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1 **Comparison of virulence gene profiles of *Escherichia coli* isolates**
2 **from sows with Coliform mastitis and healthy sows**

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24

25 **Abstract**

26 Coliform mastitis (CM) is not only a serious economical and animal welfare touching
27 problem in dairy cattle, but also in sows after farrowing. Due to this disease, the
28 essential adequate supply with colostrum for the growth and the health of the piglets
29 is not ensured. Besides other influencing factors, *Escherichia (E.) coli* is of great
30 importance as a causative agent of this multifactorial disease. In this study, *E. coli*
31 isolates from milk samples of healthy and CM-affected sows were examined for the
32 presence of virulence genes associated with extraintestinal *E. coli* strains,
33 enterotoxigenic *E. coli* and other pathogenic *E. coli*.

34 The isolated *E. coli* harbored mainly virulence genes of extraintestinal *E. coli* strains
35 (especially *fimC*, *ompA*, *traT*, *hra*, *kpsMTII*, *iroN*). The virulence gene spectrum for
36 both samples from CM-affected and healthy sows did not differ significantly.
37 Particular virulence gene profiles of *E. coli* isolates from diseased sows were not
38 detected.

39 This study provides novel insights into the role of *E. coli* in association with mastitis in
40 sows since it is the first time *E. coli* isolates from CM-affected sows' milk were
41 analysed for virulence genes. Because there were no differences in the prevalence of
42 *E. coli* and their virulence-associated genes between healthy and diseased sows,
43 other causative factors seem to have greater influence on the pathogenesis of
44 porcine CM.

45

46 **Keywords**

47 ETEC, ExPEC, multiplex PCR, swine, virulence factors

48

49 Introduction

50 'Coliform mastitis' (CM) is the main symptom of puerperal disorders occurring in sows
51 after farrowing which are subsumed under the term postpartum dysgalactia
52 syndrome (PPDS or PDS) (Gerjets and Kemper, 2009; Klopfenstein et al., 2006). The
53 etiology of CM is multifactorial with husbandry, management, feeding and hygiene as
54 influencing factors (Klopfenstein et al., 2006), but mainly bacteria are the causative
55 agents for the inflammation. In bacteriological analyses, especially *Escherichia (E.)*
56 *coli* was isolated, but the strains were not further investigated for virulence-
57 associated genes. Strains of *E. coli* can be broadly classified into three groups by
58 their location and their characteristic virulence genes: commensal *E. coli*, intestinal
59 pathogenic *E. coli* (IPEC) colonizing the intestine, and extraintestinal pathogenic *E.*
60 *coli* (ExPEC) that reach extraintestinal niches like the urinary tract (Russo and
61 Johnson, 2000). In swine, especially enterotoxigenic *E. coli* (ETEC) as a pathotype of
62 IPEC are well described as causal agents for severe diseases like diarrhea in
63 neonatal and weaned piglets (Casey and Bosworth, 2009). The ExPEC pathotypes
64 are e.g. causative for urinary tract infections (uropathogenic *E. coli* (UPEC)) or
65 septicaemia in pigs (Daigle et al., 1997; Krag et al., 2009; Shpigel et al., 2008). A
66 selection of virulence genes known to be associated with ETEC, ExPEC pathotypes
67 and shiga toxin-producing *E. coli* (STEC) is listed in Table 1.

68 A new putative pathotype of ExPEC was proposed by Shpigel et al. (2008):
69 mammary pathogenic *E. coli*, with a specific set of virulence genes, which are
70 associated with mastitis in dairy animals. However, up to now epidemiological studies
71 have not shown a common virulence gene profile for these *E. coli* so far (Kaipainen
72 et al., 2002; Srinivasan et al., 2007; Wenz et al., 2006).

73 The aim of our study was to analyse the occurrence of different virulence genes in *E.*
74 *coli* isolates associated with Coliform mastitis in sows.

75

76 **Materials and methods**

77 *Animals and study design*

78 The investigation was carried out between April 2008 and August 2010 on five
79 multiplication herds in Germany (A - E), supervised by PIC Germany GmbH
80 Schleswig (Table 2). The farms were of high health status and tested free from
81 enzootic pneumonia, rhinitis, *Actinobacillus pleuropneumoniae* and dysentery. The
82 number of sows housed in the farms ranged from 700 to 1,800. The sows were in
83 different parities (1–9) and of different lines (Landrace, Large White and crossbreds,
84 partly with Duroc).

85 They were identified as CM-affected when their rectal temperature was above 39.5°C
86 24 h post partum (Furniss, 1987) and the mammary glands showed symptoms of
87 inflammation. In addition, the appearance and the performance of the piglets were
88 evaluated with regard to their behavior and body condition. Healthy half- or full-sib
89 sows from the same farrowing group that farrowed closest in time served as controls.
90 The half-sib design was chosen due to further studies on the genetic background via
91 genotyping (Preißler et al., unpublished data). In total, 2,005 milk samples were
92 examined (1,026 milk samples from sows with CM and 979 from healthy sows).
93 Before gathering a collective sample of several teats, mammary glands were cleaned
94 and disinfected with disinfection swabs containing 70% isopropyl-alcohol. The first
95 streams of milk were discarded whereas the followings were milked on transport
96 swabs with Amies medium (transwab, medical wire & equipment, Corsham,

97 England). The milk samples were stored at 4°C before sending them to the
98 laboratory within 72 hours.

99 *Bacteriological analysis*

100 The swabs were incubated in Caso broth for 24 h at 37°C. With a plastic loop, 10 µL
101 of the enrichment were streaked onto Columbia blood agar and Endo agar (both
102 Oxoid, Cambridge, United Kingdom) and incubated aerobically another 24 h at 37°C.
103 The grown bacteria were differentiated by their morphology, haemolysis on blood
104 agar and Gram staining. Pure cultures were grown on blood agar after another 24 h
105 incubation at 37°C before biochemical confirmation to species level with the
106 identification system API (bioMérieux, Craaponne, France).

