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***Research Article** (Open Access)*

IN VITRO EVALUATION OF SHRIMP CHITOSAN'S EFFICACY AGAINST *ESCHERICHIA COLI* FROM POULTRY

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ABSTRACT
The objective of this study is to produce chitosan from the shell waste of Shrimp (Penaeus indicus) via a chemical process that involves the stages of deproteinization, demineralization, and deacetylation. Chitosan's inhibitory effect was evaluated against a multidrug-resistant *Escherichia coli* strain and was isolated from poultry litter samples from various districts of Tamil Nadu. The chitosan extract showed more effective activity against *Escherichia coli* at different concentrations when Agar well diffusion and Minimum Inhibitory Concentration (MIC) tests were conducted. Therefore, the naturally occurring bioactive substance chitosan can also be used as a better substitute for the antibiotics that are currently in use. Another issue that needs to be addressed is the spread of antimicrobial resistance from poultry sources to humans, which is currently a cause for concern. To address this, the most affordable source of bioactive substances like chitosan from shrimp waste, can also be used as an alternative source of antibiotics to minimize the spread of antimicrobial resistance from poultry to the human population.

***Keywords**: Chitosan, Antibacterial Activity, Escherichia coli, Poultry Litter, Tamil Nadu*

INTRODUCTION
The second most common natural polymer in the world is chitin. Shrimp and crabs, two types of marine crustaceans, are the primary sources. Chitin, or poly-N-acetylglucosamine, shares structural similarities with cellulose; however, at the C-2 atom, the substituent is an acetylated amino acid (-NH–CO–CH3) rather than a hydroxyl (–OH) group (Friedman *et al*., 2010). The amount of deacetylation (DD) in chitosan is shown by the ratio of glutamine to N-acetyl-D-glucosamine. There are two different kinds of chitosan and chitin straight-chain copolymers (Rinaudo, 2006). Chitin is a useful suture material in large part because of its biocompatibility, biodegradability, non-toxicity, antibacterial activity, and low immunogenicity. This implies that there's a lot of space for more advancement (Pillai *et al.*, 2009).
To determine the Minimum Inhibitory Concentrations (MIC) and the Minimum Bactericidal Concentration (MBC) of chitosan, the antibacterial activity was evaluated against Gram-positive *Bacillus subtilis* and Gram-negative bacteria (*Stenotrophomonas maltophilia* and *Enterobacter cloacae*). The Minimum Bactericidal concentration (MBC) demonstrated the process's viability and the good antibacterial potential of extracted chitosan against both Gram-positive and Gram-negative bacteria (Vilar Jr *et al*.,2016).
The dissemination of dangerous bacteria poses a major risk to public health, and pathogenic *E. Coli* especially its antibiotic-resistant strains is a zoonotic problem, as has been well-recognized in recent years (Mohamed *et al.,* 2022). Considerable associations between the majority of the genotypes and antibiotic resistance characteristics are seen in the *E. coli* isolates. research revealed that to reduce the emergence of bacterial strains resistant to antibiotics, antimicrobials must be used sparingly in hens (Rahman *et al*., 2020). Resistance *E. coli* was found in a large percentage of samples, and it was found in turkeys and broilers at significantly greater rates than in the population of laying hens. Those who raised turkeys and broilers frequently encountered multi-resistant isolates, whereas laying hen farmers did not. The study exhibited that it is frequent for people to come into contact with resistant *E. coli* clones and plasmids from poultry

(Van den Bogaard *et al*., 2001). In light of organic poultry litter and traditional chemical fertilizer, the study offered quantitative data on the prevalence and longevity of antibiotic-resistant Escherichia coli. Supervised throughout the entire corn-growing season. Comparing poultry litter to chemical fertilizer or unfertilized soil, Antibiotic-resistant *E. coli* levels increased dramatically one week after amendment. It was only a transient impact, though, lasting about a month (Agga *et al.,* 2024).

