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Detection and quantification of methicillin-resistant *Staphylococcus aureus* in fresh broiler meat at retail in Germany☆

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ABSTRACT

The aim of this study was to detect, to quantify and to characterize MRSA in broiler meat samples with skin. Furthermore, we compared an isolation method using a second selective enrichment step (method A) with a simpler method omitting this step (method B). For quantification we used a direct plating method on selective agar plates and a "Most probable number" (MPN) technique for estimation of low numbers of MRSA. Presumptive MRSA colonies were confirmed by MALDI-TOF and by PCR. After confirmation the isolated MRSA were characterized by *spa*-typing and, if necessary, by multi-locus sequence typing.

Method B detected more MRSA-positive samples (16.7%, n = 215) than method A (12.1%). However, method B also produced more false positive results (28.4%).The highest estimated number of MRSA in fresh broiler meat with skin was 1100 MPN/g, but in most positive samples (80.1%) the estimated numbers of MRSA were lower than 10 MPN/g. Thus, the numbers of MRSA in the samples were too low to detect using the spread plate technique. Ten different *spa*-types were identified. Six of these with 69% of the isolates were assigned to the clonal complex CC398 (t034; t011; t2576; t571; t5452; t1457). *Spa*-types t1430, t13177 and t899 can be assigned to CC9. *Spa*-type t304 was identified as MLST-type ST6.

In conclusion, we provide quantitative data on low level contamination of fresh broiler meat with MRSA. Most isolated MRSA were from livestock associated *spa*-types. Omitting the second enrichment step was associated with an increase in sensitivity but lower specificity of the cultural method.

1. Introduction

Livestock associated methicillin-resistant *Staphylococcus aureus* are widespread in animal populations and meat from livestock in Germany. However, the role of MRSA in food for the spread of LA-MRSA to humans is still considered negligible (Goerge et al., 2017; Kock et al., 2014; Petinaki and Spiliopoulou, 2012). Previous studies revealed that the occurrence of LA-MRSA in humans is mainly associated with a close contact of humans to livestock, especially among veterinarians or farmers being occupationally exposed to pigs (Sahibzada et al., 2018). Poultry meat has been reported to be much more frequently contaminated with MRSA as compared to red meat (de Boer et al., 2009). Although the prevalence of MRSA in these food products is relatively high, there is still no evidence that LA-MRSA from food is responsible for infections in humans. The reasons for the overt discrepancy are not fully understood. One hypothesis is that the number of bacteria in meat is very low and therefore not sufficient to cause colonization in humans handling or consuming meat.

Data on the quantity of MRSA on food are, however, scarce. Previous studies have used enrichment culture techniques that have a very low detection threshold. In one study the colony counts of MRSA on meat were described as below 10 cfu/g product with no further categorization (de Boer et al., 2009). A Danish study found sporadically MRSA in chicken and turkey meat using direct plating of an unusually large amount of meat (144 ± 69 g). However, they did not further quantify MRSA in the positive samples (Tang et al., 2017). A Canadian study found low levels of MRSA in different types of meat. However, the number of quantifiable samples from chicken meat was too small (only 3 quantifiable samples to derive distributions from it and the limit for quantification was 20 cfu/g). Moreover, the authors did not detect the livestock associated strains but a common human MRSA-clone (USA100). Therefore, comparability to the European situation is

