Evaluation of virulence factors profiles and antimicrobials resistance of *Escherichia coli* isolated from bulk tank milk and raw milk filters

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ABSTRACT

Data on the presence of pathogenic *Escherichia coli* in bulk tank milk (BTM) and raw milk filters (RMF) are not available in Italy and there are few studies worldwide. Therefore, a study under field condition was conducted to assess the presence of *E.coli* pathogenic and commensal (CoEC) strains in BTM and RMF samples and their associated AMR pattern.

One hundred forty-nine *E.coli* isolates were characterized. Among all the isolates, 53 (35.6%) were classified as pathogenic while the other ones were classified as CoEC. Among the pathogenic ones, 23 (54.7%) were classified as enterotoxigenic *E.coli* (ETEC), 6 (11.3%) as enteroinvasive *E.coli* (EIEC), 2 (3.8%) as enteroaggregative *E.coli* (EAEC), 12 (22.6%) harboured virulence factors (VF) common to ETEC+EIEC, and 2 (3.8%) common to ETEC+EAEC. To our knowledge, it is the first time that ETEC isolates harboring VF associated with EAEC or EIEC are observed in raw milk. These data support the presence of transmission of VFs genes among isolates.

None of the isolates showed resistance to three or more antimicrobials. The CoEC role as a vector of AMR was confirmed by the presence of 18% ampicillin- and cephalexin-resistant isolates. The presence of AMR in CoEC supports the role of these bacteria as source of resistance genes.

Monitoring raw milk by either BTM or RMF analysis, and the relatively cheap procedure applied to identify *E.coli* pathotypes can be useful to identify hazards related to the spread of enteric

diseases and antimicrobial resistance.

Keywords: *Escherichia coli*, antimicrobials resistance, bulk tank milk, raw milk filters, pathogenic *E.coli*.

INTRODUCTION

Escherichia coli is a commensal organism that colonizes the gastrointestinal tract of humans and warm-blooded animals (Drasar and Hill, 1974). However, a small proportion of *E.coli* harbors different virulence factors (VF) causing infections in humans and animals. The pathogenic strains can be identified using VF which are distinctive for each pathogenic mechanism (Nataro and Kaper, 1998; Russo and Johnson, 2000). *E.coli* pathogenic strains can be classified in two main groups based on the diseases caused: gastrointestinal and extraintestinal (ExPEC) infections (Ishii and Sadowsky, 2008). The strains causing gastrointestinal diseases can be further grouped in several pathotypes, among them the most frequently isolated in bovine are: enteropathogenic *E.coli* (EPEC), enterotoxigenic *E.coli* (ETEC), enteroaggregative *E.coli* (EAEC) and enteroinvasive *E.coli* (EIEC). The ExPEC strains are epidemiologically and phylogenetically distinct from the intestinal pathogenic strains. They possess specific genes as fimbria and multiple virulent-trait categories which allow *E.coli* to cause diseases outside of the gut reservoir (Russo and Johnson, 2000).

The One Health approach emphasize the importance of the presence of AMR in foodborne pathogens and in clinical pathogens as a major concern both in public health and in food animal production systems (Cipolla et al., 2015). Among the foodborne bacteria, *E.coli* infections are becoming of great importance, and it has been suggested that *E.coli* commensal (CoEC) strains can harbor resistance genes that may be transferred to pathogenic or opportunistic bacteria contaminating raw food (Sørum and Sunde, 2001). Indeed, the spreading of AMR depends from mobile genetic elements, such as plasmids and integrons present in pathogenic and nonpathogenic isolates of *E.coli* (Bennett, 2008; Santos et al., 2010).

Dairy cattle, milk and dairy products have been implicated in outbreaks of foodborne illness, although with a lower frequency compared to outbreaks related to meat and vegetables (Karns et al., 2007). Furthermore, the common use of waste milk and colostrum contaminated with pathogenic or AMR *E.coli* for calf-feeding could transmit the infection to calves and increase the risk of AMR among the animal population. The presence of AMR strains in milk may contribute the propagation

of AMR bacteria from animals to humans (WHO, 2002). Indeed, recent studies showed the presence of extended-spectrum beta-lactamase-producing bacteria (ESBL) suggesting that raw milk could be a potential source of exposure for the consumer (Odenthal et al., 2016; Skockova et al., 2015).

