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Within-farm prevalence and environmental distribution of livestock-associated methicillin-resistant *Staphylococcus aureus* in farmed mink (*Neovison vison*)

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ABSTRACT

The aim of the present study was to identify the animal prevalence and environmental reservoir of livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) in mink farms. LA-MRSA on mink constitutes a human health hazard to farmers and farm workers, who handle the animals and are at risk of bites and scratches from colonized sites. The primary route of LA-MRSA colonization of mink is suspected to be by ingestion of contaminated pig by-products.

We performed a cross-sectional study with repeated measurements during May-July 2017. A total of 644 mink carcasses (542 mink kits and 102 breeding animals) from five Danish farms were sampled. From each carcass, pharynx was swabbed and the right forepaw dissected. In addition, environmental samples covering feed, air, glove, cages (top and between) and nest boxes were collected on the farms. MRSA was selectively cultured from each sample and suspect colonies were assessed using matrix-assisted laser desorption ionisation (MALDI-TOF) for species confirmation. Further, from each farm, three isolates from mink and one isolate per positive environmental site were sent for whole genome sequencing.

We isolated LA-MRSA from mink in four out of the five farms, but LA-MRSA bacterium was detected on all farms. On farms with LA-MRSA positive animals, the overall apparent animal prevalence ranged from 20% [13;29] $_{CI95\%}$ to 29% [22;38]$_{CI95\%}$. LA-MRSA was isolated from kits before weaning, most likely due to a contaminated environment or transfer from the dam. Further, the apparent prevalence of LA-MRSA in kits tended to increase during the first months of age. On farms where LA-MRSA was isolated from mink, LA-MRSA was also isolated from the environment. LA-MRSA was isolated from all environmental sites tested (i.e. glove, on top of and between the cages and in the nest boxes), apart from air. The negative air samples contrast with the high concentrations of LA-MRSA in air found in the pig production. Hence, the risk of human exposure to LA-MRSA on mink-farms tends to be associated mainly with direct contact with contaminated environmental sites and the handling of colonized mink. All sequenced isolates were confirmed as LA-MRSA CC398 and genetically similar to clones previously isolated from the Danish pig production, supporting the hypothesis of LA-MRSA being transmitted by contaminated pig by-products.

1. Introduction

In 2013, livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) was isolated for the first time from Danish mink (*Neovison vison*), with paws and pharynx being the most frequent anatomical sites of isolation (Hansen et al., 2017). Subsequent testing of all mink, submitted for necropsy and diagnostics in 2015 at the national Danish reference laboratory, revealed that 34% (20/58) of pooled mink samples were positive for LA-MRSA. In addition, healthy mink from 40% (20/50) screened Danish mink farms were found positive (Hansen et al., 2017). In 2016, contact to mink farms was officially included as a risk factor for human MRSA carriage meaning that certain precautions have to be taken prior to admission to the healthcare system (Hansen, 2017).

MRSA is resistant towards most ß-lactams (Ito et al., 2001). The distinct clone, LA-MRSA, belonging to the clonal complex 398 (CC398)

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has its major reservoir in production animals, mainly pigs, cattle and poultry (Graveland et al., 2011) and have additionally been associated with the tetracycline resistance genes *tet*(K) and *tet*(M) (Sieber et al., 2018). In Denmark, LA-MRSA was first isolated from a human case in 2004 (DANMAP, 2016), and subsequently from the Danish pig production in 2005 (Guardabassi et al., 2007). Hereafter, the prevalence of Danish LA-MRSA positive pig farms has gradually increased, to reach 88% in 2016 (DANMAP, 2017). Furthermore, LA-MRSA has sporadically been isolated from Danish horses (Islam et al., 2017), poultry, organic pigs and veal calves (DANMAP, 2016). In these species the herd prevalence ranges from 2% to 10% and are in the latter three species likely due to spill-over from the pig industry. In Danish pig farms, three major genetic lineages have been identified, L1-L3, of which L3 has become the most dominant (Sieber et al., 2018). LA-MRSA can be introduced to pig farms by purchase of colonized pigs, while indirect transmission routes such as colonized human visitors or lorries have also been suggested (Grøntvedt et al., 2016; Sieber et al., 2018). In mink farms, contaminated feed is regarded as the most likely source of LA-MRSA (Hansen et al., 2017).

Denmark is the World's largest producer of mink with an annual production of almost 18 mill pelted skin per year (Kopenhagen Fur, 2017). In Denmark, mink are either kept in open sheds or closed halls. Both types of housing may be present on the same premise. Sheds typically host two rows, while halls may host up to twenty rows, of connected mesh-cages and consequently, the mink are in direct contact with neighbors. Each cage holds a straw-bedded nesting box (Danish Ministry of Environment- and Food, 2015). The whelping season starts in the beginning of May. Kits gradually start eating the same feed as the female from around four weeks of age. They are weaned at eight weeks of age (Danish Ministry of Environment- and Food, 2015) and pelted in November. In Denmark, mink farms receive feed from one of 13 mink feed producers (Lyhs et al., 2018), which deliver freshly produced moist feed with 1–2 days interval. On-farm, the feed is administered separately once or twice daily on top of the mesh wires. The composition of the feed varies throughout the year to match changing dietary needs of the growing mink, but is partly composed by non-heated pig by-products from the Danish pig production (Lyhs et al., 2018).

To estimate the risk of human exposure to LA-MRSA on mink farms three factors are important: The proportion of colonized animals on positive farms, the persistence on mink after exposure and the spread and persistence of LA-MRSA contamination in the farm environment. Hence, the objective of the present study was to identify the animal prevalence of LA-MRSA in five Danish mink farms and to identify environmental reservoirs for LA-MRSA on-farm.

