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Antibiotic Resistance of *Escherichia coli* and *Salmonella* Species Isolated from Table Eggs in Morocco

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ABSTRACT

The development of antimicrobial resistance has become a severe global public health emergency. Foods of animal origin are considered possible drivers of resistant bacteria, including *Escherichia coli* (*E. coli)* and *Salmonella* spp. It is associated with the indiscriminate use of antibiotics, resulting in the inability to treat patients infected with antibiotic-resistant pathogens and a high risk of transmission of these resistant pathogens. The current study aimed to determine the prevalence and antibiotic resistance of *E. coli* and *Salmonella* spp. in raw table eggs in Morocco. A total of 870 table eggs resulting from 290 samples (3 eggs = 1 sample), were purchased from ambulatory sellers, street vendors, kiosks, and neighborhood markets from different cities in Morocco and transferred to the laboratory in the Hassan II Agronomy and Veterinary Medicine Institute of Rabat, Morocco. The egg shells and contents were tested separately then the isolation and identification of bacterial pathogens were performed according to the Moroccan Standard Norms. The bacterial isolates were tested for susceptibility to six commonly used antibiotics, namely nalidixic acid (30 µg), kanamycin (30 µg), gentamycin (15 µg), ciprofloxacin (15 µg), tetracycline (30 µg), and amoxicillin (10 µg). The findings revealed that 38 samples (13%) tested positive for *E. coli* of which 9% were on egg shells, and 4% were in egg content, while for *Salmonella enteritidis* (*S. enteritidis*), 5 samples (2%) tested positive and only in the egg contents. *Escherichia coli* showed the highest resistance to amoxicillin, followed by tetracycline and nalidixic acid with 92.10%, 84.21%, and 50%, respectively, and was sensitive to ciprofloxacin (84.21%), kanamycin (65.79%), and gentamicin (60.54%). *Salmonella enteritidis* had the highest resistance against tetracycline (80%), followed by ciprofloxacin and nalidixic acid with 40% each. The highest sensitivity rates of *S. enteritidis* were for gentamicin, amoxicillin, and kanamycin at 80%, 80%, and 40%, respectively. Given that these resistant bacteria could potentially be transferred to humans through eggs or egg products, it is necessary to employ strict hygiene measures and provide a wise and legal use of antibiotics in animal breeding.

Keywords: Antibiotic resistance, *Escherichia coli*, *Salmonella enteritidis*, Table egg

INTRODUCTION

Antimicrobial resistance (AMR) is one of the world's most challenging problems today (WHO, 2021). The inappropriate use of antibiotics, including their use in animal production systems as growth promoters and their overuse in clinical treatments, has created selective pressure on bacteria in recent years leading to defense systems against these antibiotics and a therapeutic impasse (Zhao et al., 2012; Roca et al., 2015; Zwe et al., 2018). It is estimated that AMR leads to the annual death of approximately 700,000 people worldwide (Clifford et al., 2018). These resistant bacteria may contaminate humans directly through cross-contamination or/and handling of contaminated food or indirectly when contaminated food or food products are consumed (Collignon et al., 2016; Lambrecht et al., 2018).

Among the foods responsible for AMR transmission, poultry, and poultry products act as the primary vector for transferring antimicrobial-resistant bacteria and antimicrobial-resistance genes to humans (de Mesquita Souza Saraiva et al., 2022) since the inappropriate use of antibiotics as a treatment and growth promoters at sub-therapeutic doses can lead to their development. However, many bacteria are isolated from poultry meats and products, WHO considers *Salmonella* spp. and *Escherichia coli* (*E. coli*) as the most responsible bacteria for AMR transmission (WHO, 2021). These two bacteria have been shown to cause major infectious diseases in both poultry and humans. The *E. coli* is the main causative agent of cellulitis, septicemia, and aerosacculitis in poultry, and *Salmonella* spp. is the causative agent of

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pullorum disease, avian typhoid, and avian paratyphoid (Gomis et al., 1997). In addition to mild to severe gastrointestinal diseases, *E. coli* can cause urinary tract infections, pneumonia, meningitis, and peritonitis in humans (Schoeni and Doyle, 1994). *Salmonella* spp. can also cause human foodborne gastroenteritis, a disease characterized by intestinal inflammation and self-limited diarrhea (Winter et al., 2010).

