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Antimicrobial resistance patterns in *Salmonella enterica* subsp. *enterica* and *Escherichia coli* isolated from bivalve molluscs and marine environment

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ABSTRACT

The current study presents data on the antimicrobial resistance (AMR) patterns of 102 *S. enterica* subsp. *enterica* (72 *Salmonella* ser. Typhimurium and 30 monophasic *S*. Typhimurium serovar) and 79 *Escherichia coli* (and their phylogenetic group determination) isolates from different species of bivalve molluscs and from the water samples collected from the sub-areas of a mollusc production area near Ferrara (Italy). These areas were classified as Long-line, Lupini, B-Out, B-in, and Sacca. A retrospective evaluation was performed to assess the spatial trends of the resistance patterns of *Salmonella* and *E. coli* and the temporal trend for *Salmonella*; the role of molluscs as AMR indicators and the potential use of *E. coli* as a microorganism indicator of AMR occurrence in a seawater environment were also investigated. Overall, 81% of *Salmonella* spp. and 75% of *E. coli* isolates were resistant to, at least, one antimicrobial agent (AA) and 44% and 38% of the isolates were multidrug resistant (MDR), respectively. The resistance levels of *Salmonella* were influenced by the investigated serovars. Monophasic *S*. Typhimurium serovar showed the highest resistance value with 70% of MDR isolates, in contrast with only 33% in *S*. Typhimurium. In monophasic *S*. Typhimurium versus *S*. Typhimurium, twofold resistance levels were observed to streptomycin (97 versus 43%), ampicillin (80 versus 40%) and tetracyclines (67 versus 36%). Regarding the temporal trend for *Salmonella*, strains were resistant to, at least, one AA, but this resistance was significantly lower during the first years of this 17-year sampling; however, in parallel MDR isolates, the resistance increased from 23% to a maximum level of 57% during the 2008-2012 period. On assessing the spatial trends, the Sacca area was found to show the lowest number of *Salmonella* spp. and *E. coli* strains resistant to, at least, one AA and MDR. For *E. coli*, the most commonly observed resistance was towards ampicillin (56%), streptomycin (52%), sulphonamides (30%) and ceftiofur (24%). The great majority (65%) of *E. coli* isolates belonged to the commensal phylogroups A and B1, with B1 as the dominant one, whereas most MDR isolates belonged to phylogroup C. Molluscs may be an efficient tool for antimicrobial resistance monitoring, and *E. coli* could be used as a microorganism indicator of the occurrence of antimicrobial resistance in seawater environment.

1. Introduction

Bivalve molluscs represent an important tool for monitoring antibacterial-resistant *Escherichia coli* and other members of the Enterobacteriaceae family in a coastal environment (Bighiu et al., 2019; Grevskott et al., 2017). Antimicrobial resistance (AMR) is a natural phenomenon, but the misuse and overuse of antibacterial agents in human and veterinary medicine, as well as in agriculture, have increased the release of these substances to the environment, which threatens global public health (Davies & Davies, 2010). Marine environments play an important role in accentuating antimicrobial resistance, as an unknown amount of these drugs ends up either indirectly in the receiving waters, or directly, as a result of intensive fish farming. As a consequence, living organisms could be exposed to a variety of these compounds present in the environment at low concentrations (Chiesaet al., 2018). Besides, coastal areas are subjected to faecal contamination of human and animal waste coming from a variety of sources, including rivers, runoff from agricultural and industrial activities and urban wastewater, resulting in the pollution of marine habitats (Vignaroli et al., 2016; Grevskott et al., 2017). In this context, marine ecosystems are not only an important reservoir for AMR, but also drive its emergence (Al-Sarawi et al., 2018; Taylor, Verner-Jeffreys, & Baker-Austin, 2011; Williams et al., 2016).

In the European Union (EU), the microbiological quality of coastal waters (Directive 2006/7/EC), as well as the sanitary control of shellfish produced and sold for human consumption, are consistently monitored by measuring the abundance of faecal indicator bacteria, *E. coli* and enterococci, in waters and the content of *E. coli* in the soft parts, flesh and intravalvular liquid of harvested bivalves for the classification of the mollusc production areas as A, B or C (Regulation EC 853/2004; Regulation EC 2017/265; Regulation EC 2019/265; Regulation EC 2019/267). In addition, the food safety criteria concerning bivalves entering the market states that the absence of *Salmonella* and the enumeration of *E. coli* are requested (Regulation EC 2073/2005; Regulation EC 2285/2015; Regulation 229/2019). In the European Union, *Salmonella* is the second most common

cause of human gastroenteritis (EFSA & ECDC, 2018). A considerable amount of epidemiological data regarding the presence of *Salmonella* in seafood and its related illnesses is available, and the risks of foodborne diseases associated with *Salmonella* in molluscs are classified as low (Davies & Davies, 2010; NACMCF, 1992). Even if the microorganisms of the genus *Salmonella* are not natural inhabitants of aquatic environments, several *Salmonella* serovars are widely distributed in water (sea, estuarine, river) and in a variety of seafood, with the highest prevalence in molluscs, shrimps, clams, and various fish species (Novoslavskij et al., 2016). *E. coli* is a common inhabitant of the human and animal intestinal tract. *E. coli* may inhabit a host as a harmless symbiont or, depending on the spectrum of encoded virulence factors, it can cause either intestinal or extraintestinal infections (Logue et al., 2017). *E. coli* is a common indicator organism of faecal contamination in aquatic systems, and it is recognized as an important player in the spread of antibiotic resistance (Henriques et al., 2006; Szmolka & Nagy, 2013) due to its plasticity and high aptitude to acquire genetic information through horizontal gene transfer. These characteristics enable *E. coli* to exchange genetic material with other bacterial species (Araújo et al., 2017).

