Pakistan J. Zool., pp 1-9, 2023. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20220802110804

Genetic Analysis of Antimicrobial Resistance Genes in *Salmonella* Isolated from Diseased Broilers in Egypt

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ABSTRACT

Salmonella spp. are known to be a major cause of foodborne infection; it primarily spreads from poultry to humans, significantly burdening public health, especially with the currently high rates of antimicrobial resistance and the emerging multidrug-resistant strains. As a result, this study determined the patterns of antibiotic resistance in *Salmonella* spp., which was isolated from sick broilers from different farms in Egypt. Then, we investigated the presence of extended-spectrum beta-lactamases and plasmid-mediated quinolone resistance genes in *Salmonella* isolates. First, 800 internal organs (heart, liver, intestine, and yolk sac) were collected from 200 infected broilers to genetically analyze their recovered *Salmonella* antimicrobial resistant genes. Ten isolates of *Salmonella* were recovered: two (20%) for each *S. enterica* serovar Grampian, *S. enterica* serovar Kentucky, and *S. enterica* serovar Blegdam and then one (10%) for each *S. enterica* serovar Hadar, *S. enterica* serovar Anatum, *S. enterica* serovar Kirkee, and *S. enterica* serovar Tranoroa in the serotypes of isolated biochemically identified *Salmonella*. As per the results of this study, *Salmonella* isolates demonstrated multidrug-resistant phenotypes, with the highest resistance being against ampicillin, cefoxitin, cefpodoxime, and oxacillin (100%) and then against cefotaxime (80%), ceftazidime (70%), ciprofloxacin, ceftriaxone, and nalidixic acid (60%), including amoxicillin–clavulanic acid (50%). Furthermore, antimicrobial resistance genes, such as ESBL (bla_{TEM}, bla_{SHV}, and bla_{CMY-2}), and quinolone resistance genes (*qnrA*, *qnrB,* and *qnrS*) were examined in these isolates. Results showed that although all isolates tested were found negative for *qnrA* and *qnrB* and positive for the *qnrS*, they were positive for the ESBL genes bla_{TEM} and bla_{SHV} but negative for bla_{CMY-2}. In conclusion, the multidrug-resistant bacteria, *Salmonella*, demonstrated a high incidence in the diseased broiler chickens, with a possibility of human infection and treatment failure. Therefore, it is highly recommended that developing countries drastically reduce the overuse of antibiotics in poultry.

Article Information
Received 02 August 2022
Revised 06 August 2022
Accepted 10 August 2022
Available online 22 May 2023
(early access)

Authors' Contribution
MFS and AMR collected the samples, conducted the experiments and analyzed the results. SMH and AMR analyzed the data. All authors discussed the results and wrote the manuscript.

Key words
***Salmonella enterica*, Broiler, Antibiotic resistance, Quinolone resistance genes, multidrug-resistant bacteria**

INTRODUCTION

Consumption of tainted food poses the risk of various foodborne diseases with the possibility of outbreaks, making food safety a global public health issue. The yearly cases of food poisoning are around 600 million (approximately 1 in 10 people worldwide) with 420,000 cases ending with death losing 33 million disability adjusted life according to a recent report from the WHO (WHO, 2020). Poultry and its products are major prevalent sources of non-typhoidal *Salmonella* infections in human (Eguale, 2018). *Salmonella* is one of the most prevalent bacteria that cause gastrointestinal illnesses in livestock and poultry. *Salmonella* infections are highly linked to the consumption of tainted poultry products (Cogan and Humphrey, 2003). Controlling *Salmonella* in poultry, on the other hand, is difficult; for broiler chickens, this has historically relied on a balance of farm biosecurity and antibiotic usage (Davies, 2005). Since the early 1960s, *Salmonella* isolates with clinically relevant antibiotic resistance have been documented as majority of the resistance was restricted to a single antibiotic (Bulling *et al*., 1973; Cherubin, 1981; Van leeuwen *et al*., 1979). However, since the mid-1970s, *Salmonella* isolates with MDR characteristics have been on the rise all across the world. Antimicrobial-resistant *Salmonella* has been found in foods of animal sources,