107 *Escherichia coli* isolates were distinguished due to individual morphology on blood
108 agar and API 20E. All isolates were selected for further investigations.
109 Desoxyribonucleic acid of the identified *E. coli* strains was prepared by solving a few
110 colonies in 200 µL distilled water. After boiling for 10 min and centrifugation, 3 µL of
111 the supernatant was taken for PCR analysis. The presence of virulence genes
112 associated with ExPEC strains, ETEC and other pathogenic *E. coli* was determined
113 by multiplex PCR (mPCR) assays for all *E. coli* isolates, as described by Ewers et al.
114 (2007) and Casey and Bosworth (2009).

115 In total, 2,403 isolates were tested for the presence of 27 virulence genes for the
116 following virulence factors (Table 1): heat labile toxin (*LT*), heat stable toxin a and b
117 (*STI*, *STII*), Shiga toxin (*Stx2e*), capsular polysaccharide (*neuC*), group II capsule
118 antigen (*kpsMTII*), outer membrane protein (*ompA*), transfer protein (*traT*), heme
119 receptor gene (*chuA*), catecholate siderophore receptor (*iroN*), iron transport system
120 genes (*sitD* chr., *sitD* ep.), haemolysin A (*hlyA*), invasins (*gimB*, *ibeA*), serin protease

121 autotransporter (*pic*), different adhesins and fimbrial genes (*afa/draB*, *fimC*, *hra*, *iha*,
122 *sfa/foCD*, *K88*, *K99*, *987P*, *F41*, *F18*) and pathogenicity-associated island marker
123 (*RPai (malX)*). Controls for molecular assays were avian pathogenic *E. coli* (APEC)
124 strain IMT2470, UPEC strains IMT7920 and IMT9267 and ETEC strains IMT204,
125 IMT19, IMT4830 and IMT3838 (Casey and Bosworth, 2009; Ewers et al., 2007),
126 kindly provided by the Institute of Microbiology and Epizootics of the Free University
127 Berlin.

128 *Statistical analysis*

129 The statistical analysis was performed using the procedures FREQ and CORR from
130 the Statistical Analysis System (SAS Institute Inc., 2005). Chi square-tests were used
131 to analyse differences in virulence gene frequencies between diseased and healthy
132 sows. Statistical significance was indicated in two levels: $P < 0.05^*$ and $P < 0.01^{**}$.

133 Pearson correlation coefficients, calculated to show associations between virulence
134 genes, were presented as heatmaps. Heatmaps representing gene prevalence were
135 generated to allow assessment of the virulence genes regarding occurrence and
136 distribution (R Development Core Team, 2009). Virulence genes in the heatmaps
137 were arranged automatically according to their means. Genes with similar means are
138 ordered close together.

139 Correlations and heatmaps were performed with only those virulence genes detected
140 in more than 1 % of the analysed *E. coli* strains.

141

142

143

144

145 **Results**

146 *Escherichia coli* strains

147 *Escherichia coli* was found in 70.6 % (n=724) of the milk samples of CM-affected and
148 in 77.9 % (n=762) of the milk samples of non-infected sows. In total, 1,132 *E.coli*
149 isolates from CM-positive samples and 1,271 isolates from CM-negative samples
150 were identified and further examined by mPCR. The median number of isolates in
151 milk samples of both diseased and healthy sows was one (Figure 1).

152 Of the 2,403 *E. coli* isolates, 593 harbored one virulence gene, 983 two, 357 three
153 and 369 four or more virulence genes. In 101 *E. coli* isolates, no virulence-associated
154 genes were found. The *E. coli* isolates from CM-positive as well as from negative
155 sows had a median number of two virulence genes.

156 *Comparison of virulence gene profiles*

157 A variety of virulence genes was identified consisting of mainly those associated with
158 ExPEC (98.9 % of *E. coli* isolates from diseased and 99.0 % of *E. coli* isolates from
159 healthy sows) (Table 1). The highest prevalence was found for the type 1 fimbriae
160 *fimC* (in 84.7 % of the isolates of diseased and 82.3 % of the isolates of healthy
161 sows) and for the protectins *ompA* and *traT* (in 35.3 % and 52.1 % of the isolates
162 from CM-positive, and 37.6 % and 49.8 % of the isolates from CM-negative sows,
163 respectively). Other genes identified in 9.3 to 14.8 % of the *E. coli* isolates were *hra*,
164 *kpsMTII* and *iroN*. Almost all of the virulence-associated factors were more often
165 detected in *E. coli* isolates of CM-affected sows than in isolates of healthy sows,
166 except *987P*, *neuC*, *ompA* and *gimB*.

