Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Veterinary Medicine and Animal Science
Department of Clinical Sciences

Antibiotic resistance in *Staphylococcus aureus* and *Staphylococcus pseudintermedius* isolated from milk, cattle and dogs in and around Kampala, Uganda

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Antibiotikaresistens hos *Staphylococcus aureus* och *Staphylococcus pseudintermedius* från mjölk, nötkreatur och hundar i Kampala, Uganda

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SUMMARY

Development of antimicrobial resistance is internationally recognized as a major concern to public health and veterinary medicine. Methicillin-resistant *Staphylococcus aureus* (MRSA) and Methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) can cause a variety of diseases and have limited options for treatment. Studies on the occurrence and prevalence of MRSA in cattle on the African continent are few, and reports of MRSP are even fewer. This pilot study hoped to shed some light on how widespread antibiotic resistance in *S. aureus* and *S. pseudintermedius* might be in animals in Kampala.

The study included (a) 26 samples from commercially sold raw milk, (b) 66 samples of fresh milk pooled with nasal and perineal swabs from dairy cattle, and (c) 40 nasal and perineal swabs from dogs. After enrichment in selective broth, *S. aureus* was isolated from (a) five, (b) tree and (c) five of the samples respectively, and *S. pseudintermedius* was isolated from ten of the dog samples. One MRSA was identified from the (a) raw milk category, according to phenotypic characteristics, but the genes *mecA* or *mecC* PCR could not be amplified on Real-Time PCR. All ten isolated *S. pseudintermedius* had MICs < 0.25 μg/mL for oxacillin, and where thus not regarded as MRSP, but three isolates were resistant to ≥ 3 classes of antibiotics, thus these isolates might be regarded as multi-drug resistant. The results warrant further investigations to establish the prevalence of these bacteria.

SAMMANFATTNING

Utveckling av antibiotikaresistens är ett internationellt allvarligt problem inom både human- och veterinärvården. Meticillin-resistenta *Staphylococcus aureus* (MRSA) och Meticillin-resistenta *Staphylococcus pseudintermedius* (MRSP) kan orsaka en mängd olika sjukdomar och har i vissa fall få behandlingsalternativ. Studier kring förekomst och prevalens av MRSA hos nötkreatur från den afrikanska kontinenten är få, och rapporter om MRSP är ännu färre. Vi ville med denna pilotstudie få en uppfattning om hur utbredd antibiotikaresistens är hos isolat av *S. aureus* och *S. pseudintermedius* från djur i Kampala.

I studien ingick (a) 26 prover tagna från opastöriserad mjölk såld i området, (b) 66 prover från noshåla, perineum och mjölk från mjölkkor och (c) 40 svabbprover från nos och perineum på hundar. Efter anrikning i selektiv buljong kunde *S. aureus* isoleras från (a) fem, (b) tre och (c) fem prover i respektive kategori och från tio av hundarna isolerades *S. pseudintermedius*. Från den förstnämnda kategorin (a) med mjölkprover isolerades en fenotypisk MRSA, där generna *mecA* eller *mecC* dock inte kunde påvisas. Alla tio isolerade *S. pseudintermedius* hade MIC < 0,25 μg/mL för oxacillin, vilket betyder att ingen definierades som MRSP men tre isolat uppvisade resistens mot ≥ 3 antibiotikaklasser, vilket gör att de kan betraktas som multiresistenta. Dessa resultat lägger en grund för vidare studier rörande prevalensen av dessa bakterier.

CONTENTS

INTRODUCTION

The population of Uganda is one of the world's fastest growing and youngest, and also one of the world's poorest when counted as GDP (gross domestic product) per capita. The population is estimated (Jul 2017) to be 39 million and out of these about 25% is undernourished and 6.5% living with HIV/AIDS. With the majority of people living in rural areas, agriculture is the most important source of livelihood for a large proportion of the Ugandan population (*FAOSTAT*; CIA, 2018). The most commonly held domesticated mammals are goats with an estimate of 12.5 million heads in a census in 2008, followed by cattle at 11.4 million and sheep at 3.4 million. Dogs were in the same census estimated to be 1.6 million (UBOS, 2010). Dogs in Uganda have traditionally been kept for hunting, herding, security and guarding livestock (Millán *et al.*, 2013). Many tropical diseases are still common in cattle, and these are often treated with antibiotics (UNAS, 2015).

Development of antimicrobial resistance is internationally recognized as a major concern to public health. Highlighting its importance, antimicrobial resistance was raised as a topic for the UN General Assembly in Sept 2016, as one of only four subjects relating to a public health problem ever to be lifted in this way (*PRESS RELEASE: High-Level Meeting on Antimicrobial Resistance*, 2016). The prevalence of different antibiotic-resistant strains of bacteria in various countries of Africa has been shown to be intermediate to high, but the studies are relatively few, as in many developing countries (Rothe *et al.*, 2013; Abdulgader *et al.*, 2015; Lozano *et al.*, 2016; Najjuka *et al.*, 2016; Pires Dos Santos *et al.*, 2016). The studies published on Methicillin-resistant *Staphylococci* in Uganda have almost exclusively been related to human healthcare (Asiimwe *et al.*, 2017b) and to our knowledge, none has been done on Methicillin-resistant *S. pseudintermedius* (MRSP).

This pilot study aimed to investigate the presence of Methicillin-resistant *S. aureus* (MRSA) or MRSP in dogs, cattle and milk in Kampala, by definition of phenotype or by presence of the genes *mecA* or *mecC*.

LITERATURE REVIEW

Conditions for animals and animal industry in Uganda

The dairy industry in Uganda

Cattle are the major milk-producers in Uganda and an average cow produces 8.5 L of milk per week. Uganda has five geographical regions, whereof the central and western regions have both the highest number of milked cows and the highest milk yield per cow. These regions also have the highest percentage of and are suitable for temperate cow breeds such as the Holstein-Friesian. These breeds or crossbreeds produce about 30% of the total milk produced in Uganda and have a 2-3 times higher average milk-yield per cow than the indigenous breeds such as Ankole. Still, indigenous cows yield most of the milk produced in Uganda. They are more resistant to diseases (e.g. spread by the tsetse-fly), adaptable to local conditions and are more versatile, also producing beef, draft power and various social functions (Balikowa, 2011; UBOS 2010).

The dairy cows in Uganda are mostly kept in intensive farming systems in the urban and peri-urban settings. In the rural settings the extensive systems are prevailing and are largely based on small herds, mostly managed by family members. Intensive systems require a higher input e.g. hired labour, food or AI-services, but generally also generate higher output and larger herds. A recently expanding sector that is an exception to this are the zero-grazing smallholder farms where 1-3 dairy cows are kept in enclosures and fed concentrates and grass or parts from cash crops (Garcia *et al.*, 2008). In total, smallholder producers own over 90% of the national herd and the average herd is only 6.9 cows (UBOS, 2010; UNAS, 2015). Milk is mostly produced in the so-called "cattle-belt" extending from South-western to Central Uganda. The Kiruhura and Mbarara region in South Western Uganda produces a significant part of the national milk production (Mwebaze & Kjaer, 2013). In these areas, the dominating production types are pastoral or agro-pastoral, the latter being semi-intensive (Grimaud *et al.*, 2007a).

The milk in Uganda is sold either through the formal or informal market. The formal market includes large-scale processing companies, which place strict quality controls both on the raw milk and on the processing, usually including pasteurization and packing. The informal market constitutes an estimated 87% of the marketed milk in Uganda and most of this milk is sold without previous processing or packaging (Balikowa, 2011). Farmers are, according to numbers from the 2008 national census, only selling approximately 35% of the milk they produce, thus the producing household is consuming most of the product (UBOS, 2010). In the Mbarara region 85.7% of the respondents stated that they consumed milk at least once a day and 37.9% consumed it without previous boiling (Nasinyama *et al.*, 2014). Both figures were slightly lower in the Kampala region, with 53.6% consuming milk daily and 16.7% reporting consumption of unboiled milk (Nasinyama *et al.*, 2014). Another study found consumption of raw milk in 15% of the respondents in Gulu district, Northern Uganda, and 42% of the respondents in Soroti district, Eastern Uganda (Rock *et al.*, 2016). The milk can be sold directly from the farm, to nearby households or to milk collection centres, sometimes through a chain of middlemen. From the milk collection centres, the milk can be sold to consumers or be collected for transport in insulated road tankers and distributed among milk collection centres in urban areas, for example Kampala (Balikowa, 2011).

Most farmers rely on hand milking, milking machines are rare and milking hygiene is often of substandard quality (Kateete *et al.*, 2013). The base levels of contamination on the farms is

unacceptably high and according to one study, total plate count (TPC) was around 2×10^6 colony forming units per millilitre (cfu/mL) in the dry season and 8×10^6 cfu/mL in the rainy season (Grimaud *et al.*, 2009). By the time the milk reached the milk collecting centre (in Mbarara) the TPC was around 83×10^6 cfu/mL and when it reached the urban market in Kampala the value was 1419×10^6 cfu/mL in the dry season and 953×10^6 cfu/mL in the rainy season (Grimaud *et al.*, 2009). However, some uncertainty exists regarding these figures, since in an earlier publication Grimaud *et al.* (2007b) stated that the study was conducted in 2004, before the 2006 ban on long-range transport of milk in aluminium cans (Mwebaze & Kjaer, 2013). However, another study found TPC to be 245-324 $\times 10^6$ cfu/mL in milk from milk cooling points in central Kampala (Mugampoza *et al.*, 2011) and a Kenyan study stated similar figures of 117×10^6 cfu/mL in raw can milk on arrival to the first milk collection centre (Teresiah *et al.*, 2016). Another Kenyan study found that most milk had $< 2 \times 10^6$ cfu/mL at the farm level but 43-70% of milk sold had $> 2 \times 10^6$ cfu/mL (Orregård, 2013).

As a reference value, most Swedish farms are consistently delivering milk with less than 15 000 cfu/mL (Christiansson *et al.*, 2011) and according to the European Commission regulation (EC) No 1020/2008 of 17 October 2008[1], actions must be made to ensure that the total plate count is under 300 000 cfu/ml in raw cows' milk used to prepare dairy products. Acceptable microbial standards for raw milk according to East African Standards are $< 2 \times 10^6$ cfu/mL for TPC in raw milk (EAS 67:2006).