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0030-9923/2023/0001-0001 $ 9.00/0

raising worries that treatment of human salmonellosis may be jeopardized because strains with antimicrobial resistance tend to be more frequently linked with severe illness than susceptible isolates (Helms *et al*., 2002; Varma *et al*., 2005). As antibiotic-resistant bacteria proliferate, curiosity in the genetics and resistance mechanisms that bacteria have developed to fend off antimicrobial drugs has increased (Ahmed and Shimamoto, 2012). Antibiotic misuse, abuse, and overuse have resulted in inefficiency and exacerbated the seriousness of this zoonotic disease (Cruchaga *et al*., 2001). The resistance to antimicrobial medications has risen over the past years creating a significant concern and challenge for public health professionals worldwide. However, the condition is much more severe in developing countries because strategies to prevent antimicrobial resistance are only of minor concern (Da Costa *et al*., 2013). Hence, such a high incidence of antimicrobial resistance in *Salmonella* spp. necessitates the determination of a resistance dissemination route, horizontally or vertically, in the evolution of MDR strains (Nemati and Ahmadi, 2020). However, as we gain a better understanding of the genome's molecular fluidity, any attempt to combat bacteria results in more bacterial adaptation or evolution to occur in the new free ecological niche (Velge *et al*., 2005). Resistance molecular basis in *Salmonella* isolates from livestock and poultry worldwide have been identified in several investigations (Ahmed *et al*., 2009; Zhao *et al*., 2007). In different *Salmonella* serovars, studies have reported that the rapid improvement in resistance to extended-spectrum cephalosporin was related to the plasmid-mediated manufacturing of β-lactamase-producing bacteria (EFSA, 2008, 2009; Authority, 2018). TEM genes (bla_{TEM}) and SHV genes (bla_{SHV}) are the main genes involved for ESBL production (Habeeb *et al*., 2013). This ongoing evolution poses a serious threat to public health by causing bacterial infections treatment limitation (Sharma *et al*., 2013; WHO, 2013). Quinolone resistance genes mediated by plasmids have recently been discovered in several Enterobacteriaceae, and their incidence is increasing worldwide (Poirel *et al*., 2012). Although the PMQR genes expression only provides a limited amount of quinolone resistance, it can enable the additional chromosomal resistance mechanisms selection, resulting in the emergence of highly resistant quinolone-resistant bacteria (Strahilevitz *et al*., 2009; Tamang *et al*., 2011). Of particular concern is the recent plasmid-mediated quinolone resistance development in various parts of the world, which is encoded by a large number of *qnr* genes. Furthermore, both clinical and food isolates of *Salmonella* have recently sharply increased ciprofloxacin resistance (Lin *et al*., 2015). The relevant gene, *qnr*, was shown to be unique from other quinolone resistance genes previously identified (Tran *et al*., 2002). Therefore, in this study, we investigated how widespread the resistance genes for broad-spectrum beta-lactamase and quinolone antibiotics are in *Salmonella* isolates from diseased broilers.

MATERIALS AND METHODS

Sampling

A total of 800 internal organs (heart, liver, intestine, and yolk sac) were collected from 200 diseased broiler chickens from various farms in Egypt in a poultry lab. The broilers have clinical signs of salmonellosis as pasty vent, whitish diarrhea, roughed feather and poor general condition and their postmortem examination revealed bronze discoloration and enlargement of liver with necrotic foci and pericarditis with enlarged heart, peritonitis, perihepatitis, intestinal and caecal inflammation and unabsorbed yolk sac in young chicks. Sterile plastic bags were used to preserve the samples which were then transported in an icebox directly to the Animal Health Research Institute, Tanta branch.

Isolation and identification of Salmonella *(ISO 6579-1: 2017)*

The organs' surface was scorched by hot spatula, then a sterilized loop was inserted through scorched part of the organ. All samples (liver, heart and yolk sac) were obtained aseptically and enriched in buffered peptone water for non-selective enrichment. Pre-enrichment is essential to allow the detection of low number of *Salmonella* or injured *Salmonella.* At room temperature, 10 ml of buffered peptone water were inoculated with 1 gm of the tested material using a 1/10 dilution (weight to volume). Then incubated at 37°C for 18 h. After that, all samples (Intestine, liver, heart and yolk sac) were inoculated into tubes containing Selenite F broth for inhibition of coliforms and certain other microbial species and thus, was beneficial in the restoration of *Salmonella* species. A tube containing 10 ml of selenite F broth and 1 cm of the pre-enrichment culture were combined, and they were incubated at 37 °C for 18 h. A 10 µl loop-full of selenite F broth was spread on the surface of xylose lysine desoxycholate (XLD) agar and incubated for 24 h at 37°C. By inoculating into triple sugar iron agar slopes, *Salmonella*-typical morphology in the form of doubtful colonies was verified biochemically. For upcoming research, the probable colonies were collected and preserved on semisolid agar.