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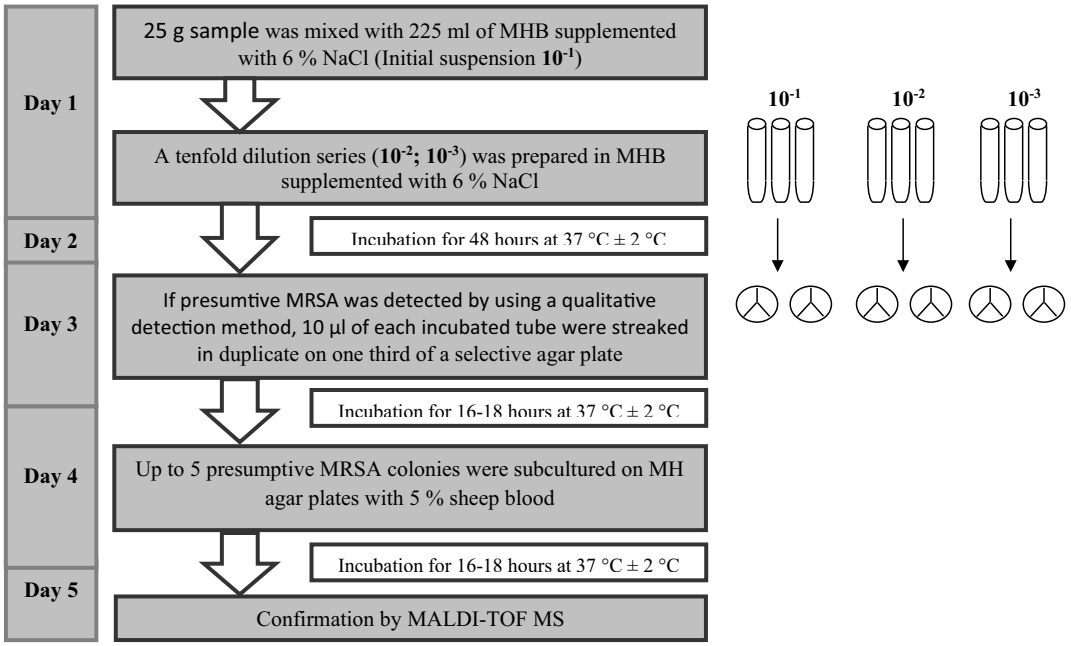

Fig. 1. Flowdiagram of the MPN-procedure to estimate the MRSA concentration in food samples.

doubtful (Weese et al., 2010).

We set up a study to quantify MRSA in fresh broiler meat at retail using the MPN technique (Munoz and Silverman, 1978) to generate quantitative estimates of low contamination levels that can be used in models of exposure and cross-contamination at the kitchen level. Moreover, we investigated the effect of one and two selective enrichment steps on the accuracy of MRSA-detection in fresh broiler meat samples. Detected MRSA isolates were characterized by *spa*-typing.

2. Material and methods

2.1. Food samples

Samples of fresh broiler meat with skin (wings, chicken legs with or without a part of the back and entire carcasses) were purchased once a week from January 2018 to March 2018 from nine different supermarkets located in Berlin and surrounding regions (Germany). During one shop visit only samples from different manufacturers and lot-numbers were selected. Altogether, 215 samples were purchased. Each test sample of 25 g contained approximately 20 g of meat and 5 g of skin. It was mixed with 225 ml of Mueller Hinton broth (MHB) supplemented with 6% NaCl in a sterile stomacher bag (nerbe plus, Winsen, Germany) and homogenized for one minute using a Smasher™ (bioMérieux, Marcy l'Etoile, France) to prepare the initial suspension (10^{-1}).

2.2. Detection methods and MRSA isolation

2.2.1. Detection methods

MRSA were isolated according to the previously described method of the National Reference Laboratory (NRL) for staphylococci including *S. aureus* at the German Federal Institute for Risk Assessment (BfR). This method was based on Vossenkuhl et al. (2014). However, the NaCl concentration was reduced to 6% after validation experiments at the BfR. After pre-enrichment of the initial suspension for 16–18 h at 37 °C ± 2 °C, 1 ml of each culture was transferred into 9 ml tryptone soy broth (TSB) supplemented with 3.5 mg/l cefoxitin and 50 mg/l aztreonam (CM0129, Oxoid, Germany). This selective enrichment was incubated for another 16–18 h at 37 °C ± 2 °C. In a final step, 50 µl of the incubated selective enrichments were plated onto chromogenic selective agar plates (ChromID® MRSA SMART – bioMérieux, Germany) (Method A). After an incubation period for 16–18 h at 37 °C ± 2 °C, MRSA colonies typically show a green colored growth on the chromogenic selective agar plates. Up to five presumptive MRSA colonies per sample were subcultured on Mueller Hinton agar plates with 5% sheep blood (Oxoid, Germany) for subsequent confirmation by MALDI-TOF MS and molecular typing. For all broiler meat samples, the same procedure was additionally carried out using the same approach without the selective enrichment step and direct plating of 50 µl of the incubated MHB supplemented with 6% NaCl onto chromogenic selective agar plates (Method B).