2. Materials and methods

2.1. Study design

We performed a cross-sectional study with repeated measurements in three consecutive months starting at the whelping period (mid May 2017). As we expected an animal prevalence similar to pig herds (90%) (Dewaele et al., 2011; Espinosa-Gongora et al., 2012) and aimed at a precision of 5%, we calculated the required sample size to 138 mink per farm (Dohoo et al., 2010).

2.2. Study herds

Participating farms were selected in order for them to represent deliveries from three various feed producers. Practicing veterinarians aided in the recruitment of study farms, as they presented the study to mink producers and identified farmers interested in participation.

Information on farm demographics and antimicrobial use were based on registrations in the two national registers: the Central Husbandry Register (CHR) and VetStat. CHR holds information on the total number of mink females on-farm as annual registrations made by the farmer, while VetStat holds information on all prescriptions of drugs at the farm-level (Birkegård et al., 2018).

2.3. Antimicrobial use

Prescriptions of antimicrobials registered in VetStat for the participating farms were used as a proxy for the actual used amount of antimicrobials on-farm. To account for seasonal variation in antimicrobial use (Jensen et al., 2016), we included all prescribed antimicrobials from 12 months prior to the sampling period, 01 August 2016 to 31 July 2017. Antimicrobials were aggregated on a monthly basis and quantified as animal daily doses per kg biomass ($DADD_{kg}$/kg biomass), accounting for the weight of the estimated on-farm mink population present at the time (Jensen et al., 2016). The unit $DADD_{kg}$/kg biomass proxies the number of days with which the mink population present on-farm can be treated with the prescribed amount of antimicrobials.

2.4. Carcass samples

Each farm was instructed to collect up to 100 mink carcasses for each of the consecutive months (May–June–July 2017), resulting in a maximum of 300 carcasses per farm. The carcasses were sealed in plastic bags and frozen. If more than one mink was found dead in the same cage, the farmer was asked to compile all mink from the respective cage in a single plastic bag. The plastic bags were marked with date of collection and date of birth, from which age of the mink carcasses was calculated. At the National Veterinary Institute one mink carcass was selected from each plastic bag (cage), from which the pharynx was swabbed with a sterile cotton swab and the right forepaw dissected at the carpal joint. Individual pharyngeal swabs and paws were transferred to separate clean plastic containers and processed as one-step selective enrichments in line with Hansen et al. (2017). Pharyngeal swabs were added 3 ml while paws were added 30 ml Mueller-Hinton broth with 6.5% NaCl (Oxoid, Basingstoke, United Kingdom), to cover the sampled material completely. Subsequently, the samples were incubated at 37 °C for 18–24 hours before they were plated on a selective Brillance MRSA 2 agar (Oxoid). MRSA suspect colonies were assessed using matrix-assisted laser desorption ionisation (MALDI-TOF mass spectrometry) for species confirmation.

Mink were categorized as positive for MRSA, if the bacterium was isolated from either paws or pharynx.

2.5. Environmental samples

At the end of the study period a number of environmental samples were collected from each farm. All samples were collected by one person. Gloves were changed between each sample to reduce the risk of carry-over. On each farm the following environmental samples were collected:

- **Feed**: One feed sample was collected in a plastic bag directly from the feed tank or from the top of a cage where fresh feed had been distributed. In the laboratory, 10 g of feed was put into a clean plastic container and added 90 ml of Mueller-Hinton broth with 6.5% NaCl (Oxoid) prior to incubation at 37 °C for 18–24 hours, and following plated on a selective Brillance MRSA 2 agar (Oxoid).
- **Air samples**: Three air samples were taken, covering both open sheds and halls if present on-farm. The air sampler was placed on-top of the mink cages in height of an adult person at three different locations. We used a Sartorius Airport MD8 sampling device set at its maximum sampling rate for the current conditions (volume = 750 l, speed = 50 l/min), resulting in a minimum detection limit of <1 cfu/m^3 air. Following each air sampling, the gelatine filter was placed directly on selective Brillance MRSA 2 agar (Oxoid) prior to incubation at 37 °C for 18–24 hours.
- **Glove:** One of the gloves used by the farmer for handling the mink

Table 1
Farm demographics of five Danish mink farms and findings of livestock-associated methicillin resistant *Staphylococcus aureus* in environmental samples, May-September 2017. Estimates of the total number of females on-farm based on registrations in Central Husbandry Register. N = total number of environmental samples taken per farm.

	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5
Geographical location	Zealand	Zealand	Jutland	Jutland	Jutland
Housing type tested	Open sheds	Open sheds + Hall	Open sheds	Open sheds + Hall	Open sheds + Hall
Feed producer	A	A	B	C	B
Mink females on-farm	4100	1200	3600	3110	3296
LA-MRSA-isolated from environmental samples (number of positive samples)					
Feed (n = 1)	1	1	0	0	0
Air (n = 3)	0	0	0	0	0
Glove (n = 1)	0	0	1	1	1
Top cages (n = 10)	1	0	7	9	1
Between cages (n = 10)	1	0	7	6	0
Nest boxes (n = 10)	1	0	2	5	0

was thoroughly swabbed with a sterile cloth (Sodibox). After sampling, the cloth was reintroduced into its original plastic bag and sealed for transportation. In the laboratory, the plastic bag was added 100 ml Mueller-Hinton broth with 6.5% NaCl (Oxoid), stomached for 30 s at 230 rpm (Stomacher 400 circulator, Seward) prior to incubation at 37 °C for 18–24 hours, and subsequently plated on a selective Brillance MRSA 2 agar (Oxoid).

