

1 Genotypic Characterization and Biofilm Formation of Shiga-toxin producing  
2 *Escherichia coli*

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8 biofilm formation; fecal isolates; goat's milk

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25 **ABSTRACT**

26 Shiga toxin producing *Escherichia coli* (STEC) are recognized as one of the most dangerous food-borne  
27 pathogens. The production of Shiga toxins together with intimin protein are among the main virulence  
28 factors. However, the ability to form biofilm can protect bacteria against environmental factors (i.e.  
29 exsiccation, UV rays' exposure, predation, *etc.*) and sanitization procedures (cleaning, rinsing, chlorination),  
30 increasing their survival on food products and in manufacturing plants. Forty-five isolates collected from  
31 food and fecal samples were genotyped by Pulsed Field Gel Electrophoresis (PFGE) analysis with *Xba*I  
32 restriction enzyme and investigated by searching for toxins (*stx1*, *stx2*) and intimin (*eae*) genes and  
33 serogroup (O157, O26, O145, O111, O103 and O104). Afterward, the ability to develop biofilm in microtiter  
34 assay and the production of adhesive curli fimbriae and cellulose in agar plates were tested. Our study  
35 demonstrated that biofilm formation has a great variability among STEC strains and can not be related to a  
36 specific pulsotype nor even to serogroup or presence of virulence genes.

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## 38 INTRODUCTION

39 *Escherichia coli* is a constituent of the natural gastrointestinal microbiota of humans and warm-blooded  
40 animals; hence, it is used as a reliable indicator of fecal contamination for food and water. Most *E. coli* are  
41 harmless commensal; however, some strains are involved in critical foodborne infections including for  
42 example, hemolytic uremic syndrome (HUS), pyelonephritis, septicemia and gastroenteritis (Kaper, Nataro  
43 and Mobley 2004). These pathogenic strains are characterized by their ability to cause illness through  
44 genetically controlled mechanisms such as toxin production, adhesion and invasion of host cells, interference  
45 with cell metabolism and tissue destruction (Croxen *et al.* 2013). Shiga-toxin producing *E. coli* (STEC) are  
46 often associated with sporadic infections and outbreaks linked to the ingestion of contaminated food and  
47 water, direct contact with animals (especially ruminants), or via person-to-person transmission (Kaper,  
48 Nataro and Mobley 2004). In 2013, 6,043 confirmed cases of STEC infections were reported in the EU, with  
49 a notification rate 5.9 % higher than in 2012. Serogroup O157 was the most commonly reported one  
50 followed by O26 (EFSA (European Food Safety Authority) and ECDC (European Centre for Disease  
51 Prevention and Control) 2015). Beyond toxin production, these strains can colonize the host intestinal  
52 epithelium contributing to the pathogenesis of the disease. The ability of colonization was also observed on  
53 biotic and abiotic surfaces often through the formation of complex communities of cells called biofilms (Ryu  
54 and Beuchat 2005; Nesse *et al.* 2014). Microbial cells in a biofilm are embedded in a layer of extracellular  
55 polysaccharides (EPS) and diverse additional constituents, such as nucleic acids, proteins, glycoproteins and  
56 lipoproteins offering to the cell an improved resistance to the environmental stresses and a protection against  
57 different substances (i.e. antibiotics, sanitizing agents) (Ito *et al.* 2009; Wang *et al.* 2013). This phenomenon  
58 worries the food industry due to the potential ineffectiveness of both washing treatment of incoming raw  
59 materials and cleaning and disinfection procedures for the equipment sanitation. Furthermore it was  
60 already demonstrated that biofilms can be an ideal environment for the dissemination of *stx* genes through  
61 bacteriophages (Solheim *et al.* 2013). The production of adhesive curli fimbriae (Olsén, Jonsson and  
62 Normark 1989) together with cellulose are two of the main colonization factors. Typically, environmental  
63 factors such as temperature below 30°C, microaerophilic conditions, low osmolarity, and nutrient limitation  
64 (nitrogen, phosphate and iron) support the expression of curli (Barnhart and Chapman 2006).  
65 The aim of this work was to characterize potentially pathogenic *E. coli* strains collected from food (goat's  
66 milk and milking filters) and fecal samples. Isolates were discriminated by PFGE patterns analysis and  
67 investigated for virulence genes coding for Shiga toxins, intimin and O-serogroup. Since biofilm formation  
68 can be a significant feature for the persistence of STEC strains outside human or animal host, we tested the  
69 ability of the isolates to produce factors involved in the adhesion mechanism in order to understand if these  
70 traits can be associated to specific genotypic profiles.