A system to monitor the presence of pathogenic and AMR bacteria can be based on bulk tank milk -BTM (Berge et al., 2007) and on raw milk filter (RMF) analysis. The RMF is a component of milking machines, which blocks the entry of debris, large particles of organic material and foreign objects in BTM. The material of the filter is similar to a tissue with a variable weight of 60 to 80 g/m^2 and the analysis of the filter showed to be useful in identifying the presence of foodborne pathogens (Albonico et al., 2017; Murphy et al., 2005).

Data on the presence of pathogenic *E.coli* in BTM and RMF are not available in Italy and there are very few studies worldwide (Lambertini et al., 2015; Sonnier et al., 2018). The presence of AMR bacteria in milk was mainly focused on the use of waste milk from antibiotic-treated cows to feed calves (Aust et al., 2013; Brunton et al., 2012), but there are very few studies addressing the presence of AMR in BTM (Berge et al., 2007), and to our knowledge, none in RMF.

In order to gain epidemiological data on these aspects, a study was conducted to assess the presence of *E.coli* pathogenic strains in raw milk in BTM and RMF samples and the associated antimicrobial resistance pattern both in pathogenic and CoEC isolates.

MATERIALS AND METHODS

Samples collection

Bulk tank milk samples and RMF samples were collected in 67 dairy farms located in two Italian regions: Lombardy and Trentino-Alto Adige during a period of 6 months (September-February). Herds were selected at random, within the ones delivering samples to the respective regional diagnostic lab of Dairy Farmer Association (ARA). Each farm supplied a single BTM and RMF.

The BTM samples were collected at the end of the milking, while RMF were obtained just before the cleaning and disinfection procedures. All the samples were stored at 4 °C and delivered under refrigeration to the Department of Veterinary Medicine of the University of Milan within 8 h after collection. Once arrived in the laboratory, the samples were frozen at −20 °C until processed (Albonico et al., 2017).

Microbiological isolation

The samples were treated differently depending on their BTM or RMF origin. The BTM samples were defrosted at room temperature and serially diluted using peptone water. The RMF were chopped using sterile scissors, introduced in plastic bags with 50 mL of sterile peptone water and processed in a Stomacher for 4 minutes. The obtained preparation was serially diluted using peptone water. After dilutions, all samples were spread on plates for the enumeration of *E.coli* (EC 3M™ Petrifilm ™) and the plates were incubated overnight at 37 °C. Then, a maximum of 10 suspected *E.coli* colonies were picked up randomly from each plate and plated on nutrient agar, then incubated at 37°C for 18h. All the suspected colonies were identified by VITEK® 2 system (bioMérieux, Marcy l'Étoile, France), using the VITEK® 2 GN ID card for identification of Gram-negative bacilli, and the results of the analyses were recorded for additional phenotypic characterization. Each single colony confirmed to be *E.coli* was transferred in 100 µl of molecular grade water using a loop with a needle end.

DNA extraction

The bacterial DNA was extracted from the isolates using alternative cycles of hot and cold break, consisting in 15 minutes at 95 °C and 15 minutes at -80°C. This method allowed the cells lysis and DNA extrusion from bacterial cells.

Virulence factors detection by conventional PCR

The isolated colonies were analyzed using conventional PCR to identify VF genes encoding for specific pathogenic profiles. The procedure described by Franz *et al.*, (2015) was followed for the detection of EAEC, EIEC, ExPEC and for the heat label enterotoxin (*elt*), while for the detection of heat stable enterotoxin (*est*) the procedure described by Muller *et al.* 2007 was followed. The selected VF genes were: aggregative virulence regulator (*aggR*) for EAEC; invasion plasmid antigen (*ipaH*) for EIEC; *eltB, estIa* and *estIb* for ETEC. An *E.coli* isolate was defined as ExPEC if having three or more of the following virulence genes (Jang et al., 2013): F1C fimbria (*focG*), group 2 polysaccharide capsule (*kpsMII*), P fimbria (*papA*), d S fimbria (*sfaS*), afimbrial adhesion (*afa*), cytolytic protein toxin (*hlyD*) and iron acquisistion system (*iutA*) for ExPEC. The primer sequence, concentration and annealing temperature are reported in **Table S1**. The PCR reactions were carried out in a total volume of 20 µl containing 2 µl of DNA, 1X Taq buffer (containing 1.5 mM MgCl2), 0.2 mM dNTPs, 0.2-0.4 µM of each primer, and 1.25 U TaqPromega; H_20 was added to reach a total volume of 20 µl. The PCR reactions were performed on T100™ Thermal Cycler (Bio-Rad). The thermo-cycling condition was as follows: denaturation step at 95° C for 10 min; 35 amplification cycles at 95°C for 30 s, annealing T °C depending on each primer pair for 30 s and 72°C for 30s; and the final elongation step at 72°C for 10 min. The amplification products were run on 2% agarose gels and visualized under an UV-transilluminator.