2.2.2. Enumeration methods

For enumeration of MRSA in the analyzed samples a spread plating technique was used. To this end, 100 µl of each initial suspension (10^{-1}) were directly transferred to chromogenic selective agar plates (chromID® MRSA SMART – bioMérieux, Germany) and spread over their surfaces using a Drigalski spatula followed by incubation for 16–18 h at 37 °C ± 2 °C. Therefore, the detection limit of this method is 100 cfu/g. After incubation up to five presumptive MRSA colonies per sample were counted and subcultured on Mueller Hinton agar plates with 5% sheep blood (Oxoid, Germany) for subsequent confirmation.

For enumeration of low numbers of MRSA in the analyzed samples a MPN technique using three successive dilutions with three replicates according to ISO 7218:2007 was used. For every sample, a tenfold dilution series of the homogenized sample (10^{-1}; 10^{-2}; 10^{-3}) was prepared in MHB supplemented with 6% NaCl and three parallel tubes per dilution (3 × 0.1 g, 3 × 0.01 g and 3 × 0.001 g) were incubated for 48 h at 37 °C ± 2 °C. If at least one of the qualitative detection methods detected presumptive MRSA in a sample, 10 µl of every incubated tube were streaked in duplicate on one third of a chromogenic selective agar plate (chromID® MRSA SMART – bioMérieux, Germany) and incubated for 16–18 h at 37 °C ± 2 °C. Up to five presumptive MRSA colonies per dilution were subcultured on Mueller Hinton agar plates with 5% sheep blood (Oxoid, Germany) for subsequent confirmation by MALDI-TOF MS and molecular typing (Fig. 1). Using the numbers of MRSA-positive tubes per dilution the MPN/g was estimated according to ISO 7218. The minimum quantification limit of the MPN-procedure is 3 MPN/g with a

confidence interval of 0.1 to 9.5 MPN/g. The MRSA-concentration was estimated as < 3MPN/g if all tubes per dilution of a MRSA-contaminated sample were MRSA-negative using the MPN-procedure.

2.3. Typing

2.3.1. MALDI-TOF MS

Whenever possible, five MRSA-colonies per method were chosen for typing. Presumptive MRSA isolates were cultured on Mueller Hinton agar plates with 5% sheep blood for 16–18 h at 37 °C ± 2 °C. One colony was spotted on a well of the MALDI target. The bacteria on the wells were covered with 0.8 µl of α-Cyano-4-hydroxycinnamic acid (HCCA, Bruker, USA). After drying, the samples were analyzed with a MALDI Biotyper (Bruker Daltonics, Bremen, Germany). Instrument parameters for the Microflex were: mass range, 1960–24,100 m/t; ion source (IS) 1, 19,96 kV; IS2, 18,16 kV; lens, 0 kV; detector gain, 2966 V. Spectra were acquired at maximum frequency with 240 shots per spot.

2.3.2. Molecular typing of MRSA

Presumptive MRSA isolates were confirmed by an in-house multiplex PCR simultaneously targeting the nuclease gene *nuc* which is specific for *S. aureus*, and the resistance gene *mecA* (Poulsen et al., 2003). Template DNA was extracted by enzymatic lysis. For this extraction method, MRSA isolates were cultured on Mueller Hinton agar plates with 5% sheep blood for 16–18 h at 37 °C ± 2 °C. One to three MRSA-colonies were mixed with 45 µl TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.6) and 5 µl lysostaphin. This solution was incubated for 45 min at 37 °C ± 2 °C. Afterwards, the solution was incubated for 12 min at 95 °C. At the end, 200 µl TE buffer was added. All MRSA isolates were further characterized using *spa*-typing (Shopsin et al., 1999). Multilocus sequence typing (MLST) according to Enright et al. (2000) was performed for all MRSA with *spa*-types that could not be assigned to CC398 based on previous typing of isolates in the National Reference Laboratory. *Spa*-type and MLST were determined using Ridom Staphtype software (Ridom GmbH, Germany) and the *S. aureus* MLST database (http://www.saureus.mlst.net).

