

27 diseases and antimicrobial resistance.

28

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

30 *E.coli*.

31 **INTRODUCTION**

32 *Escherichia coli* is a commensal organism that colonizes the gastrointestinal tract of humans
33 and warm-blooded animals (Drasar and Hill, 1974). However, a small proportion of *E.coli* harbors
34 different virulence factors (VF) causing infections in humans and animals. The pathogenic strains
35 can be identified using VF which are distinctive for each pathogenic mechanism (Nataro and Kaper,
36 1998; Russo and Johnson, 2000). *E.coli* pathogenic strains can be classified in two main groups
37 based on the diseases caused: gastrointestinal and extraintestinal (ExPEC) infections (Ishii and
38 Sadowsky, 2008). The strains causing gastrointestinal diseases can be further grouped in several
39 pathotypes, among them the most frequently isolated in bovine are: enteropathogenic *E.coli*
40 (EPEC), enterotoxigenic *E.coli* (ETEC), enteroaggregative *E.coli* (EAEC) and enteroinvasive *E.coli*
41 (EIEC). The ExPEC strains are epidemiologically and phylogenetically distinct from the intestinal
42 pathogenic strains. They possess specific genes as fimbria and multiple virulent-trait categories
43 which allow *E.coli* to cause diseases outside of the gut reservoir (Russo and Johnson, 2000).

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

52 Dairy cattle, milk and dairy products have been implicated in outbreaks of foodborne illness,
53 although with a lower frequency compared to outbreaks related to meat and vegetables (Karns et al.,
54 2007). Furthermore, the common use of waste milk and colostrum contaminated with pathogenic or
55 AMR *E.coli* for calf-feeding could transmit the infection to calves and increase the risk of AMR
56 among the animal population. The presence of AMR strains in milk may contribute the propagation

57 of AMR bacteria from animals to humans (WHO, 2002). Indeed, recent studies showed the
58 presence of extended-spectrum beta-lactamase-producing bacteria (ESBL) suggesting that raw milk
59 could be a potential source of exposure for the consumer (Odenthal et al., 2016; Skockova et al.,
60 2015).

61 A system to monitor the presence of pathogenic and AMR bacteria can be based on bulk
62 tank milk -BTM (Berge et al., 2007) and on raw milk filter (RMF) analysis. The RMF is a
63 component of milking machines, which blocks the entry of debris, large particles of organic
64 material and foreign objects in BTM. The material of the filter is similar to a tissue with a variable
65 weight of 60 to 80 g/m² and the analysis of the filter showed to be useful in identifying the presence
66 of foodborne pathogens (Albonico et al., 2017; Murphy et al., 2005).

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

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

75 **MATERIALS AND METHODS**

76 *Samples collection*

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

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

87 *Microbiological isolation*

88 The samples were treated differently depending on their BTM or RMF origin. The BTM
89 samples were defrosted at room temperature and serially diluted using peptone water. The RMF
90 were chopped using sterile scissors, introduced in plastic bags with 50 mL of sterile peptone water
91 and processed in a Stomacher for 4 minutes. The obtained preparation was serially diluted using
92 peptone water. After dilutions, all samples were spread on plates for the enumeration of *E.coli* (EC
93 3M™ Petrifilm™) and the plates were incubated overnight at 37 °C. Then, a maximum of 10
94 suspected *E.coli* colonies were picked up randomly from each plate and plated on nutrient agar,
95 then incubated at 37°C for 18h. All the suspected colonies were identified by VITEK® 2 system
96 (bioMérieux, Marcy l'Étoile, France), using the VITEK® 2 GN ID card for identification of Gram-
97 negative bacilli, and the results of the analyses were recorded for additional phenotypic
98 characterization. Each single colony confirmed to be *E.coli* was transferred in 100 µl of molecular
99 grade water using a loop with a needle end.

