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► **To cite this version:**

Leena Suojala, Tarja Pohjanvirta, Heli Simojoki, Anna-Liisa. Myllyniemi, Anna Pitkälä, et al.. Phylogeny, virulence factors and antimicrobial susceptibility of isolated in clinical bovine mastitis. *Veterinary Microbiology*, 2010, 147 (3-4), pp.383. 10.1016/j.vetmic.2010.07.011 . hal-00654949

HAL Id: hal-00654949

<https://hal.science/hal-00654949v1>

Submitted on 24 Dec 2011

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Accepted Manuscript

Title: Phylogeny, virulence factors and antimicrobial susceptibility of *Escherichia coli* isolated in clinical bovine mastitis

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PII: S0378-1135(10)00346-9
DOI: doi:10.1016/j.vetmic.2010.07.011
Reference: VETMIC 4967

To appear in: *VETMIC*

Received date: 12-4-2010
Revised date: 12-7-2010
Accepted date: 14-7-2010

Please cite this article as: Suojala, L., Pohjanvirta, T., Simojoki, H., Myllyniemi, A.-La., Pitkälä, A., Pelkonen, S., Pyörälä, S., Phylogeny, virulence factors and antimicrobial susceptibility of *Escherichia coli* isolated in clinical bovine mastitis, *Veterinary Microbiology* (2010), doi:10.1016/j.vetmic.2010.07.011

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1 **Phylogeny, virulence factors and antimicrobial susceptibility of *Escherichia coli* isolated in**
2 **clinical bovine mastitis**

3

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21

22 **Abstract**

23 The aim of this study was to identify specific phylogeny groups, virulence genes or antimicrobial
24 resistance traits of *Escherichia coli* isolated in bovine mastitis associated to clinical signs,
25 persistence of intramammary infection in the quarter and recovery from mastitis. A total of 154 *E.*

26 *coli* isolates from bovine clinical mastitis, 144 from the acute stage and 10 from follow-up samples
27 three weeks later, originating from 144 cows in 65 dairy herds in Southern Finland were
28 investigated. Phylogeny groups and virulence genes of the isolates were determined using
29 polymerase chain reaction, and antimicrobial susceptibility using the VetMICTM microdilution
30 method. In ten cows (11.8%), infection persisted, confirmed by re-isolation of the same pulsed-field
31 gel electrophoresis type from the affected quarter at three weeks post-treatment. The majority of
32 isolates, 119 (82.6%), belonged to phylogeny group A, which mainly consisted of commensal
33 strains. Altogether 56 isolates (38.9%) had at least one virulence gene detected. Most common
34 virulence genes detected were *irp2*, *iucD*, *papC* *iss*; genes *svg*, *stx1*, *stx2*, *cnf1* and *hlyA* were not
35 found. Combinations of virulence genes varied greatly. Forty (27.8 %) of the 144 *E. coli* isolates
36 showed resistance to at least one antimicrobial tested. None of the studied phylogeny groups,
37 virulence factors or antimicrobial resistance traits was associated with clinical signs, persistence of
38 intramammary infection or clinical recovery from mastitis. The results support the conclusion that
39 mastitis-causing *E. coli* bacteria are typical commensals.

41 **Keywords:** Antimicrobial susceptibility; Bovine; *Escherichia coli*; Mastitis; Phylogeny; Virulence
42 factors

44 **1. Introduction**

46 *Escherichia coli* is a mammary pathogen originating from the environment of the cow and entering
47 the udder via the teat canal (Nemeth et al. 1994, Lipman et al. 1995). Intramammary infection (IMI)
48 caused by *E. coli* is commonly considered to be of short duration and is mostly spontaneously
49 eliminated (Burvenich et al. 2003). *E. coli* IMI can also be associated with severe clinical signs and
50 marked tissue damage of the mammary gland (Pyörälä et al. 1994, Wenz et al. 2001).

51

52 In their review, Burvenich et al. (2003) concluded that the severity of *E. coli* mastitis is mainly
53 determined by cow factors, rather than specific features of the bacterial strain. However, some
54 authors have suggested that some *E. coli* causing bovine mastitis may have characteristics of an
55 invasive animal-pathogenic *E. coli*, which might help bacteria to invade the bovine mammary gland
56 and also explain why some *E. coli* can cause persistent IMI (Lam et al. 1996, Bradley and Green
57 2001, Blum et al. 2008, Shpigel et al. 2008).