In Italy, a continuous microbiological monitoring of compliance with both the shellfish harvesting areas and food safety criteria has been established by the official Veterinary Authorities, as required by the EU regulations. Therefore, a continuous baseline data on the presence of *Salmonella* and *E. coli* enumeration in water, shellfish flesh and intravalvular liquid is available. However, this monitoring does not include the isolation and typing of *E. coli* isolates, as well as the AMR evaluation of *Salmonella* and *E. coli*. The current study presents data on the AMR patterns of *Salmonella* isolated with a continuous sampling history, from 2001 to 2017, and of *E. coli*, isolated from 2016 to 2018, in the mollusc production area in the province of Ferrara, Emilia-Romagna Region, Northern Italy. Here, even the distribution of *E. coli* in the phylogenetic groups was investigated. A retrospective evaluation was performed with the following objectives: i) to determine and compare the antimicrobial susceptibility among *Salmonella* serovars and the most prevalent *E. coli* phylogenetic groups identified from bivalve molluscs and sea and brackish water,

as well as from different sampling areas; ii) to assess the spatial trends of resistance patterns of *Salmonella* and *E. coli* and the temporal trend for *Salmonella* in the mollusc production area of the province of Ferrara; iii) to investigate the role of molluscs as AMR indicators and to evaluate the potential use of *E. coli* as a microorganism indicator of AMR occurrence in a seawater environment.

2. Materials and methods

2.1 Salmonella spp. strain collection

In the present study, 102 *S. enterica* subsp. *Enterica* isolates from a strain collection of *Salmonella* isolates were selected: a total of 72 *Salmonella* ser. Typhimurium and 30 monophasic *S.* Typhimurium serovar (4,[5],12:i:-), isolated from 2001 to 2017, were considered. All these strains were isolated during the official monitoring performed by the regional Veterinary Authority and during a shellfish monitoring program using live bivalve molluscs and water samples from the seashore and inland channels of the province of Ferrara, Emilia-Romagna region, Italy. Most of these strains belong to a previous study (Rubini et al., 2018), which involved a total of 237 *Salmonella* isolates identified as *S. enterica* subsp. *enterica* (collected from a total of 10,757 seawater and molluscs samples from 1997 to 2015), in which 53 different serovars were observed. *S.* Typhimurium was the dominant serovar (26.9%), followed by its monophasic variant 4,[5],12:i:- (11.8%), Derby (6.3%), Newport (5.5%) and Thompson (4.6%). The *Salmonella* isolates considered in this study represent about 50% of isolates and almost all the serovars monophasic *S.* Typhimurium and *S.* Typhimurium collected in the investigated molluscs area. Each isolate refers to a specific area of classification (A or B), a different area of sampling (namely, specific sampling points within the considered area), a different source (Manila clams (*Ruditapes philippinarum*) (n = 30 isolates), striped clams (*Chamelea gallina*) (n = 1 isolate), mussels (*Mytilus galloprovincialis*) (n = 3 isolates) and water samples (n = 68 isolates), and a different time of sampling (see Table 1 of supplemental material). All the *Salmonella* strains were isolated in the North Western area of the

Adriatic Sea, facing the Southern area of the Po river delta, the major Italian river which, from spring to estuary, flows through the Po Valley (Pianura Padana) for a total of 652 km. The Po Valley is a densely populated area with a high number of large intensive animal farms. The Po river, near its end, in the Adriatic Sea, creates a wide delta with a surface area of 31 km^2 and an average depth of 1.5 m; its hydrographic network is mostly artificially regulated and, as a consequence, its freshwater flows are partially independent of rainfalls. More than one third of the lagoon surface is exploited for clam farming, with an annual production that has reached a maximum of 87,000 t/year in 2011 (Bison, 2012). This area has been divided into five sub-areas, following the mollusc production areas: I) long-line: the marine class A area used to breed mostly mussels and secondary oysters; ii) Lupini: the coastal marine area, including seawater between 1 and 2 nautical miles, that is classified as a class A area in which natural banks of striped clams are present and harvested; iii) B-Out: the narrow sea coastal area and inland waters, classified as a class B area; iv) B-In: class B area that includes the inner channels directly connected to the sea, together with the internal waters; v) Sacca: the class B area included between the Po river and the marine coastline (figure 1). These last three sub-areas are used to breed mussels, Manila clams and oysters.

2.2. E. coli isolation and phylogenetic group determination

The isolation of *E. coli* was performed starting from the official samplings of Manila clams (*R. philippinarum*) (n = 54), striped clams (*C. gallina*) (n = 3) and mussels (*M. galloprovincialis*) (n = 11), collected between 2016 and 2018 in the same aforesaid areas, and analysed for *E. coli* enumeration using the Most Probable Number (MPN) technique specified in ISO 16649-3, and from water samples (n = 11), using a membrane filtering method (APAT, 2003a. 2003b, 2003c). Each sample refers to a different area of classification, namely A and B, a different area of sampling, namely specific sampling points within the considered areas, a different source and a different time of sampling (see Table 2 of supplemental material). The MPN method utilizes Minerals Modified Glutamate Broth (MMGB) as the growth medium, and the material from

positive tubes, i.e., tubes whose colour has changed due to acid production, which was confirmed on Tryptone Bile with X-glucuronide (TBX) agar (Oxoid, Basington, UK); the suspected *E. coli* isolates were streaked onto MacConkey agar (Oxoid, Basington, UK) and incubated aerobically at 37 ± 1°C. After 24 hours of incubation, colonies of Gram-negative rods were streaked onto Tryptone Soya Agar (Oxoid, Basington, UK). All the isolates were subjected to a DNA extraction using the REDExtract-N-Amp tissue PCR kit (Sigma, St Louis, MO) and were identified using the PCR procedure described by Clermont and colleagues (Clermont et al., 2008). Overall, 79 *E. coli* strains were considered in this study and were subjected to phylogenetic group determination. *E. coli* isolates were assigned to one of the 7 phylogroups (A, B1, B2, C, D, E or F) based on the PCR amplification of *chuA*, *yjaA*, *arpA* and *trpA* genes and of the TspE4.C2 DNA fragment (Clermont et al., 2013). The reference *E. coli* strain ATCC 25922 and sterile water were used as the quality and negative control samples throughout the procedure, respectively.