167 The virulence genes *hra*, *chuA*, *iroN* and *kpsMTII* occurred significantly more
168 frequently in isolates of diseased animals. The same applied for particular

169 combinations of these genes (Table 3), except for the profiles *chuA* - *iroN*, *kpsMTII* –
170 *chuA* - *iroN* and *kpsMTII* – *hra* – *chuA* - *iroN*. Those combinations were also less
171 prevalent in all *E. coli* isolates. The greatest difference between diseased and healthy
172 sows was found for the virulence gene profile *chuA* - *hra* (2.7 % in *E. coli* of CM-
173 positive and 0.9 % in *E. coli* of CM-negative sampled sows, respectively). In total,
174 there were no obvious patterns specific for either diseased or healthy sows.

175 *Correlations between virulence genes*

176 Statistical analysis of associations between all virulence factors of the *E. coli* isolates
177 is shown in Figure 2. Several similar patterns in the heatmaps were visible for
178 virulence genes of strains from CM-positive and negative sows: the gene *hlyA* is
179 positively associated with *chuA* and *pic*; *iroN* is positively associated with *ompA* and
180 *sitDepi*, respectively. Highest positive correlations existed between the genes *iroN*
181 and *sitDepi* for both isolates from diseased and healthy sows. The genes *traT* and
182 *fimC* were also highly positive correlated, but only in *E. coli* isolates of CM-negative
183 sows.

184 *Gene prevalence with regard to different seasons and farms*

185 The gene *traT* was more often found in *E. coli* isolates of samples of CM-positive
186 sows in winter whereas *STI* (heat stable toxin a) was only found in summer (Figure
187 3). The gene *chuA* occurred more frequently in *E. coli* isolates of positive sows in
188 winter and autumn and *iroN* in summer, autumn and winter as well as *kpsMTII* was
189 always more prevalent in samples of diseased sows. All virulence genes were found
190 more often in *E. coli* isolates of diseased sows in all seasons except for *ompA* and
191 *traT* which were more prevalent in isolates of healthy sows in spring.

192 However, the differences in occurrence of the genes were greater between the
193 seasons than between CM-positive and negative sows.

194 The same held true for the influence of the farms on the occurrence of virulence
195 genes. The gene *STI* was only found in *E. coli* isolates sampled from farm A whereas
196 *kpsMTII* was more prevalent in samples from farm D. The gene *traT* occurred more
197 often in isolates from diseased sows. The gene prevalence on the farms differed only
198 slightly between CM-infected and healthy sows. Differences regarding the
199 occurrence of the mentioned virulence genes in the seasons and farms were
200 significant ($P<0.05$).

201

202 **Discussion**

203 The aim of the study was to analyse and compare virulence genes of *E. coli* isolates
204 from milk samples of CM-positive and CM-negative sows, because virulence gene
205 profiles of *E. coli* isolates associated with mastitis has not been described so far
206 (Kaipainen et al., 2002; Srinivasan et al., 2007; Wenz et al., 2006). *Escherichia coli* is
207 the pathogen most frequently isolated in association with porcine puerperal disorders
208 (Armstrong et al., 1968; Awad Masalmeh et al., 1990; Bertschinger et al., 1977a;
209 Ross et al., 1981). It was also isolated in high frequencies in milk samples of
210 diseased sows in this investigation as well as in milk from healthy sows.

211 The detailed analysis of virulence-associated genes of the *E. coli* isolates revealed
212 only slight differences between isolates of diseased and healthy sows ($P<0.05$).

213 Although there were single genes or gene combinations with a greater linkage to *E.*
214 *coli* isolates from milk samples of CM-affected sows, there were no specific virulence
215 gene patterns detectable. Heatmaps were performed to allow a visualization of

216 correlations among virulence genes of isolates of different CM-status, seasons and
217 farms.

218 The *E. coli* strains were isolated using an enrichment of the milk samples. This
219 qualitative culture procedure was used to promote the growth of the *E. coli* strains, as
220 described before for faecal samples (Hussain et al., 2010; Wu et al., 2010).
221 Regarding the actual presence of virulence genes, an influence of enrichment
222 procedures has only been described in detail for STEC (Vimont et al. 2007), but has
223 not been proven for incubation in Caso-Broth for the applied duration. However, a
224 possible influence on the quantitative proportion of different strains cannot be
225 excluded though faecal contamination of the samples was minimized by a strict
226 sampling protocol.