The Dairy Development Authority, Uganda requires registration and annual registration fees from all individuals and companies intending to handle or process milk, a practice that was established to enable them to carry out inspections (*Dairy Development Authority*). To increase profits, however, many farmers and vendors still add water to the milk, a claim that can be validated by abnormally low density in 30-36% of milk samples in urban milk coolers and up to 86% of milk from cyclist mobile vendors (Balikowa, 2011; Grimaud *et al.*, 2009). Again, Kenyan studies show similar numbers, with unacceptably low density in 27% (Orregård, 2013) or 23.8%-36.8% (Teresiah *et al.*, 2016) of samples at shop level. Against regulations, to counteract the contamination of the milk, milk is sometimes boiled in large open metallic containers or various chemicals are added to the milk (Balikowa, 2011).

Dog ownership in Uganda

Information portraying dog ecology or management in Uganda has seldom been captured in studies, but a study including 799 respondents identifying 175 dog owners in five districts distributed from Western to Eastern Uganda reported that 31.4% of the dogs were always allowed to roam freely, whereas 21.7% were always confined to the owner's property. Overall 74.3% of the dogs were allowed to roam freely to some degree. Less than half of the owners provided their dogs with veterinary care (43.7%) or shelter (37.9%). Based on stated number of deaths during the last five years, the rate of suspected canine rabies was 5.1 per 1000 dogs per year and the mortality rate was 101 deaths per 1000 dogs per year. In this study the number of unowned dogs in the villages could not be ascertained and these dogs were thus not included (Wallace *et al.*, 2017). A cohort study following 61 dogs during fifteen months in rural Western Uganda, showed that crude mortality rate was 168 per 1000 dogs per year, with infectious disease causing 46.1% of the deaths, followed by culling (euthanasia) performed by the owners (30.8%), and attacks by baboons, *Papio anubis* (23.1%). Interviews indicated that 98.4% of the

[1]) OJ L 277/8, 18.10.2008, p. 7, Celex 32008R1020.

dogs were fed human food and most dogs also hunted or scavenged wildlife and ate dead livestock at least once a year. Over half (54.1%) had poor body condition score (< 2/5) and all interacted daily with other dogs (Hyeroba *et al.*, 2017).

Suburban areas of developing countries generally have a higher dog density but lower dog-to-human ratios as compared to rural areas (Davlin & VonVille, 2012). The overall dog-to-human ratio in Africa has been estimated at 1:21 in urban areas and 1:7 in rural areas (Knobel *et al.*, 2005) but Wallace *et al.* (2017) claims that the dog-to-human ratio in Uganda is probably better estimated as almost the double (1:47) since he found that poverty affects the likelihood of dog-ownership. With this consideration he estimates the Ugandan dog population to include 729 486 dogs.

Diseases of the cattle and their treatment

Common diseases in Ugandan cattle include Foot-and-Mouth-disease and Contagious Bovine Pleuropneumonia. East Coast Fever is a substantial problem in some areas and other tick- borne diseases like Lumpy Skin Disease are also frequent. Further, mastitis and subclinical mastitis are common, and cause significant loss of milk production and profits (UNAS, 2015). In a worldwide perspective, mastitis is the most prevalent disease in the dairy industry. It has severe economic impact through decrease in milk production, drug- and veterinary costs and sometimes by premature culling of cows (Seegers *et al.*, 2003).

Mastitis is by definition inflammation of the mammary glands. It can manifest itself as anything from a severe, systemic illness to a mild inflammation that only affects the quality of the milk. Clinical mastitis (CM) is diagnosed when the milk looks abnormal and/or the animal displays signs of infection or inflammation of the udder. Subclinical mastitis (SCM) cannot be found through a physical examination and is usually diagnosed through an elevated somatic cell count (SCC; more than 200 000 cells/mL) in the milk. Infection, tissue injury or stress to the mammary gland results in an increase of both epithelial cells and leucocytes (mostly neutrophils), modulated by inflammatory mediators. However, SCC is affected by *e.g.* the stage of lactation, breed, time of the day and season, and there is an overlap between SCC-values of the normal and the infected udder. Milk with high SCC has altered properties which results in reduced cheese yield and can disrupt fermentation processes (e.g. of yogurt). Causative infective agents are frequently also a zoonotic concern, through viable organisms or their toxins in the milk (Harmon, 1994; Sharma *et al.*, 2011).

In a survey on subclinical mastitis in the Kiboga district located in central Uganda, 87.9% of the 124 dairy cattle from 12 farms suffered from subclinical mastitis. Over half of the cows (54.8%) were affected in all four quarters whereas 4% of the cows were negative in all quarters. Out of 163 isolates, the most commonly isolated bacteria were coagulase-negative staphylococci (64.4%) and *S. aureus* (16.6%) (Kasozi *et al.*, 2014). Byarugaba (2008) reports a lower frequency of 60.7% cases of subclinical mastitis in the eastern district of Jinja, with bacteria isolated in 51.9% of 688 quarter samples. The most commonly isolated bacteria in this study were coagulase-negative staphylococci, coliforms and *S. aureus* (Byarugaba, 2008). In the Kiruhura district, southwestern Uganda, a study found subclinical mastitis in 76.1% out of 71 cows tested, with *Staphylococcus* spp. isolated in 30.8%. *Proteus* spp. was the next most commonly found bacteria with a prevalence of 13.8% (Ssajjakambwe *et al.*, 2017). A study looking at clinical mastitis in the Kampala area identified 20 out of 58 (34.5%) strains to be coagulase negative staphylococci (CNS), 12 (20.7%) strains to be *E. coli* and only one (2%) to

be *S. aureus*. The most commonly found CNS strains were *S. saphrophyticus, S. xylosus* and *S. sciuri* (Kateete *et al.*, 2013).

Another study on subclinical mastitis in the urban and suburban region of Kampala showed that using the California mastitis test (CMT), 86.2% of 195 tested cattle had ≤ 3 in at least one quarter. This study found coagulase-negative staphylococci to be the most commonly isolated bacteria, and isolated *S. aureus* in 8 (0.9%) cases. In this study, all clinical cases of mastitis were excluded (Abrahmsén *et al.*, 2014). A complementary study in the same area reported clinical mastitis in 13% and CMT ≤ 3 in at least one quarter in 90% of the 138 cows tested. Bacteriological sampling of all quarters with CMT ≤ 4 revealed the most prevalent cause of clinical and subclinical mastitis to be coagulase-negative *staphylococci*, of which *S. epidermidis* was the most commonly isolated. *S. aureus* were isolated in 8.5% of the clinical and 4% of the subclinical cases of mastitis (Björk, 2013).

A thesis study on milk quality in South-western Uganda reported the average SCC in milk from 100 farms to be 507 000 cells/mL, with 34% of the farms being under 200 000 cells/mL and 7% being over 1000 000 cells/mL (Rutaro, 2015). All the Ugandan studies judged SCC according to scores described by Mellenberger & Roth (2008), where a score of "one" or more is considered sublinical mastitis and "three" is the maximum score. Abrahmsén *et al.* (2014) and Björk (2013) judged subclinical mastitis according to the Nordic grading system where "three" roughly corresponds to score "one" according to Mellenberger & Roth (2008; Table 1).

Table 1. *The two grading systems used for studies on subclinical mastitis and their corresponding SCC/mL*

Interpretation	Mellenberger & Roth	Corresponding SCC/ml	Nordic System	Corresponding SCC/ml
Negative	N	100 000	1	0 - 200 000
Trace	T	300 000	2	150 000 - 500 000
Weakly positive	1	900 000	3	400 000 - 1 500 000
Positive	2	2.7 million	4	800 000 - 5 000 000
Strongly positive	3	8.1 million	5	> 5 000 000

Many authors relate the high frequency of mastitis on Ugandan farms to poor milking hygiene and absence of milking order in relation to udder health status (Byarugaba, 2008; Kateete *et al.*, 2013; Abrahmsén *et al.*, 2014; Björk *et al.*, 2014; Ssajjakambwe *et al.*, 2017) and some authors also relate it to the suboptimal use of antibiotics (Byarugaba, 2008; Kateete *et al.*, 2013; Kasozi *et al.*, 2014; Ssajjakambwe *et al.*, 2017). The most commonly used antibiotics were penicillin and tetracycline (Byarugaba, 2008; Kateete *et al.*, 2013; Kasozi *et al.*, 2014; Ssajjakambwe *et al.*, 2017), an observation that is true for the veterinary sector at large in Uganda (UNAS, 2015).

Antibiotic usage in agriculture in Uganda is legally only permitted after prescription from veterinary surgeons, but the authorities' means for control are insufficient and most antibiotics are readily available over-the-counter for anyone in community pharmacies (Mukonzo *et al.*, 2013). Sales numbers are thereby hard or impossible to estimate. Usage of tetracycline in the vaccination procedure for *Theileria parva Lawrencei* described by Radley *et al.* (1979) is still widely practiced in Uganda (Perry, 2016). Knowledge of the accelerators of antibiotic resistance is low, and even students in health sciences are not using it appropriately (Nambatya *et al.*, 2011).

Antibiotic resistance

It is important to remember that usage of antibiotics against one pathogen is not only selecting for resistance in that specific pathogen, but also in all other bacteria exposed. Every time an antimicrobial agent is used it will select for overgrowth of bacteria that expresses genes for resistance towards that agent. Resistant strains will have less competition and can multiply with great speed. Since they might not cause disease, commensals that become resistant are likely to be selected and maintained without detection, giving them ample opportunity to serve as reservoirs of resistance genes (O'Brien, 2002).

Antibiotic resistance can be intrinsic, a natural quality in the organism in absence of selective pressure of antibiotics (Sefton, 2002). Of greater consequence is the acquired resistance, where the genome of the bacteria is altered either by spontaneous mutations and selection or by acquisition of extrinsic DNA (Sefton, 2002; Tenover, 2006). The three main mechanisms of gene transfer between microorganisms are conjugation of plasmids, transformation and bacteriophage transduction. In short, transduction occurs when bacteriophages (viruses that infects bacteria) accidentally carry bacterial genes between bacterial hosts and transformation may occur when a bacterium encounters free DNA in the environment and incorporates it into its chromosome (Holmes *et al.*, 2016). Plasmids are circular DNA mobile genetic elements that can transfer directly between two bacteria through a pore or tube connecting the two bacterial cells. Plasmid conjugation is the most efficient way of horizontal gene transfer and is considered one of the major reasons for spread of antibiotic resistance (Grohmann *et al.*, 2003).

The natural resistance of bacteria can be referred to as the "wild type" of that bacteria. The natural resistance of a bacterial species is relevant in the determination of epidemiological cut-off values (ECOFFs) for resistance, which are used in this study. The ECOFF value is not necessarily equivalent to the clinical resistance breakpoint, and an ECOFF value for a certain antibiotic in a bacterium that is naturally sensitive to that antibiotic can thus be lower than the MIC that is considered as clinical resistance (Swedres-Svarm, 2016).