2.3 Antibiotic susceptibility testing

All the isolates were tested for their antimicrobial susceptibility to twelve antibiotic agents, according to the agar disk diffusion method described by the Clinical and Laboratory Standard Institute (CLSI, 2016). The antimicrobial panel was chosen considering the importance of antimicrobial classes in the treatment of human and animal infections and the intrinsic resistance of *E. coli* reported by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The antimicrobials tested and the resistant breakpoints used in this study are reported in Table 1. For the breakpoint selection, when available, the epidemiological cut-off values proposed by EUCAST were used as the first choice. The EUCAST clinical breakpoints for Enterobacteriaceae were used as the second choice, and finally, for antimicrobials/bacterial species breakpoints not defined by EUCAST, the CLSI breakpoints for Enterobacteriaceae were chosen. Strains were considered multidrug resistant (MDR) when they showed resistance to three or more antimicrobial classes (Schwarz et al., 2010).

2.4 Statistical analysis

Descriptive statistics (absolute frequencies and percentages) have been provided regarding the number of isolates: isolates resistant to, at least, one AA and MDR isolates. Findings were presented by area of production, period of sampling, tested antimicrobial agent, *Salmonella* serovars, and phylogenetic group of *E. coli*. The monitoring part was not planned and designed in this study because it followed the real official activities performed by the Veterinary Authority. Given the long period of official monitoring considered in this study, these 17 years of sampling were arbitrarily divided into three different periods, namely, from 2001 to 2007 (17 isolates), from 2008 to 2012 (28 isolates) and from 2013 to 2017 (57 isolates), in order to have similar periods with enough data to be compared. Furthermore, a limited number of samplings was performed for Long-line, Lupini and B-out areas (11 for *Salmonella* and 19 for *E. coli* isolates), and therefore, the overall isolates belonging to these areas were merged and, in the aggregate analysis, three definitive areas were considered to assess the spatial resistance differences, namely in B-in, Sacca and the area including Long-line, Lupini and B-out areas. Significant caution should be exerted for data belonging to this merged area.

Pearson's chi-squared test and Chi-square test for trend were used to compare the temporal trends of resistance patterns of *Salmonella*, whereas Chi-square test or Fisher's exact test were used to compare the spatial trends for both *Salmonella* spp. and *E. coli* in the mollusc production areas of the province of Ferrara. The significance limit was set at a $p < 0.05$.

3. Results and Discussion

In total, the antibiotic sensitivity tests showed that 19 (18.62%) *Salmonella* and 20 (25.31%) *E. coli* isolates were susceptible to all the antimicrobials tested. Among the 102 *Salmonella* isolates, encompassing the two different serovars, a total of 83 isolates (81.37%) showed resistance to, at least, one antimicrobial agent and 45 isolates (44.12%) were MDR. More specifically, detailing the patterns of resistance associated with the two considered serovars, monophasic *S.* Typhimurium

serovar showed the highest rates of resistance, with 96.67% and 70% of the isolates being resistant to, at least, one antimicrobial agent and having MDR, respectively, whereas 75% and 33.33% of *Salmonella* ser. Typhimurium isolates were resistant to, at least, one antimicrobial agent and possessed MDR, respectively. Tables 2 and 3 detail the antimicrobial susceptibility findings of *Salmonella* isolates of both serovars. The most common resistance in *Salmonella* isolates was towards streptomycin (58.8%), ampicillin (52%) and tetracyclines (45.1%), but different resistance levels were observed in the two considered serovars. The monophasic variant and *S.* Typhimurium showed resistance to streptomycin (97 and 43%, respectively), ampicillin (80 and 40%, respectively) and tetracyclines (67 and 36%, respectively). A very different behaviour was observed for other AAs: very high rates of resistance to sulphonamides (60%) were reported for the monophasic variant, whereas high resistance to carbapenems (23.6%) and chloramphenicol (21%) was observed in *S.* Typhimurium isolates. Over the years, the trend of resistance to, at least, one antimicrobial agent, significantly decreased ($p < 0.05$) from 94% to 72% and, in parallel, the proportions of MDR *Salmonella* isolates increased from 23.53% (4/17) between 2001 – 2007 to 57.14% (16/28) and 43.86% (25/57) in the 2008-2012 and 2013-2017 periods, respectively, even though no significant differences were observed. More details are presented in Table 4.

Among the 79 *E. coli* considered from 2016 to 2018, a total of 59 isolates (74.68%) showed resistance to, at least, one antimicrobial agent and 30 isolates (37.97%) were MDR. The most common types of resistance of *E. coli* isolates were to ampicillin (56%), streptomycin (52%), gentamycin (35,4%), sulphonamides (30%) and ceftiofur (23%). Regarding the phylogenetic group determination, most isolates belonged to phylogroup B1 (n = 40; 50.63%), followed by phylogroups A (n = 11; 13.92%) and C (n = 11; 13.92%), and even to other less frequent phylogroups, D (n = 7; 8.86%), B2 (n = 6; 7.59%) and E (n = 2; 2.53%). Two isolates were untypable and were classified as “unknown” (2.53%). The highest resistances were observed in phylogroup C, in which 90.91% and 72.73% of the *E. coli* isolates showed resistance to, at least, one antimicrobial agent and were MDR, respectively. Phylogroup C showed the highest resistances to almost all the considered

antimicrobial agents (data not shown). Tables 5 and 6 detail the antimicrobial susceptibility findings of *E. coli* isolates, also regarding the distribution of the frequent *E. coli* phylogroups.

