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

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Abstract

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

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

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

Keywords

ETEC, ExPEC, multiplex PCR, swine, virulence factors

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Introduction

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

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

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

Materials and methods

Animals and study design

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

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

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

Bacteriological analysis

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

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

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

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

Statistical analysis

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

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

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

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Results

Escherichia coli strains

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

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

Comparison of virulence gene profiles

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

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

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

Correlations between virulence genes

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

Gene prevalence with regard to different seasons and farms

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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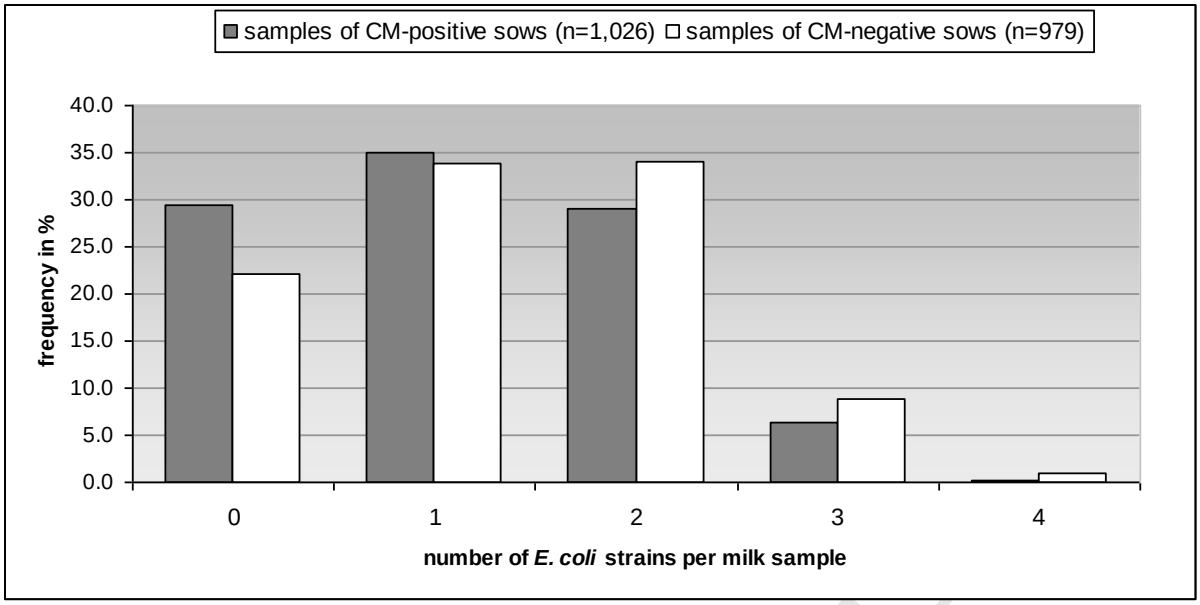

Figure 1: Number of *E. coli* isolates in milk samples from CM-positive and negative sows