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## 72 MATERIALS & METHODS

### 73 Bacterial strains

74 Twenty-eight *E. coli* isolates were collected from 14 fecal samples gathered at Center for HUS Control,  
75 Prevention and Management -Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico (Milano, Italy)  
76 from patients with diarrheic syndrome and hemorrhagic colitis that showed to be positive to *stx* and/or *eae*  
77 virulence factors. Besides, 17 potentially pathogenic *E. coli* isolates where collected from 12 samples of  
78 milking filter and raw goat milk in Lombardy region (Table1).

### 79 Detection of virulence factors gene by PCR analysis

80 After DNA extraction (Sambrook and Russell 2001), the isolates were screened for the presence of *stx1*, *stx2*  
81 (Gannon *et al.* 1992) and *eae* genes (Gannon *et al.* 1997) and for the serogroup (Paton and Paton 1998;  
82 Monday, Beisaw and Feng 2007; Bielaszewska *et al.* 2011). PCR reactions were carried out with T-Gradient  
83 PCR thermal cycler (Biometra GmbH, Göttingen, Germany). After each PCR assay, 10 µl of the  
84 amplification product were analyzed on 1.2% agarose gels containing 0.4 µg/ml ethidium bromide,  
85 visualized with UV illumination, and photographed. Each agarose gel electrophoresis run included DNA  
86 molecular size standards (100bp XL Ladder; MBI Fermentas, St Leon-Rot, Germany).

### 87 Genotyping by PFGE analysis

88 Pulsed-field gel electrophoresis (PFGE) was performed as previously described (Picozzi *et al.* 2005). Briefly,  
89 DNA was digested with *XbaI* and separated on 1.2% agarose gel with a CHEF DR II apparatus under the  
90 following conditions: voltage gradient of 6 V/cm, increasing pulse time from 2.4 to 52.4 over a 23h period.  
91 The CDC strain G5244, was included as reference. The gels were stained with ethidium bromide and  
92 photographed. Images were analyzed with BioNumerics software (Applied Maths N.V., Sint-Martens-Latem,  
93 Belgium). The band based Dice similarity coefficient and the unweighted pairs geometric matched analysis  
94 (UPGMA) dendrogram type were used with a position tolerance setting of 0,5% for optimization and  
95 position tolerance of 1,5% for band comparison. Restriction profiles of all the isolates were normalized to the  
96 known molecular size bands of the *E. coli* G5244 standard strain. Pulsotypes were assigned based on the  
97 difference in the presence or absence of at least one band.

### 98 Detection of curli and cellulose

99 The assay for curli fimbriae expression was performed modifying the method of Kim and Kim (2004).  
100 Briefly, 40 mg L<sup>-1</sup> of Congo red dye was added to colonization factor antigen (CFA) agar consisting of 1%  
101 Casamino acids, 0.15% Yeast extract, 0.005% MgSO<sub>4</sub>, 0.0005% MnCl<sub>2</sub>, 0.002 % Coomassie Blue and 2%  
102 agar at pH 7.4. To evaluate the production of exopolysaccharides (cellulose, PNAG, chitin), 0.005% of the  
103 fluorescent whitener Calcofluor (CF) was added to the agar medium instead of Congo Red and Coomassie  
104 Blue (Tagliabue *et al.* 2010). Overnight cultures of bacterial strains grown in LB were streaked on Congo red  
105 and Calcofluor agar plates and incubated for 36-48h at 30°C and 18-24h at 37°C. After incubation, curli-

106 expressing strains (curli<sup>+</sup>) displayed red colonies and non-curli-expressing strains (curli<sup>-</sup>) showed white  
107 colonies. Polysaccharides production was instead verified by the observation of fluorescent colonies under  
108 UV light.