No differences were observed regarding the resistance proportions of both *Salmonella* and *E. coli* isolates collected from molluscs and water samples, and from isolates belonging to classes A and B areas (data not shown). However, considering the spatial trends, the *E. coli* and *Salmonella* isolates in the area of Sacca had the lowest resistance for both serovars. Significant differences ($p < 0.05$) among the three different areas were observed, probably mostly regarding the lower percentage in Sacca, for MDR *Salmonella* spp. (20% in Sacca versus 51% in B-in and 73% in Long-line, Lupini B-out) and *S*. Typhimurium strains (9% in Sacca versus 67% in B-in and 39% in Long-line, Lupini B-out), but not for its monophasic variant. No significant differences were found regarding *Salmonella* spp. and *E. coli* strains resistant to, at least, one antimicrobial agent and even MDR among the three different areas. More details are reported in Table 7.

Extremely high resistance levels to, at least, one antimicrobial agent and high multi-resistant levels were observed in *Salmonella* spp. and *E. coli* isolates from molluscs, sea and brackish water in the investigated production area of Ferrara. The *Salmonella* data presented in this study comprise almost half of the overall *Salmonella* isolates of the last 20 years, from molluscs and water collected from the area of Ferrara, that is an important mollusc production area in Italy (Rubini et al., 2018), and therefore, they represent relevant data regarding the occurrence of antimicrobial resistance in *Salmonella* spp. isolates in molluscs. By plotting the amount of resistance of *Salmonella* for each period of the considered 17-year sampling, an evident rise in resistance from the first years of sampling with a doubling of the multi-resistance level in the following years must be mentioned, confirming the global antimicrobial resistance public health concern (ECDC, 2018; EFSA &ECDC, 2019). This increasing resistance is worrisome, particularly since several antimicrobial agents that were considered in this study are empiric or mere antibiotics which are commonly used in cases of serious human and animal infections.

Serovars monophasic *S.* Typhimurium and *S.* Typhimurium significantly contribute to the overall numbers of *Salmonella* isolates: in the European framework, they are among the most commonly reported serovars in human cases, as well as in food and animals, with the difference that *S.* Typhimurium has been associated with many food and animal sources, whereas monophasic *S.* Typhimurium has been mainly associated with pig and broiler sources (EFSA &ECDC, 2018). In molluscs, few data are available in the literature and the positive findings are usually reported as *Salmonella* spp.; however, the complexity of the global epidemiology of *Salmonella* requires improved monitoring data of those serovars which are of the highest epidemiologic importance (Rene et al., 2011).

Based on our findings, and in line with literature (EFSA &ECDC, 2019), the resistance levels for *Salmonella* spp. are greatly influenced by the serovars investigated, and therefore, the characterization at the serovar level is indispensable to address the temporal, geographic and source trends. The MDR level was high, overall, but monophasic *S.* Typhimurium had the highest MDR levels (70%) and the resistance level is twice as high for streptomycin, ampicillin, tetracyclines, and sulphonamides (see Table 3); in contrast, the highest proportion of resistance for meropenem and chloramphenicol were reported in *S.* Typhimurium isolates (see Table 3). *S.* Typhimurium and monophasic *S.* Typhimurium were the second and third most common *Salmonella* serovars identified in 2017 from human cases in Europe, respectively, in which the highest proportion of resistance was observed for ampicillin, sulphonamides and tetracyclines, with occurrences of 53, 48 and 44% in *S.* Typhimurium and of 87, 87 and 88% in monophasic *S.* Typhimurium (EFSA & ECDC, 2019). These data, even if with clear differences, is in line with our resistance findings, whereas for meropenem, a non-negligible level of resistance was observed in our study, compared to a full susceptibility found in human isolates. From a clinical point of view, fluoroquinolones and third-generation cephalosporins are classified as critically important antimicrobials (CIA) of the highest priority and represent the most important antimicrobial classes for treatment of

salmonellosis. Our findings showed moderate resistances to third-generation cephalosporins, but which were still higher than data reported in humans, pigs, calves or cattle (EFSA & ECDC, 2019).

E. coli is an important foodborne pathogen. In addition, it is used worldwide as an indicator of faecal contamination and as a hygiene indicator regarding sanitary quality or unsanitary conditions (Metz, Sheehan, & Feng, 2020). In antimicrobial resistance, *E. coli* is also considered as a sensor of the situation at each moment, and it has emerged as a major player in resistance: *E. coli* is typically chosen as the representative indicator of antimicrobial resistance in Gram-negative bacteria, and its monitoring in a specific population provides valuable data on the resistance occurring in that population (EFSA & ECDC, 2019). Regarding the data on antimicrobial resistance in *E. coli* isolated from molluscs and seafood or marine environments, available in the literature, comparisons are arduous due to the differences in antimicrobial agents and breakpoints used; in most studies, the most common resistances were observed for tetracycline, trimethoprim-sulfamethoxazole, ampicillin and streptomycin, with lower prevalence values than those found here (Changkaew et al., 2014; Grevskott et al., 2017; Ryu et al., 2012; Van et al., 2008; Vignaroli et al., 2015; Vignaroli et al., 2016; Wang et al., 2011) or with values in line with ours (Al-Sarawi et al., 2018; Bighiu et al., 2019). The prevalence of MDR strains in our study is higher than in most of the aforementioned studies, but is still in line with those of other studies (Bighiu et al., 2019; Boss, Overesch, & Baumgartner, 2016; Changkaew et al., 2014; Kumaran et al., 2010; Van et al., 2008). In addition, in the European Union, the monitoring of antimicrobial resistance using the indicator *E. coli* in food-producing animals (pigs and calves) and in their food products, has been mandatory since 2014: in both animal species, tetracycline resistance was the most common trait, followed by resistance to sulfamethoxazole and ampicillin (EFSA & ECDC, 2019).

