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Research

Antimicrobial resistance among commensal enteric bacteria isolated from healthy cattle in Libya

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Abstract

Introduction: *Enterococcus* and *E. coli* are important commensals bacteria widely used as bio-indicators to monitor the occurrence and distribution of antimicrobial resistance. **Methods:** rectal faecal samples were collected from healthy cattle from the suburban and farming areas in Tripoli within the period 2017-2018. Samples were subjected to standard and presumptive laboratory methods to isolate *E. coli* isolates and enterococci species then further subjected to Kirby-Bauer disk diffusion antimicrobial susceptibility test. A selection of multidrug resistant isolates of Enterococci and *E. coli* isolates were further investigated and analysed using the phoenix automated microbiology identification and susceptibility system. PCRs protocols were further applied to screen for *bla*TEM, *bla*SHV, *bla* CTX-M, class 1 and 2 integrons and the *qac*EΔ1-*sul*1 region of class 1 integrons. **Results:** a total of 103 healthy cattle were included in the study yielding 206 *Enterococcus* species and 162 *E. coli* antimicrobial resistant isolates. Of these, 73% and 61% were respectively characterized as multidrug-resistant strains. The MDR enterococci strains (n=27) expressed various susceptibility patterns distributed over four different enterococci species including *E. faecium, E. faecalis, E. hirae*, and VanC type enterococci. Eight enterococci isolates expressed low level susceptibility to vancomycin of which three isolates were VanC intrinsic type and five were vancomycin intermediate susceptibility represented by VanC/type (n=2), *E. faecium* (n=1), *E. hirae* (n=1) and *E. faecalis* (n=1). The investigated MDR *E. coli* strains (n=61) revealed high-level resistance to ampicillin (100%), trimethoprim/sulfamethoxazole (81%), gentamicin (30%) and ciprofloxacin (7%) but no resistance was identified to other important critical antimicrobial classes. PCRs revealed that only seventeen MDR *E. coli* isolates (n=17/25) were positive for *bla*TEM, and sixteen isolates (n=16/25) positive for the class 1 integrons. **Conclusion:** this is the first surveillance report on antimicrobial resistance among commensal bacterial organisms isolated from cattle in Libya. Healthy cattle can carry important bacterial strains expressing different and important antimicrobial resistance phenotypes that require continuous monitoring and antimicrobial stewardship in veterinary medicine.

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Introduction

Drug-resistant bacteria increasingly reported from humans, food resources and livestock including asymptomatic animals [1-8]. These are increasingly reported in the underdeveloped countries, mainly attributed to the use of antimicrobials, intensive farming production, unregulated use of antibiotics, socioeconomic factors, poor sanitation and inadequate information on antimicrobial consumption [9-13]. Food animal bacteria have major implications on public health and associated with treatment failures causing serious opportunistic and hospital acquired infections [14,15]. Commensals bacteria from food animals are exposed to antimicrobial pressure and can be a source of multidrug resistant (MDR) bacteria such as extended spectrum β-lactamases (ESBLs)-producing *Enterobacteriaceae* as well as vancomycin resistant enterococci [16-20]. *E. coli* and *Enterococcus* spp. are classically bio-indicator commensal organisms used to monitor antimicrobial resistance (AMR) providing essential data on the selective pressures from antimicrobial use [21-24]. In cattle, commensal *E. coli* isolates may serve as important reservoirs of drug resistance determinants mainly carried by mobile genetic elements, such as transposons and conjugative plasmids as well as integrons [25]. Although, the intestinal commensals of healthy population may harbour antimicrobial resistance genes that are associated with extraintestinal infections, such information particularly of animal origins are very limited [26]. The current study investigated and analysed antimicrobial resistance of commensals enterococci and *E. coli* strains isolated from faecal samples of healthy cattle in Tripoli between 2017-2018.

