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ORIGINAL ARTICLE

Occurrence of ESBL-producing *Escherichia coli* in soils subjected to livestock grazing in Azores archipelago: an environment-health pollution issue?

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Abstract

Antibiotics are successful drugs used in human and animal therapy; however, they must be considered as environmental pollutants. This study aims to isolate and characterize the extended-spectrum β-lactamase (ESBL) producing *Escherichia coli* soil from Azores Archipelago subjected to livestock agricultural practices. Twenty-four soil samples were collected from three different pasture systems with different number of cattle heads, and from a control site. Antibiotic susceptibility method was performed by Kirby–Bauer disk diffusion method against 16 antibiotics, and the presence of genes encoding lactamases, antimicrobial resistance genes, virulence factors, and phylogenetic groups was determined by polymerase chain reaction (PCR). Nine ESBLs were recovered from the three grazing sites, and all isolates presented the beta-lactamase genes $bla_{\text{CTX-M-3}}$ and bla_{SHV}. *E. coli* isolates were resistance to tetracycline and streptomycin and harbored the *tet*B, *str*A, and *str*B genes. One isolate also showed resistance to sulfonamides, and the genes *sul*1 and *sul*2 were detected. The isolates were grouped into the following phylogenic groups: B1 ($n = 6$), D ($n = 2$), and A ($n = 1$). The presence of antibiotics and resistance genes in soils may be the source to the development of antimicrobial resistance, which may have negative consequences in human and animal health.

Keywords Extended-spectrum beta-lactamase · *E. coli* · Soil · Livestock · CTX-M · Environmental pollution

Introduction

Antibiotics are one of the leading advance developments in medicine that prevent and treat bacterial infection diseases in humans and animals. However, the acceleration of propagation of multidrug-resistant bacteria contributed to the antibiotic resistance that is considered one of the most important challenges to global public health in the last decades (Tang et al. 2016).

Vanessa Silva and Fernando Peixoto contributed equally to this work.

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The extensive use of antibiotics for both farming and clinical proposes has led to an antibiotic environmental pollution. When our environment is polluted by antibiotics it represents an ecological risk since it can lead to toxic effects on plants, animals, and eventually humans that go from molecular level, organism level, and to ecosystem level (Gothwal and Shashidhar 2015). Also, the antibiotic resistance genes (ARGs) may be considered pollutants as well. *Escherichia coli* is classified as a commensal bacteria that inhabits the gastrointestinal tract of many animals (Gonçalves et al. 2013; Carvalho et al. 2017), and it is commonly found in other ecological niches such as soil (Fouhy et al. 2015), vegetables (Campos et al. 2013), and water (Bulycheva et al. 2014). Normally, these bacteria are not the source of disease, but they can act as potential reservoirs of genetic elements that contain ARGs (Radhouani et al. 2009). These genes can easily diffuse in distinct ecosystems through the food chain and water (Guerrero-Ramos et al. 2016; Boulianne et al. 2016). When antibiotics are released to the ecosystems, they can promote a selective pressure favoring the selection of resistant species and bacterial strains. For this reason, determinants of antibiotic resistance could be transferred via horizontal gene transfer to other commensal or pathogenic bacteria. The occurrence of extended-spectrum beta-lactamases (ESBLs)-producing *E. coli* is considered one of the major leading resistance mechanisms for two classes of antibiotics: penicillins and cephalosporins (Silva et al. 2012). The ESBL-producing *E. coli* strains are spreading fast, which is a concern since infections caused by these strains are extremely difficult to treat (Queenan et al. 2004). Besides, ESBL-producing *E. coli* have a resistance mechanism that causes decrease in efficacy of most frequently used antibiotics such as penicillins, first- to fourth-generation cephalosporins, combinations with beta-lactamase inhibitors, and the monobactamic aztreonam, which causes treatment failure. A few studies have been performed to investigate the prevalence of ESBLs recovered from soil environment, particularly in farming and livestock grazing soils (Hartmann et al. 2012; Blaak et al. 2015). There is only one study that analyzed the antibiotic resistance and ARGs in soils in Azores Archipelago; however, this study is not based on *E. coli* strains (Silva et al. 2018). Thus, our study aimed to characterize the antibiotic-resistant profiles in *E. coli* isolates, phenotypically and genotypically, and to determinate the existence of ESBL-producing *E. coli* recovered from distinct soils subjected to long-term livestock practices in Azores Archipelago.