- **Cages:** Ten samples were taken from each of three different sites:
  - **Top of the cages** in the area used for distribution of feed. One sterile cloth (Sodibox) was used to swab five to ten cages, each covering an area of approximately 100 cm^2.
  - **Between the cages** on sites where a gap of approximately 20 cm of space was present between neighbouring cages. One sterile cloth (Sodibox) was used to swab three to five between-cage areas, each covering an area of approximately 50 cm^2.
  - **Inner side of nest boxes.** One sterile cloth (Sodibox) was used to swab one single nest box on all four walls, covering a total area of approximately 300 cm^2.

Cloths used for cage-samples were handled in the same way as the glove-samples. All MRSA suspect colonies were further assessed using MALDI-TOF for species confirmation.

2.6. Whole genome sequencing

From each farm with positive samples, three MRSA isolates from mink (two from pharynx and one from paws) and one sample per positive environmental site were subjected to whole genome sequencing. DNA was isolated using the Maxwell® 16 LEV Blood DNA kit from Promega according to manufacturer's specification with the addition of lysostaphin and subsequently sent for sequencing on an Illumina Nextera XT in a 150 bp paired-end configuration (Statens Serum Institut, Copenhagen Denmark). Briefly, sequence analysis was performed using a custom pipeline. Sequence qualities were examined using the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) and *de novo* assemblies were made using SPAdes (Bankevich et al., 2012) and annotated using prokka (Seemann, 2014). The sequence type was found using BLAST (Altschul et al., 1990) against a database of all sequence types retrieved from pubmlst.org (Jolley and Maiden, 2010). As reference isolates, 86 LA-MRSA assemblies from mink and mink feed (Hansen, 2017) were included. Core genomes were extracted and aligned using Roary (Page et al., 2015) and FastTree 2.1.9 (Price et al., 2010) was used to produce a phylogenetic tree, which was visualized in R version 3.4.1 (R Core Team, 2014) using ggplot2 (Wickham et al., 2018) and ggtree (Yu et al., 2017).

2.7. Data handling

All observations were manually typed into Excel 2016 from the Microsoft Office Package and further exported to R version 3.4.1 for data handling (R Core Team, 2014).

To test the overall difference in positive response rates between paired samples (paw and pharyngeal swab), we performed a McNemar's test. Further, we quantified Cohen's Kappa using the psych package in R (Revelle, 2017) and calculated Chamberlain's positive and negative percent agreement (PPA = 100% * (a/(a + b + c))), and (NPA = 100% * (d/(b + c + d))) as suggested by Cicchetti and Feinstein (1990) to assess agreement between tests in the lack of a Gold Standard.

Due to lack of sensitivity and specificity of the applied tests we calculated the apparent prevalence and 95% confidence intervals (Wilson method), by means of the binom package in R (Dorai-Raj, 2014). Difference in prevalence of LA-MRSA between months for farm-specific kits/breeding mink was tested by Fisher's exact test due to few observations (≤6) in one or more of the categories.

3. Results

Two farms from Zealand and three farms from Jutland were included in the study. Farms received feed from three different feed producers (A–C), Table 1. Herd size varied from 1200 to 4100 mink female (Table 1).

All farms used prescribed antimicrobials during the LA-MRSA sampling period (Fig. 1) apart from Farm 2, which had not used antimicrobials for the previous seven months. In Sep-Oct 2016, Farm 2 received macrolides corresponding to the treatment of the whole population present for a maximum of approximately eight days (Fig. 1). The remaining farms had prescriptions during the sampling period. These farms received tetracyclines and/or penicillins for oral treatment, except Farm 5, which only received other antimicrobials for oral treatment, and very small amounts of penicillin for parenteral treatment (Fig. 1). Gastrointestinal disorders in the whelping period (summer) and respiratory disorders in the autumn are the main clinical indications for antimicrobial use in mink (Jensen et al., 2016). Cephalosporin was not prescribed by any of the study farms during the study period, in line with the zero usage in the Danish mink industry (DANMAP, 2015, 2016; DANMAP, 2017).

From the five farms, a total of 644 mink carcasses were analyzed during the study period, distributed as 542 mink kits and 102 breeding mink. The mink kits had an average age of 29 days (range 8–48) in May (n = 45), 48 days (range 23–87) in June (n = 311) and 72 days (range 37–95) in July (n = 152). Date of birth and death was missing for 21 kits, while date of birth solely was missing for another 13 kits. Hence, age could not be calculated for 34 kits; 27 originating from Farm 1 and seven from Farm 5.