In staphylococci, there are two different resistance mechanisms that mainly account for β-lactam resistance. It is achieved either through enzymatic inactivation by β-lactamase, or target site replacement by the gene products of *mec*-genes, coding for alternative penicillin-binding proteins that have a strongly reduced affinity to virtually all β-lactam antibiotics (McManus, 1997; Wendlandt *et al.*, 2015). *Staphylococcus aureus, Staphylococcus pseudintermedius* and coagulase-negative staphylococci can all carry *mec*-genes and are considered commensals with pathogenic potential (Piette & Verschraegen, 2009; Feßler *et al.*, 2010; Weese & van Duijkeren, 2010; Bannoehr & Guardabassi, 2012).

Staphylococcus aureus

Characteristics

S. aureus is a common coloniser of human and animal epithelia and may act as an opportunistic pathogen, often involved in skin and soft tissue infections (Grundmann *et al.*, 2006; Bouchiat *et al.*, 2017; Planet *et al.*, 2017). It commonly expresses a variety of different toxins and virulence factors that enable it to invade tissues and evade the immune system (Wamel *et al.*, 2006; Thammavongsa *et al.*, 2015). One of these is Panton-Valentine leucocidin (PVL), a two-component pore-forming toxin targeting human and rabbit mononuclear cells (Prévost *et al.*, 1995). It is encoded by the genes *lukS-PV* and *lukF-PV* and is transferred through bacteriophages (Boakes *et al.*, 2011). It has been linked by epidemiological data to invasive

disease in people without previous healthcare-contact (Lina *et al.*, 1999; Gillet *et al.*, 2002) but the extent to which the presence of the gene affects virulence is not clearly established (Voyich *et al.*, 2006; Boyle-Vavra & Daum, 2007; Malachowa *et al.*, 2011; van Hal *et al.*, 2012). An association between leukocidins and severity of bovine mastitis has also been suggested (Bar-Gal *et al.*, 2015). In bovine mastitis, different strains show distinct pathogenic, contagious and persistence traits, and may thus require different strategies of control and treatment (Fournier *et al.*, 2008; Graber *et al.*, 2009; Harris *et al.*, 2013; Wang *et al.*, 2017).

Different lineages of *S. aureus* generally show a certain degree of host specificity (Sung *et al.*, 2008). The differences were however found to be small, with genes between animal and human lineages showing no larger differences as compared to the differences in genes between animal lineages. Strains isolated from bovine mastitis were most commonly found to be native to the species, whereas other animals like horses and dogs were more often considered transient carriers, since strains isolated tended to originate from human lineages (Sung *et al.*, 2008). Other studies have found that human-bovine host shift can occur (Sakwinska *et al.*, 2011), that a bovine *S. aureus* strain was as capable of colonising and persisting in human nares as a human-native strain (Slingerland *et al.*, 2012) and when co-colonising gnotobiotic piglets, a human-native and a porcine-native strain were growing in equal densities (McCarthy *et al.*, 2014). Identical or near identical strains have also been isolated from humans and animals that live in close proximity, suggesting transfer of bacterial strains (Voss *et al.*, 2005; Weese *et al.*, 2006).

Different bacterial species vary greatly in the stability of the genome (from clonal to recombinogenic), and *S. aureus* displays a very clonal population structure (Vos & Didelot, 2009). Lineages are divided into clonal complexes (CCs) of strains with similar multilocus sequence types (MLSTs) or sometimes groups of CCs on an epidemiological basis (Haaber *et al.*, 2017). Another way of identifying related *S. aureus*-strains is *spa*-typing, where a variable region of the surface protein A is genotyped (Miao *et al.*, 2017). The mobile genetic elements (MGEs) comprise 15-20% of the genome in *S. aureus* (Haaber *et al.*, 2017) and the most common MGEs are bacteriophages, pathogenicity islands (SaPI), transposons, plasmids and staphylococcal cassette chromosomes (SCC) (Lindsay, 2010). All these elements may carry and transfer antibiotic resistance genes (Haaber *et al.*, 2017).

β-lactam-resistance in S. aureus

The first antibiotic widely used against staphylococci was penicillin. It binds to a penicillin-binding protein in the bacterial cell wall and ultimately kills the microbe. It was introduced in the 1940s and soon some strains were observed to resist the antibiotic. The cause of the resistance was identified as the production of penicillinases or β-lactamases, enzymes that can break down the β-lactam ring of penicillin (Kirby, 1944; Abraham *et al.*, 1941). Methicillin was the first semisynthetic penicillin and had an increased resistance towards β-lactamases (Grundmann *et al.*, 2006). Cephalosporin antibiotics are even more resistant to β-lactamases, although some β-lactamases have an increased affinity for cephalosporins (Bush & Jacoby, 2010).

Methicillin-resistant *S. aureus* (MRSA) was first identified in the early 1960's just two years after the introduction of Methicillin on the market (Jevons, 1961). The earliest described mediator of the broad β-lactam resistance that is associated with the methicillin-resistant strains of *S. aureus* is the gene *mecA* (Matsuhashi *et al.*, 1986), borne on a staphylococcal cassette chromosome *mec* (SCC*mec*) mobile genetic element. This gene induces changes in the bacterial

target of β-lactam antibiotics, the penicillin binding protein PB2, and dramatically diminishes its affinity for all β-lactam antibiotics (Hartman & Tomasz, 1981; Pinho *et al.*, 2001).

At first the strains isolated were primarily found in connection to human healthcare (Jack Benner & Kayser, 1968; Rountree & Beard, 1968; Grundmann *et al.*, 2006). These first strains became known as Healthcare associated/acquired (HA) MRSA when in the late 1990's, the community-associated/acquired (CA) MRSA started emerging (Herold *et al.*, 1998; Okuma *et al.*, 2002; Vandenesch *et al.*, 2003). These new CA-MRSA strains were isolated from humans without any apparent previous contact or connection to the healthcare system (Herold *et al.*, 1998; Stefani *et al.*, 2012). In the mid-late 2000's when livestock-associated (LA) MRSA strains were found in quick succession, it became evident that this also was a serious problem from the veterinary and OneHealth perspective (Voss *et al.*, 2005; van Loo *et al.*, 2007; Weese & van Duijkeren, 2010). Though, as strains of different clonal complexes are found in new settings, these associations are blurring (Bal *et al.*, 2016).

The gene $mecA_{LGA251}$, later renamed *mecC* (Ito *et al.*, 2012), was characterized in 2011 but has been found in clinical samples with origin from decades earlier (García-Álvarez *et al.*, 2011; Shore *et al.*, 2011). Thus, many samples and cases have potentially been missed or misdiagnosed (Guardabassi *et al.*, 2013; Paterson *et al.*, 2014). *mecC* is 70% homologous to *mecA* on a gene- and amino acid level (García-Álvarez *et al.*, 2011) but, interestingly, a slight difference in antibiotic resistance has been observed between the phenotypes associated with carriage of the two genes. The strains expressing *mecA* are most commonly resistant to both oxacillin and cefoxitin, while the strains expressing *mecC* are often relatively sensitive to oxacillin but resistant to cefoxitin. Therefore, cefoxitin is considered a better determinant of possible MRSA-strains as compared to oxacillin (Kim *et al.*, 2012; Skov *et al.*, 2014).

The provenance of the *mecA* genes is not yet known, however several studies have found *mecA* homologs in animal commensals like *Staphylococcus sciuri* (Couto *et al.*, 1996; Wu *et al.*, 2001; Monecke *et al.*, 2012; Harrison *et al.*, 2014), *Staphylococcus fleurettii* (Tsubakishita *et al.*, 2010; Monecke *et al.*, 2012), *Staphylococcus haemolyticus* (Monecke *et al.*, 2012) and *Macrococcus caseolyticus* (Baba *et al.*, 2009; Tsubakishita *et al.*, 2010; Gómez-Sanz *et al.*, 2015; Schwendener *et al.*, 2017). However, not all are associated with β-lactam resistance (Monecke *et al.*, 2012). Some of these *mecA* homologs have been proposed as the *mecA*-precursor (Couto *et al.*, 1996; Wu *et al.*, 1996; Rolo *et al.*, 2017). A recent study indicates that β-lactams and antibiotics that target DNA (e.g. trimethoprim and ciprofloxacin) can trigger a SOS-mechanism that promotes SCCmec-excision and thereby enable horizontal gene transfer (Liu *et al.*, 2017). In the absence of stressors, these excisions still occur but at a very low rate (Stojanov *et al.*, 2013; Liu *et al.*, 2017). There is evidence that SCC*mec*-elements have been exchanged between *S. epidermidis* and *S. aureus* (Wielders *et al.*, 2001; Méric *et al.*, 2015) and it has been proposed that *mecA* originated from coagulase-negative staphylococci, and was transferred through *S. epidermidis* to *S. aureus* (Méric *et al.*, 2015; Haaber *et al.*, 2017).

Transfer of *mecB* to *S. aureus* has however not been seen, until very recently when a *S. aureus* carrying *mecB* was isolated (Becker *et al.*, 2018). The isolate was recovered from a 67-year old cardiology inpatient with no signs of infection, during routine MRSA screening in Germany. The *mecB* was identical (100% complete sequence identity) to *mecB* earlier found in *M. caseolyticus* (Baba *et al.*, 2009; Tsubakishita *et al.*, 2010; Becker *et al.*, 2018) either within an SCC*mec*-like element or carried on a plasmid (Baba *et al.*, 2009; Tsubakishita *et al.*, 2010; Gómez-Sanz *et al.*, 2015). This *S. aureus* carried the *mecB* on a plasmid distantly related to the

one seen in *M. caseolyticus*, supporting possible gene transfer between the genera. Carriage on a plasmid has the potential to increase transferability of the methicillin resistance and drastically change the MRSA epidemiology (Becker *et al.*, 2018).

The prevalence of MRSA in EU/EEA countries is ranging between 1.2% and 50.5%, with the northern countries in general reporting the lower frequencies. A decreasing trend has been observed in more than a third of the countries between the years 2013-2016. Comprehensive MRSA strategies, regarding both prudent antimicrobial use and infection prevention and control across all healthcare sectors, remain essential to further slow the spread (ECDC, 2016).

Transfer and spread of resistance in S. aureus

As earlier mentioned, *S. aureus* displays a very clonal family tree (Vos & Didelot, 2009). Several barriers for gene transfer exists; The type I restriction-modification system (Waldron & Lindsay, 2006; Sung *et al.*, 2008; Cooper *et al.*, 2017) and type IV restriction systems (Corvaglia *et al.*, 2010; Xu *et al.*, 2011) are very effective and common and the CRISPR-cas-system are also present but uncommon (Cao *et al.*, 2016). There are differences in the restriction-modification systems between CCs, which has been proposed to underlie the findings that transfer within lineages rather than between lineages seem to be more common (Waldron & Lindsay, 2006; Roberts *et al.*, 2013; Planet *et al.*, 2017).