Methods

Isolation and identification of *E. coli* and *Enterococcus spp*: a fresh faecal rectal sample was obtained from each cattle and processed in the laboratory within 4 hours. Around 2-3 grams of faeces were added to 10 ml Brain Heart infusion broth (BHI) (LAP M) containing 5% glycerol and homogenized using vortex mixer. The mix was then used to streak selective mediums to characterize presumptive colonies of enterococci and *E. coli*. For enterococci, 1 ml from the faecal mix was added to 3 ml Kanamycin aesculin azide broth (KAAB) (LAP M) and incubated under aerobic condition at 37 ^{o}C for 24 hours. A loopful from KAAB was streaked onto Kanamycin aesculin azide agar (KAAA) and incubated under aerobic conditions at 35 ^{o}C for 48h. Plates were checked for white or grey typical colonies surrounded by black zones and further subjected for presumptive identification by Gram stains, catalase reactivity and tested for esculin hydrolysis. For *E. coli*, 1 ml from the faecal mix was added to 3 ml of Brilliant Green broth (Oxoid, UK) and incubated at 37 ^{o}C for 24 hours under aerobic conditions. Broths were streaked onto eosin methylene blue (EMB) agar (Oxoid, UK) and incubated under the same previous conditions. Plates were then examined for typical and presumptive round colonies of *E. coli* with a metallic sheen. Colonies were subjected for presumptive characterization using Gram stain, catalase and oxidase productivity and lactose fermentation. In house enterococci and *E. coli* strains were used as positive controls in the selection process. Two presumptive isolates of each bacterium were selected from each faecal sample and further tested for susceptibility to antimicrobial drugs.

Antimicrobial susceptibility testing: isolates were subjected to Kirby-Bauer disc diffusion method following the clinical and laboratory standards Institute guidelines [27]. *E. coli* strains were tested to ampicillin, tetracycline,

trimethoprim/sulfamethoxazole, chloramphenicol, and nalidixic acid. Enterococci isolates were tested to ampicillin, ciprofloxacin, erythromycin, tetracycline, chloramphenicol, and gentamicin. For the purpose of the current study, isolates were identified as antimicrobial resistant (AMR) strains based on the criteria of "resistance to at least one antimicrobial agent" and as multidrug resistant (MDR) strains if the criteria of "resistance to at least two different antimicrobial agents" was characterized. Epi Info™ of the Centers for Disease Control and Prevention was used to calculate the statistically significant differences of antimicrobial resistance phenotype between the studied strains using the Chi-square test (P≤0.05). A selection of MDR isolates of each bacterium were chosen based on the inclusion of ampicillin resistance within their MDR profiles for further investigation using the phoenix automated microbiology identification and susceptibility testing system (BD Phoenix (PAMS, MSBD Biosciences, Sparks Md, US). This step was used to confirm the studied selections at species level as well as determining the susceptibility to extended antimicrobial classes. In relation to enterococci, the automated system cannot fully identify and differentiate between the intrinsic VanC type of enterococci (i.e. *E. casseliflavus/gallinarum*), therefore, these bacteria are referred to as VanC type enterococci in results and discussion sections.

Molecular investigation of MDR selected *E. coli* isolates: a selection of MDR *E. coli* isolates, that were fully characterized by the automated system, were chosen based on variable and extended AMR profiles and screened by PCRs for the presence of the important and common resistant genetic mechanisms; *bla*TEM, *bla*SHV, *bla* CTX-M, class 1 and 2 integrons and the qacEΔ1-*sul*1 region of class 1 integrons [28-30]. DNA was extracted by boiling a suspension of overnight- grown cultured bacterial cells in 200 µl of sterile distilled water. Each PCR mix (25 µl) contained 12.5µl HotStarTaq® Master Mix (QIAGEN, France) and 0.5µM of each primer. All PCRs were performed using an Eppendorf thermal cycler, and each run included a negative and positive inhouse control. After electrophoresis, PCR products (20 µl) were resolved on a 2% (wt/vol) agarose gels containing ethidium bromide (0.5 µg/mL). A 100-bp DNA marker (Invitrogen™ 100 bp DNA Ladder) was used to determine amplicon size.