It is widely acknowledged that conventional antibiotics have been used in the production of livestock, particularly poultry, as a therapeutic or prophylactic treatment against illnesses caused by pathogenic bacteria. Unfortunately, the inappropriate use and use of these drugs has led to the creation and spread of antibiotic resistance, which is currently a serious public health concern. Since multidrug-resistant bacteria are becoming more common and can infect humans and animals with deadly infections, the study evaluates new and innovative methods to counteract antimicrobial resistance (Abreu *et al.,* 2023).

The bacteria isolated from poultry litter, feces, neck, and skin revealed genes encoding resistance to β-lactams (blaTEM, blaCTX-M), fluoroquinolones (qnrA, qnrB, qnrS), aminoglycosides (strA-strB, aphA1, aac(3)-II), sulfonamides (sul1, sul2, sul3), trimethoprim (dfr1, dfr5, dfr7/17), and tetracyclines (tetA, tetB). 75% of the Multi-drug resistant *E. coli* isolates had confirmed class 1 and 2 integrons; of these, 60% had class 1 integrons, 15% had class 2 integrons, and 11.7% had both classes of integrons. Therefore, it can be said that at critical phases of the production of poultry, commensal multidrug-resistant Escherichia coli share integrons as shared mediators of antibiotic resistance (Racewicz *et al.,* 2022).

MATERIALS AND METHODS

SAMPLE COLLECTION

Shrimp shell waste collected from the local market in Chennai, Tamil Nadu. The gathered shrimp shell waste was thoroughly cleaned to remove any soft tissues, and then it was rinsed with distilled water. Samples were then dried under sunlight for 2 days and further dried in a hot air oven at 80^0 c for 24 hours. Then the dried samples were blended into small pieces for the preparation of chitosan.

PREPARATION OF CHITOSAN

Chitosan powder was prepared from shrimp shell waste followed by the reported data by Boudouaia *et al*.,2019. The three Basic steps involved in the production of chitosan from shrimp shell waste are 1) Deproteinization is the removal of protein from the chitin. 2) Demineralization consists of the removal of minerals from chitin.3) The Deacetylation step is involved to remove some or all the acetyl groups from the chitin.

ISOLATION AND IDENTIFICATION OF *E. COLI*

A total of 132 poultry litter samples were collected from the poultry farms which are located in various districts of Tamil Nadu. The samples were collected by using a sterile scalpel and forceps in plastic zip lock cover bags and stored at 4^0 c. The samples were then thawed to room temperature and further serially diluted in 0.85%Nacl. The two tube dilutions (10^{-5}, 10^{-6}) were spread plated into the Nutrient agar plates. To obtain a pure culture the individual colonies were picked up from a spread plate and streaked into nutrient agar plates separately. The *E.coli* colonies were screened by the colony morphology and gram staining. The isolated culture was confirmed by inoculating into the selective medium and Biochemical identification (Dadheech *et al.,* 2016).

ANTIMICROBIAL SUSCEPTIBILITY TESTING

Disk Diffusion Method

The *E. coli* isolates were tested for antibiotic susceptibility according to the Kirby- Bauer disk diffusion method followed by the guidelines of the clinical and laboratory standard institute (CLSI; M100,29th edition, January 2019). The selected antibiotic disc of different classes used for this study is ampicillin (10 µg), amoxicillin (10 µg), vancomycin (30 µg), penicillin (10 µg), amikacin (30 µg), gentamicin (30 µg), cefixime (5 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), imipenem (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), and erythromycin (15 µg). The *E. coli* isolates are described as multidrug-resistant when they show resistance to three or more antimicrobial agents of different classes.

Antibacterial activity determination by Agar well diffusion method

The *E. coli* culture was grown in 1ml of Muller-Hinton broth for 2-3 hours and its turbidity was adjusted with 0.5 McFarland standard. The grown bacterial culture was then swabbed aseptically on the surface of the Muller-Hinton agar plate. An agar well with a 6mm diameter was cut on the agar plate aseptically and the chitosan solution prepared with 0.2% acetic acid (Shanmugam et al.,2016) with different concentrations was loaded in the agar wells. The antibiotic standard Gentamycin was used as a Positive control and 0.2% acetic acid was used as a negative control. The plates were then incubated at $37^{0\,c}$ for 24 h and zones of inhibition were recorded after incubation.