2.4. Statistics

Results of the qualitative detection method were expressed as proportion of positive samples among the tested samples. Sensitivity and specificity of the methods with and without the selective enrichment step were compared. To this end, the combined result of the two methods was used as the gold standard. An analyzed sample was considered positive if either of the methods produced isolates that were confirmed as MRSA using MALDI-TOF MS and molecular typing. False positives using the combined result were considered highly unlikely on account of the confirmation methods used. The sensitivity was expressed as the proportion of the positive samples correctly identified by the respective cultural method. The specificity was calculated as the proportion of the MRSA-positive samples after confirmation among the presumptive MRSA-positive samples. The positive predictive value describes the probability, that a positive result is truly positive, i.e. the proportion of true positives among the positives. Conversely, the negative predictive value gives the probability that a sample tested negative is truly negative, i.e. the proportion of true negatives among the negatives.

3. Results

3.1. Proportion of positive samples using the detection methods

Method A detected fewer MRSA-positive samples (26/215, 12.1%) than method B (36/215, 16.7%, Table 1). All samples identified as positive by method A were also identified by method B. However, method A yielded fewer false-positive samples (19/215, 8.8%) compared to method B (61/215, 28.4%). By using MALDI-TOF MS the isolates from the false-positive samples were among others identified as other *Staphylococcus* spp., *Enterococcus* spp. or *Arthrobacter woluwensis*.

Table 1
Comparison of the qualitative detection methods A and B with regard to sensitivity, specificity, positive and negative predictive values and accuracy.

	With selective enrichment Method A	Without selective enrichment Method B
Fresh broiler meat samples tested (n)	215	215
No. of food samples with presumptive MRSA (n, %)	45 (20.9%)	97 (45.1%)
No. of food samples with confirmed MRSA (n, %)	26 (12.1%)	36 (16.7%)
Sensitivity	72.2%	100%
Specificity	89.4%	65.9%
Positive predictive value	57.8%	37.1%
Negative predictive value	93.1%	100%
Accuracy	86.5%	71.6%

The sensitivity of the alternative method B was 100%, i.e. higher than the sensitivity of the standard method A (72.2%). Conversely, the specificity of the standard method A (89.4%) was higher than that of the alternative method B (65. 9%). In consequence the positive predictive value of method A was higher while the negative predictive value was lower than for method B. Overall, accuracy was better for method A (Table 1).

3.2. Enumeration of MRSA in fresh broiler meat samples

MRSA-positive samples and the associated estimated MRSA-concentrations (MPN/g) are listed in Table 2. Few MRSA-positive samples (7/36, 19.4%) had MRSA concentrations of > 10 MPN/g. The highest MRSA concentration was determined in a chicken wing sample with 1100 MPN/g. All samples that were only MRSA-positive by one of the two qualitative detection methods had very low MRSA-concentrations (< 3 to 3.6 MPN/g). None of the samples was MRSA-positive using the quantitative spread plate technique. Using this technique, 19 samples showed growth of typically green colored colonies on the chromogenic selective agar plates. However, these presumptive colonies could not be confirmed as *S. aureus* using MALDI-TOF MS.

3.3. Typing

Ten different *spa*-types were identified among the 393 confirmed MRSA-isolates. Six of these *spa*-types with 69% of the isolates were assigned to the CC398 (t034; t011; t2576; t571; t5452; t1457). The other four *spa*-types (t1430; t13177; t899; t304) were not assigned to the CC398 group. *Spa*-types t1430, t13177 can be assigned to CC9, the *spa*-type t899 was identified as MLST-type ST9, and the *spa*-type t304 was identified as MLST-type ST6. In 24 of 36 MRSA-contaminated samples only one *spa*-type was detected. In twelve samples two or three different *spa*-types were detected (Table 2). The *spa*-type t034 was isolated most frequently. It was identified in 39% of the isolates and isolated from 52.8% of the positive and 8.4% of all 215 samples. t1430 from CC9 was the second most prevalent type, with 23% of isolates, one third of positive samples and 8.8% of all samples (Table 3).

4. Discussion

The prevalence of MRSA determined in the examined samples confirms previous results on broiler meat in Germany (Federal Office for Consumer Protection and Food Safety, 2017). Both qualitative detection methods can be used for detecting MRSA in broiler meat samples. However, both have their drawbacks in terms of sensitivity

Table 2
MRSA-contaminated broiler meat samples using the qualitative methods A and B, estimated MRSA-concentrations (MPN/g) with their confidence intervals (according to ISO 7218) and results of *spa*-typing and assignment to MLST-clonal complexes.

