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Abstract

This study was conducted to investigate the occurrence and genetic characteristics of extended spectrum β-lactamase (ESBL) and methicillin-resistant *Staphylococcus aureus* (MRSA) in 80 samples of Swiss (n=36) and imported (n=44) raw chicken meat collected at retail level. In addition, ESBL-producers were screened for the presence of the plasmid-mediated colistin resistance gene *mcr-1*. Countries of import included Argentina (n=2), Austria (n=1), Brazil (n=3), Denmark (n=5), France (n=1), Germany (n=13), Hungary (n=5), Italy (n=8), and Slovenia (n=6). Forty ESBL-producing *E. coli* strains were isolated from 33 (41.3%) of the 80 samples, comprising seven (19.4%) of the Swiss and 26 (59%) of the imported samples. The most common bla_{ESBL} among the isolates were $bla_{CTX\text{-}M\text{-}1}$ (n=14) and $bla_{SHV\text{-}12}$ (n=16). Other genes comprised $bla_{TEM\text{-}52}$ (n=4), $bla_{CTX\text{-}M\text{-}2}$ (n=3), $bla_{CTX\text{-}M\text{-}8}$ (n=1), $bla_{CTX\text{-}M\text{-}14}$ (n=1) and a novel $bla_{CTX\text{-}M\text{-}14}$-like variant (n=1). Two ESBL-producers isolated from samples from Germany (n=1) and Italy (n=1) tested additionally positive for the plasmid-mediated colistin resistance gene *mcr-1*. Six (7.5%) samples, all imported from Germany, were found to contain MRSA. Three isolates belonged to the livestock-associated CC398-MRSA-V-t034, and 3 to CC9-MRSA-IV-t13177, described here for the first time in chicken meat.

Keywords: antibiotic resistance, *Escherichia coli*, *Staphylococcus aureus*, food, chicken meat

Molekulare Charakterisierung von ESBL-Bildnern und Methicillin-resistenten *Staphylococcus aureus* (MRSA) isoliert aus Schweizer und importierem Geflügelfleisch erhoben auf Detailhandelsstufe

Für die vorliegende Studie wurden insgesamt 80 rohe Geflügelfleischproben auf das Vorkommen von extended-spectrum β-Laktamase (ESBL)- produzierenden Enterobacteriaceae und Methicillin-resistenten *Staphylococcus aureus* (MRSA) untersucht. Zusätzlich wurden die ESBL-Bildner auf das Vorhandensein des plasmid-kodierten Gens *mcr-1* untersucht. Das Fleisch stammte aus der Schweiz (n=36), sowie aus Argentinien (n=2), Österreich (n=1), Brasilien (n=3), Dänemark (n=5), Frankreich (n=1), Deutschland (n=13), Ungarn (n=5), Italien (n=8), und Slovenien (n=6). Insgesamt wiesen 33 Proben (41.3%) ESBL-produzierende Enterobacteriaceae auf, wobei sieben (19.4%) aus Schweizer Fleisch und 26 (59%) aus importiertem Fleisch isoliert wurden. Am häufigsten wurden $bla_{CTX\text{-}M\text{-}1}$ (n=14) und $bla_{SHV\text{-}12}$ (n=16) gefunden, gefolgt von $bla_{TEM\text{-}52}$ (n=4) $bla_{CTX\text{-}M\text{-}2}$ (n=3), $bla_{CTX\text{-}M\text{-}8}$ (n=1), $bla_{CTX\text{-}M\text{-}14}$ (n=1) und eine neue $bla_{CTX\text{-}M\text{-}14}$ Genvariante (n=1). Bei 2 ESBL-Bildnern von Proben aus Deutschland (n=1) und Italien (n=1) wurde zusätzlich das *mcr-1*, ein plasmid-kodiertes Resistenzgen gegen Colistin, nachgewiesen. Sechs Proben (7.5%), welche alle aus Deutschland stammten, waren MRSA positiv. Genotypisierung ordnete 3 der Stämme dem Nutztier-assoziereten Klon CC398-MRSA-V-t034, und 3 weitere dem CC9-MRSA-IV-t13177 zu. Letzterer wird in dieser Arbeit erstmals in Geflügelfleischproben beschrieben.