58

59 Phylogenetic grouping of *E. coli* has been used in epidemiological virulence studies (Picard et al.
60 1999, Russo and Johnson 2000, Silva et al. 2009), but the phylogeny of isolates in bovine mastitis
61 has not been evaluated earlier in larger extent. Most *E. coli* causing mastitis have been serum-
62 resistant (Carrol and Jasper 1977, Nemeth et al. 1991, Fang and Pyörälä 1996) and different
63 virulence factors and their combinations have been distributed widely (Nemeth et al. 1991, Nemeth
64 et al. 1994, Lipman et al. 1995, Kaipainen et al. 2002), but the majority of isolates have not
65 possessed any of the virulence factors evaluated. Virulence factors can also be linked with
66 antimicrobial resistance (Martinez and Baquero 2002). Pathogenic *E. coli* strains are classified into
67 different pathotypes according to their ability to cause specific diseases (Kaper et al. 2004). No
68 special virulence factors or pathotypes have been reported in *E. coli* isolated in bovine mastitis, nor
69 have any associations been shown between the virulence factors evaluated and the clinical severity
70 of mastitis caused by the isolate (Lehtolainen et al. 2003, Wenz et al. 2006). However, some
71 evidence of strain selection in the mammary gland during infection has been found (Bradley and
72 Green 2001, Blum et al. 2008).

73

74 The aim of this study was to identify possible specific phylogeny groups, virulence genes or
75 antimicrobial resistance traits of *E. coli* isolated in bovine mastitis in relation to clinical signs at the
76 acute stage, persistence of IMI in the quarter and recovery from mastitis.

77

78 **2. Materials and methods**

79

80 *2.1. Origin of isolates, clinical examination and assessment of cure*

81

82 The material consisted of 154 *E. coli* isolates, 144 from initial samples and 10 from follow-up
83 samples taken three weeks later, originating from bovine clinical mastitis in 65 dairy herds in
84 Southern Finland (mean 2.2 cows/herd, 1 quarter/cow). At the time that the initial milk sample was
85 taken (day 0) from the affected quarter, the cow was examined clinically by the attending
86 veterinarian. Clinical signs were recorded on the data collection form. Clinical signs (local and
87 systemic) were scored according to a 3-point scale (1=no signs to 3=severe signs), as described
88 earlier (Suojala et al. 2010). Clinical signs with scores ≤ 1.5 were considered mild and those with
89 scores ≥ 2 severe. After 3-4 weeks (day 21), the farmer estimated the milk production (returned
90 /diminished /blind) and clinical recovery of the affected quarter, carried out California Mastitis Test
91 (CMT) and recorded the results on the data collection form. Half of the cows were treated with
92 enrofloxacin and the other half served as untreated controls; no differences between the treatment
93 groups were found in the outcome of mastitis (Suojala et al. 2010). The outcome was evaluated by
94 bacteriology and assessment of clinical cure. A cow was defined as clinically cured if no clinical
95 signs were present and milk appearance was normal and acceptable for delivery to the dairy plant.

96

97 *2.2. Bacterial isolates and laboratory methods*

98

99 A 4-ml milk sample was collected aseptically from the affected quarter at the acute stage of mastitis
100 (day 0) and a second sample 3 weeks (day 21) later for bacteriological and milk N-acetyl-β-D-
101 glucosaminidase (NAGase) analyses. Ten µl of milk was cultured on blood agar (6-10% sheep
102 blood) (Tammertutka, Tampere, Finland). Isolates were identified as *E. coli* by colony morphology,
103 Gram-stain and typical growth on eosin methylene blue (EMB) agar (Hogan et al. 1999);
104 identification was confirmed by the API 20E test (bioMérieux, Marcy l'Etoile, France). Follow-up
105 milk sample on day 21 were stored frozen and cultured within a month. Altogether 154 isolates, 144
106 from the pre-treatment samples (day 0) and 10 isolates from the follow-up samples at 21 days post-
107 treatment, were examined. Isolates were stored frozen before examination.