Moreover, *E. coli* and *Salmonella enterica* subsp. *enterica* serovars are the most common avian pathogens that can be vertically transmitted through eggs (Singh et al., 2010). Many studies have isolated *Salmonella* spp. and *E. coli* from eggs (Adesiyun et al., 2005; Stepien, 2010; El ftouhy et al., 2022), and several egg-borne outbreaks of salmonellosis have been reported (Guerra-Centeno et al., 2020), considering eggs a possible vehicle for resistant bacteria and genes.

In Morocco, despite the increasing demand for the production (5.5 billion units in 2020, according to FISA) and consumption of table eggs, there is a lack of information on their microbiological quality, foodborne pathogens, and AMR, particularly *E. coli* and *Salmonella enteritidis* (*S. enteritidis*), isolated from eggs or egg products. Also, previous studies have demonstrated that treatment failures were linked to reports of increased antimicrobial resistance (Filali et al., 1988; Amara et al., 1995). Therefore, the current study aimed to investigate the occurrence and the antimicrobial resistance of *Salmonella* spp. and *E. coli* in fresh table eggs in Morocco.

MATERIALS AND METHODS

Ethical approval

All procedures in the present study were carried out following the Hassan II Agronomic and Veterinary Institute of Rabat, Morocco, and Moroccan Ministry of Agriculture recommendations, which are in accordance with international ethical standards (European Union Directive 2010/63/EU) legislation and ARRIVE (Animal Research Reporting of *in vivo* Experiments) guidelines.

Sample collection

A total of 870 table eggs, resulting from 290 samples of 3 eggs each, were purchased from ambulatory sellers, street vendors, kiosks, and neighborhood markets from January to September 2021. Eggs were kept under ambient temperature on the markets. Samples were collected from different locations in Morocco, namely Kenitra, Sale, Rabat, Temara, Mohammedia, Casablanca, and Benslimane. Once collected, eggs were transferred aseptically to the microbiology laboratory of the Avian Pathology Unit at the Hassan II Agronomy and Veterinary Medicine Institute in Rabat, Morocco, to run different analyses for egg shells and contents.

Egg shells

A swab technique was applied. The surface of the whole eggs was aseptically swabbed with a sterile cotton swab moistened in a sterile distilled water solution (Adesiyun et al., 2005).

Egg content

Eggs were soaked in 70% ethanol for 5-10 seconds to disinfect and then air-dried near the Bunsen burner. Then, the egg contents (3 eggs) were decanted and pooled into a sterile stomacher bag before finally being mixed manually for 30 seconds to obtain a homogeneous mixture. Both swabs and a mixture of egg contents were used separately to inoculate 9 ml of water peptone buffer and incubated at 37°C for 18-24 hours (Adabara et al., 2020).

Isolation of *Salmonella*

For *Salmonella* spp. isolation, 0.1 ml of the pre-inoculated buffered peptone water (CM 0509 Oxoid, Oxoid LTD, Basingstoke, Hampshire, England) was transferred to 10 ml of Rappaport-Vassiliadis Soja (RVS; BK148HA Biokar diagnostics, Zac de Ther, France) and incubated at 42°C for 24 hours. A loopful of RVS was transferred to Xylose lysine deoxycholate (BK058HA Biokar diagnostics, Zac de Ther, France) agar and incubated at 37°C for 24-48 hours according to the Moroccan standard NM ISO 6579, 2007 (NM 08.0.103), which is similar to the international Norm (ISO, 2002).