100

101 *DNA extraction*

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

105

106 *Virulence factors detection by conventional PCR*

107 The isolated colonies were analyzed using conventional PCR to identify VF genes encoding for
108 specific pathogenic profiles. The procedure described by Franz *et al.*, (2015) was followed for the
109 detection of EAEC, EIEC, ExPEC and for the heat labile enterotoxin (*elt*), while for the detection of
110 heat stable enterotoxin (*est*) the procedure described by Muller *et al.* 2007 was followed. The
111 selected VF genes were: aggregative virulence regulator (*aggR*) for EAEC; invasion plasmid
112 antigen (*ipaH*) for EIEC; *eltB*, *estIa* and *estIb* for ETEC. An *E.coli* isolate was defined as ExPEC if
113 having three or more of the following virulence genes (Jang *et al.*, 2013): F1C fimbria (*focG*), group
114 2 polysaccharide capsule (*kpsMII*), P fimbria (*papA*), d S fimbria (*sfaS*), afimbrial adhesion (*afa*),
115 cytolytic protein toxin (*hlyD*) and iron acquisition system (*iutA*) for ExPEC. The primer sequence,
116 concentration and annealing temperature are reported in **Table S1**. The PCR reactions were carried
117 out in a total volume of 20 µl containing 2 µl of DNA, 1X Taq buffer (containing 1.5 mM MgCl₂),
118 0.2 mM dNTPs, 0.2-0.4 µM of each primer, and 1.25 U TaqPromega; H₂O was added to reach a
119 total volume of 20 µl. The PCR reactions were performed on T100™ Thermal Cycler (Bio-Rad).
120 The thermo-cycling condition was as follows: denaturation step at 95° C for 10 min; 35
121 amplification cycles at 95°C for 30 s, annealing T °C depending on each primer pair for 30 s and
122 72°C for 30s; and the final elongation step at 72°C for 10 min. The amplification products were run
123 on 2% agarose gels and visualized under an UV-transilluminator.

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

126

127 *Antimicrobial resistance*

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

138

139 *Statistical analysis*

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

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

148

149 **RESULTS**

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

151 Bulk tank milk and RMF have been collected from 67 herds (47 from Lombardy and 20
152 from Trentino Alto Adige). Overall, 149 *E.coli* were identified: 64 (43%) collected from BTM and
153 85 (57%) from RMF. Among BTM, *E.coli* was isolated in 22 out of 67 samples (22 from Lombardy
154 herds and none from Trentino herds). Among RMF, *E.coli* was isolated from 19 out of 67 samples
155 (17 from Lombardy herds and 2 from Trentino herds). The frequency of isolates among herds was
156 in the range 1-6 in both BTM and RMF. Fifty-three isolates (35%) were classified as pathogenic, of
157 which 29 were detected in RMF and 24 were detected in BTM. More in details, 31 isolates were
158 characterized as ETEC, 6 as EIEC, 2 as EAEC (**Figure 1**). In 14 isolates a combination of VF were
159 observed, indeed ETEC VF were also identified together with EIEC VF in 12 isolates and with
160 EAEC VF in 2 isolates. The ExPEC VFs genes were detected only in 3 isolates that were positive
161 for only 2 target genes, therefore they were not defined as ExPEC (Jang et al., 2013). Pathogenic
162 *E.coli* were recovered from 11 BTM, all from Lombardy herds, and from 18 RMF (17 from
163 Lombardy herds and 1 from Trentino herds).

164

165 *E.coli MCI values*

166 The *E.coli* isolates were tested for MIC using VITEK[®] 2 system (**Table 1**). The recorded
167 MICs were generally low for all the antimicrobials tested with few important exceptions. More than
168 50% of EIEC showed the highest MIC values (≥ 32 $\mu\text{g/ml}$) for AMP, as well as 25% of
169 ETEC/EIEC, 20% of ETEC and 50 % of EAEC isolates. Among the commensal *E.coli* isolates
170 (CoEC), 18% of them showed MIC ≥ 32 $\mu\text{g/ml}$ for AMP.

171 Overall, a single isolated classified as CoEC showed the highest MIC value for CFX (≥ 32
172 $\mu\text{g/ml}$), while 50% of EAEC had a MIC of 16 $\mu\text{g/ml}$. The same MIC value was observed for 21%
173 of CoEC, 29% of EIEC, 10% of ETEC and 25% of ETEC/EIEC.