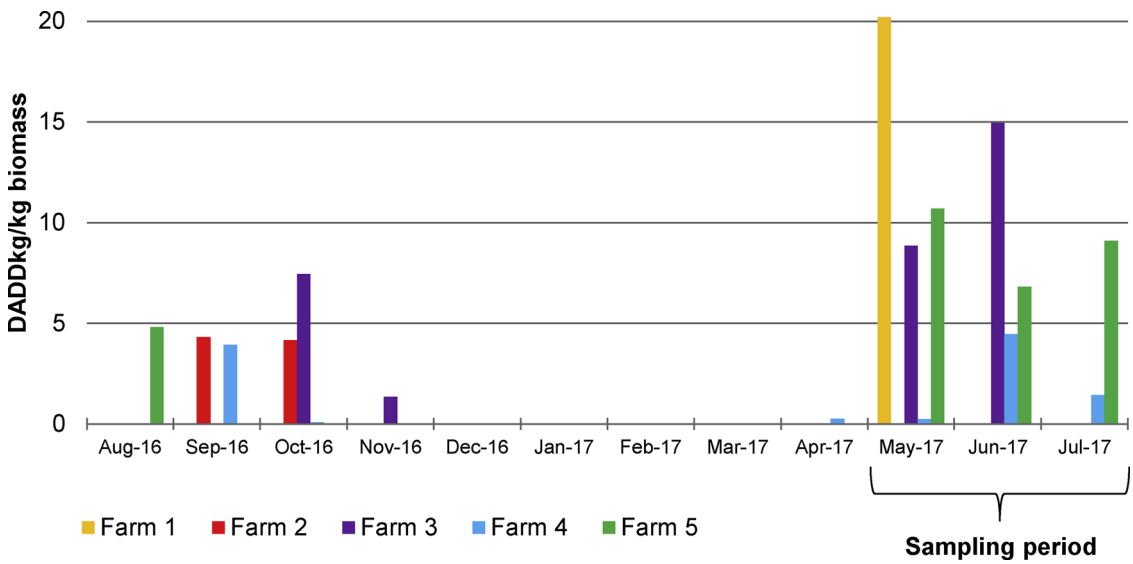

Fig. 1. Antimicrobial use in five Danish mink farms, August 2016 – July 2017. For each month, the amount of antimicrobials prescribed was quantified as $DADD_{kg}$/kg biomass. This unit proxies the number of days with which the population at the farm present can be treated with the prescribed amount of antimicrobials.

Table 2
Livestock-associated methicillin resistant *Staphylococcus aureus* isolated from paws and/or pharynx in 644 mink carcasses originating from five Danish mink farms, sampled May-July 2017. Stated is the apparent prevalence (%) [95% confidence interval] (number of positive LA-MRSA samples/total number of tested samples). Significant difference in prevalence of LA-MRSA between months for both kits and breeding mink was tested by Fisher's exact test.

		May prevalence %	June prevalence %	July prevalence %	p-value	Prevalence % (age)	p-value	Prevalence % (overall)
Farm 1	Kits	**0** [0;35] (0/7)	**6** [2;17] (3/49)	**36** [16;61] (5/14)	0.01	**20** [13;29] (18/91)*	1	**20** [13;29] (18/92)
	Breeding mink	–	**0** [0;79] (0/1)	–	–	**0** [0;79] (0/1)		
Farm 2	Kits	**0** [0;30] (0/9)	**0** [0;3] (0/117)	**0** [0;6] (0/62)	1	**0** [0;2] (0/188)	NA	**0** [0;2] (0/241)
	Breeding mink	**0** [0;66] (0/2)	**0** [0;9] (0/37)	**0** [0;22] (0/14)	1	**0** [0;7] (0/53)		
Farm 3	Kits	**0** [0;28] (0/10)	**33** [22;46] (19/58)	**46** [31;61] (17/37)	0.02	**34** [26;44] (36/105)	<0.001	**26** [20;34] (36/136)
	Breeding mink	**0** [0;39] (0/6)	**0** [0;14] (0/23)	–	1	**0** [0;11] (0/31)*		
Farm 4	Kits	**0** [0;79] (0/1)	**13** [5;32] (3/23)	**47** [25;70] (7/15)	0.05	**25** [15;41] (10/39)	1	**25** [16;38] (14/55)
	Breeding mink	**0** [0;49] (0/4)	**0** [0;39] (0/6)	**80** [38;96] (4/5)	0.01	**25** [10;49] (4/16)*		
Farm 5	Kits	**33** [16;56] (6/18)	**24** [16;35] (17/70)	**39** [24;56] (12/31)	0.30	**29** [22;38] (35/119)	1	**29** [22;38] (35/120)
	Breeding mink	–	**0** [0;80] (0/1)	–	–	**0** [0;79] (0/1)		

* Date of death was missing from 21 kits from Farm 1, two and one breeding mink from Farm 3 and Farm 4, respectively. Results from these mink are only included in the overall LA-MRSA prevalence at the farm (and not for individual months).

LA-MRSA was isolated from mink in four out of five farms. On positive farms, the overall apparent animal prevalence, ranged from 20% $[13;29]_{CI95\%}$ to 29% $[22;38]_{CI95\%}$ (Table 2). The apparent prevalence of LA-MRSA in kits tended to increase over time (Farm 1, Farm 3, Farm 4; Table 2). In these three farms, penicillins and/or tetracyclines were prescribed in the sampling period. The youngest kit from which LA-MRSA was isolated was 28 days of age and originated from Farm 5. From this kit, LA-MRSA was isolated from both paws and pharynx.

We found a moderate agreement between paw and pharyngeal test results (Cohen's Kappa = 0.56 $[0.46;0.66]_{CI95\%}$). However, the McNemar's test suggested a significant difference in positive response rates between paw and pharyngeal swabs (McNemar's chi-squared test = 23.6, p-value = 1.18×10^{-6}), suggesting a higher sensitivity for detecting LA-MRSA in paw swabs compared to pharyngeal swabs. The positive percent agreement was 44% (45/103), while the negative percent agreement was 90% (541/599) (Table 3).

On farms where LA-MRSA was isolated from mink (Farm 1, Farm 3, Farm 4, and Farm 5), LA-MRSA could also be isolated from the environment. In general, LA-MRSA was isolated from all environmental sites tested (glove, top and between the cages and in the nest boxes), except air (Table 1). As the air sampling volume was 750 l, the concentration of LA-MRSA in air was found to be <1 cfu/m^3 on all five mink farms.

From each LA-MRSA-positive sample site within each farm, one isolate was send for whole genome sequencing. All sequenced isolates were found to be CC398. All newly sequenced isolates grouped into the L3 lineage (Fig. 2), which is among one of the three major lineages isolated from the Danish pig production (Islam et al., 2017; Sieber et al., 2018). In general, isolates from each farm tended to cluster. On Farm 1, all three isolates from mink (paw and/or pharynx), two isolates from top/between cages and one isolate from the nest boxes clustered in one subclade, while the isolate from feed located further apart. Isolates from Farm 3 showed a similar pattern. These isolates originated from three mink, two isolates from top/between cages and one isolate from nest boxes. Isolates from Farm 4 and Farm 5 had a slightly other genomic structure as they grouped into two to three sub-clusters. From Farm 4, two isolates from one mink (paw and pharynx), one isolate from the

Table 3
Variation in the anatomical site of isolation (pharynx or paws) of livestock-associated methicillin resistant *Staphylococcus aureus* isolated from 644 Danish mink carcasses, originating from four farms, May–July 2017.