227 *Escherichia coli* strains causing acute coliform mastitis in dairy cattle originate from
228 the animal's faecally contaminated environment and infect the udder via the teat
229 canal (Eberhart, 1984). Experiments by Bertschinger et al. (1990) and Bertschinger
230 et al. (1977b), where the mammary glands of sows were protected against faecal
231 contamination, support the theory of a galactogenous route of infection via the teat
232 duct. Like bovine mastitis, porcine mastitis may also resemble urinary tract infection
233 as the infection may be ascending (Kaipainen et al., 2002). Among others, causative
234 agents of urinary tract infections (UTI) are UPEC, a pathotype of ExPEC. In contrast
235 to commensal *E. coli* isolates, UPEC harbor more virulence genes encoding virulent
236 capsule antigens, iron acquisition systems, adhesions and secreted toxins (Wiles et
237 al., 2008). The virulence genes *iroN* and *fimC* are reported as urovirulence factors
238 (Russo et al., 2002; Wiles et al., 2008) and were also identified in high percentages
239 in our study. In a survey by Won et al. (2009), the presence of 19 virulence-

240 associated genes in avian pathogenic *E. coli* (APEC), another pathotype of ExPEC,
241 was determined, and approximately 95 % of the APEC isolates possessed *fimC*.
242 However, *fimC* has also been frequently detected in non-pathogenic *E. coli* and is
243 proposed to be not highly associated with the pathogenesis of APEC-infections
244 (Kawano et al., 2006). We also found the fimbrial gene *fimC* in high prevalence in
245 isolates of healthy sows, confirming this theory.

246 The *traT* gene, detected in half of the examined *E. coli* strains, was found in milk of
247 CM-affected dairy cattle, too. Out of 160 Finnish isolates from cows with mastitis, 37
248 %, and out of 113 Israeli isolates, 41 % harbored *traT* (Kaipainen et al., 2002).
249 Nemeth et al. (1991) identified the gene in 43 % of *E. coli* strains isolated from the
250 milk of cows with mastitis. In another study by Acik et al. (2004), milk samples from
251 healthy cows and sheep were analysed and *traT* was present in 62.3 % of all isolates
252 (62.5 % of the isolates from cows and 60 % of the isolates from sheep).

253 All in all, a spectrum of virulence genes was present in bovine mastitis strains of *E.*
254 *coli*, but those strains do not possess specific virulence factors contributing to clinical
255 disease. Serum resistance was the only virulence property of *E. coli* consistently
256 associated with isolates of coliform mastitis in dairy cattle (Barrow and Hill, 1989;
257 Fang and Pyorala, 1996). A relationship between *traT* and serum resistance,
258 however, could not be confirmed (Nemeth et al., 1991; Vandekerchove et al., 2005).

259 The results and conclusions concerning the virulence genes related to bovine
260 mastitis are comparable to the findings of our study in sows. Specific sow factors,
261 e.g. the individual disposition of the animal, are probably more important and the host
262 defense status is generally accepted as key factor determining the outcome of the
263 disease (Burvenich et al., 2003). Current investigations deal with the genetic

264 background of CM via genotyping of diseased and healthy sows (Preißler et al.,
265 unpublished data).

266 In conclusion, a variety of virulence genes was detected among the *E. coli* isolates
267 for both samples from CM-positive and negative sows. The identified virulence genes
268 belonged mainly to the large group of genes related to ExPEC, but a categorization
269 into the pathotype ExPEC only by virulence gene typing was not possible. Many
270 virulence-associated factors (e.g. for iron-uptake systems, fimbriae and other
271 adhesions) are fitness factors which help the bacteria to adapt to and successfully
272 colonize the host so that the line between virulence and fitness properties of *E. coli*
273 strains is very thin (Dobrindt, 2005).

274 The results of our study support the hypothesis that any given *E. coli* strain, even
275 those considered to be non-pathogenic, can cause coliform mastitis in sows, if further
276 adversely environmental, genetic or other influencing factors promoting infection are
277 present.

278

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289

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Table 1: Prevalence of virulence-associated genes in *E. coli* isolates from healthy/ diseased sows (P*<0.05)**

Gene(s)/categories		prevalence of virulence-associated genes (%)				<i>P</i> -value
		<i>E. coli</i> isolates (n = 1,271) of CM-negative sows	no. of farms with isolates with the respective gene	<i>E. coli</i> isolates (n = 1,132) of CM-positive sows	no. of farms with isolates with the respective gene	
Adhesins						
<i>afa / dra</i>	ExPEC	-	-	-	-	-
<i>fimC</i>	ExPEC	82.30	4	84.72	4	0.1112
<i>hra*</i>	ExPEC	11.33	4	14.84	4	0.0106
<i>iha</i>	ExPEC	0.16	2	0.18	2	0.9077
<i>sfa / foc</i>	ExPEC	0.08	1	0.18	2	0.4971
<i>K99 (fanA)</i>	ETEC	-	-	-	-	-
<i>K88 (faeG)</i>	ETEC	0.08	1	0.09	1	0.9367
<i>987P (fasA)</i>	ETEC	0.08	1	-	-	0.3443
<i>F18 (fedA)</i>	ETEC	-	-	0.09	1	0.2892
<i>F41 (fedA subunit)</i>	ETEC	-	-	-	-	-
Iron acquisition						
<i>chuA*</i>	ExPEC	4.80	4	6.71	4	0.0434
<i>iroN*</i>	ExPEC	9.28	5	12.37	5	0.0148
<i>sitD chr.</i>	ExPEC	0.24	3	0.62	3	0.1461
<i>sitD epi.</i>	ExPEC	5.74	5	6.27	5	0.5858
Protectins						
<i>neuC</i>	ExPEC	0.39	2	0.18	2	0.3251
<i>kpsMT II*</i>	ExPEC	9.99	4	13.07	4	0.0178
<i>ompA</i>	ExPEC	37.61	5	35.34	5	0.2480
<i>traT</i>	ExPEC	49.80	5	52.12	5	0.2568
Toxins						
<i>hlyA</i>	ExPEC	1.65	4	2.56	3	0.1189
Enterotoxins						
<i>STII</i>	ETEC	-	-	0.18	1	0.1338
<i>STI</i>	ETEC	2.28	2	1.94	1	0.5658
<i>LT</i>	ETEC	-	-	0.09	1	0.2892
Shiga Toxins						
<i>Stx2e</i>	STEC	-	-	-	-	-
Invasins						
<i>gimB</i>	ExPEC	0.08	1	0.00	-	0.3452
<i>ibeA</i>	ExPEC	0.63	3	0.97	2	0.3443
Miscellaneous						
<i>pic</i>	ExPEC	0.63	4	1.33	3	0.0804
<i>malX (RPai)</i>	ExPEC	-	-	0.18	1	0.1338