E. coli is a common commensal inhabitant of the gastrointestinal tract, but it is also a common cause of severe infections in humans, being the most frequent cause of bloodstream infections and urinary tract infections among the Gram-negative bacteria. *E. coli* is involved in infections of both community and healthcare origin, as well as being associated with intra-abdominal infections and

causing neonatal infections, such as early and late neonatal sepsis (ECDC, 2018; Wang et al., 2011). *E. coli* is among the infectious agents whose antimicrobial resistance has reached an extremely worrisome situation (WHO, 2014). According to the last report of the European Center for Disease Control (ECDC) (ECDC, 2018) on antimicrobial resistance obtained through the European Antimicrobial Resistance Surveillance Network database, that included only data from invasive isolates (blood and cerebrospinal fluid), in Italy, in 2017, the prevalence of *E. coli* resistant to aminopenicillins (ampicillin or amoxicillin), fluoroquinolones and to third-generation cephalosporins was 67% (EU mean value, 58%), 45% (EU mean value, 26%) and 29.5% (EU mean value, 15%), respectively. These data roughly reflect our findings regarding the resistance to ampicillin (56%) and to third-generation cephalosporins (24%), except for enrofloxacin (5%) (see Table 5). By contrast, while carbapenem resistance remains rare in Europe, and in Italy (0.3%) (ECDC, 2018), in our study, worrisome percentages (above 10%) of this type of resistance were observed. Carbapenems are among the most frequently prescribed antibiotics for the treatment of bacterial infections in humans, even if their utility is being threatened by the worldwide rise of carbapenem-resistant Enterobacteriaceae. Although the use of carbapenems is prohibited in food-producing animals and restricted to pets in most European countries, these findings illustrate the continuous spread of these highly resistant bacteria, accompanied by emerging public health problems (Roschanski et al., 2017). In our study, resistance to ceftiofur was the fifth most common resistance type. Ceftiofur is used in human and veterinary medicine and it has an activity against both Gram positive and negative bacteria; however, third-generation cephalosporins have been categorized as CIA and substances of the highest priority, while 3rd and 4th-generation cephalosporins, as well as fluoroquinolones and polymyxins, are included in category 2, according to the European Medicines Agency. Thus, those veterinary antimicrobials represent a higher estimated risk for public health than other classes of antimicrobials. Our findings confirm that bivalve molluscs are an efficient tool for identify antimicrobial resistance, being able to detect antimicrobials used in the past and still used in food-producing animals and human beings, as well

as a useful opportunity to drive emergent threats to humans in terms of both antimicrobial resistance and clinical aspects.

Given the worldwide phylogenetic analyses, *E. coli* showed an apparent clonal population structure and a clear phylogenetic signal that appropriately reflect the relationship between strains (Tenaillon et al., 2010). Different phylogenetic groups play distinct ecological roles and, therefore, the classification of *E. coli* in the phylogenetic groups is important to understand its pathogenesis and interaction with hosts. Hitherto, a robust phylogeny, was built and 8 phylogenetic groups were identified (A, B1, B2, C, D, E, F, and the *Escherichia* cryptic clade I). The *E. coli* strains responsible for extra-intestinal infections were far more likely to be members of phylogroups B2 or D, rather than of A or B1, that usually lack a distinct virulence profile and are classified as commensal or diarrheagenic strains (Clermont et al., 2013). In general, strains belonging to different phylogenetic groups show different phenotypic and genotypic traits (Gordon & Cowling, 2003); differences in the phylogroup distribution are mainly ascribable to geographical (location and climate) and host (diet, gut morphology, body mass) factors, explaining variable animals and human phylogroup identification in several studies in the literature. The phylogroups that are considered to contain highly virulent extra-intestinal *E. coli* strains are the B2 phylogroup, whose microorganisms are a major cause of bacteraemia and neonatal sepsis (Cole et al., 2019; Vila et al., 2016) and, to a lower extent, the D phylogroup, albeit it was detected as the most common in urinary tract infections in humans (Farajzadeh Sheikh, 2019). Particular *E. coli* clones, more frequently belonging to the phylogroup B2, are more prone than others to cause bacteraemia, but they cannot be considered as having a deterministic causation relationship with extra-intestinal diseases (Vila et al., 2016); the B2 group exhibits the highest diversity at both the nucleotide and the gene content level, supporting its early emergence in the species lineage and suggesting that it has a subspecies status. Considering the findings obtained in our study using Clermont typing, the great majority (65%) of *E. coli* isolates belong to the commensal phylogroups A and B1 (these groups appear as sister groups), but phylogroup B1 strains dominated with a prevalence of 51%,

both in general and in each of the three considered sampling areas. These data are in agreement with several studies reporting a higher prevalence of A and B1 phylogroups in molluscs, with A being the dominant one (Luna et al., 2010; Vignaroli et al., 2016; Vignaroli et al., 2012) in surface water, in several animals (Johnson et al., 2017; Tomazi et al., 2018) and in water and vegetables (Araújo et al., 2017). Unlike the studies performed by Vignaroli in *Chamelea gallina* clam in Marche Region, Italy, and in marine sediments in the Adriatic Sea, in which the prevalence of MDR strains was higher in phylogroup A, in our study, most MDR isolates belonged to phylogroup C, followed by B2. Phylogroup C was the only one resistant to chloramphenicol and trimethoprim-sulfamethoxazole, and for which the highest resistance values were reported for ampicillin, streptomycin, sulphonamides, and tetracycline (data not shown). In addition, it should be noted that, whereas isolates belonging to A and B1 phylogroups were observed to be MDR, depending on the considered area of sampling, isolates belonging to phylogroup C were MDR, in spite of the area considered. It has been observed that strains of these phylogroups vary in their phenotypic and genotypic characteristics, ecological niche, lifestyle, and propensity to cause disease (Tenaillon et al., 2010); therefore, these findings confirm that waters receive contamination from a variety of sources. In this context, there is a growing body of evidence suggesting that *E. coli* can not only survive for extended periods of time, but also proliferate in several different environments, such as water, soil, algae, plants, and manure, also in the absence of faecal inputs, thus supporting its use as a water quality indicator (Nanayakkara, O'Brien, & Gordon, 2019) and as an indicator organism of faecal contamination (Vignaroli et al., 2015). In addition, *E. coli* isolates that are responsible for elevated counts (blooms) in freshwater reservoirs carry a capsule originating from *Klebsiella* spp.; overall, about 7% of *E. coli* strains have acquired these capsules, which were observed to be non-random distributed and restricted to A, B1 and C phylogroups (Nanayakkara, O'Brien, & Gordon, 2019). Given the limited number of isolates used in this study, and also the absence of any isolate characterization, authors could not resolve these doubts. However, the diversity in both the