### 109 **Biofilm formation assays**

110 Biofilm formation in microtiter plates was verified as previously described (O'Toole and Kolter 1998; Dorel  
111 *et al.* 1999). *E. coli* strains were grown overnight in M9 salts (3.39% w/v Na<sub>2</sub>HPO<sub>4</sub>, 1.5 % KH<sub>2</sub>PO<sub>4</sub>, 0.25%  
112 NaCl and 0.5% NH<sub>4</sub>Cl) supplemented with 0.5% (w/v) glucose, 0.02% peptone, and 0.01% yeast extract  
113 (M9 Glu-sup medium) (Brombacher *et al.* 2006) and in TSB medium at 30° and 37°C in flat-bottomed  
114 polystyrene microtiter plates (200µL). The cell densities of the cultures were determined  
115 spectrophotometrically at 600 nm (OD600). Cells attached to the microtiter plates were washed gently with  
116 water and stained for 20 min with 1% crystal violet (CV) in ethanol, thoroughly washed with water and then  
117 dried. For semiquantitative determination of biofilms, CV-stained cells were resuspended in 0.2 ml 95%  
118 ethanol by vigorous pipetting. The OD600 of each sample was determined and normalized to the OD600 of  
119 the corresponding liquid cultures (adhesion units- AU).

120

## 121 **RESULTS AND DISCUSSION**

### 122 **Genotypic characterization**

123 The ability of STEC strains to cause disease in human is mainly due to the production of one or both Shiga  
124 toxins and then, to other virulence factor like *eae* gene coding for an intimin which mediates the intimate  
125 attachment to epithelial cells and invasion of host intestinal wall (Kaper, Nataro and Mobley 2004). In this  
126 study we analyzed 45 potentially pathogenic *E. coli* isolated both from food sources and human stools to  
127 compare the distribution of virulence genes and their possible link with the production of biofilm or other  
128 colonization factors. It is accepted that most of STEC infections are mainly due to the ingestion of raw or  
129 undercooked contaminated food of animal origin (EFSA (European Food Safety Authority) and ECDC  
130 (European Centre for Disease Prevention and Control) 2015). Given the growing consumption of goat milk  
131 and its derivatives in Europe and the fact that this animal has already been found to be a natural reservoir of  
132 STEC (Picozzi *et al.* 2005; Espié *et al.* 2006), we considered this product as a potential source of pathogenic  
133 strains.

134 PCR analyses (Table 2) highlighted the presence of Shiga toxin genes in 41 isolates out of 45 (91%). Of the  
135 remaining four, three (7%) showed only the presence of the *eae* gene and one (2%) showed no amplification  
136 signal for any of the tested virulence genes but was lately ascribed to serogroup O26 and therefore been  
137 considered in the subsequent investigations. Stx1 was the predominant toxin being highlighted in 38 isolates  
138 out of 41 (93%): 16 (42%) were positive only to *stx1*, only 3 (8%) have been shown to possess both toxins  
139 while none had only the *stx2*. The prevalence of *stx1* is clear even if we separately consider clinical and food

140 isolates (93% and 76% respectively). Noteworthy, it is known that both toxins are directly implicated in  
141 systemic infections and hemorrhagic colitis (Kaper, Nataro and Mobley 2004), but also that patients infected  
142 with STEC strains that produce stx2 (often in association with intimin) are more likely to be associated to the  
143 onset of HUS (Tozzi *et al.* 2003; Johnson, Thorpe and Sears 2006). Taking into consideration also intimin as  
144 virulence factor, 25 isolates out of 45 (56%) presented a positive result for the presence of *eae*: the  
145 association Stx1/*eae* was highlighted in ten isolates (22%), Stx1/Stx2/*eae* in 9 (20%), while only 3 (7%)  
146 presented both Stx2 and *eae*. Serogroup O157 is the most commonly involved in the onset of hemorrhagic  
147 diarrheas and in particular in HUS syndrome. However, outbreaks were often found to be caused by non-  
148 O157 strains including O26, O145, O111, O103 and O104, the serogroup responsible for the main EHEC  
149 outbreak in 2011 (EFSA Panel on Biological Hazards (BIOHAZ) 2013). Our data showed that the most  
150 frequently recovered serotype is O26 (33%), being identified in 7 out of 17 (41%) food isolates and in 8 out  
151 of 27 (30%) human isolates. Moreover, this was the only serogroup detected among food isolates,  
152 confirming the last EFSA Scientific report where serogroup O26 was the second most reported serogroup in  
153 both food and animal samples, with an increasing trend in the last few years (EFSA (European Food Safety  
154 Authority) and ECDC (European Centre for Disease Prevention and Control) 2015). Five out of seven  
155 isolates showed the presence of Stx1 while one O26 (F93-3) presented an amplification product only for the  
156 *eae* gene and one (L12-2) resulted to be negative for all the tested virulence factors (L12-2). As concern  
157 human isolates, apart from O26 serogroup, 7 (26%) samples were ascribed to O157 serotype, 1 (4%) to O145  
158 and 1 (4%) to O111. No isolates showed a positivity for serotype O103 nor for O104 serotype. Noteworthy,  
159 the presence of all the three virulence genes analyzed was found in 86% (6 out of 7) O157 isolates as well as  
160 for O145 serotype. Anyway, as far as we know, no literature works indicate a possible correlation between a  
161 serogroup and a particular combination of virulence genes.