	LA-MRSA positive (pharynx)	LA-MRSA negative (pharynx)
LA-MRSA positive (paws)	45 (0.07)	48 (0.07)
LA-MRSA negative (paws)	10 (0.02)	541 (0.84)

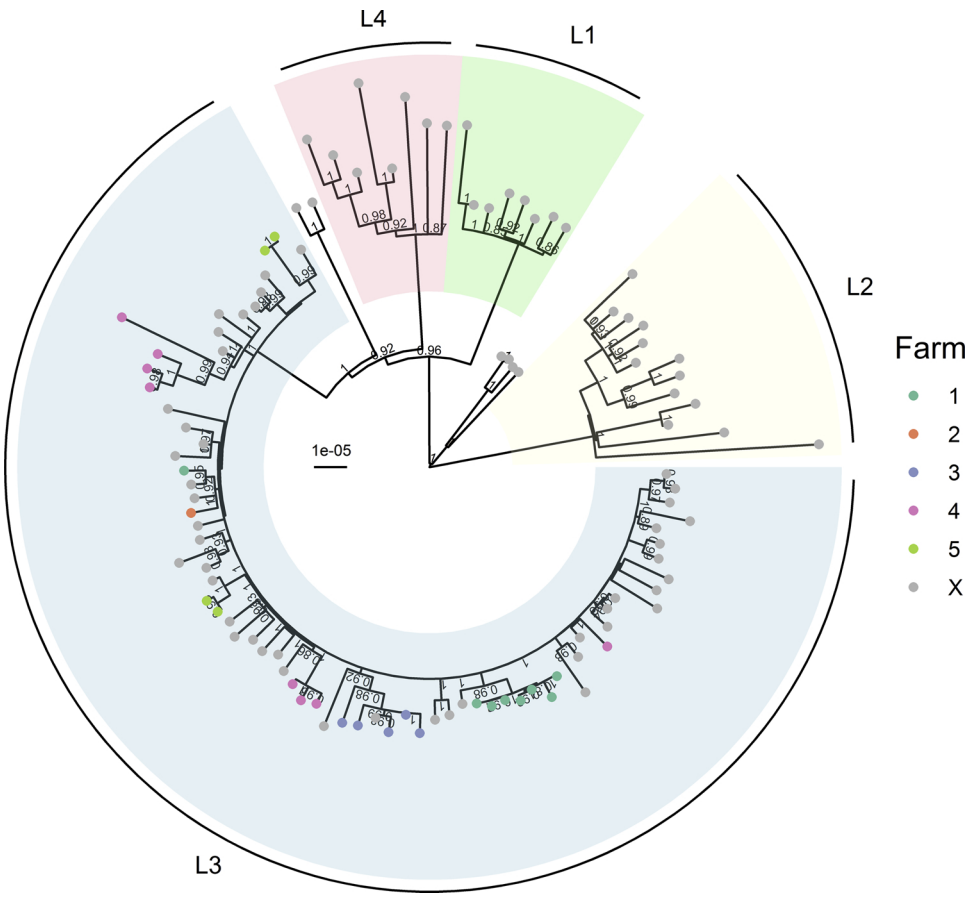

Fig. 2. Phylogenetic relationship of 26 sequenced LA-MRSA isolates from five Danish mink farms, 2017. The colour of points denotes the farm of origin. For comparison, isolates are illustrated along with 82 reference isolates, 65 originating from mink and 17 from mink feed, from Hansen (2017), referred to as "X". The three lineages previously associated with LA-MRSA from the Danish pig production are presented as L1-L3, while the remaining lineage, L4 is in accordance with mink isolates found by Hansen (2017). The scale bar shows substitutions per nucleotide and bootstrap values above 0.8 are shown at the nodes.

nest boxes and one isolate from the surface of the cages clustered together, while isolates from another two mink, one isolate from between the cages and the glove clustered separately. Farm 5 isolates grouped into two sub-clusters. One sub-cluster holding isolates from two mink (one pharyngeal swab and one paw) and another sub-cluster with an isolate from one mink (pharynx) and one isolate from the surface of cages.

4. Discussion

In the present study, LA-MRSA was isolated from mink and environmental sites in four out of five mink farms. On positive farms, the total apparent animal prevalence ranged from 20% $[13;29]_{CI95\%}$ to 29% $[22;38]_{CI95\%}$. Breeding mink were only found positive in one out of four positive farms (25% positive breeding mink on Farm 4), possibly due to a relatively low number of submitted adult mink carcasses from the remaining three farms (n = 49). On positive farms, the prevalence of LA-MRSA isolated from kits ranged from 20% $[13;29]_{CI95\%}$ to 34% $[26;44]_{CI95\%}$, and tended to increase with increased age of the kits. The proportions of kits tested positive were significantly different between months for Farm 1 ($p = 0.01$), Farm 3 ($p = 0.02$) and Farm 4 ($p = 0.05$) (Table 2). Possibly, the increasing proportion of LA-MRSA positive kits correlates with the increased consumption of feed. However, as the youngest kit tested positive was 28 days of age, direct transfer from the dam may also occur. A similar increase in the animal prevalence within the first months of life have been indicated in six pig herds; where the overall LA-MRSA animal prevalence tended to increase from piglets (67%) to weaners (99%) and subsequently reduce in the finisher (67%) and pregnant sow unit (34%) (Dewaele et al., 2011; Espinosa-Gongora et al., 2012). However, relatively small sample sizes were taken in both referred studies, resulting in large confidence intervals of the referred prevalence.