108
109 Isolates were assigned to a phylogeny group by the polymerase chain reaction (PCR) method of
110 Clermont *et al.* (2000), which is a rapid phylogenetic grouping technique based on triplex PCR. The
111 virulence genes to be examined were selected based on those of extra-intestinal pathogenic *E. coli*
112 and entero-hemorrhagic *E. coli* (EHEC). PCR was used to analyse the genes of colicin V plasmid
113 (*cva*), vacuolating autotransporter protein (*vat*), temperature-sensitive hemagglutinin (*tsh*),
114 aerobactin (*iucD*), P-fimbria (*papC*), yersinia bactin (*irp2*), increased serum survival (*iss*) and
115 enteroaggregative heat-stable toxin (*astA*) (Ewers et al. 2005). PCR described by Paton and Paton
116 (2002) was used to detect genes of shigatoxin 1 and 2 (*stx1* and *stx2*), intimin (*eaeA*),
117 enterohemolysin (*ehxA*) and autoagglutinating adhesin (*saa*) and that by van Bost et al. (2003) to
118 detect S-fimbria (*sfaD*), F17 fimbria (*f17A*) and afimbrial adhesin AFA8 (*afa8E*). Gene for
119 cytotoxic necrotizing factor 2 (*cnf2*) was analysed as described in Kaipainen et al. (2002).
120 Antimicrobial susceptibility of the *E. coli* isolates was tested by the VetMICTM microdilution
121 method (SVA Uppsala, Sweden) (CLSI 2004) using the epidemiological cutt-offs of EUCAST
122 (2008), except for ciprofloxacin, for which a cut-off > 0.06 µg/ml was used. Susceptibilities to

123 ampicillin, streptomycin, tetracycline, sulfametoxazol, trimethoprim, kanamycin, cefotaxime,
124 nalidixin acid, ciprofloxacin, gentamicin, florfenicol and ceftiofur were evaluated.

125

126 If growth of *E. coli* was detected in both pre- and post-treatment samples, isolates were genotyped
127 using pulsed-field gel electrophoresis (PFGE) to evaluate the genetic similarity (Olive and Bean
128 1999). DNA was extracted, digested with *Xba*I and subjected to PFGE essentially according to the
129 CDC/PulseNet protocol for *E. coli* O157:H7 (CDC 2008). *Salmonella* Braenderup strain H9812
130 DNA digested with *Xba*I was used as a DNA size standard. The PFGE patterns were analysed
131 visually. When the same pulsed-field type (100% band match) was found in both pre- and post-
132 treatment samples, the cases were defined as persistent IMI.

133

134 Milk NAGase activity, an indicator for inflammation and tissue damage of the affected quarter, was
135 measured from the initial milk samples taken on day 0, using the fluorogenic method with a
136 microplate modification developed by Mattila and Sandholm (1985). Milk NAGase activity values
137 higher than 24.49 pmol 4-MU/min/ μ l were recorded as 24.49 pmol 4-MU/min/ μ l.

138

139 2.3. Statistical analysis

140

141 After validation checks for data inconsistencies and missing entries, statistical analyses were carried
142 out using Stata Intercooler version 9.0 (Stata Corporation, Texas, USA). The unit for the analysis
143 was the cow. Correlations of virulence genes and antimicrobial susceptibilities were studied with
144 phi correlation test.

145

146 Three logistic regression models were used as described below. Parity (1, 2, \geq 3 times), phylogeny
147 groups (A, B1, B2, D) and days in milk (DIM) (1 week *ante partum* to 3 weeks *post partum*, $>$ 3

148 weeks to 120 days *pp*, > 120 days *pp*) were measured on a nominal scale and used as categorical
149 variables. Dichotomous variables (yes vs. no) were virulence genes, phylogeny groups,
150 antimicrobial susceptibilities, and additionally the following variables: clinical signs on day 0 (mild
151 vs. severe), NAGase activity on day 0 (values reflecting mild mastitis < 10 pmol 4-MU/min/ μ l vs.
152 values reflecting severe mastitis \geq 10 pmol 4-MU/min/ μ l) and antimicrobial treatment (yes vs. no).
153 Each of the virulence genes and antimicrobial resistance traits were included in the statistical
154 models only if data consisted of at least 10 positive findings: variables *irp2*, *iucD*, *papC*, *iss*, *cva*,
155 *AFA8*, ampicillin, streptomycin, tetracycline, sulphamethoxazol, trimethoprim and clorphenicol were
156 included in the models. Other virulence genes and antimicrobial resistance traits were included only
157 in descriptive data.