162 The PFGE is still considered one of the best molecular technique to type strains especially pathogenic ones,  
163 tracking food-borne infections through specific network like PulseNet international (Swaminathan *et al.*  
164 2006). In this work the analysis was performed on all isolates, grouping 31 different PFGE profiles at a  
165 similarity level of 55% (Figure 1). As concern food samples, isolates F10-4 and F11-4, sampled from two  
166 different milking filter in two distinct farms, showed a similarity of 100% and the same virulence profile  
167 (Stx1 toxin). Furthermore, they are related also to L12-2 (67%) that, on the other side, presented no virulence  
168 genes but shares the same O26 serogroup with the previous isolates. A 100% homology was also observed  
169 between F80-1 and F80-4 isolates and between F80-2 and F80-3 and this can be explained by the fact that  
170 the isolates were collected from the same milking filter and therefore are likely to be the same strain. This  
171 hypothesis is further sustained by the presence of the same virulence genes. A similar consideration can be  
172 done also for F95-2 and F95-3 PFGE profiles. Moreover, isolates F95-2 and F95-3 are showing a homology  
173 of 78% with F93-3 and a 62% similarity with profiles obtained from fecal samples of a unique patient  
174 (239PCH-A and 239R-A). All these strains were ascribed to O26 serogroup, but human isolates had the three  
175 virulence genes, while F95-2 and F95-3 lack of Stx2 and F93-3 has only the intimin gene. A similarity of  
176 63% was then found for F1-1 and L36-2 profiles that showed also a 55% homology with F90-1. The PCR

177 analysis pointed out that all the isolates have both toxins genes but only F1-1 showed an amplification for the  
178 O26 serogroup gene. Most of the clinical isolates constitutes a single pulsotype. An exception is given by  
179 isolates 214R-MCH-B and 214CH that are clustering together at 66% similarity level with 227MCH sharing  
180 the same serogroup (O157) and the same virulence factor profile. Besides, 229B-ACH and 229PRal-AS  
181 present a homology of 85% and the presence of the Stx1 toxin gene but only the latest isolate was ascribable  
182 to serogroup O26.

183 Biofilm formation on solid surfaces is a very common phenomenon among bacteria and has important  
184 economic and health consequences (Ryu and Beuchat 2005; Nesse *et al.* 2014). The ability of producing  
185 biofilm was evaluated in the isolates that were distinguishable after PCR and PFGE analysis, at different  
186 temperatures (30° and 37°C) and nutrients availability (minimal and rich medium). Values obtained from  
187 adhesion tests (AU) (Table 3) were compared to the ones obtained from standard strains EB1.3 and PHL628  
188 (Vidal *et al.* 1998; Prigent-Combaret *et al.* 2001). Accordingly, AU values above 1 at 30°C and above 2.5 at  
189 37°C are related to an evident staining of the well and therefore to the production of a biofilm. Considering  
190 these thresholds, we found that at 30°C, 9 out of 36 isolates (25%) produced biofilm in M9 medium and 26  
191 (72%) in TSB medium. On the other side, at 37°C only 6 (17%) isolates in M9 and 11(31%) in TSB  
192 evidenced biofilm production. Most of the isolates that showed the ability to produce biofilm at 30°C were  
193 not able to reproduce the same phenomenon at 37°C. Furthermore this seems not to be directly related to  
194 curli fimbriae expression as already demonstrated by other authors (Gualdi *et al.* 2008; Uhlich *et al.* 2014).  
195 Overall, 30 isolates (83%) showed an appreciable biofilm production at least at one incubation temperature,  
196 whereas only 2 (6%) (242CH and F90-3) gave valuable AU values for both temperatures and both media.  
197 Only in seven cases in TSB and in 2 in M9, the formation of biofilm was observed independently of  
198 temperature. Among these 30 strains, 12 (33%) were attributable to serogroup O26. However, no particular  
199 correlation with the presence of virulence factors nor with PFGE patterns was observed.