The spread of resistant clones can also be limited by fitness of the clones, illustrated by reports of clones outcompeting and replacing endemic clones (Knight *et al.*, 2012; Baldan *et al.*, 2015). *S. aureus* can exist on abiotic surfaces for months to years (Kramer *et al.*, 2006) and therefore clones that acquire genes that make them more successful at surviving on surfaces might spread more easily (Baldan *et al.*, 2015; Planet *et al.*, 2017). Fitness in relation to the survival on or in the host can be illustrated by bacteriophages that carry human-specific immune-evasion genes and that have been associated with human rather than animal isolates (Wamel *et al.*, 2006; Sung *et al.*, 2008). The largest SCC*mec*-element have also proven to affect fitness (Ender *et al.*, 2004) but others do not appear to have a fitness cost (Lee *et al.*, 2007; Knight *et al.*, 2013).

Interestingly, the mechanism of transfer of the SCC*mec*-elements between staphylococci remains unknown (Haaber *et al.*, 2017). Yet, the elements are widely dispersed in the genera (Haaber *et al.*, 2017) and mobile SCC*mec*-elements may be imported more frequently by different *S. aureus* clonal lineages than previously thought, judging from frequent emergence of new clones with limited geographic dispersal observed in global collections of isolates (Nübel *et al.*, 2008) and regional distribution of MRSA lineages in hospital settings in Europe (Grundmann *et al.*, 2010). The variance in *spa*-types and SCC*mec*-elements of non-Methicillin resistant *S. aureus* lineages is still far greater (Grundmann *et al.*, 2010; Becker *et al.*, 2017) but genome analysis suggests that some mobile genetic elements may even cross species boundaries, represented by the likely acquisition of vancomycin resistance from enterococci (Noble *et al.*, 1992; Weigel *et al.*, 2003; Zhu *et al.*, 2008; Rossi *et al.*, 2014).

Within lineages, in a co-colonisation model using gnotobiotic piglets, McCarthy *et al.* (2014) revealed horizontal gene transfer within just four hours. The study lasted a total of 16 days and notably, the strains co-cultured *in vitro* showed a considerably lower frequency of horizontal gene transfer than the *in vivo* strains (McCarthy *et al.*, 2014). In a co-colonisation model in nares of human volunteers, the bovine *S. aureus* strain 5062 (CC398) and human *S. aureus* strain 1036 (CC8), showed no horizontal gene transfers (Slingerland *et al.*, 2012), which McCarthy *et al.* (2014) postulates owed to that they were of different clonal complexes. Another recent study showed great inter-host variability of mobile genetic elements and antimicrobial

resistance in screened human patients in London, UK, with isolates of variable profiles isolated on a single sample occasion. Out of 38 MRSA carriers, only one carried isolates from different CCs. The results thereby were consistent with the piglet-colonisation model and suggested frequent horizontal gene transfer within lineages. It also puts the accuracy of antibiotic resistance profiles of other studies that only analyse a small number of colonies per sample in some uncertainty (Stanczak-Mrozek *et al.*, 2015).

Staphylococcus pseudintermedius

Characteristics and resistance genes

S. pseudintermedius is an opportunistic pathogen and the most prevalent cause of canine bacterial infections, but can also be found on cats and humans (Bannoehr & Guardabassi, 2012; McCarthy *et al.*, 2014; Pires Dos Santos *et al.*, 2016). *S. pseudintermedius* is part of the intermedius group, that used to be considered one species as they were indistinguishable phenotypically, but was characterized into three genera by molecular methods in 2007 (Sasaki *et al.*, 2007). They are now referred to as *S. delphini;* originally isolated from dolphins, *S. intermedius*; most commonly isolated from pigeons, and *S. pseudintermedius*; most commonly isolated from dogs (Bannoehr *et al.*, 2007; Sasaki *et al.*, 2007). After this discovery, it was proposed that any studies that isolated *S. intermedius* from dogs before 2007 should be considered to have found *S. pseudintermedius*, unless genomic investigations proved otherwise (Devriese *et al.*, 2009).

S. pseudintermedius produces a wide array of virulence factors, of which many have functional and structural similarities to those of *S. aureus* (Lindsay, 2008). Alike *S. aureus*, it also seems to have acquired methicillin resistance through the gene *mecA* on multiple occasions (Bannoehr *et al.*, 2007; Pires Dos Santos *et al.*, 2016). Some clones of MRSP have spread with great success (Perreten *et al.*, 2010; Ruscher *et al.*, 2010) and are a big impediment in the treatment of infected dogs (Perreten *et al.*, 2010; Weese & van Duijkeren, 2010; Kadlec *et al.*, 2016), potentially causing a shift of antibiotics used towards off-label use of critically important human antibiotics (Weese & van Duijkeren, 2010). Prevalence of MRSP among *S. pseudintermedius*-isolates from clinical cases varies considerably (Haenni *et al.*, 2014; Kjellman *et al.*, 2015; Grönthal *et al.*, 2017; Ventrella *et al.*, 2017; Worthing *et al.*, 2018) and has been reported to be as high as 67% (Kawakami *et al.*, 2010).

Human infection with MRSP is rare (Van Hoovels *et al.*, 2006; Stegmann *et al.*, 2010; van Duijkeren *et al.*, 2011), but may be overlooked by false identification in diagnostic laboratories, where the organism with some protocols gets misclassified as MRSA (van Duijkeren *et al.*, 2011; Guardabassi *et al.*, 2013; Börjesson *et al.*, 2015). Human carriage of *S. pseudintermedius* has been assumed to be unusual and transient (Weese & van Duijkeren, 2010) but varying degrees of *S. pseudintermedius* and MRSP-carriage in veterinarians or veterinary staff (Morris *et al.*, 2010; Paul *et al.*, 2011; Chanchaithong *et al.*, 2014) and dog owners (Chanchaithong *et al.*, 2014; Han *et al.*, 2016) have been reported. A study in Thailand however reported that in a group without pet contact, no carriers of either *S. pseudintermedius* or MRSP were found, whereas 10.5% of veterinarians and 13% of owners carried MRSP (Chanchaithong *et al.*, 2014). Reports of MRSP in Africa and South America are still too sparse to allow any conclusions to be drawn (Blunt *et al.*, 2013; Quitoco *et al.*, 2013; Pires Dos Santos *et al.*, 2016).

Diagnosis

Methods for isolation, determination of species and bacterial resistance

Many different methods have been used in the aforementioned studies, both regarding the mode of sampling and for the choice of medium for culture, the method for antibiotic resistance determination, and the methods used for identification of bacterial species or studies on the epidemiological background. The latter includes genotyping methods such as Multilocus sequence typing (MLST), *S aureus* Protein A *(spa)* typing, Pulsed field gel electrophoresis (PFGE), Restriction fragment length polymorphism (RFLP), Whole-genome sequencing and SCC*mec*-typing (Miao *et al.*, 2017).

The most sensitive sampling site for *S. aureus* in humans (Armstrong-Esther & Smith, 1976) and horses (Bergström *et al.*, 2013) is the nasal mucosa. In dogs, the nose is the second most common carrier-site for *S. pseudintermedius* and it is most reliably isolated by swabbing both the oral mucosa and the perineum (Bannoehr & Guardabassi, 2012).

For cultivation, chromogenic agars have been developed to facilitate rapid and accurate screening for MRSA. These agars use colourless enzymes that are hydrolysed by MRSA to produce a visible colour-change in or around growing colonies (Xu *et al.*, 2016). The chromogenic agar used in this study, Brilliance^{TM} MRSA 2 agar, has been reported to have both high sensitivity (100%) and specificity (99.1%), when adding a broth enrichment step before the screening of clinical samples (Veenemans *et al.*, 2013). Usage of enrichment broth before culturing has also been recommended in MRSP-screening, with subsequent culturing on blood agar being the most sensitive method (Saab *et al.*, 2017). The protocol used in this study is closely adapted from the method used at the Swedish National Veterinary Institute (SVA) and the Swedish University of Agricultural Sciences (SLU).

Using MLST and sequencing of the partial *hsp60* gene respectively as the Gold standard, Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry has been recognised as a quick and reliable identification method for *Staphylococci* (Decristophoris *et al.*, 2011; Murugaiyan *et al.*, 2014). Both MLST (Quitoco *et al.*, 2013; Haenni *et al.*, 2014; Kjellman *et al.*, 2015) and MALDI-TOF (Schwendener *et al.*, 2017; Becker *et al.*, 2018; Worthing *et al.*, 2018) were used in several of the earlier mentioned studies.

For detection of antibiotic resistance in *S. aureus* in veterinary medicine, the most commonly used phenotypic methods are agar diffusion and broth microdilution. Genotypic tests, like PCR screening for specific antibiotic resistance genes (e.g. *mec*-genes) can also be utilized. Agar disc diffusion utilizes paper discs infused with a known amount of antibiotics that are placed on agar plates where the bacteria that is to be tested has been evenly spread. The amount of bacteria on the plate, the time and temperature in the incubator etc. are predetermined and the antibiotics will diffuse out from the disc in the agar creating zones where the bacteria are inhibited from growing. These zones are measured and translated to levels of resistance (susceptible-intermediate-resistant) for the bacteria tested, with smaller zones equalling higher resistance. This method is cost-efficient but does not yield minimum inhibitory concentration (MIC) values for the antibiotics and standardisation can be problematic. Broth microdilution instead utilizes microtiterplates where the wells are lined with freeze-dried antibiotics in decreasing amounts. Two or three wells on each plate contain no antibiotics and serve as controls. Broth with a certain concentration of the bacteria that is to be tested is placed in each well, the wells are then sealed to prevent drying-off and incubated (in a predetermined time and

temperature). The antibiotics dissolve into the broth producing known concentrations of the antibiotic in each well, and the MIC is then set as the lowest concentration of antibiotics that produce no growth. The growth in the wells can be evaluated by visual inspection or by semi- or fully automated systems. The advantages to this method over disc diffusion is quantitative values of the resistance (MIC values) and that it is easier to standardise and automatize (Kadlec *et al*., 2015).

Spread of antibiotic resistance - What are the challenges and what is the extent of the problem?