Table 2: Number of milk samples and *E. coli* isolates of five different farms

farm	number of milk samples		number of <i>E. coli</i> isolates	
	CM-negative	CM-positive	CM-negative	CM-positive
A	498	501	600	477
B	13	15	16	21
C	276	323	460	481
D	25	20	32	27
E	167	167	163	126
total	979	1,026	1,271	1,132

Table 3: Prevalence of virulence gene profiles in *E. coli* isolates from clinically CM-diseased and healthy sows (* $P < 0.05$, ** $P < 0.01$)

Virulence gene profile	prevalence of <i>E. coli</i> isolates (%) with respective gene profile from:		P-value
	samples (n = 979) of CM-negative sows	samples (n = 1,026) of CM-positive sows	
<i>kpsMTII</i> *	9.99	13.07	0.0178
<i>chuA</i> *	4.80	6.71	0.0434
<i>hra</i> *	11.33	14.84	0.0106
<i>iroN</i> *	9.28	12.37	0.0148
<i>kpsMTII, chuA</i> **	0.87	2.21	0.0068
<i>kpsMTII, hra</i> **	2.44	4.42	0.0073
<i>kpsMTII, iroN</i> **	1.42	3.09	0.0052
<i>chuA, hra</i> **	0.94	2.65	0.0014
<i>chuA, iroN</i>	0.24	0.44	0.3823
<i>hra, iroN</i> *	1.34	2.65	0.0204
<i>kpsMTII, chuA, hra</i> *	0.63	1.59	0.0231
<i>kpsMTII, chuA, iroN</i>	0.08	0.27	0.2634
<i>kpsMTII, hra, iroN</i> **	0.31	1.33	0.0052
<i>chuA, hra, iroN</i> *	-	0.35	0.0339
<i>kpsMTII, chuA, hra, iroN</i>	-	0.27	0.0663

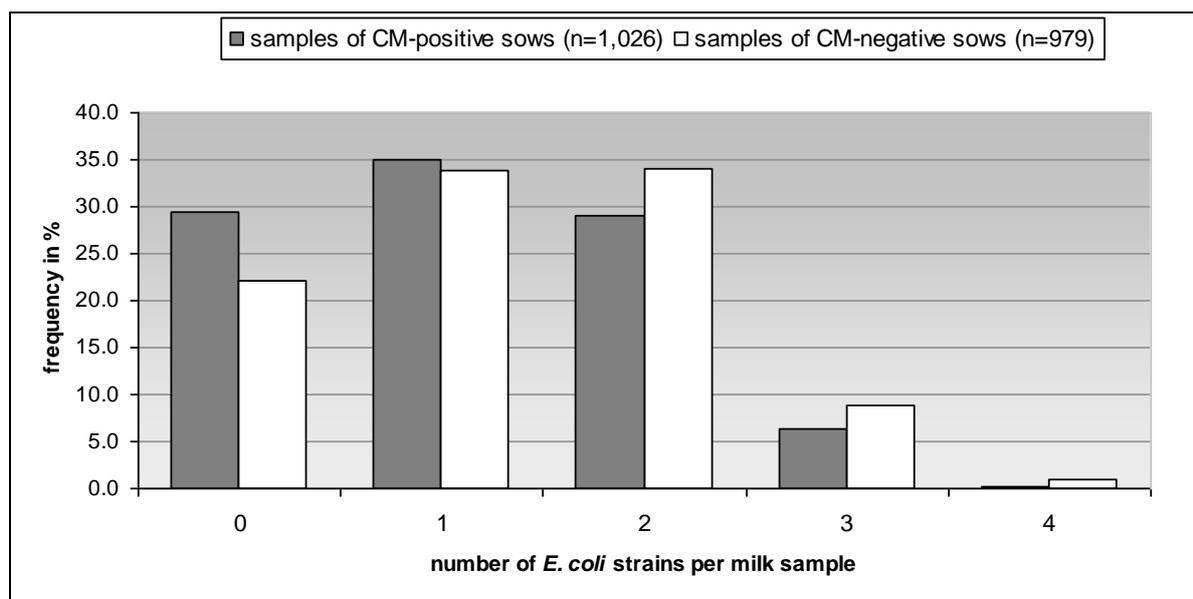


Figure 1: Number of *E. coli* isolates in milk samples from CM-positive and negative sows