Schlüsselwörter: Antibiotikaresistenz, *Escherichia coli*, *Staphylococcus aureus*, Lebensmittel, Geflügelfleisch

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Introduction

The contamination of raw meat with antimicrobial resistant bacteria such as extended-spectrum-β-lactamase (ESBL)-producing Enterobacteriaceae or methicillin resistant *Staphylococcus aureus* (MRSA) is a potential risk for consumers and food handlers to get colonized or infected (Marshall and Levy, 2011). ESBLs are enzymes that hydrolyze the β-lactam ring of extended-spectrum cephalosporins, reducing the therapeutic efficacy of modern expanded-spectrum cephalosporins. The most relevant bla_{ESBL} genes harbored by Enterobacteriaceae are plasmid-encoded $bla_{\text{CTX-M}}$- bla_{SHV}- and bla_{TEM} -variants (Bush and Jacoby, 2010). It is well recognized that food animals and meat (particularly chicken meat) are a source of ESBL-producing bacteria (Leverstein-van Hall et al., 2011; Seifert et al., 2013; Abgottspon et al., 2014; Vogt et al., 2014) and the Federal Food Safety and Veterinary Office (FSVO) national monitoring system tracks antibiotic resistance in livestock and retail meats in Switzerland (Müntener and Overesch, 2015). Furthermore, the very recent emergence of Enterobacteriaceae harboring the plasmid-mediated colistin resistance gene *mcr-1* in food animals and retail meat including chicken (Liu et al., 2015; Webb et al., 2015), is highly alarming, since colistin is a last-line antimicrobial drug for treating infections caused by multidrug resistant Gram-negative bacteria.

Resistance to β-lactams in *S. aureus* is caused by the production of the modified penicillin-binding protein 2a (PBP2a or PBP2'), which is encoded by the *mecA* or *mecC* gene located on the mobile genetic element SCC*mec* (Paterson et al., 2014). Based on multilocus sequence typing (MLST) and sequencing of repeating units within the staphylococcal protein A (*spa*) gene, MRSA isolates can be assigned to clonal complexes (CC) and *spa* types (t-types), which allows their classification as hospital-associated (HA)-, community-associated (CA)- or livestock-associated (LA)-MRSA. In Europe, the majority of MRSA in food of animal origin belong to CC398 (European Food Safety Authority (EFSA), 2009; Fessler et al., 2011). In Switzerland, LA-MRSA CC398-t034 is currently present in 26.5% of nasal swabs of healthy pigs at slaughter (Müntener and Overesch, 2015). Little is known about the prevalence of MRSA in chicken meat. In Germany and the Netherlands, MRSA was detected in 25% and 16% of raw chicken meat samples, respectively (De Boer et al., 2009; Fessler et al., 2011), whereas in 2014, only 1% of Swiss poultry meat, but 16% of imported was contaminated with MRSA (Müntener and Overesch, 2015). These data suggest that different types of ESBL and MRSA are present to varying degrees in chicken meat in different countries. Furthermore, little is known about the occurrence of *mcr-1* in Enterobacteriaceae in retail meat in Switzerland. The aim of this study was to characterize bla_{ESBL} harboring Enterobacteriaceae and MRSA in Swiss and imported raw poultry meat samples, collected at retail level. The isolates obtained during this study were subjected to molecular analysis with particular regard to their bla_{ESBL} genes and MRSA types, respectively. Moreover, ESBL-producers were screened for the presence of *mcr-1*.