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Figure 2

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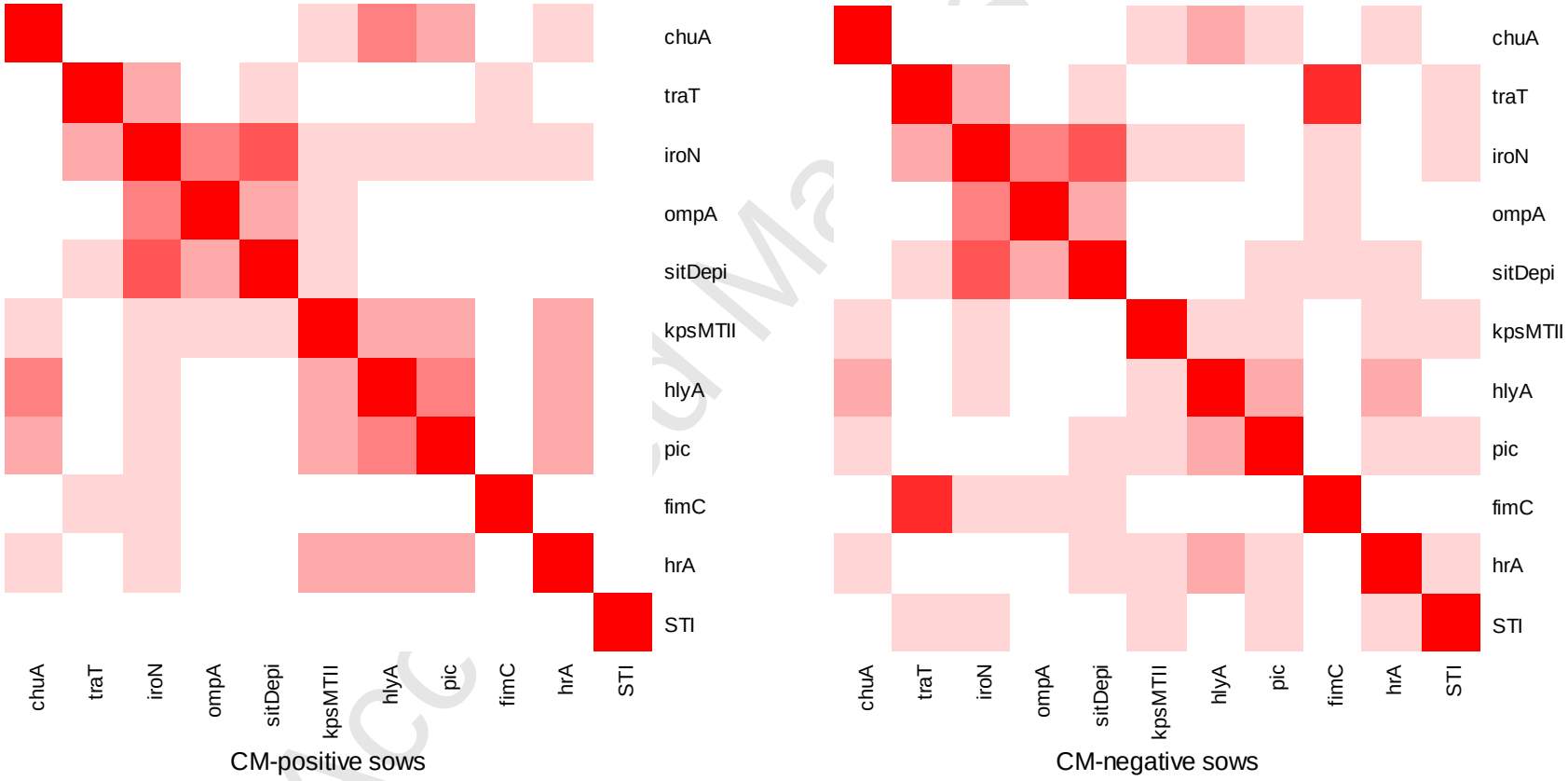

Figure 2: Statistical associations between 12 virulence-associated genes from *E. coli* isolates of CM-positive and CM-negative sows. Colours range from light red (little associated) to dark red (highly associated) ($p<0.05$). Gaping spaces indicate no significant correlation between virulence genes.

Figure 2 printed publication only
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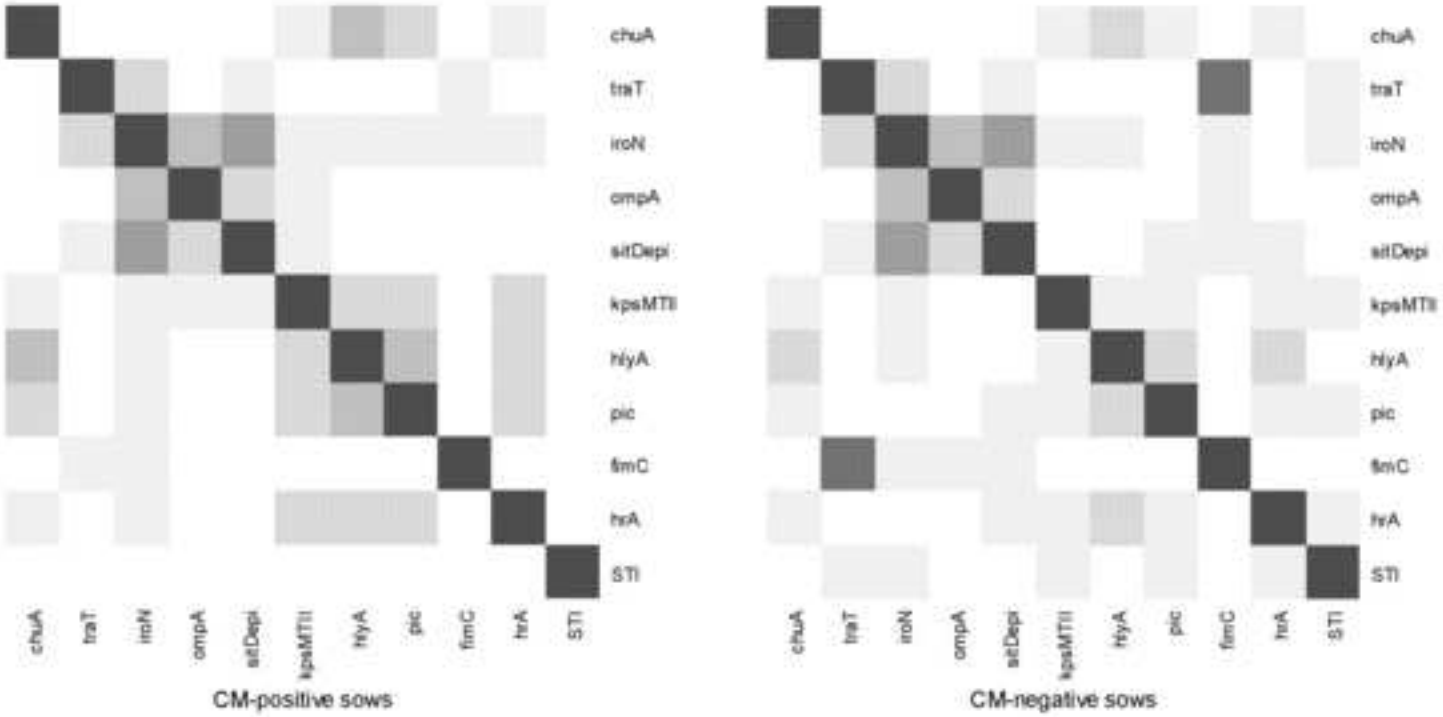