Isolation of *Escherichia coli*

For *E. coli*, a loopful of the broth water peptone buffer was subcultured on Eosin Methylene Blue agar (EMB agar; CM 0069 Oxoid, Oxoid LTD, Basingstoke, Hampshire, England) and then incubated at 37°C for 24 hours (Siriporn et al., 2015). All isolated bacteria were identified according to their colony, color, shape, morphology, and color change of the culture media. They were also dye-stained with Gram stains and examined under a × 100 light microscope (OPTIKA B-151, ITALY) using oil immersion. In addition, the performed biochemical tests included coagulase (6BR0020, Biokar diagnostics, Zac de Ther, France), catalase (1840, SOLVAPUR**,** SOLVACHIM, Morocco), oxidase (MICROBAT Oxoid LTD, United Kingdom), and motility tests using Mannitol Motility Nitrate Medium (M1320, HI Media laboratories,

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Mumbai, India). The semisolid nature of this medium helps to detect motility due to 0.35% agar. This detection was also confirmed by API 20E (20 100, biomerieux, Marcy-l'Etoile, France) for further biochemical identification (Abdullah, 2010).

Antibiotic sensitivity tests

Individual colonies of confirmed isolates of *S. enteritidis* and *E. coli* spp. were suspended in normal saline to McFarland standards of 0.5 and then were inoculated onto the Mueller-Hinton agar surface (Bk048HA Biokar diagnostics zac de ther, France). Antibiotic discs were aseptically placed on the inoculation medium with sterile forceps and incubated at 37°C for 24 hours. After incubation, the inhibition zone diameter around the antibiotic discs was measured, and sensitivity was determined. Results were interpreted later according to the criteria of the Clinical and Laboratory Standards Institute Performance Standards for Antimicrobial Susceptibility Testing (CLSI, WEINSTEIN, 2018). Antibiotic susceptibility testing was performed against six antibiotics of nalidixic acid (30 µg), kanamycin (30 µg), gentamycin (15 µg), ciprofloxacin (15 µg), tetracycline (30 µg), and amoxicillin (10 µg) purchased from Oxoid LTD, England. These antibiotics were selected according to their availability on the market and everyday use in the poultry industry worldwide.

Statistical analysis

The data obtained in this study were analyzed using the Statistical Package for the Social Sciences (SPSS, version 22). One-way analysis of variance (ANOVA) and Duncan's test were used to determine significant differences between eggshell and egg content contamination for each bacterium studied. The $p < 0.05$ was considered statistically significant. Results were also calculated and presented as a percentage using Excel spreadsheets.

RESULTS AND DISCUSSION

The table egg is a major food in the human diet due to its high nutritional value, availability, and low cost. However, it can be contaminated with harmful bacteria leading to serious foodborne diseases. The obtained results of the current study revealed that 15% of the samples tested positive for microbial contamination. Among them, 38 samples (13%) tested positive for *E. coli*, and 5 samples (2%) for *S. enteritidis* (Table 1).

The presence of *E. coli* in eggs taken from different sites in the present study may result from poor sanitary practices and conditions since *E. coli* is an indicator of hygienic quality (Carter and Cole, 1990). The results indicated that the prevalence rates of *E. coli* isolated from egg shells and egg contents were 9% and 4%, respectively. There was a significant difference between the contamination of the shells and the contents ($p < 0.05$) since this bacterium commonly contaminates the surface of eggs. As previously reported, the bacteria most frequently isolated from egg shells are Gram-negative bacteria, such as *E. coli* (Papadopoulou et al., 1997; Musgrove et al., 2004). The presence of this bacterium in the eggshell more than in the egg content can be explained by the fact that the eggs are supposed to be sterile due to their defense system (USDA, 2011).

The detection of *E. coli* in the shell may be due to the presence of feces, soil, dust, poor hygienic conditions during manipulation, contaminated egg crates, transportation, and commercialization, while the contamination recorded in the egg contents of the examined samples may be attributed to the fact that the laying hens are carriers of the pathogen before the shell formation (Gantois et al., 2009). Dirt in the nests can also contaminate the egg shells; therefore, the pathogen can move from the outside to the inside of the egg (USDA, 2011).

Several studies on different parts of the world, including Bangladesh (Haque et al., 2021), India (Arathy et al., 2009), Egypt (Mansour et al., 2015), Nigeria (Adabara et al., 2020), and South Africa (Jambalang et al., 2017) have revealed the presence of *E. coli* in table eggs. The same prevalence of *E. coli* in egg shells found in this study was previously reported in farm eggs in Nigeria (9.1%, Adabara et al., 2020). Regarding the egg shells, the prevalence of *E. coli* was detected in Zambia (Kapena et al., 2020), and Thailand (Siriporn et al., 2015). Besides, *E. coli* was isolated from egg contents in several previous studies conducted in Trinidad, Egypt, and Nigeria (Adesiyun et al., 2005; Mansour et al., 2015; Adabara et al., 2020).