Various biochemical tests such as oxidase reaction, urea hydrolysis test, triple sugar iron agar, indole reaction, methyl red test, reaction of Voges Proskauer, citrate utilization test, lysine decarboxylation test identification of *Salmonella* according to Quinn *et al*. (2002).

Serological typing of Salmonella

Using particular O and H agglutinating antisera, standard *Salmonella* isolates were further serotyped (USA, Difco, NJ, Franklin Lakes) in accordance with the Kauffmann White serotyping scheme (Grimont and Weill, 2007). Specifically, bacterial motility was detected following a previous study (Cruickshank *et al*., 1975). Then, Gram staining was used to microscopically identify suspected colonies under an oil immersion lens to observe the Gram-negative bacilli morphological traits (rod-shaped).

Antimicrobial susceptibility tests

Mueller-Hinton agar medium (Oxoid) is used according to the Clinical Laboratory Standard Institute (CLSI, 2011). According to the manufacturer's instructions, the Mueller-Hinton agar was produced. *Salmonella* isolates were tested in vitro for quinolone resistance and extended-spectrum beta-lactamase. The following list of antibiotics in use: ampicillin (AMP), 30 µg; amoxicillin-clavulanic acid (AMC), 20/10 µg; cefotaxime (CTX), 30 µg; cefoxitin (FOX), 30 µg; cefpodoxime (CPD), 10 µg; ceftriaxone (CRO), 30 µg; ceftazidime (CAZ), 30 µg; ciprofloxacin (CIP), 5 µg; oxacillin (OXA), 30 µg and nalidixic acid (NAL), 30µg.

PCR screening for antimicrobial resistance genes in Salmonella

In our study, for DNA extraction from samples, we used the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with definite changes depending on the manufacturer's instructions. Part of the sample suspension (200 µl) was treated with 10 µl of proteinase K and 200 µl of lysis buffer for 10 min at 56°C. 200 µl of 100 percent ethanol was then added to the lysate following incubation to be followed by sample washing and incubation based on the manufacturer's instructions. Using a kit and 100 µl of elution buffer, the nucleic acid was eluted. This is an oligonucleotide primer. Metabion (Germany) contributed the primers, which are shown in (Table I). *qnrA*, *qnrB*, *qnrS*, bla_{TEM}, bla_{SHV}, and bla_{CMY-2} genes PCR amplification: To test the primers a 25 µl reaction that includes 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 5.5 µl of water, 1 µl of each forward and reverse primers at 20 pmol concentration, and 5 µl of DNA template. 2720 thermal cyclers were applied to proceed the reaction. 5V/cm gradients in 1x TBE buffer were used to separate the PCR products electrophoretically at room temperature on a 1.5% agarose gel (Applichem, Germany, GmbH). Each gel slot received 15 µl of the goods for analysis. For determining the fragment sizes, a gene ruler 100 bp ladder (Fermentas, Germany) was used. For gel photography, a gel documentation system (Alpha Innotech, Biometra) was used. Computer software was used to evaluate the data. Time conditions and temperature of the two primers during PCR are presented. *S. enteritidis* was used as positive control, while DEPC-treated pure water was used as negative control.

RESULTS

For all genes the 35 thermal cycles comprised each of primary denaturation at 94°C for 5 min, secondary denaturation at 94°C for 30 s, annealing at different temperatures (57°C for *qnr* A, 53°C for *qnr* B, 48°C for *qnr* S, 54°C for bla_{TEM} and bla_{SHV}, and 55°C for bla_{CMY-2}) for 45 s, Extension at 72 °C for 45 s and final extension at 72 °C for 10 min.

Table I. Oligonucleotide primer sequences for detecting resistant *Salmonella* genes.