In the present study, LA-MRSA was generally isolated from environmental samples on all four farms where LA-MRSA was isolated from mink carcasses. We isolated LA-MRSA from glove, mesh-wires on the cages (top and side) and inside the nest boxes, indicating that LA-MRSA is widely distributed in the environment and may persist on-farm after the source of introduction has ceased. Similarly, LA-MRSA tends to distribute widely on LA-MRSA positive pig farms, where boot swabs, dust, feces, feed, floor, walls, drinking nipples, ventilation system and air all have been found positive (Dewaele et al., 2011; Friese et al., 2012). Opposite to pig farms, we were in the present study not able to isolate LA-MRSA from air (Table 1), indicating a concentration of <1 cfu/m^3 air. Airborne transmission of MRSA to human visitors therefore seems unlikely on mink farms, compared to pig farms where concentrations of LA-MRSA in the area of 5000 cfu/m^3 have been reported and a positive correlation between LA-MRSA concentration in air and positive human nasal swabs immediately after leaving a pig stable has been shown (Angen et al., 2017). One explanation of the divergence in concentration of LA-MRSA in air between species may be differences in anatomic location. LA-MRSA on pigs tends to be located on the surface of the skin and in the nose (Friese et al., 2012) covering a larger external area compared to mink where the bacterium primarily is located on paws and pharynx (Hansen et al., 2017). Another explanation may be found in the housing system where open sheds and halls on mink farms are naturally ventilated. Previous studies have indicated an effect of ventilation, as LA-MRSA positive pigs in four out of five farms spontaneously eliminated the bacterium three months after they were moved into free-range conditions (DANMAP, 2017).

LA-MRSA was isolated from the mink feed on Farm 1 and Farm 2. The feed originated from the same feed producer (Table 1), but was collected on various days. The presence of LA-MRSA in feed may shift over time, depending on the presence of LA-MRSA in the feed components at the time present. However, LA-MRSA was only re-isolated from

one of the farms feeding LA-MRSA positive feed. It may be suggested that LA-MRSA is intermittently present in feed, i.e. that some batches delivered are positive while others are not, but this needs further investigation. Likewise, we have no information on LA-MRSA levels in positive batches. This would be an important information. Sampling of feed took place only at one point in time, and the results do not exclude that other feed batches prior to sampling have been positive. LA-MRSA in mink is hypothesized to originate from contaminated mink feed due to three main reasons. Firstly, the anatomical site of isolation from the mink (pharynx and paws) are in close contact with the moist feed. Secondly, the most prevalent LA-MRSA *spa*-types (t034 and t011) found in mink correspond to those isolated from Danish pig herds and pig slaughter byproducts are used in the mink feed production. Thirdly, 19% (20/108) of mink feed samples analyzed in 2016 were found positive for LA-MRSA (Hansen et al., 2017). A recent clinical trial confirmed feed contamination as a likely route of transmission and confirmed that LA-MRSA in mink may persist for more than 26 days after the source of contamination has ceased (Fertner et al., accepted). However, further studies are needed to test whether LA-MRSA in mink represents a true colonization or simple contamination. Various *spa*-types have previously been isolated at individual mink farms indicating multiple introductions of the bacterium to the respective farms (Hansen et al., 2017). Likewise, two to three different molecular subclusters were found on Farm 4 and Farm 5 in the present study (Fig. 2) suggesting the establishment of several clones on-farm or, alternatively, a continuous introduction of new clones from an outside source. However, isolates from Farm 1 and Farm 3 grouped together which could indicate a single introduction of LA-MRSA, which subsequently disseminated throughout the farms. This would also explain the molecular divergence between the feed isolate and the remaining isolates from Farm 1. Isolates from mink, mink feed and human cases with mink-contact was previously identified as belonging to one of the three dominating pig lineages, L1-L3 (Hansen, 2017; Sieber et al., 2018), while Hansen (2017) further found a substantial number of isolates (18%) belonging to a separate sub-clone of potentially mink-adopted LA-MRSA, presented as L4 in Fig. 2. In the present study, all sequenced isolates were genetically closely related and belonged to the L3 cluster, suggesting that the L3 lineage has increased in prevalence on Danish mink farms in line with the development seen in Danish pig farms (Sieber et al., 2018).

Despite the isolation of LA-MRSA in feed from Farm 2 (Table 1), we did not isolate LA-MRSA from neither mink carcasses nor environmental sources (Table 2). In the present study it was not possible to quantify the amount of LA-MRSA in the feed. Thus, it cannot be ruled out that the concentration of LA-MRSA in feed was below a level where colonization of the animals is likely to take place. However, as Farm 1 received feed from the same feed producer as Farm 2, the difference in LA-MRSA animal prevalence between the two farms seems more likely to be explained by other factors than the variation in concentration of LA-MRSA in the feed. Large herds (Broens et al., 2011) and antimicrobial use (Bos et al., 2012) have earlier been identified as risk factors for the presence of LA-MRSA in pigs and veal calf herds. The effect of herd size may be due to characteristics in management (antimicrobial use, biosecurity, purchase of animals, hygiene) rather than the number of animals present (Broens et al., 2011). In the present study, Farm 2 was substantially smaller than the other four farms included in the study but was also the farm sampling the highest number of carcasses in the study period (Table 1). Additionally, Farm 2 had not used any antimicrobials for the current production cycle, in contrary to the remaining four farms (Fig. 1) which all used prescribed antimicrobials during the sampling period (May-July 2017). This indicate that use of antimicrobials selecting for LA-MRSA in temporal proximity to feeding of the contaminated feed, could affect the likelihood of colonization and/or influence the persistence of LA-MRSA. However, this needs further investigation. However, the application of group treatments have previously been speculated as a predisposing factor for the persistence of LA-MRSA on veal calf farms given that the bacterium is introduced to the farm (Bos et al., 2012).