200 As concern the assays on CR and CF media, 19 (53%) isolates showed a significant expression of phenotype  
201 at least at one temperature and /or medium, while only two isolates (6%) presented undetectable levels of  
202 curli and polysaccharides production. A remarkable contemporary expression of curli fimbriae and  
203 polysaccharides at 30°C and 37°C was noticed both in stool (44%) and in food (55%) isolates, but this was  
204 not always linked to biofilm formation. CR affinity seems to be related to the temperature: among the 27  
205 isolates where the expression of the phenotype is detectable, all (100%) produce curli at 30°C and 17 (63%)  
206 also at 37°C. This result was already observed in a recent work (Uhlich *et al.* 2014), although in our study we  
207 did not find strong differences between O157 and non-O157 strains. Noteworthy, the greater phenotype  
208 expression was revealed by the analysis of fluorescence on CF plates where 33 strains out of 36 (92%)  
209 presented a production of polysaccharides at 37°C, and 22 (61%) also at 30°C. Also adhesive curli fimbriae  
210 and exopolysaccharide production traits do not present specific correlation with pulsotypes.

211 Our findings seem to prove that there are no apparent correlations among toxin and intimin genes expression,  
212 serotype, pulsotype and curli and cellulose or other polysaccharide production. STEC strains had already

213 been demonstrated to have a great inconstancy in biofilm formation (Chen *et al.* 2013), and this property is  
214 highly dependent on strain rather than serotype (Wang *et al.* 2013). However, this topic is worth further  
215 consideration and deepening because of the high phenotypic variability and the potential pathogenicity of  
216 these microorganisms.

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Table 1. List of the *E.coli* isolates used in this study collected from fecal and food samples in Lombardy region.

Isolate code	Source of isolation	Eae/stx genes	Isolate	Date
375	Human stool	STX1-2-EAE	214CH	2011
	Human stool		214RACH	
	Human stool		214R-MCH-B	
646	Human stool	STX1-2-EAE	224SMA-GS	2012
	Human stool		224SMA-PS	
684	Human stool	STX1-EAE	225R-A	2012
725	Human stool	STX1-2-EAE	226B-B	2012
756	Human stool	STX1-2-EAE	227MCH	2012
	Human stool		227Rosa	
773	Human stool	STX2-EAE	228GS	2012
	Human stool		228P-CH	
783	Human stool	STX1-2-EAE	229B-ACH	2012
	Human stool		229M-AS	
	Human stool		229PRal-ACH	
	Human stool		229PRal-AS	
	Human stool		229R-ACH	
	Human stool		229Rosa-A	
819	Human stool	STX2-EAE	231PCH-A	2012
821	Human stool	STX1-2-EAE	232AS-B-LUC	2012
833	Human stool	STX1-2-EAE	233P-CH-A	2012
901	Human stool	STX2-EAE	239PCH-A	2012
	Human stool		239R-A	
834	Human stool	STX2	242CH	2012
	Human stool		242Rossa	
333	Human stool	STX2-EAE	243R-ACH	2011
	Human stool		243Ral-A	
	Human stool		243R-AS	
124	Human stool	STX1-2-EAE	245A-CH-A-	2010
1520868	Milking filter	n.d.	F1-1	2009
1030908	Milking filter	n.d.	F10-4	2009
1041186	Milking filter	n.d.	F11-4	2009
1060201	Raw milk	n.d.	L12-2	2009
1534025	Milking filter	n.d.	F27-2	2009
25022	Raw milk	n.d.	L36-2	2009
90809	Milking filter	n.d.	F69-4	2009
71519	Milking filter	n.d.	F80-1	2009
	Milking filter		F80-2	
	Milking filter		F80-3	
	Milking filter		F80-4	
1351523	Milking filter	n.d.	F90-1	2009
	Milking filter		F90-3	
42403	Milking filter	n.d.	F93-3	2009
31700	Milking filter	n.d.	F95-2	2009
	Milking filter		F95-3	
LC	Raw milk	n.d.	ECLC	2012