Results

In total, 103 healthy cattle were included originated from six dairy farms. Of these, 100% (n=103) of faecal samples were positive for at least one antimicrobial resistant enterococci species and 93% (n=96) were positive for at least one antimicrobial resistant *E. coli* strains. Also, 57% (59/103) of faecal samples were positive for each multidrug (MDR) *Enterococcus* and *E. coli* isolates. The process yielded a total of 206 enterococci species and 162 *E. coli* isolates expressing antimicrobial resistance to different antimicrobial agents. No significant differences of antimicrobial resistance expressions were identified between both bacteria for the studied and compared antimicrobial classes, except for tetracycline (P=0.00001; OR=0.3). In addition, 73% (n= 151/206) of enterococci species and 61% (n=99/162) of *E. coli* isolates expressed multidrug resistance (MDR) phenotypes revealing significantly higher detection rate of MDR among the studied *Enterococcus* species (Table 1). A selection of 27 MDR enterococci isolates were further analysed by the automated microbiological system and found to be distributed over four enterococci species; *E. faecium* (n=15), *E. hirae* (n=5), VanC/type (n=5) and *E. faecalis* (n=2). All isolates were susceptible to ampicillin, amoxicillin-clavulanate, ciprofloxacin, teicoplanin, nitrofurazone, erythromycin, daptomycin, mupirocin and linezolid. All isolates were resistant to trimethoprim-

sulfamethoxazole, clindamycin, gentamicin, fusidic acid and cephalosporins (i.e. cefoxitin and cefotaxime) (Table 2).

Three isolates were indicated by the automated system as VRE (i.e. VanC intrinsic resistant enterococci) and five were indicated as vancomycin intermediate susceptible represented by VanC/type (n=2), *E. faecium* (n=1), *E. hirae* (n=1), and *E. faecalis* (n=1). Also, five enterococci were intermediately susceptible to ciprofloxacin (MICs=2 µg/mL) represented by *E. faecium* (n=4) and *E. faecalis* (n=1). A selection of 61 MDR *E. coli* isolates were subjected to the phoenix automated system of which 41% (n=25/61) expressed resistance to various antibiotic classes including ampicillin (100%), trimethoprim/sulfamethoxazole (81%), gentamicin (30%), and ciprofloxacin (7%). Only one isolate was identified to be resistant to cephalothin, cefuroxime, ceftriaxone, intermediate to cefepime and aztreonam but susceptible to cefoxitin and ceftazidime. Also, three strains showed intermediate susceptibility to amoxicillin-clavulanic acid. None of the MDR *E. coli* strains expressed resistance to amoxicillin-clavulanic acid, carbapenems (i.e. ertapenem, imipenem, meropenem), nitrofurazone, polymyxin B and colistin. The MDR *E. coli* isolates were further tested by PCRs revealing that seventeen MDR *E. coli* isolates (n=17/25) were positive for *bla*TEM gene, sixteen (n=16/25) isolates were positive for the *intI*1 gene. None of the isolates presented the *bla*SHV, *bla*CTX-M or the *intI*2 gene and the VR of the class 1 integron was detected only in four isolates (Table 3).