158

159 Multivariable logistic regression models were used for the outcomes: clinical signs on day 0, milk
160 NAGase activity on day 0, bacterial persistence on day 21. Clustering effect of a herd was estimated
161 with a logistic mixed model. The herd was included as a random effect with models of clinical signs
162 on day 0 and bacterial persistence. All variables were subjected first to univariate, then
163 multivariable analyses. Variables with a P-value of less than 0.15 in a univariate analysis were
164 retained in the multivariable analysis after testing the variables for correlation with a pair-wise
165 correlation test. Manual stepwise backward procedures were used in model-building. Possible
166 confounding factors (treatment, DIM, parity) were kept in the analysis when there was a possibility
167 of an effect on the results. Significant correlations (> 0.65) or interactions were taken into account
168 in a final model as the most significant variable was chosen to be a representative of the group. In
169 the logistic regression model of milk NAGase activity on day 0, full and nested models were
170 compared with a likelihood ratio test. Model fit was assessed with the assumptions of normality and
171 homogeneity of variances, which were evaluated by checking the residuals. Cows with missing data

172 for any variable in the model were excluded from these analyses. For all tests, $P < 0.05$ was
173 considered significant.

174

175 **3. Results**

176

177 *3.1. Phylogeny groups and virulence genes*

178

179 The majority of initial 144 isolates, 119 (82.6%), belonged to phylogeny group A, followed by
180 group D ($n = 16$, 11.1%), B1 ($n = 7$, 4.9%) and B2 ($n = 2$, 1.4%). Of the 144 *E. coli* isolates, 56
181 (38.9%) had at least one virulence gene detected by PCR. The combinations of virulence genes
182 varied greatly. Each combination was present mainly in one isolate (Table 1). Genes *irp2*, *papC*,
183 *iucD* and *iss* were the most common (Table 2). Genes *iucD*, *cva*, *iss* and *papC* correlated together
184 ($r_{\phi}=0.56-0.74$). Only *astA*, *eaeA*, *irp2* and genes for F17 were present alone; all other genes were
185 detected in different combinations (Table 1). Genes *svg*, *stx1*, *stx2*, *cnf1* and *hlyA* were not found in
186 any of the isolates (Table 2).

187

188 *3.2. Antimicrobial resistance*

189

190 Altogether 27.8% (40/144) of the initial isolates of *E. coli* showed resistance to at least one
191 antimicrobial tested (Table 3). Resistance to more than two antimicrobials was seen in 20.1% of the
192 isolates. The most frequent resistances were for ampicillin (18.6%), streptomycin (16.4%),
193 tetracycline (15.7%) and sulfametoazol (13.6%). No resistance was detected to gentamicin,
194 florfenicol or ceftiofur. Resistance to streptomycin, ampicillin, sulfametoazol and trimethoprim
195 correlated with each other ($r_{\phi} = 0.65-0.88$). The virulence gene *iucD* was correlated with resistance
196 to streptomycin, ampicillin, sulfametoazol and trimethoprim ($r_{\phi} = 0.60-0.81$).

197

198 *3.3. Clinical signs and milk NAGase activity*

199

200 The majority of cows, 76.6%, had severe signs (severity score ≥ 2) and 23.4% (n = 32) mild signs
201 (score ≤ 1.5) at the acute stage of mastitis. None of the phylogeny groups, virulence factors or
202 resistances to antimicrobials had any association with the clinical signs of mastitis. Phylogeny
203 groups, virulence genes or antimicrobial resistance traits of the causative isolate had no significant
204 effect on milk NAGase activity.