Drivers of antibiotic resistance in developing countries

Reasons for antibiotic overuse and misuse in developing countries are many. Lack of knowledge by both "prescribers" (that may not be qualified in the first place) and by the users often results in faulty time of treatment or type of antibiotic. As laboratories of good standard are few, antimicrobial susceptibility patterns are usually unknown and leads to treatment based on empirical experience. Country-specific guidelines are often absent and continuing education programmes few. Opportunistic infections in people with severe malnutrition or HIV/AIDS, sometimes widely dispersed in these countries, may also increase the use (Okeke *et al.*, 2005a; UNAS, 2015).

Substandard quality of the antibiotics can also be a problem; they might be counterfeit, stored improperly or have passed their expiration date. Lower-than-stated doses cause suboptimal concentrations, which apart from causing therapeutic failure might result in selection of less drug-sensitive strains. A similar problem occurs when poor farmers sometimes cannot afford the prescribed full dose or treatment time-period. Economic factors also influence the prescribers, who sometimes even represent the manufacturers, and can cause excessive simultaneous use of multiple antibiotics, broad-spectrum antibiotics, high dosage and/or long treatment times (Okeke *et al.*, 2005b; UNAS, 2015).

Exposure to antibiotics by handling or ingesting milk-, egg- or meat-products is also present, since economic value of the products causes the farmers to ignore withdrawal periods (UNAS, 2015). In a review by Darwish *et al.* (2013) the most commonly found antibiotic residues in food sold for human consumption in studies from eight African countries, Uganda not included, was tetracyclines and β-lactam antibiotics. In many cases, the residues exceeded the WHO limits (Darwish *et al.*, 2013). A few reported frequencies of detectable penicillin in food products in African countries includes 14% of tested meat from a slaughterhouse in Nigeria (Ibrahim *et al.*, 2009), 2% of farm bulk milk in Ethiopia (Abebew *et al.*, 2014), 23% of locally produced beef in Egypt (Abdelrahman *et al.*, 2017) and 14% of milk in milk collection centers in Kenya (Shitandi & Sternesjö, 2001).

Besides the misuse of antimicrobials, the concurrent situation in many developing countries with shortfalls in the supply of clean water, vaccination coverage, sewage systems and safe food supplies increase the burden of infectious diseases that might need treatment with antibiotics (UNAS, 2015) and may also enable the spread of resistant microbes (Okeke *et al.*, 2005b). There are studies that suggest that transfer of MRSA between patients is more likely to occur in low-resource healthcare as compared to highly resourced hospitals (Price *et al.*, 2014; Tong *et al.*, 2015). Within the community, household subsistence farming is common in developing countries, and the close interaction between humans and animals likely increase the risk of transmission of any resistant organisms (Okeke *et al.*, 2005b). Animals in communal

grazing systems are also closely interacting (Asiimwe *et al.*, 2017b) and one study reported that the same individual milked separately owned cows on community grazing (Kateete *et al.*, 2014).

Staphylococcal antibiotic resistance in Uganda

Drug resistance is a prevalent and rapidly emerging problem in hospital settings in Uganda. In Mulago National Hospital, Kampala, SA accounted for 20.4% of 314 surgical site infections and out of these 37.5% were confirmed as MRSA by phenotype and by PCR targeting the *mecA* gene (Seni *et al.*, 2013). In the same hospital, a screening for *S. aureus* on patients (wounds or nostrils), healthcare workers (hands or nostrils) and environment (frequently touched surfaces) showed that all 41 *S. aureus*-isolates (41% of 100 samples in total) were MRSA. Of these, 73% were PVL-positive and all (100%) had *mecA* and displayed oxacillin resistance (Kateete *et al.*, 2011). In Kiruhura, South-western Uganda, out of 253 screening nasal swab cultures, *S. aureus* was isolated in 73 (28.8%) swabs and out of these 48 (65.7%) samples were positive for *mecA*. PVL-encoding genes were present in 36 (49.3%) samples and 25 (34.2%) samples were positive for both PVL and *mecA* (Asiimwe *et al.*, 2017a). Another screening of 499 rural in- and outpatients in the Mbarara Regional Referral Hospital for nasal carriage of MRSA by PCR (Cepheid Xpert SA Nasal Complete assay) found 2.8% of the samples to be positive, representing 9.7% of total *S. aureus* positives. Pig contact, open wounds and surgical ward admission was found to be associated with carriage (Bebell *et al.*, 2016).

Studies on *staphylococci* in Uganda using molecular typing methods are scarce in the veterinary sector. Asiimwe *et al.* (2017b) tested fresh bulk can milk in individual households in Kiruhura district, southwestern Uganda, which to their knowledge was the first study to demonstrate MRSA in raw milk in Uganda. After non-selective culturing on 5% sheep blood agar, *S. aureus* was isolated in 30/148 (20.3%) bulk can milk samples, of which 50% carried the *mecA*-gene and none carried *mecC*. When also including tested milk products (ghee and sour milk), 23/41 (56.1%) carried the *mecA* gene, five were positive for *lukS-PV* or *lukF-PV*, and 90% had at least one gene coding for enterotoxins. Interestingly, only two strains had zones of inhibition ≤ 22 mm for cefoxitin, making 21 strains of confirmed MRSA by *mecA*-carriage phenotypically sensitive to cefoxitin.

Out of 163 isolates from non-selective aerobic culture, *S. aureus* was isolated from 27 (16.6%) samples, in a survey on subclinical mastitis including 124 dairy cattle from 12 farms in Kiboga district, central Uganda. The *S. aureus* was not tested for oxacillin or cefoxitin resistance, but all (100%) were highly resistant to penicillin and most also had resistance patterns for other antibiotics according to the Kirby-Bauer disk diffusion method (Kasozi *et al.*, 2014). In Jinja district, eastern Uganda, coagulase-negative staphylococci was isolated in 30.5% and *S. aureus* was isolated in 11.9% of 688 quarter milk samples collected. Of all *S. aureus* and coagulase-negative staphylococci isolated, 29.7% were resistant to oxacillin and 86.8% were resistant to penicillin according to the Kirby-Bauer disk diffusion method (Buyarugaba *et al.*, 2008). An earlier MFS study on subclinical mastitis in the suburban region of Kampala isolated *S. aureus* in 8 (0.9%) of the samples, but out of these 50% were penicillinase producing (Abrahmsén *et al.*, 2014). Yet another MFS study found that out of isolated CNS and *S. aureus*, 9 isolates (100%) from clinical mastitis, and 22 isolates (81%) from subclinical mastitis, were β-lactamase producing. Of the nine *S. aureus* isolated, one had MIC ≥ 4 μg/mL for oxacillin and was thereby classified as an MRSA, one was not tested for oxacillin since it was negative for β-lactamase production, and resistance to cefoxitin was not evaluated in any of the isolates

(Björk, 2013).

A study in The Kampala area comparing clinical mastitis isolates and their milkmens' nasal isolates found coagulase-negative staphylococci resistant to both oxacillin and cefoxitin, interpreted as Methicillin resistance in both cows (12 of 21 isolated CNS, 57%) and humans (7 of 11 isolated CNS, 64%). However, out of 58 included strains only one *S. aureus* was isolated from cows, and 4 out of 31 (13%) from nasal swabs. All five had the same *spa*-type and were sensitive to oxacillin and cefoxitin but differed in sensitivity patterns to other antibiotics. Consequently, transmission was deemed unlikely (Kateete *et al.*, 2013).

MATERIAL AND METHODS

Sample collection

The proposal for the study was approved prior to commencement of the fieldwork by the Institutional Ethical Review Committee, College of Veterinary Medicine, Animal Resources and Biosecurity, Makerere University. An informed consent form was explained, signed and handed out to all animal owners or managers.

Samples from milk collection centers

26 samples from 21 milk collection centres were taken from various retailers in urban and suburban Kampala; some samples were collected repeatedly from the same retailers but on separate occasions and with several days in-between. The origin of the milk was recorded and only one sample sold in the same area and of similar origin was included per day, to decrease the risk of sampling milk from the same road-tanker. The milk was aspirated using sterile 10 cc syringes directly from the thin plastic bag that the milk is normally sold in, and transferred to a sterile test tube. The person aspirating was wearing disposable nitrile gloves disinfected with 70% alcohol. The samples were kept in a cooling box with ice clamps during the transport to the laboratory.

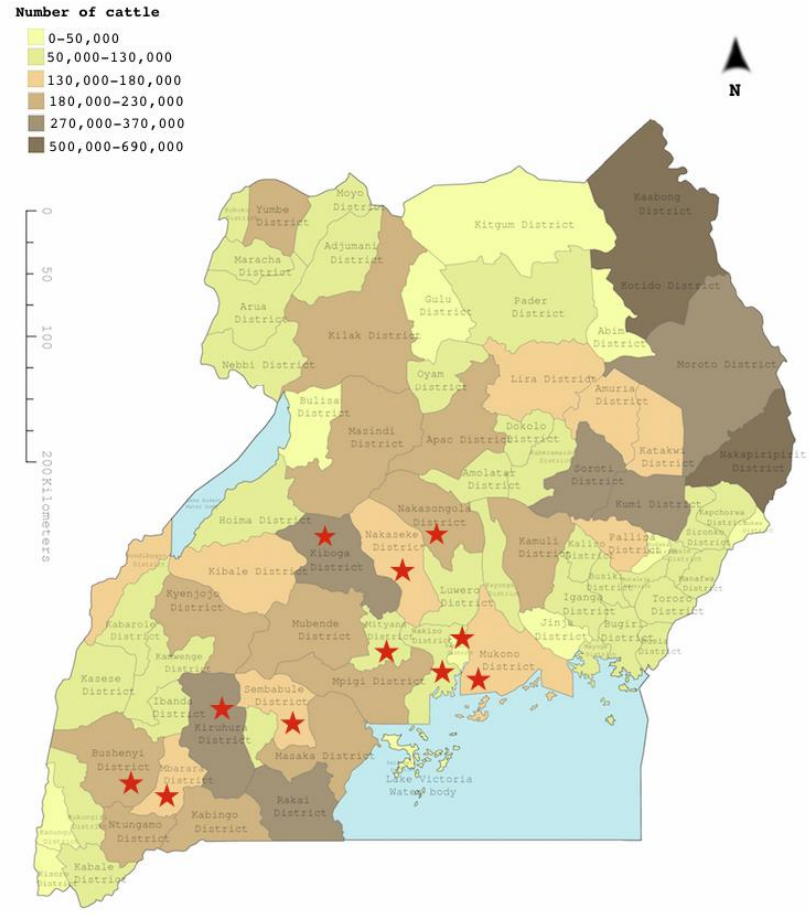

Figure 1. *Map of Uganda, illustrating the total amount of cattle in each district, based on numbers from the 2008 national census (UBOS, 2010).*

Stars represent the stated origin (district) of the milk sampled from milk collection centres.