The isolation rate of *S. enteritidis* in the table eggs tested was 2%. This prevalence was only observed in the egg contents since the shells were *S. enteritidis* free; therefore, the difference was not statistically significant ($p > 0.05$). The findings of the present study suggest that during the egg-laying process, the egg passes through the common part of the reproductive and digestive tracts where contamination may occur. In addition, the existence of *S. enteritidis* in the hen's ovary or oviduct (prior to shell formation) may also result in its presence in the egg contents (Gantois et al., 2009).

The absence of *S. enteritidis* on the shell can be attributed to the fact that poultry farmers practice strict hygiene during handling, transport, and marketing. These findings are supported by previous studies that showed the presence of *Salmonella* spp. and *Salmonella enteritidis* in table eggs (Islam et al., 2018; Hai et al., 2020; Haque et al., 2021).

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In Nigeria, analyses of egg contents from farms revealed the presence of 1.5% *Salmonella* spp. (Okorie-Kanu et al., 2016). In contrast to the present findings, some studies demonstrated the presence of *Salmonella* spp. in the egg shells purchased from street vendors and directly from farms (Arathy et al., 2009; Siriporn et al., 2015; Kapena et al., 2020).

The higher contamination of *E. coli* compared to *S. enteritidis* observed in this study could be explained by poor sanitary practices of farmers during egg handling, by the presence of excreta on eggs or by dust in the environment.

The presence of these microorganisms (*E. coli* and *S. enteritidis*) outside and inside of the egg (content and eggshell) is significant for public health since people consume table eggs at all ages (Réhault-Godbert et al., 2019).

Egg contamination can happen due to different factors, including poultry droppings, moist and warm bedding, dirt in the nest, dust, a highly contaminated environment, poor handling of eggs by farmers and their dirty clothes, poor transportation method, poor storage in stores where these eggs are sold (humidity), unhygienic handling conditions by sellers (De Reu et al., 2005).

In addition, the hen may carry the pathogen and transfer it to the egg contents inside the reproductive system. Contaminated egg crates may also contaminate the shell, and consequently, the bacteria may translocate to the egg contents through the pores of the shell over time (Musgrove et al., 2009).

Table 1. Distribution of *Escherichia coli* and *Salmonella enteritidis* contaminations in raw table eggs of Morocco from January to September 2021

Number of samples	Isolation of pathogens (%)			
	Escherichia coli		*Salmonella enteritidis*	
	Eggshell (%)	Egg content (%)	Eggshell (%)	Egg content (%)
290	9%	4%	-	2%

Antibiogram study

The *in-vitro* antibiotic sensitivity test results indicated that the isolated *E. coli* had the highest resistance to amoxicillin, followed by tetracycline and nalidixic acid with 92.10%, 84.21%, and 50%, respectively. On the other hand, this bacterium showed the maximum sensitivity to ciprofloxacin (84.21%), followed by kanamycin (65.79%), and gentamicin (60.54%, Table 2).

In the present study, *S. enteritidis* had the highest resistance patterns against tetracycline (80%), followed by ciprofloxacin and nalidixic acid with 40% each. While gentamicin, amoxicillin, and kanamycin had the highest sensitivity rates of 80%, 80%, and 40%, respectively (Table 3).