Material and Methods

Chicken meat samples

Between July 2015 and August 2015, 80 raw chicken meat samples were collected from 6 supermarket stores (five different companies) in Chur and Zürich, Switzerland. All samples were in their original packages and were frozen until further analysis. The chicken meat originated from domestic (n=36) and imported poultry (n=44). Imported samples originated from Argentina (n=2), Austria (n=1), Brazil (n=3), Denmark (n=5), France (n=1), Germany (n=13), Hungary (n=5), Italy (n=8), and Slovenia (n=6).

Microbiological methods

Screening for ESBL-producers: Of each sample, 25 g were homogenized in 225 ml buffered peptone water in a stomacher (IUL Instruments, IG Zürich, Switzerland) and incubated for 18-24 h at 37 °C for enrichment of Enterobacteriaceae. Screening was performed using chromogenic medium Oxoid Brilliance™ ESBL Agar (Oxoid, Hampshire, UK) and the β-LACTA™ Test (BIO-RAD, Cressier, Switzerland) according to the manufacturers' instructions and as described previously (Zurfluh et al., 2015).

Screening for MRSA: Of each sample, 25 g were homogenized in 225ml Mueller Hinton broth containing 6.5% NaCl and incubated for 18-24 hours at 37 °C for pre-enrichment. One ml of pre-enriched culture was enriched in 5 ml Tryptone Soya Broth (TBS) containing 4 mg/L cefoxitin and 75 mg/L aztreonam for 18-24 hours at 37 °C as described by the European Food Safety Authority (2009). Screening was done using Oxoid Brilliance™ MRSA 2 Agar (Oxoid Ltd., Hampshire, UK) according to the manufacturer's instructions.

Molecular biological methods

Molecular biological analysis of *bla* and *mcr-1* genes

Screening for $bla_{\text{CTX-M}}$, bla_{TEM} and bla_{SHV} was carried out using primers described previously (Geser et al., 2012; Woodford et al., 2006; Pitout et al., 1998; Zurfluh

et al., 2015). Screening for *mcr-1* was performed using primers described previously (Liu et al., 2015). Resulting amplicons were custom sequenced by Microsynth (Balgach, Switzerland).

Phylogenetic classification of *E. coli* isolates

Each isolate was assigned by PCR to one of the four phylogenetic groups designated A, B1, B2 or D as described previously, whereby group A and B1 typically contain commensal *E. coli* strains while groups B2 and D consist of virulent extra-intestinal strains (Clermont et al., 2000).

MRSA genotyping

Sequencing of the polymorphic X region of the *spa* gene of the isolates was performed as described previously (Aires-de-Sousa et al., 2006; Johler et al., 2011). The amplicons were custom-sequenced. The obtained repeat sequences were then compared to known *spa* types on the *spa* server (http:// www. spaserver.ridom.de/). Multilocus sequence typing was performed as described by Enright et al. (2000). For the microarray-based genotyping the Genotyping Kit 2.0 (Alere, Jena, DE) following the manufacturer's instructions was applied. The samples were profiled by the platform ArrayMate Reader.

Results

Of the 80 collected samples, 33 tested positive for ESBL-producing *E. coli*, including 7 of the Swiss and 26 of the imported samples, as shown for each country in Table 1. Seven samples contained two isolates each. A total of 40 isolates were retrieved, whereof 20 (50%) harbored $bla_{\text{CTX-M}}$-, 16 (40%) bla_{SHV}- and four (10%) bla_{TEM}-variants, which are listed in Table 1. Notably, one strain harbored a new CTX-M β-lactamase derived from CTX-M-14 through a single amino acid substitution Glu(274)→Lys. Its nucleotide sequence is filed as GenBank accession number KT944354. Two ESBL-producing *E. coli* isolates from samples imported from Italy and Germany (sample ID 38 and 51, respectively) harbored *mcr-1* (Tab. 1).

Phylogenetic typing assigned nine (22%) *E. coli* isolates to group A, 11 (26.8%) to group B1 and 21 (51.2%) to group D. None of the isolates belonged to group B2.

