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RESEARCH ARTICLE

Efficacy of Chitosan Nanoparticles as a Natural Antibacterial Agent against Pathogenic Bacteria causing Omphalitis in Poultry

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A B S T R A C T

Omphalitis is a hatchery-borne disease responsible for first-week chick mortality. The present study aimed to isolate and investigate the antimicrobial resistance profile of bacteria causing omphalitis in broiler farms and then investigate the antibacterial properties of chitosan nanoparticles against isolated bacteria. In this study, 100 living diseased chicks were collected from 10 broiler chicken farms. Overall, 86 bacterial isolates were successfully recovered and then identified as 44 *E. coli*, 21 *Salmonella*, 13 *Pseudomonas*, and 8 *Staphylococcus*. *E. coli* isolates were serotyped as EPEC (O_2H_6, $O_{121}H_7$ & $O_{55}H_7$), ETEC ($O_{128}H_2$ & O78), EHEC ($O_{26}H_{11}$ & $O_{91}H_{21}$) and EIEC (O_{159}). Meanwhile, *Salmonella* isolates were serotyped as *S. enteritidis, S. larochelle, S. typhimurium, S. kentucky* and *S. takoradi*. *Pseudomonas* and *Staphylococcus* isolates were serologically confirmed as 13 *Pseudomonas aeruginosa* and 6 *Staphylococcus aureus*. The antimicrobial resistance profile of tested isolates against sixteen commercial antibiotics showed a high multiple antibiotic resistance index. Agar well diffusion test indicated that chitosan nanoparticles were *in vitro* effective against the most resistant bacteria isolates. The minimum inhibitory concentration of chitosan nanoparticles for *E. coli* (O78), *S. kentucky, P. aeruginosa* and *S. aureus* were 1.25, 2.5, 2.5 and 10 mg. ml^{-1}, the present results indicated that chitosan nanoparticles are effective *in vitro* against the bacterial pathogens inducing omphalitis in broilers chicks.

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INTRODUCTION

Chicken is the most economical source of animal protein in comparison with red meat, particularly in developing countries, global broiler production exceeded 100.5 million tons in 2021 and is expected to further increase by 2% in 2022 (Maharjan *et al*., 2021). Pathogenic bacteria are the primary causes of poultry diseases worldwide, they are responsible for the most significant economic losses resulting in a huge annual financial loss exceeding 50 billion USD (Swidan *et al*., 2020).

Omphalitis, also referred to mushy chick disease and navel ill is a hatchery-born disease that is the major cause of first-week chick mortality. Mortality usually begins within 24 hours of the hatch and peaks within five to seven days (Jalob *et al*., 2015). Omphalitis is one of the most significant and critical infectious diseases responsible for early chick mortality with considerable financial loss, it is characterized by inflammation of the navel with improper closure. The disease spreads to the newly hatched birds with unhealed navels from the contaminated hatchery equipment and also vertically from carrier broiler breeder folks. The bacteria-induced omphalitis resulted in subsequent deterioration and decomposition of the residual yolk sac (Jawad *et al*., 2020). The residual yolk represents 20–25% of one-day-old chick's weight then gradually decrease and completely disappeared within the first week of life (Jalob *et al*., 2015). Many bacterial pathogens are responsible for navel illness including *Bacillus cereus, Clostridium, E. coli, Enterobacter spp*., *Enterococcus spp*., *Klebsiella spp*., *Proteus spp*., *Pseudomonas spp*., *Salmonella spp*., *Staphylococcus spp*., and *Streptococcus* (Oliveira *et al*., 2021).

Exaggerated antimicrobial resistance arises from the frequent application of antimicrobial agents for treating diseased animals, poultry and fish (Ali *et al*., 2021). Drug resistance is becoming a growing threat to public health, and the environment, it also induced therapeutic failure and

economic losses in animal production (Aboyadak *et al*., 2016) so, there is an urgent need for substituting antibiotics with a natural antimicrobial agent to overcome bacterial resistance.

