADED NANOPARTICLES (μG/ML)
E.COLI	15 mm	30 μg/ml: 8 mm (± 1) 15 μg/ml:7.5 mm (±0.5) 7.5 μg/ml: 8 mm (±1) 3.75 μg/ml: 6.8 mm (±0.2) 1.875 μg/ml: 7 mm (±0.5) 0.937 μg/ml: 8 mm (±1.5)
ENTEROCOCCUS FAECALIS	18 mm	30 μg/ml: 6 mm 15 μg/ml: 6 mm 7.5 μg/ml: 6 mm 3.75 μg/ml: 6 mm 1.875 μg/ml: 6 mm 0.937 μg/ml: 6 mm
ENTEROBACTER CLOACAE	17 mm	30 μg/ml: 7 mm (± 1.5) 15 μg/ml: 6.5 mm (± 1) 7.5 μg/ml: 6 mm (±1.8) 3.75 μg/ml: 7.5 mm (±1.8) 1.875 μg/ml: 6.8 mm (±1.2) 0.937 μg/ml: 6 mm (±1.5)

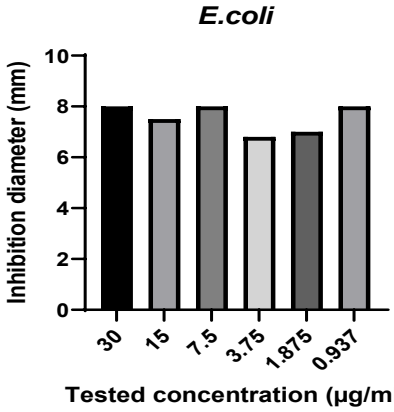
2.5 Interpretation of results: The interpretation of the sensitivity testing results was performed by measuring the diameter of inhibition zones formed around the susceptibility disks and nanoparticle-loaded wells. The diameter measurements were recorded for each concentration of florfenicol tested. Statistical analysis was carried out using GraphPad Prism 9.3.0 to determine the significance of the differences observed between the susceptibility of the bacterial strains to florfenicol and the nanoparticle-loaded formulation. The results were analyzed, and relevant statistical parameters such as mean, standard deviation, and p-values were calculated.

3. Results

3.1. Isolation and identification of bacterial strains: from the examined chickens, three bacterial strains were isolated and identified as follows: *Escherichia coli*, *Enterococcus faecalis*, and *Enterobacter cloacae*. It is important to note that these bacteria can also be part of the normal gut bacterial flora. However, further investigation is required to determine whether these isolated strains have any pathogenic effects.

3.7. Sensitivity testing results: the susceptibility testing was performed to evaluate the effectiveness of florfenicol and nanoparticle-loaded formulations against the isolated bacterial strains. The inhibition zone diameters were measured and are summarized in Table 1.

3.2 Data analysis: statistical analysis (one-way ANOVA test) was performed to assess the significance of the differences observed between the susceptibility of the bacterial strains tested at six different concentrations of florfenicol-loaded nanostructures. The analysis revealed no statistical significance between them ($p>0.05$), as shown also in figures 1, 2 and 3. It is worth noting that the bacterial strains exhibited a significantly higher susceptibility to the florfenicol disk compared to the nanoparticles loaded with florfenicol.



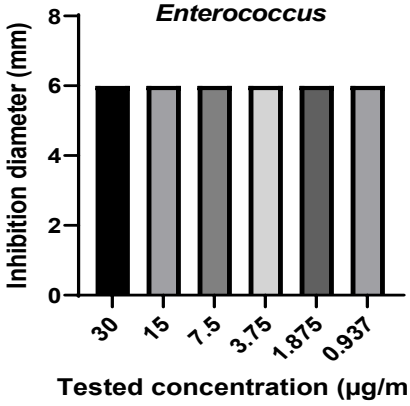


Figure 1 and 2: Graphical representation of the *E.coli* (1) and *Enterococcus* (2) susceptibility to different concentrations of florfenicol-loaded nanoparticles

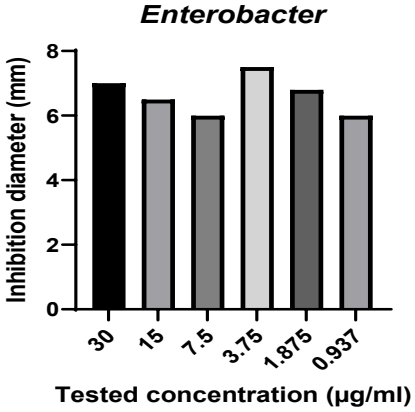


Figure 3: Graphical representation of the *Enterobacter* susceptibility to different concentrations of florfenicol-loaded nanoparticles

4. Conclusions

The main objective of this study was to investigate the susceptibility of bacterial strains (*Escherichia coli*, *Enterococcus faecalis*, and *Enterobacter cloacae)* isolated from chickens exhibiting non-specific clinical signs to florfenicol and nanoparticle-loaded formulations. The identified bacterial strains represent bacteria commonly found in poultry and fresh chicken meat and their presence suggest that they may have a significant effect on human colonization and the dissemination of antibiotic resistance in the environment. The results of the susceptibility testing revealed a higher susceptibility to he florfenicol disk compared to the nanoparticle-loaded formulation. However, it is important to consider that the aqueous solution used for the nanoparticles preparation and the potential influence of variables like the release rate of florfenicol from the nanostructures, temperature variations, and other nanostructure-related properties might have influenced these results . Statistical analysis indicated no significant differences between the six different concentrations tested, suggesting that the susceptibility of the bacterial strains to the nanoparticle-loaded formulations remained consistent across the concentration range. The findings from this study highlight the potential limitations of the nanostructures in terms of antimicrobial efficacy compared to the conventional florfenicol disk. Further investigations are warranted in order to characterize the nanostructures, including their release kinetics, as well as the loading with the active substance, and the impact of various other parameters on their antimicrobial activity. This additional research will aid in optimizing the nanoparticle formulation and overcoming the observed limitations. Moreover, considering that the isolated bacterial strains (*E. coli, Enterococcus faecalis*, and *Enterobacter cloacae*) can be part of the normal gut bacterial flora, it is crucial to conduct further studies to determine whether these strains possess pathogenic properties or are associated with the observed non-specific clinical signs in the examined chickens.

In conclusion, this study provides valuable insights into the susceptibility of bacterial strains isolated from chickens to florfenicol and nanoparticle-loaded formulations. The results suggest a higher susceptibility to the conventional florfenicol disk compared to the nanoparticle formulation, highlighting the need for further investigation and optimization of the nanoparticle system. The findings also emphasize the importance of assessing the pathogenic potential of these isolated bacterial strains to elucidate their role in the observed clinical signs. Overall, this study sets the foundation for future research aiming to enhance antimicrobial strategies and promote animal health and welfare.

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