Minimum inhibitory concentration((MIC)

The Minimum inhibitory concentration was performed as per the CLSI guidelines. A stock solution of chitosan (1mg/ml) was prepared and serial dilution was performed to attain a different concentration of chitosan between (30µg/ml to 100µg/ml). To this, 100µl of test organism was added to the broth as a positive control and the test tube containing broth without culture was used as a negative control. The turbidity in the test tube was noted after incubation. The MIC value of chitosan is determined by the tube showing inhibited growth of bacteria with the lowest concentration of chitosan added.

RESULTS AND DISCUSSION

A total of 132 poultry litter samples were collected, and 83(63%) *E. coli* isolates were identified and tested for Multidrug resistance. A total of 13 antibiotics from 8 antibiotic classes were used to assess multidrug resistance patterns of *E. coli* strains (Table: 1).

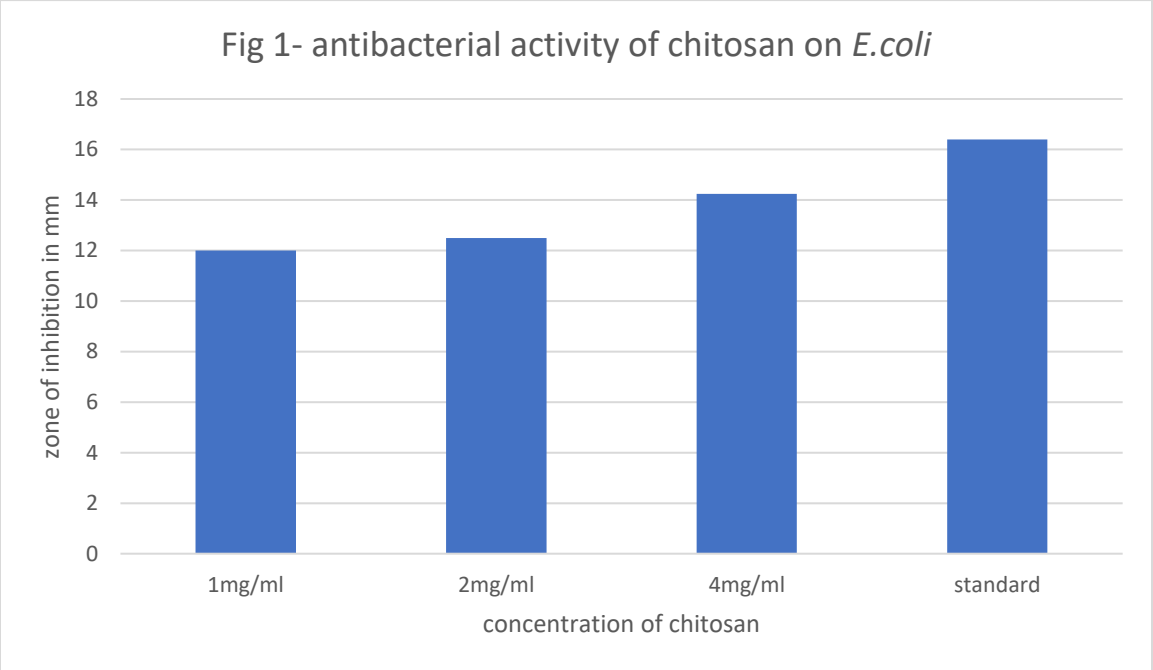
Table 1: Antibiotic resistance pattern of *E. coli* strains isolated from poultry litter. (N=83)

Class	Resistant (%)	Sensitive (%)
Penicillin	78.3	21.7
Cephalosporins	52.5	47.5
Aminoglycoside	62.6	37.4
Quinolones	30.1	69.9
Carbapenems	54.2	45.8
Phenicol	42.1	57.9
Tetracycline	48.2	51.8
Macrolides	36.1	63.9
Glycopeptide	62.5	37.5

Chitosan which is isolated from the shrimp shell waste tested against Multi-drug resistant *E. coli* strains. All the multi-drug resistant *E. coli* strains were significantly inhibited and a zone of inhibition was observed in three different concentrations. However, the highest range of zone of inhibition was observed in the 4mg/ml concentration, and the lowest range of inhibition was noted in the 1mg/ml concentration (Figure – 1).