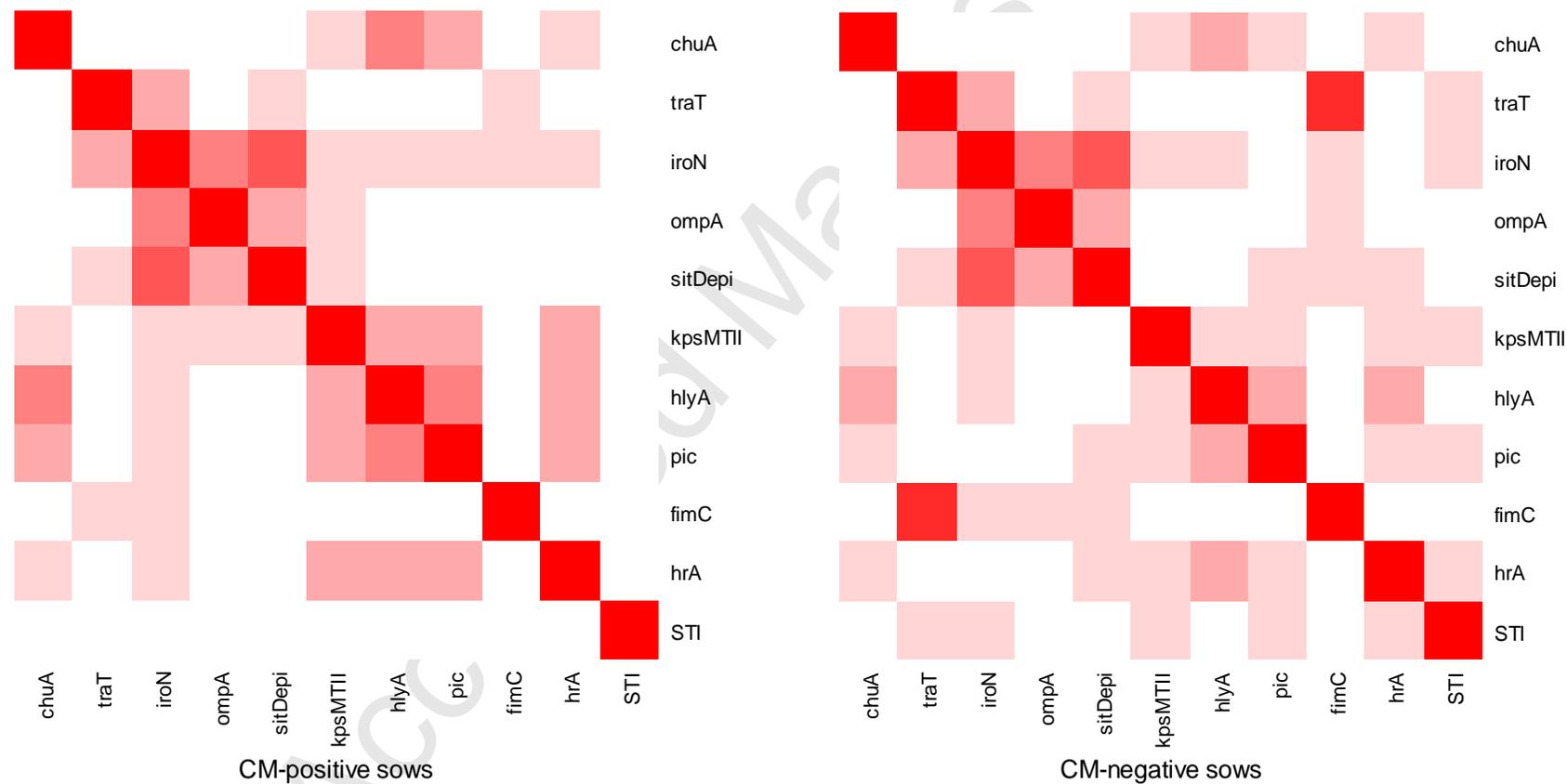


Figure 2: Statistical associations between 12 virulence-associated genes from *E. coli* isolates of CM-positive and CM-negative sows. Colours range from light red (little associated) to dark red (highly associated) ($p < 0.05$). Gaping spaces indicate no significant correlation between virulence genes.