205

206 *3.4. Persistence of IMI*

207

208 From 10 of 85 quarters sampled on day 21 (11.8%), the same PFGE type was isolated, reflecting the
209 persistence of *E. coli* IMI in the quarter. Persistent isolates belonged to phylogeny group A, except
210 one in group D. Six of the persistent isolates were susceptible to all antimicrobials evaluated; one
211 was resistant to seven different antimicrobials. Four persistent strains were negative for all virulence
212 genes studied (Table 4).

213

214 Herd had a clustering effect on the persistence of IMI (P = 0.03). Persistent isolates were found on 9
215 individual farms, each farm having a unique PFGE type. One pulsotype was associated with two
216 cases of persistent IMI in one herd. On this farm, two different cows had persistent IMI (at a 3-year
217 interval) caused by the same pulsed-field type of *E. coli*. This strain caused acute clinical mastitis
218 with severe clinical signs in both cows. In the statistical model, none of the virulence factors,
219 phylogeny groups or antimicrobial resistance traits was associated with the persistence of *E. coli*
220 IMI.

221

222 4. Discussion

223 The majority of studied *E. coli* isolates, from both persistent and non-persistent IMIs, belonged to
224 phylogeny group A. Group A consists mainly of strains that are commensally orientated and not
225 pathogenic for a healthy host (Clermont et al. 2000). They have been reported to have few virulence
226 factors. Pathogenic intestinal *E. coli* strains have been found in groups A, B1 and D (Picard et al.
227 1999, Russo and Johnson 2000), whereas pathogenic extraintestinal *E. coli* belong mainly to groups
228 B2 and D (Clermont et al. 2000). *E. coli* isolates recovered from the uterus of puerperal cows
229 belonged mainly to phylogenetic groups A and B1 (Silva et al. 2009) and had a low potential
230 virulence. Dogan et al. (2006) reported in their study with three persistent and three transient *E. coli*
231 mastitis strains, that all belonged to phylogeny group A and lacked virulence genes of invasive *E.*
232 *coli*. Commensal strains of *E. coli* commonly lack the specialized virulence traits present in
233 pathogenic strains (Russo and Johnson 2000). This supports our conclusion that the mastitis-causing
234 *E. coli* bacteria are typical commensals.

235

236 None of the examined virulence factors, phylogeny groups or antimicrobial resistance traits of *E.*
237 *coli* was associated with clinical signs of the cows at the acute stage of mastitis or with recovery
238 from mastitis. The results are consistent with those of previous studies, suggesting that severity of
239 *E. coli* mastitis is not associated with any specific known virulence factor (Lehtolainen et al. 2003,
240 Wenz et al. 2006, Dyer et al. 2007). No bacterial feature - phylogeny group, virulence factor or
241 resistance trait - was associated with persistence of *E. coli* IMI in the udder. Contradictory results
242 were presented in a previous Finnish study (Lehtolainen et al. 2003a), where genes *cnf1*, *cnf2*, *sfaD*
243 and *papC* were significantly associated with re-isolation of *E. coli* post-treatment. In their study,
244 persistence of the same strain was not confirmed by PFGE. We found only three strains with
245 virulence genes for CNF2, and none of them was a persistent strain. In our data, P-fimbria was
246 detected in 16% and S-fimbria in 2% of the isolates, and neither was linked to chronic IMI.

247 Recurrent *E. coli* mastitis has been reported to have a tendency towards milder clinical signs than
248 seen in non-recurrent cases, which was suggested to favour chronicity of the IMI (Bradley and
249 Green 2001). In an earlier Finnish study, no differences were found in the severity of clinical signs
250 of IMIs that remained persistent compared with those that were eliminated (Lehtolainen et al.
251 2003a), in agreement with our findings.

252

253 Many different virulence gene combinations were present in our *E. coli* collection, and typically
254 only one isolate represented each combination of genes, indicating that a high genotypic variability
255 is characteristic of *E. coli* strains causing clinical mastitis. This finding has also been reported in
256 earlier studies; however, the panel of virulence factors evaluated differed between the studies
257 (Nemeth et al. 1991, 1994, Kaipainen et al. 2002, Bean et al. 2004, Wenz et al. 2007).

