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Surveillance of antimicrobial resistance in *Escherichia coli* strains isolated from cattle and broiler chickens in Tunisia

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Abstract – The aim of the present study was to evaluate resistance to antimicrobial agents of bacteria isolated from mastitis cows, diarrheic and healthy calves, and healthy chickens. Therefore, a total of 679 animals were sampled; milk samples from mastitis cows (n=248), fecal specimens from calves (n=119) and fresh feces from healthy chickens (n=312) to isolate *Eschrichia coli* strains then evaluate their susceptibility to 17 antimicrobial agents. *Escherichia coli* were the commonest bacteria isolated from milk of mastitis cows (38%). The carriage rate of *E. coli* from fecal samples of diarrheic and healthy calves was 86.6% and 88.1% respectively. In poultry, the carriage rate was 90.7%. Resistance to antibiotics was higher in *E. coli* isolated from chickens then from calves and mastitis cows. The highest prevalence of ESBL producing *E. coli* was also obtained in feces samples of chickens 14.5% which are considered as a major ESBL reservoir in Tunisia.

Key words: *Escherichia coli*, mastitis cows, calves, poultry, antibiotics, ESBL, Tunisia.

1. Introduction

Antibiotics have helped to fight bacterial infections and are widely used in humans and animals. However, their inappropriate use (the use of broad spectrum antibiotics at insufficient doses and the inappropriate choice of antibiotics without referring to an antibiogram) or even their unnecessary use lead to the emergence of multidrug-resistant (MDR) bacteria which limit the treatment options. Indeed, under the selection pressure exerted by antibiotics and in order to survive in the presence of an antibiotic, bacterial organisms acquire mechanisms of resistance such as the efflux of the antibiotic from the cell via the efflux pumps, the altered membrane impermeability, the modification of properties of penicillin-binding proteins (PBPs) to prevent the interaction of the target site with antibiotics and the enzymatic inactivation of antimicrobial agents that degrades antibiotic in a way that it loses its activity. The last mechanism is mostly applied to beta-lactam antibiotics (Wright, 2005). Most resistance mechanisms are encoded by plasmid genes, which are potentially transmissible to other bacteria (Carattoli, 2009; Madec et al., 2011).

Escherichia coli is a normal inhabitant of the intestinal microbiota of animals and can also be involved as an opportunistic pathogen in animal infections (Pitout, 2010). They are used as Gram negative indicator bacteria in antimicrobial resistance surveillance programs, because of their high prevalence in the digestive tract of healthy animals and because of their ability to often harbor several resistance genes (Wray and Gnanou, 2000). Therefore, this study was conducted to identify the prevalence of *Escherichia coli* mastitis, the carrying rate of *E. coli* in diarrheic and healthy calves also in healthy poultry and to evaluate the level of antimicrobial resistance in *E. coli* isolates.

2. Materials and methods

2.1. Sample collection

From October 2015 to october 2016, 679 samples were collected from which 248 milk samples aseptically obtained from mastitis cattle, 119 fresh feces from diarrheic (n=60) and healthy (n=59) calves, and 312 cloacal swabs from healthy chickens. Samples were transported immediately to the laboratory to be analyzed.

2.2. Bacterial isolation and identification

For isolating bacteria responsible for mastitis, milk samples were plated on blood agar and incubated for 18 to 24 hours at 37°C. Milk samples were also inoculated in buffered peptone water (BPW) and after overnight incubation at 37°C, they were cultivated onto MacConkey agar supplemented with cefotaxime (CTX, 2µg/ml) to screen for ESBL-producing *E. coli*.