distribution of phylogroups among the different areas and the antimicrobial resistance patterns remains.

Regarding the spatial trends of resistance, the area of Sacca was the one with the lowest number of *Salmonella* spp. and *E. coli* isolates resistant to, at least, one antimicrobial agent and MDR; conversely, the B-in area was the area with the highest number of *Salmonella* spp. and *E. coli* strains resistant to, at least, one antimicrobial agent and MDR. However, whereas for *Salmonella* spp., isolates collected from the Sacca area were susceptible to 6 out of the 12 antimicrobials tested and showed the lowest levels of resistance, for *E. coli*, resistance was observed for all the antimicrobials, even if with the lowest levels of resistance observed. In contrast, the B-in area was the area with the highest number of *Salmonella* spp. And *E. coli* strains resistant to, at least, one antimicrobial agent and MDR, as well as having the highest levels of resistance to the antimicrobial agents. See Tables 2 and 5 for more details. Even if neither the area nor the environment could be considered truly pristine, owing to the transfer of antibiotics and resistance genes via the wind, tides, bird migration and other environmental elements (Barkovskii et al., 2012), the B-in area is directly subjected to anthropogenic activities and it is exposed to industrial, municipal, agricultural and zootechnical impacts, whereas the Sacca area is certainly less anthropogenically impacted than the others. The aforesaid occurrence frequencies of antimicrobial resistance, as well as the distributions of resistant *E. coli* isolates among the phylogroups, identify the B-in area as the main source of antimicrobial resistance. *E. coli* strains that can presumably cause human extraintestinal infections are not prominent within the *E. coli* population of the aquatic environment, but, at the same time, our findings allow us to speculate on the fact that molluscs, and therefore, water environment sites, cannot be considered risk-free. Obviously, further studies should be performed to understand and verify the ecology of allochthonous and indigenous bacteria, as well as the pathogens in aquatic environments.

4. Conclusion

In conclusion, bivalve molluscs are confirmed to be an efficient tool to detect antimicrobial resistance. This study presents data on the antimicrobial resistance in *Salmonella* and *E. coli* strains isolated from molluscs and water and showed high multi-resistant levels, as well as spatial and temporal trends of resistance; however, for trend analysis of resistance, further studies are needed. The overlap of the antimicrobial resistance data collected in this study with the previously reported data of isolates from food animals and human beings suggests that testing the isolates from water and molluscs could be a useful tool to monitor the evolution of AMR of some bacterial species. In this context, routine antimicrobial susceptibility testing could be included as a parameter to be investigated by official laboratories using isolates collected from different species of bivalve molluscs, sea and brackish water by the official Veterinary Authorities during the official monitoring of molluscs. Starting from the strength of the European microbiological monitoring of molluscs and their production areas, the implementation of this step could further allow the development of an official network at the national and/or European level, which could be able to optimize the data referring to activities already planned and which could be performed by official veterinary authorities. This monitoring could be useful to raise a constant and ongoing awareness of antimicrobial resistance in the environment.

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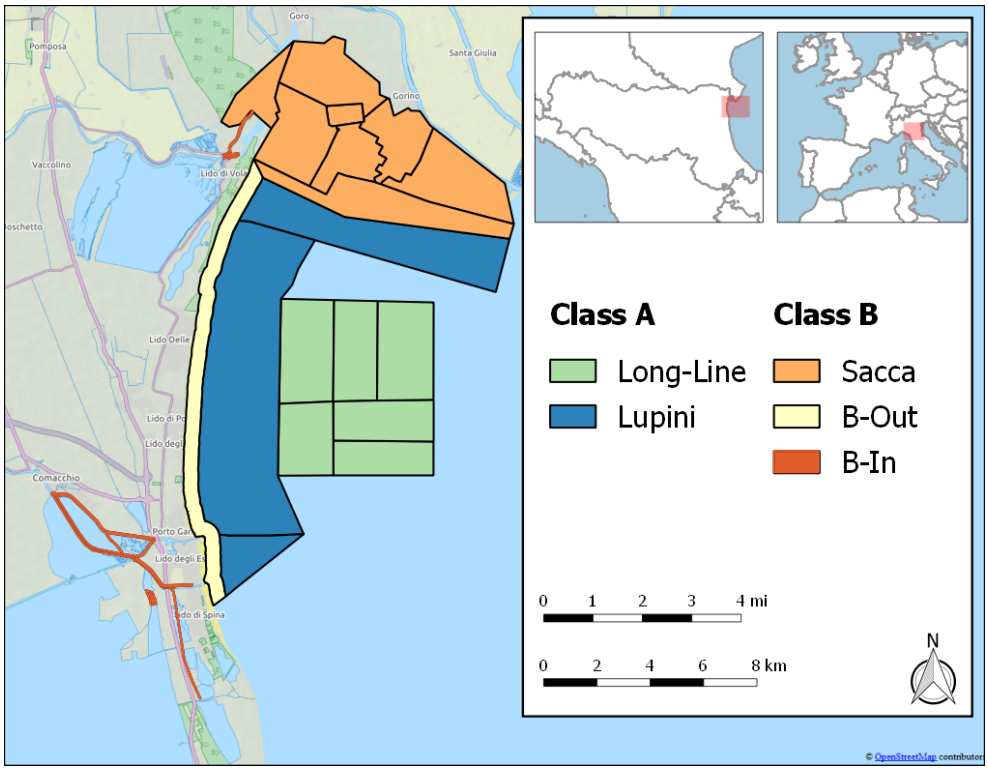
Highlights