Reference *E.coli* isolates were kindly provided by the European Union Reference Laboratory VTEC (Istituto Superiore di Sanità – Rome, Italy) and Istituto Zooprofilattico Sperimentale delle Venezie.

Antimicrobial resistance

The AMR was evaluated using the VITEK® 2 system using AST-GN65 test cards (bioMérieux, Marcy l'Étoile, France), according to the manufacturer's instructions. Susceptibility cards were inoculated and the results were interpreted according to the most recent Clinical and Laboratory Standards (CLSI, 2017). The *E.coli* isolates were tested for 18 antimicrobials: Amikacin, Amoxicillin/Clavulanic acid, Ampicillin, Cefalexin, Cefovecin, Cefpodoxime, Ceftiofur, Chloramphenicol, Enrofloxacin, Gentamicin, Imipenem, Marbofloxicin, Nitrofurantoin, Piperacillin, Polymyxin B, Tetracycline, Tobramycin, Trimethoprim-Sulfamethoxazole. Moreover, the card included the test for the presence of Extended Spectrum Beta-Lactamase. All the MIC results were recorded for additional statistical analyses. In **Table S2**, the abbreviations of antimicrobials tested and the concentrations tested are reported.

Statistical analysis

All the results of phenotypic characteristics obtained by Vitek 2 GN card as well as the MIC obtained by AST-GN65 card were collected in a database and successively the database was combined with the results of PCR analyses.

Data were analyzed by parametric and non-parametric procedures (Kruskal Wallis test) on SPSS 25 software (IBM corp., Armonk NY, USA), respectively for descriptive statistics and assessment of differences in MIC distribution. To describe the relationship among phenotypic characteristics and MIC, cluster analysis was performed on Xlstat 2018.3 software (Addinsoft, Paris, F), applying Ward’s agglomeration method with chi-square metrics.

RESULTS

Sample description and frequency of pathogenic E.coli in BTM and RMF

Bulk tank milk and RMF have been collected from 67 herds (47 from Lombardy and 20 from Trentino Alto Adige). Overall, 149 *E.coli* were identified: 64 (43%) collected from BTM and 85 (57%) from RMF. Among BTM, *E.coli* was isolated in 22 out of 67 samples (22 from Lombardy herds and none from Trentino herds). Among RMF, *E.coli* was isolated from 19 out of 67 samples (17 from Lombardy herds and 2 from Trentino herds). The frequency of isolates among herds was in the range 1-6 in both BTM and RMF. Fifty-three isolates (35%) were classified as pathogenic, of which 29 were detected in RMF and 24 were detected in BTM. More in details, 31 isolates were characterized as ETEC, 6 as EIEC, 2 as EAEC (**Figure 1**). In 14 isolates a combination of VF were observed, indeed ETEC VF were also identified together with EIEC VF in 12 isolates and with EAEC VF in 2 isolates. The ExPEC VFs genes were detected only in 3 isolates that were positive for only 2 target genes, therefore they were not defined as ExPEC (Jang et al., 2013). Pathogenic *E.coli* were recovered from 11 BTM, all from Lombardy herds, and from 18 RMF (17 from Lombardy herds and 1 from Trentino herds).

E.coli MCI values

The *E.coli* isolates were tested for MIC using VITEK® 2 system (**Table 1**). The recorded MICs were generally low for all the antimicrobials tested with few important exceptions. More than 50% of EIEC showed the highest MIC values (≥32 µg/ml) for AMP, as well as 25% of ETEC/EIEC, 20% of ETEC and 50 % of EAEC isolates. Among the commensal *E.coli* isolates (CoEC), 18% of them showed MIC ≥32 µg/ml for AMP.

Overall, a single isolated classified as CoEC showed the highest MIC value for CFX (≥32 µg/ml), while 50% of EAEC had a MIC of 16 µg/ml. The same MIC value was observed for 21% of CoEC, 29% of EIEC, 10% of ETEC and 25% of ETEC/EIEC.