258

259 No virulence genes at all were detected 60.4% of the isolates studied here. In an earlier Finnish
260 study (Kaipainen et al. 2002), in 50.5% of the isolates no virulence genes were found, but the
261 virulence genes evaluated differed from those here. In another study on *E. coli* isolates from bovine
262 mastitis, 62.5% of the isolates avoided a panel of several other virulence genes (Bean et al. 2004).
263 Shpigel et al. (2008) have suggested that *E. coli* associated with persistent bovine IMI could have
264 some unknown bacterial factors for pathogenicity. Their suggestion of a specific “mammary
265 pathogenic *E. coli*” was not supported by our results. More research is needed with new markers for
266 virulence to further explore the existence of mammary pathogenic *E. coli*.

267

268 Of the virulence genes evaluated here, only *iucD* was correlated with antimicrobial resistance to
269 streptomycin, ampicillin, sulfamethoxazol and trimethoprim. *iucD* codes for aerobactin, which is
270 involved in the iron-chelating ability of *E. coli* and may be located on either a chromosome or a
271 plasmid, where many of the antimicrobial resistance genes also reside (Hirsh et al. 1993, Johnson et

272 al. 2002). In addition, *iucD* was associated with *iss* (gene for increased resistance to serum), which
273 may also be located on a plasmid (Johnson et al. 2002). Our finding of a linkage between these
274 virulence genes and antimicrobial resistance could indicate that they are located on the same
275 plasmid or act near each other on the same pathogenicity island (Bagg et al. 1987). Plasmids are
276 major factors for the dissemination of both antibiotic resistance and virulence determinants among
277 bacterial populations (Guardabassi and Courvalin 2006). For bacteria carrying linked determinants,
278 the selection of an infective population will select for antibiotic resistance, and antibiotic selective
279 pressure will select the virulence trait (Martínez and Baquero 2002).

280

281 Antimicrobial resistance in different bacterial populations can only be compared if the same
282 methods and cut-offs have been used (Schwarz et al. 2010). The prevalence of resistance in mastitis
283 *E. coli* isolates here were found to be highest against antimicrobials that have been used for years in
284 dairy cattle (ampicillin, streptomycin, sulphonamide and tetracycline). Prevalence of resistance of
285 *E. coli* were here greater than those in the Finnish national resistance surveillance programme; in
286 addition 20% of our isolates were multiresistant as compared with only 6% in the national
287 surveillance data (FINRES-Vet 2005-2006). Our data originates from limited geographical area,
288 which may partly explain the differences when compared with national data. When compared to a
289 Swedish study, resistance proportions of *E. coli* isolates were generally only half of those seen in
290 our isolates (Bengtsson et al. 2008). Resistances to traditional antimicrobials seen here often were
291 associated, indicating that they appear in the same isolates, as also seen by Lehtolainen et al.
292 (2003b). Resistance among mastitis-causing *E. coli* may reflect the general use of antimicrobials in
293 the herd, as these bacteria are commensals. Routine antimicrobial treatment is not recommended for
294 *E. coli* mastitis in Finland (Evira 2009); this practice should in general reduce selection pressure
295 towards resistance.

296

297 **Acknowledgements**

298

299 This work was supported by grants from the Walter Ehrström Foundation, the Mercedes
300 Zachariassen Foundation, the Finnish Veterinary Foundation, the EVO Fund of Lammi–Tuulos
301 Health Centre and the Häme Regional Foundation of the Finnish Cultural Foundation. Our deepest
302 thanks are due to the veterinarians and the clients of veterinary practices for their participation in
303 the field study and to the laboratory staff at the Department of Production Animal Medicine for
304 their collaboration.

305

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Tables

Table 1. Different combinations of virulence genes detected among *Escherichia coli* isolated from bovine clinical mastitis. Number of studied isolates was 144.