Discussion

Farm animals are exposed to variable quantities of antimicrobials causing permanent alteration in the commensal microbiota [2,4,13,31]. This increases antimicrobial resistance rate up to five folds including to the same used antibiotic classes, as well as increasing the dissemination of MDR- and AMR- encoding genetic determinants [32-34]. In the current study, 93% to 100% and 57% of the studied healthy cattle were positive respectively for faecal AMR and MDR resistant strains. No significant difference was observed between both of the commensal bacterium in the resistance shown to different antimicrobial agents, however; enterococci expressed higher MDR phenotypes comparing to *E. coli*. In the current study, the 27 studied MDR enterococci isolates were found to belong to four different species mainly *E. faecium*. The *E. faecium* strains expressed resistance only to trimethoprim-sulfamethoxazole, clindamycin, gentamicin, fusidic acid and cephalosporins (i.e. cefoxitin and cefotaxime). In addition, five enterococci strains were identified as vancomycin intermediate susceptibility (MICs=8µg/mL) represented by VanC/type enterococci (n=2), *E. faecium* (n=1), *E. hirae*, (n=1) and *E. faecalis* (n=1). This corresponds with a recent pan-surveillance survey from the EU investigating the susceptibility of large collection of commensal enterococci collected from food-producing animals [24]. This report revealed variable antibiotic susceptibility and low to absent resistance to the critically important antimicrobial such as ampicillin, gentamicin, linezolid, tigecycline and vancomycin, but high resistance to quinupristin/dalfopristin, tetracycline and erythromycin [24]. This recent survey revealed similar results to our current study in regards the decreased susceptibility to vancomycin (i.e. MICs of 8µg/mL) only among few enterococci species.

In food production, the emergence of different VRE genotypes, of animal origins (e.g. cattle, poultry) has been mainly linked to the widespread use of sub-therapeutic antibiotics [35]. The VanA-VRE type is frequently associated with high level of resistance to both vancomycin (MIC≥64µg/mL) and teicoplanin (MIC≥16µg/mL), whereas VanB is associated with varying levels of resistance to

vancomycin (MIC 1-1000 µg/mL) but not to teicoplanin [20,35]. A study investigating VRE strains which were identified based on criteria of MICs≥8mg/L using an automated identification system, has reported *van*C-1 resistance gene in *E. faecium* and *E. gallinarum* characterized by low-level resistance to vancomycin [36]. Another study analysing clinical and faecal strains of 60 VRE/vancomycin intermediate enterococci (i.e. MIC of 8-16 µg/mL), has identified *van*A or *van*B genes in *E. faecalis* and *E. faecium* strains, but only *van*B gene in *E. gallinarum* strains [37]. The VanC types (i.e. *E. casseliflavus/E. gallinarum*) has attracted less attention, despite their increasing associations with human infections and outbreaks; however, these inducible types are associated with antibiotic exposure able to express various vancomycin resistant phenotypes such as VanA, VanB and VanD types [20]. In the current study, only *bla*TEM and class 1 integrons were identified respectively among 17 and 16 isolates, of the 25 studied MDR *E. coli* isolates. These findings correspond with global trends on the most reported genetic mechanisms responsible for MDR phenotypes [29]. A recent report has documented antimicrobial-resistant Gram-negative bacteria from 81% of 102 fecal rectal samples of healthy human population mainly expressing β-lactamase genes (*bla*TEM, *bla*SHV, *bla*CTX-M, and *bla*OXA) (72%) and Integron sequences (36%) [26]. Another study from North Africa investigating 174 E. coli isolates collected from healthy food producing animals including cattle reported MDR phenotypes in 44.2% of the collection but the absence of ESBL producers [9].

Class 1 integrons are mainly reported in MDR *E. coli* isolates in cattle however, class 2 integrons are reported at low frequencies [38,39]. Class 1 integrons are widely reported from different animals carried by various bacterial organisms such as *E. coli*, *K. pneumoniae and S. aureus* and associated with both vertical and horizontal transmission [40,41]. Recent reports have shown that *E. coli* isolates from healthy cattle and non-healthy calves can carry highly diverse gene cassettes encoding antibiotic resistance in the variable regions of their class 1 integrons such as *flo*, *dfrA*, and *aadA* gene cassette [42,43]. Class 1 integron has been particularly associated with various alleles of *dfr*-encoding trimethoprim resistance (e.g. *dfrA1* and *dfrA17*) and tetracycline resistance genes in commensal *E. coli* isolated from cattle and associated with antimicrobial pressure including at the subtherapeutic doses of sulfamethazine and/or chlortetracycline [44,45]. In the present study, the presumptive isolation process used in the current study have been widely documented as reliable laboratory protocols for reliable characterization of commensals *E. coli* and enterococci species [46,47]. The automated identification systems (including the phoenix system) have been increasingly used showing accurate performance for the detection and analysis of AMR and MDR pathogens (e.g. ESBLs and VRE) [48-50].