Fecal samples were enriched in BPW at 37°C for 18 to 24 hours and later cultured on two MacConkey agar plates without and with cefotaxime (CTX, 2µg/ml) to screen, respectively, for *E. coli* and ESBL-producing *E. coli,* and were incubated for 18 to 24 hours at 37°C. Bacterial colonies were identified by classical biochemical methods. *Escherichia coli* isolates were also confirmed by specific polymerase chain reaction (PCR) with amplification of *uidA* gene using specific primers (F, 5'-ATCACCGTGGTGACGCATGTCGC; R, 5-CACCACGATGCCATGTTCATCTGC) (Heininger et al., 1999). Isolates were stored in brain heart infusion broth (BHI) containing 20% glycerol at -80°C until further processing.

2.3. Antimicrobial susceptibility testing

Susceptibility testing to 17 antibiotics was carried out by disk diffusion method on Mueller Hinton agar plates according to the recommendation of the European committee on antimicrobial susceptibility testing (CASFM/EUCAST, 2016). Antibiotics tested were as follows: amoxicillin (25µg), amoxicillin/clavulanic acid (20 µg/10 µg), ticarcillin/clavulanic acid (75 µg/10 µg), ceftiofur (30 µg), cefotaxim (30 µg), ceftazidim (30 µg), cefepim (30 µg), cefoxitin (30 µg), aztreonam (30 µg), ertapenem (10 µg), chloramphenicol (30 µg), gentamicin (15 µg), colistin (50 µg), nalidixic acid (30 µg), enrofloxacin (5 µg), tetracyclin (30 µg) and sulfamethoxazole/trimethoprim (1.25 µg/23.75 µg). ESBL-producing *E. coli* strains were confirmed by the double disc synergy test. *E. coli* ATCC 25922 was used as a control strain.

2.4. Statistical analysis

Comparisons between antimicrobial resistance prevalence of *E. coli* isolated from each animal sector and even between healthy and diarrheic calves were determined by the *chi-square* test (χ^2) using Epi info (version 7.2.1.0) software.

3. Results and discussion

3.1. Mastitis cows

Udder inflammations are still the most frequent and the most economically important disease of dairy cows in the world (Kossaibati and Esslemont, 2000). Among the bacteriologically positive mastitis samples (n=202), 221 germs were isolated and *E. coli* was the most frequently bacterium isolated (38%; 84/221). *Escherichia coli* is an environmental bacteria that invade the udder after milking or after teat damage (Bradley, 2002) and can cause clinical mastitis specially in dairy cows around parturition and during early lactation (Burvenich et al., 2003). Similar results with high frequency of *E. coli* are reported in China (Cheng et al., 2010) and in England (Bradley and Green, 2001). However, in other studies *Staphylococcus aureus* was the most prevalent bacteria isolate from mastitis cows (Kalmus et al., 2011 ; Javed et al., 2015).

The highest level of resistance was observed for tetracycline (59.5%), amoxicillin (45.2%), gentamicin (27.4%), sulfamethoxazol/trimethoprim (19%), amoxicillin/clavulanic acid (15.5%), nalidixic acid (13.1%), enrofloxacin (11.9%) and chloramphenicol (9.5%) (Figure1). A rate of 2.4% (2/84) of *E. coli* strains have developed resistance to the last cephalosporins generation (3rd CG/4th CG) such as ceftazidim, cefotaxim, ceftiofur, and cefepim) and to monobactams (e.g. aztreonam) antibiotics by production of extended spectrum beta-lactamases (ESBL) (Rawat and Nair, 2010). They were confirmed for ESBL production by the synergy test. In Tunisia, only one previously study on 10 mastitis cattle has

been conducted and revealed a prevalence of 10% of ESBL producing *E. coli* (Grami et al., 2014) which led us to suppose a higher prevalence of cefotaxim-resistant *E. coli* on a larger sampling size.