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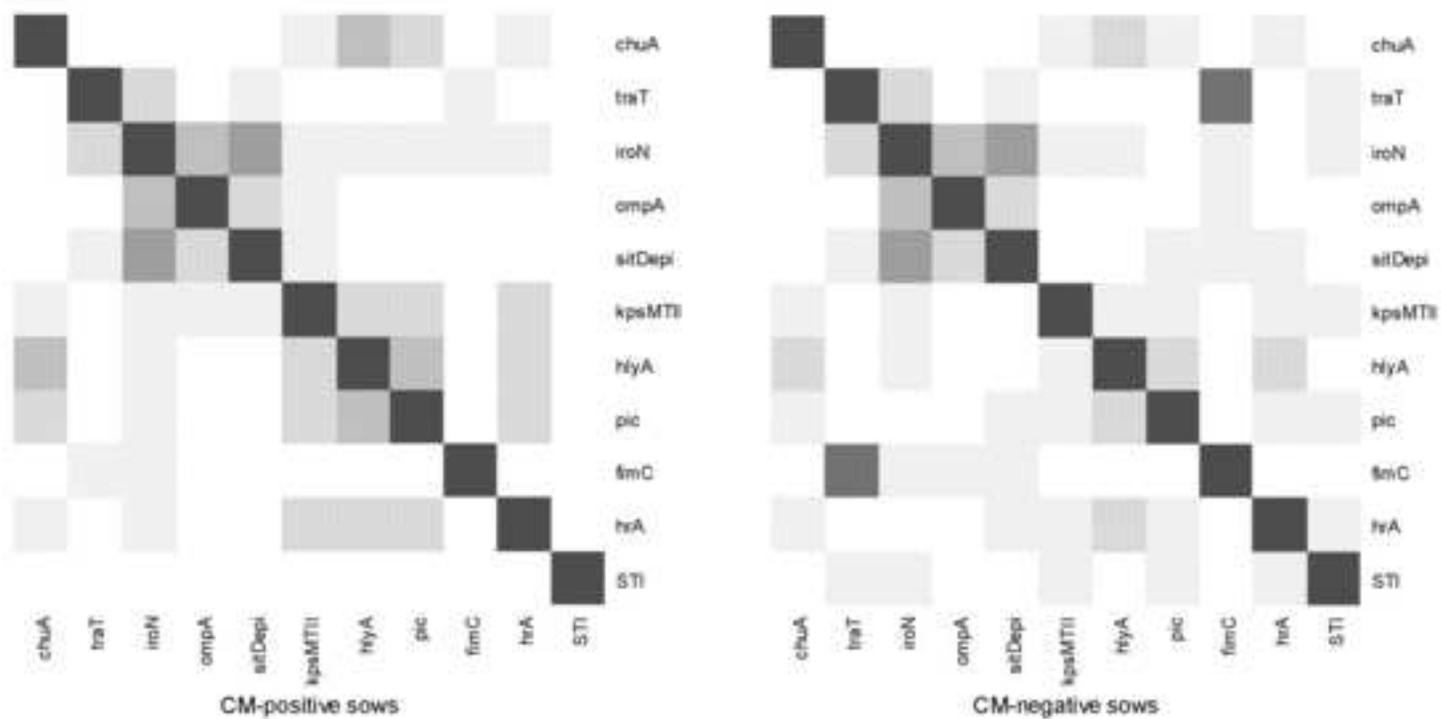


Figure 2: Statistical associations between 12 virulence-associated genes from *E. coli* isolates of CM-positive and CM-negative sows. Colours range from light grey (little associated) to dark grey (highly associated) ($p < 0.05$). Gaping spaces indicate no significant correlation between virulence genes.