Chitosan is a natural nontoxic, polycationic and biodegradable copolymer of glucosamine and N-acetylglucosamine, Chitosan is present in the shells of many crustaceans, such as crab, shrimp, squid pen and crawfish. Chitosan is a promising compound for biomedical applications as it is a very safe, non-toxic and tissue-compatible, biodegradable and mucoadhesive compound (Reshad *et al*., 2021). Chitosan is known to have a potent antimicrobial activity that may arise from binding to the negatively charged bacterial cell wall then disrupts and alters membrane permeability, chitosan is also, attached to bacterial DNA with inhibition of DNA replication followed by cell death (Li and Zhuang, 2020).

The present study aimed to identify the bacterial pathogens responsible for omphalitis in broiler farms then study the antibiotic susceptibility and determine the in-vitro chitosan nanoparticles activity against bacterial isolates.

MATERIALS AND METHODS

Synthesis of chitosan nanoparticles: Chitosan nanoparticles are synthesized by the cross-linking of chitosan to sodium tripolyphosphate solution at a final concentration of 10 mg. ml^{-1} as described by Ojagh *et al*. (2010).

Characterization of chitosan nanoparticles

Size: The morphology of chitosan nanoparticles was assayed by transmission electron microscopy (TEM), JEOL, JEM-2100, Japan as mentioned by Ali *et al*. (2022).

Electrophoretic mobility (zeta potential): Zeta potential was measured by NanoBrook Omni, Brookhaven Instruments, USA.

Study area, sampling and clinical examination: One-hundred clinical diseases chicks were collected alive from 10 broiler farms located in Kafrelsheikh governorate, Egypt (10 chicks/farm), samples were clinically examined and sacrificed humanely to record the postmortem lesions.

Initial bacterial isolation: One millilitre of yolk sample was inoculated in 9 ml of nutrient broth (Oxoid®, UK) and incubated overnight at 37°C. Nutrient agar plates supplemented with nystatin (250 U/ml) were streaked from broth and then incubated for 12 hours.

Bacterial identification

Phenotypic identification on selective media: A single colony was taken and incubated in nutrient broth for one day then sub-cultured on eosin methylene blue agar (EMB), Salmonella Shigella agar (SS agar), Baird-Parker agar (BP) and Pseudomonas agar base media (Oxoid®, UK).

The morphological characteristic of bacterial colonies grown on each selective differential media was recorded, each isolate was subjected to Gram's staining and bacterial motility was tested on a Sulfide Indole Motility medium (Oxoid®, UK).

Biochemical identification: Indole, methyl red, Vogues Proskauer, oxidase and citrate utilization, urease hydrolysis and H_2S production were for the biochemical identification of bacterial isolates.

Serological identification: Serological identification of *E. coli* was performed based on O (somatic) and (H) flagellar antigens using Denka Seiken Co. antisera kits according to the manufacturer's instructions. Serotyping of *Salmonella* was performed using Bio-Rad *Salmonella* antisera.

P. aeruginosa isolates were serotyped by the slide agglutination technique using the specific antisera (Bio-Rad®, France) and *Staphylococcus* isolates were serotyped using the Staphaurex Plus latex agglutination test, Remel Co, UK. Staphaurex test.

Antibiogram

Antibiotic susceptibility of the recovered bacterial isolates: The agar disc diffusion method was performed for determining the susceptibility of 17 *E. coli*, 10 *Salmonella*, 1 *P. aeruginosa* and single *S. aureus* isolate to Kanamycin, Clindamycin, Tetracycline, Nalidixic acid, Colistin, Sulfamethoxazole, Cefotaxime, Cefepime, Ampicillin, Ciprofloxacin, Erythromycin, Meropenem, Amikacin, Gentamicin, Oxacillin and Linezolid using the standard sensitivity discs, *Oxoid®, UK*.

Overnight broth culture was uniformly spread on the surface of Muller- Hinton Agar plates then antibiotic discs were placed over the agar surface, plates were incubated at 35°C for 24 h, and the inhibition zone was measured and interpreted as susceptible, intermediate susceptible and resistant (El-Bahr *et al*., 2019).

The multiple Antibiotic Resistance (MAR) index for each strain was determined in which

$$\text{MAR index} = \frac{\text{No. of resistance isolates}}{\text{Total No. of tested antibiotics}}$$

Antibacterial efficacy of chitosan nanoparticles against isolated bacteria

Agar well diffusion test: The agar well diffusion assay was carried out to screen the antibacterial effect of chitosan nanoparticles against the most antibiotic-resistant bacterial isolate from each pathogen (*E. coli* O78 isolate (1), *S. Kentukey* isolate, *P. aeruginosa* and *S. aureus* isolate, the test was performed according to Kadaikunnan *et al*. (2015).