Material and methods

Samples and bacterial isolates

Twenty-four samples of soil were collected from São Miguel island in Azores archipelago during the year of 2017: six samples were collected from a pasture system located in Fajã de Cima (soil subjected to 40 cattle heads), six from Arrifes (soil subjected to 60 cattle heads), six samples from Covoada (soil subjected to 80 cattle heads), and six soil samples from the forest reserve of Pinhal da Paz (reference site). All samples were collected in areas of intense trampling of the cows, from the top soil layer (0–20 cm) and three composite soil samples from each land (with three sub-samples each). The animals from Fajã de Cima and Arrifes were in a rotating system (representing a smaller manure input), and the animals from Covoada were permanently in the grazing lands. Two grams were taken randomly from the four soil samples, diluted in Brain Heart Infusion (BHI) (Oxoid, UK) broth and incubated for 24 h at 37 °C. To identify only ESBL-producing *E. coli* strains, 100 μl of inoculum were spread onto Levine (Oxoid, UK) agar plates supplemented with cefotaxime (2 μg/ml) (Sigma-Aldrich Germany) and the plates were incubated at 37 °C for 24 h. Blue–black colonies with a green metallic sheen were considered *E. coli* resistant to cefotaxime, and one colony of each plate was recovered. The identification and confirmation of *E. coli* strains were performed using microbiological, biochemical, and molecular tests, namely, TSI, urea, citrate tests, and confirmed by API 20E system (BioMérieux, La Balme Les Grottes, France) to directly distinguish colonies of *E. coli* from those that were phenotypically assimilated but belonged to another genus.

Antimicrobial susceptibility

Antimicrobial susceptibility testing was performed by the Kirby–Bauer disk diffusion method according to the criteria of European Committee on Antimicrobial Susceptibility Testing (CLSI 2017) for *E. coli* against 16 antimicrobial agents: tobramycin (10 μg), amikacin (30 μg), cefoxitin (30 μg), ampicillin (10 μg), amoxicillin + clavulanic acid (20 μg + 10 μg), aztreonam (30 μg), cefotaxime (30 μg), chloramphenicol (30 μg), ceftazidime (30 μg), imipenem (10 μg), ciprofloxacin (5 μg), gentamicin (10 μg), nalidixic acid (30 μg), sulfamethoxazole-trimethoprim (1.25 μg + 23.75 μg), streptomycin (10 μg), and tetracycline (30 μg). ESBL-phenotypic identification was performed by double-disk synergy test (CLSI 2017). *E. coli* strain ATCC 25922 (American Type Culture Collection, USA) was used as a quality control.

Characterization of antimicrobial resistance mechanisms and detection of virulence genes

The presence of encoding β-lactamases, antimicrobial resistance genes, virulence factors, and phylogenetic groups were studied by PCR and sequencing and were performed using specific primers as previously described (Table S1). The resistance genes studied were $bla_{\text{CTX-M}}$, bla_{SHV}, and bla_{TEM}