Detected virulence genes and their combinations among mastitis isolates						Number of isolates
<i>astA</i>	<i>eaeA</i>					1
<i>astA</i>	<i>irp2</i>					2
<i>astA</i>	<i>iucD</i>					1
<i>sfaD</i>	<i>papC</i>	<i>irp2</i>	<i>vat</i>			1
<i>sfaD</i>	<i>papC</i>	<i>irp2</i>				1
<i>irp2</i>	<i>iss</i>	<i>vat</i>				1
<i>tsh</i>	<i>cva</i>	<i>iucD</i>	<i>papC</i>	<i>irp2</i>	<i>iss</i>	1
<i>F17</i>	<i>irp2</i>					1
<i>F17</i>	<i>irp2</i>	<i>iss</i>	<i>CNF2</i>			2
<i>AFA8</i>	<i>irp2</i>	<i>papC</i>	<i>iucD</i>			2
<i>AFA8</i>	<i>irp2</i>	<i>papC</i>				1
<i>AFA8</i>	<i>papC</i>	<i>irp2</i>				1
<i>AFA8</i>	<i>iss</i>	<i>irp2</i>	<i>papC</i>	<i>iucD</i>		3
<i>AFA8</i>	<i>iss</i>	<i>irp2</i>	<i>papC</i>	<i>iucD</i>	<i>cva</i>	3
<i>AFA8</i>	<i>saa</i>	<i>CNF2</i>				1
<i>cva</i>	<i>iucD</i>	<i>iss</i>				3
<i>cva</i>	<i>iucD</i>	<i>iss</i>	<i>irp2</i>	<i>papC</i>		5
<i>cva</i>	<i>papC</i>	<i>iss</i>	<i>irp2</i>			1
<i>cva</i>	<i>iucD</i>	<i>papC</i>	<i>irp2</i>			1
<i>iucD</i>	<i>iss</i>					1
<i>iucD</i>	<i>iss</i>	<i>papC</i>	<i>irp2</i>			3
<i>iucD</i>	<i>papC</i>					1
<i>astA</i>						6
<i>eaeA</i>						1
<i>F17</i>						3
<i>irp2</i>						10
Total number						56

Table 2. Prevalences of virulence genes among *Escherichia coli* isolated from bovine clinical mastitis (n = 144). Number of isolates negative to detected virulence genes was 88.

Virulence gene	Number of isolates	% isolates
<i>irp2</i>	38	26.4
<i>iucD</i>	24	16.7
<i>papC</i>	24	16.7
<i>iss</i>	24	16.7
<i>cva</i>	14	9.7
<i>AFA8</i>	11	7.6
<i>astA</i>	9	6.3
<i>F17</i>	6	4.2
<i>cnf2</i>	3	2.1
<i>eeaeA</i>	3	2.1
<i>vat</i>	2	1.4
<i>sfaD</i>	2	1.4
<i>tsh</i>	1	0.7
<i>saa</i>	1	0.7
<i>stx1</i>	0	0
<i>stx2</i>	0	0
<i>hlyA</i>	0	0
<i>svg</i>	0	0

Table 3. Distributions of MICs for *E. coli* from bovine mastitis (n = 144). MICs equal to or lower than the lowest concentrations tested are given the lowest concentration. Numbers of resistant isolates for each antimicrobial tested are indicated with hatched fields. Vertical lines indicate epidemiological cut-off values for resistance.

Antimicrobial	MIC (%) distribution mg/l																	Res (%)		
	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024		2048	
Ampicillin						2	23	57	27	8	1		26							18,7
Streptomycin									18	84	16	2	1	5	9	9				18,1
Tetracycline							36	80	4			3	6	15						16,7
Sulphametoxazol											119	4							21	14,6
Trimethoprim					35	63	28	3					15							10,4
Cloramphenicol								2	78	54					10					6,9
Kanamycin								7	108	20		9								6,3
Cefotaxime			96	32	13	1	2													1,4
Nalidixin acid								2	57	80	4				1					0,7
Ciprofloxacin	12	71	60			1														0,7
Gentamicin						12	106	24	2											0
Florphenicol								2	43	92	7									0
Ceftiofur			1	1	51	87	4													0

Table 4. Virulence genes detected in 10 strains of *Escherichia coli* re-isolated in the follow-up samples 3 weeks post-treatment from bovine clinical mastitis.

Detected virulence genes and their combinations among mastitis isolates					Number of isolates
<i>irp2</i>					1
<i>eaeA</i>					1
<i>cva</i>	<i>iucD</i>	<i>papC</i>	<i>irp2</i>	<i>iss</i>	2
<i>iucD iss</i>					1
<i>iucD</i>	<i>papC</i>	<i>irp2</i>	<i>iss</i>	<i>AFA8</i>	1
Negative					4

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