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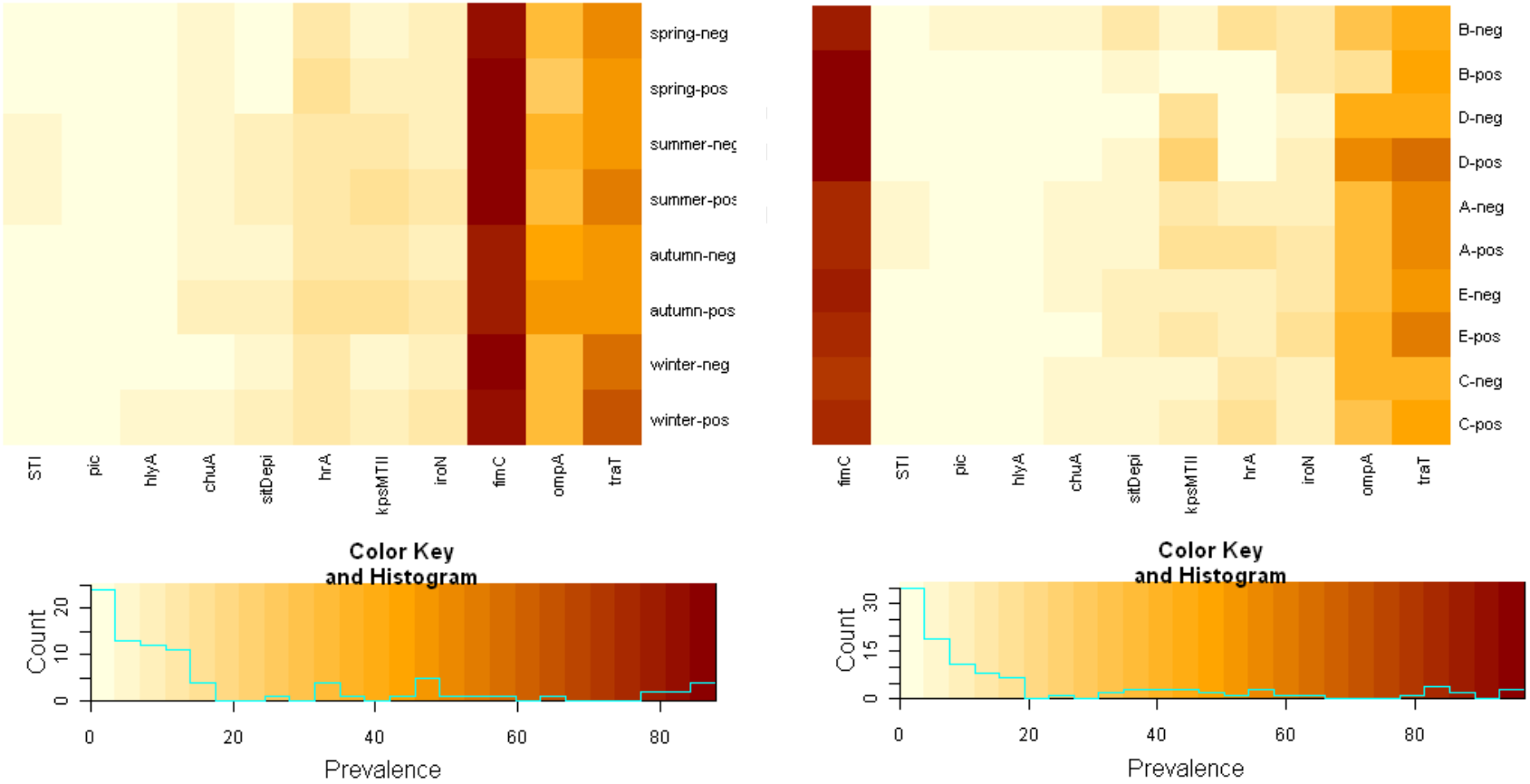