The highest MIC values for PIP and TET, respectively ≥32 µg/ml and 16 µg/ml, were

recorded in 57% of EIEC. The frequency of isolates in the highest MIC class for these antimicrobials was in the range 13-25% for all the other isolates.

All the isolates classified as EAEC, ETEC/EAEC had the highest MIC for POL (≥5 μg/ml), while about 73% of CoEC and ETEC were in the same MIC class.

Finally, MIC for CAF was relatively low for most isolates, but 43% of EIEC and 13% of ETEC showed the highest MIC values for this substance (≥64 μg/ml). Among all the isolates only 3% of them, all in the CoEC class, showed to be positive for ESBL.

To assess the presence of statistically significant differences in the distribution of MIC observed for the different pathotypes of *E.coli*, MICs were compared by Kruskal Wallis test. The results showed significant differences only for CAF (**Table 2**). The MIC in EIEC isolates had a median of 8 μg/ml and a mean of 30.86 μg/ml, which were two times higher than the values observed for all the other *E.coli* groups.

Phenotyping screening

Two thirds of the isolates were classified as CoEC, and this result suggested to investigate the association between phenotypic characterization of isolates and antimicrobial resistance. The isolates were evaluated by cluster analysis using the biochemical results of VITEK® 2 system (positive / negative) as dichotomous variable. The results of the biochemical test performed on the isolates were reported in **Table S3ab**. The analysis allowed the identification of three clusters (**Figure 2**) which have a dissimilarity > 20% (Piccinini et al., 2010).

The statistical analysis (χ^2 test) showed as the difference among clusters were related to 13 assays (ProA, SAC, ILATk, 0129R, Ado, ODC, LDC, SUCT, TyrA, ELLM, dCEL, AGAL, PHOS) (**Table S4**). Cluster A is characterized by a significant lower frequency of positive reaction to ProA, Ado, SUCT, TyrA). Cluster B is characterized by significant lower frequency for SAL and significant higher frequency for ELLM. Finally, cluster C shows significant higher frequency for SAL, ODC, LDL, TyrA and significant lower frequency for ADO.

The association between clusters and AMR were evaluated by comparing the MIC distribution in the three clusters (**Table 3**). The three clusters showed statistically significant differences in the distribution of MIC values for CAF, GEN, PIP and POL. Cluster A had the lowest mean MIC values for CAF, GEN, PIP, while cluster B had the higher MIC values for CAF and GEN, and cluster C had the highest MIC values for PIP and POL.

DISCUSSION

The importance of *E.coli* as an enteropathogenic bacteria is well known, moreover there are increasing concerns on its role for the spread of AMR both among human and animal populations. *E.coli* is usually present in raw milk as a contaminant at low concentrations, and it could be a source of both enteric diseases (Kolenda et al. 2015) and antimicrobial resistance (Sørum and Sunde, 2001). Indeed, the transmission of AMR and/or foodborne pathogen could occur when unpasteurized milk harboring commensal strains are consumed as food or feed.

Monitoring the raw milk at herd level showed to be a useful way to detect the presence of enteropathogenic strains and AMR bacteria, before being used to feed calves or to produce food for human consumption such as raw milk or raw milk products (Albonico et al., 2017; Berge et al., 2007). Besides the analysis of BTM, also RMF after milking showed to be a very useful tool to monitor the presence of hazards at milk and herd level (Albonico et al., 2017; Murphy et al., 2005).

Despite some evidence on the role of calf feeding with waste milk as a source of enteric disease in calves, and AMR in dairy farms (Aust et al., 2013; de Verdier et al., 2012), data on the presence of enteropathogenic and AMR *E.coli* in BTM and RMF are scarce.