Food product	No.	Qualitative detection of MRSA		Enumeration of MRSA		MRSA isolates	Molecular typing	
		Method A	Method B	MPN/g	Confidence interval	Number of confirmed isolates	*spa*-Type	Clonal group
Thigh	1	+	+	< 3	–	5	t1430	CC9
Thighs with back	11	–	+	< 3	–	4	t1430	CC9
Thigh	16	–	+	< 3	–	3	t034	CC398
Wing	19	–	+	< 3	–	5	t13177	CC9
Wing	24	–	+	< 3	–	5	t034	CC398
Wing	39	+	+	290	90–990	23	t034	CC398
Complete carcass	41	–	+	< 3	–	5	t034	CC398
Wing	49	+	+	3.6	0.2–17	12	t1430	CC9
Half chicken	50	+	+	3.6	0.2–17	12	t1430	CC9
Thighs with back	54	+	+	< 3	–	4	t034	CC398
chicken thigh	57	+	+	< 3	–	4	t034	CC398
Complete carcass	71	–	+	< 3	–	5	t2576	CC398
Wing	73	+	+	< 3	–	10	t2576, t034	CC398,
Wing	75	+	+	< 3	–	7	t034	CC398
Thighs with back	78	+	+	9.2	1.5–35	13	t034	CC398
Leg	81	–	+	< 3	–	5	t034	CC398
Complete carcass	84	+	+	9,2	1.5–35	7	t1430, t5452	CC9, CC398
Wing	90	+	+	< 3	–	10	t011, t034	CC398,
Thighs with back	92	+	+	7.2	1.2–17	20	t5452	CC398
Wing	98	+	+	240	40–990	19	t034, t899	CC398, CC9
Lower leg	99	+	+	< 3	–	10	t571	CC398
Leg	107	+	+	150	30–380	25	t011, t034	CC398,
Thighs with back	113	+	+	9.2	1.5–35	11	t1430	CC9
Complete carcass	116	–	+	< 3	–	1	t1430	CC9
Wing	120	–	+	< 3	–	5	t034	CC398
Thighs with back	138	+	+	< 3	–	8	t034	CC398
Lower leg	139	+	+	93	18–360	20	t304, t034	CC6, CC398
Wing	141	+	+	75	17–199	24	t034	CC398
Leg	143	+	+	7.2	1.2–17	18	t011, t1430	CC398, CC9
Complete carcass	152	+	+	< 3	–	9	t034	CC398
Thighs with back	157	+	+	9.2	1.5–35	13	t011, t2576	CC398,
Thigh	170	–	+	3.6	0.2–17	6	t571, t2576, t1430	CC398, CC9
Wing	181	+	+	1100	200–4000	20	t1457, t034	CC398,
Wing	209	+	+	15	4–38	18	t1430, t571	CC9, CC398
Wing	210	+	+	7.4	1.3–20	18	t1430, t2576	CC9, CC398
Wing	213	+	+	< 3	–	9	t1430	CC9

Table 3
Proportion of *spa*-types among 393 MRSA isolates from 36 out of 215 fresh broiler meat samples with skin.

spa-Type	Isolates [%]	MRSA-positive samples [%]	All samples [%]
t034	39	52.8	8.8
t1430	23	33.3	5.6
t011	10	11.1	1.9
t2576	8	13.9	2.3
t5452	5	5.6	0.9
t571	4	8.3	1.4
t304	4	2.8	0.5
t899	3	2.8	0.5
t1457	3	2.8	0.5
t13177	1	2.8	0.5

(method A) or specificity (method B). The comparison of the methods indicates that the selective enrichment in TSB supplemented with 3.5 mg/l cefoxitin and 50 mg/l aztreonam may impair growth of MRSA strains leading to false negative results. However, it reduces the probability of false-positive results. Skipping the selective enrichment increased the detection rate of MRSA contaminations especially at very low concentrations indicating differences in detection limits (Bocher et al., 2008; Larsen et al., 2017; Van Heirstraeten et al., 2009). The choice of methods will therefore depend on the desired detection limits and on the concentrations of MRSA that are considered biologically relevant. Using both methods in parallel would not be beneficial as it would not solve the specificity issue of Method B with the associated extra labour for confirming the isolates. However, further studies should seek to combine the advantages of the two methods and solve the challenges.