3.2. Diarrheic and healthy calves

In fecal samples, the *E. coli* carriage rate was 88.1% (52/59) in healthy calves and 86.6% (52/60) in diarrheic calves. The carriage rate of *E. coli* obtained in healthy and diarrheic was almost similar, due to the fact that *E. coli* is a common microorganism of the gut flora of calves. However, for more differentiation between pathogenic and commensal strains it is important to use additional techniques (*e.g.*, screening for virulence genes, serotyping). The overall antimicrobial susceptibility test showed a high resistance level for *E. coli* stains isolated from healthy and diarrheic calves with a high resistance level respectively against tetracycline (67.3%; 75%), amoxicillin (53.9%; 48.1), amoxicillin/clavulanic acid (51.9%; 50%), sulfamethoxazole/trimethoprim (28.9%; 36.5%), nalidixic acid (30.8%; 25%) and chloramphenicol (25%; 26.9%) (Figure 1). Furthermore, 2 ESBL-producing *E. coli* were identified, one from a healthy calf and the other was from a diarrheic one. In terms of antibiotic resistance, there is no significant difference (P> 0.05) between *E. coli* isolated from healthy or diarrheic animals.
Our results showed a high prevalence of resistance compared with previously published resistance rates of *E. coli* from fecal samples of cattle at slaughterhouse in Portugal (Ramos et al., 2012) and a lower resistance rate compared to those reported with *E. coli* isolated from feces of calves in Belgium (Catry et al., 2007; Chantziaras et al., 2014).

3.3. Healthy chickens

In chickens, the rate of carriage of *E. coli* was 90.7%. A much higher level of resistance was observed in *E. coli* strains isolated from feces of chickens (Figure 1): amoxicillin (78.5%), nalidixic acid (77.6%), tetracyclin (75.8%), chloramphenicol (75.8%), sulfamethoxazole/trimethoprim (66.9%), enrofloxacin (55.5%) and ticarcillin/clavulanic acid (53.4%).

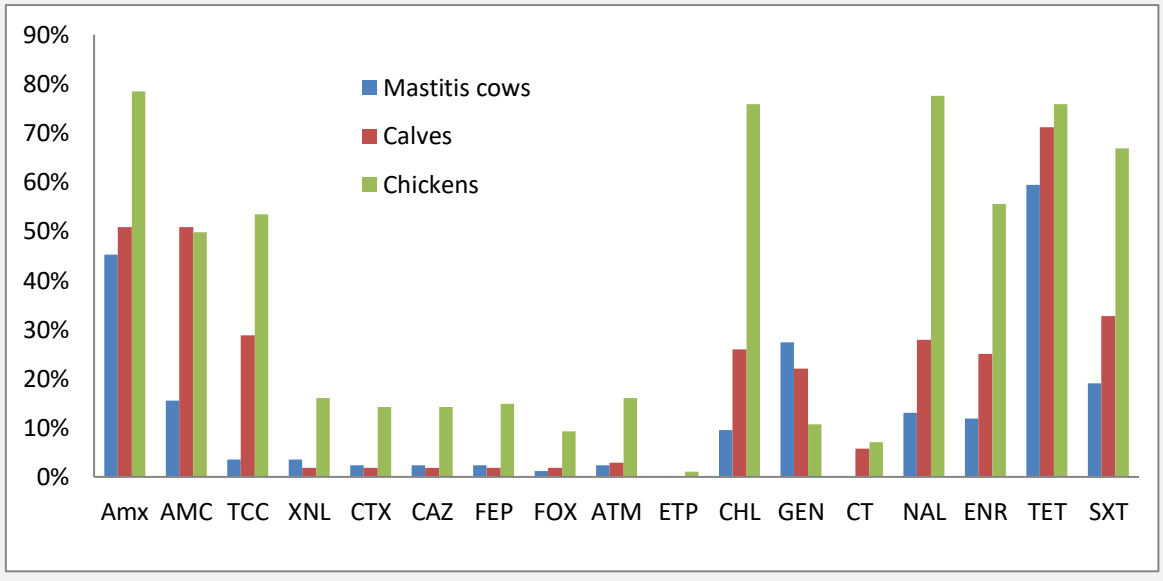

Figure 1. Prevalence of antimicrobial resistance in *E.coli* isolated from bovine mastitic milk and feces of calves and chickens.