Cattle samples

The local supervisors identified the farms selected for sampling, since local farmers only accepted my sampling in the company of their local veterinarian. However, efforts were made to scatter the locations to different areas and directions within 50 km of central Kampala. The total 16 farms included both large (15-55 animals) and small (2-5 animals) dairy farms. A majority of the farms were utilizing zero-grazing systems, but grazing and semi-grazing systems were also represented. A short questionnaire was performed to collect information on antibiotic usage and cattle history (see Appendix 1).

Swab samples were taken using ESwab™ sterile swabs with Amies media (Copan, Bresca, Italy), from perineum and nasal mucosa in the ruminants. If restraint was needed for collection of the nasal swabs, it was usually achieved by firmly holding the horns. One of the swab sites was excluded in 11 (17%) of the cases, due to excessive resistance from the animal. The 66 cows sampled were between 2.5-10 (mean 5.5) years of age and all were temperate (Friesian-Holstein) breed or temperate-crossbreed (Friesian-Holstein cross with native breed). Four calves were also sampled, with nose-only or nose and perineal swabs. A median of 4.5 cows were sampled from each farm, ranging from 2 to 7 samples.

Approximately 2-8 mL of milk from two to four teats was also collected from each cow. In the majority of cases, these were taken with the aid of the farmer who milked the cow while the sampler was catching the milk in a sterile sample tube. Some farmers washed the teats with water as a normal preparation before milking but no disinfectants were used. The farmers milking into the tube did not wear gloves but the sampler holding the tube was wearing disposable nitrile gloves that had also been disinfected with 70% alcohol. The samples were kept in a cooling box with ice clamps during the transport to the laboratory.

Dog samples

39 swabs for MRSP/MRSA screening in dogs were taken from patients seeking care in two animal clinics (13 samples from clinic A and 12 samples from clinic B), one rescue dog shelter (8 samples), two farms (3 samples) and three private homes (3 samples). Wearing disinfected gloves in an analogous manner, the dogs were swabbed on the nose and perineum using ESwab™ sterile swabs put in Amies media. A short questionnaire was performed to collect information on previous antibiotic treatment and illness history (see Appendix 2).

Culturing and analyses

A total of 96 samples from cattle and/or milk and 39 samples from dogs were included in the analyses. The methods used to determine carrier status were adapted from the methods currently employed by the Swedish National Veterinary Institute (SVA):

Swabs and/or 0.8 mL of the milk sample were at the end of the day (or within 24 hours) mixed and transferred to 8 mL of selective enrichment broth. The cattle samples were pooled by means of adding both the milk and the swab from one individual to one tube with broth. Tryptic soy broth with 4% NaCl, 1% mannitol (SVA, Uppsala, Sweden) and 75 mg/L aztreonam added were used for both milk, cow and dog samples, but to allow growth of both MRSA and MRSP in the dog samples, the concentration of cefoxitin in the broth was calculated to 1 mg/L instead of 4mg/L. The broth was incubated at 37°C for 1-1.5 days. One negative control was set for every batch of broth.

The broth was vortexed, and 20 µL per plate was cultured on 5% bovine blood agar (SVA, Uppsala, Sweden) and chromogenic selective agar for MRSA (Oxoid Brilliance^{TM} MRSA 2 agar; Oxoid, Basingstoke, Great Britain). The dog samples were cultured on 5% bovine blood agar (SVA, Uppsala, Sweden) and Mannitol salt agar with LiCl (MAST agar; SVA, Uppsala, Sweden). The plates were incubated at 37°C, and growth was interpreted after 1 and 2 days. Suspected colonies were pure-cultured on 5% bovine blood agar plates and then assessed for haemolysis and Gram-stained. They were also tested with potassium hydroxide for distinction between Gram-positive and Gram-negative bacteria and catalase for distinction between *streptococci* and *staphylococci*. Isolates with β-, broad α-, or double haemolysis that were catalase-positive, potassium hydroxide-negative and microscopically were identified as cocci were frozen in BHI with 17% glycerol added (Oxoid, Basingstoke, Great Britain) until the end of the field work. Some phenotypically commonly occurring strains in the samples were also preserved for species-identification in Sweden. At the end of the fieldwork the isolates were again subcultured and shipped to Sweden in Amie's media.

In Sweden, the isolates were cultured on 5% bovine blood agar plates. All strains were species-identified using MALDI-TOF (Matrix Assisted Laser Desorption Ionization-Time of Flight) mass spectrometry (Bruker, Bremen, Germany). The strains that proved to be *S. aureus* or *S. pseudintermedius* were again sub-cultured and tested for antimicrobial susceptibility by determination of minimum inhibitory concentration (MIC) using VetMIC CLIN staph/strept panels (E 395129; SVA, Uppsala, Sweden) and cation-adjusted Mueller-Hinton broth (SVA, Uppsala, Sweden), a microdilution method, according to Clinical and Laboratory Standards Institute (CLSI; VET01/M100) standards. The control strain *S. aureus* ATCC 29213 was tested in parallel with the isolates.

The isolates were classified as "susceptible" or "resistant" based on species-specific epidemiological cut-off values for each type of antibiotic, issued by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). For some antibiotics that were not listed by EUCAST, the cut-off values used in the annual Swedres-Svarm-report was used (Swedres-Svarm, 2016). *Staphylococcus aureus* with MIC ≤ 2 µg/mL for cefoxitin and *S. pseudinter-medius* with MIC ≤ 0.5 µg/mL for oxacillin were assayed for *mecA*, *mecC* and *lukS*-PV using Real-Time PCR following a protocol previously described (Pichon *et al.*, 2012), using the *S. aureus* strains CCUG35601, CCUG60578 and CCUG63582 as controls.

RESULTS

Samples from milk collection centers

Of the 26 samples from milk collection centres, *S. aureus* were isolated from five samples, corresponding to 19.2% of all samples in this category. One sample was identified as MRSA by phenotype (representing 3.8% of all samples in this category) but was negative for *mecA* and *mecC* (Table 2 no. 8). This isolate was also positive for PVL-toxin and resistant to tetracycline. The other four samples had a MIC of 4 µg/mL for cefoxitin and all were negative for *mecA*, *mecC* and *lukS*-PV (see Table 2).

An additional six samples from milk collection centres were tested without the broth-enrichment step. The samples were originally excluded since they were collected from retailers that stated the same region of origin of the milk (Mbarara), but the next day 20 µL of milk from each sample was streaked directly on 5% bovine blood agar (SVA, Uppsala, Sweden). One sample was suspected and later proven to grow *S. aureus* (Table 2, No. 15).

Table 2. *Distribution of MIC values (µg/ml) among the S. aureus isolates. None of the 15 isolates expressed resistance towards enrofloxacin, fusidic acid, clindamycin, gentamicin or nitrofurantoine. Isolates no 1-4 originating from cows, No.s 5-9 originating from milk collection centres, no. 10 originating from a dog in clinic B, No.s 11-12 originating from dogs in the shelter, No.s 13-14 from dogs at a farm and no 15 from a milk collection centre without broth enrichment. Abbreviations: Pen= penicillin; Cet= cefalotin; Oxa= oxacillin; Fox= cefoxitin; Ery= erythromycin; Tet= tetracycline; Tsu= trimethoprim-sulfonamide*

Isolate no.	Pen	Cet	Oxa	Fox	Ery	Tet	Tsu	PCR
1	>1	<1	1	4	1	0.5	0.5/9.5	PVL-pos.
2	>1	<1	0.5	4	<0.5	0.5	>4/76	PVL-pos.
3	>1	<1	>1	4	<0.5	0.5	0.5/9.5	PVL-pos.
4	>1	<1	<0.25	4	<0.5	>4	<0.25/4.75	Neg.
5	>1	<1	1	4	<0.5	>4	<0.25/4.75	Neg.
6	>1	<1	>1	4	<0.5	>4	<0.25/4.75	Neg.
7	0.06	<1	0.5	4	<0.5	0.5	<0.25/4.75	Neg.
8	>1	2	>1	>8	<0.5	>4	0.5/9,5	PVL-pos.
9	>1	<1	0.5	4	<0.5	>4	<0.25/4.75	Neg.
10	>1	<1	1	4	<0.5	0.5	>4/76	PVL-pos.
11	>1	<1	<0.25	4	<0.5	<0.25	<0.25/4.75	Neg.
12	<0.03	<1	<0.25	2	1	0.5	<0.25/4.75	Neg.
13	>1	<1	<0.25	2	<0.5	1	<0.25/4.75	Neg.
14	>1	<1	<0.25	2	<0.5	0.5	<0.25/4.75	Neg.
15	>1	<1	<0.25	2	<0.5	<0.25	<0.25/4.75	PVL-pos.

Cattle samples

In total, cows from 16 farms and households were sampled. The results from the questionnaire indicated that most farmers had used antibiotics historically. Five farmers stated that they used intramammal dry-cow therapy for all cows and six farmers perceived mastitis to be a reoccurring problem in their herd. During the withdrawal periods, most farmers fed the milk to the calves, pigs or dogs. Only two farmers stated to sometimes discard the milk, and two admitted that the milk sometimes was drunk by humans or even sold. Most farmers stated that their milkers washed their hands before but not after the milking. All cows were manually milked and about one out of four had problems with sores on the teats. Two farms stated that the milkers also worked on other farms, two cows sometimes used community grazing. All but one farm stated that the buyers come to the farm and the cows are not brought to the market. All respondents answered that the veterinarian prescribed the drugs used.

The cows' history of mastitis was not always known, but a third of the cows were stated to have a history of mastitis and in three cows, the mastitis was perceived as recurring. Four farmers used a combination of procaine penicillin, streptomycin sulphate, neomycin sulphate and prednisolon (Multiject IMM, Norbrook, Great Britain), intramammary suspension, as a standard treatment for clinical mastitis. Dry cow therapy for all cows with cloxacillin was reported by three farmers. Four farmers stated previous use of tetracycline and/or gentamycin and eight reported previous use of penicillin with or without streptomycin.

Four *S. aureus*-isolates were found in samples from three cows (4.5% of all samples in this category) and all had a MIC of 4 μg/mL for cefoxitin. One of the tested animals appeared to have two *S. aureus* strains (Table 2, No.s 2 and 3), both displaying cefoxitin resistance of 4 μg/mL but with distinctly different phenotypical appearance on the plate, and displaying differing resistances towards oxacillin and trimethoprim/sulphadiazin. The isolate depicted as number one (Table 2), was isolated from a cow kept at a separate location but with the same owner as No.s 2 and 3. All three isolates from that farm were PVL-positive (Table 2, No.s 1, 2, and 3).