- Bivalve molluscs are confirmed as efficient tool for antimicrobial resistance monitoring
- High multi-resistant levels were observed in *Salmonella* spp. and *E. coli* from molluscs and water
- Resistance levels for *Salmonella* are influenced by the investigated serovars
- Spatial trends of MDR *Salmonella* strains are observed

Figure 1

Figure 1. Scheme and classification of the five sub-areas in which bivalve molluscs and water samples were collected in the Ferrara area, Italy, from 2001 to 2018.



Table

Table 1. List of tested antimicrobials and resistant breakpoints used.

Antimicrobials	Abbreviations	Disk content (µg)	Resistance breakpoint (zone diameter mm)	
			Salmonella enterica subsp. *enterica*	*Escherichia coli*
Ampicillin	AMP	10	≤17	≤13
Ceftazidime	CEFTZ	10	≤19	≤21
Ceftiofur	CEFT	30	≤17	≤17
Chloramphenicol	CHL	30	≤18	≤16
Enrofloxacin	ENR	5	≤16	≤16
Gentamicin	GENT	10	≤15	≤15
Meropenem	MER	10	≤26	≤24
Nalidixic Acid	NA	30	≤13	≤13
Streptomycin	ST	10	≤11	≤11
Sulfisoxazole	SULFAX	250	n.p.	≤12
Sulphonamides	SULFA	250	≤12	n.p.
Tetracycline	TETRA	30	≤16	≤11
Trimethoprim-sulfamethoxazole	T-SULFA	25	≤10	≤15

n.p. not performed; EUCAST breakpoints were chosen for all antimicrobials agents except for ceftiofur, enrofloxacin, nalidixc acid, streptpmycin for both *Salmonella enterica* subsp. *enterica* and *Escherichia coli*, as well as for sulfonamides for *Salmonella enterica* subsp. *enterica* and sulfisoxazole and tetracycline for *Escherichia coli*, for which CLSI breakpoints were used.

Table 2. Results of susceptibility testing to antimicrobial agents of *Salmonella* spp. isolates collected in different areas of mollusk production in the province of Ferrara.

Area	No. of isolates	No. of resistant isolates (%)													
		PEN	CEP III		CARB	QUIN		AMIN		PHEN	TETRA	SULFA	POT SULFA	R at least one AA	MDR
		AMP	CEFTZ	CEFT	MER	NA	ENR	GENT	ST	CHL	TETRA	SULFA	T-SULFA		
B-in	61	37(60.7)	-	7(11.5)	13(21.3)	2(3.8)	1(1.6)	2(1.9)	41(67.2)	11(18)	28(45.9)	20(32.8)	2(3.3)	53(86.9)	31(50.8)
Long-line, Lupini, B-Out	11	8(72.7)	-	-	1(9)	-	-	-	9(81.8)	5(45.4)	8(72.7)	4(36.4)	1(9)	9(81.8)	8(72.7)
Sacca	30	8(26.7)	-	2(6.7)	6(20)	-	-	-	10(33.3)	-	10(33.3)	4(13.3)	-	21(70)	6(20)
Total	102	53(52)	-	9(8.8)	20(19.6)	2(1.9)	1(0.9)	2(1.9)	60(58.8)	16(15.7)	46(45.1)	28(27.4)	3(2.9)	83(81.4)	45(44.1)

PEN: Penicillins; CEP III; cephalosporins III; CARB: Carbapenems; QUIN: Quinolones; AMIN: Aminoglycosides; PHEN: Phenicols; TETRA: Tetracyclines; SULFA: Sulphonamides; POT SULFA: Potentiated sulphonamides; R: resistant; AA: antimicrobial agent; MDR: multidrug resistant strains; - : not detected. The results are expressed as number of resistant isolates and their percentage value (%).

Table 3. Results of susceptibility testing to antimicrobial agents of *Salmonella* ser. Typhimurium and monophasic *S.* Typhimurium isolates

Serovar	No. of isolates	No. of resistant isolates (%)													
		PEN	CEP III		CARB	QUIN		AMIN		PHEN	TETRA	SULFA	POT SULFA	R at least one AA	MDR
		AMP	CEFTZ	CEFT	MER	NA	ENR	GENT	ST	CHL	TETRA	SULFA	T-SULFA		
*S.*Typhimurium	72	29(40.3)	-	7(9.7)	17(23.6)	1(1.4)	1(1.4)	2(2.8)	31(43.1)	15(20.8)	26(36.1)	10(13.9)	3(4.2)	54(75)	24(33.3)
monophasic *S.* Typhimurium	30	24(80)	-	2(6.7)	3(10)	1(3.33)	-	-	29(96.7)	1(3.3)	20(66.7)	18(60)	-	29(96.7)	21(70)

PEN: Penicillins; CEP III; cephalosporins III; CARB: Carbapenems; QUIN: Quinolones; AMIN: Aminoglycosides; PHEN: Phenicols; TETRA: Tetracyclines; SULFA: Sulphonamides; POT SULFA: Potentiated sulphonamides; R: resistant; AA: antimicrobial agent; MDR: multidrug resistant strains; - : not detected. The results are expressed as number of resistant isolates and their percentage value (%).

Table 4. Temporal trends of resistance to antimicrobial agents of *Salmonella* spp. isolates collected in different years of mollusk production in the province of Ferrara.