Table 1 Characteristics of the isolates recovered from Azores archipelago soils

Location	Number of isolates	Phylogenetic group	Phenotypic resistance to beta-lactams	Beta-lactamase genes	Phenotypic resistance to other antibiotics	Other genes detected	Virulence genes
Pinhal da Paz	0	–	–	–	–	–	–
Fajã de Cima	1	B1	CTX-CAZ- AMP-AMC-ATM	$bla_{CTX-M-3}$, bla_{SHV}	TET, STR	*tet*B, *str*A, *str*B	*fim*A
	1	B1	CTX-CAZ-AMP-ATM	$bla_{CTX-M-3}$, bla_{SHV}	TET	*tet*B	*fim*A
	1	B1	CTX, AMP, ATM	$bla_{CTX-M-3}$, bla_{SHV}	TET, STR	*tet*B, *str*A, *str*B	*fim*A
Arrifes	1	A	CTX, AMP, ATM	$bla_{CTX-M-3}$, bla_{TEM}, bla_{SHV}	TET, STR	*tet*B, *str*A, *str*B	*fim*A
	1	D	CTX, CAZ, AMP, AMC, ATM	$bla_{CTX-M-3}$, bla_{TEM}, bla_{SHV}	TET, STR	*tet*B, *str*A, *str*B	*fim*A, *pap*C
	1	D	CTX, CAZ, AMP, AMC, ATM	$bla_{CTX-M-3}$, bla_{TEM}, bla_{SHV}	CHL, TET, CIP, STR, SXT	*tet*B, *str*A, *str*B, *sul*1, *sul*2,	*fim*A
Covoada	2	B1	CTX, CAZ, AMP, AMC, ATM	$bla_{CTX-M-3}$, bla_{TEM}, bla_{SHV}	TET, STR	*tet*B, *str*A, *str*B	*fim*A
	1	B1	CTX, CAZ, AMP, AMC	$bla_{CTX-M-3}$, bla_{TEM}, bla_{SHV},	TET, STR	*tet*B, *str*A, *str*B	*fim*A

CTX cefotaxime, *CAZ* ceftazidime, *AMP* ampicillin, *ATM* aztreonam, *AMC* amoxicillin with clavulanic acid, *TET* tetracycline, *STR* streptomycin, *CHL* chloramphenicol, *SXT* trimethoprim-sulfamethoxazole, *CIP* ciprofloxacin

(beta-lactamases); *tet*A and *tet*B (tetracycline resistance); *aad*A, *str*A, and *str*B (streptomycin resistance); *cml*A (chloramphenicol resistance); and *sul*1, *sul*2, and *sul*3 (trimethoprim-sulfamethoxazole resistance). The detection of virulence factors commonly found in pathogeny in *E. coli* (*cnf*1, *aer*, *fim*A, *pap*GIII, *pap*C, and *sxt*) and phylogenetic groups were also studied (Gonçalves et al. 2011). The positive and negative controls (from the strains collection of University of Trás-os-Montes and Alto Douro, Portugal) were used in every PCR analysis.

Results and discussion

From the twenty-four soil samples, a total of nine (37.5%, 95% confidence interval 18.8 to 59.4) ESBL-producing *E. coli* were recovered from the 3 grazing lands, whereas no ESBL was recovered from Pinhal da Paz (control site) (Table 1). These results confirmed the wide dissemination of these resistant bacteria in soils subjected to livestock grazing. As reported in many studies, manure from livestock cattle may contain antibiotic resistant *E. coli* and ESBL-producing *E. coli* associated with livestock, which is of particular concern (Reinthaler et al. 2010; Gao et al. 2015; Hosseini et al. 2016; Santman-Berends et al. 2017). The administration of antibiotics to farm animals, including livestock, may be the source of the high predominance of antibiotic resistant bacteria and ARGs and, in this specific case, ESBL-producing *E. coli* (Berendsen et al. 2015). In this study, the β-lactamase genes found were $bla_{CTX-M-3}$ ($n = 9$), bla_{SHV} ($n = 9$), and bla_{TEM} ($n = 6$). ESBL-producing *E. coli* carrying these genes have been found in different sources (Gonçalves et al. 2013; Bonnedahl et al. 2015; Rebbah et al. 2017). The presence of CTX-M type is a concern since they have emerged worldwide and gradually replaced the TEM and SHV families. The $bla_{CTX-M-3}$ gene belongs to the bla_{CTX-M} group 1, which have been previously found in soils subjected to livestock cattle (Hartmann et al. 2012). However, the most prevalent bla_{CTX-M} gene found in soils seems to be $bla_{CTX-M-14}$ (Hartmann et al. 2012; Zheng et al. 2017). The bla_{TEM} and bla_{SHV} genes have been identified in both farm and livestock grazing soils (Jones-Dias et al. 2015). The *tet*B gene was detected in all of the isolates with resistance to tetracycline. Ji et al. (2012) investigated the prevalence of antibiotic presence in soil after manure application and found out that concentrations of tetracycline residues were significantly higher than other antibiotics, including sulfonamides and chloramphenicol. However, in the same study the amount of sulfonamide resistance genes (*sul*1, *sul*2, and *sul*3) was higher than tetracycline resistance genes in both manures and soils. Resistance to sulfonamides and chloramphenicol were also detected in our study but only in one isolate, which presented the *sul*1 and *sul*2 genes. Other studies showed that the same ARGs