As sensitivity and specificity of the applied tests are unknown we were not able to calculate the true animal prevalence on-farm. Positive response rates between paw and pharyngeal swabs varied significantly. Variation in sensitivity may partly explain the discrepancy, as a swab may hold the risk of missing bacteria compared to culture of bacteria from the entire paw embedded in broth. A latent class analysis would have been applicable for the estimation of sensitivity and specificity of the applied tests but was beyond the scope of this project.

5. Conclusions

In the present study, the animal prevalence of LA-MRSA on positive mink farms was found to range from 20% $[13;29]_{CI95\%}$ to 29% $[22;38]_{CI95\%}$. LA-MRSA was isolated from kits already before weaning, most likely due to a contaminated environment or transfer from the dam. The prevalence of positive mink kits increased with age.

LA-MRSA seems to be widely distributed in the environment on positive mink farms. However, LA-MRSA could not be detected in air, and therefore human exposure through air is unlikely. However, LA-MRSA on mink may still pose a human health hazard to farmers through direct contact with contaminated environmental sites and the risk of bites and scratches when handling colonized mink.

Conflict of interest statement

None to declare.

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References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215 (3), 403–410.

Angen, Ø., Feld, L., Larsen, J., Rostgaard, K., Skov, R., 2017. Transmission of methicillin-resistant *Staphylococcus aureus* to human volunteers visiting a swine farm. Appl. Environ. Microbiol. 83, 1–10. https://doi.org/10.1128/AEM.01489-17.

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., Lesin, V.M., Nikolenko, S.I., Pham, S., Prjibelski, A.D., Pyshkin, A.V., Sirotkin, A.V., Vyahhi, N., Tesler, G., Alekseyev, M.A., Pevzner, P.A., 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J. Comput. Biol. 19, 455–477. https://doi.org/10.1089/cmb.2012.0021.

Birkegård, C., Fertner, M., Jensen, V.F., Boklund, A., Toft, N., Halasa, T., Antunes, A.C.L., 2018. Building the foundation for veterinary register-based epidemiology: a systematic approach to data quality assessment and validation. Zoon Public Heath. https://doi.org/10.1111/zph.12513.

Bos, M.E.H., Graveland, H., Portengen, L., Wagenaar, J.A., Heederik, D.J.J., 2012. Livestock-associated MRSA prevalence in veal calf productions associated with farm hygiene, use of antimicrobials, and age of the calves. Prev. Vet. Med. 105, 155–159. https://doi.org/10.1016/j.prevetmed.2012.01.002.

Broens, E.M., Graat, E.A.M., Van Der Wolf, P.J., Van De Giessen, A.W., De Jong, M.C.M., 2011. Prevalence and risk factor analysis of livestock associated MRSA-positive pig herds in the Netherlands. Prev. Vet. Med. 102, 41–49. https://doi.org/10.1016/j.prevetmed.2011.06.005.

Cicchetti, D.V., Feinstien, a R., 1990. High agreement but low kappa. J. Clin. Epidemiol. 43, 551–585. https://doi.org/10.1016/0895-4356(90)90159-M.

Danish Ministry of Environment- and Food, 2015. Regarding Protection of Fur animals (in Danish). Order (DK) 1553/2015 by 11/12/2015. https://www.retsinformation.dk/Forms/R0710.aspx?id=176306.

DANMAP DANMAP 2014 – Use of Antimicrobial Agents and Occurrence of Antimicrobial

Resistance in Bacteria from Food Animals, Food and Humans in Denmark. https://www.danmap.org/-/media/arkiv/projekt-sites/danmap/danmap-reports/danmap-2014/danmap_2014.pdf?la=en.

DANMAP DANMAP 2015 – Use of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Bacteria from Food Animals, Food and Humans in Denmark. http://viewer.webproof.com/pageflip/336/187121/index.html#/page=1.

DANMAP DANMAP 2016 – Use of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Bacteria from Food Animals, Food and Humans in Denmark. https://www.danmap.org/-/media/arkiv/projekt-sites/danmap/danmap-reports/danmap-2016/danmap_2016_web.pdf?la=en.

Dewaele, I., Messens, W., De Man, I., Delputte, P., Herman, L., Butaye, P., Heyndrickx, M., Rasschaert, G., 2011. Sampling, prevalence and characterization of methicillin-resistant *Staphylococcus aureus* on two Belgian pig farms. Vet. Sci. Dev. 1, 1. https://doi.org/10.4081/vsd.2011.e1.

Dohoo, I., Martin, W., Stryhn, H., 2010. Sampling. In: McPike, M. (Ed.), Veterinary Epidemiologic Research. VER Inc., Charlottetown, Canada, pp. 47–48.

Dorai-Raj, S., 2014. R Package 'binom' https://cran.r-project.org/web/packages/binom/binom.pdf.

Espinosa-Gongora, C., Larsen, J., Moodley, A., Nielsen, J.P., Skov, R.L., Andreasen, M., Guardabassi, L., 2012. Farm-specific lineages of methicillin-resistant *Staphylococcus aureus* clonal complex 398 in Danish pig farms. Epidemiol. Infect. 140, 1794–1799. https://doi.org/10.1017/S0950268811002391.

Fertner, M., Pedersen, K., Chriél, M., 2019. Exposure to contaminated feed is a likely route for colonizing farmed mink (*Neovison vison*) with livestock-associated methicillin-resistant *Staphylococcus aureus*. Vet. Microbiol. 45–47.