Minimum Inhibitory Concentration (MIC): The broth dilution technique was used was performed as described by Ali *et al*. (2019) to determine the MIC of chitosan nanoparticles against the recovered bacterial isolates. Chitosan nanoparticles suspension was used at a final concentration of 10, 5, 2.5, 1.25, 0.625 and 0.3125 mg. ml^{-1}.

RESULTS

Characterization of prepared chitosan nanoparticles: Chitosan nanoparticles were spherical or semi-spherical particles, 20±5 nm in diameter (Fig. 1), zeta potential of chitosan nanoparticles was 28.3 mV.

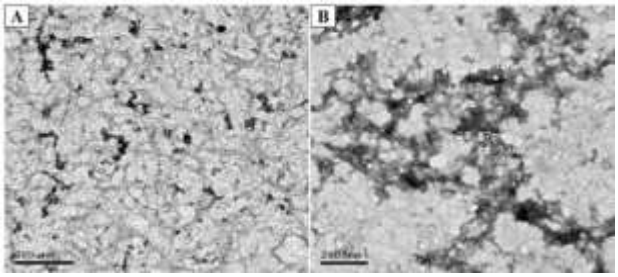

Fig. 1: TEM microphotographs showed chitosan nanoparticles.

Fig. 2: Improper closure of navel [A], wetted and inflamed navel area [B], distended abdomen with remarkable unabsorbed yolk sac [C], inflamed unabsorbed yolk sac with greenish *discolouration [D].*

Clinical, postmortem examination and mortality rate: Diseased chicks showed the typical clinical sign of omphalitis including improper closure of the navel with wet and inflamed tissues around the navel (Fig. 2A and B). Distended yolk sac with offensive odour, yolk consistency was liquid, flatulent or coagulated as in (Fig. 2C and D) and the mortality rate reached up to 15 – 20 % at one week.

Initial bacterial isolation: Eighty-six bacterial isolates were successfully recovered from the 100 chick samples that showed clinical omphalitis signs.

Identification of bacterial isolates

Phenotypic identification and biochemical characteristics of the recovered bacterial isolates: Forty-four bacterial isolates (51.16%) were grown as metallic-sheen colonies characteristic for *Escherichia coli* on the EMB agar. Isolates were motile, gram-negative short rods arranged in single, pairs or short chains. Twenty-one isolate was identified as *Salmonella spp*. through the attribute black colonies on SS agar represented 24.41% of total isolates. Isolates were motile, gram-negative rods arranged in single, pairs or short chains.

Thirteen isolates represented 15.11% of isolated pathogens were *P. aeruginosa* through the distinctive greenish colonies on Pseudomonas agar base media, bacterial cells appeared as gram-negative motile single rods. Eight isolates (9.3%) grew as black colonies on BPR agar indicating the presence of *S. aureus*. Bacterial cells are nonmotile gram-positive rounded cells arranged in grape-like clusters.

Table 1: Biochemical profile of bacterial isolates.

Tests	*E. coli*	*Salmonella*	*P. aeruginosa*	*S. aureus*
Indole Production	+	-	-	-
Methyl red	+	+	-	+
Voges Proskauer	-	-	-	+
Citrate Utilization	-	+	+	-
Oxidase Utilization	-	-	+	-
Urase Hydrolysis	-	-	-	+
H_2S Production	-	+	-	-

1.1. Serological identification:

Table 2: *E. coli* serotypes.

E. coli serotype	Characterization	No.	%
O2:H6	EPEC	7	15.9
O121:H7	EPEC	8	18.18
O55:H7	EPEC	6	13.6
O128:H2	ETEC	4	9
O78	ETEC	11	25
O26:H11	EHEC	2	4.5
O91:H21	EHEC	3	6.8
O159	EIEC	3	6.8
Total		44	100.0

Table 3: Recovered *Salmonella* serotypes.