This study was designed to contribute to fill this gap by investigating the presence of pathogenic and CoEC in 149 isolates collected from BTM and RMF and their association with AMR pattern. The higher proportion of isolates from RMF, when compared to BTM confirmed the usefulness of this sampling in monitoring the presence of hazards at dairy herd level (Albonico et al., 2017; Murphy et al., 2005). Among all the isolates, 53 (35.6%) were classified as pathogenic while the other ones were classified as CoEC. Among the pathogenic ones, 23 (54.7%) were classified as enterotoxigenic *E.coli* (ETEC), 6 (11.3%) as enteroinvasive *E.coli* (EIEC), 2 (3.8%) as enteroaggregative *E.coli* (EAEC), 12 (22.6%) harboured virulence factors (VF) common to ETEC+EIEC, and 2 (3.8%) common to ETEC+EAEC. In our knowledge, it is the first time that isolates harboring both VFs linked to ETEC and VFs associated with EAEC or EIEC are observed in raw milk. These data support the presence of transmission of VFs genes among isolates (Bennett,

2008). This is also the first study reporting the presence of *aggR* gene in *E.coli* isolated from raw milk. This gene is a transcriptional activator of aggregative adherence fimbria I expression in EAEC. In all the previous investigations on the presence of this specific gene in isolates from bovine origin gave negative results (Kolenda et al., 2015). Moreover, the relative abundance of EAEC and EIEC isolates in raw milk, when compared to ETEC, supports previous investigation suggesting that a higher attention on these specific pathotypes should be spent to prevent enteric disease in both human and animal populations (Kolenda et al., 2015).

Antimicrobial resistance pattern was evaluated by calculating MIC by the means of VITEK® 2 system. None of the isolates showed to be multi-resistant. A high frequency of high mean MIC values was observed for AMP on EIEC isolates, and with a lower frequency in all the other isolates. The role of CoEC as a potential vector of AMR was confirmed by the presence of 18% of these isolates have very high MIC for AMP and CFX. The resistance to these substances was not unexpected and confirmed the outcomes of previous studies (de Verdier et al., 2012). To be noticed the high MIC values observed for POL in all isolates, suggesting a risk also for colistin resistance, being closely related.

EIEC isolates showed also a large frequency of high MIC levels for PIP and TET, when compared to the other isolates, confirming values reported in previous studies (de Verdier et al., 2012). Taken together, the relative high frequency of EIEC, and their high MIC levels for AMP, PIP and TET, antimicrobials that are not commonly used or, in the case of PIP, that are not available in Italian dairy herds, suggest that this pathogens may have an environmental source (Santos et al., 2010). The absence of a direct association with disease outbreaks in the dairy herds considered, suggests that environmental sources may contaminate raw milk, thus representing an import factor in the spread of this pathogens to other populations.

We also observed an unusual high frequency of high MIC values for CAF in EIEC isolates, and, to a lesser degree, in ETEC isolates. The resistance to CAF in *E.coli* isolates was unexpected because the use of this drug was banned in food animals in ‘80s in Europe and in USA (Gilmore,

1986). However, this outcome was also observed in a previous study (Santos et al., 2010) and it was attributed to treatment with florfenicol, which confers cross-resistance to CAF (White et al., 2000). The presence of 65% of isolates classified in CoEC class, and the presence of high levels of MIC for several antimicrobials among these isolates confirm the potential risk represented by these bacteria in raw milk for the spread of antimicrobial resistance. Cluster analysis applied to the biochemical characteristics of the *E.coli* isolates enabled to identify potential markers associated to three separate clusters. Furthermore, these clusters are associated to a different AMR patterns. This suggests that biochemical markers can be used for a quick and cheap identification of potential AMR *E.coli*, when other methods are not available.

CONCLUSIONS

This is the first published report regarding the presence of pathogenic *E.coli* strains and the AMR pattern in BTM and RMF from Italian dairy herds, and one of the few on this topic, worldwide. The results confirm the usefulness of RMF analysis to identify the presence of zoonotic food-borne and AMR bacteria in dairy herds.

The study supports previous studies reporting an increasing frequency of EIEC and EAEC. These pathotypes, very likely of environmental origin, may find in raw milk an efficient vehicle for spreading in animal and human populations.

The observed MIC values suggest that AMR is relatively low in the studied population, but the presence of AMR in CoEC supports the role of commensal bacteria as a source of transmissible resistance genes among bacterial population and the risk of the transmission to feed and food.

E.coli were recovered with a higher frequency from BTM, while pathogenic *E.coli* were more frequently recovered from RMF. Therefore, BTM or RMF samples may be selected based the aim of the investigation. As a conclusion, monitoring raw milk either by BTM or RMF analysis and the characterization of *E.coli* by genetic or phenotypic methods can be an efficient, and relatively cheap procedure to identify hazards related both to the spread of enteric diseases and of

antimicrobial resistance.

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