Among the 5 Different concentrations of chitosan (20,40,60,80 and 100µg/ml), the MIC value of chitosan was observed in the concentration 80µg/ml against multidrug-resistant *E. coli*. The bacterial growth in the form of turbidity was noted visibly in 20,40 and 60µg/ml concentrations and bacterial turbidity was not noted in 80µg/ml concentration.

The 143 Enterobacteriaceae isolates were found in 87 pooled chicken droppings from the previously published data by Tigabie *et al.*, (2023). Out of these, 87 (60.8%) are *E. Coli*, followed by 23 (16.1%) *Salmonella* spp., 18 (12.6%) *P. mirabilis*, and 11 (7.7%) *K. pneumoniae*. Ampicillin 131 (91.6%) had the highest resistance rate, followed by tetracycline 130 (90.9%) and trimethoprim-sulfamethoxazole 94 (65.7%). 116/143 (81.1%) was the overall multidrug resistance rate (95% CI: 74.7–87.5). Extended-spectrum beta-lactamase producers made up 12/143 (8.4%; CI: 3.9–12.9) isolates in total, of which 1/11 (9.1%) and 11/87 (12.6%) were *K. pneumoniae* and *E. coli.*



In the present study penicillin class of antibiotics showed the highest rate of resistance (78.3%) followed by Aminoglycoside (62.6%) and the lowest rate of resistance was observed in the Quinolones (30.1%). Moreover, the present study also revealed the resistance rate of *E. coli* against carbapenem classes is (54.2%), the identification of carbapenem resistance in poultry litter indicates significant concern about microbial resistance to public health and the safety of our environment. Poultry litter is generally used as a fertilizer in agricultural practices, and the spread of carbapenem resistance can lead to environmental contamination, this environmental dissemination eventually reaches to human population through various ecological pathways.

Carbapenems are the last resort of the antibiotic classes used to treat several bacterial infections, particularly against the multi-drug resistance bacteria. While the use of Carbapenem in poultry production is restricted, the presence of carbapenem resistance may result from overuse or misuse of other antibiotics can promote cross-resistance by its resistance mechanism. The identification of carbapenem resistance in poultry litter emphasizes the urgent need for a varied approach to combat antimicrobial resistance.

Based on the findings reported by Jawad (2022). When chitosan made from prawn shells was used against gram-negative bacteria isolated from urinary tract infections, it demonstrated biological activity at high and varied rates in different concentrations (2,3,4,5, and 10 mg/ml). The development of harmful bacteria is significantly inhibited by increased chitosan concentrations, in various prawn treatments, the zone of inhibition for E. Coli, Proteus, Enterobacter, and Klebsiella varied from 18–24, 22–12, 15–25, and 14–21

mm, respectively. Gram-negative pathogenic bacteria were greatly inhibited by the chitosan made from prawn shells.

In the present study, the chitosan produced from shrimp shell waste was significantly effective at the varied concentrations of 1mg /ml to 4 mg/ml. The antibacterial activity of chitosan is concentration-dependent. The highest zone of inhibition was obtained in the concentration of 4mg/ml concentration. The increasing prevalence of multi-drug resistance in poultry production has raised enormous challenges to public health, chitosan a natural biopolymer derived from chitin has emerged as an alternative antibiotic due to its wide range of antimicrobial properties. Incorporating chitosan in poultry feed can help to reduce the intestinal bacterial pathogens improving the gut health and minimal the use of antibiotics. Unlike synthetic antibiotics, chitosan does not contribute to environmental pollution and it is obtained from renewable sources. With the use of chitosan disinfectants in poultry farming practices, poultry producers can achieve the greatest standards of hygiene and reduce the reliance on the continuous use of antibiotics, achieving overall health and productivity in poultry production. Future trends and developments of incorporating chitosan in poultry production will explore the key to controlling the dissemination of antimicrobial resistance, reducing the need to use antibiotics and realizing the potential of chitosan in the poultry industry.

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