Of the 80 samples, 6 (7.5%) tested positive for MRSA. All samples originated from Germany, corresponding to 46% of the 13 German samples. Three of the MRSA-positive samples (sample ID 51, 52 and 62) were also contaminated with ESBL-producers harboring $bla_{\text{SHV-12}}$, $bla_{\text{CTX-M-1}}$, and $bla_{\text{TEM-52}}$, respectively (Tab. 1 and 2). Three of the MRSA isolates were assigned to

Table 1: Origin and number of analyzed chicken meat samples, percent ESBL-positive samples, strain identities, bla_{ESBL} genes, presence of the *mcr-1* gene and phylogenetic groups of *E. coli* detected in meat samples.

Origin of meat sample (no/%ESBL-positive)	Strain ID	bla_{ESBL}	*mcr-1*	Phylogenetic group
Switzerland (36/19.4%)	71.2	$bla_{\text{CTX-M-1}}$	—[a]	D
	72.1	$bla_{\text{CTX-M-1}}$	—	D
	72.2	$bla_{\text{CTX-M-1}}$	—	D
	2	$bla_{\text{SHV-12}}$	—	D
	20	$bla_{\text{SHV-12}}$	—	D
	42	$bla_{\text{SHV-12}}$	—	D
	47	$bla_{\text{TEM-52}}$	—	B1
	54	$bla_{\text{TEM-52}}$	—	B1
Argentina (2/100%)	10.2	$bla_{\text{CTX-M-2}}$	—	D
	49.1	$bla_{\text{CTX-M-2}}$	—	A
	10.1	$bla_{\text{CTX-M-14}}$	—	A
	49.2	$bla_{\text{CTX-M-14}}$-like[b]	—	A
Austria 1(100%)	3	$bla_{\text{CTX-M-1}}$	—	B1
Brazil (3/66.7%)	45	$bla_{\text{CTX-M-2}}$	—	B1
	79	$bla_{\text{CTX-M-8}}$	—	A
Denmark (5/20%)	8.2	$bla_{\text{CTX-M-1}}$	—	A
Germany (13/38.5%)	52	$bla_{\text{CTX-M-1}}$	—	D
	46	$bla_{\text{SHV-12}}$	—	D
	51	$bla_{\text{SHV-12}}$	positive	B1
	32	$bla_{\text{TEM-52}}$	—	D
	62	$bla_{\text{TEM-52}}$	—	D
Hungary 5/60%)	75	$bla_{\text{CTX-M-1}}$	—	D
	6	$bla_{\text{SHV-12}}$	—	D
	48	$bla_{\text{SHV-12}}$	—	A
Italy (8/100%)	14.1	$bla_{\text{CTX-M-1}}$	—	B1
	14.2	$bla_{\text{CTX-M-1}}$	—	A
	38	$bla_{\text{CTX-M-1}}$	positive	B1
	59	$bla_{\text{CTX-M-1}}$	—	D
	65	$bla_{\text{CTX-M-1}}$	—	B1
	13	$bla_{\text{SHV-12}}$	—	D
	37.1	$bla_{\text{SHV-12}}$	—	B1
	60	$bla_{\text{SHV-12}}$	—	D
	66.1	$bla_{\text{SHV-12}}$	—	D
	66.2	$bla_{\text{SHV-12}}$	—	A
Slovenia (6/66.7%)	73.2	$bla_{\text{CTX-M-1}}$	—	D
	74.1	$bla_{\text{CTX-M-1}}$	—	D
	23	$bla_{\text{SHV-12}}$	—	B1
	43	$bla_{\text{SHV-12}}$	—	D
	73.1	$bla_{\text{SHV-12}}$	—	D
	74.2	$bla_{\text{SHV-12}}$	—	B1

[a] not detected.
[b] new $bla_{\text{CTX-M}}$-variant filed as GenBank accession number KT94435

Table 2: Genotyping data of 6 MRSA isolates from raw chicken meat imported from Germany.