Salmonella spp.	No. of isolates	%	O antigens	H antigens	
				Phase 1	Phase 2
Salmonella kentucky	1	4.75	8, 20	i	z6
Salmonella gallinarium	5	23.75	1, 9, 12	-	-
Salmonella newport	2	9.5	6, 8, 20	e, h	1, 2
Salmonella enteritidis	9	42.75	1, 9, 12	g, m	1, 7
Salmonella typhimurium	4	19.25	1, 4, 5, 12	i	1, 2
Total	21	100%			

All thirteen *P. aeruginosa* isolates were serotyped as group B antigen O5, six out of eight *Staphylococcus* isolates were having the clumping component and protein A (*S. aureus*).

The biochemical profile indicated the presence of *E. coli*, *Salmonella spp*., *P. aeruginosa* and *S. aureus* as represented in Table 1.

***Escherichia coli*:** Eight different serotypes of *E. coli* were classified into 4 pathogenic categories enteropathogenic (EPEC), enterotoxigenic (ETEC), enterohemorrhagic (EHEC) and enteroinvasive (EIEC) as shown in Table 2.

Salmonella *spp.*: *Salmonella* isolates were serotyped into nine *S. enteritidis* isolates (42.75%), five *S. gallinarium* isolates (23.75%), four *S. typhimurium* isolates (19.25%), two *S. Newport* isolates (9.5 %) and one *S. kentuchy* isolate (4.75%). The antigenic structure of different serotypes is shown in Table 3.

Antibiogram test results: Tested *E. coli* isolates were highly sensitive to Meropenem, Gentamicin, Colistin and kanamycin while they showed high resistance to Erythromycin, Oxacillin, Clindamycin, Nalidixic acid and Ampicillin as represented in Table 5, MAR index of tested isolates was ranged between 0.063 and 1 (Table 4).

Tested *Salmonella* serotypes were highly sensitive to Meropenem, Amikacin Ciprofloxacin and Gentamicin while all the tested isolates were completely resistant to Erythromycin, Oxacillin and Clindamycin, MAR index of tested isolates was ranged between 0.125 and 1 as represented in Table 5.

Antibiotic sensitivity test revealed that the *P. aeruginosa* isolate was sensitive to Amikacin, Linezolid, Cefepime, Meropenem and Kanamycin, this isolate was intermediate sensitive to Ciprofloxacin. On the contrary, it was resistant to Sulfamethoxazole, Tetracycline, Nalidixic Acid,

Table 4: Antimicrobial susceptibility of *E. coli* isolates.

Antimicrobial agents	Susceptible		Intermediate		Resistant	
	NO	%	NO	%	NO	%
Erythromycin	-	-	-	-	17	100
Oxacillin	-	-	1	5.9	16	94.1
Clindamycin	-	-	3	17.6	14	82.4
Nalidixic acid	2	11.8	2	11.8	13	76.5
Ampicillin	3	17.6	2	11.8	12	70.6
Cefepime	6	35.3	1	5.9	10	58.8
Tetracycline	6	35.3	2	11.8	9	52.9
Amikacin	8	47.1	-	-	9	52.9
Sulfamethoxazole	9	52.9	1	5.9	7	41.2
Cefotaxime	10	58.8	1	5.9	6	35.3
Linezolid	10	58.8	2	11.8	5	29.4
Ciprofloxacin	11	64.7	1	5.9	5	29.4
Kanamycin	12	70.6	-	-	5	29.4
Colistin	13	76.5	1	5.9	3	17.6
Gentamicin	13	76.5	2	11.8	2	11.8
Meropenem	16	94.1	-	-	1	5.9

Table 5: Antimicrobial susceptibility of *Salmonella* serotypes.

Antimicrobial agents	Susceptible		Intermediate		Resistant	
	NO	%	NO	%	NO	%
Clindamycin	-	-	-	-	10	100
Oxacillin	-	-	-	-	10	100
Erythromycin	-	-	-	-	10	100
Ampicillin	1	10	2	20	7	70
Tetracycline	1	10	2	20	7	70
Nalidixic acid	3	30	-	-	7	70
Sulfamethoxazole	2	20	2	20	6	60
Cefepime	4	40	-	-	6	60
Cefotaxime	3	30	2	20	5	50
Colistin	3	30	3	30	4	40
Kanamycin	5	50	1	10	4	40
Linezolid	6	60	-	-	4	40
Gentamicin	7	70	-	-	3	30
Ciprofloxacin	7	70	1	10	2	20
Meropenem	8	80	1	10	1	10
Amikacin	8	80	1	10	1	10

Erythromycin, Oxacillin, Cefotaxime, Ampicillin, Gentamicin, Clindamycin and Colistin with MAR index equals 0.625.