Figure 3: Heatmaps representing gene prevalence in *E. coli* isolates (n=2,403) of different CM-status (neg, pos), seasons (spring, summer, autumn, winter) and farms (A, B, C, D, E). Colours range from light yellow (gene found in 1 - 5 % of the isolates) to dark red (gene found in 80 - 88 % of the isolates).

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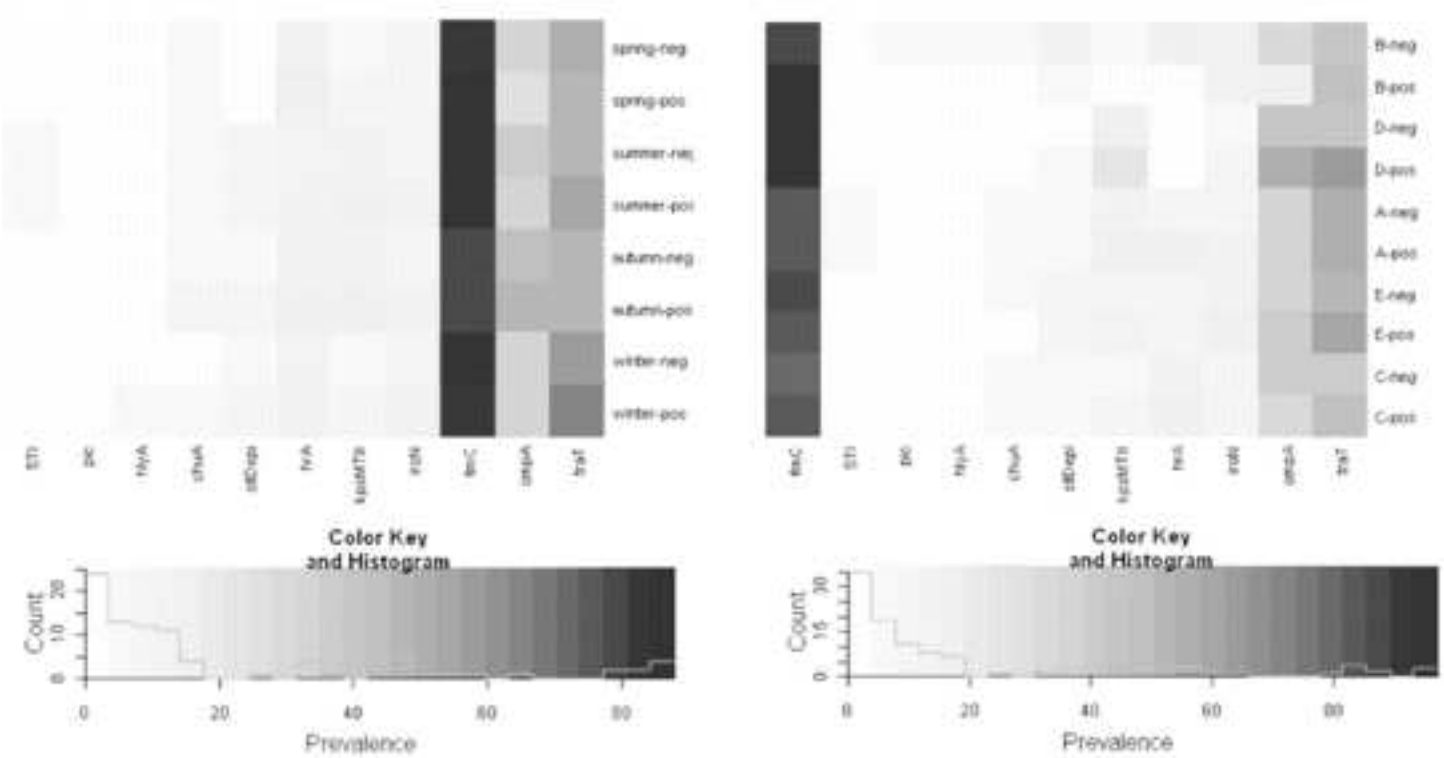

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