Gene	Nucleotide sequence 5`→3`	Amplified product	Reference
qnrA	GATAAAGTTTTTCAGCAAGAGG	543 bp	Cambau *et al*., 2006
	ATCCAGATCGGCAAAGGTTA		
qnrB	ATGACGCCATTACTGTATAA	562 bp	Azeez *et al*., 2018
	GATCGCAATGTGTGAAGTTT		
qnrS	ATGGAAACCTACAATCATAC	491 bp	Le Thi Minh Vien *et al*., 2009
	AAAAACACCTCGACTTAAGT		
bla_{TEM}	ATCAGCAATAAACCAGC	516 bp	Colom *et al*., 2003
	CCCCGAAGAACGTTTTC		
bla_{SHV}	AGGATTGACTGCCTTTTTG	392 bp	
	ATTTGCTGATTTCGCTCG		
CIT (bla_{CMY-2})	TGG CCA GAA CTG ACA GGC AAA	462 bp	Pérez-Pérez and Hanson, 2002
	TTT CTC CTG AAC GTG GCT GGC		

Prevalence of Salmonella

All suspected colonies (pink with black centers) were identified on the XLD media, including a typical colony on the *Salmonella–Shigella* agar (colorless with or without black center). Specifically, Gram-negative nonspore-forming rods were observed on Gram-stained colonies. Then, motility test revealed that the *Salmonella* isolates were extremely motile. Furthermore, biochemical analysis revealed that while all isolates were nonlactose fermenting with a negative oxidase reaction, most isolates produced hydrogen sulfide and were positive for methyl red and citrate and negative for Voges–Proskauer, indole, and urease hydrolysis tests. Nevertheless, the total percentage of *Salmonella* species identified by biochemical tests was 10%, resulting in 80/800 *Salmonella* isolates from the investigated organs (24/200 isolates from the liver, 32/200 isolates from the yolk sac, 8/200 isolates from the heart, and 16/200 isolates from the intestine) (Table II).

Table II. Prevalence of *Salmonella* isolated from diseased broiler chickens.

Examined organs in 200 broiler chickens	Positive *Salmonella*	
	No	%
Liver	24	12
Intestine	16	8
Heart	8	4
Yolk sac	32	16
Total (800)	80	10

Note: The (%) rate of each number is obtained by dividing the number by the total number of samples.

Serotyping of isolated Salmonella

The isolates were two for each *Salmonella enterica* serovar Grampian, *Salmonella enterica* serovar Kentucky, and *Salmonella enterica* serovar Blegdam and then one for each *Salmonella enterica* serovar Hadar, *Salmonella enterica* serovar Anatum, *Salmonella enterica* serovar Kirkee, and *Salmonella enterica* serovar Tranoroa.

Antimicrobial susceptibilities of different Salmonella *isolate serotypes*

Ten isolated *Salmonella* serovars were tested for their resistance to ESBL and quinolone. Results showed that 100% of the isolates were resistant to ampicillin, cefpodoxime, cefoxitin, and oxacillin, while 80% were found to be resistant to cefotaxime; 70% to ceftazidime; 60% to ciprofloxacin, ceftriaxone, and nalidixic acid; and 50% to amoxicillin–clavulanic acid.

Incidence of PMQR and β-lactamase-encoding genes in Salmonella *isolated from diseased broilers*

Plasmids are known to mediate quinolone resistance genes. At 543 and 562 bp, while all isolates tested negative for *qnrA* and *qnrB*, respectively, they all tested positive for *qnrS* (at 491 bp). Meanwhile, although all isolates tested positive for ESBL, bla_{TEM} (516 bp) and bla_{SHV} (392 bp) genes, they were negative for bla_{CMY-2} (462 bp) (Supplementary Fig. 1).