Conclusion

This is the first study that provides novel data on the status and distribution of antimicrobial resistance in food producing animals in Libya. The gastrointestinal microbiota of cattle may harbour commensal bacterial species carrying various antibiotic resistance determinants of public health concerns particularly among the enterococci species. Antimicrobial stewardship in animal medicine is very important to overcome the critical consequences of AMR and MDR on human health and further investigations and monitoring studies are required.

What is known about this topic

- Antimicrobial resistance in a global serious concern affecting humans and animals with variable geographic distribution;
- Animals particularly food-producing livestock play important role in the dissemination and development of antimicrobial resistance;
- The available data are mostly related to humans and hospital acquired bacteria with little information of zoonotic origins.

What this study adds

- The important and possible role of commensals as a reservoir of antimicrobial resistance and the importance of continuous monitoring;
- Understand the burden of bacterial drug resistance and pressure effect of antimicrobials use in important food animals;
- Data and epidemiological information particularly of zoonotic nature on the spread of drug resistant bacteria in food animals in North African region.

Competing interests

Authors declare no conflicts of interests.

Authors' contributions

Almshawt NF collected samples, presented data and performed laboratory analysis. Hiblu MA has contributed in collecting samples and data. Abid AS and Abbassi MS have equally contributed in the laboratory work and interpretation of results. Abouzeed YM and Elkady AA have equally contributed in presenting and analysing data. Ahmed MO has contributed in designing the study, interpretation of results and writing the manuscript.

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Tables

Table 1: susceptibility to antimicrobials of *Enterococcus* and *E. coli* resistant isolates

Table 2: summary and distribution of antibiotic resistance of MDR *Enterococcus* species isolated from faecal samples

Table 3: the MICs values and interpretation of MDR *E. coli* isolates and the associated genes and genetic determinants (BD Phoenix) (CLSI)

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Table 1: susceptibility to antimicrobials of Enterococcus and E. coli resistant isolates

Antibacterial agents	**No. of resistant Enterococci isolates (%)**	**No. of resistant E. coli isolates (%)**	**P (OR)**
Ampicillin	28 (14%)	114 (70%)	0 (15)
Chloramphenicol	24 (12%)	13 (8%)	0.2(0.6)
Nalidixic acid	ND	43 (27%)	--
Ciprofloxacin	56 (27%)	ND	--
Erythromycin	102 (50%)	ND	--
Tetracycline	178 (86%)	108 (67%)	0.00001 (0.3)
Trimethoprim- Sulfamethoxazole	ND	75 (46%)	--
Gentamicin	129 (63%)	ND	--
Multi Drug resistant	151 (73%)	99 (61%)	0.01 (0.6)

Abbreviation: --, Not defined

Table 2: summary and distribution of antibiotic resistance of MDR Enterococcus species isolated from faecal samples