Period	No. of isolates	No. of resistant isolates (%)													
		PEN	CEP III		CARB	QUIN		AMIN		PHEN	TETRA	SULFA	POT SULFA	R at least one AA	MDR
		AMP	CEFTZ	CEFT	MER	NA	ENR	GENT	ST	CHL	TETRA	SULFA	T-SULFA		
2001-2007	17	4(23.5)	-	-	10(58.8)	-	-	-	91(52.9)	4(23.5)	4(23.5)	4(23.5)	2(3.3)	16(94.1)	4(23.5)
2008-2012	28	18(64.2)	-	2(7.1)	7(25)	-	1(1.6)	-	22(78.6)	6(28.6)	13(46.4)	8(28.6)	-	26(92.9)	16(57.1)
2013-2017	57	31(54.4)	-	7(12.8)	3(5.3)	2(3.8)	-	2(1.9)	29(50.9)	6(28.1)	29(50.9)	16(28.1)	1(9)	41(71.9)	25(43.9)
Total	102	53(52)	-	9(8.8)	20(19.6)	2(1.9)	1(0.9)	2(1.9)	60(58.8)	16(15.7)	46(45.1)	28(27.4)	3(2.9)	83(81.4)	45(44.1)

PEN: Penicillins; CEP III; cephalosporins III; CARB: Carbapenems; QUIN: Quinolones; AMIN: Aminoglycosides; PHEN: Phenicols; TETRA: Tetracyclines; SULFA: Sulphonamides; POT SULFA: Potentiated sulphonamides; R: resistant; AA: antimicrobial agent; MDR: multidrug resistant strains; - : not detected. The results are expressed as number of resistant isolates and their percentage value (%).

Table 5. Results of susceptibility testing to antimicrobial agents of *E. coli* isolates collected in different areas of mollusk production in the province of Ferrara.

Area	No. of isolates	No. of resistant isolates (%)													
		PEN	CEP III		CARB	QUIN		AMIN		PHEN	TETRA	SULFA	POT SULFA	R at least one AA	MDR
		AMP	CEFTZ	CEFT	MER	NA	ENR	GENT	ST	CHL	TETRA	SULFAX	T-SULFA		
B-in	27	17(63)	-	9(33.3)	4(14.8)	2(14.3)	2(7.4)	12(44.4)	18(66.7)	-	2(7.4)	13(48.1)	-	23(85.1%)	15(55.6%)
Long-line, Lupini, B-Out	19	8(42.1)	-	2(10.5)	1(5.2)	3(15.8)	-	7(36.8)	12(63.2)	-	3(15.8)	5(26.3)	-	14(73.7%)	6 (31.6%)
Sacca	33	19(57.6)	1(3)	7(21.2)	4(12.1)	3(9.1)	2(6.1)	9(27.3)	11(33.3)	1(3)	2(6.1)	6(18.2)	2(6.1)	22(66.7%)	9(27.3%)
Total	79	44(55.7)	1(1.3)	18(22.8)	9(11.4)	8(10.1)	4(5.06)	28(35.4)	41(51.9)	1(1.3)	7(8.9)	24(30.4)	2(2.5)	59(74.7)	30(38)

PEN: Penicillins; CEP III; cephalosporins III; CARB: Carbapenems; QUIN: Quinolones; AMIN: Aminoglycosides; PHEN: Phenicols; TETRA: Tetracyclines; SULFA: Sulphonamides; POT SULFA: Potentiated sulphonamides; R: resistant; AA: antimicrobial agent; MDR: multidrug resistant strains; - : not detected. The results are expressed as number of resistant isolates and their percentage value (%).

Table 6. Results of susceptibility testing to antimicrobial agents in relation to the inclusion of *E. coli* isolates in more and less frequent phylogenetic groups and the sampling area in the mollusk production area in the province of Ferrara; findings were expressed as number of resistant isolates and their percentage value (%).

	Phylogenetic group							
	A		**B1**		**C**		**B2, D, E and unknown**	
Area	R at least one AA	MDR	R at least one AA	MDR	R at least one AA	MDR	R at least one AA	MDR
B-in	7(100)	5(71.4)	9(81.8)	7(63.6)	4(100)	2(50)	3(60)	1(20)
Long-line, Lupini, B-Out	1(100)	-	6(75)	2(25)	2(66.7)	2(66.7)	5(71.4)	2(28.6)
Sacca	3(33)	-	13(61.9)	3(14.3)	4(100)	4(100)	4(80)	2(40)
Total	11(81.8)	5(45.4)	28(70)	12(30)	10(90.91)	8(72.7)	12(70.6)	5(29.4)

R: resistant; AA: antimicrobial agent; MDR: multidrug resistant strains

Table 7. Spatial trends of resistance to at least one antimicrobial agent and multi-resistant strains expressed as number of resistant isolates and their percentage value (%).

	***Salmonella* ser. Typhimurium**		**monophasic *S.* Typhimurium serovar**		***Salmonella* spp.**		***Escherichia coli***	
Area	R to at least one AA	MDR	R to at least one AA	MDR	R to at least one AA	MDR	R to at least one AA	MDR
B-In	33(80.5%)	16(39%)*	20(100%)	15(75%)	53(86.9%)	31(50.8%)*	23(85.1%)	15(55.6%)
Long-line, Lupini, B-Out	7(77.8%)	6(66.7%)*	2(100%)	2(100%)	9(81.8%)	8(72.7%)*	14 (73.7%)	6 (31.6%)
Sacca	14(63.6%)	2(9.1%)*	7(87.5%)	4(50%)	21(70%)	6(20%)*	22(66.7%)	9(27.3%)

R: resistant; AA: antimicrobial agent; MDR: multidrug resistant strains. Number of isolates in columns bearing * are significantly different ($p < 0.05$).