DISCUSSION

Salmonella is known to be a major zoonotic pathogen, with poultry serving as one of its primary hosts. Therefore, infections with *Salmonella* are a significant hazard to the poultry farming sector in developing countries (Li *et al*., 2018). In this study, we demonstrated that the yolk sac had the highest rate of *Salmonella* isolates isolates (16%), followed by the liver (12%). However, this rate differed from that previously reported (El-Mohsen *et al*., 2022), which observed that *Salmonella* was more prevalent in the liver by 13.33% than in the yolk sac by 9.3%. Also Menghistu *et al*. (2011) found the prevalence of *Salmonella* was 2.7% (7/260) from 220 poultry tissue samples and 40 egg samples and the highest number of *Salmonella* isolates came from liver and intestine. The findings of our study also differ from yet another study by Eguale (2018), which observed a *Salmonella* prevalence rate of 4.7%. Finally, 14% of the understudied samples were *Salmonella* positive in the study by El-Tawab *et al*. (2019). Alternatively, results of serotyping matched those by Rady *et al*. (2020). They reported *S.* kentucky as the most common serotype of the *Salmonella* isolates and with Zhang *et al*. (2018) who found *S*. Kentucky as one of the most dominant serotypes in chicken samples by (12.6%). Whereas Ammar *et al*. (2016) disagreed with these findings because their study isolated *Salmonella enterica* serovar Kentucky in 12.5% of *Salmonella* isolates, alongside other serotypes *Salmonella enterica* serovar Enteritidis (56.25%) and *Salmonella enterica* serovar Typhimurium (18.75%). Additionally, *Salmonella* isolates in our study showed different antimicrobial resistance results, similar to a previous study by El-Tawab *et al*. (2019). While they detected that 89% of *Salmonella* species were cefotaxime-resistant, Rady *et al*. (2020) detected that many isolates were resistant to both ampicillin (90%) and nalidixic acid (88%). Nevertheless, nalidixic acid and ampicillin had the highest antibiotic resistance against *Salmonella* isolates within the chicken production chain, whereas ciprofloxacin was linked to low resistance levels (Castro-Vargas *et al*., 2020). This could partially agree with Yildirim *et al*. (2011) who found that all isolates of *Salmonella* spp., exhibited resistance to ampicillin, oxacillin and cefotaxime were evident 97%,

85.2% and 2.9%, respectively. Also Waghamare *et al*. (2018) mentioned that *Salmonella* isolates were resistant to ampicillin, ciprofloxacine and cefotaxime by 21.43%, 19.05% and 14.19%, respectively. While Singh *et al*. (2013) reported that all *Salmonella* isolates were sensitive to ampicillin. Our study finding the resistance to amoxicillin-clavulanic acid by 50 % and this higher than Khan *et al*. (2021) who found it by 2.4%. Our results for antimicrobial resistance were different from Yang *et al*. (2013) who found the resistance to ampicillin by 45.6%, nalidixic acid by 75.8%, ciprofloxacine by 12.1%, ceftriaxone by 6.0% and cefoxitin 4.0%. Regarding these findings, the careful use of antibacterial medicines in clinical, veterinary, and agricultural contexts is strongly suggested to preserve antibiotic efficacy and prevent the development of cross-resistance. Quinolones are widely used in veterinary medicine to treat *Salmonella* infections over the world Mehdi *et al*. (2018). This work looked for ESBL and PMQR genes in *Salmonella* isolates from infected broiler chickens. According to global studies, there has been an alarming increase in beta-lactam antibiotic resistance. In this study, we have showed that although *Salmonella* strains were negative for *qnrA* and *qnrB* they were positive for the *qnrS* in all isolates of this investigation partially agreeing with the study by Dembélé *et al*. (2020), who could not identify *qnrA* and *qnrS* in any *Salmonella* strain. These findings highlight the low incidence of *qnr* among *Salmonella* isolates. However, Soliman *et al*. (2017) found the plasmid-mediated quinolone-resistance gene *qnrA1*. Furthermore, we observed that *Salmonella* isolates were more fluoroquinolone-resistant, as evidenced by PCR for *qnrS*, PMQR genes, revealing that 100% of the samples tested positive for *qnrS*. This outcome was greater than what had previously been reported by Abo-Remela *et al*. (2015) who were able to identify that 18% were positive for *qnrS*. Furthermore, another study by Zhao *et al*. (2017) discovered that while *qnrA* and *qnrB* had a high incidence, *qnrS* had a low incidence. But in 2020 (Zhao *et al*., 2020) reported *qnrB* with low incidence (6/67, 9.0%). However Yang *et al*. (2013) could identify *qnrA*, *qnrB* and *qnrS* genes by (46.6%), (12.7%), (19.5%) respectively. Besides, although Dembélé *et al*. (2020) could not identify bla_{TEM} and bla_{SHV} in ESBL, dominant beta-lactamase genes detected in our investigation were similar to the previously reported data by Eguale *et al*. (2017). While Ramatla *et al*. (2022) could find high levels of β-lactamase encoding genes bla_{TEM} in their *Salmonella* isolates. Also Zhao *et al*. (2021) found the majority of isolates harbored the bla_{TEM} gene (74.4%). Shahada *et al*. (2010) also could identify the wild-type bla_{TEM-1} gene that mediated resistance to ampicillin. Soliman *et al*. (2017) also found bla_{TEM-1} in *Salmonella* isolates and Zhao *et al*. (2020) found bla_{TEM} in all *Salmonella* isolates (100%). Rady *et al*. (2020) found that all isolates were positive ESβLs genes but were negative for bla_{CMY} gene. While Ahmed and Shimamoto (2012) could identify bla_{CMY-2} in one isolate of *Salmonella enterica* serovar Enteritidis only. Moreover, Adel *et al*. (2021) reported β-lactamase-encoding genes, including $bla_{SHV-12,}$ bla_{CMY-2} (AmpC type), and bla_{TEM-1} in the *Salmonella* isolates. Sabry *et al*. (2020) found 16 of *Salmonella* isolates were ESBL-producing with the majority carrying bla_{SHV} and bla_{TEM} genes and 4 ESBL-negative isolates carried bla_{CMY-2}.