174 The highest MIC values for PIP and TET, respectively ≥ 32 $\mu\text{g/ml}$ and 16 $\mu\text{g/ml}$, were

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

177 All the isolates classified as EAEC, ETEC/EAEC had the highest MIC for POL (≥ 5 $\mu\text{g/ml}$),
178 while about 73% of CoEC and ETEC were in the same MIC class.

179 Finally, MIC for CAF was relatively low for most isolates, but 43% of EIEC and 13% of
180 ETEC showed the highest MIC values for this substance (≥ 64 $\mu\text{g/ml}$). Among all the isolates only
181 3% of them, all in the CoEC class, showed to be positive for ESBL.

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

187

188 *Phenotyping screening*

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

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

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

206

207

208 **DISCUSSION**

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

215 Monitoring the raw milk at herd level showed to be a useful way to detect the presence of
216 enteropathogenic strains and AMR bacteria, before being used to feed calves or to produce food for
217 human consumption such as raw milk or raw milk products (Albonico et al., 2017; Berge et al.,
218 2007). Besides the analysis of BTM, also RMF after milking showed to be a very useful tool to
219 monitor the presence of hazards at milk and herd level (Albonico et al., 2017; Murphy et al., 2005).

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

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

234 2008). This is also the first study reporting the presence of *aggR* gene in *E.coli* isolated from raw
235 milk. This gene is a transcriptional activator of aggregative adherence fimbria I expression in
236 EAEC. In all the previous investigations on the presence of this specific gene in isolates from
237 bovine origin gave negative results (Kolenda et al., 2015). Moreover, the relative abundance of
238 EAEC and EIEC isolates in raw milk, when compared to ETEC, supports previous investigation
239 suggesting that a higher attention on these specific pathotypes should be spent to prevent enteric
240 disease in both human and animal populations (Kolenda et al., 2015).

241 Antimicrobial resistance pattern was evaluated by calculating MIC by the means of
242 VITEK[®] 2 system. None of the isolates showed to be multi-resistant. A high frequency of high
243 mean MIC values was observed for AMP on EIEC isolates, and with a lower frequency in all the
244 other isolates. The role of CoEC as a potential vector of AMR was confirmed by the presence of
245 18% of these isolates have very high MIC for AMP and CFX. The resistance to these substances
246 was not unexpected and confirmed the outcomes of previous studies (de Verdier et al., 2012). To be
247 noticed the high MIC values observed for POL in all isolates, suggesting a risk also for colistin
248 resistance, being closely related.

249 EIEC isolates showed also a large frequency of high MIC levels for PIP and TET, when
250 compared to the other isolates, confirming values reported in previous studies (de Verdier et al.,
251 2012). Taken together, the relative high frequency of EIEC, and their high MIC levels for AMP,
252 PIP and TET, antimicrobials that are not commonly used or, in the case of PIP, that are not
253 available in Italian dairy herds, suggest that this pathogens may have an environmental source
254 (Santos et al., 2010). The absence of a direct association with disease outbreaks in the dairy herds
255 considered, suggests that environmental sources may contaminate raw milk, thus representing an
256 import factor in the spread of this pathogens to other populations.

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

260 1986). However, this outcome was also observed in a previous study (Santos et al., 2010) and it was
261 attributed to treatment with florfenicol, which confers cross-resistance to CAF (White et al., 2000).
262 The presence of 65% of isolates classified in CoEC class, and the presence of high levels of MIC
263 for several antimicrobials among these isolates confirm the potential risk represented by these
264 bacteria in raw milk for the spread of antimicrobial resistance. Cluster analysis applied to the
265 biochemical characteristics of the *E.coli* isolates enabled to identify potential markers associated to
266 three separate clusters. Furthermore, these clusters are associated to a different AMR patterns. This
267 suggests that biochemical markers can be used for a quick and cheap identification of potential
268 AMR *E.coli*, when other methods are not available.

269

270 **CONCLUSIONS**

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

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

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

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

286 antimicrobial resistance.

287

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291 *E.coli* strains.

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