None of the samples was MRSA positive by direct spread plating. This method does not foresee pre-enrichment. Therefore MRSA could not recover from potential stress factors (e.g. low temperatures in the cold chain) (Valero et al., 2009). At the same time the bacteria were exposed to the antibiotics in the agar. These stress factors may have reduced the sensitivity of the direct spread plating method in comparison to the MPN-procedure. Moreover, in consideration of the detection limit of the direct spread plating method (100 cfu/g) and the confidence intervals of the estimated MPN-results (Table 2) the MRSA-concentrations of most contaminated samples was too low for the direct spread plating method.

These very low concentrations were expected from previous reports (de Boer et al., 2009; Tang et al., 2017; Weese et al., 2010). However, no data were available that allowed for estimating the quantitative distribution of the low level contamination which is a cornerstone of modelling the quantitative development of contamination e.g. in the kitchen environment. We therefore applied the MPN technique (Munoz and Silverman, 1978) to be able to estimate the frequency distribution of the low level contamination as a baseline for modelling the spread of MRSA from fresh broiler meat in the kitchen environment via cross-contamination. The results of the MPN technique showed that most samples identified as MRSA-positive using qualitative detection methods contained very low numbers of MRSA. In 29 out of 36 MRSA-positive samples, the estimated MRSA concentration was below 10

MPN/g. Just one MRSA-positive sample harbored a higher concentration (1100 MPN/g). This is in line with the hypothesis that the number of MRSA in meat is very low and therefore not sufficient to cause colonization in humans handling or consuming meat. The challenge with this hypothesis is that neither the minimum infectious dose for humans nor the bacterial counts on retail meat or the reduction of these counts during meat handling and preparation in the kitchen are known. The infectious dose for nasal colonization has been determined in pigs and the smallest dose was 10^4 CFU (Jouy et al., 2012). Other studies reported higher doses for successful induction of colonization via the nasal route (Szabo et al., 2012).

The MRSA from 36 contaminated samples predominantly belonged to the clonal complex CC398 (69%) which is in line with previous reports on MRSA in the broiler food chain (Argudin et al., 2011; Geenen et al., 2013; Kraushaar et al., 2017). Likewise, the CC9 (27%) can be considered as LA-MRSA. It has been shown to occur frequently in East Asia (Larsen et al., 2012). On the other hand, *spa*-type t1430 from CC9 has frequently been reported from poultry food chains before (de Boer et al., 2009; Kraushaar et al., 2017; Vossenkuhl et al., 2014). The *spa*-type t304 with the MLST-type ST6 (4%), however, is considered a hospital-associated MRSA (Senok et al., 2016) indicating potential contamination with MRSA of human origin. It has not been described for the poultry food chains in Germany before.

Identification of several *spa*-types of MRSA in the same sample indicates either multiple contamination events or contamination with an MRSA-flora already made up out of several *spa*-types. Given the low detection levels of MRSA in broiler primary production and the higher contamination rates of broiler carcasses at slaughter (Federal Office for Consumer Protection and Food Safety, 2017; Kraushaar et al., 2017) the slaughterhouse is a likely source of this contamination. However, further processing steps may also have contributed to the contamination. This holds especially true for the hospital associated type. More research is needed to fully understand the dynamics of this contamination in the food chain.

5. Conclusion

The results show that omitting the selective enrichment of the samples in tryptone soy broth supplemented with 3.5 mg/l cefoxitin and 50 mg/l aztreonam may increase the detection rate of MRSA in broiler meat. However, this clearly goes at the cost of a decrease in specificity. Moreover, concentrations of MRSA were very low. Our quantitative results provide the basis for quantitative modelling of the spread of MRSA from retail broiler meat in the kitchen environment and therefore contribute to a better understanding of the potential exposure of consumers to these MRSA via food. Further research is needed to optimize the detection methods and potentially also to simplify the quantification method for a routine application.

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