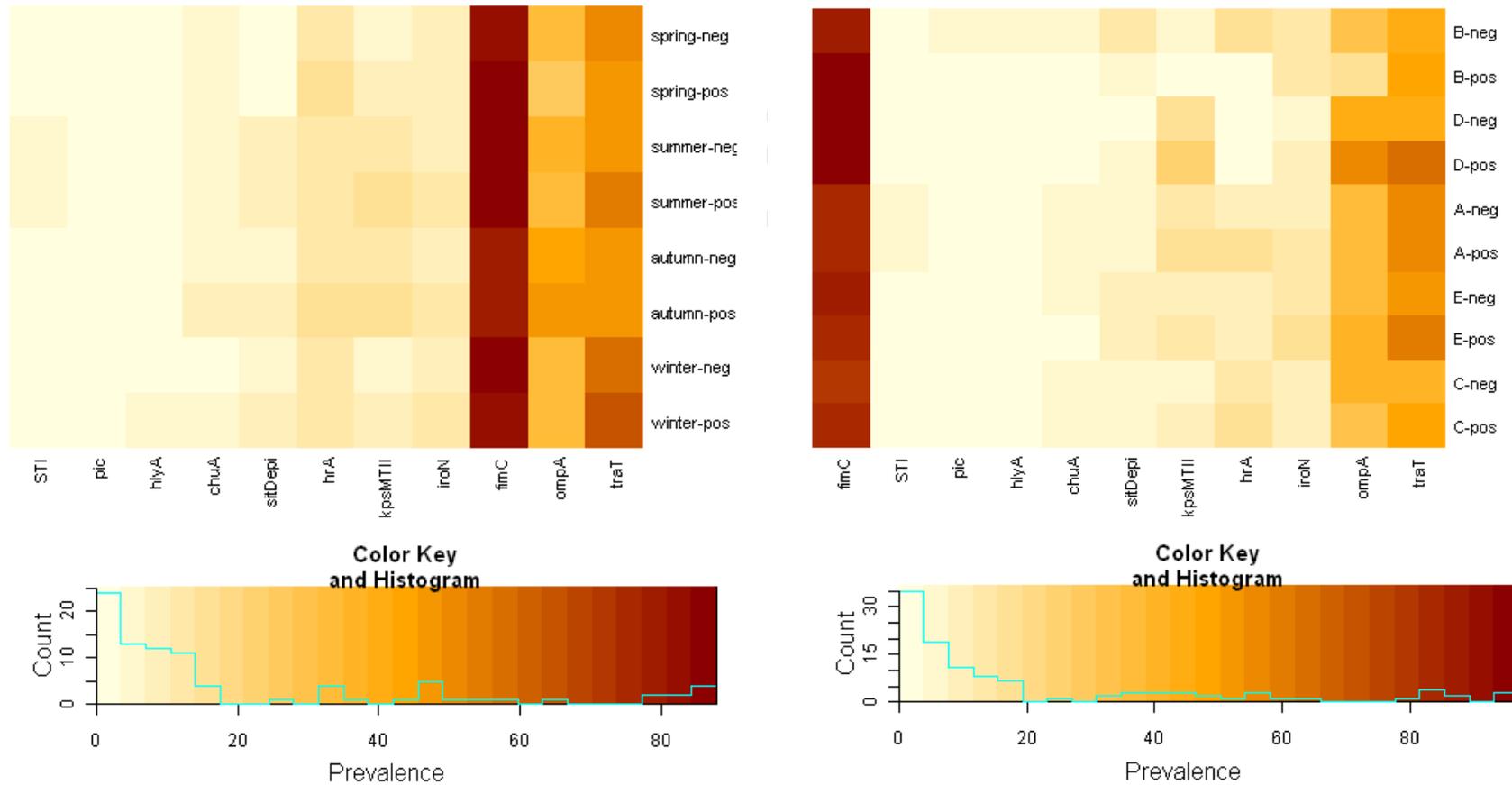


Figure 3: Heatmaps representing gene prevalence in *E. coli* isolates (n=2,403) of different CM-status (neg, pos), seasons (spring, summer, autumn, winter) and farms (A, B, C, D, E). Colours range from light yellow (gene found in 1 - 5 % of the isolates) to dark red (gene found in 80 - 88 % of the isolates).

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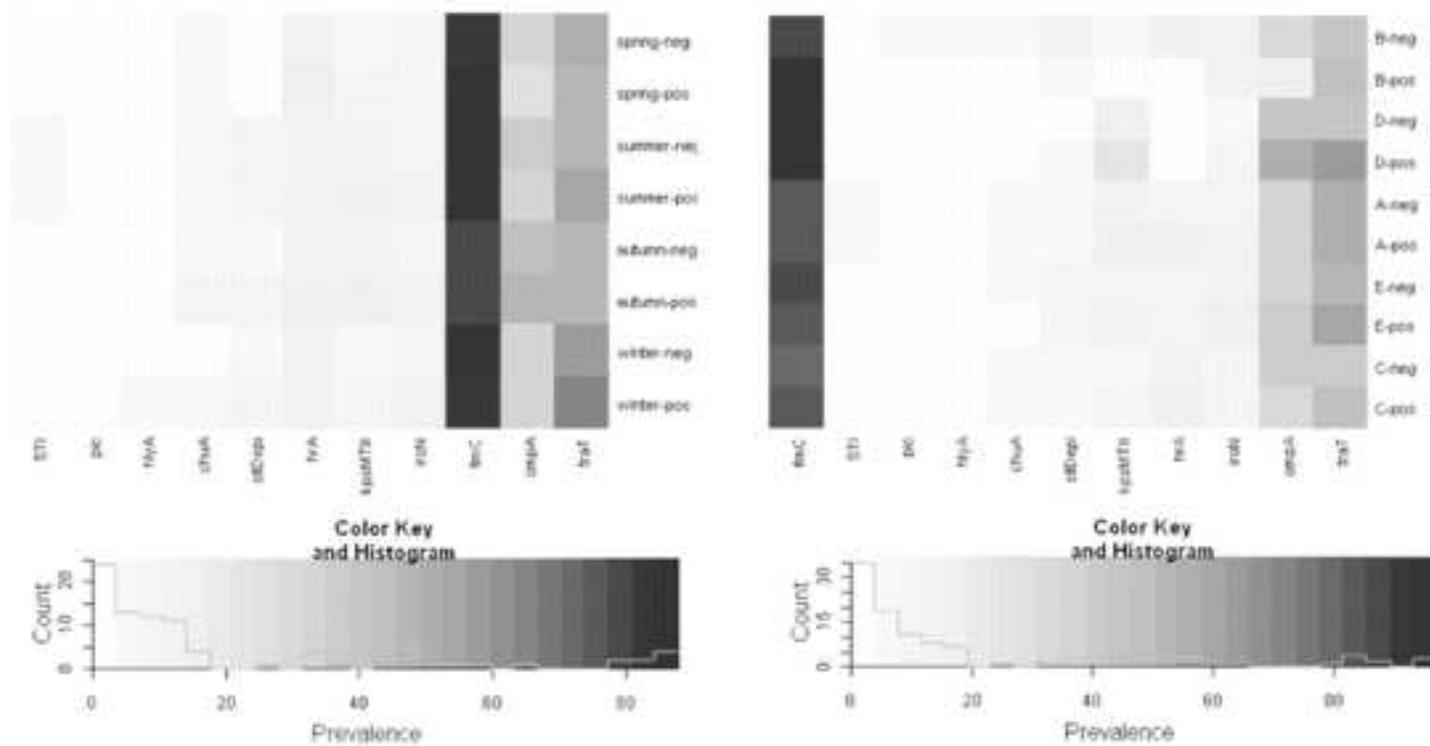


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