Dog samples

Out of 39 dog samples *S. aureus* grew in five samples (Table 2, No.s 10-14), representing 12.8% of all samples in this category. Among the five *S. aureus*- strains isolated from dogs, one strain was from clinic B, two were from the shelter-dogs and two were from a farm. Out of 10 samples that initially grew *S. pseudintermedius* (25.6% of all samples in this category, see Table 3) none showed any resistance to oxacillin and therefore none was included for PCR. However, three *S. pseudintermedius* strains had a MIC of 2 μg/mL for erythromycin and clindamycin and five strains had a MIC of > 4 μg/mL for tetracycline. One strain, from clinic A, showed resistance towards almost all tested antibiotics except the β-lactams; enrofloxacin, erythromycin, clindamycin, gentamicin, tetracycline, trimethoprim and sulphadiazin (Table 3, No. 2).

From the questionnaires, the median age of the dogs sampled (excluding 11 with unknown age) was 2 years, ranging from 2 months to 14 years. One third of the dogs (13/39) had been treated with antibiotics in the last 6 months. Out of the dogs with isolated *S. pseudintermedius*, six (60%) had been treated with antibiotics in the last 6 months (Table 3, No.s 2, 3, 4, 6, 7 and 10). No treatment failures were perceived, and the most commonly used antibiotics were penicillin-streptomycin and metronidazole. Three dogs had a history of dermatitis and of these, one grew haemolytic staphylococci that proved to be *S. pseudintermedius* (Table 3, No. 6). No dogs except for the shelter dogs were kept in a kennel with other dogs. None of the dogs with isolated

S. aureus had been treated with antibiotics, but the shelter dogs lived in close proximity and were treated with antibiotics after spaying that occurred regularly. The answers regarding prescribers of the antibiotics and whether anyone in the family worked with animals or healthcare was mostly not recorded, hence these results are not presented.

Table 3. *Distribution of MIC values (µg/mL) among the S. pseudintermedius isolated. None of the 10 isolates expressed MIC <0.5 µg/mL for fusidic acid or <16 µg/mL for nitrofurantoine. No.s 2-5 originated from clinic A, No.s 6-10 from clinic B, and isolate no 1 from a farm. Abbreviations: Pen= penicillin; Cet= cefalotin; Oxa= oxacillin; Fox= cefoxitin; Enr= enrofloxacin; Ery= erythromycin; Cli= clindamycin; Gen= gentamicin; Tet= tetracycline; Tsu= trimethoprim-sulfonamide*

Isolate	Pen	Cet	Oxa	Fox	Enr	Ery	Cli	Gen	Tet	Tsu
1	1	<1	<0.25	<0.25	0.5	>2	>2	<1	>4	0.5/9.5
2	0.25	<1	<0.25	<0.25	1	>2	>2	>4	>4	>4/76
3	0.5	<1	<0.25	<0.25	<0.25	<0.5	<0.5	<1	>4	>4/76
4	<0.03	<1	<0.25	<0.25	<0.25	<0.5	<0.5	<1	<0.25	0.5/9.5
5	<0.03	<1	<0.25	0.5	<0.25	<0.5	<0.5	<1	<0.25	0.5/9.5
6	>1	<1	<0.25	<0.25	0.5	>2	>2	<1	>4	0.5/9.5
7	>1	<1	<0.25	0.5	<0.25	<0.5	<0.5	<1	>4	1/19
8	<0.03	<1	<0.25	<0.25	<0.25	<0.5	<0.5	<1	<0.25	<0.25/ 4.75
9	0.5	<1	<0.25	<0.25	<0.25	<0.5	<0.5	<1	<0.25	0.5/9.5
10	>1	<1	<0.25	<0.25	<0.25	<0.5	<0.5	<1	>4	1/19

Profuse growth of bacterial and sometimes fungal species was found in most samples, including various kinds of *bacilli*, yeast and/or mold. All samples appeared to have at least two species of bacteria growing on the blood agar plates, and only a few samples had a single species growing on the selective Brilliance^{TM}-agar plates. Fourteen isolates that were not included in the results were transported to Sweden. Three were inconclusive or suspected on the tests in Uganda, and the rest were brought out of interest or as examples, of which four were non-haemolysing staphylococci. Species identified by MALDI-TOF included *Macrococcus caseolyticus*, *Enterococcus faecalis*, *Aerococcus viridans*, *Staphylococcus sciuri*, *Staphylococcus schleiferi* and *Staphylococcus epidermidis*. These were not tested for antibiotic susceptibility or for *mec/luk* -genes.

DISCUSSION

Samples from milk collection centers

With only one isolated MRSA (Table 2, No. 8), any predisposing factors or common variables are impossible to determine. The milk from which the MRSA was isolated had a stated origin of Nyabushozi, South-western Uganda. On two subsequent samplings within eight days from the same shop, *S. aureus* was not found. This is encouraging and could indicate that the bacteria did not persist and perhaps successfully were removed during cleaning of the tank. Possibly, it could also have been picked up from the hands of the retailer on the day of the first sampling.

A MIC of > 4 μg/L for cefoxitin in *S. aureus* is mostly due to the presence of *mecA* or *mecC* (EUCAST, 2018), so it is notable that the MRSA-isolate (Table 2, No. 8) appeared not to carry these genes. Further analyses to determine the cause of the high-level resistance towards β-lactams in the MRSA-isolate would be very relevant and interesting, preferably using whole-genome sequencing. This would illustrate any mutations that might have affected the primer attachment-sites and therefore prevented amplification or could detect the presence of other *mec*-genes (Hill-Cawthorne *et al*., 2014). As *M. caseolyticus* recently has been proved to carry and have the ability to transfer a *mecB*-element to *S. aureus* (Tsubakishita *et al.*, 2010; Schwendener *et al.*, 2017; Becker *et al.*, 2018), analysis for carriage of *mecB* might prove relevant, but is much less informative than whole-genome sequencing. The microdilution-array for the MRSA-isolate was however only done once, so an advisable first step would be to repeat the test to confirm the MIC-value.

The microdilution-array used in this study limited the determination of exact MIC values for all β-lactams concerning the MRSA strain isolated (Table 2, No. 8), except the MIC for cefalotin. As a comparison, confirmed MRSA-isolates with the same MIC (2 μg/L) for cefalotin as the isolate in this study, collected from animals in Sweden between 2006-2016, had MIC values of > 16 μg/mL for cefoxitin in 80% of the cases (range 8 to > 16), and MIC values of > 16 μg/mL for oxacillin in 60% of the cases (range 1 to > 16) (Swedres-Svarm, 2016). The recently described *mecB*-carrying MRSA UKM4229 also had a MIC value for cefalotin of 2 μg/L, and its MIC was 32 μg/mL for cefoxitin and 12 μg/mL for oxacillin (Becker *et al.*, 2018). It is possible that the isolate found in this study had comparable MIC values for cefoxitin and oxacillin. It was the only strain isolated that displayed resistance towards cefalotin.

The total percentage of milk samples from which *S. aureus* was isolated (19.2%) correlates well with the recent results of Asiimwe *et al.* (2017b; 20.3%). This study also reports that out of 23 *mecA*-positive Ugandan isolates from milk samples, only two were phenotypically resistant to cefoxitin according to EUCAST definitions. These two isolates were also resistant to oxacillin; the resistance status to oxacillin regarding the remaining isolates was however not accounted for. This could otherwise have been an interesting comparison since among the *S. aureus* isolated in this study, a variance in susceptibility to oxacillin between < 0.25 to > 1 μg/mL was noted in the strains with MIC 4 μg/mL for cefoxitin. The results of Asiimwe *et al.* (2017b) also implies that the method used in this study, with added antibiotics to the enrichment broth, could mean *S. aureus* that were not expressing their *mec*-genes were not found. All *S. aureus*-isolates in the present study were negative for the *mecA* and *mecC* genes, however six (including the single MRSA isolate, Table 2, No. 8) were positive for *lukS*-PV, a gene that possibly can potentiate the bacterium in the early stages of infection (Voyich *et al.*, 2006). All *S. aureus* isolates in this study showed some resistance to β-lactams, which in theory can affect the outcome of clinical treatment.

The scope of the study did not allow analysis of all staphylococcus-species found, even if some are known to be able to carry *mec*-genes (Couto *et al.*, 1996; Wu *et al.*, 2001; Monecke *et al.*, 2012) that have been suspected to have been transferred to *S. aureus* (Wielders *et al.*, 2001; Méric *et al.*, 2015). The absolute majority of milk and cow samples appeared to grow coagulase-negative staphylococci (and *Macrococcus caseolyticus*), including the samples that also grew *S. aureus*. In future studies, it would be relevant to analyse most of the staphylococci that appear to be able to grow in the selective antibiotic broth, because of their pathogenic potential to humans and cattle (Piette & Verschraegen, 2009; Björk, 2013; Buyarugaba *et al.*, 2008) but also for indications of possible transfer of *mec*-genes.

Cattle samples

The farmers were not asked to wear gloves or wash the teats with disinfectants/alcohol before sampling. Further, milk samples and swab samples from the same individual were pooled. This procedure was chosen since any MRSA found on the outside of the teat, on the nose or perineum, or on the milkers' hands means that it is present in the cows' immediate surroundings and are posing the cow at risk for infection. As anticipated, *S. aureus* did still not appear to be common in the samples.

The sample groups were not randomly selected, and the farms selected were dependent upon previous contacts with veterinarians, a bias that can be claimed to both favour and oppose the results in this study, since the system in Uganda does not exclusively limit the usage of antibiotics to veterinarians. Antibiotic usage could be favoured, since they have contact with a veterinarian that might observe disease and recommend therapy, or it could be causing a more directed or appropriate usage and thereby result in lower usage than a farmer without guidance would have. The sample group was also heavily biased towards exotic or crossbreed exotic cows, kept in intense systems. According to the questionnaire, most cows had limited direct and indirect contact with other cattle, which could decrease the risk of transmission of disease and bacteria.

A few individual responses might also be mentioned. For example, one farmer stated that they treated cows with suspected ECF with tetracycline just to get the fever down, and one treated all cows with penicillin-streptomycin the first few days after giving birth. Many farmers did not seem to know the difference between antibiotics and other drugs, and to make sure to not miss anything, all drugs were listed, including vaccinations and deworming. It was generally hard to establish the timeline of when the treatments had taken place, so except for in a few cases when it was recent, the treatments they usually used or had used in the past were noted. Hence records of individual treatments were too vague to produce any statistics.