Drug resistance continues to threaten public health, challenging the treatment of infectious diseases (WHO, 2021). In the current study, *E. coli* showed the highest resistance rates to amoxicillin, tetracycline, and nalidixic acid (92.10%, 84.21%, and 50%, respectively). The resistance rate to amoxicillin in this study agrees with previous studies that found that *E. coli* isolated from eggs were highly resistant to this antibiotic, with 90.1% and 88.89% of resistance (Rahmatallah et al., 2017; Ashish and Rajesh, 2017). The same is true for tetracycline resistance. Different studies recorded high rates of *E. coli* isolated from table eggs resistant to tetracycline with almost identical rates (Kapena et al., 2020, Haque et al., 2021). Regarding nalidixic acid resistance, a study conducted in Zambia showed that *E. coli* isolated from table eggs were resistant to nalidixic acid with a rate of 32.4% (Kapena et al., 2020). In contrast to the present study, a study in Bangladesh indicated that *E. coli* isolated from eggs were susceptible to this antibiotic with a rate of 44.44% (Islam et al., 2018). Of the 6 antibiotics, isolated *E. coli* showed the greatest sensitivity to ciprofloxacin, followed by gentamycin and kanamycin. Some studies have reported sensitivity to these antibiotics at a rate of 100% for each (Ashish and Rajesh, 2017; Islam et al., 2018; Adabara et al., 2020).

The resistance to tetracycline and amoxicillin could be partly attributed to the historical use of these antimicrobial classes in aviculture, resulting in antibiotic resistance development of the bacteria over time (Okorie-Kanu et al., 2016). The susceptibility to ciprofloxacin, gentamicin, and kanamycin could be attributed to the increased cost of these drugs, leading to their reduced use in poultry production (Okorie-Kanu et al., 2016).

The aforementioned results showed notable resistance rates of *S. enteritidis* to tetracycline, ciprofloxacin, and nalidixic acid: 80%, 40%, and 40%, respectively. A Moroccan study was conducted in 2016 to evaluate the antimicrobial resistance of *Salmonella* spp. isolates in Moroccan laying hens. The findings indicated that *S. enteritidis* was only resistant to nalidixic acid at a rate of 37.5%, meaning that the resistance in Morocco is increasing (Ziyate et al., 2016). Apart from tetracycline resistance, the present results disagree with reports from similar studies where *Salmonella* spp. isolated from table eggs were susceptible to ciprofloxacin and nalidixic acid (Okorie-Kanu et al., 2016; Islam et al., 2018; Kapena et al., 2020).

The noted susceptibility of *S. enteritidis* to gentamicin (80%) recorded during the present investigation was in line with the findings reported in an Egyptian study that reported a 90.9% of susceptible *Salmonella* spp. isolated from layer farms in Egypt (Diab et al., 2019). Concerning amoxicillin sensibility, a study conducted in Rajshahi found a rate of 76.47% of *S. enteritidis* isolated from eggs (Islam et al., 2018). Finally, previous studies conducted in Ethiopia and Egypt revealed the susceptibility of *Salmonella* isolated from eggs against kanamycin at 91% and 95.5% (Tessema et al.,

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2017; Diab et al., 2019). The uncontrolled, random, and repeated use of antibiotics in chicken farming worldwide may lead to the development and growth of resistant bacteria (Diab et al., 2019). For example, tetracycline was used for years to one-day-old chickens against *Salmonella* and *E. coli*, leading to its resistance to both antibiotics (Ekperigin et al., 1983; Lutful Kabir, 2010). Ciprofloxacin also belongs to the group of fluoroquinolones that have a rapid bactericidal action against *Salmonella* spp. Fluoroquinolones are widely used to treat salmonellosis in humans and animals (Folster et al., 2015) and be useful for treating infections caused by multidrug-resistant strains (Barnass et al., 1990). Therefore, its overuse may explain the high resistance observed in this study (Diab et al., 2019). Cross-resistance can also explain resistance to ciprofloxacin and nalidixic acid, as they are concrete examples of such resistance. When bacteria develop resistance to ciprofloxacin, they may also develop resistance to nalidixic acid because both drugs inhibit topoisomerase, a key enzyme in DNA replication (Périchon and Courvalin, 2009). On the other hand, some antimicrobials are rarely recommended because their high cost may explain the high susceptibility rate noted above (Okorie-Kanu et al., 2016).

Most of the *E. coli* strains (60.5%) were found to be multidrug-resistant (MDR), as they showed resistance to three antibiotics tested. In the same context, several studies have reported that *E. coli* is an MDR bacterium that has shown resistance to three, five, or more antibiotics, including tetracycline, amoxicillin, trimethoprim sulfate, streptomycin, and doxycycline (Adesiyun et al., 2007; Eid et al., 2015).