CONCLUSION

Salmonella serovars obtained from diseased broilers have a high resistance rate to quinolones and β-lactams. Accordingly, this study has detected quinolone-resistant and ESBL-producing Enterobacteriaceae in rather significant numbers. Furthermore, high frequencies of *qnrS*, bla_{TEM}, and bla_{SHV} were observed in all isolates. Thus, identifying quinolone-resistant and ESBL-producing Enterobacteriaceae is critical for effective therapy and infection management. Hence, proper use of these antibiotics will restrict the propagation of resistance genes while reserving their use for therapeutic purposes.

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20220802110804

Statement of conflict of interest

The authors have declared no conflict of interest.

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Pakistan J. Zool., pp 1-9, 2023. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20220802110804

Supplementary Material

Genetic Analysis of Antimicrobial Resistance Genes in *Salmonella* Isolated from Diseased Broilers in Egypt

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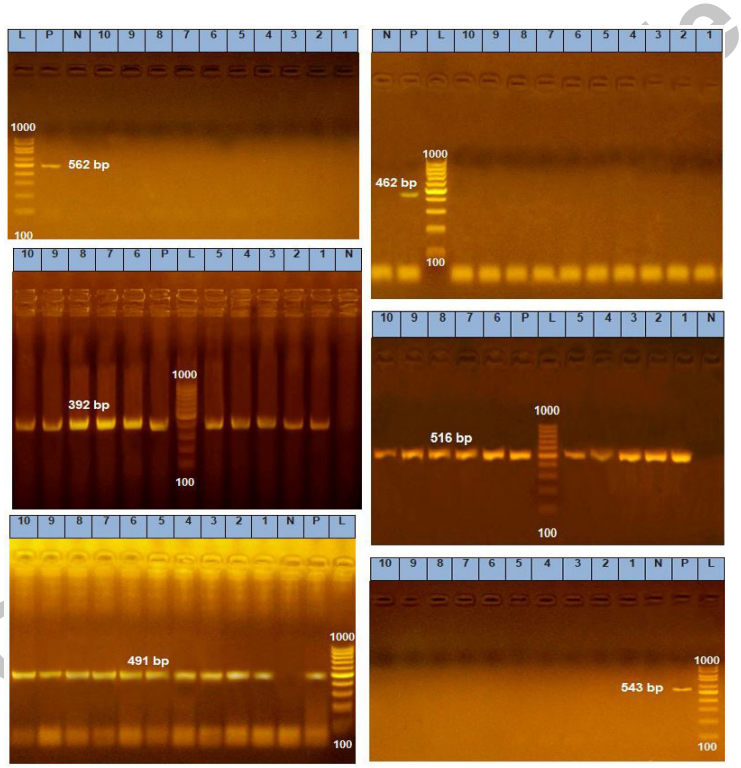

Supplementary Fig. 1. Agarose gel electrophoretic PCR pattern for detecting *qnrA*, *qnrB*, *qnrS*, bla_{TEM}, bla_{SHV} and bla_{CMY-2} at 543 bp, 562 bp, 491 bp, 516 bp, 392 bp and 462 bp respectively L: Ladder from 100 bp to 1000 bp P: Positive control N: Negative control: field isolate tested and confirmed by PCR to be negative for the related genes Lane 1 to 10: Negative amplification of *qnrA, qnrB and* bla_{CMY-2} and Positive amplification of *qnrS,* bla_{TEM} *and* bla_{SHV}.

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0030-9923/2023/0001-0001 $ 9.00/0