The tested *S. aureus* isolate was resistant to Clindamycin, Kanamycin, Tetracycline, Nalidixic Acid, Colistin, Sulfamethoxazole, Ampicillin and Erythromycin. On the other side, it was susceptible to Meropenem, Amikacin, Gentamicin, Oxacillin, Linezolid, Ciprofloxacin, Cefotaxime and Cefepime with equalised MAR index (0.5).

Antibacterial efficacy of chitosan nanoparticles: Agar well diffusion test revealed the sensitivity of four tested isolates to chitosan nanoparticles with inhibition zone diameter estimated by (16.0±1.5), (12.0±3), (13.0±1.5) and (9.0±2) mm for *E. coli* (O78), *S. kentucky*, *P. aeruginosa*, and *S. aureus* respectively. Broth dilution test also indicated similar results with MIC equals 1.25, 2.5 and 5 mg. ml^{-1} for *E. coli* (O78), *S. kentucky*, *P. aeruginosa*, and *S. aureus* respectively.

DISCUSSION

Yolk sac infection is one of the most serious poultry health issues responsible for decreased hatchability and higher chick culling rates. Bacterial contamination of eggshells, lack of hygiene in the nests, and simultaneous incubation of dirty with clean eggs are the main causes. The current research was conducted to determine the most prevalent bacterial diseases associated with yolk sac infection (omphalitis) in 10 poultry farms together with detecting the susceptibility of collected isolates to different antibiotics and to chitosan nanoparticles as well.

In the present work affected birds showed a 20% mortality rate during the first week, Taunde *et al*. (2021) also, reported a peak mortality rate at 3-5 days post-hatch. The unabsorbed congested yolk sac is the most prominent sign but, in a few cases, an unpleasant odour is raised after opening the yolk sac, the present findings come hand in hand with results reported by Jawad *et al*. (2020) and Mohibbullah *et al*. (2022). Eighty-six bacterial isolates were recovered from clinically diseased chicks, *E. coli* was the most dominant bacteria inducing omphalitis representing 51.15% of total isolates followed by *Salmonella* (24.4%), this finding was nearly similar to Abdel-Tawab *et al*. (2016) and Mohibbullah *et al*. (2022) results, they reported that *E. coli* was the most common bacterial pathogen associated with omphalitis representing 50, 47.93 and 46% of total isolates. Abdel-Tawab *et al*. (2016) and Mohibbullah *et al*. (2022) also, isolated *Salmonella* with a prevalence of 12 and 22 which is somewhat lower than our findings, these differences can be related to different study areas. *P. aeruginosa* and *S. aureus* are also important bacteria responsible for omphalitis, this finding is supported by several previous studies which linked such bacteria to yolk sac infections in Egypt (Elshraway and Abdel Hafez 2017; Abd El-Ghany 2021).

Biochemical identification has confirmed the results of bacterial isolation on selective differential media to genus level, moreover, the serological identification was also important to confirm the identity of recovered bacterial isolates to species and serotype level. In this study, eight *E. coli* serotypes have been identified, of them, the enteropathogenic pathotype was the most prevalent followed by enterotoxigenic, enterohaemorrhagic and enteroinvasive. These four pathotypes are the most fatal due to the variety of virulence factors and toxin production they have. Following our results, Hasona *et al*. (2023) found that enteropathogenic *E. coli* was the most dominant pathotype isolated from Egyptian broiler farms followed by enterohaemorrhagic and enterotoxigenic.