It was observed that most milkmen used a poor milking technique, pulling the teats instead of squeezing them, an observation that has been made in earlier studies (Kateete *et al.*, 2013). As earlier mentioned, around one out of four cows had problems with sores on the teats, which according to local veterinarians is a common side effect of this milking technique. To prevent these sores, some farmers used Norbrook Milking Salve (Noorbrook, Nairobi, Kenya) with 0.55% w/w Dichlorophen BP. Dichlorophen is a biocide that has been used as a veterinary anticestodal drug, and has antifungal, antiprotozoal and antibacterial activity (Aronson, 2016). From observations made during the study, it was highly likely to contaminate the milk during milking.

If, despite the milking salve, the sores get infected they can serve as a reservoir and cause spread of the bacteria to other milking cows. Even in the absence of clinical signs of infection, damage in the skins' natural barrier will provide a favourable environment for bacteria.

Dog samples

The sample group was probably biased towards dogs that had been treated with antibiotics since most dogs were sampled at veterinary clinics and a third of the dogs had been treated in the last six months. Since no numbers are available on antibiotic consumption for the species in Uganda, this can however not be verified. The proportion of treated dogs seemed to be higher (6 of 10) in the group where *S. pseudintermedius* could be detected, as compared with dogs where *S. pseudintermedius* could not be isolated (7 of 29). If the sample had been larger this difference would have been interesting to test for statistical significance. There also seemed to exist an association between antibiotic treatment in the last six months and the dogs carrying *S. pseudintermedius* displaying resistance to more than one antibiotic, since all but one of these were isolated from dogs that had a history of antibiotic treatment. Since the kind(s) of antibiotic(s) used were not always specified and the sample size was very small, the relevance of these findings is unclear. It is however established that antibiotic use promotes antibiotic resistance and sometimes genes for resistance to different antibiotic groups are linked (Knight *et al*., 2012; Perreten *et al.*, 2010; Bal *et al*., 2016) so it is possible that the connection would still be found in a larger sample group.

The exception was the dog from the farm, from which both *S. pseudintermedius* (Table 3, No. 1) and *S. aureus* (Table 2, No. 14) were detected and it lived on a farm where *S. aureus* was isolated from a cattle-sample (Table 2, No. 4). Another dog was sampled there, and *S. aureus* was isolated (Table 2, No. 13) from the swab. Both *S. aureus*- isolates from the dogs were susceptible upon microdilution in Sweden (2 μg/mL for cefoxitin). These results might still suggest transfer of antibiotic resistance, although then more likely of β-lactamases and tetracycline resistance. A third sample from a dog on that farm was unfortunately unaccounted for upon arrival to Sweden, but all three samples grew haemolytic staphylococci. This was the only sample that was unaccounted for.

Since cefoxitin resistance is not considered predictive for Methicillin-resistance in *S. pseudintermedius* (EUCAST, 2018), the inclusion of this antibiotic in the enrichment broth used for cultivation of the dog samples could have negatively affected the number of samples where *S. pseudintermedius* could be detected. For safety reasons, the oral mucosa was not sampled, which also could have affected the results since it is one of the most common carrier sites (Bannoehr & Guardabassi, 2012).

The dogs could easily be colonised with environmental flora since dogs tend to sniff their surroundings and the strains that were obtained could have been picked up upon arriving, or even from the samplers. Even if measures were taken to collect the swabs as soon as possible at arrival to the clinic, this was not always the case.

Confounding factors and observations

Sampling and analyses of bacteria in a developing country is a challenge to overcome, from the hardships with translation to the occasionally malfunctioning equipment and lack of electricity. The questionnaires were interpreted and conveyed by the same person on all occasions, but the answers still sometimes seemed a bit lost in translation. Not all questions were answered satisfactory, and some answers were excluded when it was suspected that the respondent had

misinterpreted them. Still, the response frequency to the questions that have declared results was over 85%, with some exceptions already mentioned. The presence of both the researchers and the local veterinarian might also have been influencing the answers.

The incubators had backup-electricity and were to our knowledge not affected by any power failures. However, in the cultivation of approximately the 20 first samples (from individual cattle), growth was slower than anticipated. This was amended when an incubator that was not opened as frequently was designated for this study, but the first samples were thus given less opportunity to grow in the broth, a possible source of error. The rest of the samples were incubated approximately 1-1.5 days and all plates were also evaluated after 2 days to ensure nothing was missed because of decreased speed of growth.

Notably, two strains of *S. aureus* displayed almost no resistance towards penicillin and still had a MIC of 2-4 μg/mL for cefoxitin (Table 2, No.s 7 and 12). β-lactamases that have a higher affinity towards cefoxitin than penicillin do exist, however they are very rare (Bush *et al.*, 1995) and the results might be faulty despite repeated analysis.

The colour and appearance of the suspected MRSA on the Brilliance-plates mostly did not appear typical, none of the isolated *S.aureus* were found through their appearance on the brilliance plates. This might be because none turned out to carry *mecA* or *mecC*, although the no. 8 (Table 2) and no. 1 (Table 2) did grow in an anticipated manner for MRSA on the Brilliance-agar, when subcultured from the suspected colonies found on the blood agar plates. Since all suspected staphylococci with haemolysis were included, this would not have affected the results, but streaking all samples on the brilliance plates seemed an unnecessary procedure.

Suspected contaminating colonies were isolated from a total of five samples. Upon resistance testing, they all had the same antibiotic resistance pattern as an example strain for MRSA that had been isolated in the Ugandan lab from a dog with an infected wound, prior to the commencement of this study. The five samples included three samples that were originally shipped to Sweden as example strains of non-haemolytic coagulase-negative staphylococci but were contaminated with *S. aureus* upon culturing in Sweden, and secondly, two isolates that were identified as contaminations in Uganda since they were found out of streak, and were brought for analysis in Sweden out of curiosity. These isolates had a MIC > 1 μg/mL for oxacillin and penicillin, 8 μg/mL for cefoxitin, > 1 μg/mL for enrofloxacin, > 2 μg/mL for erythromycin and > 4 μg/mL for gentamicin. They were sensitive to the other antibiotics tested. The MRSA example strain was negative for *mecA*, *mecC* and PVL-toxin. The occurrence of this identical antibiotic resistance pattern on multiple contaminants of the samples is suggestive of a lab-borne contagion. However, none of the other antibiotic resistance patterns clearly suggested a common origin for the rest of the samples.

Three of the samples from milk collection centres (Table 2, No.s 6, 7 and 9) derived *S. aureus* from just one colony with clear haemolysis in the primary streak. This might be explained by their MIC for cefoxitin, which was the same concentration (4 μg/ml) as the broth used in Uganda. The bacteria supposedly were able to survive but not to reproduce, making the amount of *S. aureus* scarce in the broth and thus rendering colonies only appearing in the primary streak. The massive growth of other bacteria in the broth could also have inhibited growth due to shortage of substrate. Most of the milk and cow samples had large pellets of bacteria at the bottom of the tubes by the time they were to be streaked on the agar plates. Another explanation would be that the primary streak was contaminated after streaking of the sample.

Final comments

The risk of transfer of staphylococci to humans from animals and vice versa is well documented (van Loo *et al.*, 2007; Weese & van Duijkeren, 2010; Soedarmanto *et al.*, 2011). The situation in Uganda with poor milking hygiene (Byarugaba, 2008; Kateete *et al.*, 2013), high frequency of (SCM) mastitis (Abrahmsén *et al.*, 2014; Björk *et al.*, 2014), poorly controlled use of antibiotics (Mukonzo *et al.*, 2013) and sale and distribution of unprocessed raw milk (Grimaud *et al.*, 2009) leaves both humans and animals exposed to possible future spread of and disease caused by resistant bacteria. This spread is already evident in some hospital settings in Uganda (Ojulong *et al.*, 2009; Kateete *et al.*, 2011; Seni *et al.*, 2013). With molecular characterization of strains of animal origin largely lacking, the relevance of animals as potential reservoirs cannot be assessed. More studies are needed and awareness of the problem among the public needs to increase. From the results in this study, sampling of unprocessed bulk milk seems a cost-efficient way to obtain a broad perspective on the prevalence of antibiotic resistance in the dairy industry in Uganda and is a very relevant direction for future studies. The presence of methicillin resistance seemed low in this study but resistance to other antibiotics and among other bacterial species seemed more commonplace. However, the sample size was small and the biases many, so further studies are needed to make any statements about antibiotic resistance in Kampala or Uganda.

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APPENDIX 1

Questionnaire for large and small dairy units **Sample number:**
Date:

General questions about the herd:

Herd is: Grazing/Semi-grazing/community grazing/tethering/zero grazing

1. Have your cattle been treated with medicine in the last six months? With what?

Do you use any regular treatments with antibiotics?

2. Who decided which drugs to be used? Owner/manager/local vet/pharmacist

3. What happens to the milk during withdrawal period? Sold/fed to the animals/humans drink/poured away

4. Do you think mastitis is common on your cow(s), for the last year(s)? YES/NO/Unsure

5. Do you think your herd has problems with recurring mastitis? YES/NO/Unsure

6. Do you wash your hands before Often/Sometimes/Rarely

and/or after milking? Often/Sometimes/Rarely

7. Do you use dry cow therapy? Is this on every animal, or only those that has had mastitis?

8. Are the cows sold at the market? If not sold, are they released back into the herd?

9. Do the milkers work on other farms as well? YES/NO/Unsure

10. Do you want to know the preliminary results? YES/NO

Questions about the individual cow(s): Age?

11. Has this cow been treated with medicine? Within the last three months or before that?

12. With what?

13. Did it respond, or did you have to change treatment? Using which drug? YES/NO/Unsure

14. Does this cow have a history of mastitis? YES/NO/Unsure

15. Has it had mastitis several times? YES/NO/Unsure

Notes on site and manor of sample collection:

APPENDIX 2

Questionnaire for dog owners **Sample number:**
Date:

1. Age of the dog?

2. Has your dog been treated with antibiotics, for example for a wound, in the last six months? Do you remember the name of the product?

3. … If so, who prescribed/provided it for you? Owner/manager/local vet/pharmacist

4. Did it respond, or did you have to change treatment? Using which antibiotic?

5. Has your dog shared kennel or household with another dog that was treated with antibiotics, the last six months? Do you know with what?

6. Has your dog or anyone in its surroundings had problems with wounds not healing?

… or dermatitis? YES/NO/Unsure

7. Does anyone in the family work in a hospital/care facility? YES/NO/Unsure

8. Does anyone in the family work with animals? YES/NO/Unsure

9. Do you want to know the preliminary results? YES/NO

Notes on site and manor of sample collection: