From the
Institute for Animal and Environmental Hygiene
Department of Veterinary Medicine
Freie Universität Berlin

In cooperation with the
Friedrich-Loeffler-Institut,
Federal Research Institute for Animal Health,
Institute for Bacterial Infections and Zoonoses, Jena

Epidemiology, genotyping and antibiotic resistance of zoonotic bacteria isolated from poultry in Egypt

Inaugural-Dissertation
to obtaining the degree of
Doctor of Veterinary medicine
at the
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submitted by
Amira Awad Ibrahim Moawad
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Abbreviations

***aac* (3)-(IV)**	Aminoglycoside 3-N-acetyltransferase/gentamicin resistance associated gene
aadA1	Streptomycin associated resistance gene
AmpC	Class C β-lactamases
AMR	Antimicrobial resistance
APEC	Avian pathogenic *E. Coli*
***bla*$_{CTX-M}$**	Beta-lactamase cephalosporin resistance associated gene
***bla*$_{CMY}$**	Beta-lactamase cephamycin resistance associated gene
***bla*$_{GES}$**	Guiana extended-spectrum, carbapenem-resistance associated gene
***bla*$_{SHV}$**	Beta-lactamase penicillins and first generation cephalosporins resistance-associated gene (sulfhydryl reagent variable)
***bla*$_{TEM}$**	Beta-lactamase ampicillin resistance associated gene
***bla*Z**	Beta-lactamase penicillin resistance associated gene
SCCmec	Staphylococcal chromosomal cassette mec
CDC	Centre for Disease Control and Prevention
CFU	Colony Forming Unit
cfr	Chloramphenicol-florfenicol resistance associated gene
***cit*M**	Ampicillin resistance associated gene
CLSI	Clinical and Laboratory Standards Institute
CoNS	Coagulase negative staphylococci
CoPS	Coagulase positive staphylococci
***dfr*A1**	Trimethoprim resistance associated gene
EAEC	Enteroaggregative *E. coli*
E. coli	*Escherichia coli*
EHEC	Enterohemorrhagic *E. coli*
EMB	Eosinmethyleneblue agar
EPEC	Enteropathogenic *E. coli*
ere	Erythromycin resistance associated gene
ESBLs	Extended spectrum beta-lactamases
ETEC	Enterotoxigenic *E. coli*
EUCAST	European Committee on Antimicrobial Susceptibility Testing
EXPEC	Extra intestinal pathogenic *E. Coli*
***feo*B**	Fe(2+) transporter gene
***fim*C**	Chaperone-like periplasmic protein
***fim*H**	Fimbrin D-mannose specific adhesin
***fli*C**	Flagellin encoded gene
***flo*R**	Florfenicol ressitance associated gene
FSDA	Food safety and Drug Administration
fus	Fusidic acid resistance associated gene
GIT	Gastrointestinal tract
H-antigen	Flagellar antigen
hlyF	Hemolysin F
IBV	Infectious bronchitis virus
IMI	Imipenem-hydrolyzing enzyme
IMP	Active-on-imipenem-β-lactamase resistance gene
IPEC	Intestinal pathogenic *E. Coli*
***ire*A**	Iron-regulated virulence gene
iron	Iron protein
***irp*-2**	Iron regulatory protein
iss	Increased serum survival gene
***iut*A**	Iron uptake gene
KPC	*Klebsiella pneumonia*-carbapenemase
MALDI-TOF	Matrix-assisted laser desorption/ionization time of flight assay
mcr	Colistin resistance associated gene
MDR	Multidrug–resistance
***mec*A**	Methicillin resistance associated gene
MGEs	Mobile genetic elements
MIC	Minimal Inhibitory Concentration
MLS	Macrolides, lincosamides and streptogramines group
MRSA	Methicillin-resistant *Staphylococcus aureus*

MUG	4-Methylumbelliferyl-β-D-glucuronide
NDM	New Delhi metallo-β-lactamase
NMC	Not-metallo-enzyme carbapenemase
NMEC	Neonatal meningitis *E. coli*
O-antigen	Lipopolysaccharide antigen
OM	Outer membrane
***omp*T**	Outer membrane protease
PCR	Polymerase chain reaction
PBPs	Penicillin binding proteins
PetN	Phosphoethanolamine transferase
PG	Phospholipid phosphatidylglycerol
qnr	Quinolone resistance associated gene
SAM	S-adenosyl-L-methionine methylase family
S. aureus	*Staphylococcus aureus*
SEPEC	Sepsis-associated *E. coli*
SME	Serratia marcescens enzyme
SPM	Sao Paulo metallo-β-lactamase
stx	Shiga toxin gene
tet	Tetracycline resistance associated genes
Tsh	Temperature-sensitive hemagglutination
***uid*A**	Beta-D-glucuronidase gene
UPEC	Uropathogenic *E. coli*
VAs	Veterinary antibiotics
vat	Virginiamycin A acetyltransferase gene
vga	Vga-A variant protein gene
VIM	Verona integron-encoded metallo-β-lactamase
VRE	Vancomycin-resistant enterococci
wzx	O-antigen flippase gene
wzy	O-antigen polymerase gene
16S rRNA	16Svedberg units-ribosomal RNA

Introduction

Introduction

Poultry remains one of the most important reservoirs for zoonotic multidrug resistant pathogens that cause foodborne infections in humans. The global rise of antimicrobial resistance in both Gram-negative and Gram-positive bacteria is of reasonable concern and demands intensified surveillance (Moawad et al., 2019; Moawad et al., 2017a; Moawad et al., 2018).

The prevalence of highly antibiotic-resistant *Escherichia* (*E.*) *coli* was frequently recorded in poultry meat more than all other kinds of meat (Rasheed et al., 2014).

The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been applied as a wide-range technique for bacterial identification (Croxatto et al., 2012). Microarray assays are used for rapid identification and typing of bacterial genome including resistance and virulence-associated determinants (Dunne et al., 2018). The use of a rapid molecular assay as an alternative to phenotypic detection was proved to be a useful option for detection of antibiotic resistance to frequently applied antimicrobial agents in poultry production (El-Adawy et al., 2012).

Extended-spectrum β-lactamases (ESBL)-producing *Enterobacteriaceae* have emerged as human, animal and poultry-pathogens (Founou et al., 2019). ESBLs are plasmid-encoded enzymes found in Gram-negative bacteria especially *Enterobacteriaceae* conferring resistance to first, second and third generation cephalosporins while they are inhibited by clavulanic acid (Lee et al., 2012).

Carbapenems are still the drugs of choice to treat infections caused by ESBL-producing *Enterobacteriaceae* in humans (Zhanel et al., 2007). Increasing use of carbapenems reinforces the probability of their resistance development among *Enterobacteriaceae* (Canton et al., 2012).

Colistin is a last-resort antibiotic for treatment of infections caused by multidrug-resistant Gram-negative bacteria (Timmerman et al., 2006).The irrational use of colistin in veterinary practice is a main cause of the recent emergence of colistin resistance (Biswas et al., 2012; Giamarellou, 2016).

Although infections caused by coagulase-negative staphylococci (CoNS) are less severe than *Staphylococcus* (*S.*) *aureus* infections, their treatment has been shown to be more complicated since the dramatic increase in antibiotic resistance to most of antimicrobial classes (Becker et al., 2014). CoNS has the ability to acquire, possess and alter resistance genes rapidly. This

feature furtherly promotes the transmission of these genes into different staphylococcal species or even other bacterial genera (Becker et al., 2014; Otto, 2013).

Infections caused by antibiotic-resistant CoNS have been increasing in humans worldwide. However, only few studies have discussed the presence of CoNS in humans and animals in Egypt (El-Razik et al., 2017; Mashaly and El-Mahdy, 2017; Osman et al., 2016).

Linezolid is a last-resort antimicrobial agent for the control of serious infections caused by methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE) in humans (Bender et al., 2018). Linezolid resistance is increasing more intensely in CoNS than in *S. aureus* (Balandin et al., 2016; Baos et al., 2013). However, the resistance of CoNS to linezolid has not been reported in Egypt, neither in humans nor in animals (Moawad et al., 2019).

This work aims to investigate the epidemiology and antimicrobial resistance of zoonotic bacteria in poultry production in Egypt including:

1. Investigation of epidemiology of *Enterobacteriaceae* among healthy broilers from different districts in northern Egypt.
2. Gaining insight into the emerging antimicrobial resistance of *Enterobacteriaceae*.
3. Investigation of the prevalence of ESBL/carbapenemase-producing and colistin resistant *E. coli*.
4. Determination of prevalence and antimicrobial resistance of coagulase-negative staphylococci isolated from apparently healthy turkeys housed in different governorates of the Nile Delta in Egypt.
5. Studying mechanism of linezolid-resistance among CoNS and its associated resistance genes.

C H A P T E R 1

Review of Literature

Review of literature

The poultry industry is considered one of the most important sources of support for the Egyptian economy, with an investment worth of $3.5 billion and a labor force of 16 million workers. Egyptian society mainly rely on poultry as a major source for animal protein intake. The annual consumption rate of poultry meat in Egypt is around 1,125 million tons. Despite the economic size of the Egyptian poultry industry, turkey production is still considered as a growing sector limited to a small scale production. This can be attributed to a number of challenges including the currently expensive production costs, market price compared to alternative sources of white meat, the unfavorable weather conditions, the high vulnerability of the young chicks to diseases and the lack of rearing knowledge and experience (Moawad et al., 2017b).

The massive and uncontrolled use of antimicrobial agents in poultry farms in Egypt for disease control and prevention, helped greatly in releasing of many drug-resistant bacteria, most of them are considered as a dangerous zoonotic threat to human health (Moawad et al., 2017a; Moawad et al., 2018).

Various combinations of constraints in veterinary and human medicine have been identified in Egypt, such as the lack of legislation, knowledge, resources and veterinary services. These constraints act as obstacles that hamper the prudent use of antimicrobial drugs. The antimicrobial drugs used in poultry production in Egypt are applied for growth promotion and prophylaxis besides treatment of infections. There are no proper legislations take place to regulate the sale of antimicrobial drugs used for poultry production in Egypt (Moawad et al., 2019).

Zoonotic diseases are infections that can be transmitted between animals and humans with or without vectors through direct contact, indirect environmental contact, or through food (EFSA., 2016). In the past few decades, zoonotic outbreaks have increased greatly causing health and economic impact worldwide. About 60% of pathogens causing human infections are of animal origin (Zhang et al., 2016). Zoonotic diseases are of bacterial, viral or parasitic causative agents. Bacterial diseases of animal origin, e.g., caused by *Campylobacter* spp., *Listeria* spp. and *Enterobacteriaceae* family, are major human threats in developing countries and in developed ones as well (Keerthirathne et al., 2016; Rohde et al., 2016). *Staphylococcus* spp. are emerging potential pathogens that cause zoonotic infections in humans (Somayaji et al., 2016). A wide range of zoonotic pathogens have been reported in Egypt (Helmy et al., 2017). Antimicrobial resistance (AMR) is an additional risk associated with exposure to zoonotic pathogens leaving few treatment options in both human and veterinary medicines (Agunos et al., 2016).

1. *Escherichia coli*

1.1. Organism characterisation

The Gram-negative rod-shaped *E. coli* is a member of the family *Enterobacteriaceae*. It is normally commensal in nature, even though it contains many pathogenic strains causing infections in humans and animals (Yang et al., 2004). Pathogenicity potential of *E. coli* is encoded by various virulence genes (Johnson et al., 2012b; Pires-Dos-Santos et al., 2013). There are two major groups of pathogenic *E. coli*, i.e. the intestinal pathogenic *E. coli* (IPEC) and the extra-intestinal pathogenic *E. coli* (ExPEC) (Chakraborty et al., 2015; Kaper et al., 2004).

Escherichia coli is characterized by a broad host range, mostly possessing antibiotic resistance genes in addition to the most important potential virulence genes (Olsen et al., 2014).

Escherichia coli is able to grow both aerobically and anaerobically, preferably at 37°C and can be either motile with peritrichous flagella or non motile. As not all *E. coli* strains are lactose fermenters, the change in pH due to lactose fermentation can be used to differentiate between lactose-fermenting and non-lactose-fermenting strains. Lactose-positive *E. coli* colonies appear red or pink on media such as MacConkey agar. Different methods have been employed to isolate, identify and confirm *E. coli*. To improve *E. coli* detection, samples are inoculated in broth with 4-methylumbelliferyl-β-D-glucuronide (MUG) at 44.5°C for 22-26 h as a specific pre-enrichment step. Enriched samples are then cultured on MacConkey agar enriched with 7% sheep blood agar, Chromocult Coliform agar or eosin methylene blue (EMB) agar (Croxen et al., 2013; DePaola et al., 2010).

Escherichia coli can be also directly inoculated on selective agar medium that inhibits Gram-positive bacteria growth and distinguishes *E. coli* from other coliform colonies.

After the incubation at 37°C for 24-48 h, the culture is examined for the characteristic *E. coli* colonies. Pure colonies are subjected to biochemical test to identify and differentiate from other *Enterobacteriaecae* members. The identification of *E. coli* is based on the results of diagnostic tests, which include Gram staining, colony morphology, gas production and the ability to be enriched in the MUG broth.

Matrix-assisted laser desorption/ionization time-of-flight mass spectromtry (MALDI-TOF MS) is an analytical mass spectroscopy technique that uses a laser energy to ablate and desorb molecules that create ions with minimal fragmentation. It is considered as a rapid technique for bacterial identification. Interpretation of results is performed according to the manufacturer's recommendation: score of ≥ 2.3 represents reliable species level identification; score 2.0–2.29,

probable species level identification; score 1.7–1.9, probable genus level identification, and score ≤ 1.7 was considered an unreliable identification (Paauw et al., 2015).

Further molecular identification of *E. coli* could be performed using PCR for amplification of 16S rRNA gene (Andreotti et al., 2011; Seidavi et al., 2010), genes involved in O-antigen serotyping (e.g., wzx and wzy genes), *fli*C for the H antigen (DebRoy et al., 2011; Wang et al., 2003) and other genes such as *uid*A gene encoding enzymatic activity beta-D-glucuronoside glucuronosohydrolase (Molina et al., 2015).

1.2. Zoonotic importance of*E. coli*

Escherichia coli has a potential of zoonotic transfer between animals, poultry and humans. Poultry is considered as one of the important reservoirs of antimicrobial resistant *E. coli* implicated in human infections. The transmission between poultry and humans has been supported by the fact of virulence-associated genes similarities between human and avian *E. coli* strains (Johnson et al., 2012) in addition to similarities between extended-spectrum β-lactamases in poultry and humans (Hernandez et al., 2013). However, the transmission between poultry and humans has been weakly supported by other studies (Olsen et al., 2014).

Escherichia coli is a common human pathogen implicated in many infections. Diarrheagenic strains vary in clinical signs and pathogenesis into enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC) and enteroaggregative *E. coli* (EAEC). They never cause extraintestinal infections (Vila et al., 2016).

In addition to gastrointestinal tract (GIT) infections, extraintestinal pathogenic *E. coli* (ExPEC) are implicated in many different extraintestinal infections in humans via spreading outside the digestive system. The ExPEC group includes uropathogenic *E. coli* (UPEC), sepsis-associated *E. coli* (SEPEC), neonatal meningitis *E. coli* (NMEC), and avian pathogenic *E. coli* (APEC) (Sarowska et al., 2019).

1.3. *E. coli*infections in poultry

Escherichia coli is a common inhabitant in the intestinal tract of poultry that found at high concentrations in the lower intestinal tract as well as in younger birds which haven't established normal flora yet (Clavijo and Florez, 2018). However, in the immune suppressed and debilitated hosts or in case of disrupted gastrointestinal barriers, the non-pathogenic *E. coli* can cause infections in poultry, humans and animals. Furthermore, there are certain *E. coli* strains act as Avian pathogenic *E. coli* (APEC) that infect various internal organs and cause colibacillosis characterized by systemic fatal diseases with fibrinous lesions of internal organs (airsacculitis, pericarditis, perihepatitis) (Schouler et al., 2012). *E. coli* cause septicemia in

poultry accompanied by enteritis, omphalitis, polyserositis, respiratory tract infection, yolk sac infection, cellulitis and salpingitis that causes a devastating impact on the poultry industry worldwide (Lutful Kabir, 2010).

APEC strains in poultry have different virulence factors including lipopolysaccharides, temperature-sensitive hemagglutinin (Tsh), and increased serum survival factor (*iss*). These factors mostly belong to O1, O2, O15, O55 and O78 serotypes (Lutful Kabir, 2010). APEC strains usually invade birds through inhalation of dust contaminated with APEC strains following primary infection with respiratory pathogens such as infectious bronchitis virus (IBV), *Mycoplasma gallisepticum* or *Mycoplasma synoviae* (Dziva and Stevens, 2008).

1.4. Global prevalence of antibiotic resistant *E. coli* isolated from poultry

Escherichia coli was isolated with high prevalences from poultry farms and their environment worldwide. Antibiotic resistance among *E. coli* has been recently reported in alarming rates from all types of poultry production including intensive, extensive, layers and breeders. Antibiotic resistant *E. coli* is widely associated with several foodborne outbreaks.

In India, different serotypes O1, O2, O29, O40, O60, O87 and O106 were isolated from poultry meat. The Shiga toxin (*stx*-2) virulence gene was identified in 16.12% of isolates. The isolates showed wide variation in resistance pattern against several antimicrobial agents. Highest resistance was observed against cefuroxime and penicillin while the lowest resistance was against amikacin, ciprofloxacin and ofloxacin (Kaushik et al., 2018).

In China, *E. coli* was isolated from internal organs of infected chickens with pathogenic lesions. The *fim*H, *feo*B, and *iron* genes were the most prevalent virulence-associated genes among all isolates. More than 70% of isolates also carried the *ire*A, *irp-2*, *iut*A, *omp*T and *iss* virulence genes. All isolates exhibited resistance to ampicillin and tetracycline. The frequencies of resistance to other antimicrobials including nalidixic acid, chloramphenicol, kanamycin and gentamicin ranged between 50 to 89.6%. All of the isolates were multidrug-resistant strains (Xu et al., 2019).

In Bangladesh, all collected cloacal swab samples from broilers were positive for *E. coli.* The isolates exhibited highest susceptibility to colistin sulphate followed by gentamicin and levofloxacin. The majority of isolates were harboring the antimicrobial resistance genes *tet*(A), *tet*(B) followed by bla_{TEM}, *aad*A1, *ere*A, and *dfr*A1 (Azad et al., 2019).

In Taiwan, the prevalence of *E. coli* strains isolated from poultry hatcheries was 2.0%. Isolates expressed the adhesion gene *fim*C. *E. coli* expressed multidrug resistance including 100%

resistance to ampicillin, amoxicillin, cephalexin, florfenicol, and trimethoprim-sulfamethoxazole (Zhao et al., 2019).

1.5. Epidemiology and antibiotic resistance of *E. coli* in poultry in Egypt

In Egypt, *E. coli* was isolated with high prevalences from poultry farms and their environment causing severe infections in Egypt.

In 2019, the prevalence of *E. coli* in poultry suffering from colibacillosis was 34%. The predominant serotypes were O2, O44, O111, O128 and O158. All isolates showed resistance to ampicillin, erythromycin, and sulfamethoxazole-trimethoprim, while most of them were sensitive to colistin sulfate and levofloxacin. The tetracycline resistance associated *tet*A gene was identified in 91.2% of isolates (Ibrahim et al., 2019).

Escherichia coli was isolated from turkeys in Egypt suffering from respiratory infections. The most identified serogroups were O6, O8, O119 and O169. All isolates showed phenotypic resistance against cefuroxime, tetracycline, and trimethoprim. Additionally, isolates showed massive resistance against amoxicillin/clavulanic acid, enrofloxacin, and norfloxacin. About 33.3%, 44.4% and 72.2% of isolates demonstrated the phenotypic pattern of ESBL producers with cefepime, cefotaxime, and ceftriaxone resistance, respectively. Both, bla_{TEM} and *int*1 genes were identified in majority of isolates (Eid and Samir, 2019).

The prevalence of *E. coli* in diseased poultry between 18 to 34 days old was 35%. Identified *E. coli* serotypes were O24, O44, O55, O78, O86, 124, O127 and O158. Most of isolates showed high phenotypic resistance to oxytetracycline and kanamycin. However, the resistance rates to erythromycin and oxacillin were low among isolates. Antibacterial resistance-associated genes *cit*M (ampicillin resistance), *ere* (erythromycin resistance), *aac (3)-(IV)*(gentamicin resistance), *tet*A and *tet*B (tetracycline resistance), *dfr*A1(trimethoprim resistance) and *aad*A1 (streptomycin resistance) were detected (Amer et al., 2018).

Escherichia coli identified from poultry suffering form colibacillosis showed high phenotypic resistance rates against cefazolin, streptomycin, ampicillin, doxycycline, ciprofloxacin, co-trimoxazole and gentamicin. Additionally, 6% and 12% of isolates were identified as ESBL and AmpC-producing strains. The bla_{TEM}, bla_{SHV}, bla_{CTX-M} and bla_{CMY} genes were identified (El-Shazly et al., 2017).

Among broiler poultry farms, a high prevalence of *E. coli* infection was detected. The highest phenotypic resistance was against ampicillin followed by amoxicillin, erythromycin, sulfamethoxazole-trimethoprim, tetracycline, neomycin and chloramphenicol (Dahshan et al., 2015).

Escherichia coli was isolated from healthy and diseased broiler chickens. The serotypes were O78, O86, O128 and O111. The virulence-associated gene *iss* was identified in 72.2%. Antimicrobial resistance patterns revealed a complete resistance against gentamicin, pefloxacin, amoxicillin, and enrofloxacin. The lowest resistance was recorded against colistin sulphate (Mohamed et al., 2014).

In another study, *E. coli* isolates from poultry flocks in Egypt were identified as O5, O11, O36, O141, O157 and O158 serogroups. Most of them possessed virulence associated genes *iro*N, *omp*T, *hly*F, *iss*, and *iut*A. Isolates were highly resistant to antimicrobials tested with the exception of amikacin and sulfisoxazole. More than 70% of the isolates showed high resistance against nalidixic acid, tetracycline, ampicillin, streptomycin, ciprofloxacin and trimethoprim-sulfamethoxazole (Hussein et al., 2013).

Escherichia coli was isolated from septicemic broilers, and most of pathogenic strains carried three or more of the APEC virulence-associated genes *iro*N, *omp*T, *iss*, *iut*A, and *hly*F. All isolates showed resistance to ampicillin, tetracycline, spectinomycin, streptomycin, kanamycin, and trimethoprim/sulfamethoxazole. The class 1 and class 2 integrons were identified in 46.6% and 9.6% of isolates, respectively. The β-lactamase-encoding genes (bla_{TEM-1}, $bla_{TEM-104}$, bla_{CMY-2}, bla_{OXA-30}, $bla_{CTX-M-15}$, and bla_{SHV-2}), tetracycline resistance genes (*tet*A, *tet*B, *tet*C, *tet*D, and *tet*E), the plasmid-mediated quinolone resistance genes (*qnr*A1, *qnr*B2, *qnr*S1, and *aac*(6')-*Ib-cr*) and florfenicol resistance gene, *flo*R, were also identified (Ahmed et al., 2013).

While low prevalences (37.5% and 25%) of ESBL-encoding genes (bla_{TEM} and bla_{OXA}) were detected in *E. coli* isolated from poultry meat in another study in Egypt (Younis et al., 2017).

2. *Staphylococcus* spp.

The genus *Staphylococcus* (*S.*) has decimated humans for centuries. These organisms were firstly described in1882 by the Scottish surgeon Sir Alexander Ogston as Gram-positive bacteria and classified as *Staphylococcus*. Two years later a German physician, Friedrich J. Rosenbach, described two pigmented colonies of staphylococci and proposed the nomenclature *S. albus* (Latin for “white”) and *S. aureus* (from the Latin aurum [“gold”]). Since that time, staphylococci has continued to infect and decimate millions of patients (Wilson, 1987). Additionally, the genus developed important multidrug-resistance traits such as methicillin and vancomycin-resistance (Gould, 2010; Malachowa and DeLeo, 2010).

For now, 51 species and 27 sub-species of the genus *Staphylococcus* are well described. The species having the capacity to coagulate plasma of mammals are forming the group of coagulase-positive staphylococci (CoPS). The group includes 7 species: *S. aureus*, *S.*

delphini, *S. intermedius*, S. *pseudintermedius*, *S. lutrae*, *S. schleiferi* ssp. *coagulans* and *S. hyicus*. Among this group, *S. aureus* is considered as the most virulent (Balbutskaya et al., 2017).

Staphylococcus aureus is a commensal bacterium and also a disease causing human pathogen. Around 30% of the human population is colonized with *S. aureus*. It is considered a leading cause of skin and soft tissue osteoarticular and pleuropulmonary infections as well as bacteremia and infective endocarditis (IE) (Coates et al., 2014; Stryjewski and Corey, 2014; Wertheim et al., 2005).

The other group of genus *Staphylococcus* is formed by coagulase-negative staphylococci (CoNS) which are commonly found in animals, humans, food and environment (Zong et al., 2011). CoNS were believed to be non-pathogenic bacteria until 1980. Thereafter they have gained much attention over the past years. CoNS cause serious nosocomial infections in humans, beside acting as vectors for virulence genes (Veras et al., 2008). Among CoNS, *S. epidermidis* and *S. haemolyticus* being the most significant species as infection causative agents (Becker et al., 2014). CoNS are implicated in various severe human infections including food poisoning, infections of central nervous system, surgical wound infections, peritonitis, septicemia, eye infections, neonatal sepsis, osteomyelitis, otitis media, urinary tract infections, endocarditis, soft tissue infections and abscesses in internal organs (Becker et al., 2014; Bizzarro et al., 2015; Decousser et al., 2015; Giordano et al., 2016; Kleinschmidt et al., 2015; Nanoukon et al., 2017; Piette and Verschraegen, 2009; Rivera et al., 2014).

Moreover, CoNS proved to be pathogenic also in poultry causing decreased weight gain, drop in egg production, endocarditis and increased mortality rate (Andreasen, 2013; Marek et al., 2016; Stepanovic et al., 2003; Stepien-Pysniak et al., 2017).

Although CoNS infections are less severe than *S. aureus* infections, their treatment started to be more complicated because of the dramatic increase in antibiotic resistance especially for penicillin, oxacillin/methicillin, gentamicin, clindamycin, ciprofloxacin and erythromycin (Becker et al., 2014). CoNS possessing resistance-associated genes may promote the distribution of resistance genes into different staphylococcal species or even other bacterial genera (Huang et al., 2017). Several outbreaks of antibiotic-resistant CoNS have been reported in humans worldwide (Brito et al., 2009; Hagiya et al., 2018; Mazzariol et al., 2012), while only few studies discussed presence of CoNS in poultry (Boamah et al., 2017; Marek et al., 2016; Stepien-Pysniak et al., 2017). Additionally, very few studies have investigated the presence of CoNS in humans and animals in Egypt (El-Razik et al., 2017; Mashaly and El-Mahdy, 2017; Osman et al., 2016).

2.1. Epidemiology of antibiotic resistant CoNS in poultry worldwide

CoNS are implicated in serious infections in both, humans and animals and show high resistance to wide range of antibiotics. They are associated with recent outbreaks in human all over the world (Nanoukon et al., 2017).

In Ghana, CoNS were isolated from poultry and their litter. The different species included *S. sciuri*, *S. lentus*, *S. gallinarum*, *S. xylosus*, *S. haemolyticus*, *S. saprophyticus* and *S. cohnii*. All isolates were susceptible to amoxicillin/clavulanic acid and amikacin, while they exhibited high resistance to tetracycline, doxycycline and oxacillin (Boamah et al., 2017).

In USA, the predominant CoNS species isolated from poultry and poultry litter were *S. sciuri*, *S. lentus*, *S. xylosus* and *S. simulans*. The antimicrobial resistance was primarily limited to clarithromycin, erythromycin, clindamycin and tetracycline (Simjee et al., 2007).

In Poland, *S. cohnii, S. saprophyticus, S. epidermidis, S. xylosus, S. lentus, S. sciuri, S. simulans, S. haemolyticus* and *S. hominis* were isolated from broiler chickens and turkeys. The *blaZ* and *mec*A genes were detected. Among genes responsible for resistance to tetracyclines, *tet*K was the most frequent. Few strains carried resistance genes to macrolides, lincosamides, and florfenicol/chloramphenicol (Pyzik et al., 2019). In another study in Western Poland, *S. cohnii* and *S. lentus* were isolated from poultry. More than half of the isolated staphylococci exhibited resistance for enrofloxacin (Marek et al., 2016).

In Denmark, *S. hyicus*, *S. xylosus* and *S. cohnii* were identified in poultry samples. The isolates were susceptible to most antimicrobials tested. Low resistance to sulphamethoxazole was observed among isolates. Moderate resistance against novobiocin and erythromycin was identified. Few isolates were found to be resistant to streptogramins and harbored the *vat*B, *vga*B and *tet*K resistant associated genes (Aarestrup et al., 2000).

2.2. Epidemiology and antibiotic resistance of CoNS in poultry in Egypt

Different species of CoNS including *S. epidermidis, S. lugdunensis, S. haemolyticus, S. hominis, S. schleiferi, S. cohnii,* and *S. lentus* were isolated from retail raw chicken meat in Egypt. Isolates showed high resistance against ciprofloxacin, gentamicin, erythromycin, methicillin, oxacillin, penicillin, sulfamethoxazole/trimethoprim, and vancomycin. The *mec*A gene was detected in most of isolates. (Osman et al., 2016).

CoNS were also isolated from diseased and apparently healthy poultry include *S. xylosus, S. sciuri* and *S. lentus*. All isolates showed resistance to oxytetracycline, trimethoprim-sulphamethoxazole, clindamycin and oxacillin while they were susceptible to

vancomycin. Most of isolates exhibited the resistance associated genes *mec*A, *tet*K, *bla*Z and *erm*C (Shokry et al., 2018).

From chicken meat, *S. lugdunensis, S. epidermidis, S. hominis, S. haemolyticus, S. cohnii* and *S. lentus* were isolated. The percentage of resistance to β-lactams was variable, with the highest resistance being to penicillin and clindamycin and lowest to ampicillin-sulbactam (Osman et al., 2015).

3. Antimicrobial drugs

The Gram-positive bacteria consist of cytoplasmic membrane surrounded by a tough and rigid cell wall. In contrast, Gram-negative bacteria consist of thin cell wall surrounded by second lipid membrane called outer membrane (OM). The space between the OM and cytoplasmic membrane is referred as periplasm. The OM is an additional protective layer in Gram negative bacteria and prevents entering of many substances into the bacterium. Nonetheless, this membrane contains channels called porins, which allow the entry of various molecules such as drugs. The cell wall prevents bacterial cell from mechanical and osmotic stresses. The cytoplasmic membrane prevents ions from flowing into or out of the cell and maintains the cytoplasmic and bacterial components (Kapoor et al., 2017).

Antimicrobials are basically used to treat and control infections of bacterial or fungal origin in humans and animals. The beginning of antimicrobials was through the discovery of penicillin by Alexander Fleming in 1928 and subsequent development of the fungal metabolite into a viable treatment for infections by Howard Florey. The discovery of penicillin was followed by the development of a variety of new antibiotic classes such as aminoglycosides (e.g., gentamicin, amikacin, tobramycin), glycopeptides (e.g., vancomycin, teicoplanin), cephalosporins (e.g., cefalotin, cefazolin), and quinolones (e.g., ciprofloxacin, ofloxacin). In veterinary practice, antibiotics are extensively used for many purposes such as prevention and control of diseases, growth promotion and assistance in struggling environmental stress. A noticeable increase in the use of antimicrobials in veterinary practice has been recorded in the USA with a 109-fold rise from 1950 to 2004. About 60-80% of antimicrobial drugs are used as feed supplements and non-therapeutic purposes (Arikan et al., 2009).

During the 1960s and 1970s, antimicrobial drugs were very promising choices for treatment of infections. However, less than 50 years later the WHO published its first Global Report on Antimicrobial Resistance (AMR) and concluded that without intervention, a post-antibiotic era will evade, where minor infections and small injuries will be fatal (WHO., 2014). A later report stated that, the deaths due to antimicrobial-resistant bacterial infections would increase from 700.000 annually to 10 million annually by 2050, as the main cause of mortality and healthcare

industry losses that will cost trillions of USD (O'Neill, 2014). In 2016, a report was given to the United Nations (UN) concerning the serious consequences of antibiotic resistance threat human health (WHO., 2016).

3.1. Mechanisms of action of antimicrobials

3.1.1. Antibiotics targeting the cell wall

Bacterial cells are surrounded by a peptidoglycan cell wall that constitutes of long sugar polymers. The peptidoglycan undergoes cross-linking of the glycan strands by transglycosidases and the peptide chains extend from sugars into polymers and form cross links from one peptide to another (Kahne et al., 2005). The D-alanyl-alanine part of peptide chain is cross-linked by glycine residues in the presence of penicillin-binding proteins (PBPs) (Reynolds, 1989). This cross-linking strengthens the cell wall. The β-lactams and the glycopeptides inhibit cell wall synthesis. The primary targets of β-lactams are the PBPs that interacts with the β-lactam ring and disrupts peptidoglycan synthesis leading to lysis of bacterium (Džidić et al., 2008), while glycopeptides such as vancomycin inhibit binding of D-alanyl subunit with (PBP) leading to inhibition of cell wall synthesis (Grundmann et al., 2006).

3.1.2. Inhibitors of protein biosynthesis

Protein synthesis is catalyzed by ribosomes and cytoplasmic factors. The bacterial 70S ribosome is composed of two ribonucleoprotein subunits (30S and 50S) (Yoneyama and Katsumata, 2006). Antimicrobials inhibit protein biosynthesis by targeting the 30S or 50S subunit of the bacterial ribosome (Johnston et al., 2002; Vannuffel and Cocito, 1996).

3.1.2.1. Inhibitors of 30S subunit of ribosomes

a) Aminoglycosides

The aminoglycosides are positively charged molecules attached to the negatively charged OM leading to formation of large pores that allowing antibiotic penetration into the bacterial cell and reaching its target (bacterial ribosome). This transport requires oxygen as an energy motivator. This is the cause, that aminoglycosides work in aerobic conditions and have limited activity against anaerobic bacteria. Aminoglycosides interact with the 16S rRNA gene of the 30S subunit causing misreading and premature cessation of mRNA translation (Kapoor et al., 2017).

b) Tetracyclines

Tetracyclines act on conserved sequences of the 16S rRNA gene of the 30S ribosomal subunit and prevent binding of tRNA to the A site of ribosome (Wise, 1999; Yoneyama and Katsumata, 2006).

3.1.2.2. Inhibitors of 50S subunit of ribosomes

a) Chloramphenicols

Chloramphenicol inhibits the protein synthesis by preventing binding of tRNA to the A site of the ribosome. It interacts with the 23S r-RNA of the 50S subunit (Yoneyama and Katsumata, 2006).

b) Macrolides

Macrolides, lincosamides, and streptogramin B affect the early stage of protein synthesis by targeting the conserved sequences of the peptidyl transferase center of the 23S rRNA of the 50S ribosomal subunit resulting in a premature detachment of incomplete peptide chains (Wise, 1999; Yoneyama and Katsumata, 2006).

c) Oxazolidinones

Oxazolidinones (e.g. Linezolid) inhibit protein synthesis by binding to 23S rRNA of the 50S subunit, suppress 70S inhibition and interact with peptidyl-t-RNA (Bozdogan and Appelbaum, 2004; Lambert, 2005).

3.1.3. Inhibitors of DNA replication

The fluoroquinolones inhibit the bacterial DNA gyrase enzyme, which separates the double-stranded DNA and introduces negative supercoils and then reseals the nicked ends. This is necessary to prevent excessive positive supercoiling of the strands when they separate to permit replication or transcription. The DNA gyrase consists of two A subunits and two B subunits. The gyrase A subunit carries out the nicking of DNA, while gyrase B subunit introduces negative supercoils, and then the A subunit reseal the strands. The fluoroquinolones bind to A subunit and interfere with its strand cutting and resealing function. In Gram-positive bacteria, the major target of action for fluoroquinolones is topoisomerase IV which nicks and separates daughter DNA strand after DNA replication (Higgins et al., 2003; Wise, 1999; Yoneyama and Katsumata, 2006).

3.1.4. Folic acid metabolism inhibitors

Sulfonamides and trimethoprim inhibit folic acid metabolism. Sulfonamides inhibit dihydropteroate synthase and trimethoprim inhibits the enzyme dihydrofolate reductase (Yoneyama and Katsumata, 2006).

4. Antimicrobial resistance (AMR)

The dramatic increase in the emergence of antibiotic resistant bacteria has become a worldwide nightmare and a critical health threat leaving very limited treatment choices for infection control. In the USA, antibiotic resistant bacteria cause at least 23,000 deaths and $55–70 billion costs per year (Li and Webster, 2018). The AMR economic impact is expected to cost over $105 billion annually worldwide with the largest relative economic impact in Africa with a great drop in Gross Domestic Product (GDP) (KPMG, 2014).

Currently, there is a daily notorious increase in infections caused by microorganisms that fail to respond to treatments, all over the world. Meanwhile, the discovery of antibiotics has been decreasing rapidly over the past decades: for example, 16, 14, 10, and 7 new antibiotics were approved for treatment during 1983–1987, 1988–1992, 1993–1997, and 1998–2002, respectively. Moreover only 5 and 2 antibiotics were approved for treatment during 2003–2007 and 2008–2012, respectively (Boucher et al., 2013). This decrease was due to declining antibiotic research and development in pharmaceutical companies. Development of new antibiotic has been hindered by the rising antibiotic resistance and governmental strategies and regulations (DiMasi et al., 2016).

The Centers for Disease Control and Prevention (CDC) has classified the MDR microorganisms into three different threat levels (urgent , serious, and concerning) (CDC., 2013).

Between years 1937-1940, both penicillin and sulfonamides were approved for treatment. Few years later, resistances to both drugs were recorded (Davies and Davies, 2010). Resistance to chloramphenicol, tetracycline and streptomycin was reported in the 1950s. Methicillin was firstly introduced in 1959 and methicillin-resistant *S. aureus* (MRSA) was recorded in 1961. Linezolid and daptomycin were introduced in the 2000s and their resistance was reported five years later (Li and Webster, 2018). Carbapenems were firstly introduced in 1976 and imipenem was the first commercially available carbapenem (Kropp et al., 1985), however their resistance was also reported few years later. Then colistin has been used for treatment of infections caused by carbapenem-resistant bacteria and unfortunately, the first colistin resistance was recorded in 2016 (Mediavilla et al., 2016).

In human medicine, many factors can increase the drug resistance including insufficient amounts of active ingredients, poor-quality pharmaceutical ingredients and ineffective release of compounds. These factors induce subtherapeutic doses of drugs and play a main role in antimicrobial resistance (Zaman et al., 2017).

In veterinary practice, the common regimens in animal and poultry farms are to treat sick animals or birds together with healthy ones throughout pantreatment of the whole herds and flocks. Involvement of such large numbers of animals is considered as misuse and/or inappropriate usage that increases the probability of selecting for antibiotic resistant organisms (Yang et al., 2004). Residues of veterinary antibiotics (VAs) can be released to the environment through the manure of treated poultry and animals as soil fertilizers, direct contact with colonized animals and consumption of meat (Cornejo et al., 2018).

Animal health is also threatened because of infection with antimicrobial-resistant pathogens. This can prolong illness and decrease productivity through higher fatality rates.

Different reports have documented cases of antimicrobial resistance in human that were attributed to microbes of livestock origin with the possibility that infected animals can be asymptomatic (Mamun et al., 2017; Paphitou, 2013). This correlation was strongly proofed with many examples as when the glycopeptide avoparcin was banned in the 1990s in European Union, the prevalence of vancomycin-resistant enterococci in both humans and poultry has decreased (Klare et al., 1999).

Different studies reported the antimicrobial overuse in veterinary medicine worldwide (Yang et al., 2004).

In Egypt, there have been many studies discussing the prevalence of antibiotic-resistant bacteria and antibiotic residues in poultry and poultry meat (Elkenany et al., 2019; Moawad et al., 2017a; Osman et al., 2019). The excessive and uncontrolled use of antimicrobials in veterinary practice in Egypt plays a key role in the spread of antibiotic-resistant bacteria, especially enteric pathogens (*E. coli* and *Salmonella* spp.) that increasingly threatens the successful treatment of infectious diseases (Moawad et al., 2019; Moawad et al., 2017a; Moawad et al., 2018).

4.1. Origin and transmission of antimicrobial resistance throughout bacterial species

Most of antimicrobials are naturally produced molecules to which some bacteria have developed mechanisms to overcome their action. These bacteria are considered naturally resistant to one or more antimicrobials. The problem is the acquired resistance in a bacterial population that was originally susceptible to the antimicrobial compound. The acquired

antimicrobial resistance is mainly attributed to mutations in chromosomal genes or due to the acquisition of external genetic determinants of resistance from resistant bacteria. The clinical susceptibility breakpoints (susceptible, intermediate and resistant) mainly relies on the *in vitro* activity of an antibiotic against a measurable bacterial concentration with fixed conditions (e.g., infection site concentrations of the antimicrobial). The *in vivo* susceptibility of an organism to antibiotics differ according to the size of the bacterial inoculum (CLSI., 2014). An organism is considered multidrug-resistant (MDR) when it is resistant to three or more drugs of different antimicrobial classes (Liarrull et al., 2010).

Mutations resulting in antimicrobial resistance modify the antibiotic action via decrease in the drug uptake and change of the antimicrobial target or efflux pumps that extrude the antibiotic molecule (Munita and Arias, 2016).

Bacterial acquisition of external genetic material occurs through transformation (DNA integration), transduction (phage mediated) or conjugation. Transformation "naturally" incorporate naked DNA to develop resistance while conjugation uses mobile genetic elements (MGEs) to share genetic information. The most important MGEs are plasmids and transposons. Direct chromosome to chromosome transfer has also been documented (Manson et al., 2010).

Integrons also play a main role in antibiotic resistance. They provide additional new genes into bacterial chromosomes with the necessary mechanism to ensure their expression (Thomas and Nielsen, 2005).

4.2. Mechanisms of bacterial antimicrobial resistance

4.2.1. Modifications of the antibiotic molecule

Bacteria develop the resistance through production of enzymes that are able to destroy the antibiotic molecule or making the antibiotic unable to interact with its target. The β-lactamases, aminoglycoside-modifying enzymes, and chloramphenicol acetyltransferases (AACs) are examples for these enzymes (Mims et al., 2004).

a. Antibiotic chemical alteration

Chemical alteration occurs through production of modifying enzymes that make chemical changes of the antimicrobial molecule in both Gram-negative and Gram-positive bacteria. Many types of enzymatic actions have been described: **1**) acetylation (aminoglycosides, chloramphenicol, streptogramins), **2**) phosphorylation (aminoglycosides, chloramphenicol) and **3**) adenylation (aminoglycosides, lincosamides) (Schwarz et al., 2004).

b. Destruction of the antibiotic molecule

The production of β-lactamases is the main mechanism of β-lactam resistance. These enzymes destroy the amide bond of the β-lactam ring, leaving the antimicrobial ineffective. The plasmid-encoded β-lactamase capable of hydrolyzing ampicillin was firstly found among Gram-negative bacteria and named (*bla*$_{TEM-1}$) after the patient in which it was originally found (Temoneira) (Paterson & Bonomo, 2005). This was followed by appearance of enzymes capable of destroying other β-lactam drugs.

Genes encoding for β-lactamases are called (*bla*) followed by the name of the specific enzyme (e.g. *bla*$_{KPC}$) and they have been found in the chromosome or localized in MGEs. These genes can also be found on integrons (Sultan et al., 2018).

The β-lactamases are classified based up on two main schemes; **1**) The Ambler classification that relies on amino acid sequence and divides β-lactamases into 4 groups (A, B, C, and D), and **2**) The Bush-Jacoby classification that divides β-lactamases according to their biochemical function into 4 categories (Bush, 2013).

Both classifications have some common features. For example, TEM-3 is developed from the original TEM-1 penicillinase after changes of two amino acids that leaded to the ability to hydrolyze third generation cephalosporins and aztreonam (a change from narrow spectrum into “extended spectrum β-lactamase” [ESBL]) (Paterson and Bonomo, 2005; Sirot et al., 1987).

The term ESBL means the ability to hydrolyze penicillins, 3rd generation cephalosporins and monobactams, but not cephamycins and carbapenems. Most of the ESBLs are inhibited by clavulanic acid or tazobactam. This help to differentiate them from AmpC enzymes, which are class C β-lactamases that also hydrolyze 3rd generation cephalosporins, but are not inhibited by clavulanic acid or tazobactam (Rawat and Nair, 2010).

The group carbapenemases (a β-lactamases group that hydrolyze carbapenems) are divided into serine carbapenemases (class A or D) and metallo-carbapenemases (class B). The class A carbapenemases contains five different families. Three of five families are chromosomally encoded: not-metallo-enzyme carbapenemase (NMC), imipenem-hydrolyzing enzyme (IMI) and *Serratia marcescens* enzyme (SME) and the remaining two *Klebsiella* (*K.*) *pneumoniae* carbapenemase functional group 2f enzymes (KPC) and (GES) are plasmids or other MGEs encoded (Queenan and Bush, 2007). Carbapenemases are all inhibited by clavulanic acid and tazobactam as all members of class A hydrolyze aztreonam but not cephamycins. KPC was firstly reported in 1996 from *K. pneumoniae* in USA (Yigit et al., 2001). Carbapenemases were firstly identified in *Klebsiella spp*., however they were reported in several other Gram-

negatives, including *E. coli*, *Proteus mirabilis*, *Enterobacter* spp., *Salmonella* spp. and *Pseudomonas* (*P.*) *aeruginosa*. About 22 variants of the bla_{KPC} gene have been described to date, most of them located on plasmids (Nordmann et al., 2009).

Different classes of β-lactamases

Class A β-lactamases share the serine residue in the catalytic site with class C and D enzymes. Most class A enzymes are inhibited by clavulanic acid and their spectrum of activity include monobactams but not cephamycins (cefoxitin and cefotetan). Class A enzymes include a wide range of proteins with very different catalytic activities, covering penicillinases (TEM-1 and SHV-1 that only hydrolyze penicillin), ESBLs (such as CTX-M) and carbapenemases-like KPC (*Klebsiella pneumoniae* carbapenemase).

CTX-M is a plasmid-encoded ESBL commonly found in *Enterobacteriaceae*. It is a class A Ambler, however it is not derived from TEM or SHV. It was thought to be acquired from *Kluyvera* spp. (Bonnet, 2004). It is the most prevalent ESBL worldwide and responsible for majority of cephalosporin resistance in *E. coli* and *K. pneumoniae*.

Class B β-lactamases (metallo- β-lactamases) use the metal ion (usually zinc) as a cofactor instead of serine residue for the attack of the β-lactam ring of the antimicrobial drug. They are inhibited by the presence of ion-chelating agents such as EDTA. They are active against a wide range of β-lactams, including carbapenems (class A carbapenemases). Metallo-β-lactamases are not inhibited by clavulanic acid or tazobactam and efficiently hydrolyze cephamycins and aztreonam. They were discovered 50 years ago encoded by genes usually located in the chromosome of non-pathogenic bacteria. However, in the1990's, VIM and IMP were increasingly reported in *Enterobacteriaceae*, *Pseudomonas* spp. and *Acinetobacter* spp (Queenan and Bush, 2007). There are ten types of metallo-carbapenemases, but the important ones belong to four families, Verona integron-encoded metallo-β-lactamase (VIM), active-on-imipenem (IMP), New Delhi metallo-β-lactamase (NDM) and Sao Paulo metallo-β-lactamase (SPM).

The first IMP-type enzymes were described in Japan in 1990s in *Serratia* (*S.*) *marcescens*, and since then, more than 20 different subtypes have been described worldwide in *Enterobacteriaceae*, *Pseudomonas* spp. and *Acinetobacter* spp. The bla_{IMP} genes have been found on plasmids and forming part of class 1 integrons (Poirel et al., 2007). The VIM-type enzymes were firstly reported in 1990s in Verona, Italy and then spread all over the world. These enzymes were firstly described in *P. aeruginosa*, but their association with class 1 integrons, helped their transmission to other bacterial species. VIM-2 is the most widely distributed enzyme all over the world (Cornaglia et al., 2011).

In 2008, a new carbapenemase was identified in *K. pneumoniae* in New Delhi, India, and named as NDM-1 (Kumarasamy et al., 2010). Although NDM-1 shares little amino acid identity with other members of the Ambler class B enzymes (e.g. 32% with VIM-1), its hydrolytic profile is very similar to all of them. The bla_{NDM} gene is plasmid-mediated, found in different Gram-negative species. Unlike other genes encoding metallo-enzymes, bla_{NDM} is not usually related to integrons (Nordmann et al., 2011). Moreover, MGEs-containing genes coding for NDM enzymes generally carry multiple other resistance determinants such as genes encoding other carbapenemases (e.g. VIM-type and OXA-type enzymes), ESBL, methylases conferring resistance to macrolides, the quinolone resistance Qnr protein, enzymes that modify rifampin and proteins involved in resistance to sulfamethoxazole. So, the presence of NDM-1 is usually accompanied by a multidrug-resistant phenotype.

The emergence of NDM-1 is concerning because the bla_{NDM} gene has shown to be readily transmissible among different types of Gram-negative organisms, spreading to many countries in a short time and becoming one of the most feared resistance determinants in several parts of the world (Cornaglia et al., 2011).

Class C β-lactamases confer resistance to all penicillins and cephalosporins, including cephamycins. They do not hydrolyze aztreonam and are not inhibited by clavulanic acid. The most clinically relevant class C enzyme is AmpC, which is a cephalosporinase that is generally encoded on the chromosome (however, the bla_{AmpC} gene was found in plasmids). The chromosomal AmpC is produced by *Pseudomnas* (*P.*) *aeruginosa*, *Citrobacter* (*C.*) *freundii*, *Enterobacter* (*E.*) *cloacae*, *Enterobacter* (*E.*) *aerogenes*, *Serratia* (*S.*) *marcescens*, *Providencia* spp., and *Morganella morganii* (Jacoby, 2009).

Class D β-lactamases include a wide range of enzymes that were differentiated from the class A penicillinases that they can hydrolyze oxacillin and they are poorly inhibited by clavulanic acid. Many OXA variants have been described, including enzymes with the ability to degrade third generation cephalosporins (ESBLs). OXA-48 is a widely disseminated class D carbapenemase in *K. pneumoniae*. The OXA-48 and its variants are now widely spread in *Enterobacteriaceae*, and *Acinetobacter spp.* Many other types of OXA enzymes have been described and usually encoded by genes that are often found in MGEs (Evans and Amyes, 2014).

4.2.2. Decreased antibiotic efflux and penetration

a. Efflux pumps

This mechanism is the ability of bacterial cell to eject the antimicrobial molecule. Most of efflux pumps are multidrug transporters that are capable to pump a wide range of antibiotics, ie. tetracyclines, macrolides, and fluoroquinolones in both Gram-negative and Gram-positive pathogens. The genes encoding efflux pumps can be located on MGEs (as initially described for the *tet* gene) or on the chromosome. Chromosomally encoded pumps can explain the inherent resistance of some bacterial species to a particular antibiotic (e.g. *E. faecalis* intrinsic resistance to streptogramin A) (Singh et al., 2002). Tetracycline resistance is one of the classic examples of efflux-mediated resistance.

b. Decreased permeability

Many of the antibiotics have bacterial intracellular targets. Gram-negative bacteria have developed mechanisms to prevent the antibiotic from reaching its intracellular target by decreasing the uptake of the antimicrobial molecule. Hydrophilic molecules such as β-lactams, tetracyclines and some fluoroquinolones are particularly affected by changes in permeability of the outer membrane since they often use water-filled diffusion channels known as porins to cross this barrier (Pages et al., 2008). That makes some antibiotics such as vancomycin ineffective against gram-negative organisms due to the lack of penetration through the outer membrane (prions). The same with β-lactams ineffectiveness against *Pseudomonas* and *Acinetobacter* (Hancock and Brinkman, 2002).

Modifications of porins occure through change in the level of porin expression, shift in the type of porins expressed and impairment of the porin function (Nikaido, 2003).

4.2.3. Changes in target sites

A common strategy for bacteria to develop antimicrobial resistance is to change target sites specific for these drugs that inhibit the antibiotic from reaching its binding site (Munita and Arias, 2016).

a. Target protection

Most of genes coding for proteins responsible for target protection are carried on MGEs, however some of them have been found to be chromosome-mediated. Tetracycline, fusidic acid and fluoroquinolones are examples of drugs affected by this mechanism through genes *tet*M and *tet*O (tetracycline), *fus*B and *fusc* (fusidic acid) and *qnr* (fluoroquinolones) (Munita and Arias, 2016).

b. Modification of the target site

This mechanism affects antibiotic binding of almost all families. These target changes may consist of point mutations in the genes encoding the target site, enzymatic alterations of the binding site and replacement of the original target leading to decrease in the affinity of the antibiotic for the target site (Munita and Arias, 2016).

b.1. Target site mutation

Classical example of mutational resistance is the rifampin resistance, which occurs by point mutations resulting in amino acid substitutions (Floss and Yu, 2005).

Colistin resistance is also an example of chromosomal mutation through modifications of the bacterial outer membrane by alteration of the Lipopolysaccharides (LPS) and reduction in its negative charge (Landman et al., 2008).

Oxazolidinones (linezolid and tedizolid) are good examples of antimicrobial resistance due to mutational changes. They interact with the A site of bacterial ribosomes leading to inhibition of protein synthesis. Linezolid is the most used antibiotic of this class, while tedizolid was only recently introduced for clinical use. Although linezolid resistance remains an uncommon phenomenon, it is now reported in clinically relevant Gram-positives bacteria. The most commonly characterized mechanisms of linezolid resistance include mutations in genes encoding the domain V of the 23S rRNA gene and/or the ribosomal proteins L3 and L4, and methylation of A2503 in the 23S rRNA gene mediated by the *Cfr* enzyme (Mendes et al., 2014).

b.2. Enzymatic change of the target site

The macrolide resistance is conferred to the erythromycin ribosomal methylation (*erm*) genes which are capable of methylation of the ribosome. These enzymes make adenine residue in the domain V of the 23rRNA of the 50S ribosomal subunit. Due to this biochemical change, the binding of the antimicrobial molecule to its target is impaired. Since macrolides, lincosamides, and streptogramin B antibiotics have overlapping binding sites in the 23S rRNA, expression of the *erm* genes confers cross-resistance to all members of the macrolides, lincosamides and streptogramines (MLS) group (Leclercq, 2002; Weisblum, 1995). More than 30 different *erm* genes have been described, many of them located in MGEs, including aerobic and anaerobic Gram-positive and Gram-negative bacteria.

For linezolid resistance, the *cfr*-mediated resistance is an example of enzymatic alteration of the target. The *cfr* gene has been reported in several species of human pathogens, including *S. aureus*, *E. faecalis*, *E. faecium* and some Gram-negative bacteria. This gene encodes the *cfr* enzyme, which is a member of the S-adenosyl-L-methionine (SAM) methylase family that

also confers resistance to phenicols, lincosamides, pleuromutilins, and streptogramin A. Moreover, *cfr* has been associated with various MGEs suggesting that it has an enhanced potential of spread causing transferable linezolid resistance in the future. It is worth saying that carriage of *cfr* does not appear to confer resistance to the recently FDA-approved oxazolidinone tedizolid (Locke et al., 2014).

b.3. Replacement of the target site

Some bacteria develop new targets that are similar to original target in biochemical functions but are not inhibited by the antimicrobial molecule. The most common example is the methicillin resistance in *S. aureus* due to the acquirement of an exogenous PBP (PBP2a) and vancomycin resistance in enterococci through modifications of the peptidoglycan structure mediated by the *van* gene clusters (Munita and Arias, 2016).

Resistance to methicillin in *S. aureus* results from the acquisition of a foreign *mec*A gene (likely from *S. sciuri)* often located in a large DNA fragment designated staphylococcal chromosomal cassette *mec* (SCC*mec*). The *mec*A gene encodes PBP2a, a PBP that has low affinity for all β-lactams, including penicillins, cephalosporins (except for last generation compounds) and carbapenems. Acquisition of *mec*A renders most β-lactams useless against MRSA and alternative therapies must be used in serious infections (Hiramatsu et al., 2013; Moellering, 2012).

4.2.4. Resistance due to cell adaptations

Bacteria have developed different mechanisms to adapt with environmental stressors in order to survive the wide range of hosts. Inside the host, bacterial organisms are constantly attacked by the host's immune system and bacterial pathogens have devised very complex mechanisms to avoid their cell wall and membrane destruction. Development of resistance to daptomycin and vancomycin are the most clinically relevant examples.

Daptomycin is a lipopeptide antibiotic that alters cell envelope homeostasis. The bactericidal activity requires four important steps: First, daptomycin is complexed with calcium (giving a positively charged molecule) and, subsequently, is directed to the negatively charged cell membrane (CM) (Pogliano et al., 2012). Second, the antibiotic molecules initially oligomerizes at the outer layer of the CM in the presence of phospholipid phosphatidylglycerol (PG). Third, the antibiotic molecules reach the inner layer of the CM and alter the properties of the CM leading to leakage of ions (e.g, potassium) from the cytoplasm. Finally, these structural and functional CM alterations lead to bacterial death (Zhang et al., 2014). Some bacteria have developed defense mechanisms to withstand the drug effect and protect cell envelope.

5. Identification of antibiotic resistance

The antibiotic resistance of microorganisms is detected either phenotypically or genotypically. The phenotypic resistance is determined using qualitative or quantitative methods. The presence of phenotypic resistance, which could be a result of gene transfer, is a prior step to determine the presence of genes that encode resistance (Schumacher et al., 2018).

a. Phenotypic identification

1. Qualitative methods for detection of antibiotic resistance

Kirby-Bauer or disk diffusion test is the most frequently used qualitative method for determining resistance in microorganisms. A disk diffusion method is performed by applying an inoculum containing approximately 1-2x10^8 colony forming units (cfu)/ml of the microorganism to the surface of the Petri dish with the appropriate nutrient medium. A filter-paper disk impregnated with the antibiotic to be tested is then placed on it. The results are read after incubation at 37°C for 16-24h by measuring the growth inhibition zone of the examined microorganism (Jorgensen and Ferraro, 2009). Although the disc diffusion is the most often used method in clinical practice because it is cheap, simple and well-standardized, there is no possibility of quantification of the resistance results. Isolates can only be classified as susceptible or resistant, based on the size of zone inhibition (EFSA., 2012).

2. Quantitative methods for detection of antibiotic resistance

Quantitative methods based on the determination of minimal inhibitory concentration (MIC) are more accurate and relevant (EFSA., 2012). The MIC is the lowest antibiotic concentration that has the ability to inhibit microorganism growth under precise conditions (Wiegand et al., 2008). There are several methods for determining MIC values, such as agar dilution method, broth dilution method, E-tests and more recently automated instrumental systems such as Vitek-2 system (Jorgensen and Ferraro, 2009).

2.1. **Agar dilution method** is a method in which certain concentrations of antibiotics are added directly to the agar, and then the examined microorganism is deposited on its surface. Results are based on the presence or absence of microorganism growth on agar surface after incubation (Wiegand et al., 2008).

2.2. **Broth dilution method**, a liquid medium is used with the addition of antibiotics at a precisely determined concentration to determine the presence of resistance. There are two methods of broth dilution:

   a. **Macrodilution method:** which is performed in tubes and the broth volume into which the microorganism and the antibiotic are added should be greater than 2 mL. Although the macrodilution method is the first method developed to determine the

MIC value it is no longer in use because it is more time consuming and more expensive than the microdilution method (Jorgensen and Ferraro, 2009).

b. **Microdilution method:** involves the use of microtitration plates with the total volume in each well plate not exceeding 500 µL (Wiegand et al., 2008). Microtitration plates have a standard of 96 well configuration allowing different concentration of antibiotics to be tested.

Antibiotic concentrations in the tubes or microtitration plates are usually in series of double dilutions (e.g., 1, 2, 4, 8, etc. µg antibiotics / mL substrate). Microdilution plates can be manufactured directly in a laboratory, but commercial plates with wells containing lyophilized antibiotics at appropriate concentrations are also available. Concentrations of antibiotics in microtitration plate wells are prescribed according to standards or appropriate guidelines (Jorgensen and Ferraro, 2009). The results of the macrodilution method are interpreted on the basis of the broth cloudiness and in the microdilution method they are based on the presence or absence of the sediment in the microtitration well. The lowest concentration of antibiotics in which no visible cloudiness or sediment is formed considered as the MIC value for the examined isolate (Jorgensen and Ferraro, 2009).

2.3. E-tests

E-tests are thin plastic strips with scaling numbers that indicate antibiotic concentrations. Strips are placed on the surface of a suitable agar previously inoculated with the examined microorganism. MIC values are read as the point where the growth inhibition ellipse intersects with the MIC scale on the strip. E-tests are simple and easy to perform (Jorgensen and Ferraro, 2009).

2.4. Automated and Semi-automated techniques

Recently, semi-automated and automated methods for the determination of MIC values have been developed, such as MicroScan Vitek-2 System (bioMeriéux), BD Phoenix Automated Microbiology Diagnostics (BD Diagnostics), WalkAway (Siemens Healthcare Diagnostics) and Sensititre Aris 2X (Trek Diagnostic Systems). Automated methods allow rapid and standardized reading of results but their use is still limited to human practice (Jorgensen and Ferraro, 2009).

Specificity of resistance testing differs according to the origin of isolates that is either of clinical or food isolates. Determination of resistance of a strain isolated from food is based on microbiological (epidemiological) MIC limit values, unlike clinical isolates for which resistance determination is based on clinical limit values (EFSA., 2008b).

Clinical limit values are aimed at examining the possibilities of treating a bacterial infection with an antibiotic, taking in consideration clinical studies of efficacy, antibiotic dosing, pharmacodynamics and pharmacokinetics (Silley, 2012).

Resistance monitoring programs for bacteria isolated from food set monitoring of resistance in *Salmonella* spp. and *Campylobacter* spp. as a minimum condition (WHO., 2011). Additionally, *E. coli* was most commonly included as a marker of Gram-negative commensal microflora and *Enterococcus* spp. as a marker of Gram-positive commensal microflora (Founou et al., 2016; WHO., 2011). According to the recommendations of the European Food Safety Authority (EFSA), testing of MIC values in *Salmonella* spp, *Campylobacter* spp, *Enterococcus* spp. and *E. coli* should be done according to instructions issued by the European Comittee on Antimicrobial Susceptibility Testing (EUCAST) (EUCAST., 2017) using only quantitative methods for determining MIC values, since the disk diffusion method is marked as inadequate (EFSA., 2008a). The most commonly used medium for antibiotic resistance testing is Mueller-Hinton broth (McDermott et al., 2005).

b. Molecular identification of antibiotic resistance-associated genes

The genetic basis of resistance is determined by molecular-biological methods using PCR technique. All the data necessary for the design of primers, the data on resistance genes most commonly present in the examined microorganism, as well as data on their localization and transferability can be found in literatures.

Although PCR is the most commonly used method for proving the presence of resistance genes, it requires the isolation of bacterial DNA and depends on the culture techniques and their limitations in bacterial isolation. This leads to improvement of advanced, culture-independent techniques, such as meta-genomics and sequencing of whole genome (Founou et al., 2016). These methods enable the detection and testing of the whole bacterial genome, the identification of new genetic traits and the identification of unknown genetic elements, which is not possible with routine PCR methods (Allen, 2014; Thanner et al., 2016).

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C H A P T E R 2

Antimicrobial resistance in *Enterobacteriaceae* from healthy broilers in Egypt: emergence of colistin resistant and extended-spectrum β-lactamase-producing *Escherichia coli*

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Antimicrobial resistance in *Enterobacteriaceae* from healthy broilers in Egypt: emergence of colistin resistant and extended-spectrum β-lactamase-producing *Escherichia coli*

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Abstract

Background: Poultry remains one of the most important reservoir for zoonotic multidrug resistant pathogens. The global rise of antimicrobial resistance in Gram-negative bacteria is of reasonable concern and demands intensified surveillance.

Methods: In 2016, 576 cloacal swabs were collected from 48 broiler farms located in five governorates in northern Egypt. Isolates of *Enterobacteriaceae* could be cultivated on different media and were identified by MALDI-TOF MS and PCR. *Escherichia coli* isolates were genotyped by DNA-microarray-based assays. The antimicrobial susceptibility to 14 antibiotics was determined and resistance-associated genes were detected. The VITEK-2 system was applied for phenotypical confirmation of extended-spectrum β-lactamase-producing isolates. The determination of colistin resistance was carried out phenotypically using E-test and genotypically using PCR for detection of the *mcr*-1 gene.

Results: Out of 576 samples, 72 representatives of *Enterobacteriaceae* were isolated and identified as 63 *E. coli* (87.5%), 5 *Enterobacter cloacae* (6.9%), 2 *Klebsiella pneumoniae* (2.8%) and 2 *Citrobacter* spp. (2.8%). Seven out of 56 cultivated *E. coli* (12.5%) were confirmed as ESBL-producing *E. coli* and one isolate (1.8%) as ESBL/carbapenemase-producing *E. coli*. Five out of 63 *E. coli* isolates (7.9%) recovered from different poultry flocks were phenotypically resistant to colistin and harboured *mcr*-1 gene.

Conclusions: This is the first study reporting colistin resistance and emergence of multidrug resistance in *Enterobacteriaceae* isolated from healthy broilers in the Nile Delta region, Egypt. Colistin-resistant *E. coli* in poultry is of public health significance. The global rise of ESBL- and carbapenemase-producing Gram-negative bacteria demands intensified surveillance. ESBL-producing *E. coli* in poultry farms in Egypt are of major concern that emphasizes the possibility of spread of such strains to humans. The results also reinforce the need to develop strategies and to implement specific control procedures to reduce the use of antibiotics.

Keywords: *Enterobacteriaceae*, Antibiotic resistance, DNA microarray, ESBL, Colistin, Broiler

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Background

Poultry and their products are considered the main vehicle for pathogenic bacteria such as *Salmonella* (*S.*) serovars, *Escherichia* (*E.*) *coli* and *Klebsiella* (*K.*) spp. that cause foodborne infections in humans [1–3].

The prevalence of highly antibiotic-resistant *E. coli* was recorded in poultry meat more frequently than in all other kinds of meat [4, 5].

Extended-spectrum β-lactamases (ESBLs) are plasmidencoded enzymes found in Gram-negative bacteria especially in *Enterobacteriaceae* conferring resistance to first, second and third generation cephalosporins while they are inhibited by clavulanic acid [6–10].

ESBL-producing *Enterobacteriaceae* have emerged as pathogens in both poultry and humans [7, 11]. Many ESBL-producers are additionally multiresistant to non-βlactam antibiotics, including fluoroquinolones, aminoglycosides, trimethoprim, tetracyclines, sulfonamides and chloramphenicols [12, 13]. Resistance to cephalosporins is mediated by ampicillin class C β-lactamases (AmpC β-lactamase) and encoded by bla_{CMY} genes [14, 15]. Carbapenems are still the drugs of choice to treat infections with ESBL-producing *Enterobacteriaceae* in humans [16] and their increasing use reinforces the probability of resistance development to carbapenems among *Enterobacteriaceae* [17–19]. The coexistence of multiple ESBL and carbapenemase genes as well as other antibiotic resistance determinants on mobile elements is of a major concern that might lead to the emergence of organisms with resistance to all antibiotics [6, 20, 21].

Most ESBLs encoding genes in bacteria of clinical interest are located on plasmids [22]. These plasmids may also carry genes encoding resistance to other drug classes including aminoglycosides and fluoroquinolones [23]. Transmission of ESBLs genes can occur either by emerging bacterial clones or by horizontal gene transfer. In the latter case, plasmids containing resistance genes, spread between bacteria [22]. Colistin recently gained attention as a last-resort antibiotic for treatment of infections caused by multidrug resistant Gram-negative bacteria. In veterinary practice, colistin is a drug of choice for the treatment of frequent digestive tract infections caused by *E. coli* in food-producing animals [24]. The irrational use of colistin in veterinary practice may be the main cause of the increasing rate of colistin resistance. Recently, the emergence of colistin resistance has caused great concern [25, 26] and resistance mediated by the plasmid-borne *mcr*-1 gene has been detected worldwide in *Enterobacteriaceae* [27].

In some countries, antimicrobials are used in the poultry industry for treatment of diseased animals, prevention of diseases and promotion of growth [28–30]. In Egypt, *E. coli* infections are considered as one of the most serious diseases leading to economic losses in poultry production [31].

Unfortunately, there are no legislations in Egypt regulating the use of antibiotics. Some of them such as tetracycline, quinolones and beta-lactams are still used for non-therapeutic uses [32]. This improper use of antimicrobials leads to rapid selection of multiresistant strains of *Enterobacteriaceae* in poultry and plays a key role in the spread of antibiotic-resistant bacteria along the food chain to humans [33–35].

In recent years, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been applied as a wide-range technique for bacterial identification [36]. Microarray systems are wellestablished tools for rapid genotypic characterization of bacteria and identification of resistance and virulenceassociated determinants [37]. The data can be obtained in a single experiment with the benefit of saving time and money [38–40]. The broth microdilution method proved to be an easy and reliable method for determination of the minimum inhibitory concentration (MIC) of antibiotics and can be used as an alternative technique to agar diffusion test [41–43].

Table 1 Overview about investigated poultry farms and the number of collected samples

	Governorate					Total
	Dakahlia	Sharkiya	Gharbiya	Damietta	Kafr El-Sheikh	
No. of farms	20	5	4	9	10	48
No. of birds	58,000	18,000	12,000	28,000	28,000	144,000
No. of samples	232	72	48	112	112	576

The use of a rapid molecular assay as an alternative to phenotypic detection was proved to be a useful option for detection of antibiotic resistance to frequently applied antimicrobial agents in poultry production [43].

The objective of this study was to gain insight into the antimicrobial susceptibility of *Enterobacteriaceae*, especially *E. coli* originating from healthy broilers from different districts in northern Egypt and to understand its public health significance. In addition, the prevalence of ESBL/carbapenemase-producing *E. coli* and colistin resistance were investigated.

Methods

Isolation and characterization of bacterial strains

During 2016, 576 cloacal swabs were randomly collected from apparently healthy broilers housed in 48 farms located in five governorates, namely: Dakahlia, Kafr El-Sheikh, Damietta, Gharbiya and Sharkiya, in the Nile Delta region, Egypt. An overview about investigated poultry farms, the number of birds and the number of collected samples are given in Table 1. Sampling was carried out using sterile cotton swabs. The collected samples were transported at 4 °C to the laboratory for microbiological examination. The samples were enriched in Buffered Peptone Water at 37 °C for 24 h and then streaked on MacConkey Agar and Eosin Methylene Blue (EMB) Agar (Oxoid, Manchester, UK), followed by further incubation at 37 °C for 24 h. For identification of ESBLproducing *Enterobacteriaceae*, the enriched bacterial cultures were cultivated on Brilliance™ ESBL agar (Oxoid GmbH, Wesel, Germany) at 37 °C for 24 h.

Identification by MALDI-TOF MS

Isolates were identified using MALDI-TOF MS [44]. Interpretation of results was performed according to the manufacturer's recommendation: score of ≥ 2.3 represented reliable species level identification; score 2.0–2.29, probable species level identification; score 1.7–1.9, probable genus level identification, and score ≤ 1.7 was considered an unreliable identification [45].

DNA extraction and purification

Genomic DNA was extracted from bacterial cultures using High Pure PCR Template Purification Kit (Roche Diagnostics, Mannheim, Germany) according to the instructions of the manufacturer.

Identification of *E. coli* isolates using PCR

The identified *E. coli* isolates were confirmed at species level using a specific PCR assay targeting 16S rRNA genes with primers ECO-1 (5′-GAC CTC GGT TTA GTT CAC AGA-3′) and ECO-2 (5′-CAC ACG CTG ACG CTG ACC A-3′) which geared from previous study by Seidavi et al. [46]. The PCR reaction was carried out with the following amplification conditions: An initial denaturation step at 96 °C for 60 s was followed by 35 cycles of denaturation (96 °C for 15 s), annealing (58 °C for 60 s) and extension (72 °C at 45 s) with a final extension at 72 °C for 60 s. PCR resulted in 585 bp amplicons. PCR products were analyzed on a 1.5% agarose gel, stained with ethidium bromide and visualized under UV light.

Genoserotyping of *E. coli* isolates using microarray assay

The serotypes of *E. coli* isolates were determined using the *E. coli* SeroGenoTyping AS-1 Kit (Alere Technologies GmbH, Jena, Germany). Five microliters of extracted RNA-free DNA (with a concentration of at least 100 ng/ µl) were biotin-labeled by a primer extension amplification using *E. coli* SeroGenoTyping AS-1 Kit according to manufacturer's instructions. The procedures for multiplex labelling, hybridization and data analysis were carried out as described in a previous study [47].

Phenotypic antibiotic susceptibility testing

The antibiotic susceptibility testing of all isolates was performed with the MICRONAUT system using commercial 96-well microtiter plates (Merlin, Bornheim, Germany) as recommended by the manufacturer. This system allowed the determination of minimum inhibitory concentrations (MICs) of 14 antimicrobial agents (Tables 2, 3) in serial dilutions of the antibiotics. Overnight grown bacteria were suspended in NaCl solution (0.9%) to obtain a turbidity corresponding to a McFarland standard of 0.5 (Dr. Lange, CADAS photometer 30, Berlin, Germany). One hundred microliters of the suspension were diluted with 10 ml of Mueller–Hinton broth (Oxoid GmbH) resulting in a concentration of approximately 1 0^6–10^7 colony forming units (cfu)/ ml. In total, 100 µl of the inoculum were given in each well of the plate. After sealing the plates, they were incubated for 18 h to 24 h at 37 °C. Reading of plates was done with a photometer (Merlin) at a wavelength of 620 nm. An optical density of > 0.1 was interpreted as an indication of growth. MICs were interpreted with the advanced expert system MCN-6 (Merlin) using the guidelines of the German Institute for Standardization (Deutsches Institut für Normung, Berlin, Germany). *E. coli* ATCC 25922, *E. coli* ATCC 35218 and *K. pneumoniae* ATCC 700603 were used as quality controls.

Vitek-2 system

All suspected ESBL isolates were subsequently confirmed using an automated microdilution system (VITEK-2, bioMérieux Deutschland GmbH, Nürtingen, Germany) according to the instructions of the manufacturer. For this study, the test card AST-N289 was used that included the following antibiotics: piperacillin (PIP), piperacillin/tazobactam (TZP), cefotaxime (CTX), ceftazidime (CAZ), cefepime (FEB), aztreonam (ATM), imipenem (IMP), meropenem (MEM), amikacin (AMK), gentamicin (GEN), tobramycin (TOP), ciprofloxacin (CIP), moxifloxacin

(MXF), tigecycline (TGC), fosfomycin (FOS), colistin (CT) and trimethoprim/sulfamethoxazole (T/S).

Table 2 Antibiotic susceptibility of 56 *Escherichia coli* isolates from broilers using broth microdilution test

Antibiotic	Class	S	I	R	Resistance rate (%)
Penicillin (PEN)	β-Lactam	0	1	55	98.2
Erythromycin (ERY)	Macrolide	1	1	54	96.4
Rifampicin (RAM)	Ansamycin	1	1	54	96.4
Trimethoprim/sulfamethoxazole (T/S)	Diaminopyrimidine/sulfonamide	19	1	36	64.3
Streptomycin (STR)	Aminoglycoside	12	14	30	53.6
Tetracycline (TET)	Tetracycline	24	4	28	50.0
Ceftazidime (CAZ)	β-Lactam (cephalosporin)	24	9	23	41.1
Amoxicillin/clavulanic acid (AMC)	β-Lactam/β-lactamase inhibitor	29	12	15	26.8
Chloramphenicol (CMP)	Non-classified	41	2	13	23.2
Ciprofloxacin (CIP)	Fluoroquinolone	38	6	12	21.4
Gentamicin (GEN)	Aminoglycoside	38	7	11	19.6
Levofloxacin (LEV)	Fluoroquinolone	35	13	8	14.3
Amikacin (AMK)	Aminoglycoside	43	7	6	10.7
Imipenem (IMP)	β-Lactam (carbapenem)	48	7	1	1.8

S susceptible, *I* intermediate, *R* resistant

Table 3 Antibiotic susceptibility of 9 *Enterobacteriaceae* isolates other than *E. coli* from broilers using broth microdilution test

Antibiotic	*Enterobacter* Resistance			Resistance *cloacae* (n = 5) rate (%)	*Citrobacter* spp.			Resistance (n = 2) rate (%)	*Klebsiella* *pneumoniae*			Resistance (n = 2) rate (%)
	S	I	R		S	I	R		S	I	R	
Penicillin (PEN)	0	0	5	100	0	0	2	100	0	0	2	100
Erythromycin (ERY)	0	0	5	100	1	0	1	50.0	0	0	2	100
Rifampicin (RAM)	0	0	5	100	0	0	2	100	0	0	2	100
Trimethoprim/sulfamethoxazole (T/S)	2	0	3	60.0	0	1	1	50.0	0	0	2	100
Streptomycin (STR)	1	0	4	80.0	0	0	2	100	0	0	2	100
Tetracycline (TET)	4	0	1	20.0	1	0	1	50.0	0	0	2	100
Ceftazidime (CAZ)	3	1	1	20.0	0	0	2	100	0	0	2	100
Amoxicillin/clavulanic acid (AMC)	1	0	4	80.0	1	1	0	0.0	0	0	2	100
Chloramphenicol (CMP)	4	0	1	20.0	2	0	0	0.0	0	0	2	100
Ciprofloxacin (CIP)	4	0	1	20.0	2	0	0	0.0	0	0	2	100
Gentamicin (GEN)	4	0	1	20.0	2	0	0	0.0	0	0	2	100
Levofloxacin (LEV)	4	0	1	20.0	2	0	0	0.0	0	0	2	100
Amikacin (AMK)	5	0	0	0.0	1	0	1	50.0	2	0	0	0.0
Imipenem (IMP)	4	1	0	0.0	2	0	0	0.0	1	1	0	0.0

Detection of antibiotic resistance and virulence-associated genes of E. coli isolates by microarray analysis

Antimicrobial resistance (AMR) genotypes and other resistance genes were ascertained using the CarbDetect AS-2 Kit and *E. coli* PanType AS-2 Kit, respectively (Alere Technologies GmbH). The data were automatically summarized by the "result collector", a software tool provided by Alere Technologies GmbH. An antibiotic resistance genotype was assigned to be a carrier of a group of genes which have been described to confer resistance to a family of

antibiotics (e.g., the genotype "$bla_{CTXM1/15}$, bla_{TEM}" conferring resistance to 3rd generation cephalosporins).

The detection of virulence-associated genes was performed using *E. coli* PanType AS-2 Kit (Alere Technologies GmbH). Twenty-eight different combinations of genes encoding virulence factors associated with adhesion, fimbriae production, secretion systems, SPATE (serine protease auto-transporters), toxins and miscellaneous genes were detected. The genes were detected and analysed by the "result collector", a software tool provided by Alere Technologies GmbH.

Determination of colistin resistance

All identified *E. coli* isolates were tested for presence of plasmid-mediated *mcr*-1 gene using PCR [27]. Briefly, a PCR with 25 µL reaction mixture using CLR5-F (5′-CGG TCA GTC CGT TTG TTC-3′) and CLR5-R (5′-CTT GGT CGG TCT GTA GGG-3′) was performed with the following amplification conditions: initial denaturing at 96 °C for 60 s was followed by 35 cycles of denaturing at 96 °C for 15 s, annealing at 55 °C for 60 s and extension at 72 °C for 30 s. PCR was finished by final extension at 72 °C for 60 s. Amplicons (308 bp) were analyzed on 1.5% agarose gel, stained with ethidium bromide and visualized under UV light.

For isolates possessing *mcr*-1 gene, MICs were determined with RUO E-test colistin CO 256 according to the manufacturer's guidelines (bioMérieux Deutschland GmbH). Briefly, an overnight bacterial suspension in Mueller–Hinton broth was adjusted to a density of McFarland 0.5 evenly streaked on Mueller–Hinton agar plates to ensure uniform growth. Once the agar surface was dry, an E-test® colistin strip (concentration range from 0.016 to 256 µg/ml) was applied to the plate with sterile forceps. The MIC was determined after aerobic incubation for 20 h at 37 °C as the point, where inhibition of bacterial growth intersected the E-test strip. According to clinical breakpoints given by EUCAST, an isolate was defined as resistant to colistin when the MIC value was > 2 µg/ml [48].

Results

Isolation and identification of *Enterobacteriaceae* Out of 576 samples, 72 *Enterobacteriaceae* isolates were identified by MALDI-TOF MS. The isolates were classified as 63 *E. coli* (87.5%), 5 *Enterobacter cloacae* (6.9%), 2 *K. pneumoniae* (2.8%) and 2 *Citrobacter* spp. (2.8%).

Seven out of 63 *E. coli* isolates could not be re-cultivated for testing of antibiotic resistance after applying MALDI-TOF MS (11.1%), while DNA was extracted from preserving solution for further identification.

Antimicrobial susceptibility testing

The results of phenotypic antibiotic susceptibility testing of 56 re-cultivated *E. coli* isolates were given in Table 2. *E. coli* isolates showed high resistance rates to penicillin, erythromycin and rifampicin with 98.2, 96.4 and 96.4%, respectively. Resistance rates to other tested antibiotics were between 10.7% for amikacin and 64.3% for trimethoprim/sulfamethoxazole. Only one *E. coli* isolate (1.8%) was resistant to imipenem (Tables 2 and 6). Fifty-five out of 56 *E. coli* isolates (98.2%) were resistant to antibiotics of at least three different classes of antimicrobial agents and thus they were defined as multidrug resistant isolates (Table 2).

The antimicrobial susceptibility profiles for other species of *Enterobacteriaceae* were presented in Table 3. All 5 *Enterobacter cloacae* isolates were resistant to penicillin, erythromycin and rifampicin. Two *Citrobacter* spp. isolates were resistant to penicillin, rifampicin, streptomycin and ceftazidime. Two *K. pneumoniae* strains were sensitive to amikacin and imipenem but resistant to the rest of the antibiotics tested.

Genoserotyping of *E. coli* isolates using microarray analysis

Three out of 63 *E. coli* isolates (4.8%) were determined as O91 and O15; in all other cases O type determination failed. H antigen types were identified for all isolates. Seventeen different types of H antigens (H1, H2, H4, H5, H6, H7, H8, H10, H11, H16, H19, H21, H26, H28, H32, H34 and H51) were detected. H21 (14 isolates), H28 (10 isolates) and H51 (8 isolates) are being the most common types.

Detection of antibiotic resistance determinants in E. coli by microarray analysis

Several resistance genes were identified in 15 phenotypically resistant *E. coli* using microarray analysis (Table 4). The isolates were originated from four districts located in four provinces, namely Dakahliya (n = 7), Damietta (n = 3), Gharbiya (n = 3) and Kafr El-Sheikh (n = 4). Frequently identified resistance genes were *aad*A1 associated with resistance to aminoglycosides (n = 12), *sul*2 responsible for sulphonamide resistance (n = 10) and *flo*R connected with resistance to chloramphenicol (n = 10).

In this study, five *E. coli* isolates harboured *qnr*S gene while one isolate possessed *qnr*B gene associated with quinolone resistance. In two phenotypically ciprofloxacin-resistant *E. coli*, *qnr*S gene was detected (Table 4).

The *sul* and *dfr*A genes associated with sulphonamides/ trimethoprim resistance were detected in 16 and 13 *E. coli* isolates, respectively (Table 4). Meanwhile, *sul*3 gene corresponding to sulphonamide resistance was amplified in two susceptible *E. coli* to sulphonamide/trimethoprim.

Eleven *E. coli* phenotypically resistant to tetracycline were harboured one or more *tet* genes (*tet*A, *tet*B or *tet*C) (Table 4). Chloramphenicol resistance-associated genes *cat*A1, *cat*B3, *cml*A1 and *flo*R were detected in 13 *E. coli* isolates. Out of 13 chloramphenicol resistant isolates, 10 isolates harboured one or more resistance genes. The *cml*A1 gene was detected once in an *E. coli* strain that was phenotypically susceptible to chloramphenicol.

Table 4 Genoserotypes and resistance profiles of *Escherichia coli* isolates possessed resistance genes from different farms in four districts in Egypt

District	Isolate	O-type	H-type	Virulence genes	Resistance genes	Phenotypic resistance	ESBL
Dakahliya (n=7)	16CS0049	–	5	*hemL*	*qnrS*, *tetA*, bla_{TEM}	TET, RAM, ERY, PEN	
	16CS0070	–	51	*ipfA*, *cma*, *hemL*, *intI1*, *iroN*, *iss*	*sul3*, *cmlA1*, *floR*, *aadA1*, *aphA*, *strB*, *mphA*, *mrx*, bla_{TEM}	CIP, TET, CMP, RAM, GEN, STR, ERY, PEN	
	16CS0071	–	21	*ipfA*, *cma*, *intI1*, *iroN*, *iss*	*sul1*, *sul2*, *dfrA12*, *tetA*, *floR*, *aadA1*, *mphA*, *mrx*, bla_{CMY}, bla_{TEM}	CIP, LEV, T/S, TET, CMP, RAM, GEN, STR, ERY, PEN, AMP, CAZ	+
	16CS0078	–	4	*hemL*, *intI1*, *iss*	*sul2*, *dfrA14*, *tetA*, *strB*	T/S, TET, RAM, STR, ERY, PEN	
	16CS0740	–	26	*cif*, *espA*, *espF_O103H2*, *espJ*, *nleA*, *nleB*, *O157:H7*, *tccP*, *astA*, *hemL*, *intI1*, *tir*, *eae*	*sul1*, *sul2*, *sul3*, *dfrA14*, *tetA*, *cmlA1*, *floR*, *arr*, *aadA1*, *aadB*, *ant2*, *aphA*, *strB*, *mphA*, *mrx*, bla_{OXA-7}	CIP, T/S, TET, CMP, RAM, GEN, STR, ERY, PEN, AMP	+
	16CS0744	–	51	*cma*, *hemL*, *intI1*, *iroN*	*sul3*, *dfrA1*, *dfrA14*, *tetA*, *cmlA1*, *aadA1*, *aphA*, *mphA*, *mrx*, bla_{CMY}, bla_{TEM}	CIP, T/S, TET, RAM, GEN, STR, ERY, PEN, AMP, CAZ	
	16CS0772	–	51	*ipfA*, *mchF*, *hemL*, *intI1*, *iroN*, *iss*	*qnrS*, *sul2*, *dfrA17*, *tetB*, *catA1*, *floR*, *aadA1*, *aadA4*, *strB*, bla_{TEM}, bla_{CTX-M9}	CIP, T/S, TET, CMP, RAM, GEN, STR, ERY, PEN, AMP, CAZ	+
Damietta (n=3)	16CS0069	–	51	*cma*, *intI1*, *iroN*, *iss*	*sul3*, *dfrA12*, *cmlA1*, *aadA1*, *mphA*, *mrx*	CIP, LEV, T/S, CMP, RAM, STR, ERY, PEN, AMP	
	16CS0743	–	51	*mchF*, *hemL*, *intI1*, *iroN*, *iss*	*sul2*, *dfrA17*, *tetB*, *catA1*, *aadA4*, bla_{TEM}	CIP, T/S, TET, CMP, RAM, STR, ERY, PEN	
	16CS0752	–	6	*iha*, *prfB*, *sat*, *hemL*, *intI1*	*sul1*, *sul3*, *dfrA12*, *cmlA1*, *floR*, *aac6*, *aac6 Ib*, *aadA1*, *aadA2*, *aphA*, *mphA*, *mrx*, bla_{CTX-M1}/$bla_{CTX-M15}$, bla_{OXA-1}, bla_{TEM}	not determined	
Gharbiya (n=3)	16CS0747	–	32	*hemL*, *intI1*, *ireA*	*qnrS*, *sul3*, *tetA*, *cmlA1*, *floR*, *arr*, *aadA1*, bla_{OXA-7}, bla_{SHV}, bla_{TEM}	T/S, TET, CMP, RAM, GEN, STR, ERY, PEN AMP, CAZ	+
	16CS0761	–	51	*hemL*, *intI1*, *iroN*, *iss*	*qnrS*, *sul2*, *dfrA1*, *dfrA17*, *tetA*, *tetC*, *catA1*, *floR*, *aadA1*, *aadA4*, *aphA*, *strB*, *ereA*, bla_{TEM}, bla_{LAP-1}	CIP, LEV, T/S, TET, CMP, RAM, GEN, STR, ERY, PEN, AMP	+
	16CS0762	–	51	*ipfA*, *mchF*, *hemL*, *intI1*, *intI2*, *iroN*, *iss*	*sul2*, *dfrA1*, *dfrA17*, *tetA*, *tetB*, *tetC*, *catA1*, *floR*, *aadA1*, *aadA4*, *aphA*, *strB*, *ereA*, bla_{TEM}	not determined	
Kafr El-sheikh (n=4)	16CS0067	15	1	*prfB*, *senB*, *hemL*, *iss*	*qnrS*, *sul1*, *sul2*, *dfrA7*, *dfrA17*, *dfrA19*, *tetA*, *aadA4*, *strA*, *strB*, *mphA*, *mrx*, bla_{TEM}	T/S, TET, RAM, GEN, STR, ERY, PEN	
	16CS0075	–	51	*ipfA*, *cma*, *hemL*, *intI1*, *iroN*, *iss*	*sul3*, *cmlA1*, *floR*, *aadA1*, *aphA*, *mphA*, bla_{TEM}	CIP, TET, CMP, RAM, GEN, STR, ERY, PEN	
	16CS0755	–	10	*prfB*, *cme*, *intel1*, *iroN*, *iss*	*sul2*, *sul3*, *dfrA1*, *cmlA1*, *floR*, *aadA1*, *aphA*, *ereA*, bla_{TEM}	CIP, LEV, T/S, TET, CMP, RAM, GEN, STR, ERY, PEN, AMP, CAZ	+
	16CS0774	–	1	*ipfA*, *prfB*, *tsh*, *mchF*, *hemL*, *ireA*, *iroN*, *iss*	*qnrB*, *sul1*, *sul2*, *dfrA7*, *dfrA17*, *dfrA19*, *tetB*, *aphA*, bla_{DHA-1}	T/S, TET, RAM, STR, ERY, PEN, AMP, CAZ	+

Ten different genes (*aac*6, *aac*6Ib, *aad*A1, *aad*A2, *aad*A4, *aad*B, *ant*2, *aph*A, *str*A and *str*B) associated with aminoglycoside resistance were detected in 14 out of phenotypically tested *E. coli* isolates (Table 4). All isolates harbouring at least one of described genes were phenotypically resistant to streptomycin, but four of them were

susceptible to gentamicin (Tables 3, 4). All isolates with aminoglycoside resistance-associated genes were susceptible to amikacin.

Genes associated with macrolide resistance (*ere*A, *mph*A, *mrx*) were identified in 9 phenotypically resistant *E. coli* to erythromycin. The rifampicin resistance gene *arr* was identified in only 2 phenotypically rifampicin resistant isolates.

Fifteen out of 63 *E. coli* isolates (23.8%) harboured one or more ESBL, narrow-spectrum β-lactamase (NSBL) or AmpC β-lactamase genes. The gene bla_{TEM} was found in 13 DNAs of *E. coli* isolates (20.6%), bla_{CMY} and bla_{OXA-7} were detected in 2 samples each (3.2%) and bla_{SHV}, bla_{OXA-1}, $bla_{CTX-M1/15}$ and bla_{DHA-1} were found in one isolate (1.6%).

The correlation between the genotypic and phenotypic antimicrobial resistance of *E. coli* was demonstrated in Table 4.

Thirteen out of 15 isolates harbouring *bla* genes were analyzed using the VITEK-2 (Table 5). Two samples could not be tested, as they could not be re-cultivated. All isolates possessing beta-lactam resistance genes were resistant to piperacillin, while one isolate was susceptible to moxifloxacin. All isolates were susceptible to fosfomycin. The narrow-spectrum beta-lactamase gene bla_{OXA-1} was detected once in one ESBL isolates originated from poultry farm in Damietta.

Genotypic and phenotypic identification of resistance to colistin

Plasmid-mediated colistin resistance gene *mcr*-1 was detected in 5 out of 63 *E. coli* (8.0%) using a PCR assay. All of them were phenotypically confirmed as resistant to colistin using E-test (Table 6). All colistin resistant *E. coli* isolates were phenotypically resistant to rifampicin, penicillin and erythromycin but were susceptible to carbapenems. The colistin-resistant isolates originated from different poultry flocks in Dakahliya, Kafr El-Sheikh, Damietta and Gharbiya (Table 6).

Microarray analysis concerning virulence-associated genes The virulence genes detected by microarray were differently distributed all over the isolated *E. coli*. One isolate 16CS0740 isolated from poultry farm in Dakahliya harboured 7 genes of virulence-associated secretion system: *cif, esp*A, *esp*F_O103H2, *esp*J, *nle*A, *nle*B O157:H7 and *tcc*P.

eae and *iha* genes, involved in adhesion, were identified in 16CS0740 and 16CS0752, repectively. Two isolates harboured serine protease autotransporter genes. 16CS0774 carried *tsh* while 16CS0775 had *pic* and *vat* genes.

Several toxin genes were detected in 13 *E. coli* isolates including *ast*A, *cma*, *hly*E, *mch*F, *sat* and *sen*B. Each of these isolates carried only one toxin gene except 16CS0775 which harboured *mch*F, *hly*E and *cma*. Nineteen out of 63 *E. coli* isolates (30.2%) harboured *lpf*A and 3 others carried *prf*B fimbrae virulence gene. Miscellaneous genes encoding virulence factors as *hem*L, *int*I1, *ire*A, *iro*N, *iss* and *tir* genes were identified in 45 (71.4%), 10 (15.9%), 3 (4.8%), 9 (14.3%), 36 (57.1%) and 1 (1.6%) of 63 isolates, respectively.

The distribution of virulence-associated genes in *E. coli* isolates possessed antimicrobial resistance-associated genes was demonstrated in Table 4.

Discussion

Escherichia coli is a commensal pathogen of the intestinal tract of young and adult poultry [49]. Among healthy chickens, 10 to 15% of intestinal coliform bacteria may belong to potentially pathogenic serotypes of *E. coli* [50]. The identification of bacterial foodborne pathogens of zoonotic significance using rapid, accurate and reliable tools such as MALDI-TOF MS is mandatory for public health surveillance [44, 51].

Table 5 Results of antimicrobial resistance testing using VITEK-2 system

Isolate	PIP	TZP	CTX	CAZ	FEB	ATM	IMP	MEM	AMK	GEN	TOB	CIP	MXF	TGC	FOS	CT	T/S	
16CS0049	R	I	S	S	S	S	S	S	S	S	S	I	R	S	S	S	S	No ESBL
16CS0070	R	I	S	S	S	S	S	S	S	R	R	R	R	S	S	S	S	No ESBL
16CS0071	R	I	R	R	I	R	S	S	S	R	R	R	R	S	S	S	R	ESBL
16CS0075	R	I	S	S	S	S	S	S	S	R	R	R	R	S	S	R	S	No ESBL
16CS0743	R	I	S	S	S	S	S	S	S	S	S	R	R	S	S	S	R	No ESBL
16CS0744	R	I	R	R	I	R	S	S	S	R	R	R	R	S	S	R	R	ESBL
16CS0747	R	R	R	R	I	R	I	I	S	R	R	I	R	S	S	S	S	ESBL/Carba
16CS0761	R	I	R	I	I	I	S	S	S	R	R	R	R	S	S	S	R	ESBL
16CS0772	R	R	R	R	I	R	S	S	S	R	R	R	R	S	S	S	R	ESBL
16CS0774	R	I	I	R	I	R	S	S	S	S	S	S	S	S	S	S	R	ESBL
16CS0067	R	I	S	S	S	S	S	S	I	R	R	S	R	S	S	S	R	No ESBL
16CS0740	R	I	S	S	S	S	S	S	S	R	R	R	R	S	S	S	R	No ESBL
16CS0755	R	I	R	R	I	R	S	S	S	R	R	R	R	I	S	S	R	ESBL

PIP piperacillin, *TZP* piperacillin/tazobactam, *CTX* cefotaxime, *CAZ* ceftazidime, *FEB* cefepime, *ATM* aztreonam, *IMP* imipenem, *MEM* meropenem, *AMK* amikacin, *GEN* gentamicin, *TOB* tobramycin, *CIP* ciprofloxacin, *MXF* moxifloxacin, *TGC* tigecycline, *FOS* fosfomycin, *CT* colistin, *T/S* trimethoprim/sulfamethoxazole

Table 6 Characterization of colistin-resistant and carbapenemase-producing *E. coli* isolates

Isolate	Governorate	Farm	O-antigen	H-antigen	Resistance genes	Virulence genes	Phenotypic resistance	*mcr*-1	MIC value	ESBL	Carbapenemase
16CS0744	Dakahliya	9	–	51	*sul*3, *dfr*A1, *dfr*A14, *tet*A, *cml*A1, *aad*A1, *aph*A, *mph*A, *mrx*, bla_{CMY}, bla_{TEM}	*cma*, *hem*L, *intl*1, *iro*N	CIP, T/S, TET, RAM, GEN, STR, ERY, PEN, AMP, CAZ	+	≥ 4	Positive	Negative
16CS0078	Dakahliya	16/2	–	4	*sul*2, *dfr*A14, *tet*A, *str*B	*hem*L, *intl*1, *iss*	T/S, TET, RAM, STR, ERY, PEN	+	≥ 6	nd by VITEK	nd by VITEK
16CS0075	Kafr El-Sheikh	9	–	51	*sul*3, *cml*A1, *flo*R, *aad*A1, *aph*A, *mph*A, bla_{TEM}	*cma*, *hem*L, *intl*1, *iro*N, *iss*	CIP, TET, CMP, RAM, GEN, STR, ERY, PEN	+	≥ 32	Negative	Negative
16CS0775	Damietta	4	–	4	–	*pic*, *vat*, *cma*, *hly*E, *mch*F, *hem*L, *ire*A, *iro*N, *iss*	TET, RAM, ERY, PEN	+	≥ 6	nd by VITEK	nd by VITEK
16CS0036	Damietta	6	–	21	–	*hem*L	RAM, ERY, PEN, AMC	+	≥ 4	nd by VITEK	nd by VITEK
16CS0747	Gharbiya	4	–	32	*qnr*S, *sul*3, *tet*A, *cml*A1, *flo*R, *arr*, *aad*A1, bla_{OXA-7}, bla_{SHV}, bla_{TEM}	*hem*L, *intl*1, *ire*A	T/S, TET, CMP, RAM, GEN, STR, ERY, PEN AMP, CAZ	−	nd	Positive	Positive

In 2016, 576 cloacal swabs were collected from 48 poultry farms located in 5 governorates in northern Egypt. The samples were screened for multidrug resistant bacteria and investigated for the antimicrobial resistance of *E. coli*. Seven out of 56 *E. coli* isolates (12.5%) were producing ESBLs. To analyze the underlying molecular antimicrobial resistance mechanism, all *E. coli* isolates were genotyped using the multiplex microarray technique.

The results of this study were in accordance with previous reports which demonstrated a high prevalence of *E. coli* in poultry farms and their environment in Egypt [34, 52–54].

In previous studies on broiler chickens in Egypt, high phenotypic resistance rates were found to penicillin, rifampicin, erythromycin, trimethoprim/sulphamethoxazole, streptomycin and tetracycline [53]. Antimicrobial resistance rates in this study for amoxicillin (26.8%), gentamicin (19.6%) and imipenem (1.8%) were lower than those of *E. coli* isolates from poultry reported in Egypt [53], in China [55], in United States [56], in Korea [57], in United Kingdom [58], in Australia [59] and in Portugal [60]. In the present investigation, the most striking finding was that *E. coli* isolates showed a low resistance rate to fluoroquinolones (ciprofloxacin (21.4%) and levofloxacin (14.3%)). Cephalosporins are the first-line antimicrobials for treating human bacterial infections [61]. In addition, a considerable resistance to ceftazidime was detected among *E. coli* isolates from healthy broilers (41.1%).

In this study, one carbapenem-resistant isolate (1.8%) was found within all *E. coli* isolates. A higher rate was determined with retail chicken meat, 11.3% carbapenemase-producing *Enterobacteriaceae* including *E. coli* in Egypt in a previous study [62].

Few reports discussed prevalence of ESBL-producing *E. coli* isolated from healthy birds in Egypt. Here, ESBL and/or AmpC β-lactamase-producing isolates were detected in seven out of tested 56 *E. coli* (12.5%) isolated from healthy broilers. Two ESBL-producing strains were isolated from one farm in Gharbiya governorate (Farm 4) while five other isolates could be recovered from different farms in Dakahliya and Kafr El-Sheikh governorates. Two of the ESBL-producing isolates 16CS0740 and 16CS0747 from Dakahliya and Gharbiya, respectively were additionally carrying $bla_{\text{OXA-7}}$ gene characteristic for β-lactamase-producing bacteria (Table 4).

In a previous study in 2017, only 6% ESBL-producing *E. coli* were detected in colibacillosis diseased poultry in four different Egyptian governorates [63]. In contrast, ESBL/AmpC β-lactamase-producing *E. coli* were found in all 50 investigated Dutch broiler farms [64]. In Sweden

34.0% of broilers carried ESBL/AmpC β-lactamaseproducing *E. coli* in their guts [65]. In Malaysia 48.8% of isolates which were recovered from retail poultry meat markets were ESBL-AmpC positive [66].

The prevalence of ESBLs has been found to be variable worldwide with Asian countries having the highest rates [67].

In this study, the most prevalent resistance gene was bla_{TEM}, which was identified in 85.7% of ESBL and AmpC β-lactamase-producing isolates. $bla_{\text{CMY-2}}$ was found in 2 of ESBL and AmpC β-lactamase-producing isolates. $bla_{\text{OXA-7}}$ was found in 2 of ESBL producing isolates. While $bla_{\text{CTX-M9}}$, $bla_{\text{CTX-M1-15}}$, $bla_{\text{OXA-1}}$, $bla_{\text{DHA-1}}$, $bla_{\text{LAP-1}}$ and bla_{SHV} were identified only in one ESBL-producing

E. coli isolate.

The resistance-associated genes *bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{CMY}$ were previously reported in *Enterobacteriaceae* isolated from septicaemic broilers [68] and from humans [69, 70] in Egypt.

In this study, *bla*$_{TEM}$ resistance gene was detected in 20.6% of *E. coli* isolates. This result was in accordance with previous reports in China [71, 72]. *bla*$_{CMY}$ was detected in 3.5% of 56 *E. coli* isolates while the prevalence of *bla*$_{CMY-2}$ amongst *E. coli* isolates from broilers in Japan was 69.5% [73]. In Belgium, 49.0% of ceftiofur-resistant *E. coli* isolates derived from five broiler farms carried *bla*$_{CMY-2}$ [74]. Moreover, 12.1% of avian pathogenic *E. coli* strains and 9.5% of strains recovered from meat were found positive as carriers of *bla*$_{CTX-M}$ in Palestine [75].

*qnr*B and *qnr*S genes associated with quinolone resistance were detected in one and five isolates, respectively, which is lower than described previously in *E. coli* isolated from chickens in China [72, 76]. On the other hand, *qnr*A, *qnr*B, and *qnr*S genes were detected in 0.75, 3.9 and 5.1%, respectively of *E. coli* from chicken samples in China [77].

Many studies found similarities between virulenceassociated genes in human and avian *E. coli* isolates including *iss*, *fli*C, *iha* and *ire*A genes [78].

In a previous study, the virulence genes *iro*N, *omp*T, *iss*, *iut*A, and *hly*F were detected in 80.2% of isolated *E. coli* [68]. In this study, only 9 (16.7%) of the 56 *E. coli* isolates carried 2 genes (*iro*N, *iss*) together characteristic for avian pathogenic *E. coli*.

The *mcr*-1 gene is now reported all over the world in *Enterobacteriaceae* from animals, food and humans [79]. In 2015, first time *mcr*-1 gene was detected in livestock and raw meat samples in addition to human beings in China [27]. In this study, five *E. coli* isolates (8.9%) were phenotypically resistant to colistin and harboured *mcr*-1 gene associated with colistin resistance. This result was higher than reported in *E. coli* isolates from pigs, poultry and turkey in France with 0.5, 1.8 and 5.9%, respectively [80] and 5.6% of *E. coli* isolates from broilers in Germany [81], while it was lower than in *E. coli* isolates from poultry in China [27].

In previous studies conducted in China and Austria, the majority of phenotypically colistin-resistant *E. coli* isolates carried the *mcr*-1 gene [82, 83].

Conclusion

To the best of our knowledge, this study is the first report discussing the antibiotic susceptibility profiles of *Enterobacteriaceae* and ESBL-producing *E. coli* isolated from healthy broilers in the Nile Delta in Egypt. The emergence of colistin-resistant *E. coli* isolates in poultry is of public health significance and considered as potential source of transmission of plasmid-mediated *mcr*-1 to humans. It was shown that molecular biological methods such as microarray investigation are reliable and fast tools for detection of geno-serotypes, resistance- and virulence-associated determinants.

The results reinforce the need to develop surveillance strategies and to implement specific control procedures to reduce the use of antibiotics and subsequently the development of antimicrobial resistance by over-/misuse of antibiotic agents.

Authors' contributions

AAM, HH, HMH, RE, SM and HE participated in the conception and design of the study. AAM, HH and HE performed farm and laboratory work. AAM, HH, HN, HT, RE, SM, HMH and HE analyzed the data and drafted the manuscript. HN, HT, UR and HMH participated in manuscript revision. All authors read and approved the final manuscript.

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Competing interests

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper. RE and SM are employees of Alere Technologies GmbH.

Availability of data

All the data supporting the results are presented in the main manuscript.

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C H A P T E R 3

Evolution of Antibiotic Resistance of Coagulase-Negative Staphylococci Isolated from Healthy Turkeys in Egypt: First Report of Linezolid Resistance

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Evolution of Antibiotic Resistance of Coagulase-Negative Staphylococci Isolated from Healthy Turkeys in Egypt: First Report of Linezolid Resistance

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Abstract: Coagulase-negative staphylococci (CoNS) are gaining much attention as causative agents of serious nosocomial infections in humans. This study aimed to determine the prevalence and phenotypic antimicrobial resistance of CoNS as well as the presence of resistance-associated genes in CoNS isolated from turkey farms in Egypt. Two hundred and fifty cloacal swabs were collected from apparently healthy turkeys in Egypt. Suspected isolates were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The susceptibility testing of CoNS isolates against 20 antimicrobial agents was performed using the broth microdilution test. The presence of resistance-associated genes like *mec*A, *van*A, *bla*Z, *erm*(A), *erm*(B), *erm*(C), *aac-aph*D, *optr*A, *val*S, and *cfr* was determined. Thirty-nine CoNS were identified. All isolates were phenotypically resistant to trimethoprim/sulfamethoxazole, penicillin, ampicillin, and tetracycline. The resistance rates to erythromycin, chloramphenicol, oxacillin, daptomycin, and tigecycline were 97.4%, 94.9%, 92.3%, 89.7%, and 87.2%, respectively. Thirty-one isolates were resistant to linezolid (79.5%). Low resistance rate was detected for both imipenem and vancomycin (12.8%). The *erm*(C) gene was identified in all erythromycin phenotypically resistant isolates, whereas two resistant isolates possessed three resistance-conferring genes *erm*(A), *erm*(B), and *erm*(C). The *cfr* and *optr*A genes were detected in 11 (35.5%) and 12 (38.7%) of the 31 linezolid-resistant isolates. The *mec*A, *aac-aph*D, and *bla*Z genes were identified in 22.2%, 41.9%, and 2.6% of phenotypically resistant isolates to oxacillin, gentamicin, and penicillin, respectively. This is the first study revealing the correlation between linezolid resistance and presence of *cfr* and *optr*A genes in CoNS isolates from Egypt, and it can help to improve knowledge about the linezolid resistance mechanism.

Keywords: staphylococci; linezolid; CoNS; Turkey; Egypt

1. Introduction

Coagulase-negative staphylococci (CoNS) are commonly found in animals, humans, food, and the environment. They were believed to be nonpathogenic bacteria until 1980. Thereafter, they have gained more attention as causative agents of serious nosocomial infections in humans [1].

CoNS have also proven to be pathogenic in poultry, causing decreased weight gain, drop in egg production, endocarditis, and increased mortalities [2].

Although CoNS infections are less severe than *Staphylococcus aureus* infections, their treatment has been shown to be more complicated because of the dramatic increase in antibiotic resistance, especially for penicillin, oxacillin/methicillin, gentamicin, clindamycin, ciprofloxacin, and erythromycin [1]. CoNS have a feature of rapid acquisition, possessing, and modification of resistance genes. This feature further promotes the transmission of these genes into different staphylococcal species or even other bacterial genera [1,3]. Infections caused by antibiotic-resistant CoNS have been increasing in humans worldwide. However, only few studies have discussed the presence of CoNS in humans and animals in Egypt [4–6].

Linezolid (oxazolidinone class) is a last-resort antimicrobial agent for the control of serious infections caused by methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci in humans [7]. The oxazolidinone resistance is attributed to both chromosomal mutations and the acquisition of a transferable plasmid-borne ribosomal methyltransferase gene (*cfr*) [8]. The *cfr* gene targets the adenine residue at position A2.503 in the 23S rRNA gene and prevents the binding of drugs belonging to at least five antimicrobial classes, including oxazolidinones, phenicols, lincosamides, pleuromutilins, and streptogramin A [9].

The *optr*A gene conferring resistance to oxazolidinones and cross-resistance to phenicols is associated with linezolid and tedizolid resistance [10]. Both *optr*A and *cfr* genes were identified in a multiresistance plasmid in florfenicol-resistant *Staphylococcus sciuri* isolated from pigs in China [11].

Linezolid resistance is increasing more intensely in CoNS than in *S. aureus* [12,13]. However, reports discussing this problem in animals are rarely available [14].

Moreover, resistance of CoNS to linezolid has not yet been reported in Egypt, neither in humans nor in animals. Many investigations in other countries have shown that linezolid-resistant staphylococci are still susceptible to daptomycin and tigecycline [8,12]. Daptomycin is a novel cyclic lipopeptide with great activity against most Gram-positive pathogens, including strains resistant to methicillin and vancomycin [15]. Nonetheless, the inappropriate dosing of daptomycin has resulted in the emergence of resistance in staphylococci. The resistance against daptomycin in CoNS has now been reported in humans, although it has not yet been recorded in veterinary medicine [16].

Novel formulations and potential drugs have been synthesized and evaluated for biological activity with high impact on relatively resistant microorganisms, and they exhibited a strong in vitro antimicrobial activity in susceptibility assays [17].

The aim of the present study was to determine the antimicrobial resistance of CoNS isolated from apparently healthy turkeys housed in different governorates of the Nile Delta in Egypt.

2. Materials and Methods

2.1. Sampling and Bacterial Isolation

During 2018, 250 cloacal swabs were collected from apparently healthy turkeys, aged between 6 days and 365 days and housed in 12 epidemiologically nonrelated farms located in five governorates (Dakahlia, Damietta, Kafr El-Sheikh, Sharkiya, and Gharbiya) in the Nile Delta region in Egypt (Table 1). All farms were designed as closed systems except one farm, which was an open system located in the Sharkiya governorate. The antimicrobial drugs used in turkey flocks in Egypt were applied as growth promoters (digestion-enhancing antibiotics), prophylactics, and therapeutics. The main antibiotics used in farms were chlortetracycline, fluoroquinolones, colistin, tylosin, spectinomycin, chloramphenicol, and sulfonamides. The hygienic measures in the farms were of moderate or low standards. The watering systems were designed as tap water distribution drinkers inside the farms. The workers were allowed to move between flocks for food distribution and cleaning.

Table 1. Investigated turkey farms in northern Egypt, the number of birds, and the number of collected samples.

Number of	Governorates					Total
	Dakahliya	Damietta	Kafr El-Sheikh	Sharkiya	Gharbiya	5
Farms *	4	3	2	2	1	12
Birds	5000	2100	1200	1800	800	10,900
Samples	71	44	46	46	43	250

* Each farm reared one flock.

The collected swab samples were transported at 4 °C to the laboratory for microbiological examination. Thereafter, samples were pre-enriched in buffered peptone water and incubated aerobically at 37 °C for 24 h. Enriched bacterial samples were streaked on Columbia blood agar containing 5% defibrinated sheep blood at 37 °C for 24 and 48 h as primary cultivation for the isolation of staphylococci. The suspected colonies were further streaked on Baird–Parker agar (Oxoid GmbH, Wesel, Germany) and incubated aerobically at 37 °C for 24 h to identify *S. aureus* and other

Staphylococcus spp. The black colonies were subsequently picked and identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

2.2. Identification of Bacterial Isolates by MALDI-TOF MS

Suspected colonies were identified using MALDI-TOF MS using an Ultraflex instrument (Bruker Daltonik GmbH, Bremen, Germany). Analysis was carried out using the Biotyper 3.1 software (Bruker Daltonik GmbH). Interpretation of results was performed according to the manufacturer^{0}s recommendations: a score of ≥2.3 represented reliable species-level identification, a score of 2.0–2.29 represented probable species-level identification, a score of 1.7–1.9 represented probable genus-level identification, and a score of ≤1.7 was considered an unreliable identification [18].

2.3. Phenotypic Antimicrobial Susceptibility Testing

The antibiotic susceptibility testing of all isolates to 20 antimicrobial agents was performed by the MICRONAUT system using commercial 96-well microtiter plates (Merlin, Gesellschaft für mikrobiologische Diagnostika mbH, Bornheim-Hersel, Germany) as recommended by the manufacturer. Briefly, bacteria grown overnight were suspended in NaCl solution (0.9%) to obtain a turbidity corresponding to a McFarland standard of 0.5 (Dr. Lange, CADAS photometer 30, Berlin, Germany). One hundred microliters of the suspension were diluted in 10 ml of Mueller–Hinton broth (Oxoid GmbH), resulting in a concentration of approximately 10^6–10^7 colony-forming units (cfu)/mL. In total, 100 µL of the inoculum were given in each well of the plate. The plates were aerobically incubated for 18–24 h at 37 °C. Reading of plates was operated with a photometer (Merlin) at a wavelength of 620 nm. An optical density of >0.1 was interpreted as an indication of growth. Tested antimicrobial agents, classes, concentrations, and the breakpoints are given in Table 2. The results were interpreted according to guidelines of the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [19,20]. *Escherichia coli* (*E. coli*) ATCC 25922 and *S. aureus* ATCC 29213 were used as quality controls.

Table 2. Antibiotic susceptibility of 39 coagulase-negative *Staphylococcus* isolates from turkey flocks determined by broth microdilution test.

Antibiotic	Class	0.03125	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	R (%)	MIC_{50} (mg/L)	MIC_{90} (mg/L)
Amikacin (AMK) #	Aminoglycoside								3	2	5	6	23			29 (74.4)	64	64
Ampicillin (AMP) *	β-Lactam (Penicillin)							22	3		14					39 (100)	2	16
Cefoxitin (COX) *	β-Lactam (Cephalosporin)							10	3	1	25					29 (74.4)	16	16
Chloramphenicol (CMP) *	Miscellaneous										2	2	35			37(94.9)	64	64
Ciprofloxacin (CIP) #	Fluoroquinolone					2	5	6	26							32(82)	4	4
Daptomycin (DPT) *	Cyclic lipopeptide					3	1		35							35 (89.7)	4	4
Erythromycin (ERY) #	Macrolide					1			2	36						38 (97.4)	8	8
Gentamicin (GEN) #	Aminoglycoside					6	2	5	3	23						31 (79.5)	8	8
Imipenem (IMP) *	β-Lactam (Carbapenem)			8	8	7	4	3	4	2	3					5 (12.8)	0.5	8
Levofloxacin (LEV) #	Fluoroquinolone					5	2	2	30							32 (82)	4	4
Linezolid (LIZ) #	Oxazolidinone							5	3	4	27					31 (79.5)	16	16
Moxifloxacin (MOX) *	Fluoroquinolone				2	3	2	32								32 (82)	2	2
Oxacillin (OXA) *	β-Lactam(Penicillin)				3	6	3	3	4		20					36 (92.3)	16	16
Penicillin (PEN) *	β-Lactam (Penicillin)							3	3	33						39 (100)	8	8
Rifampicin (RAM) *	Ansamycin						11		28							28 (71.8)	4	4
Teicoplanin (TPL) #	Glycopeptide						6	20	6		7					13 (33.3)	2	16
Tetracycline (TET) #	Tetracycline									4	35					39 (100)	16	16
Tigecyclin (TGC) *	Glycylcycline			3	1	1	34									34 (87.2)	1	1
Trimethoprim/Sulfamethoxazole (T/S) *	Diaminopyrimidine/Sulfonamide					39[a*]										39 (100)	4/76	4/76
Vancomycin (VAN) #	Glycopeptide					10	9	8	7	4	1					5 (12.8)	2	8

A thick black line indicates the break point between clinically sensitive and resistant strains; R—resistance rate. # EUCAST; * CLSI.

2.4. DNA Extraction and Purification

DNA was extracted from bacterial cultures using High Pure PCR Template Purification Kit (Roche Diagnostics, Mannheim, Germany) according to the instructions of the manufacturer. DNA concentration was determined photometrically using a NanoDrop ND-1000 UV–VIS spectrophotometer (NanoDrop Technologies, Wilmington, NC, USA). The DNA was stored at −20 °C for further investigations.

2.5. Identification of Resistance-Associated Genes

The potential mechanisms underlying the antimicrobial resistance of methicillin, erythromycin, penicillin, and aminoglycosides were detected by amplifying the *mec*A, *erm*(A), *erm*(B), *erm*(C), *blaZ*, and *aac-aph*D genes, respectively, as described previously [21]. The *van*A gene associated with vancomycin resistance was amplified according to Okolie et al., 2015 [22].

The presence of three different genes (*optr*A, *val*S, and *cfr*) responsible for linezolid resistance was determined by PCR. The primer sequences are given in Table 3. PCR for detection of the *val*S gene was developed in this study as follows: an initial denaturation step at 96 °C for 60 s was followed by 35 cycles of denaturation (96 °C for 15 s), annealing (51 °C for 60 s), and extension (72 °C at 30 s), with a final extension at 72 °C for 60 s. PCR amplicon of 339 bp was considered positive. PCRs for detection of *optr*A and *cfr* genes have been described previously [23]. PCR products were analyzed on 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light.

Table 3. Primers and their sequences used in this study.

Gene	Antimicrobial Agent	Primer	Primer Sequence (5′–3′)	Reference
*mec*A	Methicillin/Oxacillin	*mec*A-F	TCC AGA TTA CAA CTT CAC CAG G	[21]
		*mec*A-R	CCA CTT CAT ATC TTG TAA CG	
erm(A)	Erythromycin	*erm*A-F	TAT CTT ATC GTT GAG AAG GGA TT	
		*erm*A-R	CTA CAC TTG GCT TAG GAT GAA A	
erm(B)		*erm*B-F	CTA TCT GAT TGT TGA AGA AGG ATT	
		*erm*B-R	GTT TAC TCT TGG TTT AGG ATG AAA	
erm(C)		*erm*C-F	CTT GTT GAT CAC GAT AAT TTC C	
		*erm*C-R	ATC TTT TAG CAA ACC CGT ATT C	
blaZ	Penicillin	*blaZ*-F	ACT TCA ACA CCT GCT GCT TTC	
		blaZ-R	TGA CCA CTT TTA TCA GCA ACC	
*aac-aph*D	Gentamicin, amikacin	*aac-aph*D-F	TAA TCC AAG AGC AAT AAG GGC	
		*aac-aph*D-R	GCC ACA CTA TCA TAA CCA CTA	
*van*A	Vancomycin	*van*A.F	GCT GTG AGG TCG GTT GTG	[22]
		*van*A.R	GCT CGA CTT CCT GAT GAA TAC G	
optrA	Linezolid, chloramphenicol	*optr*A-F	AGG TGG TCA GCG AAC TAA	[23]
		*optr*A-R	ATC AAC TGT TCC CAT TCA	
*val*S	Linezolid	*val*S-F	GTA ACG ATC ATC ATT TGG G	This study
		*val*S-R	CTT TAT TAG AGC TCA ATG GGC	
cfr	Oxazolidinones	*Cfr*-F	TGA AGT ATA AAG CAG GTT GGG AGT CA	[23]
		Cfr-R	ACC ATA TAA TTG ACC ACA AGC AGC	

3. Results

3.1. Isolation and Identification of Staphylococcus spp. Isolated from Turkeys

Out of 250 cloacal samples, 39 (15.6 %) CoNS isolates were identified from 12 turkey flocks in five governorates in Egypt using MALDI-TOF MS (Table 4). The species were classified as

Staphylococcus lentus (*n* = 16), *Staphylococcus xylosus* (*n* = 8), *Staphylococcus saprophyticus* (*n* = 5), *S. sciuri* (*n* = 3), *Staphylococcus condimenti* (*n* = 2), *Staphylococcus cohnii* (*n* = 2), *Staphylococcus simulans* (*n* = 1), *Staphylococcus epidermidis* (*n* = 1), and *Staphylococcus arlettae* (*n* = 1).

Table4. Presence of antibiotic resistance genes in coagulase-negative *Staphylococcus* spp. and phenotypic linezolid resistance.

Isolate 17CS	Age (d)	Governorate	Species	Resistance-Associated Genes	Linezolid Resistance (mg/L)
0271-1	365	Dakahlia	*S. lentus*	*erm*(B), *erm*(C), *val*S	8
0275-1	365		*S. sciuri*	*mec*A, *erm*(C), *optr*A, *val*S	8
0275-2	365		*S. lentus*	*mec*A, *erm*(C)	1
0281-1	365		*S. condimenti*	*erm*(C)	1
0283-1	365		*S. sciuri*	*mec*A, *erm*(B), *erm*(C), *aac-aph*D, *val*S, *cfr*	8
0286-2	365		*S. lentus*	*erm*(B), *erm*(C), *val*S, *cfr*	8
0288-2	365		*S. condimenti*	*erm*(C)	2
0294	6		*S. xylosus*	*erm*(B), *erm*(C), *optr*A, *val*S	8
0298	6		*S. saprophyticus*	*erm*(C), *val*S, *cfr*	8
0300	6		*S. saprophyticus*	*erm*(C), *optr*A, *val*S, *cfr*	8
0303	6		*S. lentus*	*erm*(A), *erm*(B), *erm*(C), *aac-aph*D, *optr*A, *val*S, *cfr*	8
0306	240	Damietta	*S. lentus*	*erm*(C), *optr*A, *val*S	8
0307-2	240		*S. xylosus*	*erm*(C), *val*S	8
0310-2	240		*S. xylosus*	*erm*(C), *val*S	8
0311	240		*S. lentus*	*mec*A, *erm*(C), *val*S, *cfr*	8
0312-1	240		*S. xylosus*	*erm*(C), *val*S	8
0314	240		*S. xylosus*	*erm*(C), *aac-aph*D, *val*S	8
0314-1	240		*S. lentus*	*erm*(B), *erm*(C), *aac-aph*D, *optr*A	8
0316	240		*S. lentus*	*erm*(B), *erm*(C), *optrA*, *val*S, *cfr*	8
0317	240		*S. lentus*	*erm*(B), *erm*(C), *optr*A, *val*S, *cfr*	8
0318-1	240		*S. lentus*	*erm*(C), *val*S	8
0321-1	240		*S. xylosus*	*erm*(C), *aac-aph*D, *val*S	8
0322-2	240		*S. sciuri*	*mec*A, *erm*(C), *aac-aph*D, *val*S	8
0323-2	240		*S. xylosus*	*erm*(B), *erm*(C), *val*S	2
0327	240		*S. arlettae*	*erm*(A), *erm*(B), *erm*(C), *valS*, *cfr*	8
0330-1	240		*S. cohnii*	-	1

0336	10		*S. saprophyticus*	*erm*(B), *erm*(C), *aac-aph*D, *optr*A, *val*S	8
0338	10		*S. cohnii*	*erm*(B), *erm*(C), *aac-aph*D	1
0339-2	10		*S. xylosus*	*erm*(C), *aac-aph*D, *optr*A	8
0340	21	Sharkiya	*S. lentus*	*erm*(C), *val*S, *cfr*	8
0346	21		*S. saprophyticus*	*erm*(B), *erm*(C), *aac-aph*D, *optr*A, *val*S, *cfr*	8
0347-2	21		*S. saprophyticus*	*mec*A, *erm*(B), *erm*(C), *aac-aph*D	1
0349	21		*S. lentus*	*erm*(C), *aac-aphD*, *valS*	8
0353-1	75		*S. lentus*	*erm*(C), *val*S	2
0358	75		*S. lentus*	*erm*(C), *val*S	8
0366	75		*S. lentus*	*erm*(C), *val*S	8
0368	60	Kafr El-Sheikh	*S. simulans*	*mec*A, *erm*(C), *val*S	8
0370	60		*S. lentus*	*erm*(B), *erm*(C), *aac-aphD*, *val*S	8
0397	123	Gharbia	*S. epidermidis*	*mec*A, *blaZ*, *erm*(B), *erm*(C), *optr*A, *val*S	8

3.2. Phenotypic Antimicrobial Resistance Profiles

Thediversityinphenotypicantibioticsusceptibilityprofilesof39CoNSisolatesagainst20antimicrobial agents and their classes is shown in Table 2. All isolates were resistant to trimethoprim/sulfamethoxazole, penicillin, ampicillin, and tetracycline. Evident resistance rates were recorded to erythromycin 97.4%, chloramphenicol 94.9%, oxacillin 92.3%, daptomycin 89.7%, and tigecycline 87.2%. Resistance to moxifloxacin, levofloxacin, and ciprofloxacin was 82% each. Thirty-one isolates were resistant to linezolid (79.5%). Low resistance rate (12.8%) was detected for both imipenem and vancomycin.

All resistant isolates to ciprofloxacin and gentamicin were additionally resistant to oxacillin. Additionally, all oxacillin-resistant isolates were resistant to chloramphenicol and tetracycline.

The resistance rates for gentamicin, cefoxitin, amikacin, rifampicin, and teicoplanin were 79.5%, 74.4%, 74.4%, 71.8%, and 33.3%, respectively. All isolates were resistant to at least three different classes of antimicrobial agents, so they were all defined as having multidrug resistance (MDR).

3.3. Prevalence of Antimicrobial Resistance-Associated Genes

The *erm*(C), *erm*(B), and *erm*(A) genes were identified in 97.4%, 41.0%, and 5.12% of all CoNS isolates, respectively. All phenotypically resistant isolates to erythromycin carried the *erm*(C) gene. Two phenotypically resistant CoNS isolates (17CS0303 and 17CS0327; ≥ 8 mg/L) were positive for the three screened genes *erm*(A), *erm*(B), and *erm*(C) (Table 4).

Eight out of 36 (22.2%) phenotypically oxacillin-resistant isolates possessed the *mec*A gene. Additionally, these eight isolates were resistant to ciprofloxacin and gentamicin. The *van*A gene could not be detected by PCR in any of the phenotypically vancomycin-resistant isolates. The *aac-aph*D gene was detected in 41.9% and 20.7% of gentamicin- and amikacin-resistant isolates, respectively. The *blaZ* gene associated with penicillin G resistance was identified only in one isolate (2.6%), which was phenotypically resistant to penicillin and ampicillin. The *cfr* gene was identified in 11 (28.2%) of all isolates. All isolates harboring the *cfr* gene were phenotypically resistant to chloramphenicol and linezolid. The *optr*A gene was found in 12 out of 36 (38.7%) isolates that showed phenotypic resistance to linezolid (Table 4). The *val*S gene

was identified in all linezolid-resistant isolates. Five CoNS isolates carried all three genes (*cfr*, *optr*A, or *val*S), while 11 isolates harbored two of these genes (Table 4).

All isolates carrying two or three of these genes were resistant to linezolid (≥ 8mg/L (Table 4)).

Two different *Staphylococcus* species (*S. xylosus* 17CS0314 and *S. lentus* 17CS0314-1), isolated from an individual bird exhibited two different phenotypic and genotypic resistance profiles (Table 4).

Eleven CoNS (28.2%) isolated from poults (6–21 days) exhibited multidrug resistance and harbored antibiotic resistance-associated genes.

4. Discussion

The poultry industry is one of the most important sources of the Egyptian economy. However, turkey production is still limited to a small scale. Very few data are available about turkey production and diseases in Egypt [24].

CoNS are implicated in serious infections in both humans and animals and show high resistance to several antibiotics [25].

Few studies have investigated the presence of CoNS in poultry [26–28] and studies discussing the presence of CoNS in turkeys are rare globally [29]. Moreover, no data about their prevalence in turkeys in Egypt exist at all.

The current perceptions and approaches to antibiotic resistance in food animal production, especially in poultry in Egypt, are not like in other countries with developed commercial poultry farming sectors. In Egypt, various combinations of constraints in veterinary and human medicine have been identified, such as the lack of legislation, knowledge, resources, and veterinary services. These constraints act as obstacles that hamper the prudent use of antimicrobial drugs. The antimicrobial drugs used in poultry production in Egypt are applied for growth promotion (digestion-enhancing antibiotics) and prophylaxis besides treatment of infections. There are no proper legislations in place to regulate the sale of antimicrobial drugs used for poultry production in Egypt.

The current study showed that CoNS isolated from healthy turkeys had high phenotypic resistance to all β-lactams except imipenem, which is prescribed as one of the first line of defense drugs against clinical infections caused by staphylococci. The rates of resistance were as follows: 100% for penicillin and ampicillin, 92.3% for oxacillin, 74.4% for cefoxitin, and low resistance to imipenem (12.8%). These results are in accordance with previous reports stating that β-lactam resistance in CoNS is greatly increasing [30].

The *bla*Z gene is responsible for penicillin resistance [31]. Although all isolates in the present investigation were phenotypically resistant to penicillin and ampicillin, the *bla*Z gene was detected in only one isolate (2.6%), showing discrepancy between phenotypic resistance and detection of β-lactamase gene *bla*Z. β-lactamase phenotype could be the result of expression of more than one gene. Moreover, there is more than one mechanism that grant staphylococci β-lactam resistance other than the expression of *bla*Z gene [32]. On the contrary, previous studies found higher prevalence (23.0% and 20.0%) for the *bla*Z gene in CoNS isolated from mastitis in cattle in Argentina [33].

In the current study, it was strongly noticed that all isolates showed MDR to at least three different classes of antimicrobial agents. The β-lactam resistance was additionally associated with resistance to other clinically important antibiotics, including tetracycline (100%), fluoroquinolones (82% for levofloxacin, moxifloxacin, and ciprofloxacin), aminoglycosides (gentamicin 79.5% and amikacin

74.4%), macrolides (erythromycin 97.43%), and glycopeptides (teicoplanin 33.3% and vancomycin 12.8%). This could be attributed to the fact that resistance mechanisms for these classes of antibiotics are similar and usually carried with the genetic elements responsible for β-lactam resistance on the same plasmids [34]. Resistance rates to β-lactams in this study were significantly higher than results of previous studies discussing antibiotic resistance of CoNS in humans [12,35] and poultry [26,36].

In this study, an evident resistance rate (92.3%) against oxacillin (β-lactam) was detected. This result was significantly higher than those reported in previous studies in Egypt discussing methicillin resistance in CoNS of human sources (75.9%) [5] and those from chicken meat (37%) [6].

The *mec*A gene is associated with resistance to methicillin/oxacillin, often in combination with ciprofloxacin, gentamicin, and vancomycin resistance [37]. It was detected in only 8 out of 36 isolates that showed phenotypic oxacillin resistance. This incompatible result is similar to findings of oxacillin resistance with absence of *mec*A gene that was reported earlier [38].

Previously, fluoroquinolones, such as ciprofloxacin and levofloxacin, were effective against methicillin-resistant bacteria. However, the misuse of these drugs has resulted in decreasing effectiveness [39].

Phenotypic resistance to trimethoprim/sulfamethoxazole was detected in all isolates in this study, which could be attributed to the massive use of these antibiotics as growth promoters in poultry farms in Egypt [24].

Phenotypic resistance to vancomycin was detected in 30.8% of the CoNS isolates, which is higher than what was previously reported in Egypt in CoNS from chicken meat (27.8%) [6] and human clinical isolates (15.5%) [5]. Globally, much lower or no resistance to vancomycin was recorded in CoNS, both in humans [35] and broiler chicken isolates [36]. Despite the phenotypic resistance to vancomycin, the *van*A gene was not detected in any of the isolates by PCR, similar to that described in previous studies [21,22].

The *aac-aph*D gene is associated with aminoglycoside resistance [40]. In this study, *aac-aph*D gene was detected in 41.9% and 20.7% of gentamicin- and amikacin-resistant isolates, respectively.

A previous investigation detected *aac-aph*D in 30.0% of CoNS isolated from clinical samples [21].

The inconsistency between phenotypic antibiotic resistance and the presence of resistance-associated genes is supported by previous studies documenting that both are not typically linked [41]. This phenomenon could be attributed to many factors, such as the presence of other resistance-associated genes, the absence of expression of some resistance-encoding genes, or multidrug resistance efflux pumps [42].

On the other hand, there was a perfect correlation between phenotypic resistance to erythromycin and the carriage of the *erm*(C) gene. All isolates resistant to erythromycin were carrying the *erm*(C) gene. Additionally, 41% of these isolates were positive for presence of *erm*(B) by PCR. Moreover, two isolates (*S. arlettae* and *S. lentus*) carried *erm*(C), *erm*(B), and *erm*(A) genes. Different rates were recorded for *erm*(C) and *erm*(A) in CoNS isolated from clinical samples [43] and for *erm*(B) and *erm*(C) in CoNS isolated from chickens, ducks, and pigs in China [44].

Interestingly, resistance was recorded in this investigation against drugs that are not used in veterinary medicine in Egypt, such as daptomycin, tigecycline, moxifloxacin, and linezolid, with resistance rates of 89.7%, 87.2%, 82%, and 79.5%, respectively.

Linezolid is one of the last-resort antimicrobial agents for the control of serious infections caused by methicillin-resistant staphylococci in humans [8]. Incidence of linezolid resistance in CoNS is growing faster than in *S. aureus*. This increased resistance in CoNS could be attributed to the higher and easier ability of CoNS to acquire and develop resistance determinants following linezolid exposure [8]. More worrisome is the very limited treatment options for linezolid-resistant isolates, which include daptomycin and tigecycline [8].

Here, the phenotypic resistance to linezolid was recorded among different CoNS isolated from apparently healthy turkeys. The rate was much higher than what was recorded in all previous studies reporting drug resistance in CoNS isolated from humans (8.9%) [12] or total sensitivity [35]. No resistance against linezolid was detected previously in CoNS isolates from poultry, calves, and pigs [14,36]. It is worth mentioning that linezolid resistance has never been reported in Egypt, neither in humans nor in animals.

The *cfr* gene is associated with linezolid resistance [8] as well as resistance to other classes of antibiotics (oxazolidinones, phenicols, lincosamides, pleuromutilins, and streptogramin A) [9]. CoNS were identified as the most common organisms harboring the *cfr* gene, and the gene was previously identified among them [45].

In this study, 35.4% and 29.7% of isolates resistant to linezolid and chloramphenicol carried the *cfr* gene, respectively. The *cfr* gene was identified in 25.0% of linezolid-resistant CoNS isolated from pigs [46] and 1.4% from humans [47]. The *cfr* gene was identified in CoNS isolated from chicken meat in Egypt [6] and in methicillin-resistant CoNS (MRCoNS) isolates obtained from chickens, ducks, and pigs in China [44].

The *optr*A gene proved to be associated with linezolid and phenicol resistance [10]. Previousstudies have reported the correlation between linezolid resistance and the carriage of *optr*A gene in CoNS of porcine origin [11]. In this study, the *optr*A gene was carried by 38.7% of linezolid-resistant CoNS.

Coexisting carriage of *optr*A and *cfr* genes in CoNS isolated from pigs in China [11] was confirmed in this study.

The *val*S gene was identified in 79.5% of isolates. It was noticed here that all isolates harboring two or three genes of the *optr*A operon (*cfr*, *optr*A, and *val*S) were highly resistant to linezolid. All isolates possessing the *cfr* gene were carrying the *val*S gene.

The U.S. Food and Drug Administration (FDA) approved daptomycin and tigecycline as alternatives to linezolid as treatment options for infections caused by methicillin- and vancomycin-resistant organisms [12,13].

Many studies have stated that linezolid-resistant *Staphylococcus* isolates are still susceptible to daptomycin and tigecycline [8,12,13]. However, alarming studies have now reported the resistance to daptomycin in CoNS in human medicine [16]. It is important to mention that daptomycin resistance is still not reported in veterinary practice. Hence, the resistance rate of daptomycin in CoNS from healthy turkeys was very high (89.7%) in this study. It might be assumed that a transmission of resistant CoNS from human source to poultry has occurred.

Tigecycline is the first glycylcycline antimicrobial agent that is highly active against many multi-drug-resistant bacteria, including MRSA. Most of the recent studies recorded no resistance to tigecycline among CoNS isolated from clinical isolates of human origin [21]. Inthisstudy, thephenotypic resistance to tigecycline among turkey isolates was 87.2%.

The emergence of high antimicrobial resistance to linezolid, daptomycin, and tigecyclin in the CoNS isolates in this study is suspected to be from human source as the hygienic measures in these poultry farms were of moderate or low standards and permitted workers to move between flocks.

This hypothesis is strongly supported by the isolation of CoNS of human origin (*S. epidermidis* and *S. saprophyticus*) from turkey samples in this study.

Furthermore, the improper use and availability of antimicrobial agents, especially linezolid, daptomycin, and/or tigecyclin in human practice in Egypt without any prescription as a last choice for infection treatment may lead to the rapid development of resistance against these groups.

On the other hand, the cross-resistance and transfer of resistance genes from other resistant bacteria and the role of mobile genetic elements spreading among CoNS cannot be ignored.

In veterinary practice, linezolid, daptomycin, and tigecyclin are not frequently applied in commercial poultry production in Egypt as these drugs are still highly expensive when compared to other available antimicrobial agents. Although the resistant CoNS isolated in this study were from poultry flocks, it does not prove that poultry is the source of infection, and it could have actually originated from other sources, such as humans or the environment. Therefore, further investigations should be performed to study the molecular epidemiology of the CoNS strains in order to prove the genetic relationship between CoNS isolated from poultry, the environment, and humans.

The potential of natural products is significant in the efforts to bridge the large gap between needs and available treatments, especially in terms of antimicriobial drugs, and may serve as an alternative for the treatment and/or prevention of resistant pathogens [48].

5. Conclusions

Despite the fact that research interest in CoNS has been increasing in recent years, there are very few data available on the prevalence and resistance profiles of CoNS in Egypt. The misuse of antibiotics in turkey farms and bad sanitary conditions could lead to selection

pressure, development, and spread of resistant strains between turkeys and humans. This study has demonstrated that poultry can act as a vector for CoNS harboring antimicrobial resistance genes. Multi-drug-resistant CoNS are a threat in both humans and veterinary medicine.

This study is the first report on antimicrobial resistance in CoNS isolated from healthy turkeys in
Egypt. The data obtained can be used to develop guidelines for monitoring and prevention programs. The study highlights the detection of highly linezolid-resistant CoNS and its associated resistance genes. Whole-genome sequencing would be an important tool to expand our knowledge about linezolid and daptomycin resistance and their genetic basis.

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General discussion

General discussion

The poultry industry in Egypt ranks as the first in the Middle East. It is considered one of the most important sources of support for the Egyptian economy, with an investment worth $3.5 billion and a labor force of 16 million workers that. Egyptian society mainly rely on poultry as a major low cost source for animal protein intake. Egypt's annual consumption of poultry is around 1,2 billion birds, the equivalent of around 1125 million tons of poultry meat (Moawad et al., 2017b).

Despite the huge financial measure of the Egyptian poultry industry, turkey industry is still considered a developing sector restricted to a small scale production. This may be ascribed to a number of challenges as the costly production compared to other elective sources of white meat, the unfavorable weather condition, the high vulnerability of the young chicks to diseases, and the lack of rearing knowledge and experience.

There are very few data on turkey production and diseases in Egypt (Moawad et al., 2017b).

Massive use of antimicrobial and globally banned drugs as a protocol in poultry farms in Egypt for disease control and prevention, helped greatly in releasing of many drug-resistant bacteria, most of which are considered as a dangerous zoonotic threat to human health (Moawad et al., 2017a; Moawad et al., 2018).

Massive antibiotic usage plays a major role in the emerging public health crisis of antibiotic resistance, which has gotten to be an alarming issue. The majority of antibiotics are used in agricultural and veterinary practice. It was found that high percentage of clinical antibiotic resistance is associated with agricultural antibiotics (Landers et al., 2012). An anual rate of 1500 deaths in the European Union is related to antibiotic resistant pathogens of poultry sources (Collignon et al., 2013). Globally, billions of chickens receive third-generation cephalosporins, starting from one day-old chicks, a practice that made poultry as large reservoirs of resistant bacteria (Collignon et al., 2013).

The Codex Alimentarius Commission has established an ad hoc Intergovernmental Task Force on antimicrobial resistant pathogens in the food chain and the potential for these pathogens. The main task of this commission is to apply a complete risk assessment strategy on the use of antimicrobials belonging to both clinical and veterinary classes (CAC., 2011).

The current perceptions and approaches to antibiotic resistance in food animal production, especially in poultry in Egypt, are not like in other countries with more developed commercial poultry farming sectors. In Egypt, various combinations of constraints in veterinary and human medicine have been identified, such as the lack of legislation, knowledge, resources and veterinary services. These constraints act as obstacles that hamper the prudent use of antimicrobial drugs. The antimicrobial drugs used in poultry production in Egypt are applied for

growth promotion and prophylaxis besides treatment of infections. There are no proper legislations to regulate the sale of antimicrobial drugs used for poultry production in Egypt (Moawad et al., 2019).

In Egypt, several factors have contributed to the variable estimates of pathogenic bacteria and their antibiotic resistance prevalences. The methods used for collecting samples, the differences in methodology between studies, such the enrichment and selective media and isolation techniques have attributed to the variation in these reports (Boulianne et al., 2016). Additionally, the geographical variation in Egypt with different density of food animal production and the frequently used antimicrobial drugs that can be changable from district to another, can act as a common source or selective pressure (Moawad et al., 2017a; Moawad et al., 2018).

In the past few decades, foodborne zoonotic outbreaks have increased in Egypt with the additional risk of transmission of AMR genes between humans and animals (Helmy et al., 2017). Antimicrobial resistant *Enterobacteriacea* and *Staphylococcus* spp. are global and frequently reported zoonotic pathogens that cause public health problems (Keerthirathne et al., 2016; Rohde et al., 2016; Somayaji et al., 2016). Application of fast, accurate and reliable tools such as MALDI-TOF MS, Microarrayand and genome sequencing for identifying foodborne pathogens of zoonotic importance, became mandatory in public health surveillance (Bizzini and Greub, 2010; O'Hara, 2005).

Escherichia coli is a normal inhabitant of the intestinal tract of poultry. About 10-15% of intestinal colonizing *E. coli* in healthy chickens belong to pathogenic serotypes (Nolan et al., 2013).

The current work is divided into two studies. In the first study, in 2016, 576 cloacal swabs were collected from 48 poultry farms located in 5 governorates in northern Egypt. The samples were screened for multidrug resistant bacteria and investigated for the antimicrobial resistance of *E. coli*. All *E. coli* isolates were genotyped using the multiplex microarray technique to analyze the underlying molecular antimicrobial resistance mechanisms.

The isolates in this investigation showed low resistance rates to fluoroquinolones (ciprofloxacin (21.4%) and levofloxacin (14.3%)) and on the contrary a considerable resistance to ceftazidime (41.1%). Ceftazidime is a member of cephalosporins group, that are considered the first-line antimicrobials for treating human bacterial infections (Lei et al., 2010).

carbapenem-resistance was detected in (1.8%) of isolates. A higher rate (11.3%) of carbapenemase-producing *Enterobacteriaceae* including *E. coli* was determined in retail

chicken meat in Egypt, in a previous study (Abdallah et al., 2015). This could be mainly because *E. coli* strains in this study were obtained from apparently healthy poultry.

A variable prevalence of ESBLs has been reported worldwide with the highest rates in Asian countries (Hawkey, 2008). Few reports discussed prevalence of ESBL-producing *E. coli* among healthy birds in Egypt. Here, ESBL and/or AmpC β-lactamase-producing isolates were confirmed in (12.5%) of all isolates from healthy broilers located in geographically unrelated farms.

In a previous study in 2017, only 6% ESBL-producing *E. coli* were detected in colibacillosis diseased poultry in four different Egyptian governorates (El-Shazly et al., 2017). In contrast, 34.0% of broilers In Sweden carried ESBL/AmpC β-lactamase-producing *E. coli* (Börjesson et al., 2013). In Malaysia, ESBL-AmpC *E. coli* was confirmed in 48.8% of isolates recovered from retail poultry meat markets (Aliyu et al., 2016).

In this study, the most prevalent resistance gene was bla_{TEM}, which was identified in 85.7% of ESBL and AmpC β-lactamase-producing isolates. While bla_{CMY-2}, bla_{OXA-7}, bla_{CTX-M9}, $bla_{CTX-M1-15}$, bla_{OXA-1}, bla_{DHA-1}, bla_{LAP-1} and bla_{SHV} were detected with low prevalences among ESBL and AmpC β-lactamase-producing isolates.

The resistance-associated genes bla_{TEM}, bla_{SHV}, and bla_{CMY} were previously reported in *Enterobacteriaceae* isolated from septicaemic broilers (Ahmed et al., 2013) and from humans (Al-Agamy, 2013) in Egypt.

The *qnr*B and *qnr*S genes associated with quinolone resistance were detected in few isolates which is lower than described previously in *E. coli* isolated from chickens in China (Li et al., 2014; Li et al., 2015). On the other hand, the prevalences of *qnr* genes in this study were higher than those detected in *E. coli* isolated from poultry in China (Huang et al., 2009).

The colistin resistance associated *mcr*-1 gene is now reported all over the world in *Enterobacteriaceae* from animals, food and humans (Wang et al., 2017). The gene was firstly detected in 2015 in livestock and raw meat samples in addition to human beings in China (Liu et al., 2016). In this study, five *E. coli* isolates (8.9%) were phenotypically resistant to colistin and harboured *mcr*-1 gene. This result was higher than reported in *E. coli* isolates from pigs, poultry and turkey in France (Perrin-Guyomard et al., 2016) and from broilers in Germany (Irrgang et al., 2016).

Many studies found similarities between virulence-associated genes in human and avian pathogenic *E. coli* (APEC) isolates including *iss*, *fli*C, *iha* and *ire*A genes (Johnson et al., 2012a).

Although the isolates in this study were obtained from healthy birds, both *iro*N and *iss* genes, which are characteristic for avian pathogenic *E. coli* were detected in (16.7%) of isolates.

Although CoNS are implicated in serious infections in both humans and animals and show high resistance to several antibiotics (Moawad et al., 2019), few studies have investigated the presence of them in poultry (Boamah et al., 2017; Marek et al., 2016; Stepien-Pysniak et al., 2017). Moreover, reports discussing the presence of CoNS in turkeys are rare globally (Salmon and Watts, 2000) and no data about their prevalence in turkeys in Egypt exist at all.

In the second study of this work, CoNS isolates were identified in (15.6 %) in 250 cloacal samples collected from 12 turkey flocks during 2018 in five governorates in Egypt. Isolates were classified as eight diffrent species. Isolates showed high phenotypic resistance to all β-lactams except imipenem, which is prescribed as one of the first line of defense drugs against clinical infections caused by staphylococci. These results are in accordance with previous reports stating that β-lactam resistance in CoNS is greatly increasing (Miragaia, 2018).

In this study, isolated CoNS showed MDR to at least three different classes of antimicrobial agents. It was noticed that the β-lactam resistance among the CoNS isolates was additionally associated with resistance to other clinically important antibiotics, including tetracycline, fluoroquinolones (levofloxacin, moxifloxacin and ciprofloxacin), aminoglycosides (gentamicin and amikacin), macrolides (erythromycin) and glycopeptides (teicoplanin and vancomycin). This could be attributed to the fact that resistance mechanisms for these classes of antibiotics are similar and usually carried on the same plasmids together with the β-lactam associated resistance genes (Karam et al., 2016). Resistance rates to β-lactams in this study were significantly higher than results of previous studies discussing antibiotic resistance of CoNS in humans (Balandin et al., 2016; Bora et al., 2018) and poultry (Boamah et al., 2017; Vela et al., 2012).

The evident resistance rate against oxacillin among CoNS in this study was significantly higher than those reported in previous studies in Egypt in human (Mashaly and El-Mahdy, 2017) and chicken meat (Osman et al., 2016).

The *mec*A gene associated with resistance to methicillin/oxacillin, often in combination with ciprofloxacin, gentamicin and vancomycin resistance (Lee, 2003) was detected in few methicillin resistant isolates. Additionally, the penicillin resistance associated *bla*Z gene

prevalence was low among the isolates. Both genes are examples of the discrepancy between phenotypic resistance and presence of associated genes. This discrepancy could be the result of expression of more than one gene. Moreover, there could be more than one mechanism that grant staphylococci β-lactam resistance. This phenomena is supported by previous studies documenting that both are not typically linked (Xu et al., 2014). This discrepancy is multifactorial, including the presence of other resistance-associated genes, the absence of expression of some resistance-encoding genes, or multidrug resistance efflux pumps (Kosmidis et al., 2012).

On the other hand, there was a perfect correlation between phenotypic resistance to erythromycin and the carriage of the *erm* genes.

The high phenotypic resistance to some drugs such as trimethoprim/sulfamethoxazole and fluoroquinolones in this study could be attributed to the massive use of these antibiotics as growth promoters in poultry farms in Egypt (Moawad et al., 2018).

Phenotypic resistance to vancomycin detected in CoNS isolates in this study was higher than what was previously reported in Egypt in chicken meat (Osman et al., 2016) and human clinical isolates (Mashaly and El-Mahdy, 2017). Globally, much lower or no resistance to vancomycin was recorded in CoNS, in human (Bora et al., 2018) or broiler chicken (Vela et al., 2012).

The most interesting finding among CoNS isolates in this study was the high resistance against drugs that are not used in veterinary medicine in Egypt such as daptomycin, tigecycline, moxifloxacin and linezolid.

Linezolid is one of the last-resort antimicrobial agents for the control of serious infections caused by methicillin-resistant staphylococci in humans (Gu et al., 2013). Incidence of linezolid resistance in CoNS is growing faster than in *S. aureus*. This increased resistance in CoNS could be attributed to the higher and easier ability of CoNS to acquire and develop resistance determinants following linezolid exposure (Gu et al., 2013). More worrisome is the very limited treatment options for linezolid-resistant isolates, which include daptomycin and tigecycline (Gu et al., 2013).

Here, the prevalence of phenotypic resistance to linezolid among different CoNS isolated from apparently healthy turkeys was much higher than what was recorded in previous studies in humans (Balandin et al., 2016; Bora et al., 2018). No resistance against linezolid was detected previously in CoNS isolates from poultry, calves and pigs (Cuny et al., 2017; Vela et al., 2012). It is worth mentioning that linezolid resistance has never been reported in Egypt, neither in humans nor in animals.

The *cfr* gene is associated with linezolid resistance (Gu et al., 2013) as well as resistance to other classes of antibiotics (oxazolidinones, phenicols, lincosamides, pleuromutilins and

streptogramin A) (Long et al., 2006). CoNS were identified as the most common organisms harboring the *cfr* gene (Wang et al., 2013).

In this study, the *cfr* gene was identified in both linezolid and chloramphenicol resistant isolates. The *cfr* gene was previously identified in linezolid-resistant CoNS isolated from pigs (Schoenfelder et al., 2017) and from humans (Decousser et al., 2015).

The *optr*A gene has also proved to be associated with linezolid and phenicol resistance (Li et al., 2016; Wang et al., 2015). In this study, the *optr*A gene was identified among linezolid-resistant CoNS.

Coexisting carriage of *optr*A and *cfr* genes in linezolid resistant CoNS isolated from pigs in China (Li et al., 2016) was confirmed in this study.

The U.S. Food and Drug Administration (FDA) approved daptomycin and tigecycline as alternatives to linezolid as treatment options for infections caused by methicillin- and vancomycin-resistant organisms (Balandin et al., 2016; Baos et al., 2013).

Although linezolid-resistant Staphylococcus isolates are still susceptible to daptomycin and tigecycline (Balandin et al., 2016; Baos et al., 2013; Gu et al., 2013), few studies have now reported the resistance to daptomycin in CoNS in human medicine (Jiang et al., 2019). Worth mentioning that daptomycin and tigecycline resistances are still not reported in veterinary practice. The evident resistance rates of daptomycin and tigecycline resistance among CoNS in this study might be assumed that a transmission of resistant CoNS from human source to poultry has occurred. This hypothesis is strongly supported by the isolation of CoNS of human origin from turkey samples in this study (*S. epidermidis* and *S. saprophyticus*).

Conclusion

Both studies were considered as first reports discussing the emergence of antibiotic resistance among both Gram-positive and Gram-negative microorganisms against two important antimicrobial drugs such as Colistin and linezolid. In addition to discussing susceptibility profiles of *Enterobacteriaceae* and ESBL-producing *E. coli* isolated from healthy broilers in the Nile Delta in Egypt. The emergence of colistin-resistant *E. coli* isolates in poultry is of public health significance and considered as potential source of transmission of plasmid-mediated *mcr*-1 to humans.

Despite the fact that research interest in CoNS has been increasing in recent years, there are very few data available on the prevalence and resistance profiles of CoNS in Egypt. This study is the first report on antimicrobial resistance in CoNS isolated from healthy turkeys in Egypt. The data obtained can be used to develop guidelines for monitoring and prevention programs. The study highlights the detection of highly linezolid-resistant CoNS and its associated

resistance genes. Whole-genome sequencing would be an important tool to expand our knowledge about linezolid and daptomycin resistance and their genetic basis.
The misuse of antibiotics in poultry farms and bad sanitary conditions could lead to selection pressure, development and spread of resistant strains between humans and poultry. Both studies have demonstrated that both human and poultry can act as a vector for harboring antimicrobial resistance genes. Multi-drug-resistant pathogens are a threat in both humans and veterinary medicine. The results reinforce the need to develop surveillance strategies and to implement specific control procedures to reduce the use of antibiotics and subsequently the development of antimicrobial resistance by over-/misuse of antibiotic agents.

The potential of natural products is significant in the efforts to bridge the large gap between needs and available treatments and may serve as an alternative for the treatment and/or prevention of resistant pathogens.

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Summary

Summary

Poultry and poultry products are considered as major reservoirs for many zoonotic pathogens that are incriminated in human foodborne diseases globally. Moreover, these pathogens have contributed to the actual global challenge of antimicrobial resistance (AMR) with the use of antibiotics in the food production industry.

The poultry industry is one of the mainstay sources of Egyptian economy. Nonetheless, turkey production is still limited to a small scale. Few studies have discussed the probability of transmission of zoonotic pathogens and AMR between turkeys and humans in Egypt.

The global rise of ESBL- and carbapenemase-producing Gram-negative bacteria demands intensified surveillance. ESBL-producing and colistin-resistant *Escherichia* (*E*.) *coli* in poultry farms in Egypt are of major concern that emphasizes the possibility of spread of such strains and associated resistance genes including plasmid-mediated *mcr*-1 gene to humans.

Enterobacteriaceae strains were isolated and identified as *E. coli* (87.5%), *Enterobacter cloacae* (6.9%), *K. pneumoniae* (2.8%) and *Citrobacter* spp. (2.8%). ESBL-producing was confirmed in 12.5% of *E. coli* isolates while only 1.8% of isolates were confirmed as ESBL/carbapenemase-producing *E. coli*. The *mcr*-1 gene was identified in 7.9% of phenotypically colistin resistant *E. coli* isolates. The most prevalent resistance gene was bla_{TEM}, which was identified in 85.7% of ESBL and AmpC β-lactamase-producing isolates, while bla_{CMY-2} and bla_{OXA-7} were detected in 3.2% of ESBL and AmpC β-lactamase-producing isolates. Only 1.6% of ESBL-producing *E. coli* isolates possessed bla_{CTX-M9}, $bla_{CTX-M1-15}$, bla_{OXA-1}, bla_{DHA-1}, bla_{LAP-1} and bla_{SHV} genes. The virulence-associated genes detected by microarray analysis were differently distributed among the isolated *E. coli* including secretion system (*cif, esp*A, *esp*F_O103H2, *esp*J, *nle*A, *nle*B O157:H7 and *tcc*P), adhesion-involved genes (*eae* and *iha* genes) and serine protease autotransporter genes (*tsh, pic* and *vat*).

Several toxin genes were detected including *ast*A, *cma*, *hly*E, *mch*F, *sat* and *sen*B and fimbriae virulence genes (*lpf*A and *prf*B). Miscellaneous genes encoding virulence factors including *hem*L, *int*I1, *ire*A, *iro*N, *iss* and *tir* genes were identified. It was shown that molecular biological methods such as microarray investigation are reliable and fast tools for detection of geno-serotypes, resistance- and virulence-associated determinants **(Chapter 2).**

The coagulase negative staphylococci (CoNS) are implicated in serious infections in both, humans and animals. They show high resistance to several antibiotics and gain a great research interest. Nevertheless, there are very few data available on the prevalence and resistance profiles of CoNS in Egypt.

Different CoNS species were isolated from apparently healthy turkeys located in five governorates in Egypt. The CoNS species were identified using MALDI-TOF MS as *S. lentus* (41%), *S. xylosus* (20.5%), *S. saprophyticus* (12.8%), *S. sciuri* (7.7%), *S. condimenti* (5.12%), *S. cohnii* (5.12%), *S. simulans* (2.6%), *S. epidermidis* (2.6%) and *S. arlettae* (2.6%). The susceptibility testing of CoNS isolates was performed using the broth microdilution test. All isolates were phenotypically resistant to trimethoprim/sulfamethoxazole, penicillin, ampicillin and tetracycline. The resistance rates to erythromycin, chloramphenicol, oxacillin, daptomycin and tigecycline were 97.4%, 94.9%, 92.3%, 89.7% and 87.2%, respectively. Thirty-one isolates were resistant to linezolid (79.5%). Low resistance rate was detected for both, imipenem and vancomycin (12.8%). The presence of resistance-associated genes *mec*A, *van*A, *bla*Z, *erm*(A), *erm*(B), *erm*(C), *aac-aph*D, *optr*A, *val*S and *cfr* was determined. The *erm*(C) gene was identified in all erythromycin phenotypically resistant isolates, whereas two resistant isolates possessed three resistance-conferring genes *erm*(A), *erm*(B) and *erm*(C). The *cfr* and *optr*A genes were detected in 35.5% and 38.7% of the phenotypically linezolid-resistant isolates. The *mec*A, *aac-aph*D and *bla*Z genes were identified in 22.2%, 41.9%, and 2.6% of phenotypically resistant isolates to oxacillin, gentamicin and penicillin, respectively. This is the first study revealing the correlation between linezolid resistance and presence of *cfr* and *optr*A genes in CoNS isolates in Egypt. This study has demonstrated that both human and poultry can act as a vector for CoNS harboring antimicrobial resistance genes. Multidrug-resistant CoNS are a threat in both, human and veterinary medicine **(Chapter 3).**

There are no geographical boundaries that can hamper the worldwide spread of AMR. If preventive measures are not applied from farm-to-fork locally, nationally and globally, the tottering situation in one country can easily compromise the efficacy and threaten AMR control policies in other parts of the world.

Zusammenfassung

Epidemiologie, Genotypisierung und Antibiotikaresistenz von zoonotischen Bakterien, die von Geflügel in Ägypten isoliert wurden.

Geflügel und Geflügelprodukte gelten als Hauptreservoir für viele zoonotische Pathogene, welche weltweit für humane, durch Lebensmittel verursachte Erkrankungen verantwortlich gemacht werden.
Diese Krankheitserreger erlangen eine besondere Bedeutung durch die global zunehmende Ausbildung von Resistenzen gegenüber antimikrobiell wirksamen Substanzen, wofür die Verwendung von Antibiotika in der Lebensmittelindustrie mitverantwortlich gemacht wird.
Die Geflügelindustrie ist ein wichtiger Sektor der ägyptischen Ökonomie. Dabei liefert die Puten-Produktion jedoch insgesamt nur einen geringen Beitrag. Es gibt ein paar Studien, in denen eine mögliche Übertragung von zoonotischen Erregern und der damit einhergehenden Antibiotikaresistenz zwischen Puten und Mensch in Ägypten diskutiert werden.
Der weltweite Anstieg von ESBL- und Carbapenemasen-produzierenden Gram-negativen Bakterien erfordert eine intensive Überwachung. ESBL-produzierende und Colistin-resistente *Escherichia coli*-Stämme in Geflügelfarmen in Ägypten spielen eine wichtige Rolle, weil die Möglichkeit des Verbreitens und der Übertragung solcher Isolate und der damit verbundenen Resistenzgene einschließlich des Plasmid-lokalisierten *mcr*-1-Gens auf Menschen besteht. Im Rahmen dieser Untersuchungen wurden Vertreter der *Enterobacteriaceae* isoliert und identifiziert: *Escherichia coli* (87,5 %), *Enterobacter cloacae* (6,9 %), *Klebsiella pneumoniae* (2,8 %) und *Citrobacter*-Spezies (2,8 %). Unter den *Escherichia coli*-Isolaten befanden sich 12,5 % ESBL-Produzenten, während nur 1,8 % ESBL/Carbapenemasen-produzierend waren. Das *mcr*-1-Gen konnte in 7,9 % der phänotypisch resistenten *Escherichia coli*-Isolate nachgewiesen werden. Das am häufigsten nachgewiesene Resistenzgen war bla_{TEM}, welches 85,7 % der ESBL- und AmpC β-Lactamase-produzierenden Isolate trugen, während bla_{CMY-2} und bla_{OXA-7} in 3,2 % dieser Isolate gefunden wurden. Jeweils 1,6 % der ESBL-produzierenden Isolate trugen folgende Gene: bla_{CTX-M9}, $bla_{CTX-M1-15}$, bla_{OXA-1}, bla_{DHA-1}, bla_{LAP-1} und bla_{SHV}. Die durch Mikroarray-Analyse identifizierten Virulenz-assoziierten Gene waren unterschiedlich in den *Escherichia coli*-Isolaten verteilt. Unter den untersuchten Genen befanden sich solche des Sekretionssystems (*cif*, *esp*A, *esp*F_013H2, *esp*J, *nle*A, *nle*BO157:H7 und *tcc*P), Gene, die im Adhäsionsprozess beteiligt sind (*eae* und *iha*) und Serinprotease-Autotransporter-Gene *tsh*, *pic* und *vat*).

Verschiedene Toxin-Gene (*ast*A, *cma*, *hly*E, *mch*F, *sat* und *sen*B) wurden ebenso nachgewiesen wie Virulenzgene, die im Zusammenhang mit Fimbrien stehen (*lpf*A und *prf*B).

Die Anwesenheit von Genen weiterer Virulenzfaktoren in den Isolaten konnte bestätigt werden (*hem*L, *intI*1, *ire*A, *iro*N, *iss* und *tir*). Es wurde gezeigt, dass molekularbiologische Nachweismethoden wie Mikroarray-Untersuchungen zuverlässige und schnelle Werkzeuge für den Nachweis von GenoSerotypen, Resistenz- und Virulenz-assoziierten Determinanten sind **(Kapitel 2)**.

Auch Koagulase-negative Staphylokokken sind bei ernsthaften Infektionen beim Menschen und bei Tieren beteiligt. Sie zeigen oftmals Resistenz gegenüber verschiedenen Antibiotika und haben großes Interesse in der Forschung geweckt. Allerdings sind für Ägypten nur wenige Daten hinsichtlich der Prävalenz und der Resistenzprofile von Koagulase-negativen Staphylokokken verfügbar.
Verschiedene Koagulase-negative Staphylokokken-Spezies wurden von gesunden Puten aus fünf Gouvernoraten in Ägypten isoliert. Die Staphylokokken wurden mittels MALDI-TOF-Massenspektrometrie identifiziert: *Staphylococcus lentus* (41,0 %), *Staphylococcus xylosus* (20,5 %), *Staphylococcus saprophyticus* (12,8 %), *Staphylococcus sciuri* (7,7 %), *Staphylococcus condimenti* (5,12 %), *Staphylococcus cohnii* (5,12 %), *Staphylococcus simulans* (2,6 %), *Staphylococcus epidermidis* (2,6 %) und *Staphylococcus arlettae* (2,6 %). Die Antibiotika-Empfindlichkeitstestung wurde mittels “Broth Microdilution Test” durchgeführt. Alle Isolate erwiesen sich als resistent gegenüber Trimethoprim/Sulphametoxazol, Penicillin, Ampicillin und Tetrazyklin. Die Resistenzraten gegenüber Erythromycin, Chloramphenicol, Oxacillin, Daptomycin und Tigecyclin betrugen 97,4 %, 94,9 %, 92,3 %, 89,7 % und 87,2 %. Einunddreißig Isolate waren resistent gegenüber Linezolid (79,5 %). Eine niedrige Resistenzrate wurde jeweils für Imipenem und Vancomycin (12,8 %) gefunden. Die Anwesenheit folgender Resistenz-assoziierter Gene wurde bestimmt: *mec*A, *van*A, *bla*Z, *erm*(A), *erm*(B), *erm*(C), *aac-aph*D, *optr*A, *val*S und *cfr*. *erm*(C) konnte in allen phänotypisch Erythromycin-resistenten Isolaten identifiziert werden, wobei zwei Isolate drei Erythromycin-Resistenz-vermittelte Gene trugen (*erm*(A), *erm*(B), *erm*(C)).
Die *cfr*- und *optr*A-Gene wurden in 35,5 % und 38,7 % der phänotypisch Linezolid-resistenten Isolate gefunden, während die Präsenz von *mec*A (Oxacillin-Resistenz), *aac-aph*D (Gentamicin-Resistenz) und *bla*Z (Penicillin-Resistenz) zu 22,2 %, 41,9 % und 2,6 % dieser Isolate bestimmt wurde.
Diese Studie war die erste, welche sich mit dem Zusammenhang von Linezolid-Resistenz und der Präsenz der *cfr*- und *optr*A-Gene in Koagulase-negativen Staphylokokken in Ägypten befasst hat. Sie zeigte eindeutig, dass Mensch und Geflügel als Vektor für Koagulase-negative Staphylokokken, welche Resistenzgene tragen, agieren kann. Multi-resistente Koagulase-negative Staphylokokken stellen damit eine Bedrohung in der Human- und Veterinärmedizin dar **(Kapitel 3)**.

Es existieren keine geographischen Grenzen, die die weltweite Ausbreitung der Antibiotika-Resistenz verhindern können. Es ist notwendig, Präventivmaßnahmen zur Verhinderung einer weiteren Verschlechterung der Resistenzlage durchzuführen. Dies muss lokal, national, aber auch international erfolgen, um so eine Verbesserung bezüglich der Antibiotika-Resistenz von Pathogenen im Lebensmittelbereich zu erreichen.

List of published articles

A) Publications in peer-reviewed journals

1. **Moawad A. A.,** Hotzel H., Awad O., Tomaso H., Neubauer H., Hafez M. H. and El-Adawy H. (2017): Prevalence, Molecular characterization and antibiotic resistance of *Salmonella enterica* and *Escherichia coli* isolates from raw chicken and beef meat marketed in Northen Egypt. Gut Pathog. 2017 Oct 18;9:57. doi: 10.1186/s13099-017-0206-9.
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7. **Moawad A. A.,** Silge A., Bocklitz T., Fischer K., Roesch P., Roesler U., Elschner M. C., Popp, J. and Neubauer, H. (2019): A Machine Learning-Based Raman Spectroscopic Assay for the Identification of *Burkholderia mallei* and Related Species. Molecules. 2019; 24(24). doi: 10.3390/molecules24244516.

B) Publications in conferences proceedings:

1. **Moawad A. A.**, Hotzel H., El-Adawy H., Neubauer H. and Hafez H. M. (2017): Turkey Production in Egypt: Current situation and challenges. Turkey Production and Health:

Challenges and opportunities. ISBN: 978-3-86387-884-9. Proceedings of 9th "Hafez" International Symposium, Berlin, Germany 18th -20th May 2017.

2. **Moawad A. A.,** Hotzel H., El-Adawy H., Neubauer H. and Hafez M. H.(2018): Production, Berlin, Germany. Prevalence and antibiotic resistance of *Escherichia coli* isolated from healthy turkey farms in northern Egypt. 12th "Hafez" International Symposium on Turkey Diseases. 31 May- 2 June 2018, Berlin, Germany.

C) Oral presentations

1. **Moawad A. A.,** Hotzel H., El-Adawy H., Neubauer H. and Hafez M.H.(2019): Linezolid resistance in CoNS isolated from healthy turkeys in Egypt. 10th "Hafez" International Symposium on Turkey Production. 6-8 June 2019, Berlin, Germany.
2. **Moawad A. A. (2019):** Linezolid resistance in CoNS isolated from healthy turkeys in Egypt. Third International Caparica Conference in Antibiotic Resistance 2019. 10th – 13th June 2019 | Caparica | Portugal.
3. **Amira A. Moawad**, Henning K., Rösler U., Neubauer H. and Mertens K. (2018) Ultra Violet-C inactivation of *Coxiella burnetii* for production of structural preserved whole cell antigens.

D) Posters:

1. **Amira A. Moawad**, Henning K., Rösler U., Neubauer H. and Mertens K. (2018) Ultra Violet-C inactivation of *Coxiella burnetii* for production of structural preserved whole cell antigens. "Junior Scientist Symposium" September 2018, Insel Riems, Germany.
2. **Amira A. Moawad**, A. Silge, P. Rösch, U. Roesler, K. Fischer, J. Popp, H. Neubauer and M. C. Elschner. (2019): Identification of *Burkholderia mallei* and related species using Raman spectroscopy. "Junior Scientist Zoonoses Meeting" June 2019, Berlin, Germany.

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Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen Anspruch genommen habe.

Berlin, den 05.10.2020 Amira Awad Ibrahim Moawad

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