Regarding *S. enteritidis*, the present study revealed that 60% of the isolates were resistant to at least one antibiotic. In comparison, no MDR was detected, which agrees with a Moroccan study that revealed that 65.6% of *Salmonella* spp. strains were resistant to at least one antibiotic tested (Ziyate et al., 2016). This rate of multidrug resistance to antibiotics is most likely due to inappropriate use of treatment, either overuse, short treatment, or even misuse of these antibiotics (WHO, 2021).

The results were not surprising considering the range of antibiotics available in Morocco. The variation in susceptibility and resistance patterns may be due to the blinded use of antibiotics in poultry feed and poultry itself as prophylactic, therapeutic, and growth promoter agents since the farmers have unlimited and free access to antibiotics in Morocco (Rahmatallah et al., 2018).

In recent years, antibiotic-resistant bacteria have received considerable attention because they constitute an immediate risk to public health by increasing the incidence of overall hospitalizations and the risk of invasive infections and mortality (Verraes et al., 2013). They can lead to many consequences, such as the failure of medical treatments, including modern medicine. This is because surgical procedures and cancer chemotherapy would be compromised, the choice of antibiotics for treatment would be limited, and resistant gastrointestinal bacteria will gain an advantage when patients are treated with antibiotics for other medical reasons (Verraes et al., 2013). Furthermore, it has been experimentally proven that the genes conferring antibiotic resistance are easily transferable between normal flora, pathogenic *E. coli* and *Salmonella* spp. (Blake et al., 2003). Hence, antibiotic resistance in microorganisms from table eggs should be considered a significant public health hazard, as eggs can serve as a vector for transferring antimicrobial-resistant bacteria and genes to humans.

Table 2. Antibacterial sensitivity and resistant pattern of *Escherichia coli* isolated from eggs in Morocco from January to September 2021

Number of samples tested	Antibiotics	Sensitivity pattern		
		Sensitive (%)	Intermediate (%)	Resistant (%)
290	Tetracycline	10.53	5.26	84.21
	Nalidixic acid	44.74	5.26	50
	Amoxicillin	5.26	2.64	92.10
	Kanamycin	65.79	13.16	21.05
	Gentamycin	60.54	13.15	26.31
	Ciprofloxacin	84.21	5.26	10.53

Table 3. Antibacterial sensitivity and resistant pattern of *Salmonella enteritidis* isolated from eggs in Morocco from January to September 2021

Number of samples tested	Antibiotics	Sensitivity pattern		
		Sensitive (%)	Intermediate (%)	Resistant (%)
290	Tetracycline	20	0	80
	Nalidixic acid	40	20	40
	Amoxicillin	80	20	0
	Kanamycin	40	40	20
	Gentamycin	80	20	0
	Ciprofloxacin	20	40	40

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CONCLUSION

This study concludes that table eggs marketed for human consumption in Morocco could be infected with antibiotic-resistant *E. coli* and *S. enteritidis*, especially tetracycline. These resistant bacteria could potentially be transmitted to consumers through eggs or egg products, which could have significant public health consequences which require a one-health approach to combat the threat. Therefore, further studies can be conducted to assess the potential spread of antibiotic resistance from foodborne pathogens to humans.

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Authors' contributions

Fatima Zahra El Ftouhy, Sabrine Nacer, Sophia Derqaoui, and Nadia Charrat collected the samples used in this study. Fatima Zahra El Ftouhy performed the bacterial isolation and antibiotic susceptibility testing. Fatima Zahra El Ftouhy and Asma Fagrach contributed to the data analysis. Fatima Zahra El Ftouhy wrote the original manuscript. Saâdia Nassik, Abdelaziz Hmyene, and Ahlam Kadiri revised and generated the final version of the manuscript. All authors contributed to the article and approved the submitted version.

Competing interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

Ethical consideration

All authors have checked and vetted the manuscript for ethical considerations, namely plagiarism, consent to publication, misconduct, fabrication and/or falsification of data, dual publication and/or submission, and redundancy.

Availability of data and materials

The authors declare that they have all the necessary data and are available where appropriate or requested by the editor.

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