Salmonella enteritidis was the most common prevalent serotype in the studied farms followed by *S. larochelle* and *S. typhimurium*, in agreement with this result (El-Sharkawy *et al*., 2017; Shalaby *et al*., 2022) reported that *S. enteritidis* and *S. typhimurium* are most common biovars in the Egyptian farms. This study indicated that *E. coli* and *Salmonella* still represent a serious challenge to the poultry industry in Egypt. Regarding *P. aeruginosa* the recovered isolates were related to group B antigen O5 and represented 15.1%, (Dawod *et al*., 2018; Shahat *et al*., 2019) isolated *P. aeruginosa* at a rate of 16.66% and 8% from newly hatched chicks in Damietta and Luxor governorate respectively. *Staphylococcus* isolates represent 9.3% of them 7% serotyped as *S. aureus* meanwhile, other studies as Elshraway and Abdel Hafez (2017) isolate *S. aureus* from New Valley broiler farms at a rate of 51.33%, Salama *et al*. (2021) also, isolated *S. aureus* at a rate of 38.3% from the yolk sac and so this pathogen is the most prevalent in such localities.

Nearly all of the *E. coli* isolates were highly resistant to Erythromycin, Clindamycin, Nalidixic acid and Ampicillin, many isolates also, resist Tetracycline,

Amikacin, Sulphamethoxazol and Ciprofloxacin. MAR index over 0.2 which indicated high risk (Ayandele *et al*., 2020), 76.5% of *E. coli* isolates have MAR index over 0.2 and were resistant to the vast majority of antibiotics used in the veterinary field, many recent studies also reported exaggerated resistance of E. *coli* isolated from chicks and poultry to many antibiotics as mentioned by Hasona *et al*. (2023). In our point of view, a high MAR index was attributed to the frequent misuse of antibiotics in poultry farms and this highlighted on increasing drug fastness problem in the poultry industry so, there is a must for the development of an alternative treatment strategy based on using the natural antibacterial as chitosan nanoparticles.

All the tested *Salmonella* isolates completely resist Erythromycin, Clindamycin and Oxacillin and more than 50% of isolates resist Tetracycline, Nalidixic acid, Ampicillin, Sulfamethoxazole, Cefepime and Cefotaxime, these results were under Helal *et al*. (2019), particularly for isolates originated from the farmed poultry in Egypt. The MAR index of 70% of *Salmonella* isolates exceeded the acceptable value (0.2) which represents a great challenge, particularly with dangerous zoonotic pathogens like *salmonella* which resists potent antibiotics used for humans like Cefepime and Cefotaxime, this increases the challenge for developing a new recent and effective antibiotic.

Tested *P. aeruginosa* and *S. aureus* also had high MAR index equal to 0.625 and 0.5 respectively, a similar antibiotic resistance profile was observed by (Abdou *et al*., 2021; Salama *et al*., 2021), current research documented high antibiotic fastness in all the recovered pathogens from omphalitis cases and recommended the responsible use of antibiotics in poultry farms.

Scanning electron microscopy indicated that chitosan nanoparticles present as small spherical particles that concur with Chandrasekaran *et al*. (2020), who reported that chitosan nanoparticles have greater antimicrobial activity than chitosan and chitin. This is because the chitosan nanoparticles have a wider surface area and low particle size that promotes antibacterial activity. Zeta potential of chitosan nanoparticles was 28.3 mV and so it could exert its antibacterial activity through binds to the negatively charged bacterial cells to disrupt and alter membrane permeability.

The microbiological assay has proved the effective antibacterial activity of chitosan nanoparticles against the multi-antibiotic resistant *E. coli* (O78), *S. kentucky*, *P. aeruginosa*, and *S. aureus* isolates, this result agreed with the results previously reported by (Ali and Bakheet 2020; Chandrasekaran *et al*. 2020). chitosan nanoparticles could be an effective and alternative method to control the multidrug-resistant bacterial pathogens that cause omphalitis in vitro and further studies is needed to determine the antibacterial efficacy in vivo.

Conclusions: *E. coli*, *Salmonella spp.*, *P. aeruginosa*, and *S. aureus* are the main cause of omphalitis in the studied broiler, infection resulted in about 20% mortality rate, and tested isolates have a high multi-antibiotic resistance index. Chitosan nanoparticles were effective against MAR isolates in vitro but further studies are needed to estimate the therapeutic activity in termination of bacterial infection in birds.

Authors contribution: N.S. performed the experiments, analyze the data and wrote the manuscript; N.G.A. wrote, reviewed and edited the manuscript; M.A, F.K. & M.I. supervised. All authors review and approve the manuscript.

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