Sample ID	Clonal complex	*spa* type	Resistance genotype	Virulence-associated genes
51	398	t034	*blaZ/I/R, erm(A), erm(C), tet(K), tet(M), sdrM*	—[a]
52	398	t034	*blaZ/I/R, tet(K), tet(M), sdrM*	—
63	398	t034	*blaZ/I/R, erm(A), erm(B), tet(K), tet(M), qacC, qacC, sdrM*	—
18	9	t13177	*blaZ/I/R, erm(B), aadD, fosB, qacC, qacC, sdrM*	*seg, sei, selm, seln, seln, selo, egc, selu,*
39	9	t13177	*blaZ/I/R, erm(B), aadD, fosB, qacC, qacC, sdrM*	*seg, sei, selm, seln, selo, egc, selu,*
62	9	t13177	*blaZ/I/R, erm(B), aadD, fosB, qacC, qacC, sdrM*	*seg, sei, selm, seln, seln, selo, egc, selu,*

Abbreviations: a complete list of target genes is available from the manufacturer of the *Staph. aureus* genotyping kit (www.alere-technologies.com).
[a] not detected.

CC398-MRSA-V-t034, and three to CC9-MRSA-IV-t13177, respectively (Tab. 2). Antimicrobial resistance gene profiles and enterotoxin genes detected by DNA microarray are listed in Table 2.

Discussion

The Swiss FSVO national monitoring system lists the prevalence of Enterobacteriaceae resistant to third-generation cephalosporins in Swiss and imported poultry meat as 65.5% and 85.6 %, respectively (Müntener and Overesch, 2015). Therefore, the occurrence of ESBL-producers in this study was lower than expected. With regard to imported meat, we observed a comparably low occurrence of ESBL-producers in meat from Denmark. This finding correlates with a recently reported decrease in occurrence of ESBL-producing *E. coli* in chicken meat in Denmark (Bager et al., 2015), attributed to a decline in the usage of third-generation cephalosporins in the top of the broiler breeding pyramid. Nevertheless, the overall prevalence of ESBL-producers in chicken meat available in Swiss retail stores must be expected to remain high. Moreover, 51.2% of the isolates analyzed in this study belonged to the virulent extraintestinal phylogenetic group D, and two isolates tested positive for the newly emerged colistin resistance gene *mcr-1*, further underscoring the health hazard posed by contaminated chicken meat. An additional threat to consumer health is the occurrence of MRSA in chicken meat. In Switzerland, the prevalence of MRSA in domestic and imported chicken meat is reported to be 1% and 16%, respectively, with the predominance of *spa*-type t-034 (Müntener and Overesch, 2015). The present study revealed a high rate of contamination of German meat with MRSA, confirming previous results (Müntener and Overesch, 2015). LA-MRSA CC398-MRSA-V-t034, detected in three samples in this study is associated with colonization of occupationally exposed humans (e.g. farmers, veterinarians, workers at abattoirs) and in rare instances may also cause disease in humans (Köck et al., 2013). Unlike the CC398 isolates, the three CC9-MRSA-IV-t13177 isolates harbored genes corresponding to major staphylococcal enterotoxins responsible for toxemia syndromes in humans (Jarraud et al., 2001). This is the first description of *spa*-type t13177 in chicken meat. Its detection suggests that this lineage may have become established in the poultry population in Germany. Further investigations are needed in order to identify potential sources of this *spa* type. This study provides further evidence that chicken meat must be considered an important source of ESBL and MCR-1 producing Enterobacteriaceae and MRSA. The data of this study underline the importance of a correct and conscious kitchen hygiene in order to avoid cross contamination of ready-to-eat food.