Table 3. Fluorescence (CF) or red color expression (CR) and biofilm formation (AU) of *E. coli* isolates at different temperatures

Isolates	CF binding at 30°C	CF binding at 37°C	CR at 30°C	CR at 37°C	M9 at 30°C (AU)	M9 at 37°C (AU)	TSB at 30°C (AU)	TSB at 37°C (AU)
245A-CH-A	±	±	±	±	0,34	0,46	0,52	<b>6,19</b>
243R-ACH	±	+	±	±	0,57	0,31	<b>1,10</b>	0,62
243Ral-A	-	±	±	±	<b>1,73</b>	1,54	<b>1,42</b>	0,55
243R-AS	±	±	±	-	0,70	0,23	<b>1,25</b>	0,96
214CH	-	±	-	-	0,58	0,00	0,66	0,89
214RACH	+	+	+	+	<b>1,63</b>	1,08	<b>1,61</b>	<b>3,43</b>
224SMA-GS	±	+	+	+	0,59	0,95	<b>1,30</b>	0,54
224SMA-PS	-	-	-	-	0,67	1,16	<b>1,48</b>	<b>2,97</b>
225R-A	-	±	-	-	0,61	1,51	<b>1,18</b>	0,14
226B-B	-	-	±	-	0,55	<b>10,55</b>	<b>2,10</b>	0,11
227Rosa	±	+	+	+	0,29	0,45	<b>1,35</b>	0,33
228GS	-	±	-	-	0,30	0,46	0,45	0,29
228P-CH	-	-	-	-	0,20	0,41	0,27	0,29
229B-ACH	±	+	±	-	0,30	0,64	<b>1,10</b>	0,39
229M-AS	+	+	±	+	0,98	1,64	<b>1,21</b>	0,22
229PRal-ACH	±	±	+	+	0,41	0,84	<b>12,72</b>	<b>11,20</b>
229R-ACH	±	±	±	-	0,27	0,41	<b>5,08</b>	0,50
229Rosa-A	-	±	±	-	0,26	0,82	0,74	0,76
231PCH-A	±	+	±	±	0,40	<b>2,60</b>	0,67	<b>9,46</b>
232AS-B-LUC	±	+	±	-	0,63	0,78	<b>1,91</b>	0,67
233P-CH-A	±	+	+	±	0,64	0,55	<b>1,33</b>	1,41
242CH	+	+	+	+	<b>1,02</b>	<b>2,88</b>	<b>1,14</b>	<b>10,04</b>
242Rossa	+	+	+	+	<b>2,31</b>	<b>2,30</b>	<b>1,53</b>	0,38
239PCH-A	-	±	±	-	0,45	1,21	<b>1,08</b>	0,46
239R-A	-	±	±	-	0,39	1,17	<b>1,15</b>	0,18
F1-1	+	+	+	+	<b>1,11</b>	0,79	0,13	<b>5,37</b>
F11-4	±	+	±	±	<b>1,87</b>	0,44	<b>1,70</b>	<b>3,05</b>
L12-2	±	±	±	±	0,88	1,08	0,49	0,55
L36-2	+	+	+	+	0,73	<b>5,93</b>	<b>1,38</b>	1,56
F69-4	-	±	-	-	0,48	0,45	<b>1,50</b>	0,71
F80-1	±	+	+	±	0,35	1,48	0,65	0,86
F80-2	±	+	+	±	0,65	1,98	<b>1,38</b>	<b>3,25</b>
F90-3	±	±	-	-	<b>9,00</b>	<b>9,48</b>	<b>3,27</b>	<b>4,88</b>
F93-3	-	±	-	-	<b>1,49</b>	0,98	0,63	0,65
F95-2	-	±	-	-	<b>1,11</b>	0,28	<b>1,17</b>	0,31
ECLC	±	+	±	-	0,52	0,41	<b>1,22</b>	0,37