Friese, A., Schulz, J., Hoehle, L., Fetsch, A., Tenhagen, B.A., Hartung, J., Roesler, U., 2012. Occurrence of MRSA in air and housing environment of pig barns. Vet. Microbiol. 158, 129–135. https://doi.org/10.1016/j.vetmic.2012.01.019.

Graveland, H., Duim, B., van Duijkeren, E., Heederik, D., Wagenaar, J.A., 2011. Livestock-associated methicillin-resistant *Staphylococcus aureus* in animals and humans. Int. J. Med. Microbiol. 301, 630–634. https://doi.org/10.1016/j.ijmm.2011.09.004.

Grøntvedt, C.A., Elstrøm, P., Stegger, M., Skov, R.L., Skytt Andersen, P., Larssen, K.W., Urdahl, A.M., Angen, Ø., Larsen, J., Åmdal, S., Løtvedt, S.M., Sunde, M., Bjørnholt, J.V., 2016. Methicillin-resistant *Staphylococcus aureus* CC398 in humans and pigs in Norway: a "one health" perspective on introduction and transmission. Clin. Infect. Dis. 63, 1431–1438. https://doi.org/10.1093/cid/ciw552.

Guardabassi, L., Stegger, M., Skov, R., 2007. Retrospective detection of methicillin resistant and susceptible *Staphylococcus aureus* ST398 in Danish slaughter pigs. Vet. Microbiol. 122, 384–386. https://doi.org/10.1016/j.vetmic.2007.03.021.

Hansen, J.E., 2017. Methicillin-resistant *Staphylococcus Aureus* in Danish Production Animals. PhD Thesis. National Veterinary Institute, Technical University of Denmark, Kgs Lyngby, Denmark Dec 2017.

Hansen, J.E., Larsen, A.R., Skov, R.L., Chriél, M., Larsen, G., Angen, Ø., Larsen, J., Lassen, D.C.K., Pedersen, K., 2017. Livestock-associated methicillin-resistant *Staphylococcus aureus* is widespread in farmed mink (*Neovison vison*). Vet. Microbiol. 207, 44–49. https://doi.org/10.1016/j.vetmic.2017.05.027.

Islam, M.Z., Espinosa-Gongora, C., Damborg, P., Sieber, R.N., Munk, R., Husted, L., Moodley, A., Skov, R., Larsen, J., Guardabassi, L., 2017. Horses in Denmark are a reservoir of diverse clones of methicillin-resistant and -susceptible *Staphylococcus aureus*. Front. Microbiol. 8, 1–10. https://doi.org/10.3389/fmicb.2017.00543.

Ito, T., Katayama, Y., Asada, K., Mori, N., Tsutsumimoto, K., 2001. Structural comparison of three types of staphylococcal cassette chromosome mec integrated in the chromosome in methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. 45, 1323–1336. https://doi.org/10.1128/AAC.45.5.1323.

Jensen, V.F., Sommer, H.M., Struve, T., Clausen, J., Chriél, M., 2016. Factors associated with usage of antimicrobials in commercial mink (*Neovison vison*) production in Denmark. Prev. Vet. Med. 126, 170–182. https://doi.org/10.1016/j.prevetmed.2016.01.023.

Jolley, K.A., Maiden, M.C., 2010. BIGSdb: scalable analysis of bacterial genome variation at the population level. BMC Bioinf. 11, 595. https://doi.org/10.1186/1471-2105-11-595.

Kopenhagen Fur, 2017. Årsberetning 2017. Glostrup. https://www.kopenhagenfur.com/da/om-kopenhagen-fur/fakta-og-noegletal/årsberetninger/.

Lyhs, U., Nonnemann, B., Hjulsager, C.K., Pedersen, K., Chriél, M., 2018. Microbiological quality of mink feed (in Danish, with English abstract), 2018. Faglig Årsberetning 2017, 111-117 Kopenhagen Forskning. Agro Food Park 15, DK-8200 Aarhus N, Denmark.

Page, A.J., Cummins, C.A., Hunt, M., Wong, V.K., Reuter, S., Holden, M.T., Fookes, M., Falush, D., Keane, J.A., Parkhill, J., 2015. Roary: rapid large-scale prokaryote pan genome analysis. Bioinformatics 31, 3691–3693. https://doi.org/10.1093/bioinformatics/btv421.

Price, M.N., Dehal, P.S., Arkin, A.P., 2010. FastTree 2 — Approximately maximum-likelihood trees for large alignments. PLoS One 5 (3), e9490. https://doi.org/10.1371/journal.pone.0009490.

R Core Team, 2014. R: a Language and Environment for Statistical Computing.

Revelle, W., 2017. R Package 'psych' https://cran.r-project.org/web/packages/psych/psych.pdf.

Seemann, T., 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30, 2068–2069. https://doi.org/10.1093/bioinformatics/btu153.

Sieber, R.N., Skov, R.L., Nielsen, J., Schulz, J., Price, L.B., Aarestrup, F.M., Larsen, A.R., Stegger, M., Larsen, J., 2018. Drivers and dynamics of methicillin-resistant livestock-associated *Staphylococcus aureus* CC398 in pigs and humans in Denmark. mBio 9, e02142–18. https://doi.org/10.1128/mBio.02142-18.

Wickham, H., Chang, W., Henry, L., 2018. R Package 'ggplot2'. Create Elegant Data Visualisations Using the Grammar of Graphics, Version 3.0.0. https://cran.r-project.org/web/packages/ggplot2/ggplot2.pdf.

Yu, G., Smith, D., Zhu, H., Guan, Y., Lam, T.T., 2017. Ggtree: an R package for visualization and annotation of phylogenetic trees with their covariates and other associated data. Meth. Ecol. Evol. 8, 28–36. https://doi.org/10.1111/2041-210X.12628.