AMX: amoxicillin, AMC: amoxicillin/clavulanic acid, TCC: ticarcillin/clavulanic acid, XNL: ceftiofur, CTX: cefotaxim; CAZ: ceftazidim, FEP: cefepim, FOX: cefoxitin, ATM: aztreonam, ETP: ertapenem, CHL: chloramphenicol, GEN: gentamicin, CT: colistin, NAL: nalidixic acid, ENR: enrofloxacin, TET: tetracycline, SXT: sulfamethoxazole/trimethoprim.
Among the 283 *E. coli* strains tested, 41 (14.5%) displayed an ESBL phenotype. Higher fecal carriages of cefotaxim-resistant *E. coli* isolated from chickens were identified in previously reports in Tunisia (Ben Sallem et al., 2012; Mnif et al., 2012; Kilani et al., 2015; Maamar et al., 2016). The majority of

cefotaxim-resistant *E. coli* showed also resistance to other unrelated families of antibiotics (Table 1) by harboring other resistance genes to non-beta-lactam antibiotics (Meunier et al., 2010). This has created a worldwide problem that reduces therapeutic options and may leads to treatment failure (Hammerum and Heuer, 2009; Pitout, 2010).

Table 1. Multidrug resistant phenotypes of ESBL producing *E. coli* isolated from bovine mastitic milk and fecal samples of calves and chickens

Origin of strains	Number of ESBL *E. coli* strains	Resistance to non-beta-lactam antibiotics
Mastitis cattle	2	NAL, ENR, TET, SXT
Calves	1	CHL, GEN, NAL, ENR, TET, SXT
	1	NAL, ENR, TET, SXT
Poultry	5	CHL, TET, NAL, ENR, SXT
	4	TET, NAL
	3	CHL, TET, NAL, ENR, SXT, CST
	3	NAL, SXT
	3	GEN, TET, SXT
	2	CHL, TET, NAL, ENR
	1	CHL, SXT
	1	GEN
	2	GEN, CHL, TET
	1	TET
	1	CHL, TET
	1	NAL
	3	NAL, ENR, SXT
	1	SXT
	1	GEN, NAL
	1	NAL, ENR
	1	TET, NAL, ENR
	1	TET, SXT
	1	TET, ENR, SXT
	1	GEN, CHL, TET, NAL, SXT
	1	GEN, CHL, TET, NAL
	1	CHL, NAL, ENR, SXT
	1	CHL, NAL, TET, ENR
	1	CHL, TET, CT, NAL, ENR, SXT

These results illustrated the fact that ESBL producing strains are known to be multidrug resistant. Genes encoding for ESBL phenotype are linked to other resistant genes to non-betalactam antibiotics (Alyamani et al., 2017). In Tunisia, chickens constitute an important reservoir of cefotaxime-resistant *E. coli* isolates (Ben Sallem et al., 2012; Kilani et al., 2015; Maamar et al., 2016). Moreover, genes that code for ESBL phenotypes are carried by mobile genetic elements, such as plasmids, that can be transmitted between and within bacterial species, in animal or humans microbiota.

4. Conclusion

The rates of antimicrobial resistance detected in this study were associated with the animal species. Antimicrobial resistance rate was higher in *E. coli* isolated from faeces of chickens then in those from faeces of calves and bovine mastitic milk, and this may be associated with level of antibiotic use in the different animal sectors. Moreover, a higher resistance level was obtained in fecal samples of calves than in milk of mastitis cattle which can be explained by the favorable ecosystem in gut for the horizontal transfer of genetic material, including plasmids carrying antibiotic resistance genes. However, in mastitis cows the healthy mammary gland is sterile and not a favorable environment for genetic exchanges of antimicrobial resistance genes. Finally, molecular studies should be conducted to characterize ESBL plasmids for cefotaxim-resistant strains and to identify genes encoding resistance to non-betalactam antibiotics.

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