that were detected in soil right after manure application were also detected several months after and that the abundance of the *tet*B gene increased after soil fertilization with manure (Kyselková et al. 2015; Peng et al. 2016). Also, the study by Lau and Ingham (2001) showed that *E. coli*, when found in soil mixed with bovine manure, was able to survive for at least 19 weeks. Therefore, these reports indicate that, regarding our study, even with the implemented rotatory system by the animals from Fajã de Cima and Arrifes, which represented a smaller input of manure, the ARGs and the ESBLs remain in the soils after long periods of time. Moreover, mobile genetic elements contribute to the preservation and propagation of antimicrobial resistance over the environment (Stokes and Gillings 2011). Resistance to streptomycin was found in a considerable number of isolates (88.9%) and all carried the *str*A and *str*B genes. In another study, *str*A and *str*B were the most frequently detected genes among other genes conferring resistance to streptomycin (Srinivasan et al. 2008). Previous studies conducted with *E. coli* recovered from wild animals of Azores Archipelago showed that resistance to tetracycline, streptomycin, ampicillin, and sulfamethoxazole-etrimethoprim was the most prevalent (Santos et al. 2013; Marinho et al. 2014). All ESBL-producing *E. coli* isolates harbored the virulence gene *fim*A, and only one isolate presented the *pap*C gene. The *fim*A gene is often identified among commensal *E. coli* isolates (Radhouani et al. 2013). In our study, the ESBL-producing *E. coli* isolates were classified in the phylogroup group B1 ($n = 6$), D ($n = 2$), and A ($n = 1$). A similar ratio of phylogenetic groups has been reported in the past in ESBL-producing *E. coli* in soil of farm environment (Ben Said et al. 2015). Furthermore, in the same study the virulence genes *fim*A and *pap*C were found in all and in one isolate, respectively. Since Azores Archipelago is a remote island of Portugal and there are no studies regarding the detection of ESBL-producing *E. coli* in soils we cannot make a direct comparison; however, a study conducted in wild birds from Azores Archipelago presented an identical distribution of the phylogenetic groups (Santos et al. 2013). The phylogenetic groups A and B1 are associated to commensal strains (Costa et al. 2009). Therefore, the high predominance of B1 group identified in our study could confirm that the ESBL-producing *E. coli* detected in soil may come from livestock grazing in the fields. The isolates presenting the phylogenetic group D, which is associated with virulent strains, presented a higher number of phenotypic and genotypic resistance compared to the other strains, and one of the strains even harbored two virulence genes (Radhouani et al. 2013).The high currency of ESBL-producing *E. coli* recovered from soils exposed to long-term livestock grazing constitutes a public health challenge. Antibiotics released on soil through manure select resistant bacteria already prevailing in soil increasing the overall antimicrobial resistance (Heuer et al. 2011). Resistance genes positioned on mobile genetic elements may be enriched in manure and preserved in soil through horizontal gene transfer events. Besides, the animals themselves may be acting as reservoirs of ARGs promoting their accumulation in soils and dissemination though the environment.

Conclusions

In this research, we identified a high presence of ESBL-producing *E. coli* in soils exposed to livestock grazing. The spread of CTX-M type ESBLs has been increasing and represents a challenge of global dimension. There was a high prevalence of resistance to streptomycin and, as expected, all isolates showed resistance to tetracycline, which is the main class of antibiotics used in farm animals. Manure may be responsible for a massive input of resistance bacteria and genes in soils and environment, increasing the antibiotics and ARG pollution that can well contribute to the increasing of antibiotic resistance in bacteria. It is very likely that the use of antibiotics in agricultural practices contributes to the spread and distribution of antibiotic resistance and ARGs through the environment reaching the human community that are continuously exposed to these exogenous bacteria. Therefore, manure should be treated before applied on land via mechanical, chemical, physical, and biological methods. Additionally, after manure treatment, the microbiological safety and the sanitary risk should be evaluated before land application.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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