Antibiotic	**No and (%) of Enterococcus species (n=27)**			
	E. faecium (n=15)	**VanC/type (n=5)**	**E. hirae (n=5)**	**E. faecalis (n=2)**
AMP	0(0)	0(0)	0(0)	0(0)
AMC	0(0)	0(0)	0(0)	0(0)
STX	15(100)	5(100)	5(100)	2(100)
TET	7(47)	1(7)	2(13)	2(100)
CIP	0(0)	0(0)	0(0)	0(0)
CLI	15(100)	5(100)	5(100)	2(100)
GEN	15(100)	5(100)	5(100)	2(100)
VAN	0(0)	3(60)**	0(0)	0(0)
TEI	0(0)	0(0)	0(0)	0(0)
NITRO	0(0)	0(0)	0(0)	0(0)
ERY	0(0)	0(0)	0(0)	0(0)
Dapt	0(0)	0(0)	0(0)	0(0)
Mupi	0(0)	0(0)	0(0)	0(0)
FUSI	15(100)	5(100)	5(100)	2(100)
LIN	0(0)	0(0)	0(0)	0(0)
CEPH*	15(100)	5(100)	5(100)	2(100)
CARB	0(0)	0(0)	0(0)	0(0)
Polym-B	NA	NA	NA	NA
Col	NA	NA	NA	NA

Abbreviations: NA; Not Available, AMP; Ampicillin, AMC; Amoxicillin and clavulanic acid, STX; Trimethoprim- sulfamethoxazole, TET; Tetracycline, CIP; Ciprofloxacin, CLI; Clindamycin, GEN; Gentamicin, Van; Vancomycin, TEI, Teicoplanin, NITRO; Nitrofurazone**,** ERY; Erythromycin, Dapt; daptomycin, Mupi; mupirocin, FUI; Fusidic acid, LIN; Linezolid, CEPH; Cephalosporins, CARB; Carbapenems (ertapenem, imipenem, meropenem), Polym-B; Polymyxin B, Col; Colistin Footnotes: *Only one E. coli isolate showed resistance to the cephalosporins: cephalothin, cefuroxime, ceftriaxone and intermediate to cefepime but susceptible to cefoxitin and ceftazidime. Enterococcus isolate were tested to only 2 cephalosporins: cefoxitin and cefotaxime. **These VanC type enterococci were identified as vancomycin resistant by the Phoenix automated system

Table 3: the MICs values and interpretation of MDR E. coli isolates and the associated genes and genetic determinants (BD Phoenix) (CLSI)

Isolate NO.	Antimicrobial Resistance Profiling	Genes and Int determinants			
		Int1	VR(bp)	qacE-sul1	blaTEM
1a	AMP	+	-	+	+
2	AMP-STX	+	-	+	+
3	AMP-STX	+	-	+	+
4	AMP-STX	-	-	-	-
5	AMP-STX	+	-	+	+
6b	AMP-STX-CIP	+	-	-	+
7	AMP	+	-	+	+
8	AMP-STX-GEN	+	-	+	+
9	AMP-STX -GEN	+	250	+	+
10c	AMP-STX	+	300	+	+
11	AMP	-	-	-	-
12d	AMP-STX-GEN	-	-	-	-
13	AMP-STX	-	-	-	-
14c	AMP-STX	-	-	-	-
15	AMP	+	300	+	+
16	AMP-STX-GEN	+	-	+	+
17	AMP	-	-	-	-
18	AMP-STX-GEN	-	-	-	-
19	AMP-STX-GEN	-	-	-	+
20	AMP-STX	+	-	+	+
21	AMP-STX	+	800	+	+
22	AMP-STX	-	-	-	-
23	AMP-STX	+	-	-	+
24b	AMP-AMC-STX-CIP-GEN	+	-	+	+
25b	AMP-AMC-STX-CIP-GEN	+	-	+	+

Abbreviation: AMP; Ampicillin, AMC; Amoxicillin and clavulanic acid, STX; Trimethoprim-sulfamethoxazole, CIP; Ciprofloxacin, GEN; Gentamicin, ND; Not determined, +; Positive, -; Negative Footnotes: a This isolate has the following MIC range for cephalosporins as follows: cephalothin (>16), cefuroxime ((>16), ceftriaxone and intermediate to cefepime and to aztreonam; b These isolates show further resistance to levofloxacin MIC=>4); c This isolate show intermediate susceptibility to tigecycline MIC=4), d This isolate show intermediate susceptibility to aztreonam