Caractérisation moléculaire des germes producteurs d'ESBL et des *Staphylococcus aureus* résistants à la méthicilline (MRSA) isolés sur la viande de volaille suisse et importée prélevée au niveau du commerce de détail

Dans le cadre de la présente étude, on a examiné au total 80 échantillons de viande de volaille crue quant à la présence d'entérobactéries productrices de bétalactamase avec spectre élargi (ESBL) ainsi que de *Staphylococcus aureus* résistants à la méthicilline (MRSA). En outre les germes producteurs d'ESBL ont été examinés quant à la présence du gène *mcr-1*. La viande provenait de Suisse (n=36) ainsi que d'Argentine (n=2), d'Autriche (n=1), du Brésil (n=3), du Danemark (n=5), de France (n=1), d'Allemagne (n=13), de Hongrie (n=5), d'Italie (n=8) et de Slovénie (n=6). Au total, 33 échantillons (41.3%) contenaient des entérobactéries producteurs d'ESBL, 7 d'entre eux (19.4%) provenant de viandes suisses et 26 (59%) de viandes importées. Les variantes de gènes les plus fréquentes étaient $bla_{\text{CTX-M-1}}$ (n=14) et $bla_{\text{SHV-12}}$ (n=16) suivis par $bla_{\text{TEM-52}}$ (n=4) $bla_{\text{CTX-M-2}}$ (n=3), $bla_{\text{CTX-M-8}}$ (n=1), $bla_{\text{CTX-M-14}}$ (n=1) ainsi que par une nouvelle variante $bla_{\text{CTX-M-14}}$ (n=1). Sur deux germes producteurs d'ESBL isolés sur des échantillons provenant d'Allemagne (n=1) et d'Italie (n=1), on a trouvé en outre le gène *mcr-1* qui code la résistance vis-à-vis de la colistine. Six échantillons (7.5%), provenant tous d'Allemagne, étaient positifs aux MRSA. La génotypisation a permis d'en classer trois dans le clone associé aux animaux de rente CC398-MRSA-V-t034 et les trois autre dans le clone CC9-MRSA-IV-t13177. Ce dernier est décrit pour la première fois dans ce travail comme associé à des échantillons de viande de volaille.

Caratterizzazione molecolare della ESBL e dello *Staphylococcus aureus* resistente alla meticillina (MRSA) isolato nella carne di pollame Svizzera e importata proveniente dalla vendita al dettaglio

Nel presente studio, un totale di 80 campioni di carne di pollame cruda sono stati esaminati per determinare la presenza di b-lattamasi a spettro esteso (ESBL), di produzione di enterobatteri e di *Staphylococcus aureus* resistente alla meticillina (MRSA). Inoltre, i produttori di ESBL sono stati esaminati sulla presenza del plasmide codificato del gene *mcr-1*. La carne proveniva dalla Svizzera (n=36), dall'Argentina (n=2), dall'Austria (n=1), dal Brasile (n=3), dalla Danimarca (n=5), dalla Francia (n=1), dalla Germania (n=13), dall'Ungheria (n=5), dall'Italia (n=8) e dalla Slovenia (n=6). Un totale di 33 campioni (41.3%) presentavano produttori di enterobatteri ESBL, di cui sette isolati (19.4%) da carne svizzera e 26 (59%) da carni importate. Più di frequente sono stati rilevate $bla_{\text{CTX-M-1}}$ (n=14) e $bla_{\text{SHV-12}}$ (n=16), seguite da $bla_{\text{TEM-52}}$ (n=4) $bla_{\text{CTX-M-2}}$ (n=3), $bla_{\text{CTX-M-8}}$ (n=1), $bla_{\text{CTX-M-14}}$ (n=1) e una nuova variante genetica di $bla_{\text{CTX-M-14}}$ (n=1). In 2 campioni di produttori di ESBL dalla Germania (n=1) e dall'Italia (n=1) è stato inoltre dimostrata la presenza di *mcr-1*, un gene del plasmide codificato resistente alla colistina. Sei campioni (7.5%), provenienti dalla Germania, sono risultati positivi allo MRSA. La genotipizzazione ha catalogato 3 dei ceppi del clone associato agli animali da reddito CC398 MRSA V T034 e altri 3 del CC9-MRSA-IV-t13177. Quest'ultimo è descritto in questo lavoro per la prima volta nei campioni di carne di pollame.

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